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Sequestered plastids in *Mesodinium rubrum* are functionally active up to 80 days of phototrophic growth without cryptomonad prey

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ABSTRACT

The red tide ciliate *Mesodinium rubrum* is an obligate mixotroph which requires feeding on cryptomonad prey mainly to retain its photosynthetic apparatus. Functionality of the sequestered plastids has been known to be lowered within a few weeks. The upper limit of the functionally active duration for the newly retained plastid, however, has been rarely estimated or determined. In parallel with genetic analysis, we investigated dynamics of population density, orange fluorescence of the plastids, and DCMU ((3-(3,4-dichlorophenyl)-1,1-dimethylurea) photosynthetic capacity of phototrophically growing *M. rubrum* (strain MR-MAL01) for 100 days. *M. rubrum* populations continued their phototrophic growth for the first 6 weeks, with gradually decreasing growth rates. Rapid decline of population density began from the 8th week. The photosynthetic capacity remained quite stable, ranging from 0.7 during the 1st week down to 0.5 during the 11th week. On day 87, the photosynthetic capacity steeply decreased to 0.05. The orange fluorescence of the retained plastids became very weak during the 4th week, to be almost undetectable on day 98. Only plastid 16S rRNA gene kept strong band intensity of PCR products throughout the whole period of 100 day experiment. Interestingly, the band intensities from *psa*A and *psb*A genes all become dramatically weakened after day 77. After new prey cryptomonads (strain CR-MAL03) were offered to *M. rubrum* starved for 80 days, 'CR-MAL03 type' 1192-bp PCR product of plastid 16S rRNA gene was detected in most experimental single *M. rubrum* cells. Here, we demonstrate that *M. rubrum* can grow for ~6 weeks in the absence of cryptomonad prey, and photosynthetic capacity of *M. rubrum* can be maintained active for ~11 weeks without prey. Additionally, *M. rubrum* starved for 80 days was shown to be physiologically healthy enough to ingest cryptomonad preys and retain new plastids.

1. Introduction

Mesodinium rubrum Lohmann, 1908 is a mixotrophic marine photosynthetic ciliate (Crawford, 1989; Ryther, 1967; Sieburth et al., 1978), and frequently causes non-toxic red-tide in estuarine and neritic pelagic environments (Lindholm, 1985; Taylor et al., 1971; Hart, 1934). *M. rubrum* requires repeated ingestion of cryptomonad preys (Yih et al., 2004; Gustafson et al., 2000) through the cytopharynx (Nam et al., 2012) mainly for the renewing retention of photosynthetic apparatus (Hansen et al., 2012; Johnson et al., 2006, 2007; Johnson and Stoecker, 2005). Sequestered plastids of *M. rubrum* are known to become functionally less efficient within a few weeks after the initial introduction into the ciliate (Myung et al., 2011; Stoecker et al., 2009; Gustafson et al., 2000). *M. rubrum* MR-MAL01 grown phototrophically for 2 months without cryptomonad prey, and then quickly resumed its fast growth when re-fed with cryptomonad strain CR-MAL01 (Myung et al., 2011).

Maximum duration for a newly retained plastid to stay photosynthetically functional, however, has been rarely estimated or determined. Dimension of the maximum duration might be critical for a *Mesodinium rubrum* population to survive long-term starvation from its cryptomonad prey (Bielewicz et al., 2011; Laybourn-Parry, 2002). Months-long functioning of the new kleptoplastids in *M. rubrum* should lead us to better understand its overwintering success or long-term survival (Yih et al., 2013; Martin et al., 2007; Laybourn-Parry et al., 2000) under extreme environments even though the formation of cyst or resting stage in *M. rubrum* has never been known (Gibson et al., 1997; Lindholm, 1985).

Here, we report on the dynamics of population density, orange fluorescence (Glazer et al., 1971) of the retained plastids (Parsons and Blackbourn, 1968), and DCMU photosynthetic capacity (Furuya and William, 1992) of phototrophically growing

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Table 1
Primers used for the PCR amplification.

Name of primer	Target gene	Sequence (5′ → 3′)	Reference
cyb101	Plastid 16S rRNA gene	GARGRGCTCGCGTCTGA	Janson (2004)
bac2		ACCTTGTTACGACTTCAC	Janson (2004)
bAf3	*psb*A gene	ATCTTCGCTCCACCAGTTGAYATHGAYGG	Zhang et al. (2000)
bAr1		GTTGTGAGCGTTACGTTCRTGCATNACYTC	Zhang et al. (2000)
psaA130F	*psa*A gene	AACWACWACTTGGATTTGGAA	Yoon et al. (2002)
psaA1600R		GCATGAATATGRTGWACCAT	Yoon et al. (2002)
cyb101	Plastid type of CR-MAL01	GARGRGCTCGCGTCTGA	Janson (2004)
g3		GCACTCTTCCCTTTCAGG	Minnhagen and Janson (2006)
cyb101	plastid type of CR-MAL03	GARGRGCTCGCGTCTGA	Janson (2004)
g7		CGACTTTGGGTGTGGCTA	Myung et al. (2011)

Mesodinium rubrum (strain MR-MAL01) for 100 days without cryptomonad prey. Simultaneously, we also analyzed the band intensity of PCR products of plastid genes such as 16S rRNA, *psa*A, and *psb*A during the starvation period. Although its population growth, orange fluorescence of the retained plastids, and DCMU photosynthetic capacity all collapsed on day 80 of the phototrophic growth, *M. rubrum* seemed to still be able to ingest cryptomonad preys having different type of plastid and retain new plastids.

2. Materials and methods

2.1. Clonal cultures of Mesodinium rubrum and cryptomoads

Clonal cultures of *Mesodinium rubrum* MR-MAL01 (small subunit (SSU) rRNA gene sequence deposited in GenBank under the accession no. EF195734) and two cryptomoads, strain CR-MAL01 (SSU rRNA gene sequence deposited in GenBank under the accession no. EF195735) and CR-MAL03 (SSU rRNA gene sequence deposited in GenBank under accession no. EF195737) were all isolated from Gomso Bay, Korea (Yih et al., 2004; Park et al., 2007). Single cell isolation and following procedures to establish the above 3 cultures are described elsewhere (Myung et al., 2011). *M. rubrum* and the two cryptomonad cultures were maintained at 15 °C and 30 psu in enriched f/2-Si seawater medium (Guillard and Ryther, 1962). Continuous illumination of 25 and 60 μE m^{-2} s^{-1} by cool-white fluorescent lamps were applied to the cultures of the two cryptomonads and *M. rubrum*, respectively. Every 5–6 days the ratio of cryptomonads to *M. rubrum* was readjusted to 5:1 (Myung et al., 2011).

2.2. Long-term starvation experiment

Stock cultures of *Mesodinium rubrum* were grown mixotrophically with cryptomonad prey strain CR-MAL01 for 3 days, and then the well-fed cultures were gravity-filtered through 11 μm Nitex mesh (Sefar, Rüschlikon, Switzerland) to filter out prey cells. The subsamples were diluted with enriched f/2-Si media, and subsequently transferred to four 500 ml polycarbonate bottles. The initial volume and concentration of ciliate culture in each bottle were adjusted to 450 ml and 500 ciliate ml^{-1}, respectively. All cultures were incubated for 100 days under continuous illumination of 60 μE m^{-2} s^{-1}, 15 °C and 30 psu in enriched f/2-Si seawater media. Each of the 4 phototrophically growing experimental cultures of *M. rubrum* was concentrated by gravity filtration and serially transferred to new 500 ml polycarbonate (PC) bottles with fresh f/2-Si seawater media on days 21 and 38. Subsamples were collected at 3–4 day intervals over 100 days to monitor the population growth, photosynthetic capacity, plastid 16S rRNA gene, *psa*A gene, *psb*A gene, and the two plastid genotypes of *M. rubrum*, g3 type and g7 type which represent partial plastid 16S rRNA genes of cryptomonad strains CR-MAL01 and CR-MAL03, respectively. The cryptomonad strain CR-MAL03 was offered to *M. rubrum* starved for 80 days, and then single cell PCR products amplified from *M. rubrum* on day 14 after the re-feeding were examined to see whether they exhibited either the g3 type or g7 type of cryptomonad genes on the 1.5% (w/v) agarose gel (Table 1; Myung et al., 2011).

To estimate the concentrations of *Mesodinium rubrum* and cryptomonad culture, a 3 ml aliquot was subsampled from each bottle to fix with 5% acid Lugol's solution. The abundances of *M. rubrum* and cryptomonad were determined by counting cells in 1 ml Sedgewick-Rafter Chambers (SRCs) under a light microscope (Olympus BH-2). The specific growth rate of each culture was calculated by averaging the daily growth rates (DGR) using:

$$\mathrm{DGR} = \frac{\ln(St2/St1)}{(t2 - t1)} \quad (1)$$

where $t_2 - t_1 = 1$ day, and St_2 and St_1 are cell concentrations in consecutive subsamples.

As described below, photosynthetic capacity of *Mesodinium rubrum* was measured by DCMU method (Fukazawa et al., 1980; Furuya and William, 1992).

2.3. DNA extraction and PCR amplification of plastid genes

2.3.1. PCR amplification of plastid genes from filter-washed samples

Nucleic acids from *Mesodinium rubrum* cultures were prepared using the AccuPrep® Genomic DNA Extraction Kit (Bioneer, Seoul, Korea) according to the supplied protocol. To extract DNA, each 3 ml subsample of *M. rubrum* was washed with 200 ml sterile seawater using the 12 μm Isopore membrane filters (47 mm in diameter, Millipore Corp., Carrigtwohill, Ireland). After each washing, we confirmed no contamination by cryptomonads in the washed subsample using a primer set cyb 101-g1 (amplicon size: 424-bp, Minnhagen and Janson, 2006): no PCR product of g1 type, which indicates the existence of strain CR-MAL01, was detectable in the PCR amplification on the re-washed mixture of the once washed subsample and introduced prey strain CR-MAL01. The membrane filter with only *M. rubrum* was put into 1.5 ml micro-centrifuge tube for the DNA Extraction Kit. Plastid 16S rRNA gene sequence of cryptomonad strain CR-MAL03 and crytomonad strain CR-MAL01 were available on Myung et al. (2011) and Park et al. (2008), respectively. Two diagnostic primer sets, cyb101-g3 (amplicon size: 802-bp, Minnhagen and Janson, 2006) and cyb101-g7 (amplicon size: 1192-bp, Myung et al., 2011), were specifically used to amplify plastid 16S rRNA genes of cryptomonad strains CR-MAL01 and CR-MAL03, respectively (Table 1). Amplification of the plastid genes was also performed using standard PCR protocols with primers cyb101 and bac2 (Janson, 2004), primers bAf3 and bAr1 (Zhang et al., 2000), and primers psaA130F and psaA1600R

(Yoon et al., 2002) for plastid 16S rRNA, *psb*A, and *psa*A genes, respectively (Table 1).

The reaction mixture contained 50–100 ng of DNA, 0.2 mM deoxynucleoside triphosphate, and each primer at a concentration of 0.4 μM, 10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$, 40 mM KCl, and 2.5 U *Taq* DNA polymerase (Bioneer, Seoul, Korea). PCR amplification for plastid 16S rRNA gene was performed according to the following protocol: an initial denaturation step (10 min, 94 °C) was followed by 35 cycles consisting of denaturation (45 s, 94 °C), annealing (1 min, 50 °C), and extension (2 min, 72 °C), with a final 7 min extension step at 72 °C. PCR amplification for *psb*A gene, which encode the D1 polypeptide of the photosystem II (PS II) reaction center complex, was performed according to the following protocol: an initial denaturation step (5 min, 94 °C) was followed by 40 cycles consisting of denaturation (30 s, 94 °C), annealing (30 s, 55 °C), and extension (2 min, 72 °C), with a final 10 min extension step at 72 °C. PCR amplification for *psa*A gene, which encode the P-700 chlorophyll a-apoproteins of Photosystem I (PS), was performed according to the following protocol: an initial denaturation step (5 min, 94 °C) was followed by 40 cycles consisting of denaturation (1 min, 94 °C), annealing (30 s, 50 °C), and extension (1 m 15 s, 72 °C), with a final 10 min extension step at 72 °C. PCR amplification for each of g3 type and g7 type was performed according to the following protocol: an initial denaturation step (4 min, 95 °C) was followed by 35 cycles consisting of denaturation (30 s, 95 °C), annealing (30 s, 60.4 °C), and extension (1 min, 65 °C) with a final 3 min extension step at 65 °C. Amplification of both g3 type and g7 type in a *Mesodinium rubrum* culture was performed using a multiplex PCR with primers cyb101, g3, and g7 at once (Table 1). The size of the PCR products was confirmed by 1.5% (w/v) agarose gel electrophoresis. Photographs of the gel were taken using a Polaroid camera (GelCam, Polaroid).

2.3.2. Single cell PCR amplification of plastid genes of re-fed M. rubrum once starved for 80 days

Single cell PCR method was applied to *Mesodinium rubrum* cells that had been grown phototrophically for 80 days, re-fed with the cryptomonad strain CR-MAL03 for 2 days, and then finally grown phototrophically (free of cryptomonad prey) for 12 days. For single cell PCR, each of the 5 single cells of *M. rubrum* was isolated using a capillary pipette under a light microscope (Olympus BH-2), and placed into a separate 0.2 ml PCR microtube. Each microtube was then used for multiplex PCR amplification using primers cyb101, g3, and g7 simultaneously (Table 1) as described in the above.

2.4. DCMU photosynthetic capacity

Photosynthetic capacity of *Mesodinium rubrum* was measured according to the DCMU (3-3,4-dichlorophenyl)-1,1-dimethylurea) method (Samuelsson and Oquist, 1977; Roy and Legendre, 1979; Fukazawa et al., 1980; Furuya and William, 1992).

Maximum quantum efficiency of photosystem II (Parkhill et al., 2001) was estimated from in vivo fluorescence of control (F_0) and DCMU-treated (F_{DCMU}) samples. Five milliliters (or 10 ml) aliquot of control samples was incubated in the dark for 30 min prior to the measurement of the control fluorescence (F_0) using a fluorometer (Turner Designs 10-AU fluorometer, Sunnyvale, California, USA). Then, DCMU was treated (final concentration of 10 μM) to measure the maximum fluorescence (F_m). The following formula was used for the calculation of DCMU photosynthetic capacity, i.e. the photosynthetic capacity estimated by DCMU-enhanced fluorescence (Furuya and William, 1992):

$$\text{DCMU photosynthetic capacity} = \frac{F_m - F_0}{F_m}$$

where F_0 and F_m are averaged steady-state fluorescence intensities before and after addition of DCMU, respectively.

3. Results and discussion

3.1. Phototrophic growth and plastid degeneration of M. rubrum during the 100-day long starvation

Phototrophic growth rate of once well-fed *Mesodinium rubrum* strain MR-MAL01 was 0.28 ± 0.02 day^{-1} (n = 4) between days 4 and 11, and maximum abundance of was 28,400 cells ml^{-1} in the experimental bottle #2 on day 18 (Fig. 1). Following the two serial transfers to enriched f/2-Si seawater media on days 21 and 38, the four *M. rubrum* cultures exhibited very slow growth (0.08 ± 0.01 day^{-1} between days 21 and 28 and 0.07 ± 0.01 day^{-1} between days 39 and 42; Fig. 1). Population densities of the experimental *M. rubrum* cultures declined sharply from d 56 to the end of the experiment (Fig. 1). Plastids of the well-fed *M. rubrum* cells on day 1 were reddish-brown and bright yellow-orange under light and epifluorescence microscopes, respectively (Fig. 2). On day 98, however, the bright yellow-orange epifluorescence was almost invisible to the naked eyes, while discrete pale-green plastids were evident under a light microscope (Fig. 2). The bright yellow-orange fluorescence of the phototrophically growing *M. rubrum* was maintained during the first 2 weeks; thereafter, gradually weakened, and finally became very much darkened after 4 weeks (Fig. 3). The yellow-orange fluorescence of *M. rubrum* cells during the 8th week (Fig. 3), however, was still better discernible than that on the 14th week (Fig. 2).

3.2. DCMU photosynthetic capacity of M. rubrum during the 100-day long starvation

The maximum quantum yield of photosystem II, (F_v/F_m), measured by the DCMU addition method (Parkhill et al., 2001) was quite stable (~0.6) up to the 11th week. Within 10 days thereafter, the photosynthetic capacity (F_v/F_m) rapidly declined to less than 10% of the previous measurements (0.05), and was close to zero on day 98 (Fig. 4). Such a rapid decrease of the DCMU photosynthetic capacity of the *Mesodinium rubrum* population (from day 80, Fig. 4) occurred far after the drastic decline in population density (from day 56, Fig. 1), which in turn is quite behind the deep-darkening of yellow-orange fluorescence (from day 28, Fig. 3) of the phototrophically growing *M. rubrum*. Although the phototrophic growth rate of *M. rubrum* is already negative, the plastids of the remaining *M. rubrum* population seemed to be still photosynthetically active (i.e. 0.6 of F_v/F_m), indicating that photosynthetic activity did not directly result in the growth of the starved *M. rubrum*.

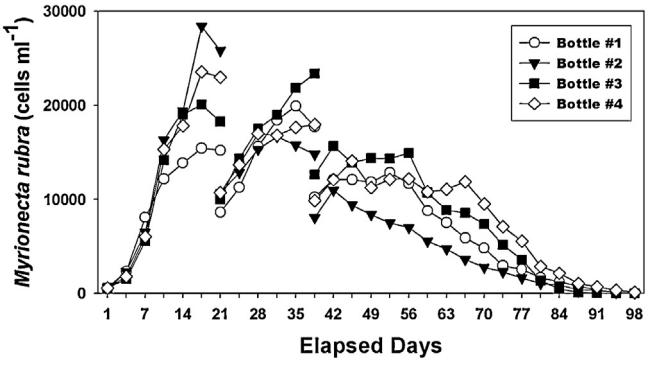

Fig. 1. Changes in cell concentration of *Mesodinium rubrum* for 100 days without addition of cryptophyte prey. (○) *M. rubrum* in bottle #1, (▼) *M. rubrum* in bottle #2, (■) *M. rubrum* bottle #3, (□) *M. rubrum* in bottle #4. Arrows indicate time of the two serial transfers.

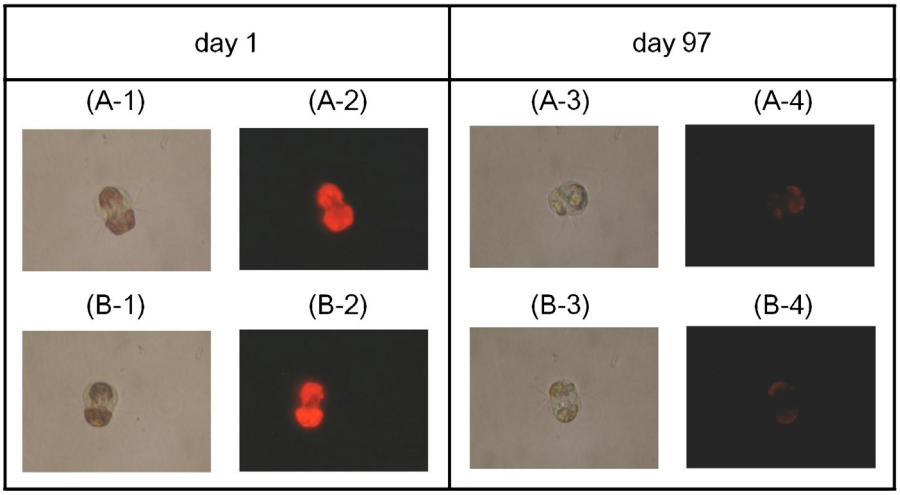

Fig. 2. Images of live *Mesodinium rubrum* cells on days 1 and 97 of phototrophic growth under a light microscope (A-1, B-1, A-3, B-3) and an epifluorescence microscope (A-2, B-2, A-4, B-4).

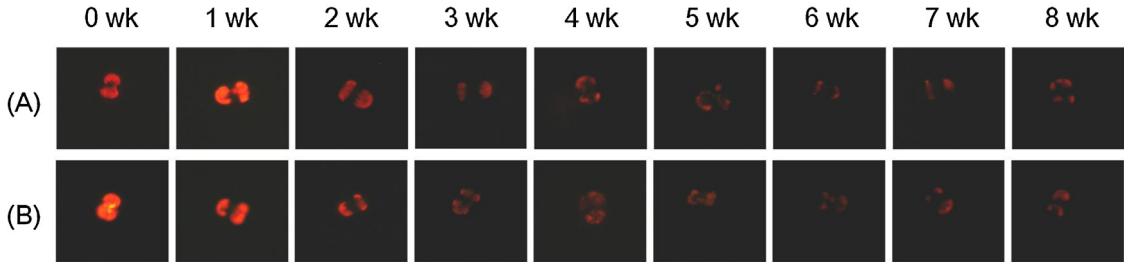

Fig. 3. Epifluorescence microscope images of live *Mesodinium rubrum* cells taken one week interval during a long-term phototrophic growth. (A) *M. rubrum* in bottle #1 and (B) *M. rubrum* in bottle #4 (Fig. 1).

3.2.1. Differential loss rate among the 3 kinds of M. rubrum *plastid genes during the 100-day long starvation*

During the 100-day long phototrophic growth of once well-fed *Mesodinium rubrum* strain MR-MAL01 with its cryptomonad prey strain CR-MAL01, the PCR products of the 3 kinds of plastid gene (i.e. 16S rRNA, *psa*A and *psb*A) were not equivocal in their time-course variation. The PCR product of plastid 16S rRNA gene amplified from each subculture of 2000 *M. rubrum* cells using the primer cyb101 and bac2 (amplicon size: 1258-bp) was quite consistent without any significant weekly variation in their band intensity on agarose gel throughout the whole period of the 100 day experiment (Fig. 5A). After day 77, however, band intensity of the PCR product of partial 16S rRNA gene (amplicon size: 802-bp) gradually decreased using the primers cyb101 and g3 (Fig. 5B). Therefore, based on the result from distinguishable band intensity pattern of the same plastid 16S rRNA gene one can conclude that specificity and affinity of the two primers are clearly different. The PCR products of the other 2 plastid genes, i.e. *psa*A and *psb*A, exhibited drastically reduced band intensities on agarose gel simultaneously after 77 days (Fig. 5B–D). This result shows a very similar pattern to the same photosynthesis-related genes of >2-month starved *Dinophysis caudata* that can feed on *M. rubrum* (Park et al., 2008). It is possible that the difference in amount of PCR products from the photosynthesis-related genes may have resulted from different copy numbers and locations in *M. rubrum* (Douglas and Penny, 1999; Park et al., 2008). Both the DCMU photosynthetic capacity (Fig. 4) and the band intensity of the PCR products of *psa*A and *psb*A gene (Fig. 5) quickly declined after 80 days of starvation. In addition, the maximum duration for a newly retained plastid of *M. rubrum* MR-MAL01 to remain photosynthetically functional might not be longer than 84 days. Without any further ingestion of cryptomonad prey after 80 days starvation, the remaining population should become photosynthetically mal-functional, and vanish finally.

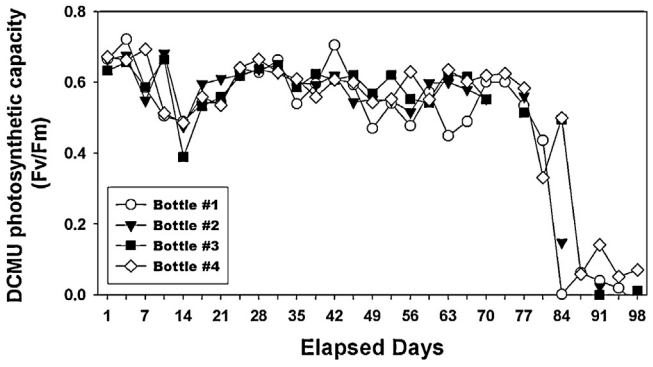

Fig. 4. Photosynthetic capacity (F_v/F_m) of *Mesodinium rubrum* during 100 days of phototrophic growth without added cryptophyte prey. (○) photosynthetic capacity of *M. rubrum* in the experimental bottle #1, (▼) in the experimental bottle #2, (■) in the experimental bottle #3, (□) in the experimental bottle #4.

The present study revealed synchronous collapse of photosynthetic signatures from plastid genes in *Mesodinium rubrum* after 80 days of starvation. All plastids examined so far, however, have small genomes that encode only a handful of proteins involved in housekeeping functions and photosynthesis (Koning and Keeling, 2004). To better estimate the time-course photosynthetic functionality of the retained plastids, we may need to analyze the

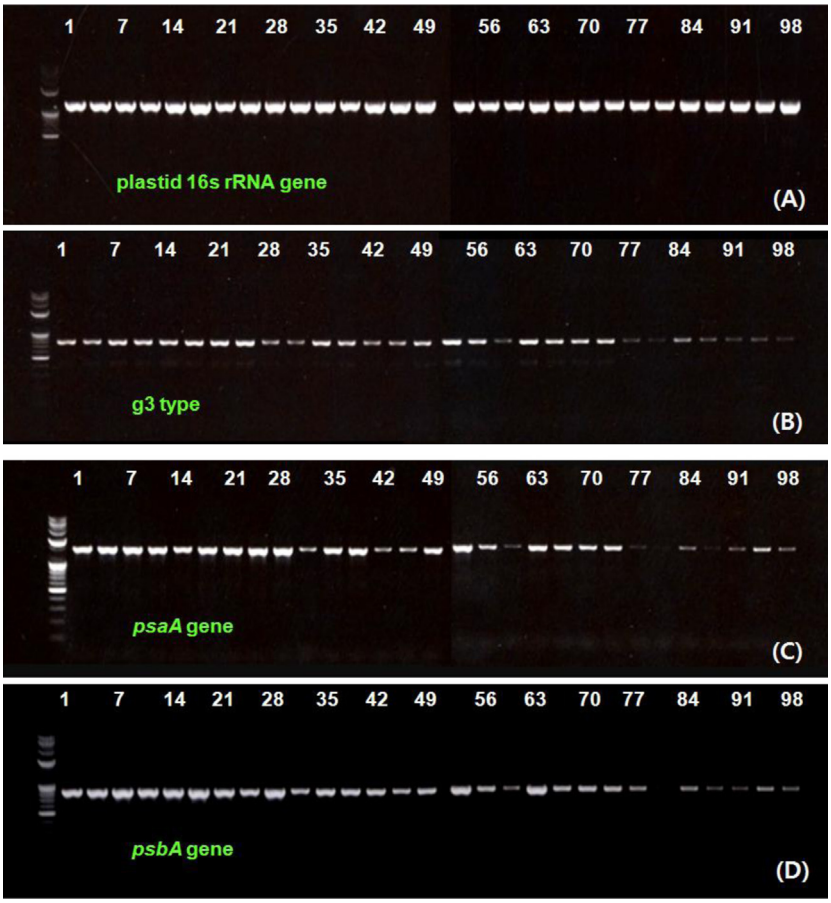

Fig. 5. PCR products amplified from (A) the plastid 16S rRNA gene, (B) the partial 16S rRNA gene (i.e. g3 type), (C) *psa*A gene, and (D) *psb*A gene amplified from *Mesodinium rubrum* during the whole experimental period.

expression of nucleus-encoded genes for plastid-targeted protein (Wisecaver and Hackett, 2010) in *M. rubrum*. Ultrastructural studies on the newly retained and aging plastids in a time-series experiment should also be of great help for the better understanding of plastid retention pattern in *M. rubrum*.

Cultures of the cryptomonad prey (strain CR-MAL03) were offered to the culture of the 80-day long starved *Mesodinium rubrum* for 48 h (Figs. 1 and 4). While only the 1192-bp PCR product (i.e. g7 type) was detected in four single cells of total five single cells, both 1192-bp PCR product and 802-bp PCR product (i.e. g3 type) were amplified from only one single cell (S2 in Fig. 6). This result leads us to the conclusion that the 80 days starved *M. rubrum* should be physiologically healthy enough to ingest cryptomonad preys, and can retain new CR-MAL03 type plastids.

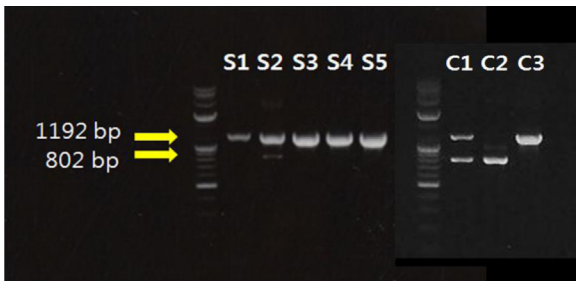

Fig. 6. PCR products of g3 type (802 bp) and g7 type (1192 bp) amplified from *Mesodinium rubrum*. Lanes S1–S5 are PCR products amplified from each single *M. rubrum* cell. C1 is PCR product amplified from a mixed culture of cryptophyte CR-MAL01 (g3 type) and CR-MAL03 (g7 type). C2 is PCR products amplified from cryptophyte CR-MAL01. C3 is PCR product amplified from cryptophyte CR-MAL03.

Photosynthetic activity of newly obtained plastids depended on the kinds of plastid, but not on the kinds of predator. Functional activity of the 'CR-MAL01 type' plastids was maintained for up to 80 days, regardless of predators (Park et al., 2008; this study). *Mesodinium rubrum* and *Dinophysis caudata* share the same 'CR-MAL01 type' plastids, and the patterns of time-course changes in their photosynthetic capacity are very similar to each other. However, other type plastids than the 'CR-MAL01 type' may exhibit completely different retention pattern in the two species. If other type plastids (i.e. CR-MAL11, see Park et al., 2010) from *M. rubrum* were taken up by the starved *D. caudata*, the newly retained plastids seemed to shortly disappear in the organism within one month (Park et al., 2010). In the present study, most of *M. rubrum* starved for 80 days can quickly replace with newly retained plastids distantly related to 'CR-MAL01 type'. Therefore, it seems reasonable to conclude that *M. rubrum* can continue to keep other type plastids originating from cryptomonad rather longer (Myung et al., 2011) when compared with *D. caudata*.

We showed that the maximum duration of 80 days should be critical for the test the capacity of *Mesodinium rubrum* strain MR-MAL01 to survive long-term starvation from its cryptomonad preys (Bielewicz et al., 2011; Laybourn-Parry, 2002). Such long-time functioning of new kleptoplastids in *M. rubrum* may help explain its overwintering success or long-term survival (Yih et al., 2013; Martin et al., 2007; Laybourn-Parry et al., 2000), even though the formation of cysts or resting stages in *M. rubrum* has not been shown (Gibson et al., 1997; Lindholm, 1985). In this study, however, re-feeding was tried on the 80-day starved *M. rubrum*

only, to confirm its quick retention of new plastids from the cryptomonad prey. Based on the quite consistent maintenance of plastid 16S rRNA gene throughout the whole 100 days starvation experiment (Fig. 5A), one may speculate on the possibility of regaining plastid retention by the photosynthetically mal-functional *M. rubrum* (Fig. 5B–D) due to severe starvation for a period over 100 or 120 days. Overwintering by vegetative forms of *M. rubrum* in temperate seas would be seen as quite natural if the 100 days or 120 days starved *M. rubrum* cells could be demonstrated to ingest newly offered cryptomonad prey and retain photosynthetically active plastids again.

Acknowledgements

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