Protist

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Molecular Identification of Sequestered Diatom Chloroplasts and Kleptoplastidy in Foraminifera

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Kleptoplastidy is the ability of heterotrophic organisms to preserve chloroplasts of algal preys they eat and partially digest. As the sequestered chloroplasts stay functional for months, the "host" becomes photosynthetically active. Although remaining a marginal process, kleptoplastidy was observed in different protist lineages, including foraminifera. Previous studies showed at least eight species of the foraminiferal genera *Haynesina* and *Elphidium* grazing on diatoms and husbanding their chloroplasts. In order to characterize more precisely the origin of kleptochloroplasts in these genera, we obtained 1027 chloroplastic 16S rDNA sequences from 13 specimens of two *Haynesina* and five *Elphidium* species. We identified the foraminiferal kleptochloroplasts using a reference phylogeny made of 87 chloroplastic sequences of known species of diatoms and brown algae. All the analyzed specimens were performing kleptoplastidy and according to our phylogenetic analyses they seem to retain exclusively chloroplasts of diatom origin. There is no apparent specificity for the type of diatom from which chloroplasts originated, however some foraminiferal species seem to accept a wider range of diatoms than others. Possibly the diversity of kleptochloroplasts depends on the type of diatoms the foraminiferans feed on.
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Introduction

First described in the sacoglossan molluscs (Kawaguti and Yamasu 1965), kleptoplastidy, also called chloroplast husbandry or sequestration, was then found to occur in some groups of protists (Stoecker et al. 2009). Although remaining a marginal process, kleptoplastidy was observed in some dinoflagellates such as *Dinophysis* (Takishita et al. 2002), *Pfiesteria piscicida* (Lewitus et al. 1999; Stoecker 1999) and *Cryptoperidiniopsis* (Eriksen et al. 2002), in at least 15 species of ciliates belonging to the genera *Laboea, Strombidium, Tontonia* and *Prorodon* (Dolan 1992) and in several foraminiferans (Bernhard and Bowser 1999). The process often defined as an "unusual symbiotic association" is characterized by the presence of foreign algal chloroplasts (also called kleptochloroplasts) inside the "host" cell while the other components of the algal prey are either discarded or digested.

Within foraminifera, at least eight different genera are known to perform kleptoplastidy: *Bulimina, Elphidium, Haynesina, Nonion, Nonionella, Nonionellina, Reophax* and *Stainforthia* (Bernhard and

Bowser 1999). The process was first described in *Haynesina* and *Elphidium,* which were shown to bear photosynthetically active chloroplasts (Lopez 1979) that remained functional for months, providing an additional "solar powered" source of nutriment to the host cell (Lee et al. 1988; Lopez 1979). Regarding chloroplast acquisition, it has been hypothesized (Bernhard and Bowser 1999) and subsequently experimentally tested (Austin et al. 2005) that the ornamental tubercles present on the surface of the test of *Haynesina* and *Elphidium* could be implicated in the kleptoplastidic process by disrupting the ingested algal prey. The complex ornamented canal systems found in *Haynesina* and *Elphidium* were proposed to function as combs or sieves to sort the different organelles and isolate the chloroplasts of the prey (Lee and Anderson 1991). Many studies, based on ultrastructural analyses (Correia and Lee 2002; Leutenegger 1984; Lopez 1979), composition of photosynthetic pigments (Knight and Mantoura 1985; Lopez 1979) and feeding experiments (Correia and Lee 2000; Lee and Lee 1989) suggested that diatoms were the source of the kleptochloroplasts. However, none of these studies could prove the exclusivity of diatoms as the donors of chloroplasts sequestered by *Haynesina* and *Elphidium*.

Furthermore, nothing is known about the precise taxonomic identity of diatoms, from which kleptochloroplasts originated in foraminifera. Different species of sacoglossan molluscs are known to have preferences for some types of algae (Clark et al. 1990; Jensen 1983), albeit these are not strictly specific associations. Within marine dinoflagellates, the genera *Pfiesteria* and *Cryptoperidiniopsis* are believed to retain exclusively chloroplasts of cryptophyte algae (Eriksen et al. 2002; Lewitus et al. 1999) although no molecular data are available to confirm this hypothesis. At least four species of the genus *Dinophysis* were cultured successfully and retained chloroplasts when fed with the ciliate *Myrionecta rubra* (Nagai et al. 2008; Nishitani et al. 2008; Park et al. 2006; Park et al. 2008), but nothing is said about their flexibility to graze on different algal types. A phylogenetic study performed on kleptochloroplasts of *Dinophysis mitra* identified the origin of sequestered organelles as haptophyte algal chloroplasts (Koike et al. 2005) without being more precise about their identity. Among marine ciliates, the situation is even more complex as for most of the species reported to perform kleptoplastidy, no data are available concerning the source of sequestered chloroplasts (Stoecker et al. 2009). Among the few species in which kleptochloroplasts

were identified, their origins vary from cryptophytes and haptophytes in the case of the genus *Laboea*, to prasinophytes, haptophytes and cryptophytes for the genus *Strombidium* and chromophytes for *Tontonia appendiculariformis* (Stoecker et al. 2009). It is important to notice that most of these studies were performed on cultured organisms, which may introduce a bias in the selection of sequestered chloroplasts.

In the present study, we analyzed kleptochloroplasts from uncultured specimens of the foraminiferal genera *Haynesina* and *Elphidium*. First, we obtained 22 complete nuclear SSU rDNA sequences of two *Haynesina* and five *Elphidium* species in order to clarify their phylogenetic relationships. Then, we identified the chloroplasts sequestered by each species by analyzing their 16S rDNA sequences. In total, we obtained and analyzed 1027 sequences from 13 specimens, using as reference, a phylogenetic tree of 84 chloroplastic 16S rDNA sequences of known species of diatoms and three sequences of brown algae. As all analyzed specimens contained chloroplasts, kleptoplastidy appears to be a ubiquitous process in the two genera analyzed. The results show that all the chloroplasts sequestered by the 13 specimens are of diatom origin. Although some species of *Haynesina* and *Elphidium* seem to preferentially retain chloroplasts from particular diatom clades, others accept a very wide range of diatoms as chloroplasts donors.

Results

SSU rDNA Phylogeny of *Elphidium*

In order to clarify the phylogenetic relationships between selected species of elphidiids and their sister genus *Haynesina*, 22 sequences of the complete SSU rDNA were obtained. This data set includes two sequences of *H. orbiculare*, one sequence of *H. germanica* and 19 sequences of elphidiids belonging to five morphospecies: *E. albiumbilicatum, E. excavatum, E. williamsoni, E. aculeatum* and *E. margaritaceum*. The geographic origins of the examined specimens are presented in Table 1 and the accession numbers of the sequences obtained are presented in the Supplementary Table S1. Maximum likelihood and Bayesian phylogenetic analyses of these sequences (Fig. 1 and Supplementary Fig. S1) allowed us to identify six clades corresponding to five studied morphospecies of *Elphidium* and the genus *Haynesina*. The tree topology of the

Table 1. Sampling locations of analyzed individuals and detailed taxonomic affinity of kleptochloroplasts found in each one. Taxonomic affinities are shown per individual and for each clade of diatom, BS (Basal Species), A, B, Db (*D. brightwellii*), C and D.

Foraminiferal species	DNA number	Sampling location	Taxonomic affinity per clade						Total
			BS	A	B	Db	C	D	
H. germanica	415	Bourg-Neuf, Atlantic, France	0	0	2	0	12	66	80
H. orbiculare	C131	Halifax, Atlantic, Canada	0	0	0	0	2	81	83
E. albiumbilicatum	10029	Unskaya, White Sea, Russia	0	0	17	0	42	0	59
E. excavatum	A217	Vilaine, Atlantic, France	6	0	77	0	3	0	86
E. excavatum	C62	Halifax, Atlantic, Canada	0	0	40	0	38	5	83
E. williamsoni	C5	Halifax, Atlantic, Canada	0	0	8	0	31	39	78
E. williamsoni	10017	Umba, White Sea, Russia	0	0	0	0	42	33	75
E. aculeatum	A53	Trebeurden, English Channel, France	0	72	9	0	1	4	86
E. aculeatum	A69	Roscoff, English Channel, France	39	3	6	7	1	19	75
E. aculeatum	A75	Roscoff, English Channel, France	0	0	49	0	5	10	64
E. margaritaceum	A13	Trebeurden, English Channel, France	0	3	1	0	6	73	83
E. margaritaceum	A17	Trebeurden, English Channel, France	4	1	21	0	36	21	83
E. margaritaceum	A109	Roscoff, English Channel, France	0	0	1	0	2	89	92
Total per clade			**49**	**79**	**231**	**7**	**221**	**440**	**1027**

elphidiids is identical in all analyses: the first diverging *E. albiumbilicatum* clade branches at the base followed by the *E. excavatum* clade and the three other *Elphidium* species (*E. williamsoni, E. aculeatum* and *E. margaritaceum*), their radiation is preceded by a long stem lineage. The clades of *H. orbiculare*, *E. albiumbilicatum* and *E. excavatum* are supported by maximal bootstrap values and posterior probabilities. The monophyletic group composed of *E. williamsoni*, *E. aculeatum* and *E. margaritaceum* also has maximal node support (NS), but the relationships within this group are not resolved, as suggested by a very weak NS (19/0.65) for the grouping of *E. aculeatum* and *E. margaritaceum*, reflecting the rapid radiation of these three species. The node support is high for the *E. margaritaceum* morphospecies (98/1), while it is moderate for *E. williamsoni* (89/0.98) and very low for *E. aculeatum* (50/0.83). Both species of our outgroup, *H. orbiculare* and *H. germanica*, form a monophyletic group with moderate NS (95/0.91).

Evolution of Morphological Patterns

Interestingly, the inferred phylogeny suggests a gradual emergence of some external morphological features, such as the ornamental teeth-like tubercles and the septal bridges containing retral processes (Fig. 1 and Supplementary Fig. S2). These projections that bridge the intercameral sutures of the foraminiferal test are a distinctive feature of the genus *Elphidium*. They are absent in the two species of *Haynesina* whose tests present only few ornamental tubercles mainly located in the umbiliculus. In the first-diverging species of elphidiids, *E. albiumbilicatum*, the septal bridges are almost invisible whereas the teeth-like structures are numerous in the umbiliculus and also visible in the intercameral sutures. The second emerging species, *E. excavatum*, shows more developed and visible septal bridges with numerous associated interiomarginal apertures. Although the surface of the chambers remains smooth, the sutures are covered by ornamental tubercles. *E. williamsoni* is the first diverging species in which the septal bridges are visible even by eye; the umbiliculus, the sutures and the interiomarginal apertures are elaborately ornamented with teeth-like structures. The last diverging group is composed of the two sister species *E. aculeatum* and *E. margaritaceum*. The overall morphology of these two species is very similar and the only distinguishable feature of *E. aculeatum* is the presence of a narrow spine of variable length at the peripheral end of each chamber. For both species the septal bridges are so
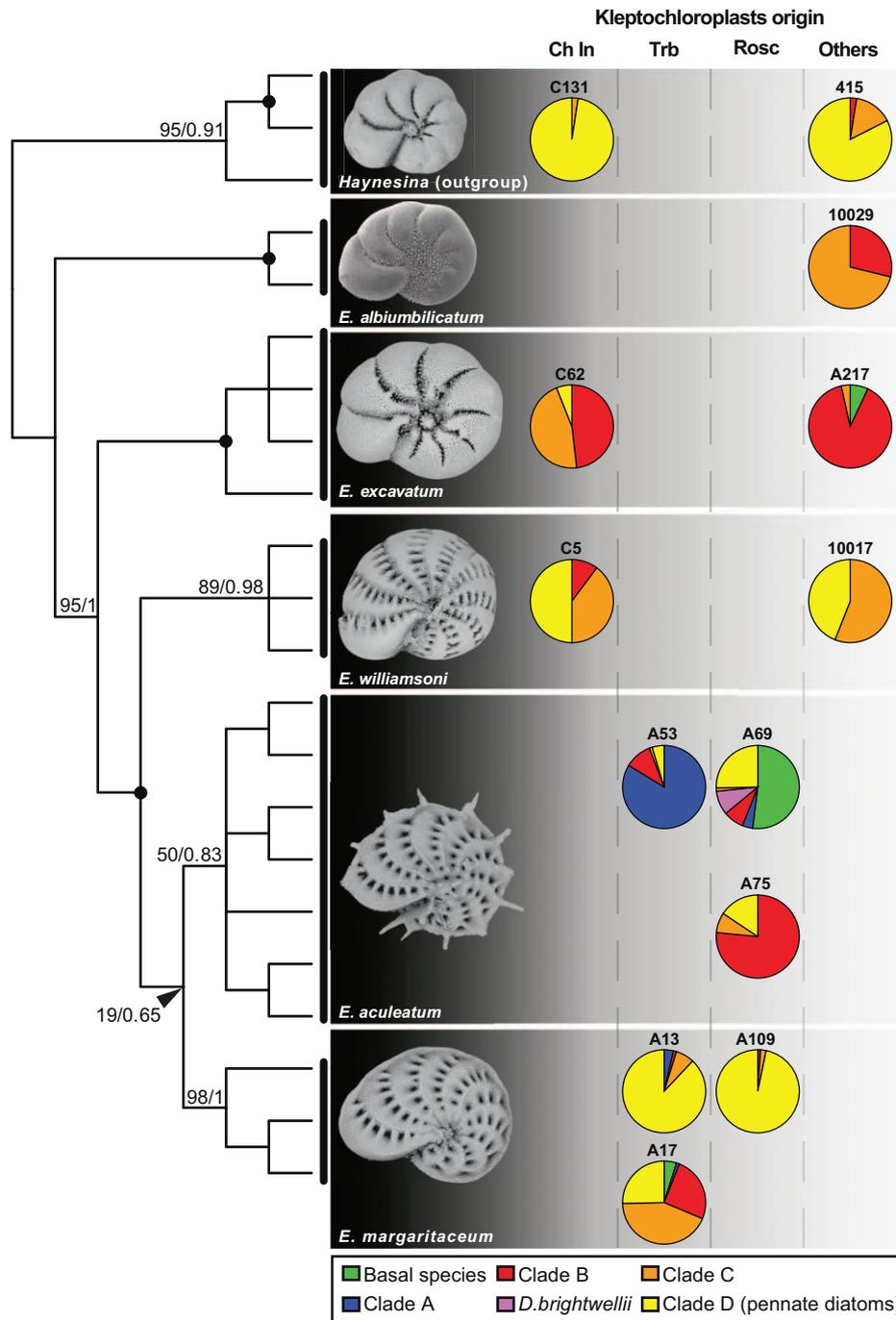
Figure 1. Elphidiids complete SSU rDNA phylogeny and content of kleptochloroplasts per individual per species. Fore more details about phylogeny, see legend of Figure S1. RAxML bootstrap values and MrBayes posterior probabilities are shown at the nodes and solid circles indicate maximum node support (100/1.0). Each pie represents a single individual, they are shown by morphospecies horizontally and by sampling location vertically, Chezzetcook Inlet (Ch In), Trebeurden (Trb), Roscoff (Rosc) and others.

developed that the intercameral apertures appear as rows of holes between two adjacent chambers and the entire surface of the test is covered by sharp ornamental tubercles.

Diatoms Chloroplastic SSU rDNA Phylogeny

In order to identify the foraminiferal kleptochloroplasts, we first inferred the phylogeny of diatoms using 84 sequences of the chloroplastic 16S rRNA gene available in the GenBank. To root this tree presented in Figure 2, we used three sequences of brown algae (*Fucus vesiculosus, Pylaiella littoralis* and *Bodanella lauterborni*). The Bayesian and the ML analyses provide similar topologies, with clear distinction of the main taxonomic groups, albeit often with no support for the relationships between these groups. Fourteen species belonging to the diatom orders Corethrales, Melosirales, Coscinodiscales, Rhizosoleniales and Paraliales branch at the base of the tree. These species are followed by four relatively well-supported clades: clade A (Hemiaulales and Chaetocerotales), clade B (Thalassiosirales), clade C (Cymatosirales and Eupodiscales) and clade D comprising all the pennate diatoms. The only representative of the Lithodesmidales, *Ditylum brightwellii*, shows no clear affinity to any of these clades and therefore is considered here as an independent lineage.

Taxonomic Affinity and Molecular Identification of Kleptochloroplasts

Because of the small size and the abundance of diatoms it was important to ensure that no diatom cells were present in our DNA extracts. In addition to thoroughly cleaning the foraminiferans prior to extraction, we also tested the foraminiferal extracts for the presence of diatom nuclear DNA. To be sure that the amplified chloroplastic genes originated from chloroplasts retained by foraminiferans and not from diatoms cells present inside or outside the test, we designed bacillariophyte-specific primers (Supplementary Table S2) to target diatom nuclear SSU rDNA. All DNA extracts (in total 46 extracts) that gave positive results with these primers were discarded.

To determine the taxonomic affinity of the kleptochloroplasts present in *Haynesina* and *Elphidium*, we selected 13 individuals representing seven morphospecies and obtained between 59 and 92 sequences of the chloroplastic 16S rRNA gene for each of them (Table 1). The sequences obtained for each individual were added to the reference diatom alignment and phylogenetic analyses were performed to determine their taxonomic affinity. The 13 trees obtained are presented in the Supplementary Material (Supplementary Figs S3 - S15) and the branching positions of the sequestered chloroplasts are summarized in Figure 2. The kleptochloroplast sequences were assigned to one of the six diatom groups, including the four identified clades (A, B, C and D), a group of basal species and *D. brightwellii*. These data are summarized in Table 1.

The results of the molecular identification of husbanded chloroplasts are correlated with the phylogeny of the elphidiids in Figure 1. Each pie represents kleptochloroplast diversity in one individual and the specimens collected in different localities are shown in separated columns. For every single locality where foraminifers were picked from the same sample, the origin of sequestered chloroplasts varies depending on the individual. The three specimens collected in Chezzetcook Inlet (*H. orbiculare* C131, *E. excavatum* C62 and *E. williamsoni* C5) (Fig. 1 Ch In) harbor chloroplasts of clades B, C or D, but the proportion of each type varies between species, with pennate diatoms (clade D) forming 98%, 6% and 50% of the total number respectively. Individuals collected in Trebeurden (Fig. 1 Trb) sequester a majority of chloroplasts from diatoms belonging to clade A (*E. aculeatum* A53), clade D (*E. margaritaceum* A13) or clades B, C and D (*E. margaritaceum* A17). The origin of kleptochloroplasts found in elphidiids collected in Roscoff (Fig. 1 Rosc) also differs between individuals: *E. aculeatum* A69 mainly bears chloroplasts from basal species of diatoms, while *E. aculeatum* A75 and *E. margaritaceum* A109 contain a majority of chloroplasts belonging to respectively clade B and clade D. The same type of differences is observed in the specimens from different localities in the White Sea and French Atlantic coast.

Although *Haynesina* and *Elphidium* sequester chloroplasts from all types of diatoms, each genus or species shows a different tendency. *H. germanica* (415) and *H. orbiculare* (C131) mainly bear chloroplasts of pennate diatoms (clade D), with few diatoms of clade B and C. This is contrary to the two early-diverging *Elphidium* species: *E. albiumbilicatum* (10029) and *E. excavatum* (C62 and A217), which sequester few or no chloroplasts of pennate diatoms but harbor mainly chloroplasts from clades B and C. The majority of kleptochloroplasts found in *E. williamsoni* (C5 and 10017) belong to diatoms from clades C and D with almost the same ratio. The six specimens of the two last-diverging species,
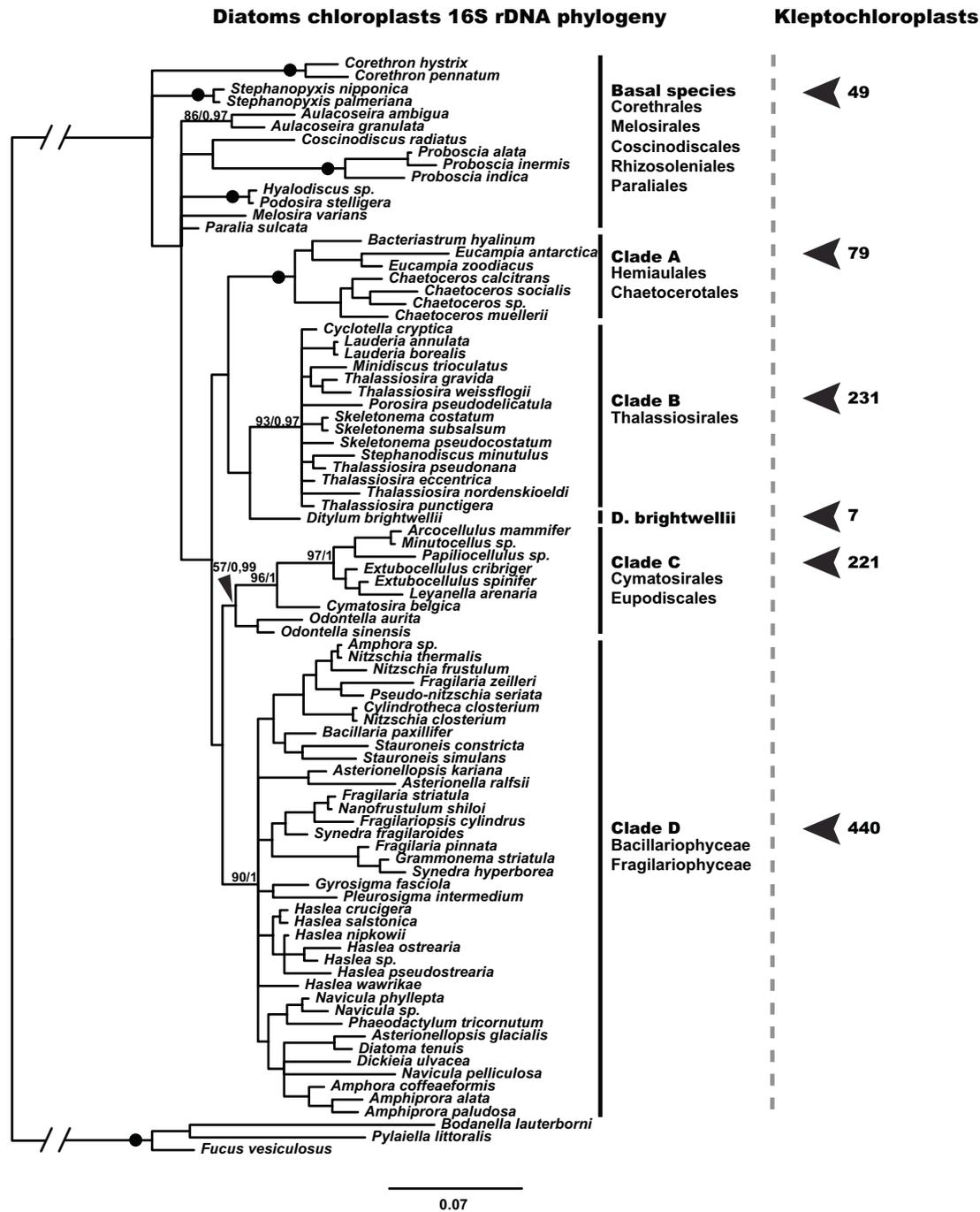
Diatoms chloroplasts 16S rDNA phylogeny **Kleptochloroplasts**

Figure 2. 16S rDNA phylogeny of diatom chloroplasts with branching positions of kleptochloroplasts. Bayesian phylogeny implemented using the GTR + Γ model of evolution. RAxML bootstrap values and MrBayes posterior probabilities are shown at the nodes and solid circles indicate maximum node support (100/1.0). The total number of kleptochloroplasts grouping in each clade is shown on the right.

E. aculeatum and *E. margaritaceum*, apparently bear chloroplasts of a greater variety of diatoms (between three and six of our clades per single individual) than the other species of *Haynesina* and *Elphidium* (between two and three clades). Inter-

estingly, the *E. aculeatum* from Trebeurden (A53) is the only specimen, whose kleptochloroplasts originate in majority from clade A, wheras one of the specimens of the same species (A69) collected in Roscoff is the only one which bears a majority of

kleptochloroplasts from the group of basal species. The two specimens of *E. margaritaceum* (A13 and A109) harbor a majority of chloroplasts from pennate diatoms, while the third specimen of the same species (A17) contains a wider range of chloroplasts.

Discussion

Characterizing kleptoplastidy is of major importance for understanding endosymbiosis and the emergence of photosynthesis among eukaryotes. Most of the hypotheses assume that the acquisition of chloroplasts by the host cell was carried through a predator-prey relationship between a heterotrophic eukaryotic cell feeding on algae (Archibald 2006; Bhattacharya et al. 2007; Keeling 2004). Kleptoplastidy could be regarded as a first step in this process and therefore it is important to understand its origin and specificity.

As shown by our study, *Haynesina* and *Elphidium* are particularly apt to perform kleptoplastidy. Eight species of these genera have been shown to bear kleptochloroplasts in previous studies (Bernhard and Bowser 1999; Lee and Lee 1989; Leutenegger 1984; Lopez 1979). Here, we add to the list four additional species that husband chloroplasts: *H. orbiculare*, *E. albiumbilicatum*, *E. aculeatum* and *E. margaritaceum*. As all the specimens belonging to the genera *Haynesina* and *Elphidium,* analyzed during this and previous studies, were found to retain chloroplasts, we believe that kleptoplastidy is a distinctive feature of the two genera, which are therefore an excellent model to study this process.

Our molecular analyses provide compelling evidence that the chloroplasts sequestered by *Haynesina* and *Elphidium* originated exclusively from diatoms. Strikingly, of the 1027 sequences of kleptochloroplasts analyzed, none has taxonomic affinity for other algae but diatoms. As the primers used to amplify the 16S rDNA of husbanded chloroplasts were designed to detect a broad range of algal types, including chlorophytes (Fuller et al. 2006; West et al. 2001) and as only ribosomal genes of diatom origin were amplified, we can exclude the presence of other types of algal chloroplasts in our total DNA extractions. Since the discovery of the kleptoplastidic process within *Haynesina* and elphidiids, many experiments have been performed to characterize the taxonomic origin of the chloroplastic "symbionts". Most of these studies led to the same suggestion that diatoms were the best chloroplast donors for these two foraminiferal genera, but because of the techniques used, the authors of these studies could not completely exclude the presence of other types of algae, mainly chlorophytes. Analyses of pigment composition (Knight and Mantoura 1985; Lopez 1979) had always detected traces of non-diatom pigments. Ultrastructural analyses using TEM (Correia and Lee 2002; Leutenegger 1984; Lopez 1979) could not exclude the possibility of missing a few husbanded chloroplasts, furthermore a proportion of organelles was mentioned as being in digestion process and therefore difficult to identify. Feeding experiments (Correia and Lee 2000; Lee and Lee 1989) concluded that diatoms were the source of kleptochloroplasts but some doubts remained because of few husbanded chloroplasts of chlorophyte origin.

In this study, we do not only confirm the exclusive diatom origin of the kleptochloroplasts sequestered by *Haynesina* and *Elphidium*, but also determine the origin of husbanded chloroplasts to a more precise taxonomic level. As our molecular phylogeny of diatoms is in agreement with previous studies (Rampen et al. 2009; Sorhannus 2007), we could identify kleptochloroplasts relatively reliably. Although the signal of the chloroplastic 16S rDNA gene is weak and does not allow the recovery of phylogenetic relationships between the main groups of diatoms, the support values obtained for these groups is sufficient to assign the kleptochloroplast sequences to different clades/lineages and to perform DNA taxonomic identification. Previous 16S rDNA phylogeny of diatoms (Rampen et al. 2009) shows the same topology and lack of resolution among the major clades (our clades A, B, C and D), including the uncertain position of *D. brightwellii*, branching separately as the unique representative of the Lithodesmidales. Furthermore, because of the weak signal of the chloroplastic marker, there is no resolution within the basal species and no structure is observed within the pennate clade (our clade D), where araphids and raphids diatoms intermingle. The nuclear 18S rDNA gene seems to be a better marker to resolve the relationships between main diatom clades (Sorhannus 2007). In fact our results are in broad agreement with this nuclear SSU phylogeny as the same main groups are recovered. Our clades A, B and C plus *D. brightwellii* are engulfed in the "bi(multi)polar centrics + thalassiosirales" group described by Sorhannus (2007). The pennate diatoms constitute the latest diverging monophyletic group in 16S rDNA and 18S rDNA phylogenies, but the use of the nuclear marker allows their partition in two sub-clades: raphid and araphid pennates. Sorhannus's "radial centrics"

group corresponds to a monophyletic clade engulfing all our basal species (Fig. 2). As this comparison suggests, albeit the signal provided by the 16S rDNA gene is weak, the topology of the obtained tree remains correct.

In view of our data, kleptoplastidy in *Haynesina* and *Elphidium* does not appear to be a selective process. All specimens harbor chloroplasts from at least two (*H. orbiculare* C131, *E. albiumbilicatum* 10029 and *E. williamsoni* 10017) and up to six (*E. aculeatum* A69) clades of diatoms. In order to exclude a possible environmental impact on our results, various sampling sites were analyzed in the present study. Within the three sets of specimens collected in the same locations, Chezzetcook Inlet (Fig. 1 Ch In), Trebeurden (Fig. 1 Trb) and Roscoff (Fig. 1 Rosc), we do not observe any similarity in the type of kleptochloroplasts retained by different species. This suggests that our results are not biased by the composition of the diatom assemblage present at the different sampling sites. However, we cannot totally exclude that the greater variety of diatoms observed in *E. aculeatum* and *E. margaritaceum* is related to a greater species richness in the diatom assemblage present in rocky shore ecosystems in Trebeurden and Roscoff, two localities only 40 kilometers away from each other.

Alternatively, the large diversity of diatoms found in *E. aculeatum* and *E. margaritaceum* could be related to the particular morphology of their tests. Considering the two clearly distinguishable morphological features of *Haynesina* and *Elphidium*, the septal bridges containing retral processes and the density of ornamental tubercles, the morphospecies analyzed in our phylogeny can be assigned to three distinct morphological types. The first one represented by *Haynesina* has few ornamental teeth; by definition this genus differs from *Elphidium* by the absence of retral process (Hayward et al. 1997; Loeblich and Tappan 1988). In the second type, represented here by *E. albiumbilicatum* and *E. excavatum*, teeth-like structures are more numerous than in *Haynesina* and retral processes are emerging but still poorly developed. The third architectural type, including *E. williamsoni*, *E. aculeatum* and *E. margaritaceum*, shows a great density of ornamental teeth and highly developed retral processes. As previously suggested (Alexander and Banner 1984; Banner and Culver 1978; Bernhard and Bowser 1999; Lee 1993; Lee and Anderson 1991), the "teeth-like" structures and the retral processes contained in the septal bridges may be involved in the acquisition of kleptochloroplasts by disassembling diatom frustules and function as combs or sieves to isolate the chloroplasts of the prey. This makes these structures important for the acquisition of diatom chloroplasts by *Elphidium* and *Haynesina* and confirms that foraminiferal test morphology has to be considered as an important feature in the development of kleptoplastidy.

One could argue that many other foraminiferal genera such as *Bulimina*, *Nonion*, *Nonionella*, *Nonionellina*, *Reophax* and *Stainforthia* have been reported to perform kleptoplastidy (Bernhard and Bowser 1999), but do not show the morphological features particular for elphidiids. First, for most of these genera there is no evidence from which source the chloroplasts originated, excepted for *Nonionellina labradorica* which was proposed to husband chloroplasts of dinoflagellate origin (Cedhagen 1991). Second, many of these genera possess internal structures, such as toothplates in *Bulimina* and *Stainforthia*, or the umbilical flap in *Nonionella* that could be involved in selection of ingested cells.

In our opinion, the elphidiids evolved their particular morphology as an adaptation to feeding on diatoms. This is confirmed by experiments showing that they feed preferentially on pennate diatoms: *Navicula musculus* and *Amphora tennerima* in the case of *H. germanica* (Lee and Lee 1989) and *Amphora coffeaeformis* in the case of *E. excavatum* (Correia and Lee 2000). Although in the later case, the type of diatom is not in accordance with the results of the present study (Fig. 1), we cannot exclude that *E. excavatum* would feed also on centric diatoms if they were provided. We think that more developed septal bridges and ornamental teeth in *E. aculeatum* and *E. margaritaceum* could possibly increase their ability to feed on a wider range of diatoms, compared to *Haynesina*, which lacks these structures. In consequence, the diversity of kleptochloroplasts retained by these two species could be higher.

As shown for other organisms (Rumpho et al. 2008; Wisecaver and Hackett 2010), we can speculate that the ancestor of elphidiids and *Haynesina* probably fed on diatoms and that possibly some of the diatom genes have been transferred to the foraminiferal nucleus (Endosymbiotic Gene Transfer). This may have enabled the functioning of diatom chloroplasts inside foraminiferal cells and gave origin to kleptoplastidy in this group. The analysis of *Elphidium* ESTs (work in progress) should help to identify these genes and contribute to further elucidation of the process leading to kleptoplastidy. However, whatever will be the outcome of these future studies, we can speculate that the acquisition of diatom kleptochloroplasts had an impact on the evolution of *Elphidium*, con-

tributing to the successful radiation of this most abundant and diverse benthic foraminiferal genus in the inner-shelf environment throughout temperate and subtropical parts of the world.

Methods

Sampling and DNA extraction: Foraminiferans analyzed in this study were collected in the North Atlantic (Canadian and French coasts), the English Channel and the White Sea. Three specimens (*H. orbiculare* C131, *E. excavatum* C62 and *E. williamsoni* C5) were collected in Chezzetcook Inlet, three more (*E. aculeatum* A53, *E. margaritaceum* A13 and A17) originated from Trebeurden and three others (*E. margaritaceum* A109, *E. aculeatum* A69 and A75) were collected in Roscoff. Four more specimens originated either from the White Sea (*E. albiumbilicatum* 10029 and *E. williamsoni* 10017) or the French Atlantic coast (*H. germanica* 415 and *E. excavatum* A217). For the three first localities, Chezzetcook Inlet (Canada), Trebeurden and Roscoff (French coast of the English Channel), the three individuals originated from the exact same portion of sediment. These sampling localities are summarized in Table 1. Algae and surface sediment were taken at low tide, washed in seawater and sieved. The fractions comprised between 500 μm and 125 μm were collected and stored at temperatures close to those of sampling sites. Live specimens were picked within a week after sampling and cleaned with small paintbrushes in several changes of filtered seawater to avoid contaminant. Total DNA was extracted from single specimens using DOC lysis buffer or guanidine buffer (Pawlowski 2000), which was modified without EDTA in order to preserve the shape of the test. Most specimens were imaged using Scanning Electron Microscopy (SEM) and morphologically determined.

PCR amplifications: To exclude the presence of contaminant diatoms in the DNA extracts, we performed a negative control by trying to amplify the SSU nuclear rDNA gene of diatoms using the DiatSSUF-DiatSSUR primers (Supplementary Table S2).

The complete foraminiferal SSU rDNA gene of seven different morphospecies of *Haynesina* and *Elphidium* was amplified in different steps using foraminiferal specific primers summarized in the Supplementary Table S2. The primers sA10 and s17 were used to amplify the 5′ end fragment and the primers s14F3 and L5.2 for the 3′ end fragment. A re-amplification of the PCR products (nested PCR) was then performed using a combination of the following primers, depending on the species: sA10-s5R; s4F-s13; s5F-s12R; s12F-s17 or Elphi12F-Elphi17R for the 5′ end fragment and s18F-L5.2; s14F1-NewB or s14F1-For2 for the 3′ end fragment. Hot Start PCR amplifications and re-amplifications were performed in a total volume of 50 μl with an amplification profile consisting of 35 cycles (25 cycles for the re-amplifications) of 30 s at 94 °C, 30 s at 52 °C, and 90 s at 72 °C, followed by 5 min at 72 °C for final extension.

The kleptochloroplast partial 16S rDNA contained in 13 different individuals of foraminifera was amplified using the PLA491F-OXY1313R primers (Fuller et al. 2006; West et al. 2001). Hot Start PCR amplifications were performed in a total volume of 50 μl with an amplification profile consisting of 35 cycles of 45 s at 95 °C, 45 s at 60 °C, and 75 s at 72 °C, followed by 7 min at 72 °C for final extension.

Cloning and sequencing: After purification using High Pure PCR Purification Kit (ROCHE DIAGNOSTICS, Basel, Switzerland) or MinElute PCR Purification Kit (QIAGEN, Basel, Switzerland), positive PCR products were cloned. They were ligated in the Topo TA Cloning vector (INVITROGEN, Basel, Switzerland), and cloned using chemically competent cells One Shot TOPO10 (INVITROGEN). Sequencing was done with an ABI PRISM Big Dye Terminator Cycle Sequencing Kit using an ABI 3130XL DNA sequencer (APPLIED BIOSYSTEM, Rotkreuz, Switzerland), according to the manufacturer's instructions. By overlapping the different fragments, a complete nuclear SSU rRNA gene was obtained for all foraminiferal morphospecies. The chloroplastic 16S rDNA sequences obtained were partial and their length ranged from 312 to 785 positions. All sequences used in this study have been deposited in the GenBank database and their accession numbers are summarized in Supplementary Table S1.

Phylogenetic analyses: Fifteen data sets of sequences were analyzed separately. The first one made of the complete SSU rDNA gene of 22 specimens from seven different species of foraminifers, was used to infer the phylogeny of the genus *Elphidium*. The second data set was made of 84 GenBank sequences of diatom chloroplastic 16S rDNA plus 3 sequences of brown algae (outgroup) and was used as a reference phylogeny. Every single set of kleptochloroplastic partial 16S rDNA sequences (one for each foraminifer analyzed) was then added to this reference diatom phylogeny, to determine the taxonomic affinity of the sequenced kleptochloroplasts. This represents the 13 other data sets. All fifteen data sets were first aligned using MAFFT v.6 (Katoh et al. 2002, 2005) and then improved manually using BioEdit Sequence Alignment Editor (Hall 1999). After having cleaned each data set and removed all ambiguous positions, bacterial contaminant or sub-quality sequences, the Collapse v.1.2 program (Posada 2004) was used to count the number of identical sequences and to transform the alignments into sets of unique haplotypes. Then, for each data set, the Perl script MrAIC 1.4.3 (Nylander 2004) in combination with PHYML v2.4.4 (Guindon and Gascuel 2003) was used to choose the best model of sequence evolution by the Akaike Information Criterion (AIC). Applying the obtained settings for each data set a Bayesian method and a Maximum Likelihood (ML) method (Felsenstein 1981) were used to infer the phylogeny. With the MrBayes program (Huelsenbeck and Ronquist 2001), two independent analyses were performed at the same time with four simultaneous chains (one cold and three heated) ran for 10,000,000 generations, and sampled every 1,000 generations. After having discarded 2,000 of the initial trees as burn-in, the consensus tree with the corresponding posterior probabilities (PP) was calculated for each data set. The ML method was implemented in the RAxML-HPC v7.0.4 software (Stamatakis 2006) and the reliability of internal branches was assessed using the RAxML rapid bootstrap method with 100 replicates (Felsenstein 1985). Most of the bioinformatic analyses were carried out on the freely available Bioportal (www.bioportal.uio.no) and the Cipres portal (http://www.phylo.org/sub_sections/portal).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.protis.2010.10.001.

References

Alexander SP, Banner FT (1984) The functional relationship between skeleton and cytoplasm in *Haynesina germanica* (Ehrenberg). J Foram Res **14**:159–170

Archibald JM (2006) Endosymbiosis: double-take on plastid origins. Curr Biol **16**:R690–692

Austin HA, Austin WEN, Paterson DM (2005) Extracellular cracking and content removal of the benthic diatom *Pleurosigma angulatum* (Quekett) by the benthic foraminifera Haynesina germanica (Ehrenberg). Mar Micropaleontol **57**:68–73

Banner FT, Culver SJ (1978) Quaternary *Haynesina* n. gen. and paleogene *Protelphidium* Haynes; their morphology, affinities and distribution. J Foram Res **8**:177–207

Bernhard JM, Bowser SS (1999) Benthic foraminifera of dysoxic sediments: chloroplast sequestration and functional morphology. Earth-Sci Rev **46**:149–165

Bhattacharya D, Archibald JM, Weber AP, Reyes-Prieto A (2007) How do endosymbionts become organelles? Understanding early events in plastid evolution. Bioessays **29**:1239–1246

Cedhagen T (1991) Retention of chloroplasts and bathymetric distribution in the sublittoral foraminiferan *Nonionellina labradorica*. Ophelia **33**:17–30

Clark KB, Jensen KR, Stirts HM, eds (1990) Survey for Functional Kleptoplasty among West Atlantic Ascoglossa (= Sacoglossa) (Mollusca: Opisthobranchia). California Malacozoological Society, Santa Barbara, CA, USA

Correia MJ, Lee JJ (2000) Chloroplast retention by *Elphidium excavatum* (Terquem). Is it a selective process? Symbiosis **29**:343–355

Correia MJ, Lee JJ (2002) Fine structure of the plastids retained by the foraminifer *Elphidium excavatum* (Terquem). Symbiosis **32**:15–26

Dolan J (1992) Mixotrophy in ciliates: A review of *Chlorella* symbiosis and chloroplast retention. Mar Microb Food Webs **6**:115–132

Eriksen NT, Hayes KC, Lewitus AJ (2002) Growth responses of the mixotrophic dinoflagellates, *Cryptoperidiniopsis* sp. and *Pfiesteria piscicida*, to light under prey-saturated conditions. Harmful Algae **1**:191–203

Felsenstein J (1981) Evolutionary trees from DNA sequences: maximum likelihood approach. J Mol Evol **17**:368–376

Felsenstein J (1985) Confidence limits on phylogenetics: an approach using the bootstrap. Evolution **39**:783–791

Fuller NJ, Tarran GA, Cummings DG, Woodward EMS, Orcutt KM, Yallop M, Le Gall F, Scanlan DJ (2006) Molecular analysis of photosynthetic picoeukaryote community structure along an Arabian Sea transect. Limnol Oceanogr **51**:2502–2514

Guindon GJ, Gascuel P (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol **52**:696–704

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser **41**:95–98

Hayward BW, Hollis CJ, Grenfell HR, eds (1997) Recent Elphidiidae (Foraminiferida) of the South-West Pacific and Fossil Elphidiidae of New Zealand. Institute of Geological and Nuclear Sciences Monograph 16. Institute of Geological and Nuclear Sciences Limited, Lower Hutt, New Zealand

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics **17**:754–755

Jensen KR (1983) Factors affecting feeding selectivity in herbivorous Ascoglossa (Mollusca: Opisthobranchia). J Exp Mar Biol Ecol **66**:135–148

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res **30**:3059–3066

Katoh K, Kuma K, Toh H, Miyata T (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res **33**:511–518

Kawaguti S, Yamasu T (1965) Electron microscopy on the symbiosis between an elysiioid gastropod and chloroplasts of a green alga. Biol J Okayama Univ **11**:57–65

Keeling P (2004) A brief history of plastids and their hosts. Protist **155**:3–7

Knight R, Mantoura RFC (1985) Chlorophyll and carotenoid pigments in Foraminifera and their symbiotic algae: analysis by high performance liquid chromatography. Mar Ecol Prog Ser **23**:241–249

Koike K, Sekiguchi H, Kobiyama A, Takishita K, Kawachi M, Koike K, Ogata T (2005) A novel type of kleptoplastidy in *Dinophysis* (Dinophyceae): Presence of haptophyte-type plastid in *Dinophysis mitra*. Protist **156**:225–237

Lee JJ (1993) "On A Piece of Chalk"-Updated. J Eukaryot Microbiol **40**:395–410

Lee JJ, Anderson OR (1991) Symbiosis in Foraminifera. In Lee JJ, Anderson OR (ed) Biology of Foraminifera. Academic Press, London, pp 157-220.

Lee JJ, Lanners E, Ter Kuile B (1988) The retention of chloroplasts by the foraminifer *Elphidium crispum*. Symbiosis **5**:45–60

Lee JJ, Lee RE (1989) Chloroplast retention in Elphidids (Foraminifera). In Nardon P, Gianinazzi-Pearson V, Greiner AM, Margulis L, Smith DC (eds) 4th International Colloquium on Endocytobiology and Symbiosis. Institut National de la Recherche Agronomique, Paris, pp 215–220

Leutenegger S (1984) Symbiosis in benthic foraminifera: Specificity and host adaptations. J Foram Res **14**:16–35

Lewitus A, Glasgow H, Burkholder J (1999) Kleptoplastidy in the toxic dinoflagellate *Pfiesteria piscicida* (Dinophyceae). J Phycol **35**:303–312

Loeblich AR, Tappan H, eds (1988) Foraminiferal Genera and their Classification. Van Nostrand Reinhold, New York

Lopez E (1979) Algal chloroplasts in the protoplasm of three species of benthic foraminifera: Taxonomic affinity, viability and persistence. Mar Biol **53**:201–211

Nagai S, Nitshitani G, Tomaru Y, Sakiyama S, Kamiyama T (2008) Predation by the toxic dinoflagellate *Dinophysis fortii* on the ciliate *Myrionecta rubra* and observation of sequestration of ciliate chloroplasts. J Phycol **44**:909–922

Nishitani G, Nagai S, Takano Y, Sakiyama S, Baba K, Kamiyama T (2008) Growth characteristics and phylogenetic analysis of the marine dinoflagellate *Dinophysis infundibulus* (Dinophyceae). Aquat Microb Ecol **52**:209–221

Nylander JAA (2004) MrAIC.pl. 1.4.3. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden

Park M, Kim S, Kim H, Myung G, Kang Y, Yih W (2006) First successful culture of the marine dinoflagellate *Dinophysis acuminata*. Aquat Microb Ecol **45**:101–106

Park MG, Park JS, Kim M, Yih W (2008) Plastid dynamics during survival of *Dinophysis caudata* without its ciliate prey. J Phycol **44**:1154–1163

Posada D (2004) Collapse: Describing Haplotypes From Sequence Alignments, v.1.2. University of Vigo, Spain

Rampen SW, Schouten S, Elda Panoto F, Brink M, Andersen RA, Muyzer G, Abbas B, Sinninghe Damsté JS (2009) Phylogenetic Position of *Attheya longicornis* and *Attheya septentrionalis* (Bacillariophyta). J Phycol **45**:444–453

Rumpho ME, Worful JM, Lee J, Kannan K, Tyler MS, Bhattacharya D, Moustafa A, Manhart JR (2008) Horizontal gene transfer of the algal nuclear gene *psbO* to the photosynthetic sea slug *Elysia chlorotica*. Proc Natl Acad Sci USA **105**:17867–17871

Sorhannus U (2007) A nuclear-encoded small-subunit ribosomal RNA timescale for diatom evolution. Mar Micropaleontol **65**:1–12

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics **22**:2688–2690

Stoecker D, Johnson M, Devargas C, Not F (2009) Acquired phototrophy in aquatic protists. Aquat Microb Ecol **57**:279–310

Stoecker DK (1999) Mixotrophy among *Dinoflagellates*. J Eukaryot Microbiol **46**:397–401

Takishita K, Koike K, Maruyama T, Ogata T (2002) Molecular evidence for plastid robbery (Kleptoplastidy) in *Dinophysis*, a dinoflagellate causing diarrhetic shellfish poisoning. Protist **153**:293–302

West NJ, Schoenhuber WA, Fuller NJ, Amann RI, Rippka R, Post AF, Scanlan DJ (2001) Closely related *Prochlorococcus* genotypes show remarkably different depth distributions in two oceanic regions as revealed by in situ hybridization using 16S rRNA-targeted oligonucleotides. Microbiology **147**:1731–1744

Wisecaver JH, Hackett JD (2010) Transcriptome analysis reveals nuclear-encoded proteins for the maintenance of temporary plastids in the dinoflagellate *Dinophysis acuminata*. BMC Genomics **11**:366