

Direct evidence for symbiont sequestration in the marine red tide ciliate *Mesodinium rubrum*

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ABSTRACT: The red tide ciliate *Mesodinium rubrum* (= *Myrionecta rubra*) is known to contain symbionts of cryptophyte origin. Molecular data have shown that the symbiont is closely related or similar to free-living species of the *Teleaulax/Plagioselmis/Geminigera* clade. This suggests that the symbiont of *M. rubrum* is either a temporary symbiont or a quite recently established symbiont. Here, we present data from a number of experiments in which we offered *M. rubrum* a phototrophic dinoflagellate and 8 different cryptophyte species belonging to 5 different clades. *Mesodinium rubrum* was only able to grow when fed the 2 cryptophyte species belonging to the genus *Teleaulax*, *T. acuta* and *T. amphioxeia*. Using the nucleomorph large subunit rDNA gene as marker, we were able to discriminate the 2 *Teleaulax* species, allowing monitoring of the exchange of the symbionts in *M. rubrum*. Over a period of 35 d, *M. rubrum* was able to exchange its symbionts from *T. amphioxeia* symbionts to *T. acuta* symbionts. This research suggests that *M. rubrum* can only utilize prey within the *Teleaulax/Plagioselmis/Geminigera* clade for sustained high growth rates and provides the first time-frame of endosymbiont replacement by *M. rubrum*.

KEY WORDS: *Mesodinium rubrum* · Symbionts · Ingestion · Cryptophytes

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INTRODUCTION

Mesodinium rubrum is a phototrophic ciliate that hosts cryptophyte symbionts consisting of chloroplasts, mitochondria, nucleomorphs, cytoplasm and a so-called 'symbiont nucleus' (Taylor et al. 1971, Hibberd 1977, Hansen & Fenchel 2006). The ciliate is well known for its ability to form dense red tides worldwide (e.g. Taylor et al. 1971, Lindholm 1985), and it has recently been identified as prey for the diarrhetic shellfish poisoning (DSP)-causing dinoflagellates *Dinophysis* spp. (e.g. Park et al. 2006, Nishitani et al. 2008a,b, Riisgaard & Hansen 2009), creating a renewal of interest within the scientific community.

Mesodinium rubrum is an obligate mixotroph requiring both food uptake and light for sustained growth. Currently, all isolates of *M. rubrum* main-

tained in culture have been fed cryptophytes belonging to the genus *Geminigera* for the cold-water strain and *Teleaulax* for the all the temperate strains. Both genera are closely related and belong to the same clade, the *Teleaulax/Plagioselmis/Geminigera* (TPG) clade (Hoef-Emden 2008). Very little is known about the growth of *M. rubrum* on cryptophytes outside of the TPG clade. Recently, 2 *Rhodomonas* strains, CR-MAL03 and CR-MAL06, have been tested as food for the Korean strain of *M. rubrum*, resulting in a very low *M. rubrum* growth rate only on CR-MAL03 (Park et al. 2007, Myung et al. 2011). However, *M. rubrum* obtains most of its carbon requirements through photosynthesis (Smith & Hansen 2007). It only needs to ingest 1 cryptophyte cell (*Teleaulax* sp.) per day to grow at its maximum growth rate (~ 0.5 d⁻¹; Smith & Hansen 2007). In terms of ingested carbon, the food

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uptake only accounts for 1 to 2% of the carbon requirements per day. Furthermore, *M. rubrum* is able to divide its chloroplasts when starved, and its symbionts can photoacclimate (Hansen & Fenchel 2006, Moeller et al. 2011), indicating some genetic control of the symbionts. Because *M. rubrum* meets most of its carbon requirements by photosynthesis, the question arises why *M. rubrum* has to eat to sustain growth. One explanation could be that *M. rubrum* harbors permanent symbionts but needs to ingest prey to obtain some essential micronutrients. Another explanation could be a need for symbiont renewal when the symbionts have undergone a certain number of divisions within the host. This hypothesis implies that the chloroplasts have to be acquired at regular intervals for sustained growth. Previous molecular analyses of the nucleomorph and chloroplast genes of the chloroplasts of *Mesodinium rubrum* and its prey have revealed identical sequences for these 2 organisms in the 3 cultured strains of *M. rubrum* (Johnson et al. 2007, Park et al. 2007, Garcia-Cuetos et al. 2010), tending to agree with the latter hypothesis.

In 2010, Park et al. (2010) studied possible renewal of symbionts using 2 strains belonging to the TPG clade (CR-MAL01=*Teleaulax amphioxeia* and CR-MAL11=*Teleaulax acuta*; Park et al. 2010). A *Mesodinium rubrum* culture fed CR-MAL11 for 5 mo still contained chloroplast markers from CR-MAL01 (see Fig. 4 in Park et al. 2010). The aim of their study was the replacement of the chloroplasts in *Dinophysis caudata*, and the results obtained for *M. rubrum* were not discussed in detail. Some ambiguity, such as the extent of plastid renewal, was left unanswered. In a recent study, Myung et al. (2011) tried to study possible renewal of the plastids by feeding *M. rubrum* with a strain belonging to the *Rhodomonas* clade. They fed cryptophyte CR-MAL03 to a 2 mo starved *M. rubrum* culture for 2 d and found chloroplast markers of both the original prey item (CR-MAL01) and the subsequent prey item (CR-MAL03) over a period of 37 d after the beginning of the experiment. The presence of both genetic markers makes it difficult to conclude whether there was full symbiont replacement and to what extent the chloroplasts inherited from CR-MAL03 were photosynthetically active.

To answer these questions, more information is needed concerning the growth performances of *Mesodinium rubrum* on a variety of cryptophyte

strains belonging to different clades as well as the possible occurrence of symbiont turnover. The aim of the present investigation was to study the growth responses of the Danish isolate of *M. rubrum* when offered 8 different species of cryptophytes belonging to 5 different clades, in addition to one dinoflagellate species. The possible exchange of symbionts was examined using transmission electron microscopy (TEM) and molecular techniques.

MATERIALS AND METHODS

Algal cultures

A total of 8 species of cryptophytes and 1 species of dinoflagellate were used as food (Table 1). The selection represents a broad range of possible prey found in *Mesodinium rubrum*'s natural environment. All cultures were grown on a glass table in h/20 medium (Guillard 1975) at a salinity of 30 and a temperature of $20 \pm 1^\circ\text{C}$. Cool white light ($100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) was provided from beneath in a light:dark cycle of 14:10 h.

Cultures of the ciliate *Mesodinium rubrum*

A culture of *Mesodinium rubrum* was established from single cells isolated from surface seawater samples collected in Frederikssund, Denmark, during a bloom event on April 17 2007, as described in Riisgaard & Hansen (2009). Prior to the experiment, the cultures, normally grown in f/2 medium (Guillard 1975), were transferred to h/20 medium and acclimated for a couple of months. The change of medium was carried out to respond to the needs of certain cryptophytes species, such as *Hanusia phi* and *Guillardia theta*.

Table 1. Protist strains used as prey in the experiments. nmSSU (nSSU) rDNA: nucleomorph (nuclear) small subunit ribosomal DNA. na: not available

Species	Culture collection ID no.	GenBank accession no.	
		nmSSU rDNA	nSSU rDNA
<i>Heterocapsa rotundata</i>	K-0483 (SCCAP)	na	na
<i>Chroomonas vectensis</i>	CCMP-432	na	HM126534
<i>Guillardia theta</i>	CCMP-2712	AF083031	X57162
<i>Hanusia phi</i>	CCMP-325	GQ396278	U53126
<i>Hemiselmis tepida</i>	CCMP-442	GQ396279	HM126533
<i>Proteomonas sulcata</i>	CCMP-321	na	HM126536
<i>Rhodomonas salina</i>	K-1487 (SCCAP)	na	HM126532
<i>Teleaulax amphioxeia</i>	K-0434 (SCCAP)	GQ396273	AJ421146
<i>Teleaulax acuta</i>	K-1486 (SCCAP)	HQ651177	HM126531

Growth and survival responses of *Mesodinium rubrum*

The 8 cryptophyte species and 1 dinoflagellate species were tested as prey for *Mesodinium rubrum* (Table 1). Initial attempts to culture *Mesodinium rubrum* on all 9 prey species, offered separately in multi-well dishes, revealed that long-term growth was only sustained with cryptophytes belonging to the genus *Teleaulax*, specifically *T. acuta* and *T. amphioxeia*.

To explore the relationship between *Mesodinium rubrum* and its prey, growth experiments were performed using all prey types. The experiments were carried out using 65 ml tissue culture bottles filled to capacity. Samples (2 ml) were drawn at Days 4, 8, 12 (or 10) and in one case 16, and bottles were refilled to capacity with fresh h/20 medium. Samples were fixed in Lugol's fixative (final concentration 2%), and cells were enumerated in a Sedgewick-Rafter chamber using an inverted microscope. Approximately one-tenth of the chamber was searched for organisms, leading to a detection limit of ~ 10 cells m^{-1} .

It was previously shown that the growth of *Mesodinium rubrum* is affected when pH exceeds 8.5 and that *M. rubrum* dies if exposed to pH > 8.8 for days (Smith & Hansen 2007). Thus, to interpret data correctly, pH was measured directly in the bottles on each sampling occasion using a Sentron® Argus pH meter equipped with a HOT-Fet line pH probe calibrated using standard buffers of pH 7 and 10. Prior to all experiments, *M. rubrum* was grown on the cryptophyte *Teleaulax amphioxeia*. Only *M. rubrum* cultures that had just depleted the prey were used for experiments. Three sets of experiments with different prey items were carried out, making each session manageable in terms of manpower.

In the first set of experiments, the cryptophyte *Teleaulax acuta* was used as prey. Initial cell concentrations were 1000 cells ml^{-1} of both prey and predator. This prey concentration was chosen based on the previous observation that maximum growth of *Mesodinium rubrum* occurs during these conditions when fed *T. amphioxeia*. Moreover, it has been shown that *M. rubrum* ingests ~ 1 prey cell (*T. amphioxeia*) d^{-1} at this prey concentration (Smith & Hansen 2007), which means that *M. rubrum* should be able to control the prey concentration in the bottles. In cases in which the prey was depleted in the experimental bottles, *M. rubrum* from that particular experiment was subcultured (i.e. diluted), and prey concentrations were increased and/or sampling frequency increased. Control experiments were carried out with a monoculture of

unfed *M. rubrum* and monocultures of the given prey species. All experiments were carried out in triplicate.

In a second set of experiments, the cryptophyte *Proteomonas sulcata* and the dinoflagellate *Heterocapsa rotundata* were used as prey. In this set of experiments, *Teleaulax amphioxeia* was used a 'control food' to demonstrate the potential performance of the *Mesodinium rubrum* isolate.

Finally, in a third set of experiments, the growth responses of *Mesodinium rubrum* were studied when offered 5 additional species of cryptophytes: *Chroomonas vectensis*, *Rhodomonas salina*, *Hanusia phi*, *Guillardia theta* and *Hemiselmis tepida*. The setup of the experiment was the same as in the 2 previous sets of experiments, except that all the mixed cultures were diluted on Day 4 to diminish effects of elevated pH on the outcome of the experiments.

DNA extraction of the cryptophytes and PCR amplification of nuclear SSU rDNA

Prior to the growth experiments, DNA of the cryptophyte species used as prey (Table 1) was extracted as described in Hansen et al. (2003). PCR amplifications of the nuclear small subunit ribosomal DNA (nSSU rDNA) of the cryptophytes were completed in 50 μl with a combination of primers CrN1F and SSUBR as outlined in Hoef-Emden et al. (2002). The nSSU rDNA gene was selected because it offers the biggest database in GenBank and allowed comparison between our strain and many other strains of cryptophytes.

Symbiont sequestration: isolation of *Mesodinium rubrum*, PCR amplification and cloning of nLSU rDNA

During the grazing and growth experiments, samples were collected (20 ml) and fixed in acid Lugol on Day 4 for all species tested as food for *Mesodinium rubrum*, on Day 12 for *Hanusia phi*, *Hemiselmis tepida* and *Teleaulax acuta* and on Days 16 and 35 for the experiment carried out with *T. acuta*. At the end of the experiments, the cells of *M. rubrum* from the experiments that sustained best growth were isolated for PCR: *H. phi*, *H. tepida* and *T. acuta* on Day 12 and cells of *M. rubrum* fed *T. acuta* on Days 16 and 35. In all cases, Lugol-fixed cells of *M. rubrum* were isolated using a drawn Pasteur glass pipette under an Olympus inverted microscope CKX31 (Olympus) and washed at least 3 times in

ddH₂O under the inverted microscope to prevent any cryptophyte present in the fixed sample from being transferred with the cell of interest. Finally, 5 washed *M. rubrum* cells were transferred into a single 0.2 ml PCR tube (StarLab) and kept frozen at –20°C until further processing.

PCR reactions were carried out in volumes of 50 µl. To determine the endosymbiont identity, PCR amplifications of the nucleomorph large subunit ribosomal DNA (nmLSU rDNA) were carried out as described by Garcia-Cuetos et al. (2010) with the primer combination nmLSUCr3F and D3B (Nunn et al. 1996). Subsequently, a semi-nested PCR amplification was performed using nmLSUCr3F and D2C (Scholin et al. 1994) and applying the same PCR profile. Ribosomal DNA genes are usually present within the telomeres of the 3 chromosomes of the nucleomorph (Douglas et al. 2001, Lane & Archibald 2006, Lane et al. 2006) and are therefore easily amplified from a single or a few cells. Prior to amplification, physical disruption was conducted using glass beads (Sigma-Aldrich) to ensure rupture of the *Mesodinium rubrum* cells within the PCR tubes (Frommlet & Iglesias-Rodríguez 2008). All PCR reactions were carried out on a PTC-200 Peltier Thermal Cycler (MJ Research).

To discriminate between possible copies of the nmLSU rDNA present in *Mesodinium rubrum* at different times during the experiment, all gene amplifications were cloned with the TOPO TA Cloning Kit (catalogue no. K4500-01) from Invitrogen®. Following plating, transformed clones were selected, and the nmLSU rDNA was amplified, as described above.

Purification and sequencing

All DNA PCR products were purified using Nucleofast, following the manufacturer's recommendations (Macherry-Nagel). PCR product (500 ng) was air-dried over night and sent to the sequencing service at Macrogen for determination in both directions using the same primers employed for amplification.

Alignments and phylogenetic analyses

nSSU rDNA alignment

To verify the phylogenetic position of the cryptophyte isolates within different marine clades and to confirm their morphological identification, a dataset with numerous cryptophyte taxa was analyzed, con-

sisting of nSSU rDNA sequences. The sequences were first aligned using MAFFT 6.624 (Katoh & Toh 2008) and then improved manually using BioEdit 7.0.5 sequence alignment software (Hall 1999). The dataset was composed of 5 new sequences and 46 sequences retrieved from GenBank. Four glaucophyte sequences were chosen as outgroup taxa based on previous phylogenetic studies of non-coding genes (Bhattacharya et al. 1995, Hoef-Emden 2008).

A Bayesian method was used to infer phylogeny, using the program MrBayes v.3.2 (Huelsenbeck & Ronquist 2001). Two simultaneous Monte Carlo Markov chains (MCMC; Yang & 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 39 000 trees. Modeltest (Posada & Crandall 1998), implemented in the PAUP* 4.0.b10 software (Swofford 2002), identified the GTR model as the best. Using these settings, a tree was reconstructed with the online version of the PhyML software (Guindon & Gascuel 2003; available on the Montpellier bioinformatics platform at www.atgc-montpellier.fr/phyml), and using the maximum likelihood (ML) method (Felsenstein 1981). The reliability of internal branches was assessed using the bootstrap method with 100 replicates (Felsenstein 1985).

nmLSU rDNA alignment

An alignment of the nmLSU rDNA sequences was completed to compare all clones from the cloning experiments with the sequences of the cryptophytes in GenBank (see accession numbers in Table 1). For the nSSU rDNA of cryptophyte alignment, the sequences were first aligned using MAFFT 6.624 (Katoh & Toh 2008) and then improved manually using BioEdit 7.0.5 sequence alignment software.

Transmission electron microscopy (TEM)

Most species of cryptophytes have chloroplasts in which thylakoids are grouped in pairs (Hill 1991). Exceptions occur within the genera *Teleaulax* and *Plagioselmis*, where the thylakoids are grouped in

triplets, as in *Mesodinium rubrum* (Hibberd 1977, Garcia-Cuetos et al. 2010). To investigate possible incorporation of chloroplasts from cryptophyte species not belonging to the TPG clade, a culture of *Mesodinium rubrum* fed *Hemiselmis tepida* was mixed 1:1 with 4% glutaraldehyde in 0.2 M cacodylate buffer at pH 7.4 and containing 0.4 M sucrose. After 1 h at 4°C, the cells were concentrated by centrifugation and rinsed twice in cold cacodylate buffer of decreasing sucrose content. Once rinsed, the material was post-fixed overnight in 2% osmium tetroxide in 0.2 M cacodylate buffer at pH 7.4 and 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 the dehydration was completed in 2 changes of absolute ethanol, 20 min in each change. Following 2 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 & 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), fitted with a Gatan 792 digital camera (Gatan).

RESULTS

Phylogeny of cryptophytes used in the present experiment

The nSSU rDNA alignment consisted of 1712 bp. The molecular phylogeny inferred from Bayesian analysis yielded the tree topology shown in Fig. 1. Glaucophytes rooted the tree, and the cryptophytes were divided into 5 clades. The first clade included 3 genera: *Hemiselmis*, *Chroomonas* and *Komma*. The

second clade comprised 4 genera: *Rhodomonas*, *Rhinomonas*, *Pyrenomonas* and *Storeatula*. The third clade was formed by 2 genera: *Hanusia* and *Guillardia*, while the fourth clade was composed of *Cryptomonas* only. Finally, the fifth clade included 3 genera: *Teleaulax*, *Plagioselmis* and *Geminigera*. Within this clade, the genus *Teleaulax* appeared to be polyphyletic. *Proteomonas sulcata* and *Falcomonas daucoides* took up isolated positions and did not belong to any of the 5 clades. Thus, the species used included species from 5 different clades (see Fig. 1).

Species	Growth rate (d ⁻¹)		
	Prey (mono)	Prey (mix)	<i>M. rubrum</i> (mix)
Expt 1			
<i>Teleaulax acuta</i>	0.66 ± 0.01	Below detection limit	0.39 ± 0.02
Expt 2			
<i>Teleaulax amphioxeia</i>	0.36 ± 0.03	Below detection limit	0.07 ± 0.02
<i>Heterocapsa rotundata</i>	1.19 ± 0.03	0.77 ± 0.03	0.08 ± 0.04
<i>Proteomonas sulcata</i>	0.91 ± 0.02	0.84 ± 0.01	0.11 ± 0.04
Expt 3			
<i>Chroomonas vectensis</i>	0.82 ± 0.03	0.60 ± 0.03	0.29 ± 0.05
<i>Guillardia theta</i>	1.00 ± 0.01	0.67 ± 0.01	0.37 ± 0.01
<i>Hanusia phi</i>	0.86 ± 0.03	0.49 ± 0.04	0.38 ± 0.02
<i>Hemiselmis tepida</i>	0.78 ± 0.01	0.33 ± 0.01	0.32 ± 0.01
<i>Rhodomonas salina</i>	1.16 ± 0.05	1.02 ± 0.06	0.28 ± 0.04

second clade comprised 4 genera: *Rhodomonas*, *Rhinomonas*, *Pyrenomonas* and *Storeatula*. The third clade was formed by 2 genera: *Hanusia* and *Guillardia*, while the fourth clade was composed of *Cryptomonas* only. Finally, the fifth clade included 3 genera: *Teleaulax*, *Plagioselmis* and *Geminigera*. Within this clade, the genus *Teleaulax* appeared to be polyphyletic. *Proteomonas sulcata* and *Falcomonas daucoides* took up isolated positions and did not belong to any of the 5 clades. Thus, the species used included species from 5 different clades (see Fig. 1).

Growth response of *Mesodinium rubrum* when fed *Teleaulax acuta* and *T. amphioxeia* as prey

In the experiments in which *Mesodinium rubrum* was fed *Teleaulax acuta* or *T. amphioxeia*, *M. rubrum* grew in the unfed control experiments during the first 4 to 8 d of the incubation (Figs. 2A, 3A & 4A, Table 2), but the growth rate leveled off. However, the control culture of *M. rubrum* used in the first set of experiments with *T. acuta* went through more cell divisions than those used in the 2 latter experiments (Figs. 3 & 4, Table 2), indicating a better feeding status of *M. rubrum* in the former culture prior to the experiments.

When *Mesodinium rubrum* was fed *Teleaulax acuta* or *T. amphioxeia* at a prey:predator ratio of 1:1,

the growth of *M. rubrum* was not significantly different from the unfed control during the first 8 or 4 d, respectively. During these periods, the prey was depleted below the detection limit (Figs. 2B & 3B).

When *M. rubrum* was re-fed *T. amphioxeia* at a prey predator ratio of 5:1 on Day 4, growth increased considerably during the subsequent 4 d of incubation (Fig. 3A), and the prey was again depleted below

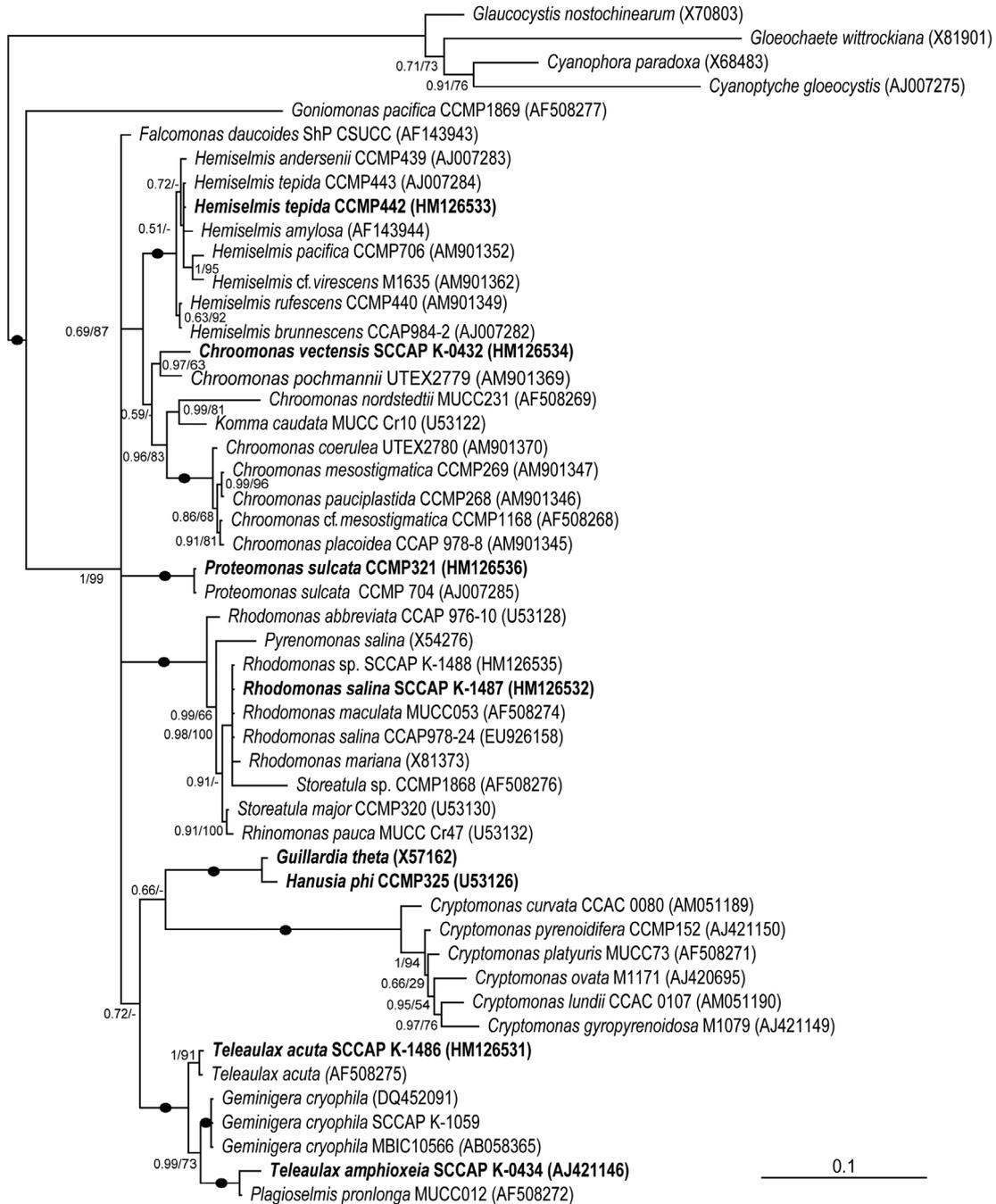


Fig. 1. Phylogeny of cryptophytes based on nuclear small subunit rDNA sequences (1712 bp) inferred from Bayesian analysis. *Glaucocystis nostochinearum*, *Gloeochaete wittrockiana*, *Cyanophora paradoxa* and *Cyanoptylche gloeocystis* were used as outgroups. Branch support was obtained from Bayesian posterior probabilities and bootstrap (100 replicates) in maximum likelihood (ML) analyses. GenBank accession numbers in parentheses. At the internodes, posterior probabilities (1) are written first, followed by bootstrap values (%) from ML. (•) Highest possible posterior probability (1.0) and bootstrap value (100%). Scale bar represents 10 substitutions per site. Species in bold face were used for the experiments in this study

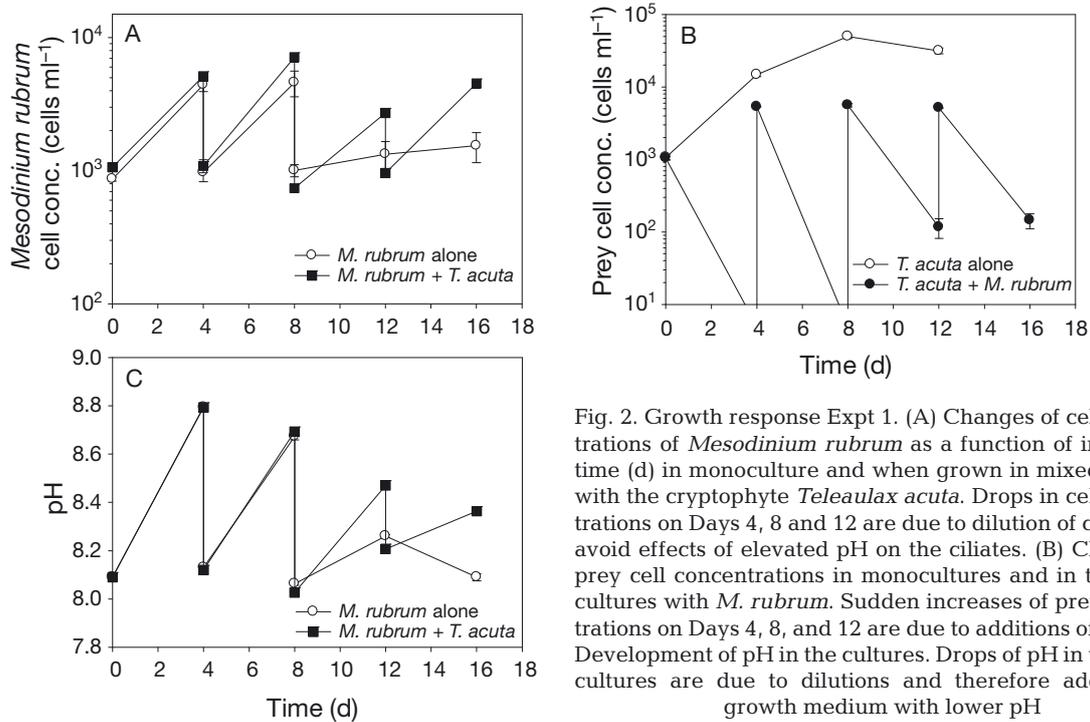


Fig. 2. Growth response Expt 1. (A) Changes of cell concentrations of *Mesodinium rubrum* as a function of incubation time (d) in monoculture and when grown in mixed cultures with the cryptophyte *Teleaulax acuta*. Drops in cell concentrations on Days 4, 8 and 12 are due to dilution of cultures to avoid effects of elevated pH on the ciliates. (B) Changes of prey cell concentrations in monocultures and in the mixed cultures with *M. rubrum*. Sudden increases of prey concentrations on Days 4, 8, and 12 are due to additions of prey. (C) Development of pH in the cultures. Drops of pH in the mixed cultures are due to dilutions and therefore additions of growth medium with lower pH

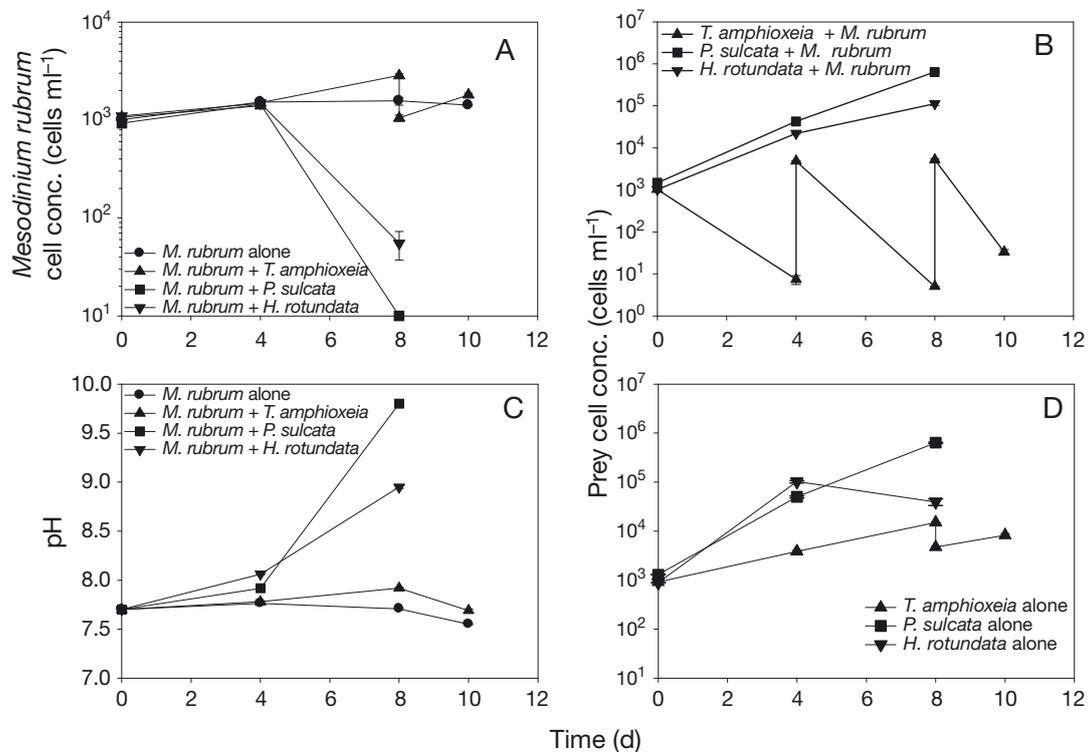


Fig. 3. Growth response Expt 2. (A) Changes of cell concentrations of *Mesodinium rubrum* as a function of incubation time (d) in monoculture and when grown in mixed cultures with the cryptophytes *Teleaulax amphioxeia* or *Proteomonas sulcata* or the dinoflagellate *Heterocapsa rotundata*. Drops in cell concentrations on Day 8 in the mixed culture of *M. rubrum* + *T. amphioxeia* are due to dilution. (B) Changes of prey cell concentrations in the mixed cultures. Sudden increases in prey concentrations on Days 4 and 8 are due to additions of prey. (C) Development of pH in the cultures. (D) Changes of prey cell concentrations in monocultures. Detection limit was ~10 cells ml⁻¹

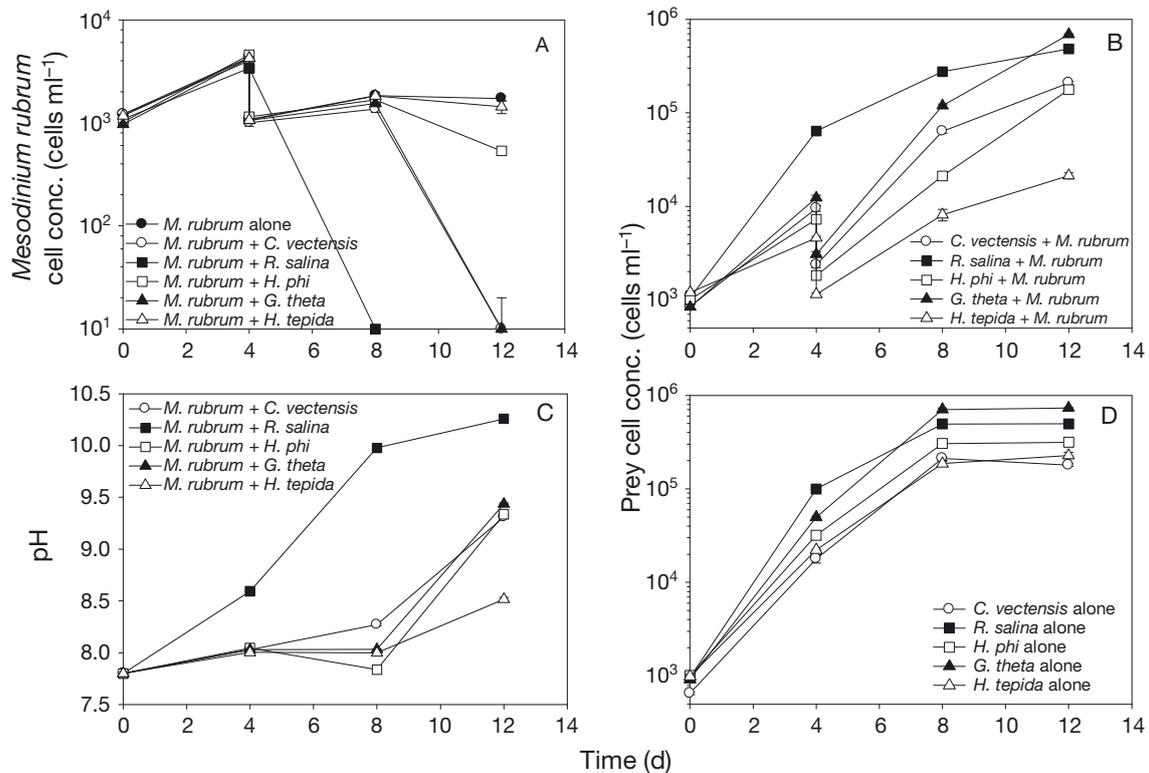


Fig. 4. Growth response experiment 3. (A) Changes of cell concentrations of *Mesodinium rubrum* as a function of incubation time (d) in monoculture and when grown in mixed cultures with the cryptophytes *Chroomonas vectensis*, *Rhodomonas salina*, *Hanusia phi*, *Guillardia theta* and *Hemiselmis tepida*. Drops in cell concentrations on Day 4 are due to dilution. (B) Changes of prey cell concentrations in the mixed cultures. Drops in prey cell concentrations on Day 4 are due to dilution of cultures. (C) Development of pH in the cultures. (D) Changes of prey cell concentrations in monocultures. Detection limit was ~ 10 cells ml⁻¹

detection limit (Fig. 3B). A second re-feeding of the *M. rubrum* on Day 8, in combination with a shortening of the incubation period to 2 d, resulted in a further increase in the *M. rubrum* growth rate (Fig. 3A). However, in both cases, the prey was not completely depleted on Day 10 in the experiments with *T. amphioxeia* or on Day 12 and 16 in the experiments with *T. acuta* (Figs. 2B & 3B). The growth of *T. acuta* and *T. amphioxeia* in monoculture was studied for comparison (Figs. 2B & 3D, Table 2).

Growth responses of *Mesodinium rubrum* when fed organisms not belonging to the TPG clade

Mesodinium rubrum could not sustain growth when fed the dinoflagellate *Heterocapsa rotundata* and the 5 non-TPG clade cryptophyte species tested (Figs. 3A & 4A). Initial growth of *M. rubrum* in the mixed cultures containing these prey types was observed for the first 4 to 8 d, but the growth rate never exceeded the growth of *M. rubrum* in monoculture. In some cases, *M. rubrum* died in the mixed cultures between

Day 4 and Day 12, depending on the prey species (Figs. 3A & 4A). The death of *M. rubrum* always coincided with an increase in pH above 9 (Figs. 3C & 4C).

In many cases, the prey items grew fast in both the algal monocultures and the mixed cultures (Figs. 3B,D & 4B,D). The pH increased above 9 after 8 to 12 d of incubation, and growth of the algae then decreased or stopped. A comparison of the growth response of the prey in the monocultures and in the mixed cultures with *Mesodinium rubrum* during the first 4 d of the incubation revealed that the prey concentrations in the mixed cultures were always lower than in the monocultures.

Sequestration of cryptophyte symbionts by *Mesodinium rubrum*

The nmLSU rDNA provided a very precise molecular signature for the plastids of the different cryptophyte species when symbiont replacement was tested (see estimated divergence between sequences in Table 3). The alignment consisted of

Table 3. Estimated evolutionary divergence (%) between nucleomorph large subunit rDNA sequences of cryptophytes

Species	<i>G. theta</i>	<i>H. phi</i>	<i>H. tepida</i>	<i>T. amphioxieia</i>	<i>T. acuta</i>
<i>Guillardia theta</i>	0.00				
<i>Hanusia phi</i>	9.7	0.00			
<i>Hemiselmis tepida</i>	31	30.5	0.00		
<i>Teleaulax amphioxieia</i>	31	29.4	23.9	0.00	
<i>Teleaulax acuta</i>	30	29.1	24.2	17.4	0.00

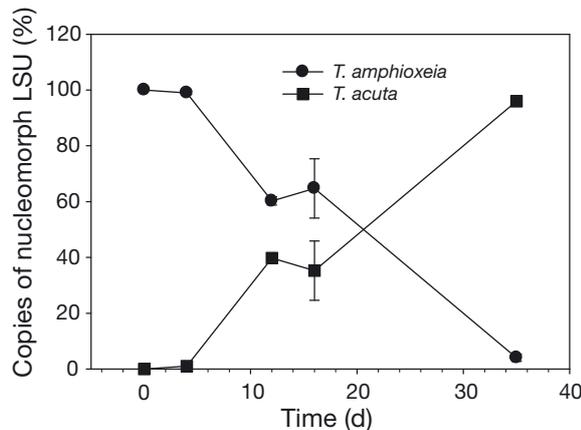


Fig. 5. *Mesodinium rubrum*. Symbiont exchange experiment. The percentage of symbiont nucleomorph large subunit (LSU) copies in an experiment in which prey was switched from *Teleaulax amphioxieia* to *T. acuta* at the start of the experiment. Values are treatment means \pm SE, $n = 4$, except for at Day 35, where $n = 3$

360 bp. When compared to the sequences retrieved from GenBank (Table 1), all of the sequences retrieved after cloning were identical to *Teleaulax amphioxieia* (GQ396273) except when *Mesodinium rubrum* was fed *T. acuta*. A total of 60 to 70 sequences were retrieved per cloning experiment replicate. When *M. rubrum* was fed *T. acuta*, the percentage of sequences belonging to *T. amphioxieia* retrieved from *M. rubrum* decreased, while that of *T. acuta* increased as the experiment went on (Fig. 5). However, the replacement rate appeared to be slow. After 15 to 18 d of exposure to *T. acuta*, only about half of the sequences retrieved from *M. rubrum* belonged to *T. acuta*. After 35 d of exposure to *T. acuta*, about 94% of the sequences retrieved from *M. rubrum* derived from *T. acuta*.

Transmission electron microscopy studies

A series of micrographs obtained from a culture of *Mesodinium rubrum* fed the cryptomonad *Hemi-*

selmis tepida are shown in Fig. 6. There are several chloroplasts in the cell (Fig. 6A), located in both the oral and the aboral end of the cell. Collectively, we designate these as chromatophores because their origins differ (see below). *Hemiselmis* is ingested at the oral end of *Mesodinium*, which here possesses, in addition to a small cytostome, bands of microtubules and a number of tentacles

(Fig. 6B). *Hemiselmis* cells are ingested whole (Fig. 6C,D), and Fig. 6C illustrates a cell in which the cryptomonad nuclei, the cryptomonad chloroplasts and the 2 types of trichocysts (ejectosomes) of *Hemiselmis* are visible. One of the 2 macronuclei of *Mesodinium* is visible in Fig. 6A, while the small nucleus in the upper part of the cell is from a prey cell. *Mesodinium* has 2 types of chromatophores (Fig. 6E,F). In one type, the permanent chromatophore or chloroplast, the thylakoids are grouped in lamellae of 3 thylakoids (Fig. 6E). For comparison, the chloroplasts from *Hemiselmis* located within *Mesodinium* showed the 2-thylakoid lamellae characteristic of *Hemiselmis* (Fig. 6F). In contrast to the 3-thylakoid lamellae, the 2-thylakoid lamellae of *Hemiselmis* were often not well preserved in the sections, indicating that they were being subjected to digestion enzymes.

DISCUSSION

Growth responses of *Mesodinium rubrum* when fed different types of prey

The present results suggest that the Danish strain of *Mesodinium rubrum* could only sustain growth for at least 12 d when supplied with *Teleaulax acuta* and *T. amphioxieia* as prey. Park et al. (2007) studied the growth response of starved *M. rubrum* (Korean strain MR-MAL01) when offered 4 cryptophytes belonging to the TPG clade and 2 belonging to the 'Rhodomonas clade' (CR-MAL03 and CR-MAL06). Except for CR-MAL06, positive growth rates were observed during the incubation time studied (6 d), and all rates were significantly different from the unfed control. The results indicate that MR-MAL01 can grow on species not closely related to *Teleaulax*, although the observed growth rate on the CR-MAL03 strain was low ($\mu = 0.16 \text{ d}^{-1}$). This observation was confirmed by Myung et al. (2011), who found low growth rates of the same strain of *M. rubrum* when starved cells

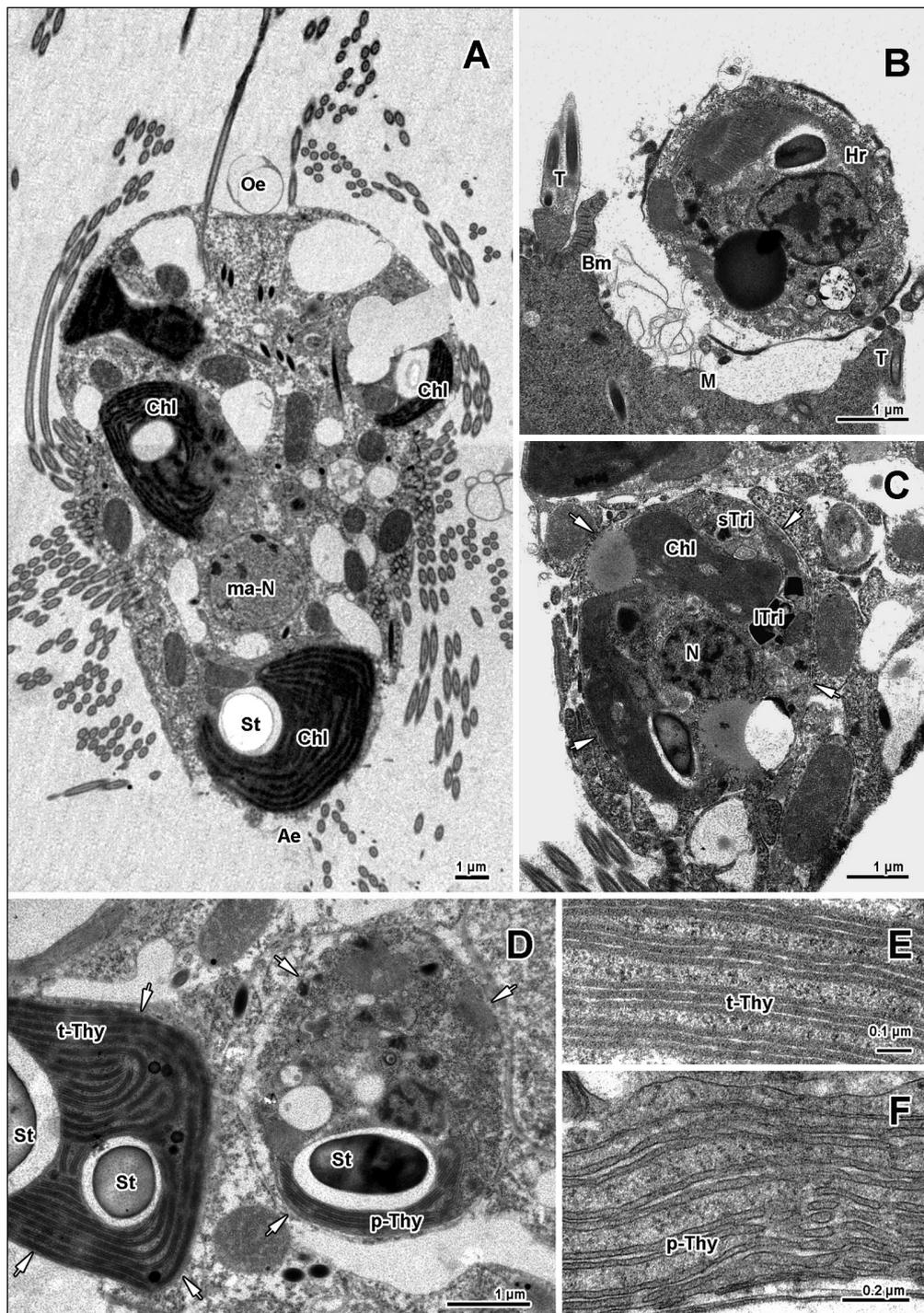


Fig. 6. *Mesodinium rubrum* grown in culture and fed *Hemiselmis tepida*. (A) Longitudinal section of *M. rubrum* from the oral end (Oe) to the aboral end (Ae) illustrating the cilia, chloroplasts (Chl), starch grains (St), macronucleus (ma-N) and the bands of microtubules around the mouth. (B) The mouth (M) of *M. rubrum* using the tentacles (T) to engulf a cell of *H. tepida* (Hr) and showing some of the bands of microtubules (Bm). (C) Engulfed cell of *H. tepida* (arrows) within *M. rubrum*. Visible organelles are the chloroplast, the small (sTri) and large trichocysts (lTri) and the cryptomonad nucleus (N). (D) Chloroplast of *M. rubrum* (arrowheads) with triplets of thylakoids (t-Thy) next to an engulfed cell of *H. tepida* (arrows) with chloroplast showing paired thylakoids (p-Thy). (E) Detail of the chloroplast of *M. rubrum* with thylakoids in triplets. (F) Detail of the chloroplast of *H. tepida*, with paired thylakoids

were fed CR-MAL03 over a longer period (10 d). Ingestion rates were not provided, but the data presented by Myung et al. (2011) indicate ingestion. *M. rubrum* was not able to control the prey populations in any of these studies, which is in agreement with our present observations.

The Danish isolate of *Mesodinium rubrum* was unable to grow on cryptophytes outside the TPG clade. However, the strain of *Rhodomonas salina* presently used as food in our studies is not identical to CR-MAL03 or CR-MAL06, and thus the possibility that the Danish *M. rubrum* strain can grow on another *Rhodomonas* species cannot be entirely ruled out.

However, no matter the strain of *Mesodinium rubrum*, it appears that cryptophytes within the TPG clade support the growth of *M. rubrum* significantly better than cryptophytes of other clades. Presently, all cultures are maintained on cryptophytes from this clade (e.g. Gustafson et al. 2000, Johnson & Stoecker 2005, Johnson et al. 2006, Park et al. 2007, the present study; Table 4). One possible explanation could be that *M. rubrum* relies on regular symbiont sequestration for sustained growth and that species within the TPG clade function best as symbionts.

A comparison of the growth response of the prey in monocultures and in the mixed cultures with *Mesodinium rubrum* during the first 4 d of the incubation revealed that prey concentrations in the mixed cultures were always lower than in the monocultures, indicating that *M. rubrum* ingested all types of the offered prey items (Table 2). However, the design of the growth experiments did not allow for estimations of ingestion rates due to the low sampling frequency (typically every 4 d) and the large change in prey

populations, which allow many types of possible interactions (e.g. nutrient limitation, allelopathy, etc.) between the prey and *M. rubrum*.

Does *Mesodinium rubrum* sequester and replace chloroplasts via prey ingestion?

Using the nmLSU rDNA gene, we were able to demonstrate a gradual shift from the *Teleaulax amphioxeia* to the *T. acuta* chloroplast type when the diet of *Mesodinium rubrum* was changed from *T. amphioxeia* to *T. acuta*, proving that replacement of chloroplasts took place. The replacement of the chloroplasts was relatively slow (>35 d for full replacement), indicating that only 'low performance' chloroplasts are renewed and that division of the chloroplast occurs within the cell. These results agree with the preliminary data published by Park et al. (2010) and provide for the first time a symbiont renewal time frame. In a previous study, the Danish strain of *M. rubrum* was believed to harbor a permanent cryptophyte endosymbiont due to the ability of the ciliate to duplicate its chloroplasts and the symbiont nucleus when starved of prey (Hansen & Fenchel 2006). The present results clearly show that the Danish strains of *M. rubrum* acquire symbiont chloroplasts from prey via ingestion, just like the Korean and Antarctic strains of *M. rubrum*.

Although *Mesodinium rubrum* cultures could not sustain growth in the long run, when fed *Hanusia phi* and *Hemiselmis tepida*, the cultures did survive for at least 12 d, which is sufficient time for chloroplast replacement to occur, based on the data collected from the experiment carried out with *Teleaulax acuta* and *T. amphioxeia*. The molecular experiment used to detect the change of plastids was therefore repeated for *H. phi* and *H. tepida*. However, we found no evidence of plastid replacement. To ensure that no plastids were present in low concentrations in the ciliate or missed due to primer bias, TEM sections of *M. rubrum* exposed to *H. tepida* were studied in detail. Prey cells taken up by *M. rubrum* were located in food vacuoles and often partly digested. In contrast to the normal endosymbionts of *M. rubrum*, which comprised plastids and mitochondria of cryptophyte origin in addition to a symbiont nucleus, only whole cells of *H. tepida* were

Table 4. List of *Mesodinium rubrum* cultures and prey that the culture can be maintained on

Place of origin of culture	Prey	Source
Antarctica	<i>Geminigera cryophila</i>	Gustafson et al. (2000), Johnson & Stoecker (2005), Johnson et al. (2006, 2007), Hackett et al. (2009)
Denmark	<i>Teleaulax</i> sp.	Hansen & Fenchel (2006), Smith & Hansen (2007)
	<i>Teleaulax amphioxeia</i>	Riisgaard & Hansen (2009)
	<i>T. acuta</i> and <i>T. amphioxeia</i>	Present study
Korea	<i>Teleaulax</i> spp., unidentified strains of the TPG clade	Yih et al. (2004), Park et al. (2006, 2007, 2010)
Japan	<i>Teleaulax amphioxeia</i>	Nagai et al. 2008, Nishitani et al. (2008a,b)

observed. Thus, we found no proof of chloroplast sequestration by *M. rubrum* when fed *H. tepida*.

Recently, the Korean isolate of *Mesodinium rubrum* was shown to contain plastids from 2 different clades. Cultures originally grown on a *Teleaulax* species (CR-MAL01) were exposed to a *Rhodomonas* species (CR-MAL03) for 2 d, after which the prey was removed. No complete exchange from the CR-MAL01 to the CR-MAL03 genetic marker was detected, but signals from both markers were maintained for at least 14 d without prey, suggesting that the chloroplasts were actually sequestered (Myung et al. 2011). The lack of a complete chloroplast turnover may perhaps be explained by the short exposure to the new cryptophyte prey. Long-term growth of *M. rubrum* on CR-MAL03 may be necessary to fully exchange chloroplasts, as observed in the present study. It therefore remains unclear whether the Korean or other isolates of *M. rubrum* can in the long term grow on cryptophytes not belonging to the TPG clade and completely exchange its symbionts.

Johnson et al. (2007) found evidence that the Antarctic strain of *Mesodinium rubrum* is able to sequester not only chloroplasts from its prey but also cryptophyte nuclei. Nuclei were retained for up to 30 d in this strain of *M. rubrum*, and the nuclei were transcriptionally active and apparently able to service plastids derived from multiple cryptophyte cells. Johnson et al. (2007) suggested that the symbiont nucleus of *M. rubrum* derived from ingested prey is necessary to control the activity of the plastids and their duplication. However, whether the symbiont nucleus regulates plastid activity is still unknown. To what extent the Danish strains of *M. rubrum* depends upon sequestration of nuclear material from its prey is currently unknown. The fate of the symbiont nuclei within *M. rubrum*, when prey is switched from one prey species to another, is likewise unknown. Therefore, future experiments should address the exact role of the symbiont nucleus and how the ciliates control the functioning and division of chloroplasts from different prey species.

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