



HOKKAIDO UNIVERSITY

Title	Ultrastructural and phylogenetic studies of unarmoured kleptochloroplastidic dinoflagellates
Author(s)	大沼, 亮
Degree Grantor	北海道大学
Degree Name	博士(理学)
Dissertation Number	甲第11803号
Issue Date	2015-03-25
DOI	https://doi.org/10.14943/doctoral.k11803
Doc URL	https://hdl.handle.net/2115/61363
Type	doctoral thesis
File Information	Ryo_Onuma.pdf



Ultrastructural and phylogenetic studies of unarmoured kleptochloroplastidic dinoflagellates

(盗葉緑体性無殻渦鞭毛藻類の微細構造学的・系統分類学的研究)

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2015

ABSTRACT

‘Kleptochloroplast’ is the temporary chloroplast ‘stolen’ from other photosynthetic algae and some dinoflagellates display the kleptochloroplast phenomenon. These dinoflagellates originally possess no chloroplast, and ingest other photosynthetic alga. The ingested chloroplast is retained in the host cytoplasm but eventually lost due to digestion or host cell division. This phenomenon can be interpreted as intermediate evolutionary step from heterotroph to phototroph that has the permanent relationship with algal symbiont.

Nusuttodinium spp. are unarmoured dinoflagellates that retain cryptomonad chloroplasts as kleptochloroplasts, and currently six species are described from both marine and fresh water habitats. They form a robust clade, indicating the single origin of the kleptochloroplastidy. According to previous descriptions, there are many more dinoflagellates possessing chloroplast in unusual colours, suggesting they are expected to be kleptochloroplastidic. Therefore, floristic study is worthwhile undertaking to explore the further diversity of kleptochloroplastidic dinoflagellates. In addition to particular interest as to their diversity, there are significant differences in regard with the degree of enlargement of kleptochloroplast and the retention of the cryptomonad organelles among *Nusuttodinium* spp. These variations might reflect differences in evolutionary steps. Although studies on general ultrastructure of *Nusuttodinium* have been reported, there is little morphological study to address how ingested cryptomonad cell is re-organised within the host cell after ingestion. Therefore, it is unclear how the dinoflagellate host retains the kleptochloroplast in the cell and what kind of factors causes the different evolutionary steps in kleptochloroplastidy in different species. Furthermore, the specificity between the dinoflagellate host and cryptomonads has never been investigated, especially in the freshwater species, which have strict relationships with cryptomonads than those of marine species.

The aims of my thesis is then, 1) to explore further diversity of kleptochloroplastidic dinoflagellates, 2) to describe morphological transition in kleptochloroplast from ingestion of cryptomonad cell and compare marine and freshwater species, which show

different strategy of kleptochloroplastidy and might represent different stages of evolutionary steps and 3) to investigate host-prey specificity using molecular approach.

In Chapter 1, I collected the samples from various marine beaches to discover novel or uninvestigated species. My survey of the diversity of *Nusuttodinium*-related dinoflagellates resulted in the discovery of two colourless dinoflagellates, referred to as DAI and DIS. Cells of DAI were found to possess a right-handed cingulum, an apical groove connecting with the sulcal extension at a right angle and nuclear chambers. Cells of DIS have a projection on their epicone encircled by an anticlockwise-directed apical groove and lack nuclear chambers. Cells of DAI digest cryptomonads directly and DIS never ingests cryptomonads, making both species non-kleptochloroplastidic. *Nusuttodinium* spp. are known to form a sister group to *Spiniferodinium* spp. which are typical photosynthetic dinoflagellates. Phylogenetic analyses showed that DIS was a member of the *Nusuttodinium*-clade, while DAI was a sister of the clade consisting of *Nusuttodinium* spp. plus DIS, and that the photosynthetic genus, *Spiniferodinium*, came to the position at the base of these dinoflagellates. Based on a unique combination of features, including the possession of unique shape to the apical groove, a right-handed cingulum, nuclear chambers and lack of a chloroplast, I described DAI as *Pellucidodinium psammophilum* gen. et sp. nov. Because of the possession of a projection on the epicone, an anticlockwise-directed apical groove and the lack of nuclear chambers, I described DIS as *Nusuttodinium desymbiontum* sp. nov. despite its inability to display kleptochloroplastidy. The phylogenetic position of *P. psammophilum* together with its shared presence of nuclear chambers with *Spiniferodinium* implies that it represents an evolutionary intermediate prior to the acquisition of kleptochloroplastidy competence, while *N. desymbiontum* appears to have lost this competence secondarily.

In Chapter 2-1, I described the morphological transition of kleptochloroplast in *Nusuttodinium poecilochroum*, *N. latum* and *N. aeruginosum*. Previous studies revealed that *N. aeruginosum* can synchronise the division of the chloroplast with its own cell division, while no simultaneous division takes place in *N. poecilochroum* and *N. latum*, which is interpreted to mean that state of kleptochloroplastidy in *N. aeruginosum* is

closer to that of the initial acquisition of the ‘true chloroplast’ within the lineage. I observed morphological changes in kleptochloroplasts of these three species following the ingestion of cryptomonad cells, using light and transmission electron microscopes. In *N. poecilochroum*, the cryptomonad ejectosomes, mitochondria and cytoplasm were all actively transferred into digestive vacuoles within 1 h of ingestion. The chloroplasts were deformed and the cryptomonad nucleus was digested after 3 h. In *N. latum*, the rapid digestion of the ejectosomes took place like in *N. poecilochroum*, while cryptomonad nucleus and nucleomorph were retained and the chloroplast encircled the organelles as if it protects some organelles from digestion. By 120 h, the chloroplast was significantly enlarged and the nucleomorph was duplicated. In *N. aeruginosum* too, the cryptomonad cytoplasm and nucleus were retained during the experimental period. The chloroplast filled the bulk of its cell, and the volume of the chloroplast increased more than 20-fold, by within 120 h of ingestion of the cryptomonad. These differences imply that the retention of the cryptomonad nucleus is important for the maintenance and enlargement of the chloroplast.

In Chapter 2-2, to understand the function of cryptomonad nucleus, I subsequently investigated the morphological transition in kleptochloroplasts and the distribution of the cryptomonad nucleus after host cell division in *Nusuttodinium aeruginosum* to address the reason why the cell ultimately loses the cryptomonad nucleus. Host cell division was not preceded by cryptomonad karyokinesis so that only one of the daughter cells inherited a cryptomonad nucleus. The fate of all daughter cells originating from a single cell through five generations was closely monitored, and this observation revealed that the cell that inherited the cryptomonad nucleus consistently possessed the largest kleptochloroplast for that generation. Therefore, this study suggests that some kind of important nuclear division mechanism in cryptomonad is suppressed under the symbiotic condition or lost during ingestion process. Furthermore, it was suggested that the cryptomonad nucleus plays an important role in the enlargement of kleptochloroplast.

In Chapter 3, I collected *Nusuttodinium aeruginosum* cells from several geographically-distant ponds and analysed the phylogenies of the dinoflagellates and

the kleptochloroplasts using internal transcribed spacer (ITS) and chloroplast 16S rDNA extracted from the same cell. The phylogenetic analysis confirmed that all dinoflagellate sequences formed a clade with the deposited sequences of *N. aeruginosum*. All sequences of kleptochloroplast were nested only in the subclade 4 of *Chroomonas/Hemiselmis* clade, while no sequences belonging to the subclades 1, 2 and 3 were obtained. The sequences of kleptochloroplasts were distributed throughout the subclade 4, indicating that *N. aeruginosum* is able to accept multiple species within the subclade 4 as a kleptochloroplast. These results suggest that *Chroomonas* spp. included in the subclade 4 can be suitable prey as a source of kleptochloroplast, and dinoflagellate-prey specificity in *N. aeruginosum* is not as strict as species specific, although the prey selection is restricted to only members of a certain clade of *Chroomonas*, i.e. subclade 4.

ACKNOWLEDGEMENTS

I would like to express my greatest gratitude to Professor Takeo Horiguchi, Department of Natural History Sciences, Hokkaido University, for his direction, guidance, and helpful suggestions on the dinoflagellate studies and critical reading of the manuscript. I am grateful to Professor Shin Tochinnai and Dr. Kazuhiro Kogame, Department of Natural History Sciences, Hokkaido University and Dr. Chikako Nagasato, Muroran Marine Station, Field Science Center for Northern Biosphere, Hokkaido University, for their helpful suggestions, technical advices and critical reading of the manuscript. I would like to show my great appreciation to Dr. Stuart D. Sym, Department of Animal, Plant and Environmental Sciences, University of the Witwatersrand, South Africa for careful reading and correction of the manuscript and helpful suggestions. I am also indebted to Dr. Tsuyoshi Abe of Hokkaido University Museum, for help in collecting samples, helpful suggestions and generous supports, and Professor Taizo Motomura, Muroran Marine Station, Field Science Center for Northern Biosphere, Hokkaido University, for his technical guidance on the transmission electron microscopy. I owe my deep gratitude to Dr. Hirohi Yamasaki for collecting samples and encouragements and Dr. Keiichi Kakui for his advice in phylogenetic analyses. I profoundly thank the members of Biodiversity laboratory II for collecting samples, invaluable discussions and constant encouragement. Finally, I am sincerely grateful to my family for giving me the opportunity to study, understanding and continuous encouragements.

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GENERAL INTRODUCTION

General characteristics of dinoflagellates

Dinoflagellates are one of the protist groups that consist of predominantly unicellular, eukaryotic, flagellated organisms, and approximately 2000 living and 2000 fossil species have been described. The group consists of both photosynthetic and non-photosynthetic members, and roughly 40-60% of the living species are photosynthetic. Dinoflagellates are distributed from marine to freshwater environments all over the world, and play an important role in aquatic ecosystems either as producer, prey or predator (Taylor 1987).

Dinoflagellates possess several characteristic morphological features (Fig. i). A typical motile dinoflagellate possesses distinct furrows. The cingulum horizontally encircles the cell, and divides the cell into an anterior part (*episome*, *epicone*) and a posterior part (*hyposome*, *hypocone*). The sulcus longitudinally indents the hypocone. The motile cell swims with whirling movement using two dissimilar flagella that lie in the cingulum (*transverse flagellum*) and the sulcus (*longitudinal flagellum*), respectively. The transversal flagellum is located within the cingulum, while the longitudinal flagellum lies in the sulcus and projects posteriorly beyond the cell. The dinoflagellates have the special cell covering called '*amphiesma*'. The amphiesma is a row of flattened vesicles supporting the plasma membrane. The armoured (= thecate) dinoflagellates form cellulosic plate, called thecal plates within each amphiesmal vesicle, while the unarmoured (= atecate) dinoflagellates possess no plates or very thin plates (Dodge 1971; Taylor 1987; Fensome *et al.* 1993). Some unarmoured dinoflagellates have a groove on the surface of epicone, called '*apical groove*'. The shape of apical groove is used for one of the taxonomic criteria in unarmoured dinoflagellates (Daugbjerg *et al.* 2000).

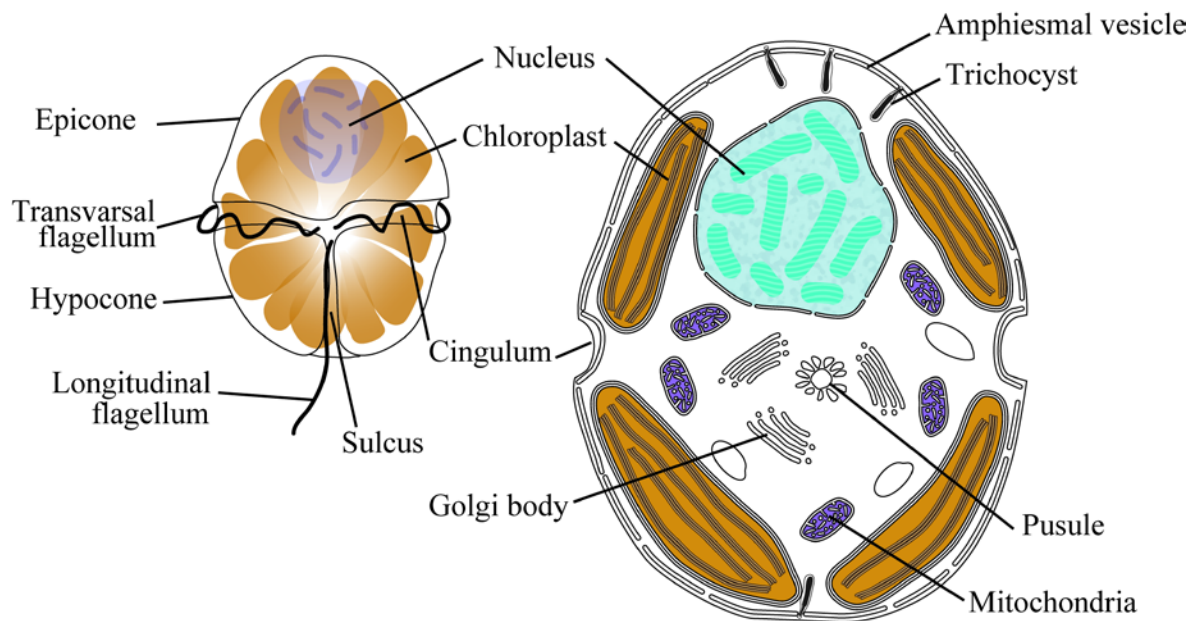


Fig. i. Schematic drawings of light (left) and transmission electron micrographs (right) of typical dinoflagellate

Dinoflagellates have also several noteworthy ultrastructural features (Fig. i). Dinoflagellates possess the unusual nucleus called *dinokaryon*, which contains condensed chromosomes throughout the cell cycle and lacks nucleosomes (Dodge 1971; Taylor 1987). A typical dinoflagellate chloroplast contains peridinin as a major carotenoid (Jeffrey *et al.* 1975). The chloroplast is encircled by three membranes, which have no obvious connections with nuclear envelope. Within the chloroplast, the lamellae are composed of three thylakoids, and the thylakoids lie parallel to each other (Schnepf & Elbrächter 1999). The chloroplast genome is unusual. Each gene is coded in a small circular DNA (2-3 kbp), called 'mini-circle' (Zhang *et al.* 1999). The mitochondria of dinoflagellate have tubular cristae (Dodge 1975; Taylor 1987). The trichocysts are ejection apparatus that located beneath the plasma membrane, which consist of two distinct parts, the neck and the body (Taylor 1987). The pusule is an organelle that is appeared to be present only in dinoflagellates. The pusule is composed of a tubule or chamber and thought to be involved in osmo-regulation (Dodge 1972). Because of these characteristic features, dinoflagellate cells can be recognised easily even with the sectioned materials for transmission electron microscopy.

Chloroplasts in dinoflagellates and kleptochloroplasts

Dinoflagellates are considered to have followed drastic evolutionary pass, especially as to their chloroplast origins. The ancestor of all dinoflagellates is considered to be photosynthetic, which acquired the chloroplast that derived from a red alga via secondary endosymbiosis, although the timing of acquisition is unclear (Keeling 2010; 2013). Typical photosynthetic dinoflagellates possess the peridinin-containing chloroplasts. However, there are a number of dinoflagellates which had lost their chloroplasts, becoming heterotrophic forms (Saldarriaga *et al.* 2001).

Although most of photosynthetic dinoflagellates possess typical peridinin-type chloroplasts, there are other dinoflagellates which possess different type of chloroplasts. These dinoflagellates had replaced their original peridinin-type chloroplasts with those of diatom or haptophyte origin via tertiary endosymbiosis, or with that of chlorophyte origin via serial secondary endosymbiosis (Saldarriaga *et al.* 2001; Stoebe & Maier 2002; Hackett *et al.* 2004; Horiguchi 2006). Thus, dinoflagellates have very complex history as to the evolution of chloroplasts.

In addition to these permanent chloroplasts mentioned above, some dinoflagellates show another unique trophic strategy. These dinoflagellates that are originally colourless ingest chloroplasts (and often other organelles) of other photosynthetic algae (Fig. iiA, B), and utilise the chloroplast for

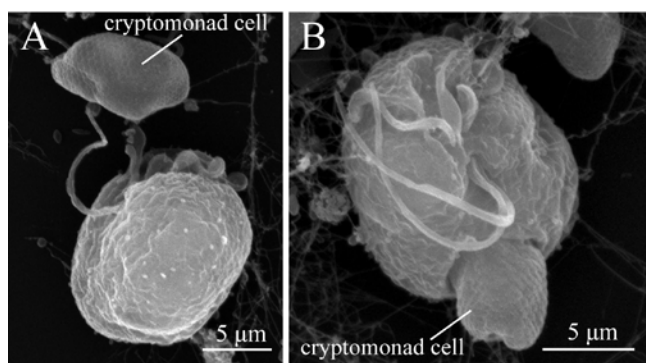


Fig. ii. Ingestion of cryptomonad cell by *Nusuttodinium poecilochroum*

photosynthesis. The ingested chloroplasts are temporarily retained in the dinoflagellate cell, but are eventually lost through cell division or digestion and the dinoflagellate needs to feed on other photosynthetic algal cells to maintain its temporary ‘chloroplast’. This type of nutrition is called “stolen chloroplast” or “kleptochloroplast” (Schnepf & Elbrächter 1992) (Fig. iii). Kleptochloroplast phenomenon is widely spread in dinoflagellates from armoured species *Amylax* spp. (Koike & Takishita 2008),

Cryptoperidiniopsis sp. (Eriksen *et al.* 2002), *Dinophysis* spp. (Schnepf & Elbrächter 1988) and *Pfiesteria piscicida* Steidinger & Burkholder (Lewitus 1999) to unarmoured species, *Gymnodinium* ‘*gracilentum*’ (Skovgaard 1998) and *Nusuttodinium* spp. (Takano *et al.* 2014). All of these unarmoured kleptochloroplastidic dinoflagellates are known to retain chloroplasts of cryptomonad origin. However, two unnamed unarmoured dinoflagellates (RS 24 and W5-1) obtain their kleptochloroplasts from the haptophyte, *Phaeocystis* (Gast *et al.* 2007).

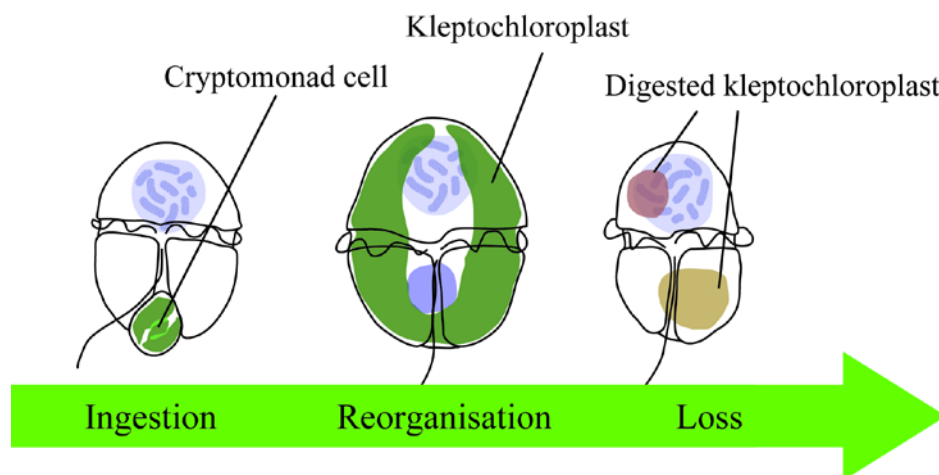


Fig. iii. Schematic drawings of kleptochloroplastidy in *Nusuttodinium aeruginosum*

Although culture of kleptochloroplastidic species is generally believed to be difficult due to necessity of co-culture with acceptable prey, some successful cultural studies have revealed an unique strategy for acquisition of kleptochloroplast. *Dinophysis* spp. keep only chloroplasts without other cryptomonad organelles in the cytoplasm, and the chloroplasts are surrounded by two membranes (Schnepf & Elbrächter 1988). They acquire kleptochloroplasts not by engulfing cryptomonad cells directly, but by sucking up the cytoplasm of ciliate *Mesodinium rubrum* Leegaard whose chloroplasts are acquired from a cryptophyte *Teleaulax*, indicating that kleptochloroplasts of *Dinophysis* spp. are resulted from serial kleptochloroplastidy mediated by *M. rubrum* (Park *et al.* 2006; Nagai *et al.* 2008). *Amylax* spp. also ingest *M. rubrum*, indicating that *Dinophysis* spp. and *Amylax* spp. share the same strategy for kleptochloroplast acquisition (Park *et al.* 2013). Thus, cultural study of kleptochloroplastidic dinoflagellates contributes to understand the strategy for acquisition of kleptochloroplast and to perform subsequent experiments.

Kleptochloroplast phenomenon is widespread not only in dinoflagellates but also in ciliates (Esteban *et al.* 2010; Johnson 2011a), foraminiferans (Lopez 1979), katablepharids (Okamoto & Inouye 2006) and sea slugs (Rumpho *et al.* 2000). Of these, the retention of chloroplast acquired from other photosynthetic organism is common feature although there are significant variations in the selection of prey species and the strategy for the retention of kleptochloroplast depending on the kleptochloroplastidic organisms (Lopez 1979; Rumpho *et al.* 2000; Okamoto & Inouye 2006; Esteban *et al.* 2010; Johnson 2011). To establish permanent endosymbiotic relationships between the host and the endosymbiont (the chloroplast), for the first step, the host cell must ingest the endosymbiont and retain the chloroplast just like kleptochloroplast. Therefore, the kleptochloroplastidy is considered to be the earliest stage of endosymbiosis (Okamoto and Inoue 2006; Nowack & Melkonian 2010; Johnson 2011b).

Genus *Nusuttodinium* and aim of this thesis

Nusuttodinium spp. are widely distributed in marine and freshwater habitats. *Nusuttodinium latum* (Lebour) Takano & Horiguchi (Fig. ivA), *N. myriopyrenoides* (Yamaguchi, Nakayama, Kai & Inouye) Takano & H. Yamaguchi and *N. poecilochroum* (Larsen) Takano & Horiguchi (Fig. ivB) are marine, sand-dwelling species (Larsen 1988; Horiguchi & Pienaar 1992; Yamaguchi *et al.* 2011), while *N. acidotum* (Nygaard) Takano & Horiguchi, *N. aeruginosum* (Stein) Takano & Horiguchi (Fig.

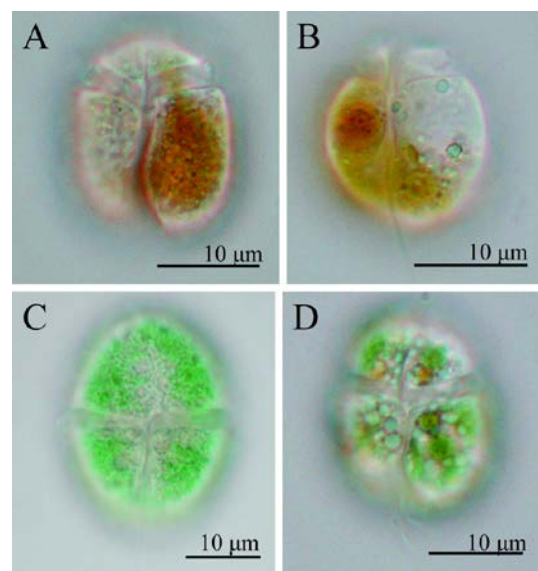


Fig. iv. Light micrographs of *Nusuttodinium* spp.

ivC) and *N. amphidinioides* (Geitler) Takano & Horiguchi (Fig. ivD) are known as freshwater representatives (Schnepf *et al.* 1989; Fields & Rhodes 1991; Takano *et al.* 2014). The chloroplasts of all these dinoflagellates possess unusual colour, such as blue-green, blue-grey or reddish brown, because they prey on cryptomonads and retain

them (Fig. iv) (Larsen 1988; Schnepf *et al.* 1989; Fields & Rhodes 1991; Horiguchi & Pienaar 1992; Yamaguchi *et al.* 2011; Takano *et al.* 2014). In addition, they share two morphological features, i.e, apical groove running in an anticlockwise direction on the epicone and the lack of nuclear chambers, the small chambers located in the nuclear envelope (Takano *et al.* 2014). A phylogenetic study revealed that all members of *Nusuttodinium* are monophyletic, with a sister clade consisting of *Spiniferodinium* spp. and *Gymnodinium palustre* Schilling (Takano *et al.* 2014). This phylogenetic relationship suggests that the unique feeding strategy of kleptochloroplastidy (Schnepf & Elbrächter 1992) evolved in photosynthetic dinoflagellates (Takano *et al.* 2014). Because all these dinoflagellates are monophyletic and share several important morphological and physiological features as mentioned above, the new genus *Nusuttodinium* was established (Takano *et al.* 2014). The species now included in the genus were originally described as members of either *Amphidinium* or *Gymnodinium*. It is an interesting question that in addition to these known species, if there are any other *Nusuttodinium* species or other kleptochloroplastidic dinoflagellates. In fact, several species of unarmoured dinoflagellates, described as either members of the genera *Amphidinium* or *Gymnodinium*, are known to possess unusually coloured chloroplasts (Conrad & Kufferath 1954, Calado & Moestrup 2005). The presence of anomalous coloured dinoflagellates suggests that there are many more possible kleptochloroplastidic dinoflagellates still to be confirmed. To understand evolutionary history of kleptochloroplastidic dinoflagellates, it is important to explore further diversity of such dinoflagellates.

Most of the studies have focused on the general ultrastructure and these studies used the cells collected from the natural populations. Therefore, it was not possible to study morphological transition of kleptochloroplast sequentially under controlled conditions and we know little about dynamic interaction between the host dinoflagellate and its prey, i.e. it is unclear how the kleptochloroplast is retained and how the morphological changes take place within the host cell after ingestion. To understand the significance of kleptochloroplast phenomenon in the evolutionary context, it is important to investigate what is actually happening during the process of

kleptochloroplastidy and compare it between the species which show different evolutionary steps.

It has been documented that freshwater kleptochloroplastidic dinoflagellates are much stricter than the marine species as to the choice of prey (cryptomonad species) (Fields & Rhodes 1991). It is intriguing question that how strict the freshwater dinoflagellates can be when they choose their prey. No detailed studies on this point have been made so far.

Based on these backgrounds, the aims of my thesis are then,

- 1) to explore further diversity of kleptochloroplastidic dinoflagellates (Chapter 1)
- 2) to describe detailed morphological transitions of cryptomonad cell after ingestion based on cultured materials and compare the results between the *Nusuttodinium* species (Chapter 2).
- 3) to investigate host-prey specificity using molecular approach (Chapter 3).

In Chapter 1, I studied the phylogeny of two novel dinoflagellates. During the course of my study of searching further diversity of kleptochloroplastidic species, I discovered two novel species that are closely-related to *Nusuttodinium*, but showed no apparent kleptochloroplast phenomenon. I investigated these new species using light, scanning and transmission electron microscope (LM, SEM and TEM) and undertook phylogenetic analysis. Then, I discussed possible evolutionary history of kleptochloroplastidy.

In Chapter 2-1, I investigated the morphological changes of kleptochloroplast at different timing after ingestion using LM and TEM in marine species *Nusuttodinium poecilochroum*, *N. latum* and freshwater species *N. aeruginosum*, which display different stages of evolutionary steps, from primitive to advanced, and discuss significance of kleptochloroplast in chloroplast evolution. In Chapter 2-2, I traced the morphological changes of kleptochloroplast that derived from single cell through cell division in *N. aeruginosum* using LM and TEM. In addition to the simultaneous division, it is reported that *N. aeruginosum* loses cryptomonad organelles such as nucleus and nucleomorph (Schnepf *et al.* 1989). I traced the distribution pattern of these

cryptomonad organelles and I discussed the importance of the cryptomonad nucleus for establishment of permanent chloroplast.

In Chapter 3, I collected *Nusuttodinium aeruginosum* cells at several different ponds and analysed the phylogenies of the dinoflagellates and the kleptochloroplasts using internal transcribed spacer (ITS) and chloroplast 16S rDNA extracted from the same cell. Then I estimated the specificity of cryptomonad in *N. aeruginosum*.

Chapter 1. A phylogenetic study of two novel heterotrophs closely-related to kleptochloroplastidic forms of *Nusuttodinium*; *Pellucidodinium psammophilum* gen. et sp. nov. and *Nusuttodinium desymbiontum* sp. nov. (Dinophyceae)

INTRODUCTION

A phylogenetic study revealed that all the unarmored kleptochloroplastidic *Nusuttodinium* spp. are monophyletic, with a sister clade consisting of *Spiniferodinium* spp. and *Gymnodinium palustre* Schilling (Takano *et al.* 2014). Although all currently-known species of *Nusuttodinium* are kleptochloroplastidic, some other unarmoured dinoflagellates have been described with chloroplasts of unusual colour. These include *Amphidinium bidentatum* Schiller, *A. bourrellyi* Wawrik, *A. caerulescens* Schiller, *A. glaucum* W. Conrad, *A. lacunarum* Skuja, *A. oculatum* Schiller, *A. phthartum* Skuja (Calado & Moestrup 2005), *A. carbunculus* Conrad & Kufferath, *A. coeruleum* Conrad, *A. corallinum* Conrad & Kufferath, *A. cyaneoturbo* Conrad & Kufferath, *A. dubium* Conrad & Kufferath and *A. salinum* J. Ruinen, (Conrad & Kufferath 1954). The existence of all these unusually-coloured dinoflagellates suggests that there may be many more dinoflagellates that are potentially kleptochloroplastidic. In order to understand the evolution and diversity of kleptochloroplastidic dinoflagellates, more floristic surveys are required to explore the diversity of this group. Hence, I sampled at various sites, focusing on Hokkaido Prefecture, Japan, in search of further new kleptochloroplastidic dinoflagellates.

As a result, I discovered two marine, sand-dwelling dinoflagellates considered to be novel species which I temporarily referred to as DAI (Dinoflagellate collected from Aininkappu) and DIS (Dinoflagellate collected from IShikari). These dinoflagellates, despite being colourless and lacking either chloroplasts or kleptochloroplasts, were included in a clade formed by *Nusuttodinium* spp. and the typical photosynthetic dinoflagellate *Spiniferodinium* spp. Moreover, DAI digested any ingested cryptomonad within a short period of time, while DIS was never witnessed to ingest cryptomonad

cells. Thus, this study suggested that these dinoflagellates were phagotrophic rather than kleptochloroplastidic. In this paper, for the purposes of convenience, organisms that immediately digest their prey following ingestion are referred to as phagotrophic, while those that retain their prey's chloroplast are referred to as kleptochloroplastidic.

In this study, I investigated the morphology of each of these dinoflagellates using the light, scanning electron and transmission electron microscopes, and estimated their phylogenetic position by SSU rDNA, partial LSU rDNA, cytochrome oxidase 1 (*cox1*) and cytochrome b (*cob*) gene sequence analyses. Based on these studies, I concluded that the novel species, DAI, should be described as a new genus *Pellucidodinium psammophilum* Onuma & Horiguchi gen. et sp. nov. and that DIS is a new species of *Nusuttodinium*, *N. desymbiontum* Onuma, K. Watanabe & Horiguchi sp. nov. This study suggests that *Pellucidodinium psammophilum* represents an intermediate evolutionary position between photosynthetic and kleptochloroplastidic dinoflagellates, while *N. desymbiontum* appears to have lost its kleptochloroplastidic ability secondarily.

MATERIAL AND METHODS

Sampling

Pellucidodinium psammophilum was collected at Aininkappu beach, Akkeshi Town, Hokkaido Prefecture, but was also recovered from samples taken at Cape Nosappu, Nemuro City, Hokkaido Prefecture and at Usutaibe-Senjoiwa shore, Esashi Town, Hokkaido Prefecture, (Table 1). *Nusuttodinium desymbiontum* was collected at Ishikari beach, Ishikari City, Hokkaido Prefecture, at a sandy beach in Fukaura Town, Aomori Prefecture and at a sandy beach in Abuta Town, Hokkaido Prefecture (Table 1). In all the cases, wet sand samples were collected at the edge of the surf. Individual cells of *P. psammophilum* and *N. desymbiontum* were isolated from the samples by micropipetting while being viewed with an inverted microscope (CKX-40, Olympus, Tokyo). The isolated cells were used for subsequent observations and sequencing.

I also attempted to establish cultures of *Pellucidodinium psammophilum* collected at Aininkappu beach and *Nusuttodinium desymbiontum* collected at Ishikari Beach by

feeding them with cells of a *Chroomonas* sp. (strain Ak01; collected from Aininkappu beach), or, in the case of the latter, with *Rhodomonas* sp. (strain Mr06). To keep prey alive, such cultures had to be grown in Daigo IMK medium (Wako, Osaka, Japan) at 20 °C in a culture cabinet under fluorescent lighting at 30-50 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ with a 16L/8D cycle.

In addition, I collected some other species of *Nusuttodinium* to obtain DNA sequences, the sampling sites and dates of which are supplied in Table 1.

Light microscopical (LM) observations

For LM observations, cells were observed using the ZEISS Axioskop2 Plus (Carl Zeiss Japan, Tokyo) and photographs were taken with a CCD camera DS-Fi1 (Nikon, Tokyo, Japan) or ZEISS AxioCam ERc 5s (Carl Zeiss Japan, Tokyo). To observe the autofluorescence of cryptomonad chloroplasts, a Fs 15 filter of the same microscope was used.

Scanning electron microscopical (SEM) observations

For SEM observations, the isolated cells were transferred into a drop of fresh medium on a poly-L-lysine coated glass plate (Oken, Tokyo, Japan) and 4% OsO_4 was added and mixed to the drop to obtain a final concentration of 1%. After 30 min of fixation, the cells on the glass plate were rinsed three times with distilled water and dehydrated through a graded ethanol series (25, 50, 80, 90, 95 and 100%) for 10 minutes in each solution. The sample was dried using a critical point dryer HCP-1 (Hitachi, Tokyo, Japan) and coated with gold in an E-1045 ion-sputter coater (Hitachi, Tokyo, Japan). Cells were observed with a S-3000N scanning electron microscope (Hitachi, Tokyo, Japan).

Transmission electron microscopical (TEM) observations

In this study, I used the single-cell TEM method for all TEM samples. The medium containing the incubated cells mentioned above was mixed with an equal volume of Karnovsky fixative (5% glutaraldehyde and 4% paraformaldehyde) in 0.1M cacodylate

buffer at pH 7.0 containing 0.3 M sucrose. The samples were pre-fixed at room temperature for 2 h. After fixation, the cell was transferred by micropipette onto a poly-L-lysine coated Thermanox plastic coverslip (Thermo Scientific, Yokohama, Japan), pre-cut to a size that can be accommodated in a 1.5 ml microtube, and the cell was allowed to settle and attach to the coverslip. The coverslip with attached cell was placed in a 1.5 ml microtube filled with 0.1M cacodylate buffer, and rinsed 3 times by exchanging the buffer on ice. The cell on the coverslip was then post-fixed for 1 h with 1% OsO₄ in the buffer. After fixation, the cell was dehydrated through a graded acetone series (25, 50, 80, 90, 95 and 100%). Post-fixation and dehydration were performed on ice. The dehydrated cell was infiltrated with Agar LV resin (AGAR Scientific, Essex, UK) and polymerized at 65°C for 16 h. The polymerised block that contained the cell was removed from the coverslip and sectioned on an ME-Ultracut S ultramicrotome (Leica, Wetzlar, Germany). Serial sections were placed onto formvar-coated copper one-slot grids, and then observed under a transmission electron microscope Hitachi H-7650 (Tokyo, Japan) without staining.

Single cell Polymerase Chain Reaction (PCR)

A single cell that was not fed was picked up from the samples and rinsed by transferring it through a series of drops of sterilized fresh IMK medium on a depression slide glass. The cell was then transferred to a 200 µl PCR tube containing 10 µl of Quick ExtractTM FFPE DNA Extraction Solution (Epicentre, Madison, WI, USA). The PCR tube containing the single cell was incubated at 56 °C for 60 min and then 94 °C for 3 min, and 1 µL of the resulting extract was used as DNA template for each PCR amplification.

In the first round of PCR for SSU rDNA, almost the complete SSU rDNA was amplified using the primers SR1 and SR12b (Nakayama *et al.* 1996; Takano & Horiguchi 2004). In the second round of PCR, 0.3 µl of a 1% solution of the first PCR product diluted with distilled water was used as DNA template, and four sets of primers (SR1 and SR5, SR4 and SR7, SR6 and SR11, SR8 and SR12b; Table 2) were used. In the first round of PCR of LSU rDNA, approximately 1400 bp of LSU rDNA were amplified using the terminal primers D1R (Hansen *et al.* 2000) and 28-1483R

(Daugbjerg *et al.* 2000). In the second round of PCR, 0.3 µl of a 1% solution of the first PCR product was used as DNA template with two sets of primers (D1R and D3B, D3A and 28-1483R) (Hansen *et al.* 2000; Daugbjerg *et al.* 2000; Table 2). In the first round of PCR for a partial *cox1* gene, amplification was achieved using the primers Dinocox1F and Dinocox1R (Lin *et al.* 2002; Zhang *et al.* 2007). In the second round of PCR, 0.3 µL of the first PCR product, diluted 1/100, was used as DNA template, together with two sets of primers (Dinocox1F and Dinocox1R2, Dinocox1F2 and Dinocox1R; Table 2) (Lin *et al.* 2002; Zhang *et al.* 2007). For amplification of the *cob* gene, the primer set PMCOBF (Lin *et al.* 2002) and Dinocob6Rk was used for the first round of PCR, and then 1/100-diluted first PCR product was used as DNA template with the internal primer sets, PMCOBF and Dinocob5R, and Dinocob4F and Dinocob6Rk (Zhang *et al.* 2007; Table 2) for the second round of PCR.

The conditions for the first round of PCR were one initial step of denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min. The final extension step was at 72 °C for 7 min. The conditions for the second round of PCR were the same as those for the first PCR except that the extension step was for 30 sec. For the amplification of the *cox1* and *cob* gene, the annealing temperature was set at 58 °C, and the other conditions were same as that of PCR for rDNA.

The purified PCR products of SSU rDNA, LSU rDNA, the *cox1* and *cob* gene were directly sequenced using the ABI PRISM BigDye Terminator Cycle Sequence Kit (Applied Biosystem, Tokyo, Japan) and a DNA autosequencer ABI PRISM310 Genetic Analyzer (Applied Biosystem, Tokyo, Japan). Both forward and reverse strands were sequenced.

Phylogenetic analyses

Both SSU rDNA and LSU rDNA sequences were aligned based on the secondary structure of the rRNA molecule and aligned manually. Due to ambiguous alignment, the highly variable D2 domain of the LSU rDNA sequences were excluded from the alignment. The *cox1* and *cob* gene sequences were aligned and refined manually.

Perkinsus marinus (Mackin, Owen & Collier) Levine and *P. andrewsi* Coss, Robledo, Ruiz & Vasta were used as an outgroup in the analyses of SSU rDNA and LSU rDNA respectively. For analyses of the *cox1* gene, the *cob* gene and the concatenated dataset, *Gymnodinium aureolum* (Hulburt) Hansen and *Lepidodinium chloropholum* (Elbrächter & Schnepf) Hansen, Botes & de Salas were used.

To explore the choice of model of sequence evolution for maximum-likelihood (ML), the program jModeltest version 2.1.4 (Darriba *et al.* 2012), which uses the Akaike information criterion (AIC), was used. The model selected for the ML analysis by AIC for the datasets of SSU rDNA, LSU rDNA, *cox1* and *cob* was GTR + G, GTR + G, GTR + I and GTR + I, respectively. The aligned sequences were analysed using maximum likelihood (ML) with PAUP* version 4.0b10 (Swofford 2002). In the ML analysis, an heuristic search was performed with a TBR branch-swapping algorithm, and the starting tree was obtained by the neighbor joining (NJ) method. Bootstrap analysis for ML was calculated for 100 replicates. For Bayesian analyses of the datasets SSU rDNA, LSU rDNA, *cox1* and *cob*, the GTR + I + G, GTR + I + G, GTR + I, GTR + I models were selected respectively as the suitable evolutionary models by MrModeltest 2.2 (Nylander 2004) and they were undertaken using MrBayes version 3.2.1 (Huelsenbeck & Ronquist 2001). Markov chain Monte Carlo iterations were carried out until 10,000,000 generations, 5,000,000 generations, 1,000,000 generations and 1,000,000 generations were attained for SSU rDNA, LSU rDNA, *cox1* and *cob* phylogeny, respectively, when the average standard deviations of split frequencies (ASDSF) fell below 0.01, indicating a convergence of the iterations.

Two sets of datasets were analysed, i.e. dataset1 (SSU rDNA + LSU rDNA + *cox1* + *cob*) and dataset2 (SSU rDNA + *cox1* + *cob*). Concatenation of the datasets and selection of the optimal model were performed with Kakusan4 version 4.0.2012.12.14 (Tanabe 2011). The ML analyses of the concatenated datasets (dataset1; 18 taxa, 4688 sites, dataset2; 18 taxa, 3586 sites) were calculated under separate model conditions in RAxML version 8.0.0 (Stamatakis 2014), assisted by phylogear2 version 2.0.2013.10.22 (Tanabe 2008a), and the corresponding bootstrap support values were calculated through ML analysis of 1000 pseudoreplicates. No significant nucleotide compositional

heterogeneity was detected for any of the datasets (Chi-square test in Kakusan4: dataset1; $P = 1.00000$, dataset2; $P = 0.99996$). For Bayesian analysis, the optimal model was selected by Kakusan4, which uses the Bayesian information criterion (BIC). For dataset1, the GTR + G, K80 + G, HKY85 + G and HKY85 + G models were selected for SSU rDNA, LSU rDNA, *cox1* and *cob* gene partitions, respectively. For dataset2, the GTR + G, GTR + G, HKY85 + G model were selected for SSU rDNA, *cox1* and *cob* gene partitions, respectively. Bayesian analysis of the concatenated dataset under the proportional model was calculated with MrBayes5D (Tanabe 2008b; a modified MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003)). Markov chain Monte Carlo iterations were carried out until 1,000,000 generations were attained, when ASDSF fell below 0.01

RESULTS

Description

Pellucidodinium Onuma & Horiguchi gen. nov.

Unarmoured dinoflagellates; apical groove running counterclockwise in a semicircle forming a right-angle with the sulcal extension; cingulum slightly right-handed, its right side narrower than that of left side; nuclear chambers present; no chloroplast present.

TYPE SPECIES: *P. psammophilum* Onuma & Horiguchi

ETYMOLOGY: Latin *pellucidus* (transparent), in reference to the lack of any chloroplast

***Pellucidodinium psammophilum* Onuma & Horiguchi sp. nov.**

Figs 1-3

Cells dorsoventrally flattened, elliptical, 20-36 μm long, 15-28 μm wide; epicone about one-third of total cell length, with a counterclockwise apical groove; posterior of hypocone bi-lobed, right side larger than left; width of hypocone larger than width of epicone; cingulum slightly right-handed, right side narrower than that of the left; no chloroplast present; nucleus spherical and anterior.

HOLOTYPE: SEM stub used to take photographs in Fig. 2A was deposited in the Herbarium of Faculty of Science, Hokkaido University as SAP114773. The specimen was collected at 22th June 2012.

TYPE LOCALITY: Aininkappu Beach, Akkeshi Town, Hokkaido Prefecture, Japan (43°00.20N: 144°51.32E)

PARATYPE: Fig. 1A

HABITAT: marine and sand-dwelling

ETYMOLOGY: Greek *psammo* (sand-) and *philus* (-lover), in reference to its habitat

Morphological observations of *Pellucidodinium psammophilum* gen. et sp. nov. (DAI)

The cells of *Pellucidodinium psammophilum* are dorsoventrally flattened, and elliptical in ventral view, measuring 20.1-36.7 μm (mean = $25.7 \pm 4.4 \mu\text{m}$, $n=18$) in length and 15.0-28.0 μm (mean = $20.0 \pm 3.4 \mu\text{m}$, $n=18$) in width. The cells swim without a whirling movement. The epicone is hemispherical or dome-shaped and its height is approximately one-third of the total cell length (Fig. 1A-D). The epicone has a projected apex produced by the edge of the apical groove (Fig. 1A, C, D). The width of the epicone is slightly smaller than that of the hypocone (Fig. 1A, C, D). The hypocone is somewhat rounded and bi-lobed at the posterior of the cell (Fig. 1B). The right side of the hypocone is larger than the left side (Fig. 1A, C, D) and curves gently at the junction of the cingulum and sulcus, while the left side of the hypocone is angulated (Fig. 1A, C, D). The cingulum is deeply indented, completely encircling the cell, and is slightly right-handed – when the lower edge of the cingulum on either side of the sulcus is compared, the left end of the cingulum border is lower than that of the right end. Both ends of the cingulum curve posteriorly at the junction with the sulcus, forming a wide V-shape (Fig. 1A, C, D) and with the right side of the cingulum being narrower than the left side (Fig. 1A, C, D). The sulcus is narrow and deep in the middle of the cell and expanded posteriorly. The sulcus in the epicone is deep and extends in an anterior direction slightly to the right before angling into a fine apical groove (Fig. 1A-D).

The cell possesses a nucleus at the anterior of the cell and large transparent starch

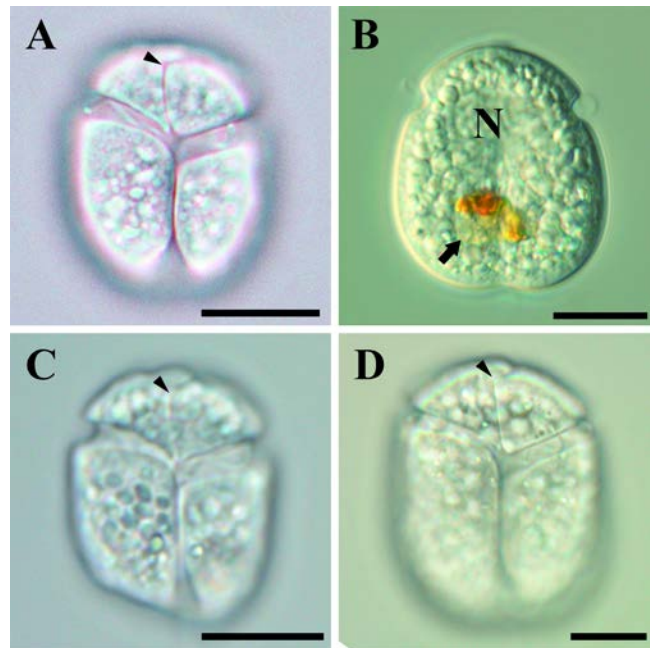


Fig. 1. Light micrographs of *Pellucidodinium psammophilum* gen. et sp. nov. Bar = 10 µm.

A. Cell collected from Aininkappu. Note the fine apical groove with the characteristic right-angled corner (arrowhead).

B. Another cell from Aininkappu showing the anterior nucleus (N) and food vacuole-like granules (arrow).

C & D. Cells from Cape Nosappu and Senjoiwa respectively, showing the fine apical groove (arrowhead).

granules are present throughout the cytoplasm (Fig. 1B). The cells possess neither chloroplast nor kleptochloroplast (Fig. 1A-D), but occasionally, yellow-brown granules or food-like granules are observed in the cell (Fig. 1B).

SEM observation shows that *Pellucidodinium psammophilum* has no apparent thecal plates but a large number of small, polygonal amphiesmal vesicles appear to cover the cell surface (Fig. 2A, D). The left side of the cingulum is broader than the right side and the slight right-handed displacement observed by light microscopy is confirmed (Fig. 2A). The sulcus is deep, broadening toward the anterior of the cell (Fig. 2A) and extends to the antapex where it widens abruptly, resulting in the notched hypocone (Fig. 2A). The sulcus extends anteriorly from the junction of the cingulum, with a slight deflection to the right side, and connects with the apical groove (Fig. 2A, B). The apical groove starts near the terminal point of the sulcal extension and runs in a counterclockwise direction, forming an indented semicircle around the apex (Fig. 2B). The anterior leading margin of the apical groove produces a distinct edge that projects

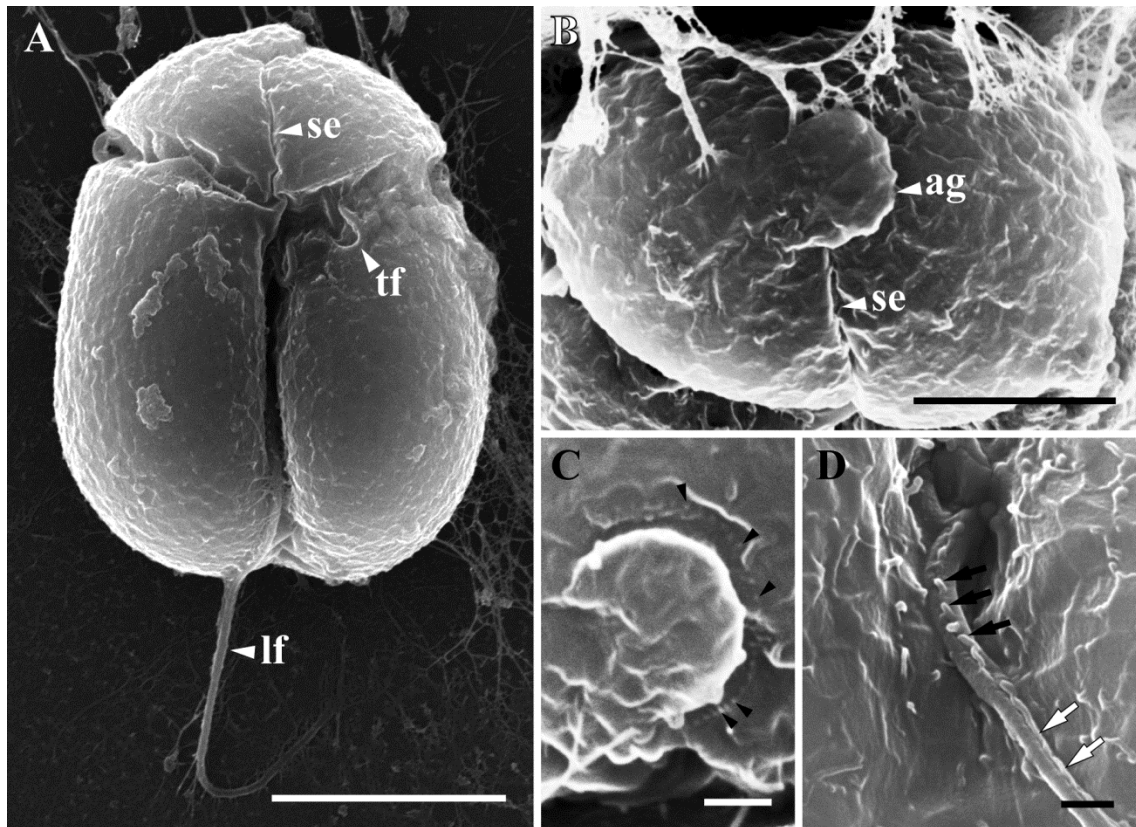


Fig. 2. Scanning electron micrographs of *Pellucidodinium psammophilum* gen. et sp. nov.

A. Ventral view of the cell showing the two flagella (tf; transverse flagellum, lf; longitudinal flagellum), the notched posterior part of the hypocone and the fine sulcal extension in the epicone (se). Bar = 10 μ m.

B. Apical view of the cell showing the anti-clockwise apical groove (ag). Bar = 5 μ m.

C. Detail of an apical groove showing small knob-like projections (arrowheads) along its length. Bar = 1 μ m.

D. Detail of the longitudinal flagellum showing the flagellar ridge running along the flagellum (white arrows) and that is replaced more proximally by a line of papillae (black arrows). Bar = 1 μ m.

slightly from the epicone (Fig. 2A, B). Along the apical groove, small ‘knobs’ of uncertain number are observed (Fig. 2C). The longitudinal flagellum possesses projecting ridge along the length of the longitudinal flagellum that becomes discontinuous and papillate near its proximal insertion (Fig. 2D).

The general ultrastructure of *Pellucidodinium psammophilum* is shown in Fig. 3. The cell has typical dinoflagellate structures, including the typical dinokaryon that contains condensed chromosomes. The nucleus is located in the upper part of the cell (Fig. 3A, B). *Pellucidodinium psammophilum* possesses vesicular chambers in the nuclear envelope (Fig. 3C). The pusule composes of a collecting chamber and tubular pusular vesicles, located below the nucleus (Fig. 3A). The hypocone of the cell contains

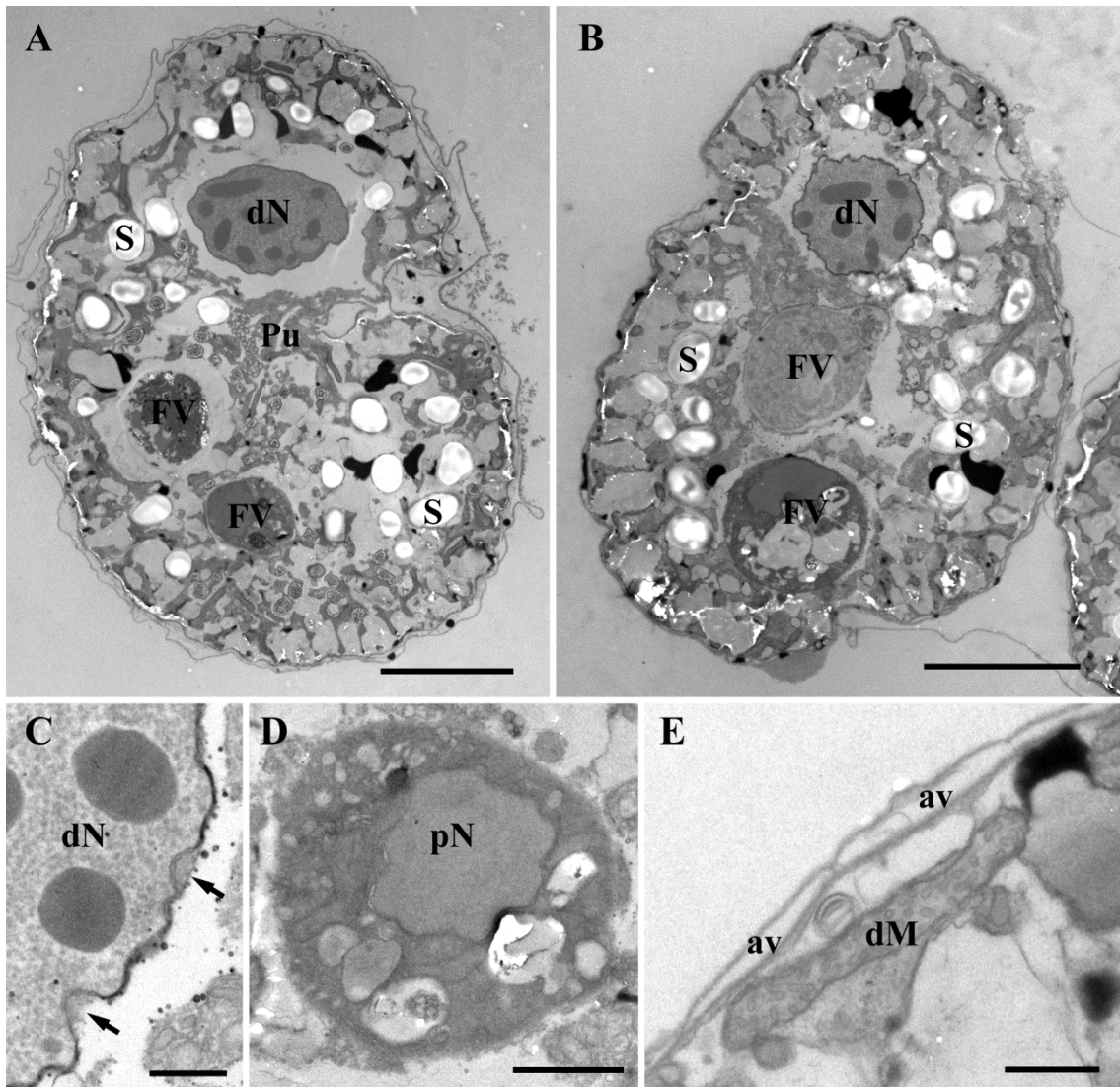


Fig. 3. Transmission electron micrographs of *Pellucidodinium psammophilum* gen. et sp. nov.

A. Longitudinal section (LS) showing dinoflagellate nucleus (dN), pusule (Pu), starch grains (S) and food vacuole with homogenous contents (FV). Bar = 5 μ m.

B. LS of a cell that has ingested a non-photosynthetic protist (FV). Bar = 5 μ m.

C. Nuclear envelope showing nuclear chambers (arrow). Bar = 500nm.

D. Food vacuole with nucleated (pN) but non-photosynthetic protist. Bar = 1 μ m.

E. Periphery of a cell showing the amphiesmal vesicles (av) without thecal plates and mitochondria with tubular cristae (dM). Bar = 500 nm.

numerous starch granules and occasionally several food vacuoles are observed (Fig. 3A, B). Most of food vacuoles in cells from natural populations contain homogeneous contents making the identity of prey organisms impossible to determine (Fig. 3A, B). One vacuole is observed with an ingested organism that possesses a clear nucleus and apparently lacks a chloroplast (Fig. 3D), but details of its ultrastructure are insufficient to allow certain identification. The amphiesmal vesicles contain no apparent plate-like structure (Fig. 3E). A mitochondrion with tubular cristae is observed (Fig. 3E).

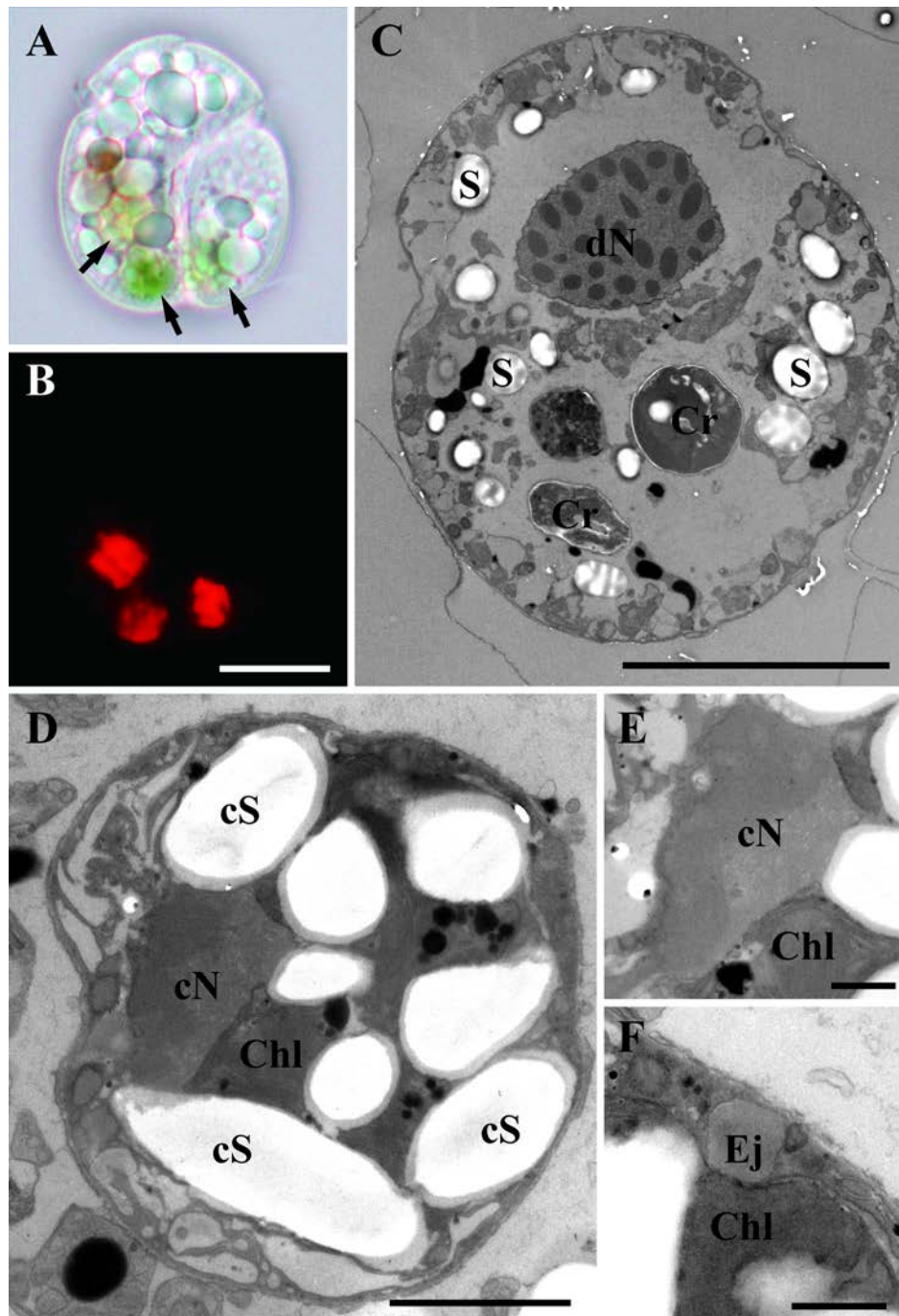


Fig. 4. Light and transmission electron micrographs of *Pellucidodinium psammophilum* collected from Aininkappu after ingestion of *Chroomonas* sp. (strain Ak01).

A-B. Bright-field and fluorescence micrographs of the same cell, showing three ingested cryptomonad cells (arrow) with irregular outline. Bar = 10 μ m.

C. Transmission electron micrograph (TEM) of a cell that has ingested cryptomonad cells (Cr). Note the dinoflagellate nucleus (dN) and starch granules (S). Bar = 10 μ m.

D. TEM of an ingested cryptomonad cell showing cryptomonad nucleus (cN), chloroplast (Chl) and large cryptomonad starch grains (cS). Bar = 2 μ m.

E. Detail of the cryptomonad nucleus (TEM), showing homogenous nucleoplasm. Bar = 500 nm.

F. Detail of the periphery of the ingested cryptomonad cell (TEM), showing digested chloroplast (Chl) and ejectosome (Ej). Bar = 500 nm.

LM and TEM observations of *Pellucidodinium psammophilum* after ingestion of cryptomonad cells

When *Pellucidodinium psammophilum* was fed with *Chroomonas* sp. (strain Ak01), it actively ingested several cells of prey (Fig. 4A) although the process of the ingestion was never directly observed. The cryptomonad cells in the dinoflagellate became spherical (Fig. 4A, B) but never enlarged. Although the ingested cryptomonad initially autofluoresced (Fig. 4B), the cryptomonad cells became granulated and were digested within 2 days. A single dinoflagellate cell was successfully cultured in the presence of *Chroomonas* sp. (strain Ak01) until approximately 30 cells were present, but the cells then stopped feeding and eventually died off within about 10 days. As a result culture strains could not be established.

Approximately 48 h after the cryptomonad cells had been ingested, the prey cells accumulated large starch granules (Fig. 4C). Although they contained a chloroplast, a nucleus (Fig. 4D, E) and the large gullet ejectosomes (Fig. 4F), they showed clear signs of degeneration relative to those of the original cryptomonad. The contents of cryptomonad nucleus became homogeneous due to digestion (Fig. 4D, E) and the chloroplast was so degenerated that the membranes of the thylakoids and the envelope could not be clearly defined (Fig. 4E, F). Other cryptomonad organelles were not distinguishable.

Description

***Nusuttodinium desymbiontum* Onuma, K. Watanabe & Horiguchi sp. nov.**

Figs 5-7

DESCRIPTION: Unarmoured dinoflagellate. Cells dorsoventrally flattened, elliptical, 21-30 μm long, 19-24 μm wide; epicone about one-quarters of total cell length, narrower than hypocone; apical groove running in a counterclockwise direction and creating a raised apical region, reminiscent of a beret; the anterior part of hypocone rounded; cingulum deep and broad, with no displacement; sulcus deep, broader at the posterior end and only covering three-quarters of the hypocone length; peduncle present; nucleus spherical, centrally-located but leaning to the left; nuclear chambers

absent; neither chloroplast nor kleptochloroplast present.

HOLOTYPE: The SEM stub used to prepare Fig. 6A deposited in the Herbarium of Faculty of Science, Hokkaido University as SAP114774. The specimen was collected at 2nd March 2012.

TYPE LOCALITY: Ishikari beach, Ishikari City, Hokkaido Prefecture, Japan (43°15'26"N: 141°21'26"E)

PARATYPE: Fig. 5A

HABITAT: marine and sand-dwelling

ETYMOLOGY: Latin *de* (away from) and *symbiontum* (symbiont), in reference to the lack of a kleptochloroplast (cryptomonad symbiont)

Morphological observations of *Nusuttodinium desymbiontum* sp. nov. (DIS)

The cell collected at Ishikari beach, Ishikari City, Hokkaido Prefecture and the cell collected at a sandy beach at Abuta Town, Hokkaido Prefecture are shown in Fig. 5A-B and Fig. 5C-D, respectively (No LM data of the cell collected from Fukaura Town exist because the cell was directly used for single-cell PCR). The cells of *Nusuttodinium desymbiontum* are dorsoventrally flattened, and elliptical in ventral view, measuring 16.3-21.3 μm (mean = $18.9 \pm 1.7 \mu\text{m}$ $n=8$) in length and 11.5-17.0 μm (mean = $14.5 \pm 1.7 \mu\text{m}$ $n=8$) in width. The epicone is broadly rounded, about one-fourth of the total cell length in height and narrower than the hypocone (Fig. 5A, C). The apex of the epicone, outlined by the edge of the apical groove, protrudes in a manner reminiscent of a beret (Fig. 5B, D). The hypocone is rounded (Fig. 5B, D) with its right side somewhat angulated at the junction of the cingulum and sulcus while the junction on the left side is gently curved (Fig. 5A, C). The broad and deeply-indented cingulum encircles the cell completely (Fig. 5A, C). The upper side of the cingulum is left-handed, while the lower side of the cingulum right-handed (Fig. 5A, C). The right side of the cingulum tapers toward the junction of the cingulum and the sulcus, and resulted in the left end of the cingulum being twice as wide as the right end (Fig. 5A, C). The sulcus is broad and deep, wider toward the antapex of the cell, but only approximately three-quarters of the length of the hypocone. Although the sulcus does not reach the antapex, a vaguely



Fig. 5. Light micrographs of *Nusuttodinium desymbiontum* sp. nov. **A, B & C, D.** A through-focus of two cells, one from Ishikari beach and the other from Abuta Town respectively. Note the projection on the epicone (arrowhead) and the nucleus (N). Bar = 10 μ m.

notched posterior end has been occasionally observed in the hypocone (Fig. 5A-D). However, especially in smaller cells, this very slight indentation could not be observed. The deep sulcus extends from the junction with the cingulum into the epicone (Fig. 5A, C).

The nucleus is located on the left side of mid-region of the cell (Fig. 5C, D). The cells possess neither chloroplasts nor kleptochloroplasts (Fig. 5A-D), but contain numerous starch granules (Fig. 5C, D). No autofluorescence was detected in field-sampled cells of *N. desymbiontum* (not shown). When *N. desymbiontum* was cultured with *Chroomonas* sp. (strain Ak01) and *Rhodomonas* sp. (strain Mr06), the cells were never witnessed to ingest the cryptomonad cells. I attempted to maintain *N. desymbiontum* cells in crude culture, and found that the cells frequently bumped with the epicone into sand grains or the bottom of culture vessel first, which resulted in them immediately attaching to the substrate and ceasing any swimming activity. This behaviour was reminiscent of the feeding behaviour often found in species of *Nusuttodinium* (R. Onuma personal observation), but I could not identify any potential prey. As a result I was unable to establish any cultures of *N. desymbiontum*.

SEM observation reveals that the cell is elliptic in shape without any apparent thecal plates (Fig. 6A). The cingulum is broad and deep, with a right end narrower than the left (Fig. 6A). The sulcus is deep, up to three-quarters the length of the hypocone (Fig. 6A). The hypocone is rounded, and a notched antapex observed in LM observation is difficult to be recognised (Fig. 6A). The peduncle projects anteriorly from the junction of the sulcus and the cingulum and the longitudinal flagellum emerges from the centre of the cell (Fig. 6A). The sulcus extends toward the apex from the junction of the cingulum and the sulcus where it connects with the apical groove (Fig. 6B). The apical groove extends from a point slightly to the right of the cell's midline in the apex,

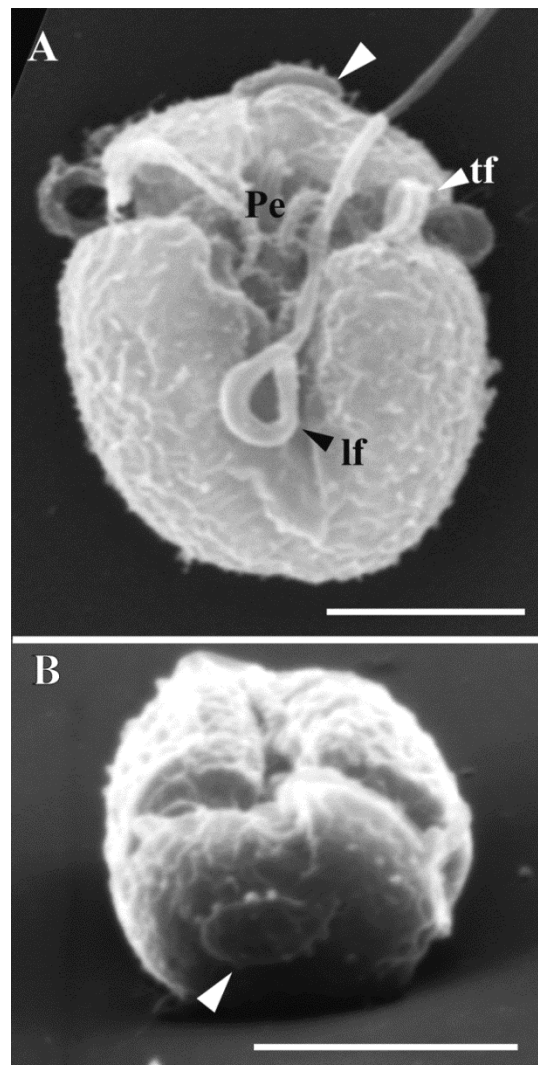


Fig. 6. Scanning electron micrographs of *Nusuttodinium desymbiontum* sp. nov.

A. Ventral view of the cell showing the two flagella (tf; transverse flagellum, lf; longitudinal flagellum), the peduncle (Pe) and the projection on the epicone (white arrowhead). Bar = 5 µm.

B. Apical view of the cell showing the anti-clockwise apical groove (white arrowhead). Bar = 5 µm.

running in an anticlockwise direction as an encircling ellipse in the apex (Fig. 6B), to terminate near the starting point, but not coincident with it (Fig. 6B). The apex encircled by the apical groove is elevated relative to the rest of the epicone, presenting a beret-like appearance in ventral view (Fig. 6A).

TEM observation shows that the cell of *Nusuttodinium desymbiontum* has typical dinoflagellate structures. The nucleus is located in the left side of the cell (Fig. 7A).

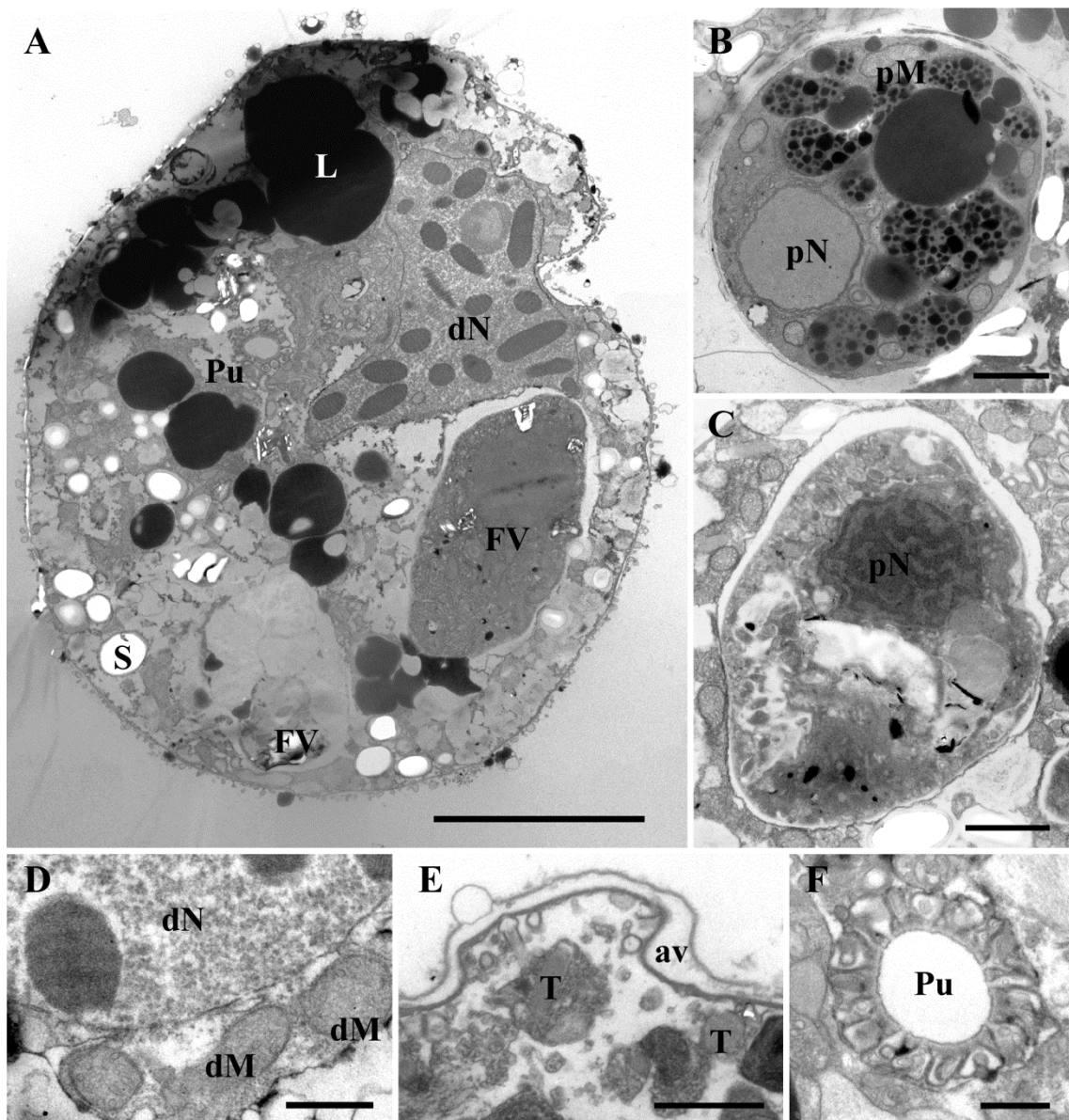


Fig. 7. Transmission electron micrographs of *Nusuttodinium desymbiontum* sp. nov.

A. LS of a cell showing dinoflagellate nucleus (dN), pusule (Pu), starch grains (S), lipid granule (L) and food vacuole (FV). Bar = 5 μ m.

B. Ingested organism, showing the nucleus (pN) and mitochondria with tubular cristae (pM). Bar = 1 μ m.

C. Ingested organism in the food vacuole, showing its nucleus (pN) with heterochromatin. Bar = 1 μ m.

D. Dinoflagellate nuclear envelope without nuclear chambers and dinoflagellate mitochondria with tubular cristae (dM). Bar = 500 nm.

E. Periphery of the dinoflagellate cell showing trichocysts (T) and amphiesmal vesicles (av) without thecal plates. Bar = 1 μ m.

F. Pusule (Pu) consisting of a collecting chamber and pusular vesicles. Bar = 500 nm.

The cells have numerous lipid and starch granules distributed throughout the cell, and have several food vacuoles restricted to the hypocone (Fig. 7A). The food vacuoles contain at least two species of protists, all of them apparently lacking a chloroplast, i.e. *N. desymbiontum* ingested heterotrophic organisms (Fig. 7A-C). The ingested organisms in the food vacuole are either uncharacterisable due to digestion (Fig. 7A), or possess mitochondria with tubular cristae and a nucleus without condensed chromatin (Fig. 7B), or have a nucleus with heterochromatin (Fig. 7C). The protists shown in Fig. 7B and C were apparently different species each other based on the structure of the nucleus (Fig. 7B, C). None of the features are sufficient to accurately identify the prey species. The nuclear envelope of *N. desymbiontum* has a smooth surface with no nuclear chambers (Fig. 7D). The mitochondria have typical tubular cristae (Fig. 7D) and trichocysts are located throughout the cell, just internal to the amphiesmal vesicles (Fig. 7E). No thecal plates are detected in the amphiesmal vesicles (Fig. 7E) and the pusule is composed of a collecting chamber with radiating tubular pusular vesicles (Fig. 7F).

Phylogenetic analyses

The phylogenetic analysis of the SSU rDNA data was performed using currently-determined sequences *Pellucidodinium psammophilum*, *Nusuttodinium desymbiontum*, *N. aeruginosum*, *N. amphidinioides*, *N. latum* and *N. poecilochroum* together with many other sequences of various taxa downloaded from Genbank. All the currently-determined sequences were included in the so called *Gymnodinium*-clade *sensu* Daugbjerg *et al.* (2000) (*Gymnodinium* s.s. clade) (Fig. 8), supported by a high bootstrap value (BS) (BS; 89%) but not by posterior probability (PP). In the *Gymnodinium* s. s. clade, *P. psammophilum* and *N. desymbiontum* formed a clade with the unarmoured kleptochloroplastidic *Nusuttodinium* spp. and the photosynthetic *Spiniferodinium* spp. and *Gymnodinium palustre* (this clade, with high support, is hereafter referred to as the SPN clade). The subclades consisting of *Spiniferodinium* spp. and *G. palustre*, and *P. psammophilum* and *Nusuttodinium* spp. were similarly well supported. Within the *Pellucidodinium/Nusuttodinium* clade, there was significant

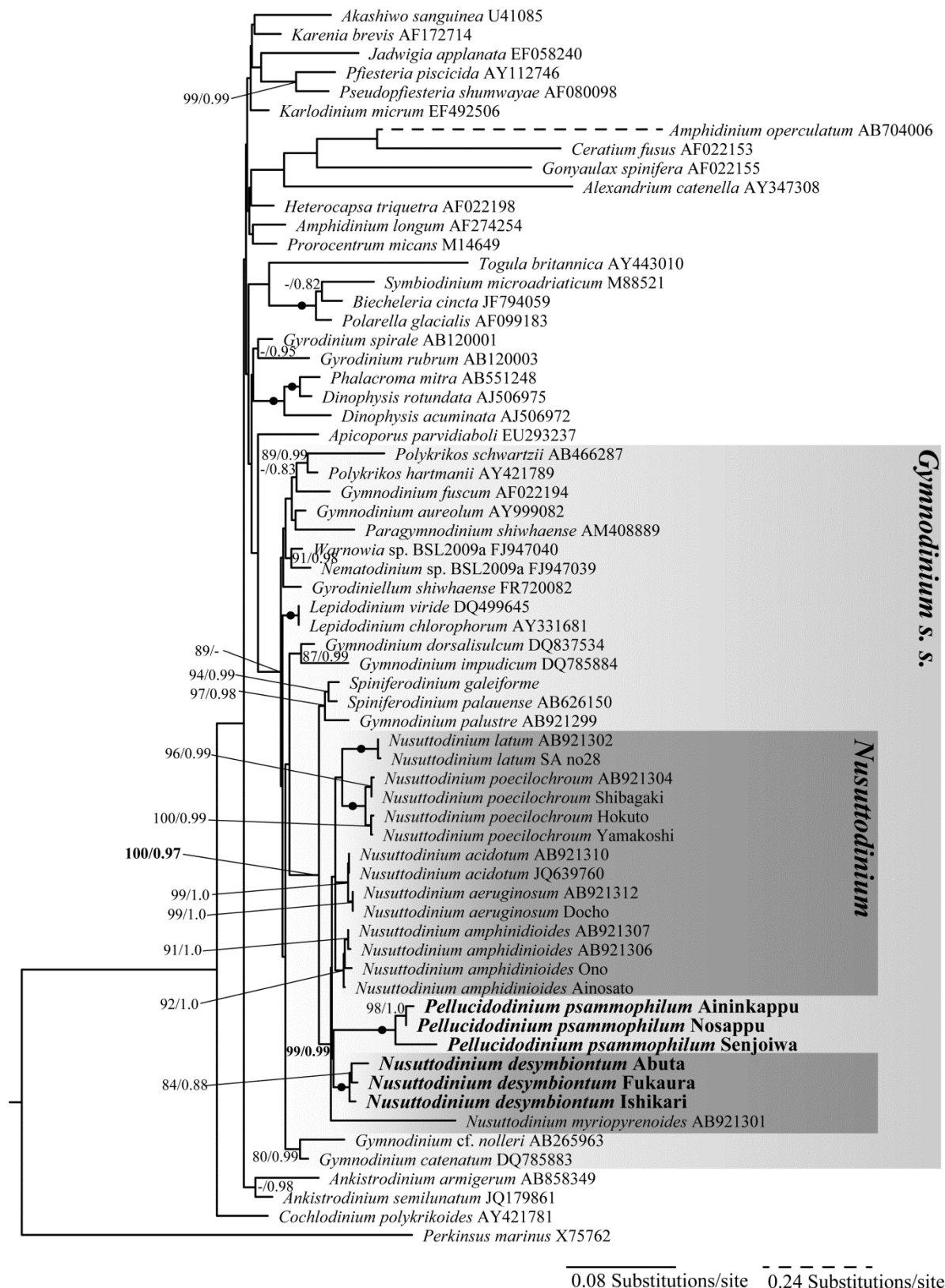


Fig. 8. Maximum likelihood tree inferred from SSU rDNA sequences. Various isolates of *Pellucidodinium psammophilum* and *Nusuttodinium desymbiontum* are indicated in bold. The bootstrap (BS) and Bayesian posterior probability values (PP) are provided at each node (BS/PP). Black circles at nodes indicate full support by BS and PP (BS/PP = 100%/1.0). The dashed line has been shortened to one-third of its length.

support (both BS and PP) for each species clade. Although *P. psammophilum* and *N. desymbiontum* formed a clade, the support was not as convincing and, likewise, the relationships between the various species could not be resolved. The clade of *P. psammophilum* and *Nusuttodinium* was only distantly related to *Amphidinium sensu stricto* that includes the type species, *A. operculatum* (Fig. 8).

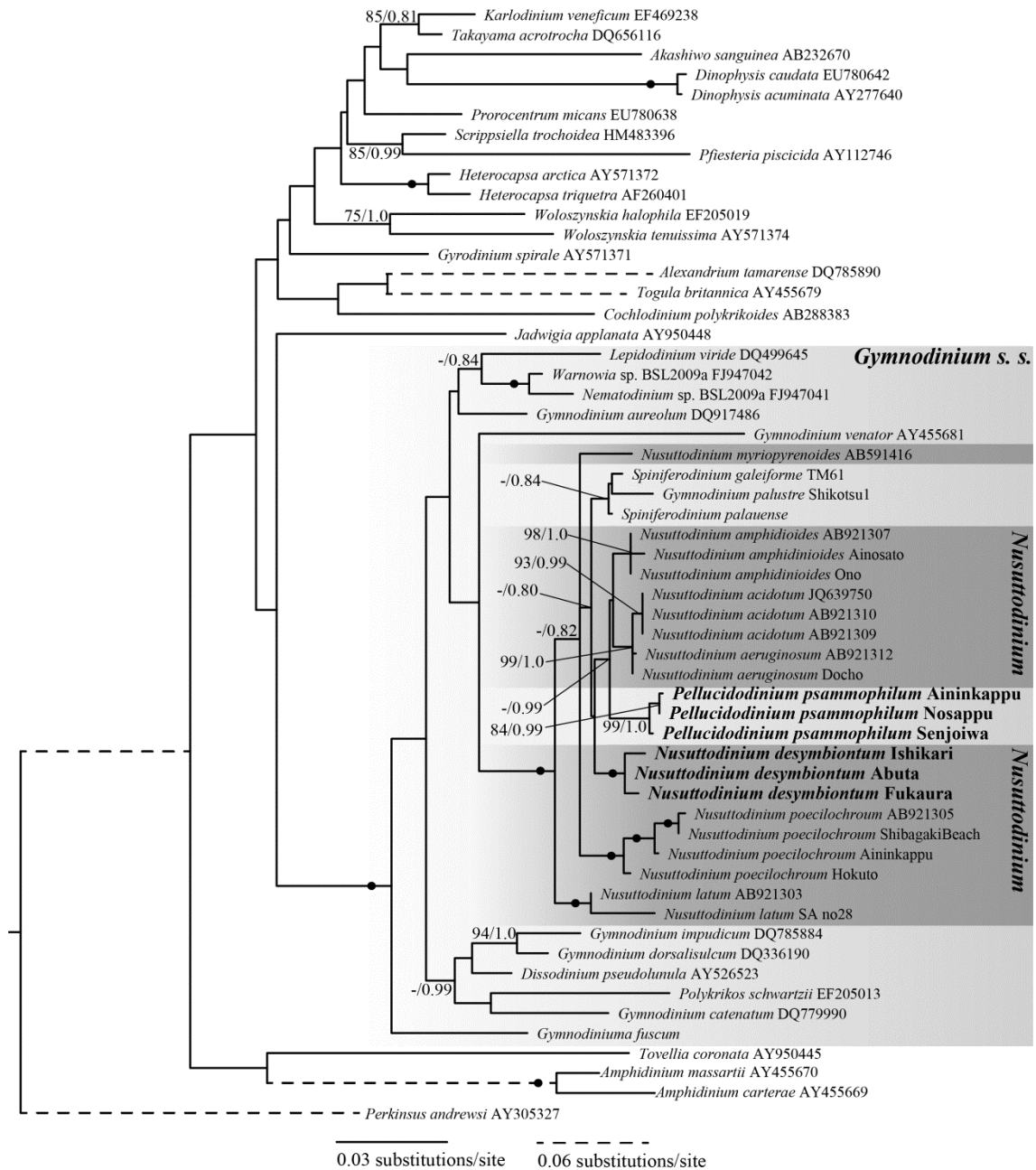


Fig. 9. Maximum likelihood tree inferred from LSU rDNA sequences. *Pellucidodinium psammophilum* and *Nusuttodinium desymbiontum* are indicated in bold. The bootstrap (BS) and Bayesian posterior probability values (PP) are provided at each node (BS/PP). Black circles at nodes indicate full support by BS and PP (BS/PP = 100%/1.0). The dashed line has been shortened to a half of its length.

For the analysis of LSU rDNA (Fig. 9), the sequences of *Pellucidodinium psammophilum*, *Nusuttodinium desymbiontum*, *N. aeruginosum*, *N. amphidinioides*, *N. latum* and *N. poecilochroum* represent newly-determined data. This analysis also recovered a SPN clade with full BS and PP support, but *Spiniferodinium* spp., *G. palustre* and *P. psammophilum* were nested within species of *Nusuttodinium* (Fig. 9). *Pellucidodinium* and freshwater representatives of *Nusuttodinium* formed a clade with relatively high support (PP; 0.99), with *N. desymbiontum* rooted at the base of this clade, but once again, the branching order within this clade was not clearly determined. For the analyses of *cox1* and *cob* genes, all sequences except those of the outgroup were new. In the phylogenetic tree inferred from *cox1* gene (Fig. 10), each of the marine kleptochloroplastidic species *N. latum* and *N. poecilochroum* formed a fully supported clade. In this phylogenetic tree, too, *Spiniferodinium* and *Pellucidodinium* were each

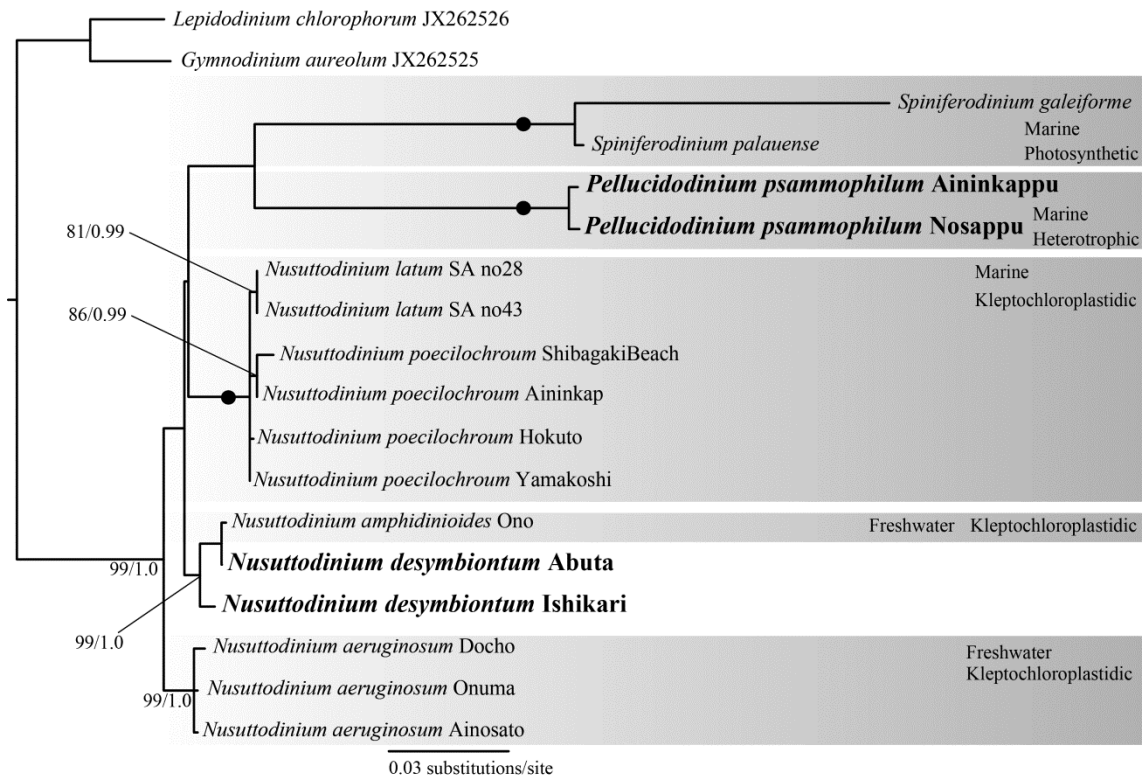
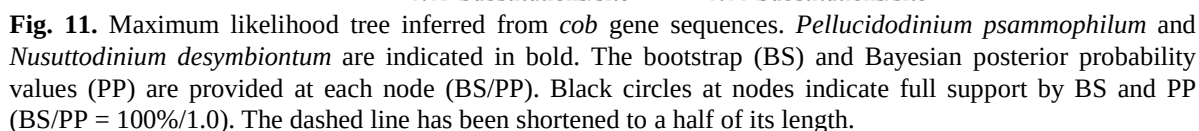


Fig. 10. Maximum likelihood tree inferred from *cox1* gene sequences. *Pellucidodinium psammophilum* and *Nusuttodinium desymbiontum* are indicated in bold. The bootstrap (BS) and Bayesian posterior probability values (PP) are provided at each node (BS/PP). Black circles at nodes indicate full support by BS and PP (BS/PP = 100%/1.0). The dashed line has been shortened to a half of its length.



kleptochloroplastidic species was well-supported (BS; 98 %, PP; 1.0). *N. desymbiontum* was a sister to the marine kleptochloroplastidic clade, but this did not allow reasonable support. *P. psammophilum* positioned as a sister of *Nusuttodinium* spp. although the BS was less than 50 % (PP; 0.91).

To ascertain the branching order of *Pellucidodinium psammophilum* and *N. desymbiontum* in relation to *Nusuttodinium* spp. and *Spiniferodinium* spp., the phylogenetic analysis of concatenated genes (dataset1; SSU rDNA + LSU rDNA + *cox1* + *cob*) was performed (Fig. 12). This analysis also recovered *N. desymbiontum* as a monophyletic clade together with other members of *Nusuttodinium*. Although this clade was supported with full PP, the BS support was not high (64 %). Within this clade, the marine kleptochloroplastidic species formed a robust clade (BS; 100 %, PP; 1.0), while the freshwater kleptochloroplastidic clade was supported by moderate BS value (BS; 71 %, PP; 1.0). *N. desymbiontum* positioned as a sister to the marine kleptochloroplastidic clade, but with a low BS support (BS; 48 %, PP; 0.95). This

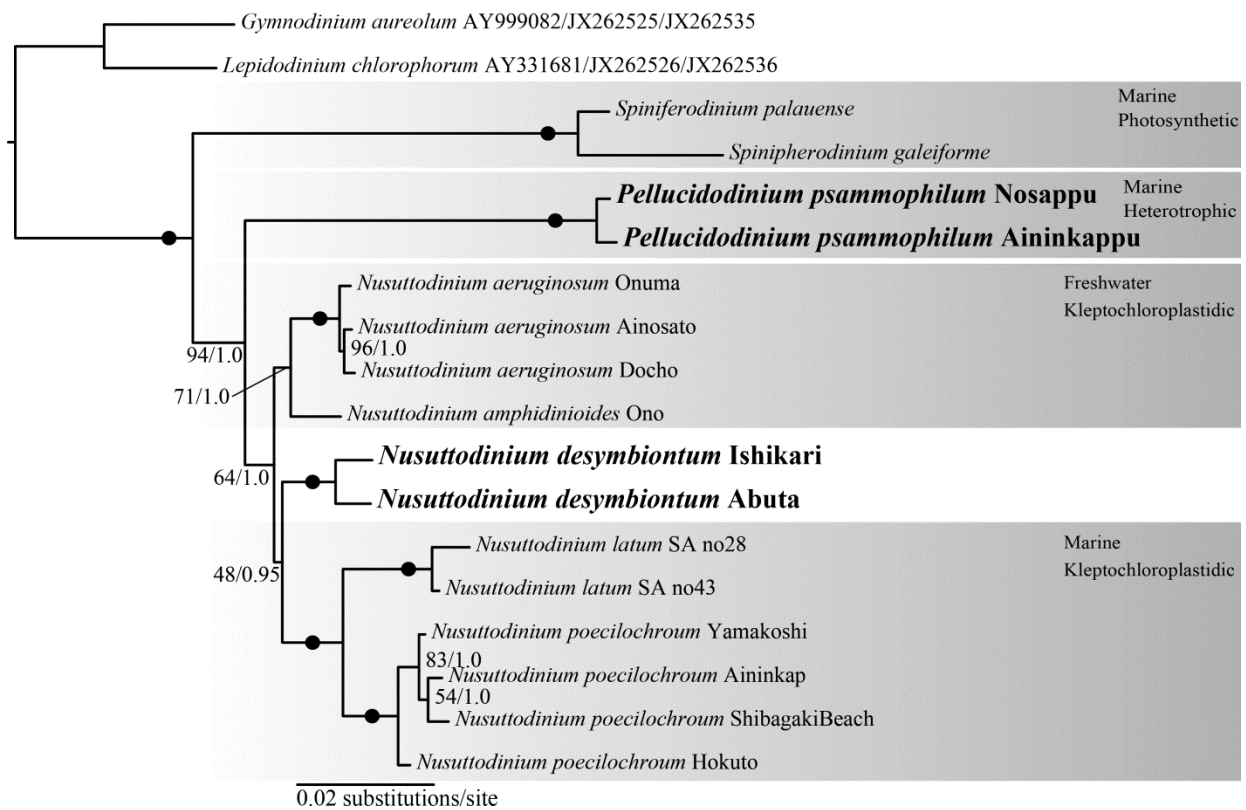


Fig. 12. Maximum likelihood tree inferred from SSU rDNA + partial LSU rDNA + *cox1* + *cob* gene sequences. *Pellucidodinium psammophilum* and *Nusuttodinium desymbiontum* are indicated in bold. The bootstrap (BS) and Bayesian posterior probability values (PP) are provided at each node (BS/PP). Black circles at nodes indicate full support by BS and PP (BS/PP = 100%/1.0).

analysis also indicated that *Pellucidodinium* and *Nusuttodinium* were monophyletic with high support values (BS; 94 %, PP; 1.0) and that *Spiniferodinium* spp. rooted, with full support, at the base of the *Pellucidodinium*/*Nusuttodinium* clade (Fig. 12). I also performed the phylogenetic analysis inferred from another concatenated dataset (dataset2; SSU rDNA + *cox1* + *cob*) (Fig.13). The tree topology is similar to that of the 4 gene concatenated tree (Fig. 12) in that it recovers a robust SPN clade (BS; 100%, PP; 1.0) and a *Pellucidodinium*/*Nusuttodinium* clade (BS; 97 %, PP; 1.0). However, the *Nusuttodinium* clade here has strong support (BS; 95 %, PP; 1.0). The marine kleptochloroplastidic species also find strong support (BS; 100 %, PP; 1.0), but the freshwater species are not well supported. *Nusuttodinium desymbiontum* was recovered as a sister to the freshwater species but with very limited support (Fig. 13).

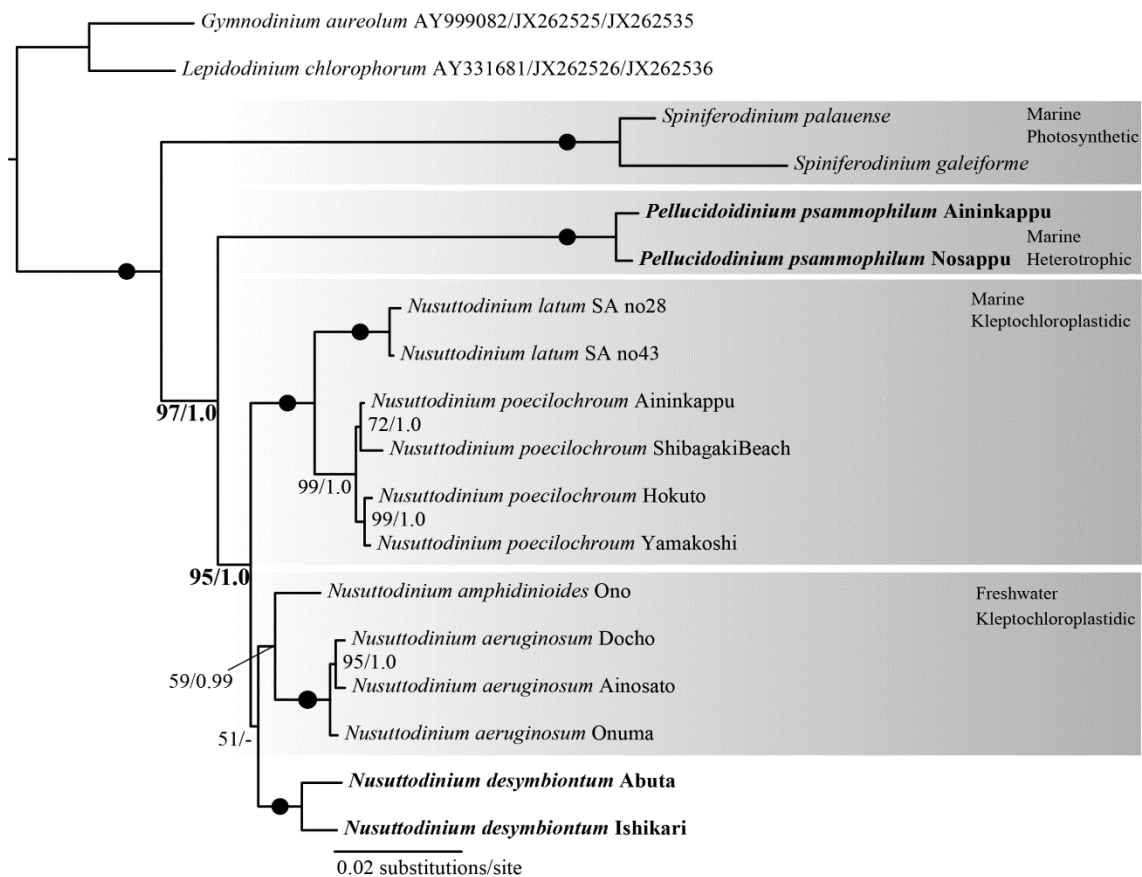


Fig. 13. Maximum likelihood tree inferred from SSU rDNA + *cox1* + *cob* gene sequences. *Pellucidodinium psammophilum* and *Nusuttodinium desymbiontum* are indicated in bold. The bootstrap (BS) and Bayesian posterior probability values (PP) are provided at each node (BS/PP). Black circles at nodes indicate full support by BS and PP (BS/PP = 100%/1.0).

DISCUSSION

Taxonomy of *Pellucidodinium psammophilum* gen. et sp. nov. (DAI)

According to the traditional classification of unarmoured dinoflagellates, DAI should belong to the genus *Amphidinium sensu lato*, because the epicone is almost one-third of the total cell length (e.g. Kofoid & Swezy 1921; Lebour 1925) and therefore, on morphological grounds, it needs to be compared with other species of *Amphidinium s. l.*. The following species may be related to DAI, *A. flexum* Herdman, *A. dentatum* Kofid & Swezy, *A. lissae* Schiller and *Gymnodinium venator* Flø Jørgensen & Murray (= previously named as *A. pellucidum* Herdman). *Amphidinium flexum* is colourless and has the bi-lobed hypocone that is slightly notched by the sulcus. However, this species possesses a twisted hypocone (i.e. the left side of the hypocone is deflected dorsally while the right side is deflected ventrally) and the nucleus lies in the posterior region of the cell. Moreover, *A. flexum* is larger (42-60 µm) than DAI, and the hypocone is twisted (Herdman 1924, Dodge 1982). *Amphidinium dentatum* and *A. lissae* are described as possessing 'blue-green' chloroplasts and in this respect, they are different from DAI. However, morphologically they have several similarities (Kofoid & Swezy 1921; Schiller 1933). *Amphidinium dentatum* has the same narrow and abruptly-widened sulcus as DAI, but differs by having a much larger cell and a triangular epicone (Kofoid & Swezy 1921). *Amphidinium lissae* resembles DAI in having an asymmetrical shape of the cingulum edge of the hypocone side, i.e., the right side of the hypocone curves gently at the junction of the cingulum and sulcus, while the left side is angulated. *Amphidinium lissae* differs from DAI, by the possession of an eyespot and a rounded hypocone. Furthermore, its sulcus dose not reach up to the hypocone end and extends into the epicone and tapers as it extends into the epicone (Schiller 1933). On the other hand, DAI possesses a bi-lobed hypocone and the sulcus reaches the antapex of the cell. The sulcal extension is apparent and the base of the sulcal extension does not narrow as it invades the epicone. Moreover, an eyespot is absent in DAI. *Gymnodinium venator* has an epicone that is one third the length of the cell, an apical groove with a right-angled turn to it, a hypocone notched by the sulcus

and it lacks chloroplasts (Herdman 1924; Flø Jørgensen *et al.* 2004). Although all these characters are reflected in DAI, *G. venator* differs from DAI by having the left side of its hypocone overlapping the sulcus (Herdman 1924; Flø Jørgensen *et al.* 2004).

Of the members of the genus *Nusuttodinium*, *N. amphidinioides* and *N. latum* resemble DAI. The sulcus of *N. amphidinioides* is narrow, extending deeply to the apex and the position of the cingulum along the length of the cell is almost same as that of DAI. However, it differs from DAI in its habitat (freshwater) and in having a rounded hypocone without a notch formed by the sulcus (Takano *et al.* 2014). *Nusuttodinium latum* is a marine sand-dwelling species with a hypocone notched by the sulcus, but it has a broadly-rounded epicone (Horiguchi & Pienaar 1992), which is different from the dome-shaped epicone of DAI. Although the *Nusuttodinium* spp. mentioned above possess some morphological similarities with DAI, the phylogenetic analysis clearly indicated that DAI is different. In addition, DAI has a larger right side to its hypocone and a right-handed cingulum, both characters that are never observed in *Nusuttodinium*, and DAI can be distinguished from other species that morphologically resemble it (see Table 3). Therefore, I conclude that DAI should be described as a new species.

As shown in this study, DAI possesses a counterclockwise apical groove and vesicular chambers in its nuclear envelopes, characters which affiliate it with *Gymnodinium sensu stricto*. Daugbjerg *et al.* (2000) redefined the genus *Gymnodinium* as unarmoured dinoflagellates possessing a horseshoe-shaped apical groove running in a counterclockwise direction, cingulum displacement, vesicular chambers in the nuclear membranes and the presence of a nuclear or dorsal fibrous connective. According to recent studies, several genera including *Chytriodinium*, *Dissodinium*, *Gymnodinium sensu stricto*, *Gyrodiniellum*, *Lepidodinium*, *Nematodinium*, *Paragymnodinium*, *Polykrikos*, *Spiniferodinium* and *Warnowia* were found to form a robust clade, the so-called *Gymnodinium* clade (Daugbjerg *et al.* 2000; Gómez *et al.* 2009; Hoppenrath *et al.* 2009; Kang *et al.* 2010; Horiguchi *et al.* 2011; Kang *et al.* 2011). These genera share a counterclockwise apical groove (Daugbjerg *et al.* 2000). DAI is included in the *Gymnodinium* clade based on its morphological similarity and the phylogenetic analyses. Of the other representatives of this clade, *Chytriodinium* and *Dissodinium* have a

parasitic stage in their life cycle (Gómez *et al.* 2009). *Lepidodinium* possesses a chloroplast derived from a green alga (Watanabe *et al.* 1990). *Nematodinium* is characterised by the possession of nematocysts and an ocelloid, the latter of which is shared by *Warnowia* (Dodge 1982; Hoppenrath *et al.* 2009). *Polykrikos* is defined by the possession of a pseudocolonial organisation (Dodge 1982). It is clear that DAI is not parasitic, does not possess nematocysts, chloroplasts or a pseudocolonial organisation. Therefore, DAI does not belong to any of the genera *Chytriodinium*, *Dissodinium*, *Lepidodinium*, *Nematodinium*, *Polykrikos* and *Warnowia*. *Paragymnodinium* and *Gyrodiniellum* are also contained within the *Gymnodinium sensu stricto* clade, but the both genera lack nuclear chambers and possess nematocysts (Kang *et al.* 2010; Kang *et al.* 2011) and thus are not appropriate candidates to accommodate DAI. *Spiniferodinium* has strong affinities with DAI in respect of the morphology of its motile stage and its phylogenetic position, but is not an appropriate genus for DAI because it is photosynthetic and has a crustose stage in its cell cycle (Horiguchi & Chihara 1987). DAI has two unique characters, a right-handed cingulum and an apical groove originating as a right-angled extension of the sulcus. These characters differentiate it from species of *Gymnodinium* which have a left-handed cingulum displaced by one or more cingulum widths and a horseshoe-shaped apical groove (Daugbjerg *et al.* 2000). DAI is also not closely related to the type species of the *Gymnodinium*, *G. fuscum* (Ehrenberg) Stein, and so it is not appropriate to accommodate it in the genus. In summary, DAI has the following combination of characters; 1) a right-handed cingulum, 2) a right-angled apical groove, 3) no chloroplast and 4) nuclear chambers in the nuclear envelope. The shape of the apical groove resembles those of *Spiniferodinium palauense* Horiguchi, Hayashi, Kudo & Hara and *G. venator* (Murray & Patterson 2002; Horiguchi *et al.* 2011), but the right-handed cingulum is rarely found in any dinoflagellate described as *Gymnodinium* (Kofoid & Swezy 1921; Lebour 1925; Schiller 1933). I therefore believe that this unique combination of features together with the distinct phylogenetic position warrants the establishment of a novel genus for DAI. Here, I propose the new genus *Pellucidodinium* and classify DAI as a new species, *P. psammophilum*.

Taxonomy of *Nusuttodinium desymbiontum* sp. nov. (DIS)

DIS should be also compared with *Amphidinium sensu lato* because of the relatively-small epicone, and *A. flexum*, *A. globosum* Schröder and *Gymnodinium venator* may be related morphologically to this species. *Amphidinium flexum* is colourless and has a similar ratio of epicone length to cell length, but it differs from DIS in that it possesses a posterior nucleus, a larger cell size (42-60 μm) and an obvious notched hypocone. In addition, the left side of the hypocone overhangs to the right side and the sulcus is obscured by the overhanging left side of the hypocone (Herdman 1924, Dodge 1982). *Amphidinium globosum* is pigmented (yellow-brown), but nevertheless has morphological affinities with DIS and deserves comparison. It possesses a rounded hypocone, a deep cingulum and a broad sulcus as in DIS, but its sulcus tapers toward the hypocone while that of *N. desymbiontum* does not (Schiller 1933). *G. venator* has a similar elliptic cell and rounded hypocone, but its sulcus is overhung by the projection from the left side of the hypocone (Herdman 1922), which is not evident in DIS.

Within the genus *Nusuttodinium*, *N. amphidinioides* and *N. poecilochroum* have a similar morphology to DIS in that they have a small epicone and a cingulum that encircles the cell without any displacement. *Nusuttodinium amphidinioides* further resembles DIS in its cell size and the rounded hypocone. However, unlike DIS, it has a symmetrical epicone and is found in freshwater habitats (Schiller 1933; Takano *et al.* 2014). *Nusuttodinium poecilochroum* is also a marine sand-dwelling species, has a similar elliptic cell with a rounded hypocone, but the epicone to total cell length ratio is markedly reduced relative to that of DIS. In addition, DIS has the unique character of a beret-like projection at its apex. This is a novel character for all the dinoflagellates currently under discussion, and because DIS cannot be affiliated with any described dinoflagellate (Table 3), it should be described as a new species.

DIS is an unarmoured dinoflagellate that possesses a counterclockwise apical groove and that is nested in the *Gymnodinium sensu stricto* clade (Daugbjerg *et al.* 2000), but it can be distinguished from others in this clade because it lacks any of their key characters, such as nuclear chambers, chloroplasts, nematocysts and parasitic or

non-motile spine-bearing crustose cell stage. *Nusuttodinium* is a genus included in *Gymnodinium sensu stricto* that is characterised by retaining a kleptochloroplast, possessing an apical groove running in a counter-clockwise direction and having no nuclear chambers (Takano *et al.* 2014). This study indicated that DIS is phylogenetically nested in the *Nusuttodinium*-clade, but that it lacks a kleptochloroplast. The phylogenetic analysis suggests the assignment of DIS to the genus *Nusuttodinium*, but this would result in conflict with the current definition of the genus. However, two key characters for the genus *Nusuttodinium* were found in DIS, i.e. the shape of apical groove and the lack of nuclear chambers, and these, together with its phylogenetic position compel us to accommodate DIS within the genus *Nusuttodinium*, despite its phagotrophic nature. The trophic condition seen in DIS could be interpreted as the secondary loss of kleptochloroplastidy. There are examples of the loss of full chloroplasts within the same genus. For example, *Amphidinium incoloratum* Campbell is a heterotrophic species in *Amphidinium*, a mostly photosynthetic genus, which led to the suggestion that this species lost its chloroplast independently (Flø Jørgensen *et al.* 2004). The freshwater mixotrophic dinoflagellate, *Esoptrodinium*, has been found to exhibit geographic variation in respect of the presence or absence of an obvious chloroplast, suggesting the independent loss of the chloroplast at various ponds (Fawcett & Parrow 2012; 2014). These instances indicate that the presence or absence of a chloroplast is probably not a definitive character for classification at the generic level. Similarly, the absence of a kleptochloroplast in *N. desymbiontum* could be interpreted to be the result of a secondary loss of kleptochloroplastidy. I suggest emendation of the description of the genus *Nusuttodinium* to include species that lack kleptochloroplast (see below). With this new description I describe DIS as a new species of *Nusuttodinium*; *N. desymbiontum* sp. nov.

Nutritional strategy of *Pellucidodinium psammophilum* and *Nusuttodinium desymbiontum*

Pellucidodinium psammophilum and *Nusuttodinium desymbiontum* are here shown to be

closely-related to a clade comprising kleptochloroplastidic dinoflagellates of the genus *Nusuttodinium* spp. However, it is also demonstrated that the cells of both species were colourless, and never possessed a kleptochloroplast. All unarmoured kleptochloroplastidic dinoflagellates in natural populations have been reported to possess a kleptochloroplast (Wilcox & Wedmayer 1984, 1985; Larsen 1988; Schnepf *et al.* 1989; Farmer & Roberts 1990; Horiguchi & Pienaar 1992; Yamaguchi *et al.* 2011), and this situation suggests that *Nusuttodinium* spp. are substantially dependent on their prey (cryptomonad) chloroplast, and they have a certain degree of specificity regarding the selection of their cryptomonad prey. In fact, *Nusuttodinium aeruginosum*, *N. amphidinioides* and *N. poecilochroum* can be successfully cultured over extended periods in the presence of cryptomonads and they are frequently observed to ingest cryptomonad cells (Larsen 1988; Onuma & Horiguchi 2013; Takano *et al.* 2014). The ingested cryptomonad chloroplast tends to be modified or enlarged in the host cell cytoplasm for certain periods (Horiguchi & Pienaar 1992; Yamaguchi *et al.* 2011; Onuma & Horiguchi 2013). In contrast, *P. psammophilum* directly transfers the cryptomonad cell into a food vacuole without the concomitant enlargement of the chloroplast, and all organelles of the ingested cells are completely digested, including the chloroplast. *Nusuttodinium desymbiontum* in culture has never been observed to ingest a cryptomonad cell. Transmission EM observations confirmed that the food vacuoles of *P. psammophilum* and *N. desymbiontum* appeared to be homologous with those of other phagotrophic dinoflagellates (Jacobson & Andersen 1994). In addition, I noted that natural populations of both species have ingested non-photosynthetic protists. These facts suggest that, unlike members of *Nusuttodinium*, *P. psammophilum* and *N. desymbiontum* do not have a specific diet requirement restricted to cryptomonads and can phagocytose various protists. For these reasons, both species are considered to be phagotrophic rather than kleptochloroplastidic.

The evolution of the unarmoured kleptochloroplastidic dinoflagellates

The morphological observations and phylogenetic analyses in this study provided some insights into the evolution of the SPN clade. *Pellucidodinium psammophilum* is

apparently closely-related to the photosynthetic *Spiniferodinium palauense*. In this regard, it is worthwhile mentioning that the apical groove of *S. palauense* has a similar shape to that of *P. psammophilum*, i.e. it is semicircular, running in an anticlockwise direction and intersects at a right angle with the sulcal extension (Horiguchi *et al.* 2011). This suggests that the ancestor of *Spiniferodinium* and *Pellucidodinium* might have had this type of apical groove.

The nuclear chambers are a character shared by members of *Gymnodinium sensu stricto* and thought to be ancestral for this group (Daugbjerg *et al.* 2000). *Spiniferodinium*, positioned at the base of SPN clade, also has nuclear chambers (Horiguchi *et al.* 2011). Given this, the character of nuclear chambers must have been present in the common ancestor of *Pellucidodinium* and *Nusuttodinium* spp., although it was subsequently lost in the *Nusuttodinium* lineage as discussed by Takano *et al.* (2014), but retained in *Pellucidodinium*. The lack of nuclear chambers in *N. desymbiontum* supports its position in the phylogenetic trees recovered, as all other *Nusuttodinium* spp. also lack this feature.

The branching order within the SPN clade is difficult to interpret because different genes used for phylogenetic analyses showed different topologies. Previous studies showed that *Gymnodinium palustre* was nested in the *Nusuttodinium* clade in phylogenetic trees inferred from LSU rDNA (Yamaguchi *et al.* 2011; Xia *et al.* 2013). The phylogenetic tree of LSU rDNA also concurs with the position of *G. palustre* (including *Spiniferodinium galeiforme*) although no strong support (BS/PP) was obtained. Because *S. galeiforme* possesses a peridinin-containing chloroplast (Norico Yamada, pers. comm. 2013), The topologies inferred from LSU rDNA like in Fig. 9 implies that a peridinin-containing chloroplast was lost by the ancestor of the SPN clade and gained again by *Spiniferodinium* and/or *G. palustre*. Moreover, the phylogenetic tree inferred from LSU rDNA is not parsimonious with regard to colonisation of the freshwater habitat. Therefore, I believe that the LSU rDNA gene is not appropriate for the phylogenetic analysis of this group. Of all the analyses undertaken here, I believe that the phylogenetic tree inferred from SSU rDNA + *cox1* + *cob* has the most reasonable topology, because each node had high support and the evolution of character

states could be plotted on this phylogenetic tree in a most parsimonious fashion (Fig. 14). It should be mentioned that the concatenated tree including LSU rDNA (Fig. 12) and the SSU rDNA tree alone (Fig. 8) showed the same topology, although support for the branching order was not always high.

The phylogenetic analyses using concatenated datasets showed that the clade of photosynthetic *Spiniferodinium* spp. was rooted at the base of the SPN clade and that *Pellucidodinium psammophilum* was a sister to the *Nusuttodinium* clade, with *N. desymbiontum* nested within the *Nusuttodinium*-clade. This topology suggests that the two heterotrophic dinoflagellates considered followed different evolutionary histories. It is also suggested that the common ancestor of *Pellucidodinium* and *Nusuttodinium* spp. lost the chloroplast and photosynthetic ability (Fig. 14). It is well known that various dinoflagellates have lost their chloroplasts multiple times and independently became heterotrophic (Saldarriaga *et al.* 2001). Therefore, it is plausible that the common ancestor of *Pellucidodinium* and *Nusuttodinium* lost the chloroplast and then separated into the two genera, *Pellucidodinium* and *Nusuttodinium*. After this split, the ancestor of *Nusuttodinium* may have gained the ability of the kleptochloroplastidy, whereas *Pellucidodinium* remained heterotrophic (Fig. 14). Based on the topology inferred from the concatenated dataset, it cannot be ruled out the possibility of two independent gain

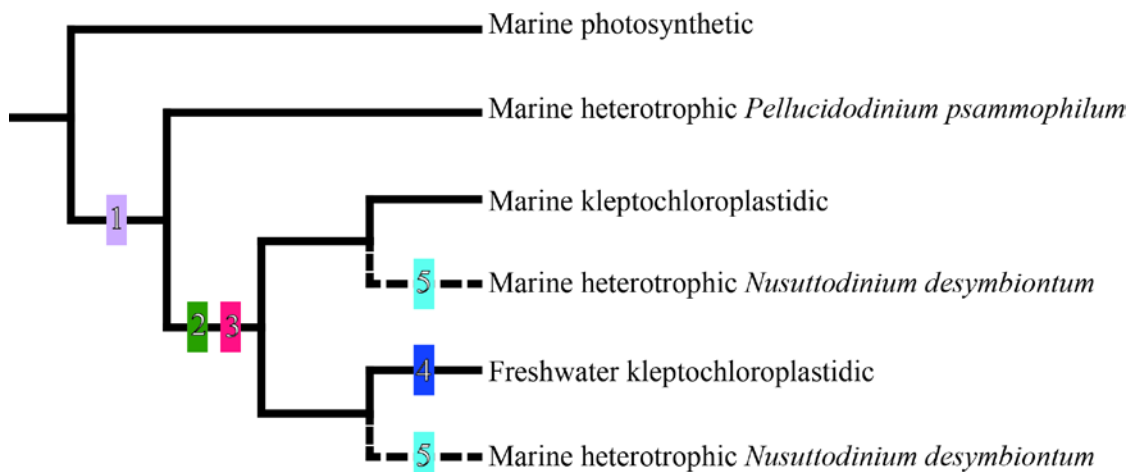


Fig. 14. Putative evolutionary history of the SPN clade. Dashed lines represent nodes without conclusive evidence. Numbers at nodes indicate the following evolutionary events, 1; loss of chloroplast, 2; gain of kleptochloroplast, 3; loss of nuclear chambers, 4; colonisation into freshwater habitat, 5; loss of kleptochloroplast.

of kleptochloroplastidy in marine and freshwater lineages, respectively. However, I believe that the single origin of the kleptochloroplastidy would be the most parsimonious. If the latter is the case, *N. desymbiontum* must have lost kleptochloroplast ability secondarily (Fig. 14).

In conclusion, the phagotrophic *Pellucidodinium psammophilum* can be interpreted to represent an intermediate position during the evolution of kleptochloroplastidic dinoflagellates from the ordinary photosynthetic dinoflagellates, whereas *N. desymbiontum* may exhibit a heterotrophic character by secondarily losing competence in kleptochloroplastidy.

Taxonomic and nomenclatural changes

Class Dinophyceae

Order Gymnodiniales

Nusuttodinium Takano, Yamaguchi, Inouye, Moestrup & Horiguchi emend. Onuma & Horiguchi

Unarmoured dinoflagellates with temporary chloroplasts (kleptochloroplasts) derived from ingested cryptomonads or heterotrophic as a result of the secondary loss of competence in kleptochloroplastidy. Apical groove curves around the apex in an anticlockwise direction. Nuclear chambers absent.

Chapter 2-1. An ultrastructural study on the morphological transitions of the kleptochloroplasts in *Nusuttodinium* after ingestion of cryptomonad cell

INTRODUCTION

Three species of marine *Nusuttodinium* species have been known (see Chapter 1). Of these, I investigated *N. latum* and *N. poecilochroum* in this study. *N. latum* is a sand-dwelling species and it can retain several cryptomonad cells as kleptochloroplasts, which are often of different colours or structures (Horiguchi & Pienaar 1992). Another marine species, *N. poecilochroum* also is a sand-dwelling representative and usually possesses 4-8 blue-green or yellow-green cryptomonad chloroplasts in a single cell, the colour depending on the species of cryptophyte engulfed (Larsen 1988). Therefore, these marine kleptochloroplastidic species are capable of ingesting more than one species (or genera) belonging to the class Cryptophyceae. These kleptochloroplasts are surrounded by four membranes; two chloroplast membranes, two chloroplast endoplasmic reticulum (ER) membranes, which is the same membrane composition as that of free-living cryptomonads. In addition to the chloroplast, the dinoflagellate engulfs cryptomonad cytoplasm and a single membrane (referred as 'phagotrophic' vacuole in Larsen (1988)) separates the cryptomonad cytoplasm from the dinoflagellate cytoplasm. The cryptomonad cytoplasm contains the cryptomonad nucleus, mitochondria and the periplastidal compartment (PPC), which contains the nucleomorph (Larsen 1988; Horiguchi & Pienaar 1992). When the dinoflagellates divide, the cryptomonad kleptochloroplasts are randomly distributed between the daughter cells, and no synchronisation of divisions of the kleptochloroplasts and host cell has been observed.

By contrast, the freshwater dinoflagellates *Nusuttodinium acidotum* and *N. aeruginosum* possess only blue-green kleptochloroplasts (Wilcox & Wedemayer 1984; Schnepf *et al.* 1989). In fact, the cryptomonads that *N. acidotum* can ingest are members of the genus *Chroomonas* (which are blue-green) only, and no other cryptophytes, such

as *Cryptomonas*, can be ingested (Fields & Rhodes 1991). It appears that the specificity of the dinoflagellate for its cryptomonad prey is stricter in freshwater dinoflagellates than it is in marine species. Usually only one kleptochloroplast is retained at a time in field-sampled freshwater dinoflagellates and it is usually so enlarged that it pervades most of the host cell. The kleptochloroplast membranes and the cryptomonad organelles are intact, as found in marine species (Wilcox & Wedemayer 1984; Schnepf *et al.* 1989; Farmer & Roberts 1990). Moreover, the division of the kleptochloroplast of the freshwater *Nusuttodinium* is synchronised with the host cell division and each half of the kleptochloroplast is inherited by each daughter cell (Fields & Rhodes 1991). Considering all above, the kleptochloroplasts in *N. acidotum* and *N. aeruginosum* represent a much more advanced stage of development of a true chloroplast from an endosymbiont than those seen in *N. poecilochroum* and *N. latum* (Fields & Rhodes 1991; Yamaguchi *et al.* 2011).

It is well known that there are significant differences between the structure of a free-living cryptomonad cell and that of the kleptochloroplast in the dinoflagellate cell. An ultrastructural study of *Nusuttodinium poecilochroum* revealed that the cryptomonad ejectosomes and mitochondria are sequestered in an ‘accumulation body’ (Larsen 1988 fig. 14, referred as ‘digestive vacuole’ in this study). Cryptomonad ejectosomes, periplasts and basal bodies have never been observed in the cells of *N. aeruginosum* and *N. acidotum* (Wilcox & Wedemayer 1984; Schnepf *et al.* 1989; Farmer & Roberts 1990). Moreover, all kleptochloroplastidic species possess chloroplasts that are considerably enlarged relative to the original cryptomonad chloroplast, especially in *N. acidotum*, where it is additionally highly-lobed and ramifies throughout the dinoflagellate cytoplasm (Wilcox & Wedemayer 1984; Larsen 1988; Schnepf *et al.* 1989; Farmer & Roberts 1990; Horiguchi & Pienaar 1992). However, it is unknown how and when organelles such as the ejectosomes are eliminated, and how the kleptochloroplasts are modified to become highly-lobed. Although previous studies revealed the general ultrastructure of the host and the kleptochloroplast (Larsen 1988; Fields & Rhodes 1991; Horiguchi & Pienaar 1992), few studies examine structural changes in the kleptochloroplasts over time, from ingestion to their disappearance.

Recently, morphological changes of kleptochloroplasts in *Dinophysis caudata* Saville-Kent were investigated over time from ingestion of prey (Kim *et al.* 2012). The study of the kleptochloroplasts in *D. caudata* revealed that the structural differences between the chloroplasts within the host and those of the original cryptomonad cell were caused by a rearrangement in the host cell (Kim *et al.* 2012). On the other hand, morphological changes in kleptochloroplasts with time have never been observed in *Nusuttodinium*, especially using the TEM. Of particular interest is how the dinoflagellates develop their kleptochloroplast from the original organization of the chloroplasts of free-living cryptomonads.

This study focuses on *Nusuttodinium poecilochroum*, *N. latum* and *N. aeruginosum*, three dinoflagellates exhibiting different possible evolutionary stages. The morphological changes in the prey of these three organisms at different times after ingestion were compared using the LM and TEM.

MATERIAL AND METHODS

Sampling and establishment of culture strains

Nusuttodinium poecilochroum was collected at Shibagaki Beach, Hakui City, Ishikawa Prefecture. Sand samples were collected at the edge of the surf. *N. latum* was collected at Port Edward, KwaZulu-Natal, South Africa (Table 1). The sand samples were then placed in plastic cups and enriched with Daigo IMK medium (Wako, Osaka, Japan). These crude cultural samples were cultured at 20 °C in a culture cabinet using fluorescent light with a photon flux density of 30-50 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ and a 16 h L/8 h D cycle. Individual cells of *N. latum* and *N. poecilochroum* in the enrichment culture were picked up by fine capillary pipette under an inverted microscope (CK X41, Olympus, Tokyo) and each single cell was placed into a compartment of a 48-well microplate containing IMK medium. *Rhodomonas* sp. (strain Mr06; collected at Denshin-Beach, Muroran-City, Hokkaido Prefecture in June 2010) was added to each well of microplate as prey. After the number of cells of *N. latum* and *N. poecilochroum* increased sufficiently to allow their successful transfer, the cells were placed in a plastic

petri dish together with *Rhodomonas* sp. and maintained as a culture strain under the conditions described above.

A sample containing *Nusuttodinium aeruginosum* was collected at the South pond of the Municipal building (Docho), Sapporo-City, Hokkaido Prefecture (Table 1), using a plankton net with a pore size of 25 μm . Cells of *N. aeruginosum* were isolated as described for marine species, but were placed in individual wells of a microplate filled with AF-6 medium (Kato 1982). As prey, *Chroomonas* sp. (strain Dc01; isolated from the same sample as *N. aeruginosum*) cells were added to each well. *N. aeruginosum* was cultured under the same conditions as those of marine taxa. After successful growth, the cells were transferred to a 24-well microplate with *Chroomonas* sp. and maintained as culture strains.

Feeding of cryptomonad cells for experiments

In order to compare the morphological changes of prey at different times after feeding, the following methods were used. For *Nusuttodinium poecilochroum*, colourless cells (= without kleptochloroplasts) were picked up from the culture strain and placed in a drop of fresh medium on a depression glass slide, and *Rhodomonas* sp. cells were added to the drop. After *N. poecilochroum* ingested the *Rhodomonas* sp., they were again isolated into a drop of fresh culture medium placed in a plastic petri dish (35mm in diameter) and incubated under the same conditions for different durations (0, 20, 30 minutes, 1, 2, 3, 4, 6 and 12 hours, respectively). Directly after ingestion (0 min), *N. poecilochroum* was observed or fixed without isolation. After the incubation period, the sample was observed using the LM and fixed for TEM observation (see below for detail).

For *Nusuttodinium latum*, starved and colourless cells were transferred in a well of a 48-well microplate containing fresh IMK medium and subsequently cells of *Rhodomonas* sp. were added into the microplate. After *N. latum* ingested the *Rhodomonas* sp., they were isolated and cultured with same methods as *N. poecilochroum*. Directly after ingestion (0 min), and after the incubation period (1, 4, 24, 48, 120 hours), the sample was observed using the LM and fixed for TEM observation.

For *Nusuttodinium aeruginosum*, cells were placed in a well of a microplate without

adding *Chroomonas* sp. and allowed to starve. When the cells became colourless, cells of *Chroomonas* sp. were added into the microplate well and *N. aeruginosum* was isolated after confirming the ingestion of *Chroomonas* sp. Incubation for different durations was performed by the same way as that described for cells of *N. poecilochroum*. To clarify the degree of the chloroplast enlargement from single cryptomonad chloroplast, I use the cells that have ingested single cryptomonad cell from the morphological observation at 48 h after ingestion.

The number of cells used for each observation is listed in Table 4.

Light microscopical (LM) observations

For LM observations, the cells of *Nusuttodinium poecilochroum*, *N. latum* or *N. aeruginosum* were observed using the ZEISS Axioskop2 Plus (Carl Zeiss Japan, Tokyo) and photographs were taken with a CCD camera DS-Fi1 (Nikon, Tokyo, Japan). To observe the autofluorescence of kleptochloroplast, a Fs 15 filter of the same microscope was used.

Transmission electron microscopical (TEM) observations

The samples for TEM observation were fixed for 2 h with Karnovsky fixative (2.5% glutaraldehyde and 2% paraformaldehyde) in 0.1 M cacodylate buffer at pH 7.0. For the fixation of cells of *N. poecilochroum* and *N. latum*, 0.3M sucrose was added in the same fixative. An equal volume of the fixative was mixed with a drop of culture medium in plastic petri dishes (35 mm in diameter). After fixation, the cell was transferred by micropipette onto a poly-L-lysine coated Thermanox plastic coverslip (Thermo Scientific, Yokohama, Japan), pre-cut to a size that can be accommodated in a 1.5 ml microtube, and the cell was allowed to settle and attach to the coverslip. The rest of the method was as described in Chapter 1.

Measurements of kleptochloroplast volumes with confocal laser scanning microscope

To measure precise chloroplast volumes, I fed colourless cells of *Nusuttodinium latum*,

N. poecilochroum and *N. aeruginosum* with a single cryptomonad cell with the same method as feeding experiment shown above. I measured the volume of kleptochloroplast at 0 min, 1 h, 4 h, 6 h, 12 h and 24 h after ingestion of cryptomonad in both *Nusuttodinium poecilochroum* and *N. aeruginosum*, and further estimates of volume in *N. aeruginosum* were conducted at 72 h and 120 h. In *N. latum*, the volume was estimated at 0 min, 1 h, 4 h, 24 h, 48 h and 120 h after ingestion. For observation of the cell right after ingestion, cryptomonad-ingesting cells were quickly isolated to 3 μ l of IMK or AF-6 medium on cover glass, and 3 μ l of 5% glutaraldehyde was added to the medium. For observation of the cells at 1 h, 4 h, 6 h, 12 h, 24 h, 72 h and 120 h after ingestion, the cells isolated on cover glass were cultured respectively in a humidified petri dish to prevent drying up, and fixed at each period. The fixed samples were observed using confocal laser scanning microscope Zeiss LSM-DUO (Carl Zeiss Japan, Tokyo) and obtained serial sectioning images (0.5 μ m interval) of chloroplast autofluorescence. The images were stored as TIFF files using software IMARIS (Carl Zeiss Japan, Tokyo), and TIFF files were put into ImageJ (<http://rsb.info.nih.gov/ij/>). According to manufactures' instruction, the volumes of chloroplast were estimated using ImageJ plugin Sync Measure 3D downloaded from ImageJ website (<http://rsbweb.nih.gov/ij/plugins/sync-windows.html>). The number of samples I measured the volume is shown in Table 4.

RESULTS

I have observed more than one cell of *Nusuttodinium poecilochroum* and *N. latum* and *N. aeruginosum* at each stage and ultrastructure of these species were investigated using serial sections. The number of the cells used for each observation was listed in Table 4.

Morphological changes in the ingested cryptomonad cells of *Nusuttodinium poecilochroum*

LM observations

The ingested cryptomonad cells (= the entire ingested cryptomonad cytoplasm including the cryptomonad chloroplast) were located in the posterior of the dinoflagellate cell, and their original shape was retained until the 10 min stage. During this period, a pyrenoid was detectable in the chloroplast at the LM level, but other structures or newly formed structures (like the digestive vacuole) in the dinoflagellate cell could not be observed (Fig. 15A-D). At 20 min from ingestion, most of cryptomonad cells were elliptic but some were slightly deformed (Fig 15E). By this stage, the host cell had formed several spherical digestive vacuoles that were visible under the LM. These digestive vacuoles were transparent, and occasionally small granules were observed in them (Fig. 15E), but no autofluorescence was detectable in them (Fig 15F). The ingested chloroplasts

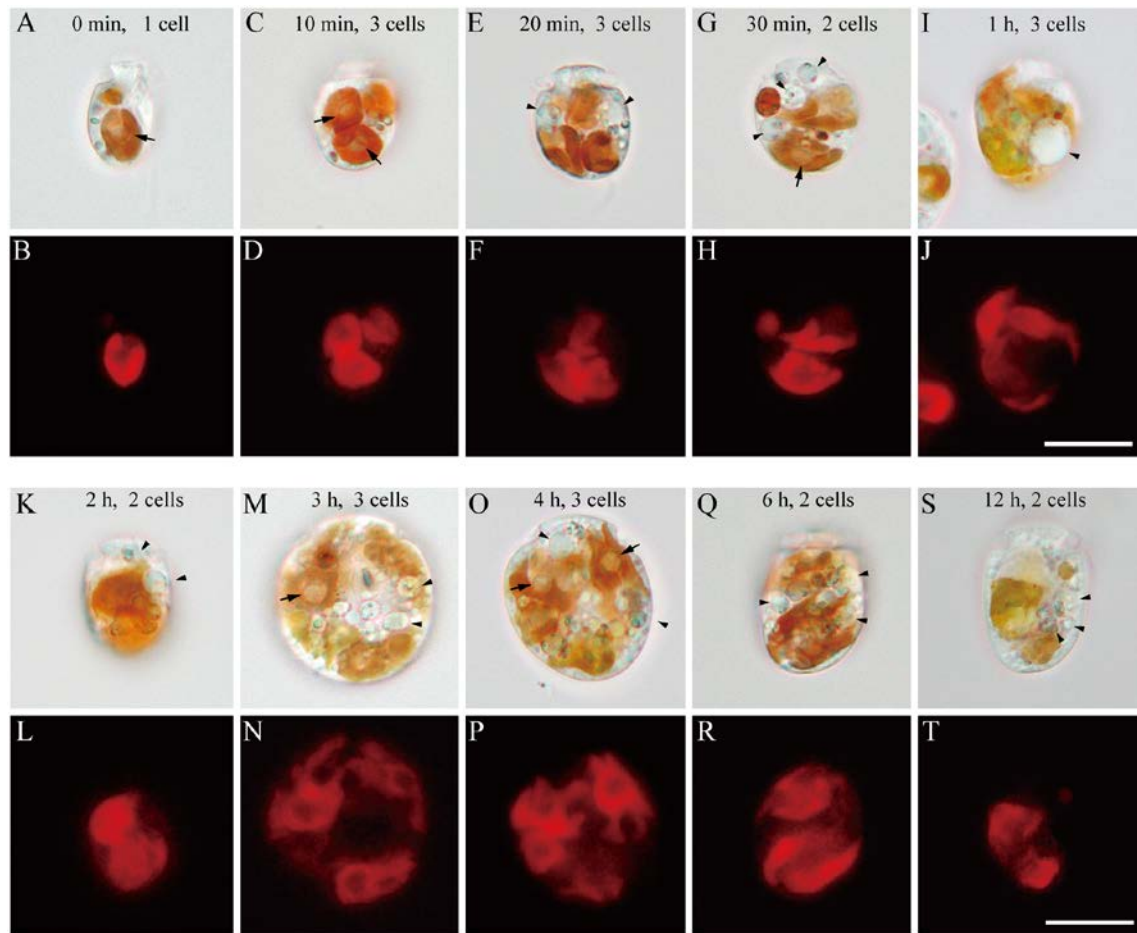


Fig. 15. Bright field and fluorescence micrographs of the morphological change in the cryptomonad cells ingested by *Nusuttodinium poecilochroum*. Times and cell numbers shown on bright field micrographs indicate the elapsed times after the ingestion of *Rhodomonas* sp. and the numbers of the ingested cryptomonad cells, respectively. Each fluorescence micrograph corresponds to the bright field micrograph directly above it. Note that the cryptomonad cells have deformed gradually after the 20 min stage. Digestive vacuoles (arrowheads) are formed after 20 min, and remain visible until the 12 h stage. Arrows indicate pyrenoids. Bar = 10 μ m.

deformed gradually beyond 20 min of ingestion and by 2 h they had lost their original cup-shape seen in Fig. 1B (Fig. 15E-L). At the 3 h and 4 h stage, the cryptomonad cells became elongated, developed lobes and were restricted to the periphery of the cell (Fig. 15M-P). Although the dinoflagellate cells at these stages seem larger than those of other stages, this is simply reflecting the fact that these particular cells engulfed more prey cell than other cells and this enlargement of cell size is not specific phenomenon for this timing. At the 6 h and 12 h stage, the cryptomonad cells were still deformed, but less so than at the 3 h and 4 h stage (Fig. 15Q-T). Digestive vacuoles could be observed from the 20 min stage to 12 h stage. At the 12 h stage, relatively large cells underwent cell division (data not shown), but the morphological change of the cryptomonad cell after host cell division was not monitored in this study.

TEM observations

Membranes of the cryptomonad cell: Right after ingestion, the ingested cryptomonad chloroplast was surrounded by two chloroplast membranes (Fig. 16A white arrowhead) and two chloroplast ER membranes (Fig. 16A arrowhead), just like in the free-living condition. The cryptomonad cytoplasm, which had less electron-dense than the dinoflagellate cytoplasm and contained a chloroplast and additional organelles, was separated from the dinoflagellate cytoplasm by a single membrane (Fig. 16A arrow). Twelve hours after ingestion, the cryptomonad cytoplasm had been removed around the chloroplast and the dinoflagellate cytoplasm was detected in close proximity with the chloroplast (Fig. 16B). Several membranes were observed around the chloroplast, probably the result of digestion of membranes or an artifact of fixation. This made it impossible to determine how many membranes were retained of the original chloroplast (the two chloroplast membranes and the two chloroplast ER membranes) or of the membrane between the cryptomonad cytoplasm and the dinoflagellate cytoplasm (Fig. 16B).

The chloroplast and additional organelles: Immediately following ingestion, the chloroplast was cup-shaped, like that of the free-living cryptomonad. It was confirmed that the cryptomonad organelles and their arrangement were well-retained. The

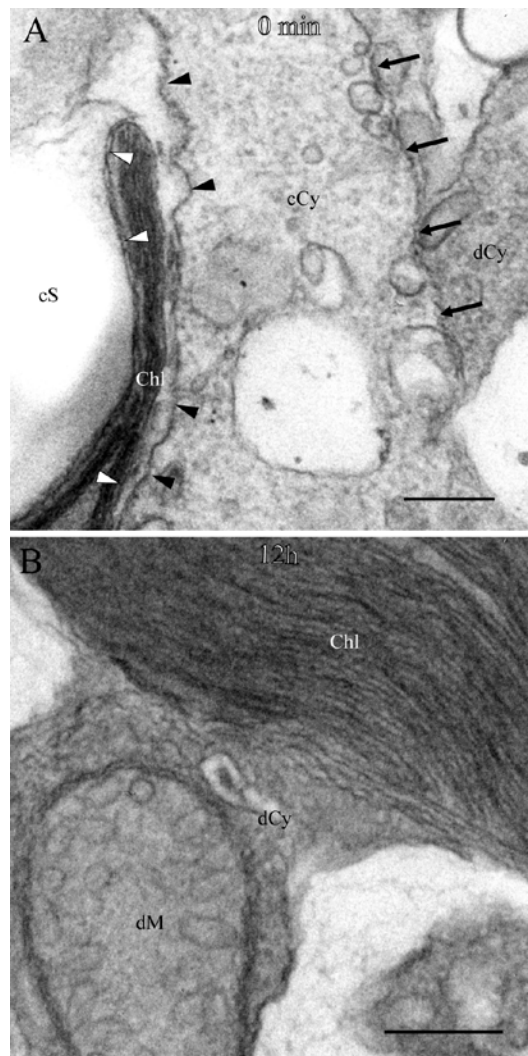


Fig. 16. TEM micrographs of membranes surrounding the kleptochloroplast in *Nusuttodinium poecilochroum*.

A. Membranes directly following ingestion. Ingested chloroplast is enclosed by four membranes; two chloroplast membranes and two chloroplast ER membranes. The cryptomonad cytoplasm is separated from the dinoflagellate cytoplasm by a single membrane.

B. Membranes 12 h after ingestion. Membranes of the chloroplast and the single membrane between the cryptomonad cytoplasm and the dinoflagellate cytoplasm cannot be resolved because they are very close to each other. The dinoflagellate cytoplasm is adjacent to the chloroplast, and the cryptomonad cytoplasm is not visible even under the TEM.

White arrowheads, arrowheads and arrows indicate chloroplast membranes, chloroplast ER membranes and the membrane between the cryptomonad and the dinoflagellate cytoplasm. Abbreviations: Chl, chloroplast; cS, cryptomonad starch; cCy, cryptomonad cytoplasm; dCy, dinoflagellate cytoplasm; dM, dinoflagellate mitochondria. Bar = 200 nm.

cryptomonad cytoplasm contained a chloroplast, a cryptomonad nucleus, a nucleomorph, ejectosomes (gullet-surrounding ejectosomes only) and mitochondria with flat cristae (Fig. 17A). At this stage, the larger amount of the cryptomonad cytoplasm appeared to be observed around the chloroplast than free-living cryptomonad cell, probably due to disorganisation of the peripheral structures (Fig. 17A). No cryptomonad periplast,

flagella or basal bodies were observed in 7 cells used for TEM observation. The peripheral ejectosomes of the cryptomonad (= smaller ejectosome; see Fig. 35B) were accumulated (see below for more detail). At the 20 min stage, the chloroplast became slightly irregular in shape, and the cryptomonad nucleus and the nucleomorph were still observed (Fig. 17B, the nucleomorph is not shown). In contrast, the cryptomonad mitochondria and most of its cytoplasm were removed from the vicinity of the chloroplast and the cryptomonad nucleus. As a result, the dinoflagellate cytoplasm approached a direct connection with the chloroplast (Fig. 17B). At the 30 min stage, the gullet-surrounding ejectosomes (= larger ejectosomes; see Fig. 35B) were still retained in a small pocket of cryptomonad cytoplasm rather than within a digestive vacuole (Fig. 17C). At the 1 h stage, the cryptomonad mitochondria and ejectosomes were removed, and no cryptomonad cytoplasm could be discerned around the chloroplast (Fig. 17D). At the 3 h stage, the chloroplast was located at the periphery of the host cell and the cryptomonad cell had lost its original organelles (Fig. 17E). At the 12 h stage, the chloroplast was significantly modified and large starch granules had accumulated in the periplastidal compartment of the chloroplast (Fig. 17F). The nucleomorph was still detected at this stage, although the cryptomonad nucleus could no longer be observed in the host cell (Fig. 17F).

Cryptomonad ejectosomes and the digestive vacuole: The peripheral ejectosomes accumulated in the space between the cryptomonad cytoplasm and dinoflagellate cytoplasm right after ingestion. The membranous material, which encircles the accumulated ejectosomes could be observed (Fig. 18A arrow). At the 20 min stage, the accumulated peripheral ejectosomes together with cryptomonad mitochondria became surrounded by a distinct membrane, which is thought to be a digestive vacuole membrane (Fig. 18B). At this stage, the gullet-surrounding ejectosomes were not contained in a digestive vacuole but remained in the cryptomonad cytoplasm (not shown). The gullet-surrounding ejectosomes were transferred into the digestive vacuole at the 1 h stage together with the peripheral ejectosomes and the cryptomonad mitochondria (Fig. 18C). At the 4 h stage, the peripheral ejectosomes were difficult to recognise due to digestion (Fig. 18D). At the 6 h stage, all ejectosomes were digested or

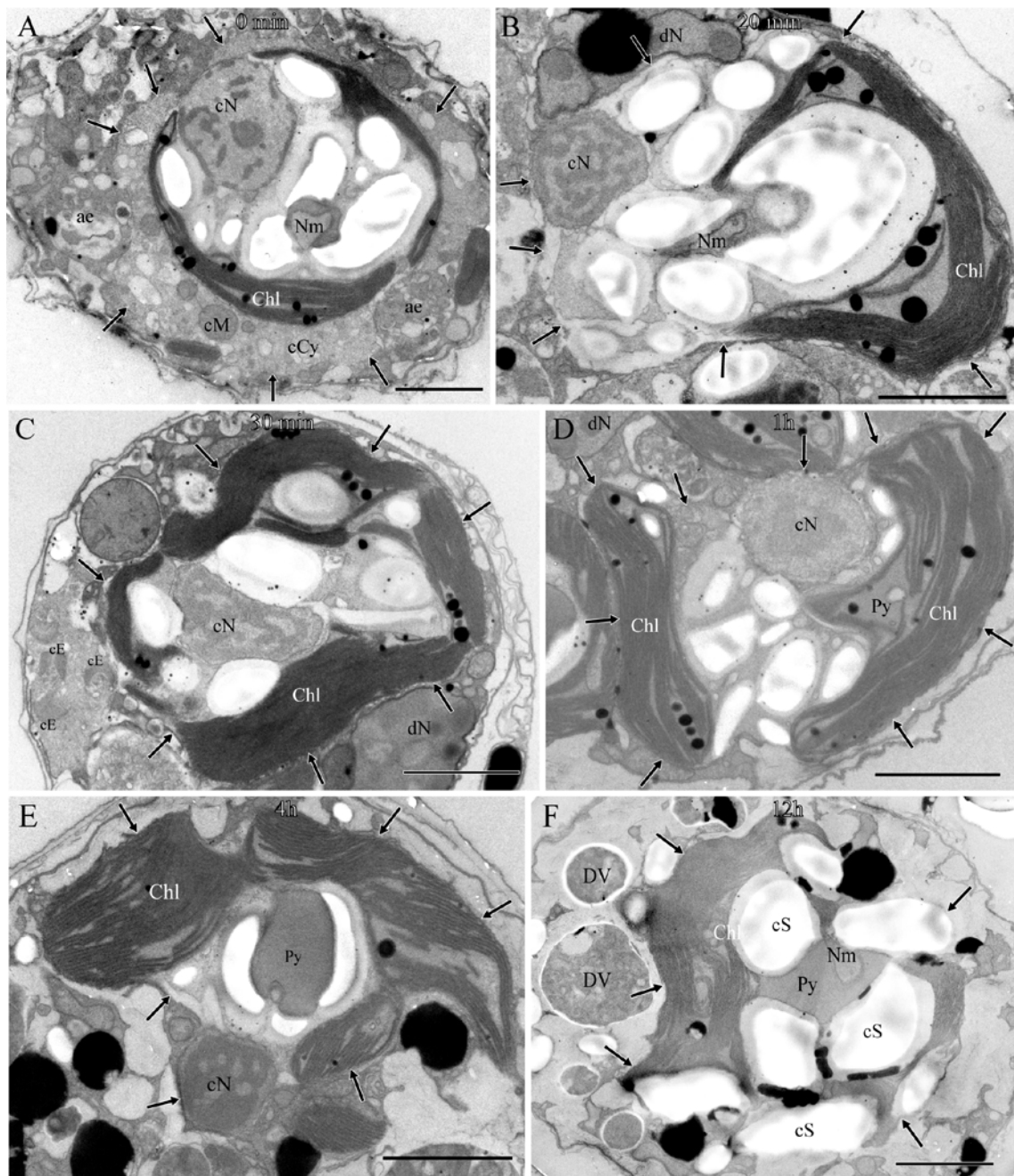


Fig. 17. TEM micrographs of the morphological change in the cryptomonad cell at various times following ingestion by *Nusuttodinium poecilochroum*.

A. Ingested cryptomonad cell directly after ingestion. The cell of *N. poecilochroum* contains a chloroplast, cryptomonad nucleus, nucleomorph, cryptomonad mitochondria and cytoplasm. The peripheral ejectosomes of the cryptomonad are accumulated. (ae; and see Fig. 18A).

B. Cryptomonad cell 20 min after ingestion. Most of cryptomonad cytoplasm is removed from cryptomonad cell. The dinoflagellate nucleus is close to the chloroplast.

C. Cryptomonad cell 30 min after ingestion. The gullet-surrounding ejectosomes of the cryptomonad are retained in the cryptomonad cytoplasm.

D. Cryptomonad cell 1 h after ingestion. Almost all the cryptomonad's mitochondria, ejectosomes and cytoplasm are removed from the vicinity of the chloroplast.

E. Cryptomonad cell 4 h after ingestion. Note that the chloroplast is elongated and on the periphery of the dinoflagellate cell and that the cryptomonad nucleus has become more electron dense than during the previous stage (see D).

F. Chloroplast 12 h after ingestion. The chloroplast completely loses its original shape. Large cryptomonad starch granules and digestive vacuoles are observed. No cryptomonad nucleus is found around the chloroplast.

Arrows indicate that the boundary between the cryptomonad and the dinoflagellate cytoplasm. Abbreviations: Chl, chloroplast; cN, cryptomonad nucleus; Nm, nucleomorph; cM, cryptomonad mitochondria; Py, pyrenoid; cE, gullet-surrounding ejectosome; cS, cryptomonad starch; cCy, cryptomonad cytoplasm; dN, dinoflagellate nucleus; DV, digestive vacuole. Bar = 2 μm.

had lost their original shape, but the mitochondrial membranes were not digested (Fig. 18E). By the 12 h stage, the cryptomonad organelles in the digestive vacuole had become unrecognizable and the contents of digestive vacuole were homogenous (Fig. 18F).

The cryptomonad nucleus and nucleomorph: TEM observations indicated that the cryptomonad nucleus was unchanged at ingestion (0 min stage) (Fig. 19A). It was still unchanged at the 2 h stage (Fig. 19B), but at the 3 h stage, its surface was undulated and the nucleoplasm was more electron dense than during the previous stages (Fig.

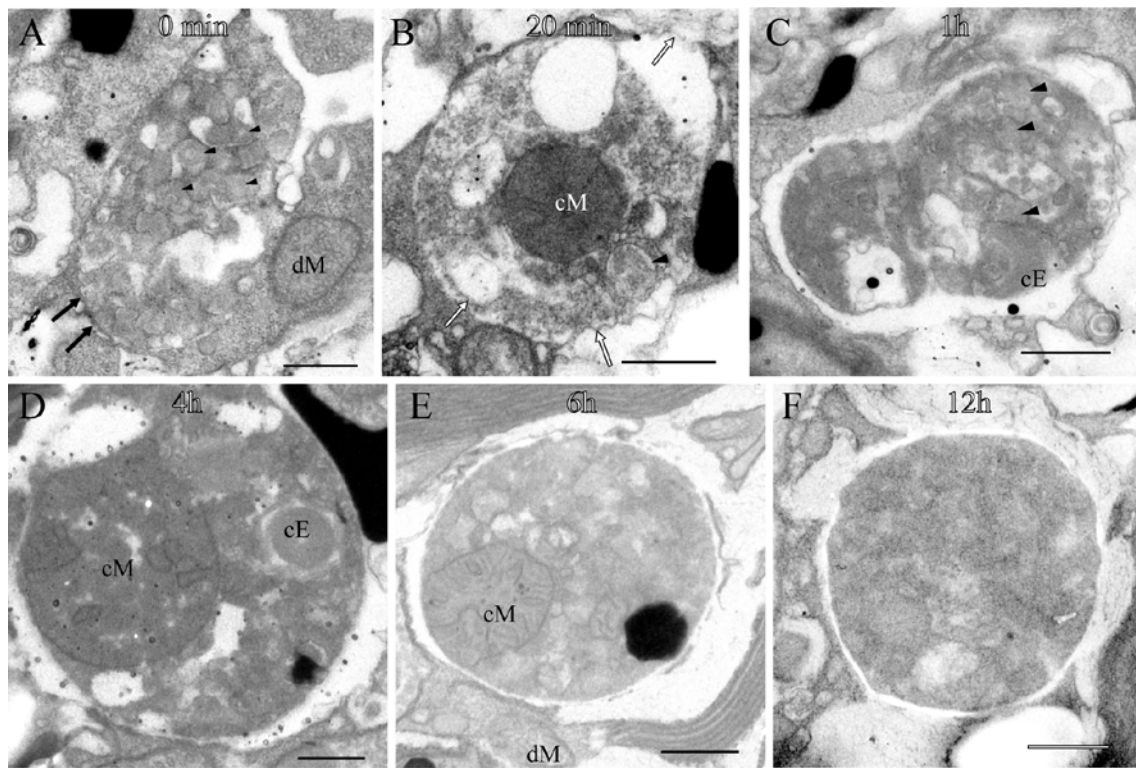


Fig. 18. TEM micrographs of digestive vacuole formation in *Nusuttodinium poecilochroum* cell.

A. Cryptomonad peripheral ejectosomes directly following ingestion. Note that the ejectosomes are accumulated between the cryptomonad and dinoflagellate. Membranous material can be seen (arrow).

B. Digestive vacuole at the 20 min stage containing accumulated ejectosomes and cryptomonad mitochondria. White arrows indicate the digestive vacuole membrane.

C. Digestive vacuole at the 1 h stage containing the peripheral ejectosomes and the gutlet-surrounding ejectosomes.

D. The digestive vacuole at the 4 h stage with digested contents. Peripheral ejectosomes cannot be discerned due to digestion.

E. The digestive vacuole at the 6 h stage showing the digested cryptomonad mitochondria and gutlet-surrounding ejectosomes.

F. The digestive vacuole at the 12 h stage containing homogeneous material. The cryptomonad organelles cannot be recognised.

Arrowhead indicates an individual peripheral ejectosome. Abbreviations: cM, cryptomonad mitochondria; cE, gutlet-surrounding ejectosomes; dM, dinoflagellate mitochondria. Bar = 500 nm.

19C). At the 4 h stage, two different states of modification in nuclei were observed; either the majority of the cryptomonad nucleus existed separated from the vicinity of the chloroplast, or it remained in close proximity with the chloroplast. Where the cryptomonad nucleus was kept near the chloroplast, the nucleoplasm and the surface of nuclear membranes were as described at the 3 h stage (Fig. 19D). The separated cryptomonad nucleus should have been transferred to digestive vacuoles, but I could not detect it in the cells at 4 h stage. At the 6 h stage, a distinct cryptomonad nucleus was never observed but the homogenous content of the digestive vacuole was reminiscent of cryptomonad nucleus. This is because it contained the same spots of low electron-density typical of the cryptomonad nucleus, suggesting that the damaged nucleus have transferred to the digestive vacuole (Fig. 19E). At the 12 h stage, trace of the cryptomonad nucleus was no longer detected because the content of digestive vacuole was completely digested (Fig. 19F).

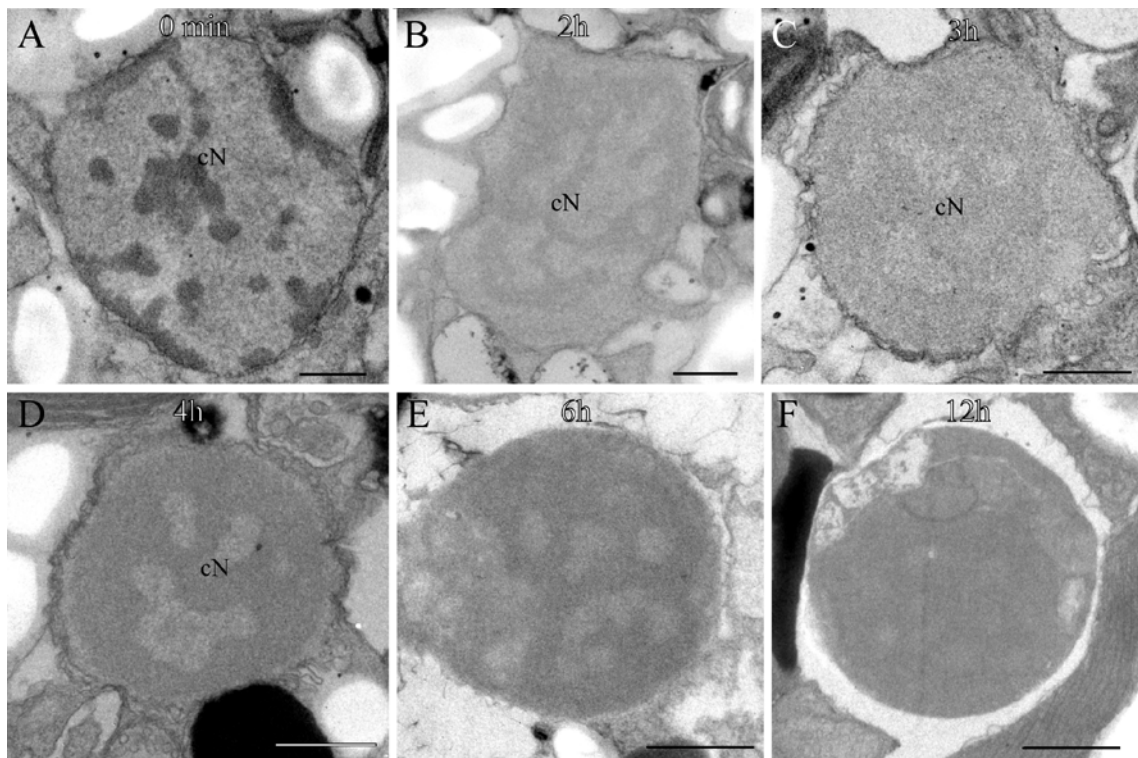


Fig. 19. TEM micrographs of the cryptomonad nucleus (cN) in *Nusuttodinium poecilochroum*.

A. Cryptomonad nucleus showing intact structure at the time of ingestion.

B. Cryptomonad nucleus at the 2 h stage showing intact structure.

C-D. The surface of cryptomonad nucleus becomes undulated at the 3 h and 4 h stages. Note that increase in electron density of the cryptomonad nucleus relative to that in the previous stages (see B).

E. The digestive vacuole at the 6 h stage containing a cryptomonad nucleus-like structure.

F. The content of digestive vacuole at the 12 h stage becoming homogeneous. No cryptomonad nucleus can be found in the digestive vacuole. Bar = 500 nm.

By contrast, the nucleomorph was highly preserved throughout the experimental period. It was intact at the time of ingestion (Fig. 20A) and later, even up to the 12 h stage, its membranes were obvious, indicating that the nucleomorph was not modified by the dinoflagellate (Fig. 20B-F). Division of the nucleomorph was not observed in this study.

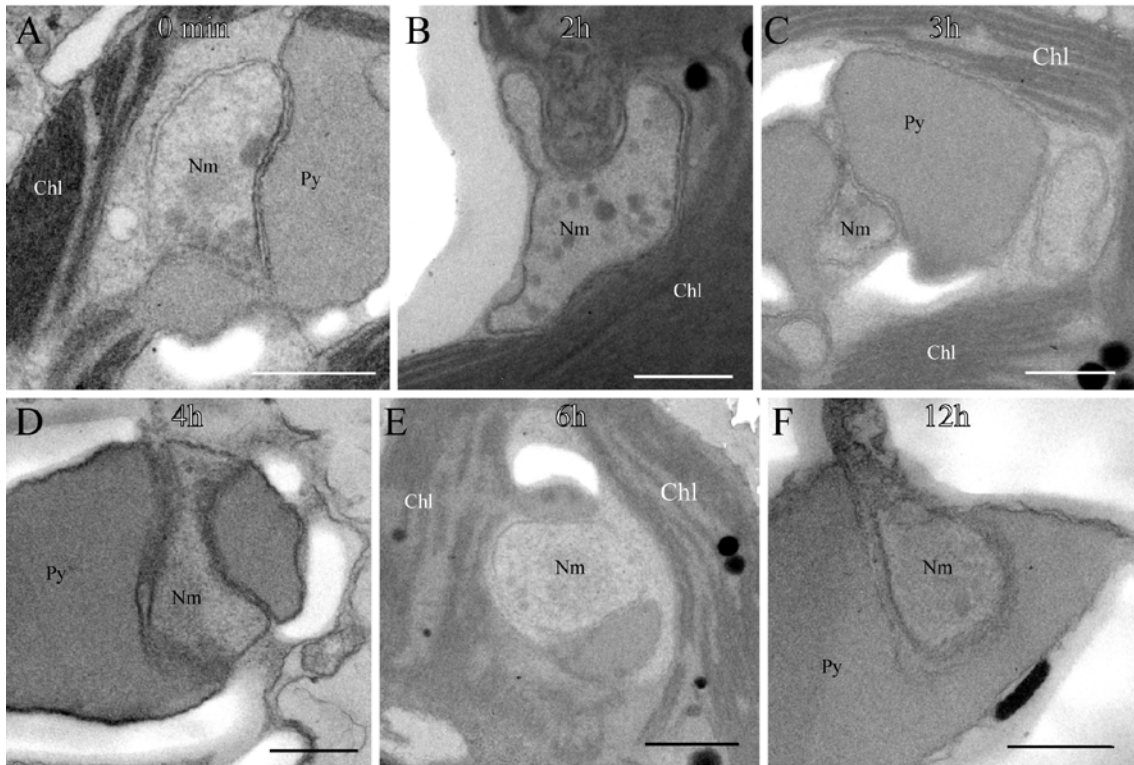


Fig. 20. TEM micrographs of the nucleomorph in *Nusuttodinium poecilochroum*.

A. Ingested nucleomorph showing intact structure within pyrenoid matrix.

B-F. Nucleomorphs at each stage investigated showing that its structure remains intact and with no visible signs of digestion.

Abbreviations: Chl, chloroplast; Nm, nucleomorph; Py, pyrenoid. Bar = 500 nm.

The morphological change of the kleptochloroplast in *Nusuttodinium latum*

LM observation

The ingested cryptomonad cell immediately following ingestion was oval and elliptic, the chloroplast was cup-shaped, indicating the original shape of the cell was well-retained (Fig. 21A, B). Digestive vacuole was not observed at this stage. At 1 h after ingestion, the chloroplast was elliptic and cup-shaped, i.e., the shape of the chloroplast was not changed (Fig. 21C, D). By 1 h, digestive vacuoles were formed in the cell. The digestive vacuoles were transparent and the contents of them could not be identified in the LM level (Fig. 21C). At 4 h after ingestion the chloroplast was not changed morphologically, retaining elliptic or oval form (Fig. 21E, F). The pyrenoid was detected inside of the chloroplast and several digestive vacuoles were still observed in the cell (Fig. 21E). By 24 h, digestive vacuole was no longer present in the cell (Fig. 21G). The cryptomonad chloroplast was elliptic and cup-shaped, and the pyrenoid was located in the chloroplast (Fig. 21G, H). At 48 h, the shape of chloroplast was retained as in the previous stages (Fig. 21I, J). By 120 h, chloroplast was apparently enlarged and occupied almost half bulk of the cell (Fig. 21K, L). The chloroplast was deformed to crescent shape, however, the pyrenoid was sheathed by the chloroplast (Fig. 21K, L). The dinoflagellate nucleus, originally located in the centre of the cell, was positioned in the left side of the cell at this stage, probably due to the enlargement of the chloroplast (Fig. 21K).

TEM observation

Membranes of the cryptomonad cell: Right after ingestion, the ingested cryptomonad cytoplasm was encircled by a single membrane (Fig. 22A). The cryptomonad chloroplast was surrounded by two chloroplast membranes (Fig 22B white arrowhead) and two chloroplast ER membranes (Fig. 22B arrowhead), and this composition was same as chloroplast of free-living cryptomonad. At 120 h after ingestion, two membranes were clearly observed around the chloroplast and at the boundary area between the cryptomonad and dinoflagellate cytoplasm (Fig. 22C, D).

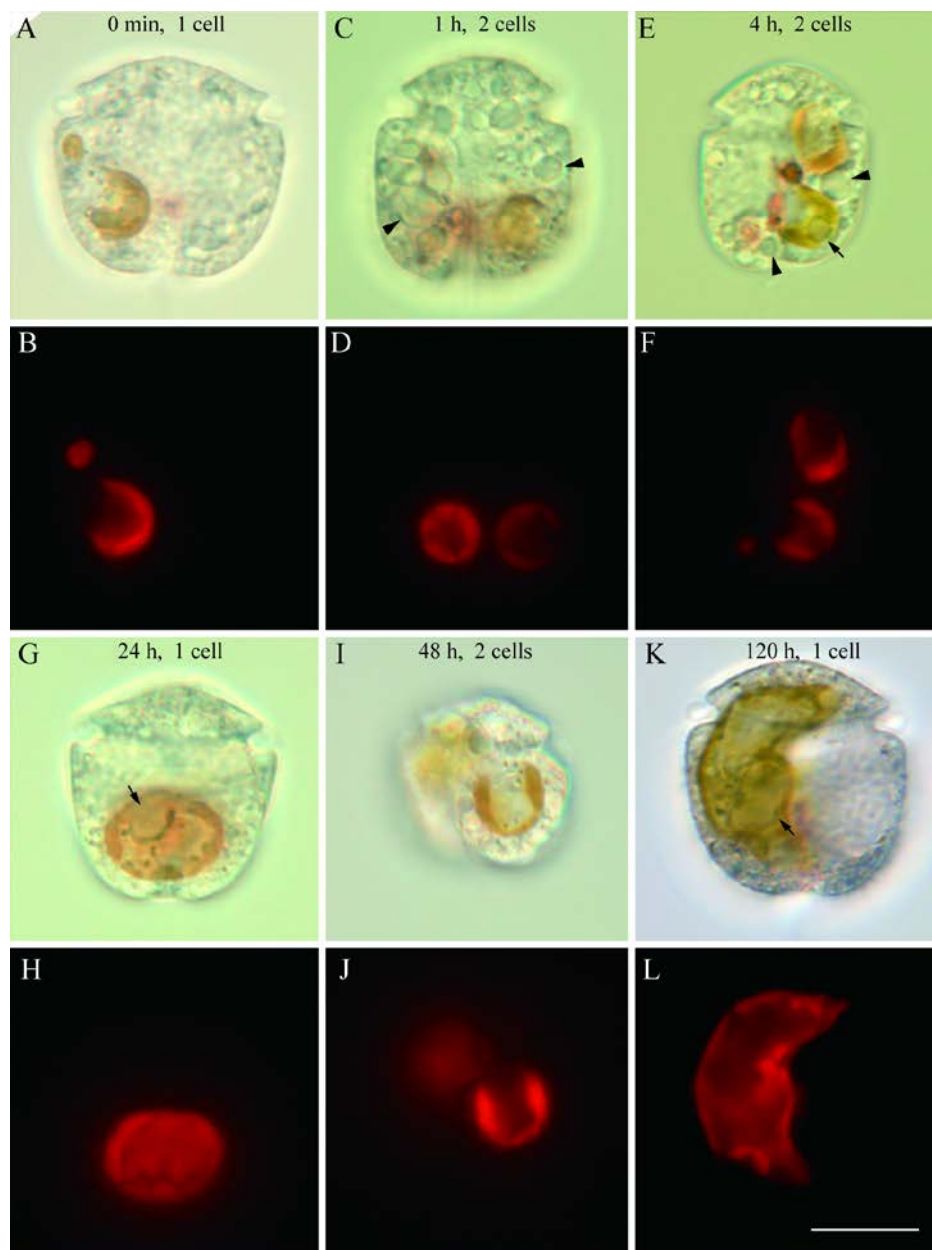


Fig. 21. Bright field and fluorescence micrographs of the morphological change in the cryptomonad cells ingested by *Nusuttodinium latum*. Times and cell numbers shown on bright field micrographs indicate the elapsed times after the ingestion of *Rhodomonas* sp. and the numbers of the ingested cryptomonad cells, respectively. Each fluorescence micrograph corresponds to the bright field micrograph directly above it. Note that digestive vacuoles (arrowheads) are formed after 1 h. Arrows indicate pyrenoids. Bar = 10 μ m.

However, the precise number of the membranes and the origin of the two membranes could not be determined because the cryptomonad cytoplasm had been removed around the cryptomonad cell and the membranes were too close to each other (Fig 22C, D).

The chloroplast and additional organelles: Directly after ingestion, the chloroplast retained the original cup-shaped form, and a nucleus, a nucleomorph and a

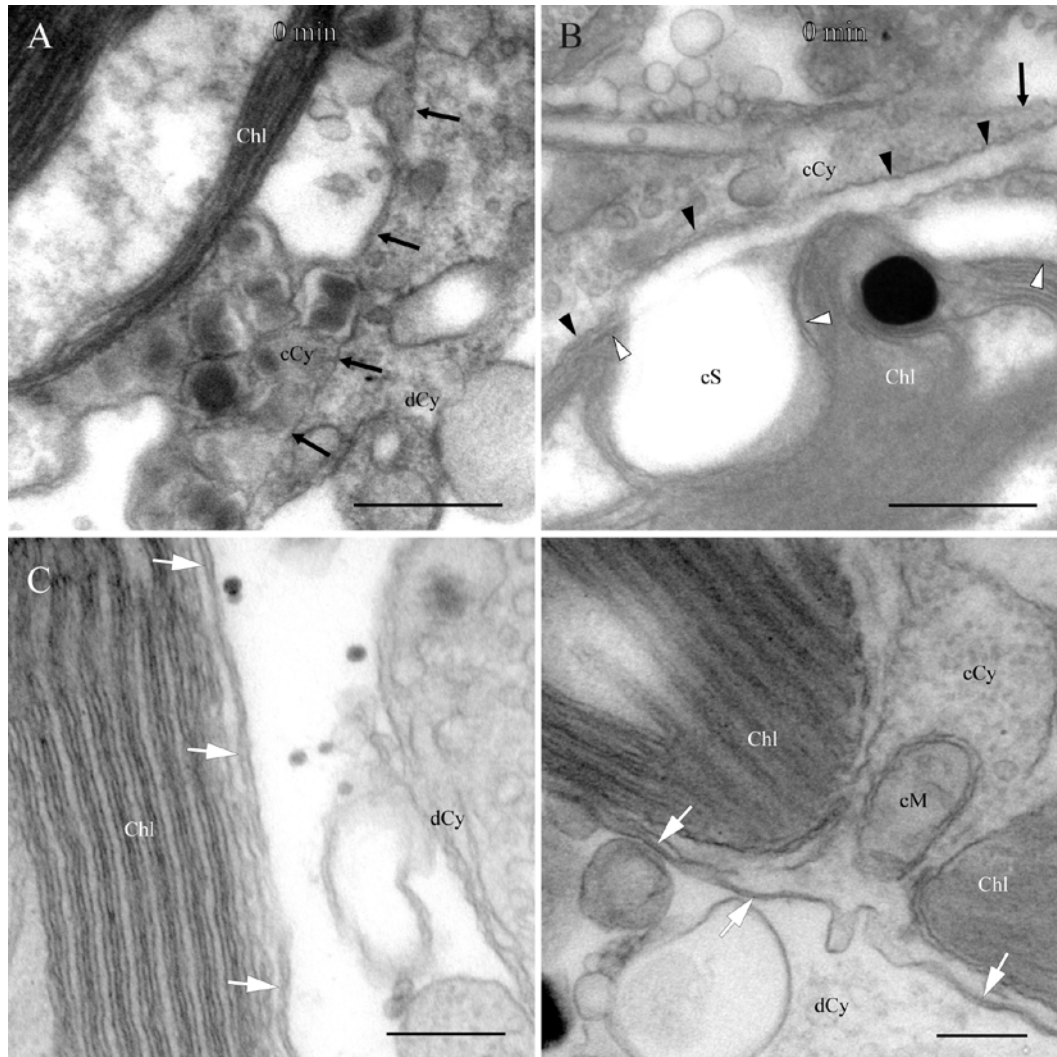


Fig. 22. TEM micrographs of membranes surrounding the kleptochloroplast in *Nusuttodinium latum*.

A, B. Membranes directly following ingestion. Ingested chloroplast is enclosed by four membranes; two chloroplast membranes and two chloroplast ER membranes. The cryptomonad cytoplasm is separated from the dinoflagellate cytoplasm by a single membrane. Bar = 500 nm.

C, D. Membranes 120 h after ingestion. Two membranes were observed between the cryptomonad cell and the dinoflagellate cytoplasm. Bar = 200 nm.

White arrowheads, arrowheads and arrows indicate chloroplast membranes, chloroplast ER membranes and the membrane between the cryptomonad and the dinoflagellate cytoplasm. Abbreviations: Chl, chloroplast; cS, cryptomonad starch; cCy, cryptomonad cytoplasm; dCy, dinoflagellate cytoplasm; cM, cryptomonad mitochondria.

pyrenoid were contained in the cryptomonad cytoplasm (Fig. 23A). No periplasts were observed. At this stage, peripheral ejectosomes and gullet-surrounding ejectosomes were located within the cytoplasm, but the original arrangement and direction of the gullet-surrounding ejectosomes were disorganised because the gullet was collapsed probably due to ingestion. By 1 h after ingestion, the cryptomonad cytoplasm containing ejectosomes was no longer observed from the vicinity of the chloroplast and

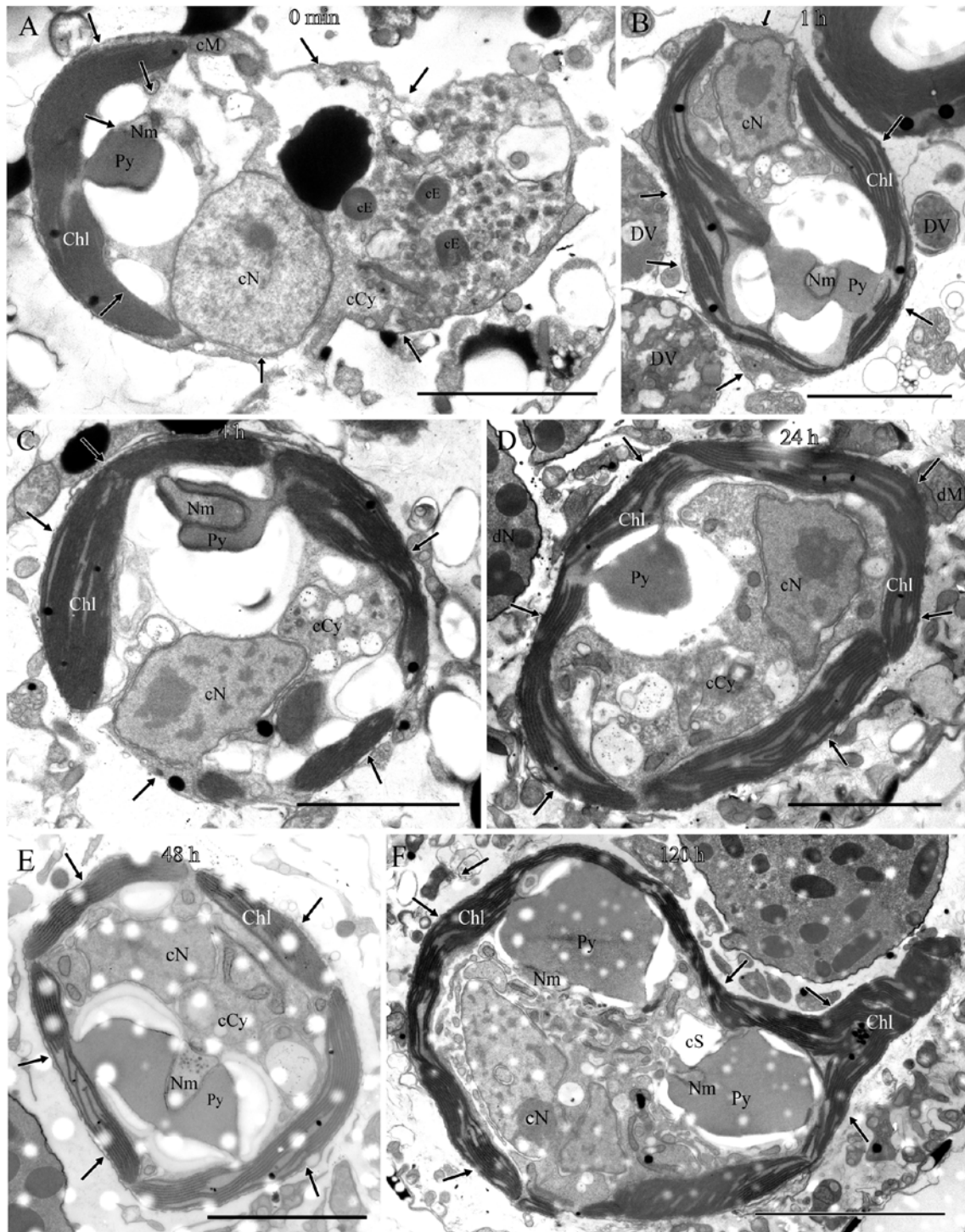


Fig. 23. TEM micrographs of the morphological change in the cryptomonad cell at various times following ingestion by *Nusuttodinium latum*.

A. Ingested cryptomonad cell directly after ingestion. The cell of *N. latum* contains a chloroplast, cryptomonad nucleus, nucleomorph, cryptomonad mitochondria and cytoplasm. Bar = 3 µm.

B. Cryptomonad cell 1 h after ingestion. The cryptomonad cytoplasm containing ejectosomes is removed. Bar = 3 µm.

C. Cryptomonad cell 4 h after ingestion. The cryptomonad organelles are retained in the cup-shaped chloroplast. Bar = 3 µm.

D. Cryptomonad cell 24 h after ingestion. The chloroplast encircles cryptomonad cytoplasm containing nucleus and pyrenoid. Bar = 3 µm.

E. Cryptomonad cell 48 h after ingestion. The cryptomonad nucleus, nucleomorph and pyrenoid are located in the chloroplast. Bar = 3 µm.

F. Chloroplast 120 h after ingestion. The nucleomorph and pyrenoid are duplicated in the chloroplast. Note that the lobe of chloroplast grew up from the periphery of the chloroplast. Bar = 5 µm.

Arrows indicate that the boundary between the cryptomonad and the dinoflagellate cytoplasm. Abbreviations: Chl, chloroplast; cN, cryptomonad nucleus; Nm, nucleomorph; cM, cryptomonad mitochondria; Py, pyrenoid; cE, gullet-surrounding ejectosome; cS, cryptomonad starch; cCy, cryptomonad cytoplasm; dN, dinoflagellate nucleus; DV, digestive vacuole.

several digestive vacuoles were formed around the chloroplast (Fig. 23B). In addition, no gullet-surrounding ejectosomes were observed at this stage. By contrast to this tendency to remove or digest the outside organelles of cryptomonad, the chloroplast was still cup-shaped, and the cryptomonad organelles, such as nucleus, nucleomorph and pyrenoid were retained inside the chloroplast (Fig. 23B). At 4 h stage, the shape of the chloroplast and the composition of cryptomonad organelles were not changed (Fig. 23C). At 24 h after ingestion, the chloroplast was elliptic and appeared to completely encircle the cryptomonad organelles (Fig. 23D). The cryptomonad nucleus, pyrenoid and nucleomorph with cryptomonad cytoplasm were retained (Fig. 23D; nucleomorph is not shown). At 48 h stage, the situation was not changed since 24 h stage (Fig. 23E). At

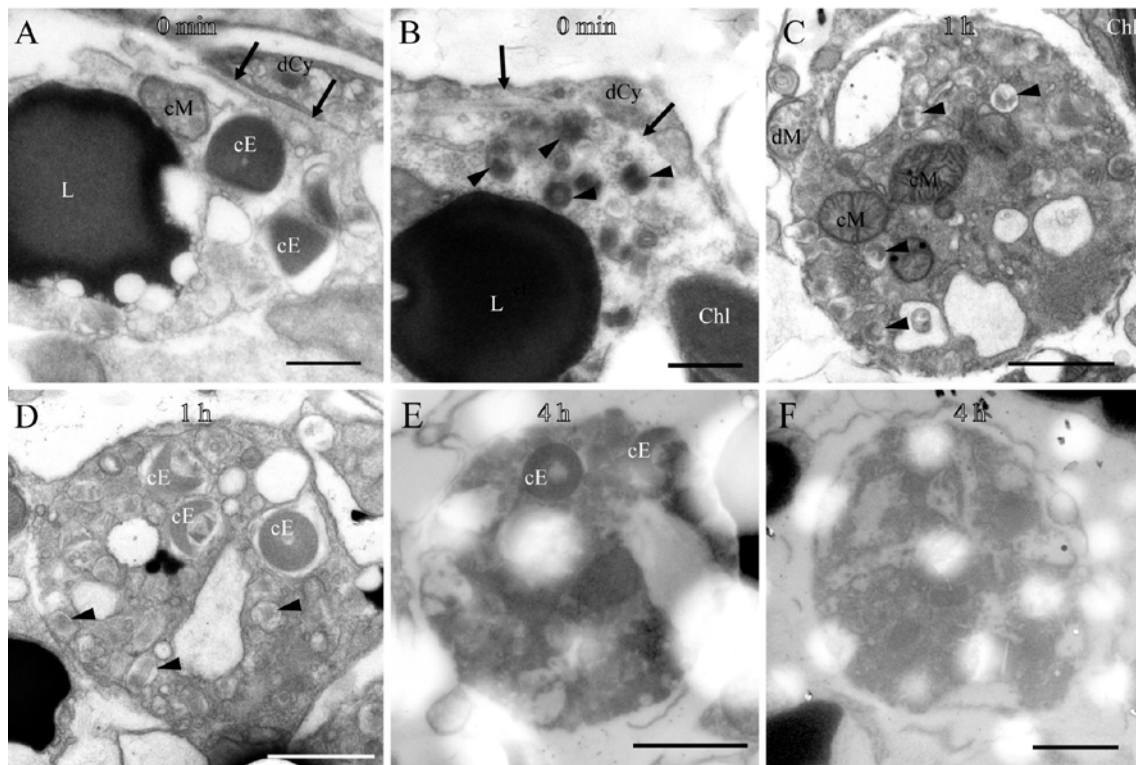


Fig. 24. TEM micrographs of digestive vacuole formation in *Nusuttodinium latum* cell.

A. Cryptomonad gullet-surrounding ejectosomes directly following ingestion. Note that the gullet-surrounding ejectosomes located in the cryptomonad cytoplasm. Bar = 500 nm.

B. Cryptomonad peripheral ejectosomes directly following ingestion. The peripheral ejectosomes also situated in the cryptomonad cytoplasm. Bar = 500 nm.

C. Digestive vacuole at the 1 h stage containing the peripheral ejectosomes and cryptomonad mitochondria. Bar = 1 μ m.

D. Digestive vacuole at the 1 h stage containing the peripheral and gullet-surrounding ejectosomes. Bar = 1 μ m.

E, F. The digestive vacuole at the 4 h stage showing gullet-surrounding ejectosomes. The other cryptomonad organelles cannot be recognised. Bar = 1 μ m.

Arrowhead and arrow indicates an individual peripheral ejectosome and the membrane separating the cryptomonad and dinoflagellate cytoplasm, respectively. Abbreviations: Chl; chloroplast, cM, cryptomonad mitochondria; cE, gullet-surrounding ejectosomes; L; lipid granule, dM; dinoflagellate mitochondria, dCy, dinoflagellate cytoplasm.

120 h after ingestion, the chloroplast was expanded and grown to form the lobe (Fig. 23F). Although the chloroplast was no longer elliptic, the cryptomonad organelles were still surrounded completely by the chloroplast (Fig. 23F). Inside the chloroplast, the nucleus, cryptomonad mitochondria could be observed, and the pyrenoid and nucleomorph had been duplicated (Fig. 23F).

Cryptomonad ejectosomes and digestive vacuoles: Right after ingestion, gullet-surrounding ejectosomes, peripheral ejectosomes and mitochondria were located in the cryptomonad cytoplasm, and digestive vacuole was not detected. At this stage, the membrane separating cryptomonad and dinoflagellate cytoplasm could be observed (Fig. 24A, B). By 1 h, digestive vacuoles were formed and encircled ejectosomes and mitochondria (Fig. 24C, D). The digestive vacuoles contained both gullet-surrounding and peripheral ejectosomes (Fig. 24C, D). At 4 h, although the shape of gullet-surrounding ejectosomes retained its shape, the peripheral ejectosome and mitochondria could not be recognised due to digestion (Fig. 24E, F). By 24 h after ingestion, no digestive vacuole was detected in the host cell.

The cryptomonad nucleus and nucleomorph: Directly after ingestion, the cryptomonad nucleus retained its double membranes and nucleolus, just like that of free-living cryptomonad (Fig. 25A). At 120 h after ingestion, the nucleus was elongated in elliptic shape, but the nuclear envelopes and nucleolus was still clearly observed (Fig. 25B). In the proximity of the chloroplast, the outer membrane ran toward the chloroplast at short intervals (Fig. 25B).

The nucleomorph immediately following ingestion was unchanged through ingestion and located nearby the pyrenoid (Fig. 25C). At 120 h, the nucleomorph was duplicated up to two, and the both nucleomorphs were situated in the periplastidal compartment, and no sign of digestion was detected (Fig. 25D, E).

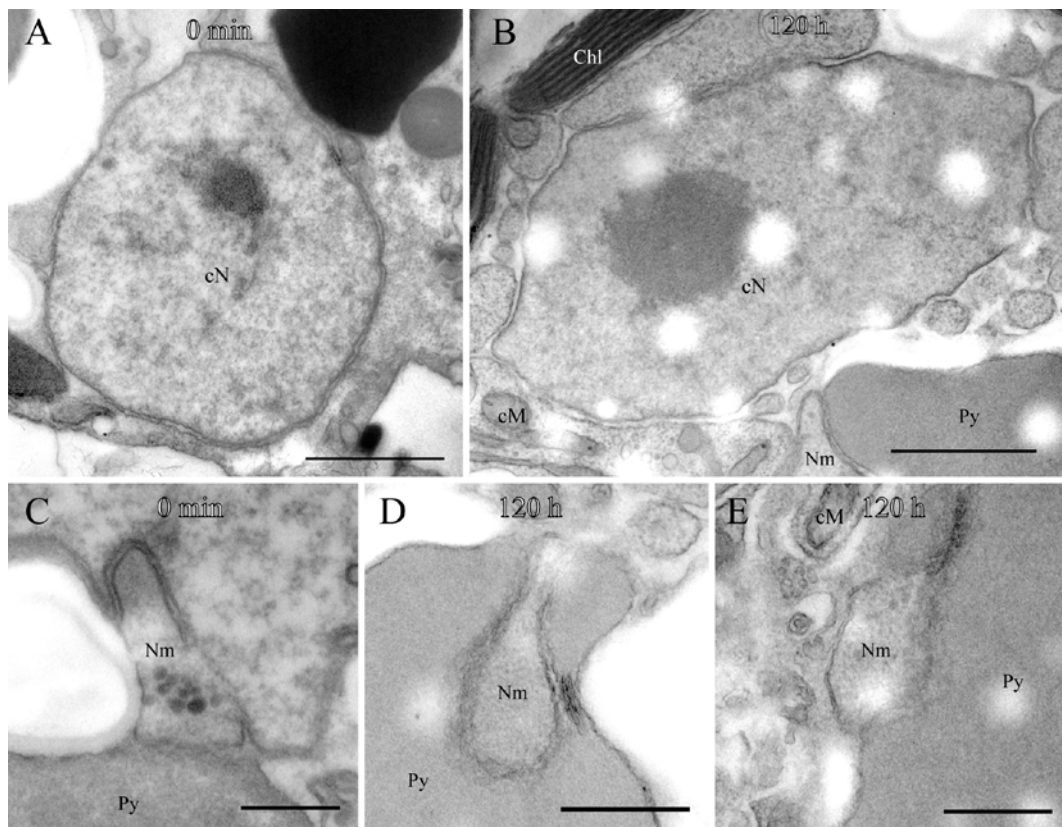


Fig. 25. TEM micrographs of the cryptomonad nucleus and nucleomorph in *Nusuttodinium latum*.
A. Cryptomonad nucleus showing unchanged structure right after ingestion. Bar = 1 μ m.
B. Cryptomonad nucleus at the 120 h stage showing unchanged structure. Bar = 1 μ m.
C. Nucleomorph showing unchanged structure directly after ingestion. Bar = 500 nm.
D, E. The duplicated nucleomorphs showing no sign of digestion at the 120 h stage. Bar = 500 nm.
 Abbreviations: Chl; chloroplast, cN; cryptomonad nucleus, Nm; nucleomorph, Py; pyrenoid, cM, cryptomonad mitochondria.

The morphological change of the kleptochloroplast in *Nusuttodinium aeruginosum*

LM observations

Newly-ingested cryptomonad cells retained their original shape in the hypocone of the dinoflagellate cell and kept their eyespot and pyrenoid (Fig. 26A, B). The cryptomonad cells were oval or elliptic and were not deformed or modified up to the 2 h stage (Fig. 26C-L). At the 3 h stage, they started to be elongate to tear-drop shape (Fig. 26M, N) and, at the 4 h and 6 h stages, they were gradually expanded and its cup-shape were no longer observed (Fig. 26O-Q). At the 12 h and 24 h stages, the cryptomonad cells became highly lobed and took on complex shapes, making their original shape unrecognisable (Fig. 26S-V, see Fig. 36C). No digestive vacuole was detected under LM in *Nusuttodinium aeruginosum*. By the 24 h stage, the newly-ingested cryptomonad cells had enlarged gradually, but they were restricted to the periphery of the hypocone of the dinoflagellate cells rather than being situated throughout the host cell. Thus, the chloroplasts, by the 24 h stage, were not of the same dimensions as those of kleptochloroplasts assimilated in the field, which enlarge into every part of the host cell.

From the experiment at 48 h after ingestion, the dinoflagellate cells have ingested single cryptomonad cell were observed. At 48 h after ingestion, the chloroplast was enlarged into cup shape and located in the peripheral of the host cell. The periphery of the kleptochloroplast was ramified into small lobes (Fig. 27A, B) and the pyrenoid and cryptomonad nucleus were visible (Fig. 13A), but no cryptomonad eyespot was detected. By 72 h after ingestion, the kleptochloroplast almost filled the host and the lobes of the kleptochloroplast increased in size and number (Fig. 27C, D). At this stage, the pyrenoids had also multiplied (Fig. 27C). By 96 h of ingestion, the lobes had extended to the cell periphery, filling the vast majority of the cell interior (Fig. 27E, F). By 120 h of ingestion, the kleptochloroplast further enlarged and its periphery became corrugated (Fig. 27G, H). The dinoflagellate cell grew with the progressive enlargement of the kleptochloroplast (Fig. 27). The cryptomonad nucleus was always located in the hypocone of the host cell. (Fig. 27A, C, E, G).

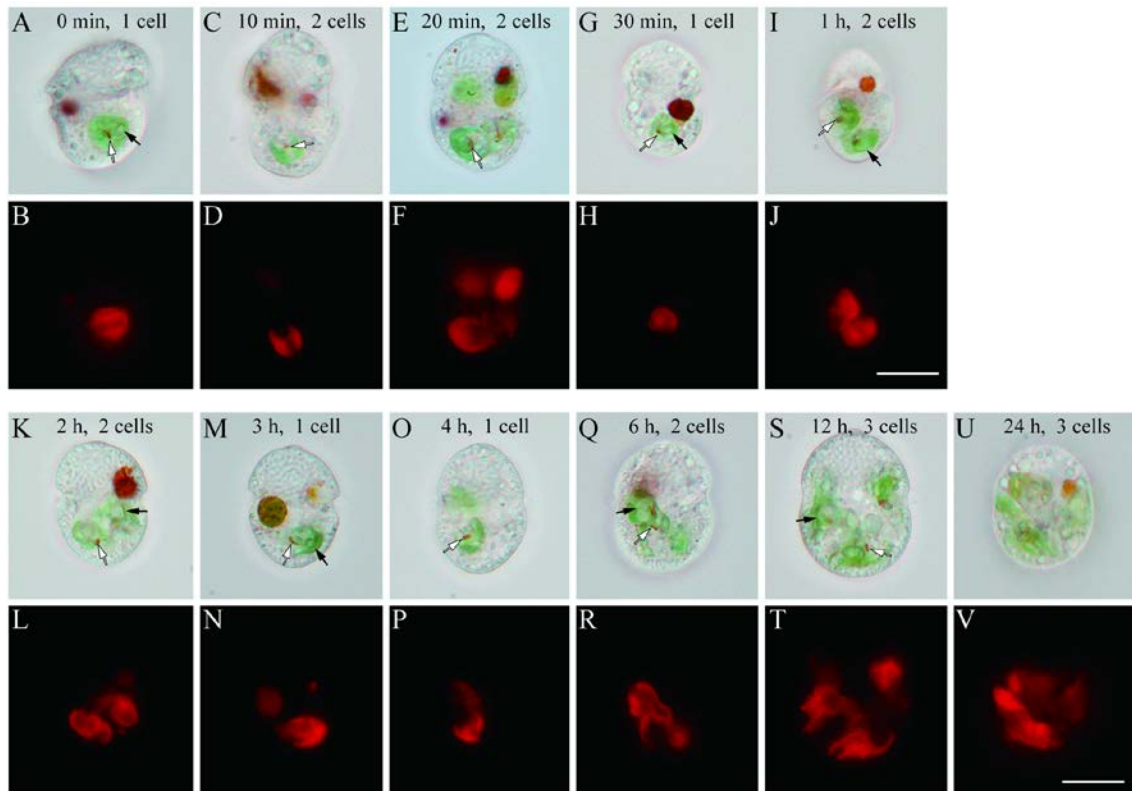


Fig. 26. Bright field and fluorescent micrographs following the morphological change of cryptomonad cells in *Nusuttodinium aeruginosum* with time after ingestion. Times and cell numbers shown on the bright field micrographs indicate that elapsed times after ingestion of *Chroomonas* sp. and the numbers of the ingested cryptomonad cells, respectively. Each fluorescent micrograph corresponds to the bright field micrograph shown above it. Note that cryptomonad cells are deformed significantly by the 6 h stage. White arrows and arrowheads indicate an eyespot and a pyrenoid of cryptomonad respectively. A digestive vacuole is not observed in *N. aeruginosum*. Bar = 10 μ m.

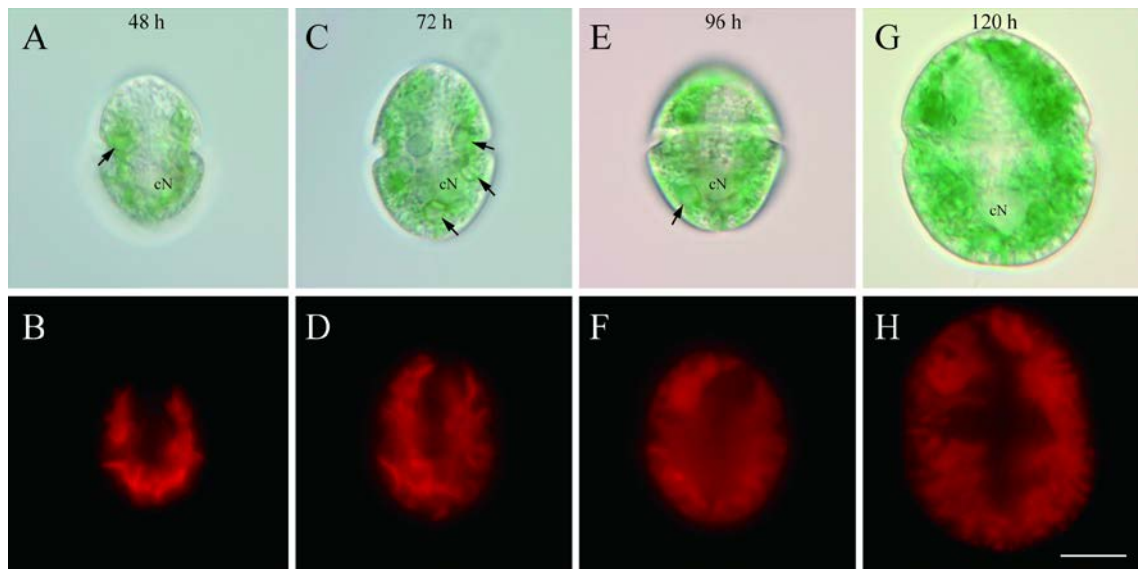


Fig. 27. Bright field and fluorescence micrographs following the morphological change in the single kleptochloroplast ingested by *Nusuttodinium aeruginosum*. Times shown in the bright field micrographs indicate the times since the ingestion of *Chroomonas* sp. Each fluorescence micrograph corresponds to the bright field micrograph directly above it. Note that the kleptochloroplast enlarged gradually in cells that retained the cryptomonad nucleus (cN). Arrows indicate pyrenoids. Bar = 10 μ m.

TEM observations

Membranes of the cryptomonad cell: Right after ingestion, there were four surrounding membranes to the chloroplast: two chloroplast membranes (Fig. 28A white arrowhead) and two chloroplast ER membranes (Fig. 28A arrowhead), and the cryptomonad cytoplasm was separated from the dinoflagellate cytoplasm by a single membrane (= the perisymbiont membrane) (Fig. 28A arrow). During kleptochloroplast enlargement, up to 120 h, the four kleptochloroplast membranes and the perisymbiont membrane were retained (Fig. 28B).

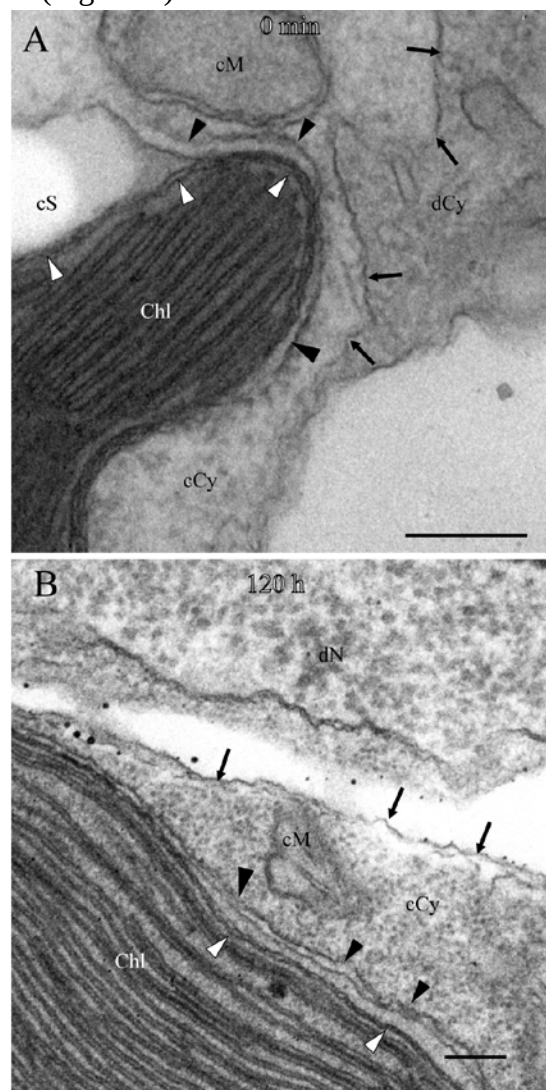


Fig. 28. TEM micrographs of membranes of the chloroplast in *Nusuttodinium aeruginosum*.

A. The ingested chloroplast is surrounded by four membranes; two chloroplast membranes and two chloroplast ER membranes. The cryptomonad cytoplasm is separated from that of the dinoflagellate by a single membrane.

B. Surrounding chloroplast membranes 120 h after. The four membranes and the cytoplasmic boundary membrane are still intact.

White arrowheads, arrowheads and arrows indicate chloroplast membranes, chloroplast ER membranes and the membrane between the cryptomonad and the dinoflagellate cytoplasm. Abbreviations: Chl, chloroplast; cCy, cryptomonad cytoplasm; cM, cryptomonad mitochondria; cS, cryptomonad starch; dCy, dinoflagellate cytoplasm; dS, dinoflagellate starch. Bar = 200 nm.

The chloroplast and additional organelles: The ingested chloroplast was nearly cup-shaped and the cryptomonad cell kept its original arrangement of organelles (Fig. 29A, see Fig. 36C). It was confirmed that almost all cryptomonad organelles were preserved at this stage; i.e. a cryptomonad nucleus, a nucleomorph, ejectosomes, cryptomonad mitochondria and basal bodies (Fig. 29A, 31A, B). Flagella and periplasts were not observed. The digestive vacuole was absent. At the 20 min stage, the shape of the chloroplast had not changed significantly and the cryptomonad organelles were preserved in their original positions. The cryptomonad nucleus was located in the posterior position, surrounded by the cup-shaped chloroplast. The nucleomorph was near the eyespot. At this stage, the cryptomonad cytoplasm was still present around the chloroplast (Fig. 29B). At the 3 h stage, the chloroplast had slightly changed by losing its original cup shape and the cryptomonad cytoplasm was still present (Fig. 29C). By the 6 h stage, although the cryptomonad nucleus and mitochondria were in the cytoplasm of the cryptomonad cell and the nucleomorph was still observed, the chloroplast had obviously deformed from the original cup-shape and the relative position of the nucleus, the pyrenoid and the nucleomorph was changed, indicating the original dorsiventrality in the arrangement of the cryptomonad organelles was lost (Fig. 29D). By the 12 h stage, the chloroplast was much more deformed and irregularly elongated than it was at the 6 h stage (Fig. 29E). Even this late, the cryptomonad cytoplasm was retained, and the nucleus, mitochondria and a nucleomorph were also observed (Fig. 29E). At the 24 h stage, the chloroplast continued to be deformed in a more irregular fashion than witnessed at the 12 h stage. The cryptomonad cytoplasm was still present around the chloroplast, and a cryptomonad nucleus and a nucleomorph could still be discerned (Fig. 29F).

At the 48 h stage, the kleptochloroplast was enlarged and randomly ramified (Fig. 30A). The cryptomonad cytoplasm was contained within the cup-shaped kleptochloroplast, and contained the cryptomonad nucleus, nucleomorph(s), and cryptomonad mitochondria (Fig. 30A). By 72 h after ingestion, the kleptochloroplast had almost reached the periphery of the host cell on all sides (Fig. 30B). The pyrenoids

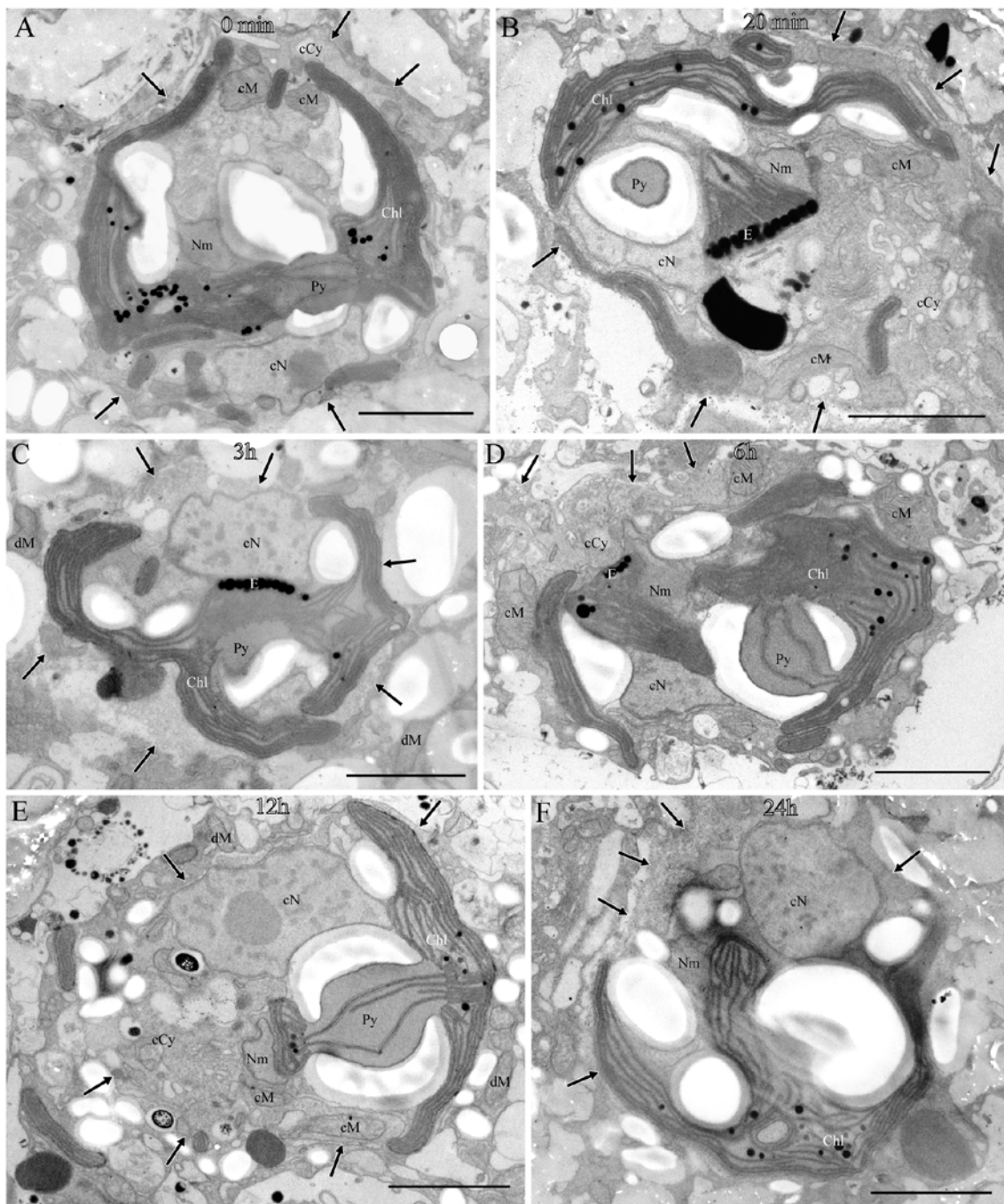


Fig. 29. TEM micrographs following the morphological change of cryptomonad cells in *Nusuttodinium aeruginosum* with time after ingestion.

A. Ingested cryptomonad cell directly after ingestion. The cell of *N. aeruginosum* possesses a chloroplast, a cryptomonad nucleus, a nucleomorph, cryptomonad mitochondria and cytoplasm.

B. Cryptomonad cell 20 min after ingestion. The chloroplast is slightly deformed. Cryptomonad organelles are not removed by this stage.

C. Cryptomonad cell 3 h after ingestion. The chloroplast is enlarged and has lost the original elliptic shape.

D. Cryptomonad cell 6 h after ingestion. Note that the complete loss of the original dorsiventrality of the cryptomonad. Cryptomonad organelles are well retained.

E. Cryptomonad cell 12 h after ingestion. Cryptomonad organelles within cryptomonad cytoplasm can be observed.

F. Cryptomonad cell 24 h after ingestion. Note that the cryptomonad nucleus, the nucleomorph and the cryptomonad cytoplasm are retained although the chloroplast has been modified.

Arrows indicate the boundary between the cryptomonad and the dinoflagellate cytoplasm. Abbreviations: Chl, chloroplast; cN, cryptomonad nucleus; Nm, nucleomorph; cM, cryptomonad mitochondria; Py, pyrenoid; E, eyespot; cCy, cryptomonad cytoplasm; dM, dinoflagellate mitochondria. Bar = 2 μ m.

had multiplied and been distributed in both the epicone and the hypocone of the host cell (Fig. 30B). The composition of the individual cryptomonad organelles was the same as in the previous stage (Fig. 30B). At 96 h, the number of pyrenoids had significantly increased and a longitudinal section of the cell showed at least 5 pyrenoids (Fig. 30C). By 120 h after ingestion, the host cell possessed an enlarged kleptochloroplast that pervaded the entire cell, the cryptomonad nucleus and the nucleomorph (Fig. 30D). TEM observations showed that the cryptomonad nucleus was always located in the

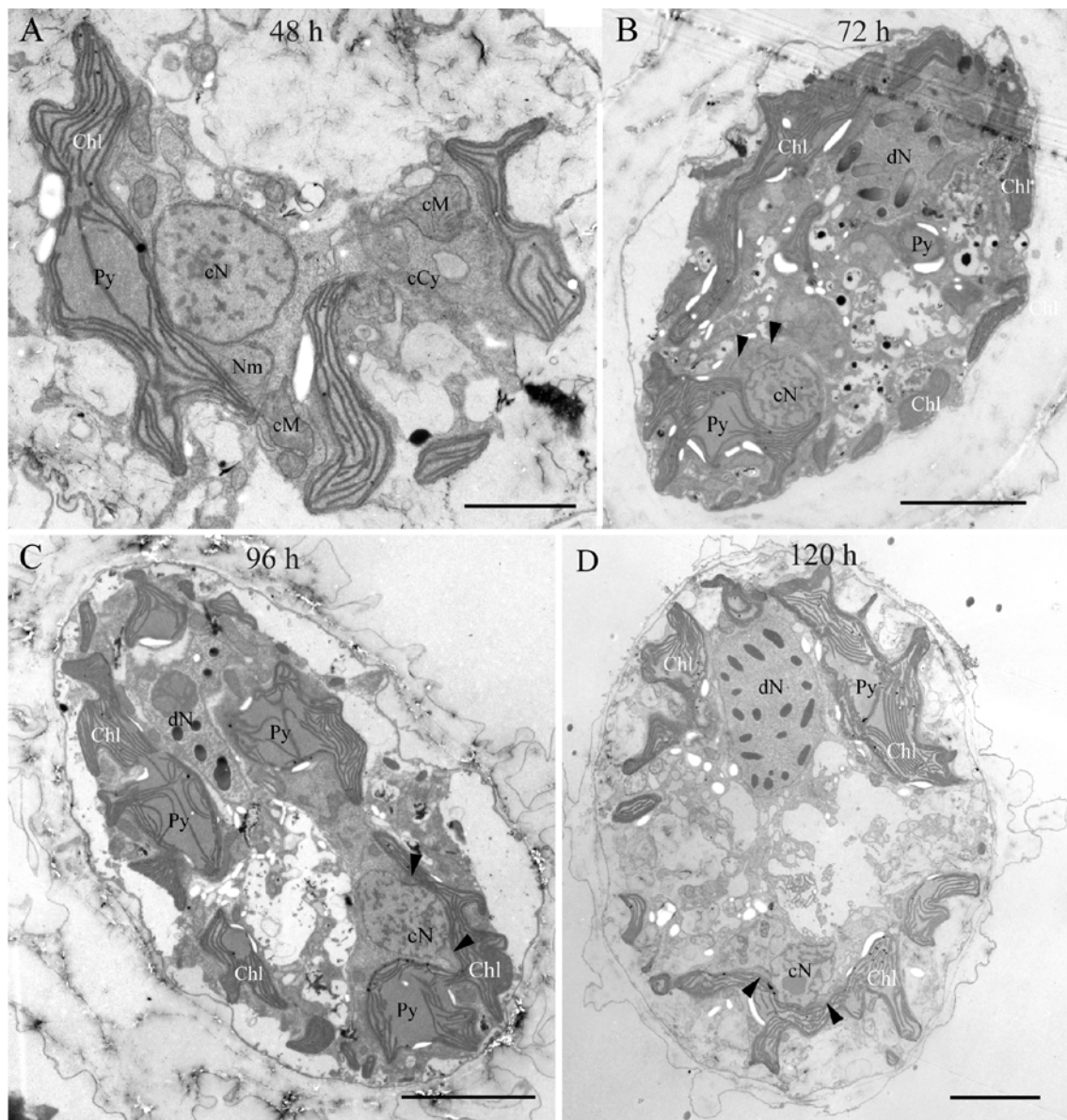


Fig. 30. TEM micrographs following the morphological changes to the ingested cryptomonad cells in *Nusuttodinium aeruginosum* over time since ingestion.

A. TEM micrograph 48 h after ingestion showing the early loss of normal cryptomonad shape caused by peripheral lobing. Bar = 2 μ m.

B-D. Cells of *N. aeruginosum* 72, 96 and 120 h after ingestion showing the elaboration of the kleptochloroplast to fill much of the host cell as well as the duplication of the pyrenoids. Note that a cryptomonad nucleus and nucleomorphs (arrowheads) are located in the hypocone. Bar = 5 μ m.

Abbreviations: Chl, kleptochloroplast; cN, cryptomonad nucleus; Nm, nucleomorph; cM, cryptomonad mitochondria; Py, pyrenoid; cCy, cryptomonad cytoplasm; dN, dinoflagellate nucleus.

hypocone of the host cell and that the nucleus, nucleomorph(s) and mitochondria were not digested during kleptochloroplast enlargement at least up to 120 h of ingestion (Fig. 30A-D). No digestive vacuole was observed. The cryptomonad nucleus and nucleomorphs were inherited to daughter cell through the host cell division, and the distribution pattern of them was traced in the next chapter.

Cryptomonad ejectosomes and the digestive vacuoles: The peripheral ejectosomes of the cryptomonad cell did not accumulated like they were in *Nusuttodinium poecilochroum* right after ingestion (Fig. 31A, B). At the 1 h stage, the gullet-surrounding ejectosomes were still present in the cryptomonad cytoplasm (Fig. 31C) and no digestive vacuole had formed in the dinoflagellate cytoplasm. Both sets of ejectosomes were not actively removed from the cryptomonad cytoplasm as they were

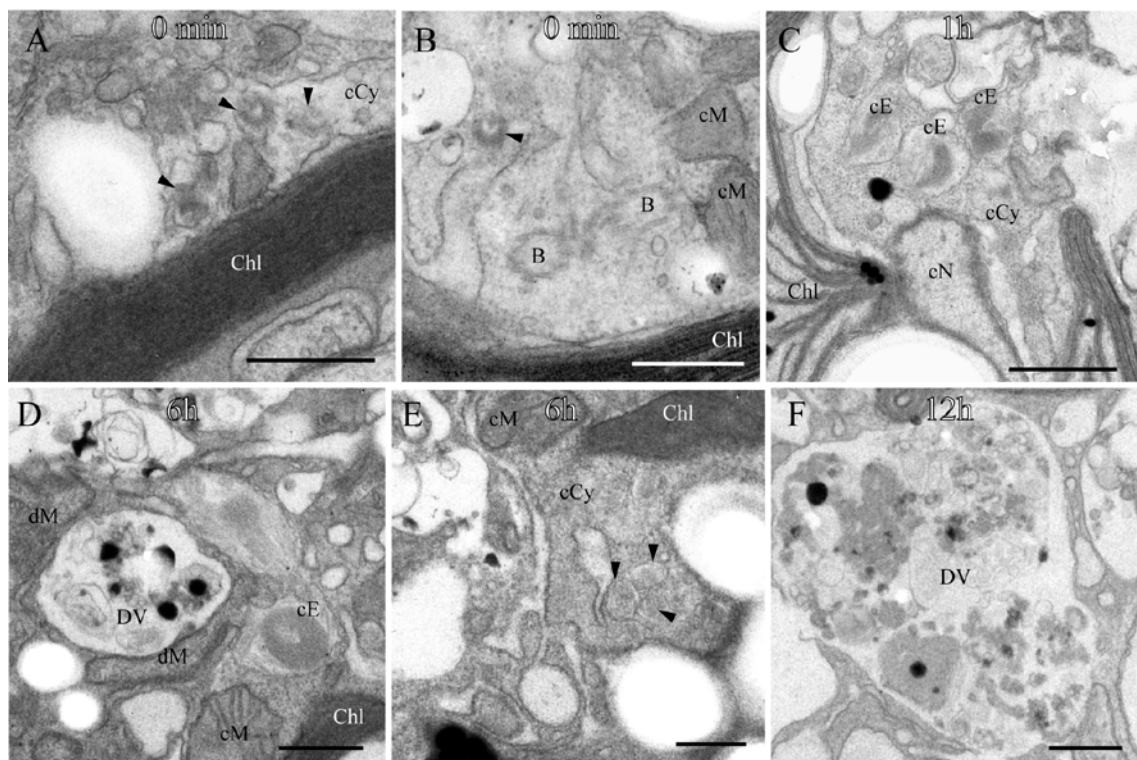


Fig. 31. TEM micrographs of cryptomonad ejectosomes and digestive vacuoles in *Nusuttodinium aeruginosum* cell. **A, B.** Cryptomonad cytoplasm containing peripheral ejectosomes, a basal bodies and cryptomonad mitochondria directly following ingestion. Note that peripheral ejectosomes are not accumulated as they are in *N. poecilochroum*. Bar = 500 nm. **C.** Cryptomonad cytoplasm containing gullet-surrounding ejectosomes at the 1 h stage. Note that gullet-surrounding ejectosomes are retained in the cryptomonad cytoplasm. Bar = 500 nm. **D.** Digestive vacuoles containing unknown material at the 6 h stage. Note that gullet-surrounding ejectosomes remain in the cryptomonad cytoplasm. Bar = 1 µm. **E.** Cryptomonad cytoplasm containing peripheral ejectosomes at the 6 h stage. Bar = 500 nm. **F.** Digestive vacuole at the 12 h stage showing an increase in size (2.5 µm in diameter) relative to that at the 6 h stage (1 µm in diameter). The contents of the digestive vacuole are unknown. Bar = 500 nm. Arrowhead indicates an individual peripheral ejectosome. Abbreviations: Chl, chloroplast; cN, cryptomonad nucleus; cM, cryptomonad mitochondria; cE, gullet-surrounding ejectosome; B, basal body; cCy, cryptomonad cytoplasm; DV, digestive vacuole; dM, dinoflagellate mitochondria.

in *N. poecilochroum*. Digestive vacuoles appeared at the 6 h stage (1 μm in diameter), but ejectosomes were not transferred into them (Fig. 31D, E). Rather they were retained in the cryptomonad cytoplasm at this stage (Fig. 31D, E). At the 12 h stage, the ejectosomes disappeared from the cryptomonad cytoplasm. Although the digestive vacuole was discernible at this stage too, neither type of ejectosomes was found in the digestive vacuole (Fig. 31F).

The cryptomonad nucleus and nucleomorph: The cryptomonad nucleus immediately following ingestion was unchanged and held in the cryptomonad cytoplasm (Fig. 32A). It was retained without any modification throughout all investigated times and its membranes were obvious, even at the 24 h stage (Fig. 32B).

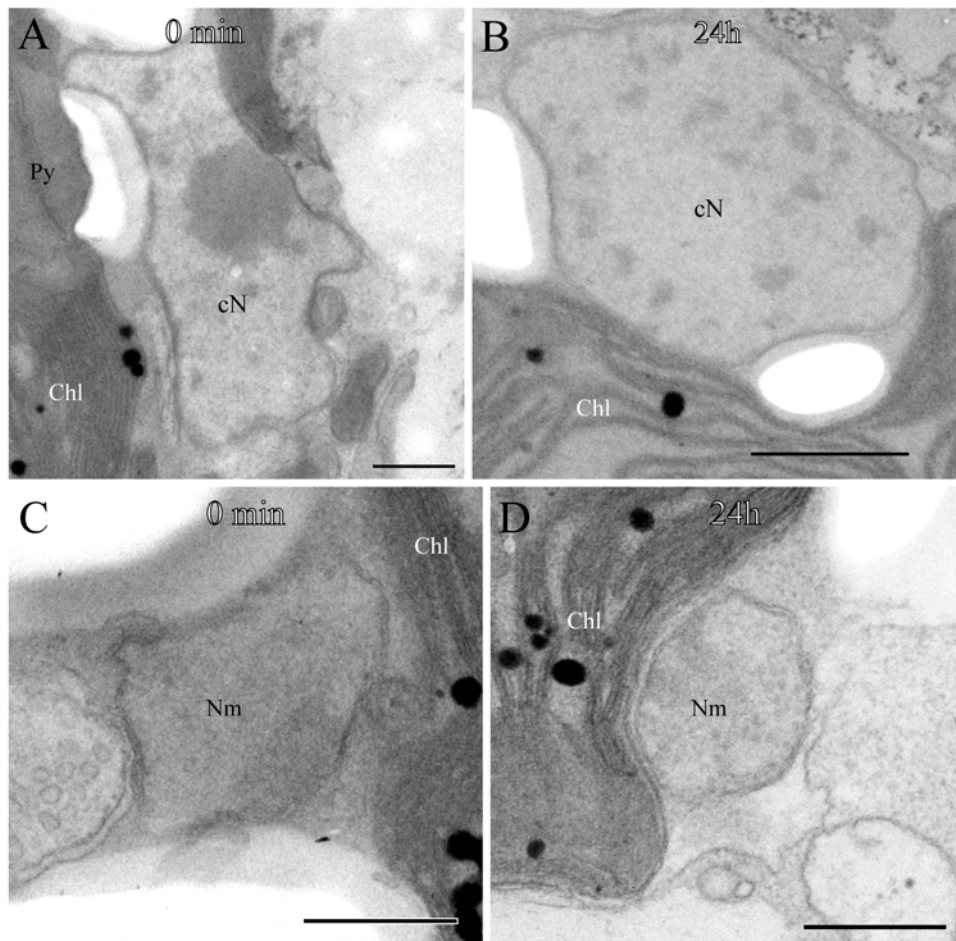


Fig. 32. TEM micrographs of the cryptomonad nucleus and nucleomorph in *Nusuttodinium aeruginosum*.
A. Cryptomonad nucleus showing its unchanged structure from the original cryptomonad nucleus at the time of ingestion. Bar = 500 nm.
B. Cryptomonad nucleus retaining its structure without any sign of digestion 24 h after ingestion. Bar = 1 μm .
C. Nucleomorph showing its intact structure directly after ingestion. Bar = 500 nm.
D. Nucleomorph at the 24 h stage, showing no effect of digestion. Bar = 500 nm.
 Abbreviations: Chl, chloroplast; cN, cryptomonad nucleus; Nm, nucleomorph; Py, pyrenoid.

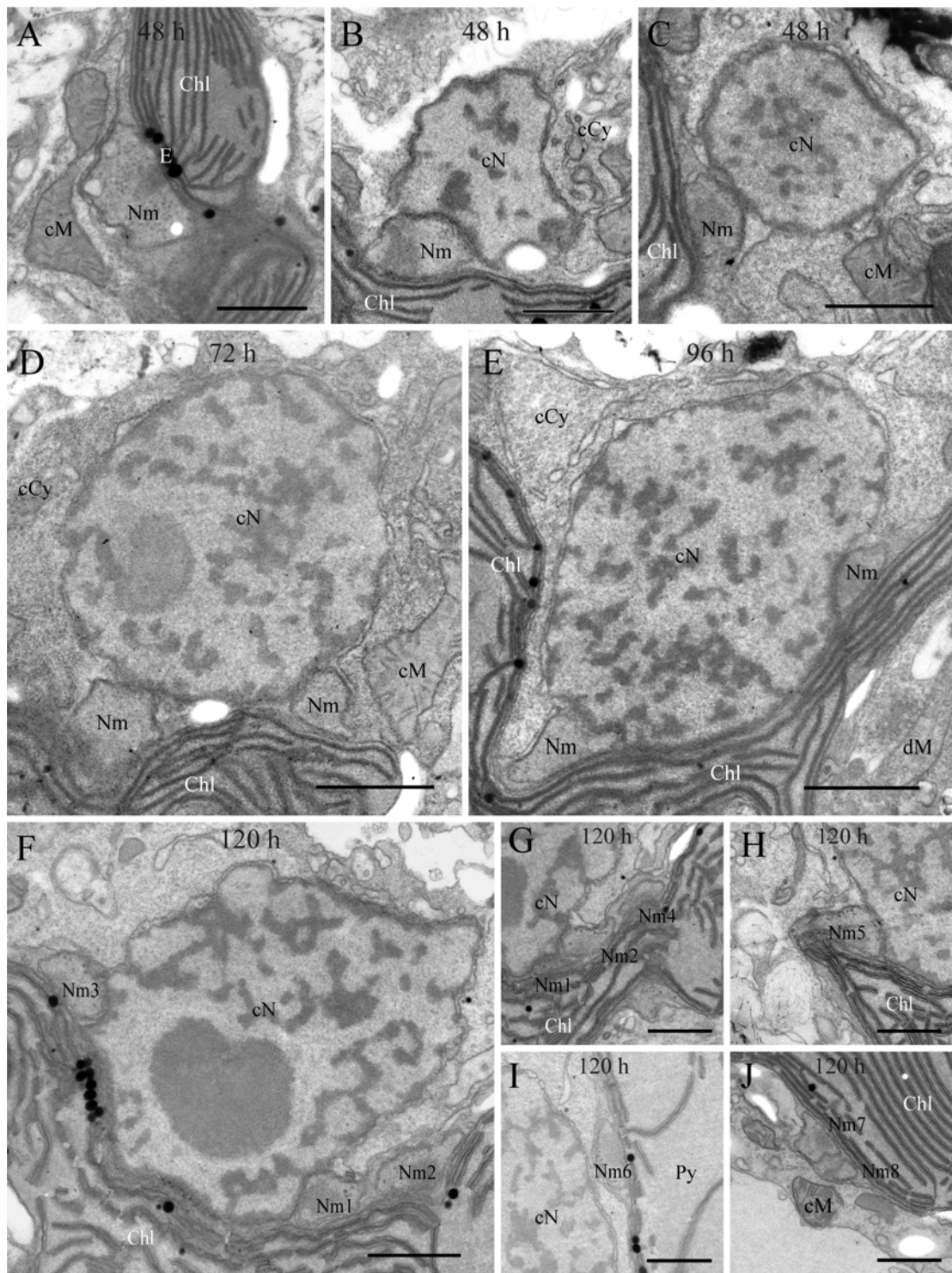


Fig. 33. TEM micrographs of cryptomonad nucleus and nucleomorphs in *Nusuttodinium aeruginosum* cell.

A. A nucleomorph 48 h after ingestion, near the eyespot.

B, C. Nucleomorphs in another cell 48 h after ingestion. The nucleomorph has replicated and is near the cryptomonad nucleus.

D-E. The cryptomonad nucleus and nucleomorphs 72 h (D) and 96 h (E) after ingestion. Note that the nucleomorphs are situated between the cryptomonad nucleus and the kleptochloroplast.

F-J. The cryptomonad nucleus and up to eight nucleomorphs (Nm1-Nm8) 120 h after ingestion.

Abbreviations: Chl, kleptochloroplast; cN, cryptomonad nucleus; Nm, nucleomorph; cM, cryptomonad mitochondria; E, eyespot; cCy, cryptomonad cytoplasm; dM, dinoflagellate mitochondria. Bar = 1 μ m.

The nucleomorph was also unchanged right after ingestion and located in the periplastidal compartment (Fig. 32C). The structure and position of nucleomorph were maintained unaltered up to the 24 h stage, and no sign of digestion was evident using the TEM (Fig. 32D). No divisions of the cryptomonad nucleus and the nucleomorph were observed.

I have observed three cells 48 h after ingesting a cryptomonad. One of these possessed a nucleomorph near the eyespot (Fig. 33A) while another had two nucleomorphs near the cryptomonad nucleus (Fig. 33B, C), indicating that the position of nucleomorphs at this stage was variable. The third cell had one nucleomorph near the cryptomonad nucleus (data not shown). By 72 h and 96 h of ingestion, the nucleomorph had divided and their products were located between the kleptochloroplast and cryptomonad nucleus (Fig. 33D, E). Serial sectioning confirmed that, the three cells we observed at the 72 h stage possessed 3, 3 and 2 nucleomorphs, respectively. I observed two cells at the 96 h stage and detected 2 nucleomorphs in each cell. Of two cells observed at the 120 h stage, one had 9 nucleomorphs while the other had 8 (all nucleomorphs in the latter cell are shown in Fig. 33F-J). Most of them were located near the cryptomonad nucleus, but some were positioned in the middle of the host cell (Fig. 33J). During kleptochloroplast enlargement, the cryptomonad nucleus retained the integrity of its nuclear envelopes and no digestion of the nucleus was detected (Fig. 33B-F). No division of cryptomonad nucleus was observed up to 120 h stage.

The estimates of kleptochloroplast volumes with confocal laser scanning microscope

In *N. poecilochroum*, the volume of chloroplast at 0 min stage was estimated as $117.72 (\pm 37.83 \text{ SD}) \mu\text{m}^3$. Despite modification of the shape of the chloroplast seen in LM and TEM observation, the volume of chloroplast remained almost constant, i.e. around $100 \mu\text{m}^3$ during the incubation time, and enlargement of kleptochloroplast was not observed based on this volume estimate (Fig. 34). In *N. latum*, the volume at 0 min was estimated as $76.30 (\pm 15.46 \text{ SD}) \mu\text{m}^3$. By 24 h, the volume reached $159.73 (\pm 30.73 \text{ SD}) \mu\text{m}^3$, indicating the kleptochloroplast grew up twice as large as 0 min stage. At 120 h, the

kleptochloroplast was considerably enlarged and the volume was estimated as $1678.22 (\pm 321.35 \text{ SD}) \mu\text{m}^3$ (Fig. 34). In *N. aeruginosum* at 0 min stage, the volume of the chloroplast was $70.49 (\pm 25.09 \text{ SD}) \mu\text{m}^3$. The volume was not changed up to 12 h (Fig. 34). The chloroplast, however, started to grow up significantly from 12 h and reached the volume of $1091.17 (\pm 378.35 \text{ SD}) \mu\text{m}^3$ at 72 h, more than ten-fold compared with that at 0 min (Fig. 34). The chloroplasts at 72 h were pervaded throughout the host cell, forming ramified shape. At 120 h after ingestion, the kleptochloroplast volume ($n = 10$) was $1694.3 (\pm 519.6 \text{ SD}) \mu\text{m}^3$, with a maximum and minimum volume of 2627.1 and $1064.4 \mu\text{m}^3$, respectively. The volume at 120 h was more than 20-fold that at the 0 min stage (Fig. 34). Unfortunately, we failed to measure volume at 72 h in *N. poecilochroum*, because the cells were disappeared after the incubation time. In my preliminary observation, it was revealed that the individual kleptochloroplast was retained only about for 3 days in *N. poecilochroum*.

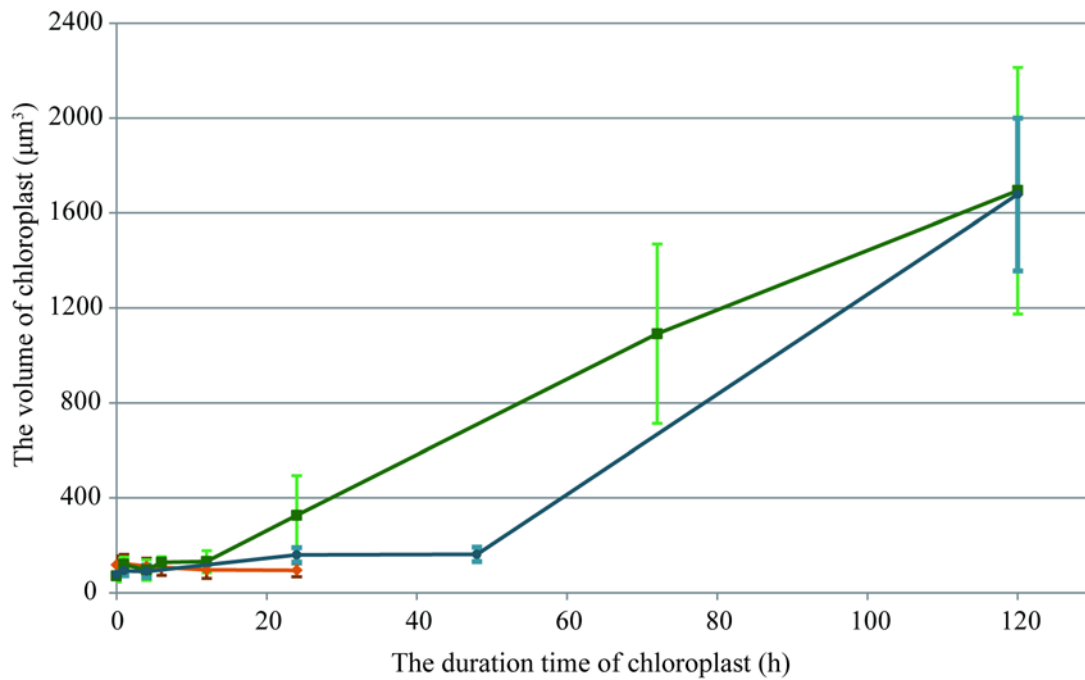


Fig. 34. Volume (μm^3) of ingested chloroplast in each stage from ingestion of cryptomonad in *Nusuttodinium poecilochroum* (diamond, orange), *N. latum* (circle, blue) and *N. aeruginosum* (square, green). Error bars mean SD.

DISCUSSION

In this study, using the single-cell LM and TEM methods, detailed structural changes of ingested cryptomonad cells were followed up to 120 h after ingestion of cryptomonad prey in *N. poeciloichroum*, *N. latum* and *N. aeruginosum* respectively (in case of *N. poeciloichroum*, only up to 12 h). These three species process their prey after ingestion in a very different fashion and might provide clues about the evolutionary steps required from simple ingestion as prey to the establishment of a true chloroplast.

Formation of the digestive vacuole and isolation of the kleptochloroplast

Directly after ingestion, the ingested cryptomonad cells lack periplast and flagella in the dinoflagellate cells. In *N. poeciloichroum* and *N. latum*, basal bodies were also not found in spite of TEM observation using serial sections of 7 cells and 4 cells, respectively. The ingested cells were encircled by a single membrane. These conditions of the ingested cryptomonad cells are same as those observed by Larsen (1988). He observed *N. poeciloichroum* ingesting a cryptomonad cell under TEM and suggested that the periplast may be digested in feeding process, and that the membrane encircling the ingested cell (referred as 'phagotrophic vacuole') was likely to be formed by the dinoflagellate (Larsen 1988). This study also suggests that outer structures of the cryptomonad were removed in the feeding process. The membrane separating the cryptomonad and the dinoflagellate cytoplasm should not be derived from the cytoplasmic membrane of the cryptomonad because the cytoplasmic membrane lies just outside the inner periplast component (Brett *et al.* 1994).

Dinophysis spp. are known to obtain kleptochloroplasts by myzocytosis from *Mesodinium rubrum* that in turn possesses kleptochloroplasts derived from the cryptomonad genus *Teleaulax* (Park *et al.* 2006; Nagai *et al.* 2008; Nishitani *et al.* 2008). *Dinophysis fortii* Pavillard, heavily fed on *M. rubrum*, possesses solitary kleptochloroplasts (= restricted to chloroplasts only) and several food vacuoles that contain membrane-like and mitochondria-like structures. The kleptochloroplasts lie directly in the dinoflagellate cytoplasm, and not in the food vacuole (Nagai *et al.* 2008).

Recently, the process of kleptochloroplast isolation in *D. caudata* was revealed ultrastructurally (Kim *et al.* 2012). The study showed that *D. caudata* takes up kleptochloroplasts together with other organelles of *M. rubrum* into a food vacuole, and then isolated the kleptochloroplasts from the food vacuole into the dinoflagellate cytoplasm (Kim *et al.* 2012). In *Nusuttodinium poecilochroum*, *N. latum* and *N. aeruginosum*, the chloroplast and cryptomonad organelles were ingested into the dinoflagellate cytoplasm directly, not via a digestive vacuole, making the mechanism of kleptochloroplast incorporation into the dinoflagellate cytoplasm clearly different from that employed by *D. caudata*.

This study reveals that digestive vacuole formation in *Nusuttodinium poecilochroum* was quite rapid, occurring within 20 minutes of ingestion. The digestive vacuoles initially (20 min) contained cryptomonad mitochondria, peripheral ejectosomes and later (1 h) the gullet-surrounding ejectosomes were added. The digestive vacuoles increased in number and volume with time, indicating that the digestive vacuole formation involves modification of the prey cell after ingestion. In *N. latum* too, the ejectosomes and mitochondria that had been located in the outside of the chloroplast were transferred into the digestive vacuole by 1 h. Larsen (1988) showed that the membrane between the cryptomonad cytoplasm and the dinoflagellate cytoplasm was very close to the chloroplast ER because of a highly reduced cryptomonad cytoplasm. He showed that *N. poecilochroum* possessed digestive vacuoles containing cryptomonad ejectosomes and mitochondria, but failed to demonstrate the process of formation of the digestive vacuoles and the resultant decrease of the cryptomonad cytoplasm. In this study, the volume of the cryptomonad cytoplasm was demonstrated to be decreased and the cryptomonad mitochondria and ejectosomes were not observed by TEM after 1 h. This supports the interpretation that *N. poecilochroum* and *N. latum* remove the cryptomonad cytoplasm together with its organelles, such as mitochondria and ejectosomes, by actively transferring them into a digestive vacuole and as a result, the cryptomonad cytoplasm is removed and the membrane between the host and the prey approaches to the chloroplast ER.

In contrast to *Nusuttodinium poecilochroum* and *N. latum*, *N. aeruginosum* did not

form a digestive vacuole rapidly, and if digestive vacuoles were formed, cryptomonad mitochondria and ejectosomes were not transferred into them until later stages; the ejectosomes being removed from the cytoplasm and disappearing at the 12 h and 24 h stages. These results suggest that *N. aeruginosum* can eliminate and digest ejectosomes between 6 h and 12 h of ingestion, transferring them into the digestive vacuole. Unlike *N. poeciloichroum* and *N. latum* which removed the prey's cytoplasm together with the non-plastidial organelles, including the nucleus, *N. aeruginosum* seemed to select and remove only the unnecessary cryptomonad organelles, because the prey's cytoplasm was retained around the chloroplast until much later. Wild material of *N. aeruginosum* has never been seen to possess cryptomonad ejectosomes, although this could be because digestive vacuoles were not encountered (Schnepf *et al.* 1989). Such a condition probably represents a stage after the selective elimination of ejectosomes, as observed in this study. The underlying mechanism behind the selective removal of particular organelles, however, remains unknown.

Enlargement and modification of the kleptochloroplast

The host organisms that undergo kleptochloroplastidy tend to enlarge or deform the ingested chloroplast. The katablepharid, *Hatena arenicola* Okamoto & Inouye, is known to possess a single kleptochloroplast derived from a *Nephroselmis* sp. (Prasinophyceae, Viridiplantae) (Okamoto & Inouye 2006). The symbiont in *H. arenicola* is enlarged after engulfment, occupying most of the host cytoplasm. The volume of the symbiont is more than ten-fold relative to the free-living *Nephroselmis*. The eyespot of the symbiont is invariably positioned at anterior of the host cell and the pyrenoid is duplicated from the single pyrenoid in the free-living *Nephroselmis* (Okamoto & Inouye 2006). In the case of *Dinophysis caudata*, kleptochloroplasts make their way into the dinoflagellate cytoplasm via the food vacuoles, and then elongate toward the periphery of the cell. At the same time, the kleptochloroplasts are highly modified not only with regard to their shape but also with regard to the arrangement of the thylakoids relative to the pyrenoid (Kim *et al.* 2012). As in *H. arenicola* and *D. caudata*, the kleptochloroplasts of *Nusuttodinium poeciloichroum*, *N. latum* and *N. aeruginosum* also become highly

modified under experimental conditions. The modification of the kleptochloroplast witnessed in *H. arenicola* and the unarmoured kleptochloroplastidic dinoflagellates represents a significant shift from the response of the prey chloroplasts during ordinary phagotrophy, where it is immediately digested. Therefore, the modification of the prey seems to be one of the characteristic features of kleptochloroplastidy and distinguishes it from the phagotrophy.

The kleptochloroplasts of currently-observed cells of *Nusuttodinium poecilochroum* (LM at 3 h and 4 h stages) were more elongated and lobed than those formerly observed (Larsen 1988), but they did not become more ramified after this stage. This implies that kleptochloroplast modification reaches its maximum state at around the 4 h stage. However, the volume of the kleptochloroplast is not increased up to 24 h, indicating *N. poecilochroum* can modify the kleptochloroplast shape but cannot enlarge it.

In *Nusuttodinium latum*, the kleptochloroplast was significantly enlarged and deformed from the original shape. It also showed tendency to encircle cryptomonad organelles such as nucleus, nucleomorph, pyrenoid and mitochondria inside cup-shaped chloroplast. Previous ultrastructural study on this species showed that the kleptochloroplast drastically deforms with a concomitant multiplication of pyrenoids and nucleomorphs. In addition, the kleptochloroplast itself divides within a single cryptomonad component (Horiguchi & Pienaar 1992). Moreover, the shape of kleptochloroplast surrounding the cryptomonad organelles in wild cells was similar with the kleptochloroplast observed at 120 h after ingestion (Horiguchi & Pienaar 1992). Although this study could not detect chloroplast division, the enlargement of the chloroplast and the duplication of nucleomorph and pyrenoids were successfully demonstrated to take place in cultural condition at 120 h after ingestion. Therefore, this study suggests that the deformed cryptomonad cell in natural population represents the cryptomonad cell that has been ingested more than five days before.

In *Nusuttodinium aeruginosum*, the kleptochloroplast grew within the host cell, by developing lobes, increasing the number of pyrenoids and extending the thylakoid membranes. The kleptochloroplast was significantly deformed and increased its volume relative to the original cryptomonad chloroplast (Fig. 36). Such enlargement and

deformation correspond to those found in kleptochloroplasts in natural population (Schnepf *et al.* 1989). Previous studies on *N. acidotum* showed that its ingested cryptomonad possesses duplicated nucleomorphs, situated near the cryptomonad nucleus (Wilcox & Wedemayer 1984; Farmer & Roberts 1990). The nucleomorphs observed in this study were in similar locality and duplicated up to 9 in the dinoflagellate cell before host cell division. The nucleomorph division demonstrated in this study suggests that the multiplication of nucleomorphs also takes place in *N. acidotum* in nature. In free-living cryptomonads, it is suggested that fibrous structures within the nucleomorph are involved in its division, and the division takes place in the absence of microtubules or a spindle, which is indicative of amitotic division of the nucleomorph (McKerracher & Gibbs 1982; Morrall & Greenwood 1982; Sato *et al.* 2014). Even after ingestion by the dinoflagellate, the nucleomorphs are located in the periplastidal compartment and remain intact, implying that the nucleomorph itself is divided with the same mechanism as that in free-living cryptomonad.

This study indicates that there are some differences in the manner of kleptochloroplastidy among *Nusuttodinium* species. The ingested chloroplast in *N. poecilochroum* is located in the dinoflagellate cytoplasm and just deformed without enlargement. By contrast to *N. poecilochroum*, *N. latum*, *N. myriopyrenoides*, *N. acidotum* and *N. aeruginosum* display remarkable enlargement of kleptochloroplast. In *Nusuttodinium latum*, although substantially enlarged, kleptochloroplast never filled the bulk of the host cell and synchronous division with the host cell was not observed (Horiguchi & Pienaar 1992). While there is limited enlargement of the kleptochloroplast in *N. latum*, another marine species, *N. myriopyrenoides* expands its kleptochloroplast throughout the host cell (Yamaguchi *et al.* 2011). The cryptomonad pyrenoid in *N. myriopyrenoides* can be multiplied, and each pyrenoid matrix is covered with a starch sheath (Yamaguchi *et al.* 2011). Although *N. myriopyrenoides* seems very similar to *N. aeruginosum* with regard to the enlargement of the kleptochloroplast, there is still uncertainty, due to a lack of careful culture study, about the number of nucleomorphs and about whether synchronous kleptochloroplast/host division occurs or not (Yamaguchi *et al.* 2011). Previous studies reported that *N. acidotum* possesses a single

kleptochloroplast that fills the bulk of the host cell, a cryptomonad nucleus and several nucleomorphs (Wilcox & Wedemayer 1984; Farmer & Roberts 1990). In unialgal culture, *N. acidotum* seems to be able to divide its single kleptochloroplast and distribute it equally to the daughter cells, although the morphology of the divided cell or the kleptochloroplast has never been investigated (Fields & Rhodes 1991). It seems, then, that kleptochloroplastidy in *N. acidotum* is at the same evolutionary stage as that in *N. aeruginosum*. Thus, kleptochloroplastidy in *Nusuttodinium* has wide variety as to the degree of kleptochloroplast enlargement and division of cryptomonad organelles including chloroplast. It is clear, therefore, a more detailed study is needed for a comprehensive comparison within the genus *Nusuttodinium*, especially in *N. acidotum* and *N. myriopyrenoides* under cultural condition.

Retention and digestion of cryptomonad organelles

Unarmoured kleptochloroplastidic dinoflagellates are known to possess, in addition to the chloroplast, organelles originating from the cryptomonad; these include the nucleus, nucleomorph and often mitochondria (Wilcox & Wedemayer 1984; Larsen 1988; Horiguchi & Pienaar 1992). However, previous studies on these dinoflagellates have never focused on fate of these organelles after ingestion with time.

This study revealed that the surface of the cryptomonad nucleus wrinkles shortly after ingestion in *Nusuttodinium poecilochroum* and that the nuclear membranes become indistinct as early as the 3 h stage. Moreover, it was completely disappeared by the 6 h stage. These results strongly suggest that the cryptomonad nucleus is selectively and rapidly digested by *N. poecilochroum*. By contrast, the nucleomorph was highly preserved in this species at least up to the 12 h stage. Cryptomonads that belong to *Rhodomonas*, position their nucleomorph within the pyrenoid matrix (Hill & Wetherbee 1989). The *Rhodomonas* sp. used in this study also has the nucleomorph embedded in the pyrenoid matrix. Previous study shows that *N. amphidinioides* possessed blue-green cryptomonad chloroplast without any other cryptomonad organelles including the nucleomorph and its chloroplast is originated from blue-green cryptomonad *Chroomonas* (Wilcox & Wedemayer 1985; Takano *et al.* 2014). These results suggest

that the cryptomonad organelles except the chloroplast were removed after ingestion (Takano *et al.* 2014), and the removal of nucleomorph might depend on its position. In case of *N. amphidinioides*, the nucleomorph of *Chroomonas* is located beside the pyrenoid, not in the pyrenoid matrix (see Fig. 36) and thus it was removed together with other organelles. By contrast, in the case of *Rhodomonas* prey, the embedded nature of this nucleomorph might contribute to its retention after ingestion by *N. poecilochroum* cell.

This study reveals that there are some differences in the strategy for retention of cryptomonad organelles between *Nusuttodinium latum* and *N. aeruginosum*. In *N. latum*, although the retention of membranes surrounding the chloroplast and cryptomonad cytoplasm could not be ascertained clearly, the cryptomonad organelles, i.e. a nucleus, nucleomorphs and mitochondria, are retained inside the cup-shaped chloroplast. Thus, *N. latum* tends to maintain cup-shape chloroplast and allows the cryptomonad organelles to stay inside the chloroplast, while the organelles locating outside the chloroplast ER are removed and digested. This strategy can be interpreted that the host cell protects cryptomonad organelles from exposing directly to the proximity of the dinoflagellate cytoplasm, probably to avoid being digested. In *N. aeruginosum*, the cryptomonad cell including cryptomonad cytoplasm is retained relatively intact. The host cell always contains a chloroplast, a cryptomonad nucleus, a nucleomorph, and cryptomonad mitochondria within the cryptomonad cytoplasm that is separated by a single membrane from its own cytoplasm. This single membrane, the double chloroplast ER membranes and the double chloroplast envelope membranes were highly retained. *N. aeruginosum* shows no active digestion like in *N. poecilochroum* and *N. latum*, and the cytoplasm containing the cryptomonad nucleus faces constantly to the dinoflagellate cytoplasm. Therefore, *N. aeruginosum* might suppress digestion of cryptomonad organelles although its mechanism is unclear.

Thus, this represents the first report of three highly divergent strategies in dinoflagellate kleptochloroplastidy: in *Nusuttodinium poecilochroum* the cryptomonad organelles other than chloroplast are actively removed, while in *N. latum* and *N. aeruginosum* the cryptomonad cell is retained more or less intact. *N. latum* and *N.*

aeruginosum, which enlarge ingested chloroplast, tend to preserve the accompanying cryptomonad organelles, implying that the retention of the cryptomonad organelles might be needed for maintenance of kleptochloroplast. Because the kleptochloroplast is originally derived from a foreign photosynthetic alga, the host most probably does not have any chloroplast genes for cryptomonad chloroplast in the dinoflagellate nucleus to maintain the stolen chloroplast, and therefore, to maximize the longevity of the chloroplast, the host needs to retain the ingested algal nucleus. Interestingly, *N. latum*, *N. myriopyrenoides*, *N. aeruginosum* and *Hatena arenicola* retain the ingested algal nucleus in addition to the enlarged kleptochloroplast (Wilcox and Wedemayer 1984; Farmer & Roberts 1990; Horiguchi & Pienaar 1992; Okamoto & Inouye 2006; Yamaguchi *et al.* 2011), implying the retained stolen nucleus plays a role for the enlargement of the kleptochloroplast, and the stolen nucleus might be critical as a first step in the evolution of a permanent chloroplast.

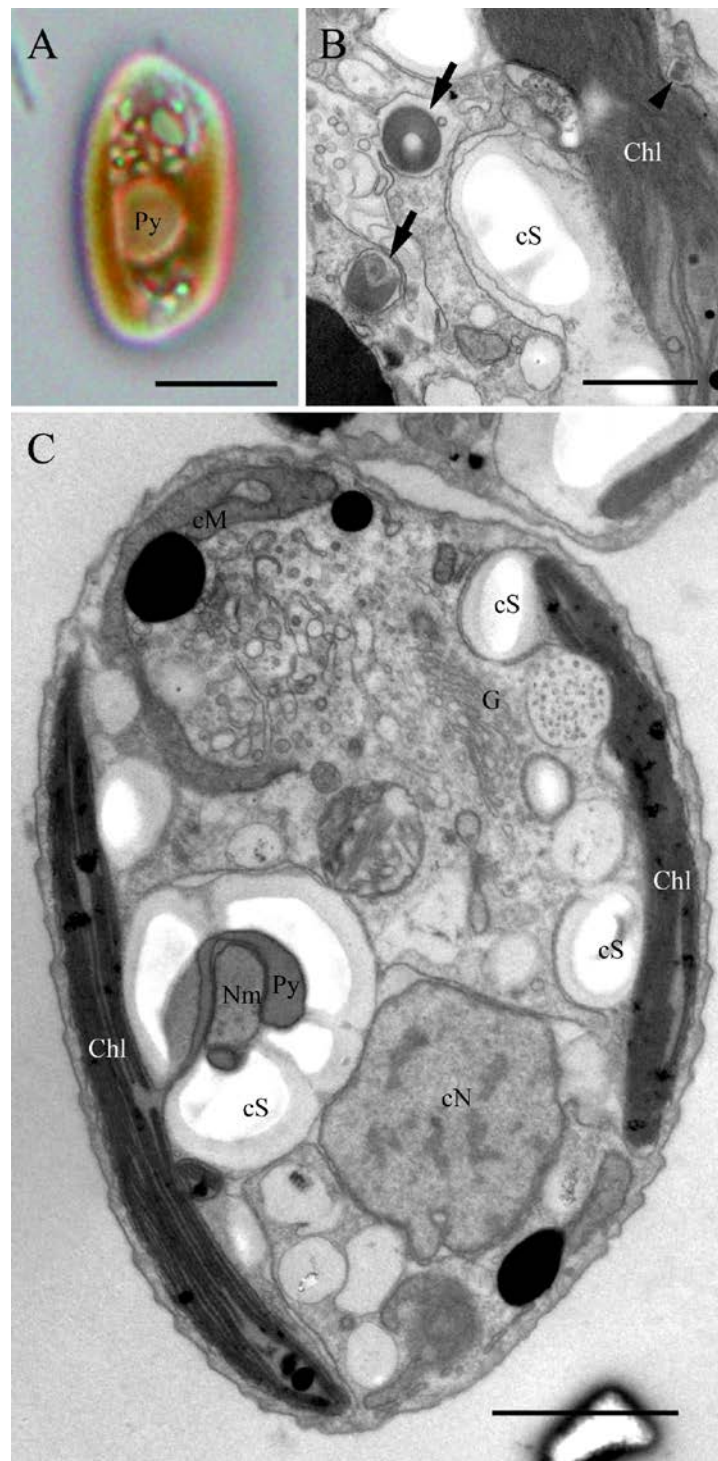


Fig. 35. light and TEM micrographs of *Rhodomonas* sp. used as prey for *Nusuttodinium poecilochroum*.

A. light micrograph of *Rhodomonas* sp. A pyrenoid (Py) is visible. Bar = 5 μm .

B. TEM micrograph of gullet-surrounding (arrow) and peripheral (arrowhead) ejectosomes. Chloroplast (Chl) and starch granule (cS) are shown. Bar = 1 μm .

C. Longitudinal section of *Rhodomonas* sp. showing a chloroplast (Chl), a nucleus (cN), a nucleomorph (Nm), mitochondria (cM), a Golgi body (G), a pyrenoid (Py) and starch granules (cS). Bar = 2 μm .

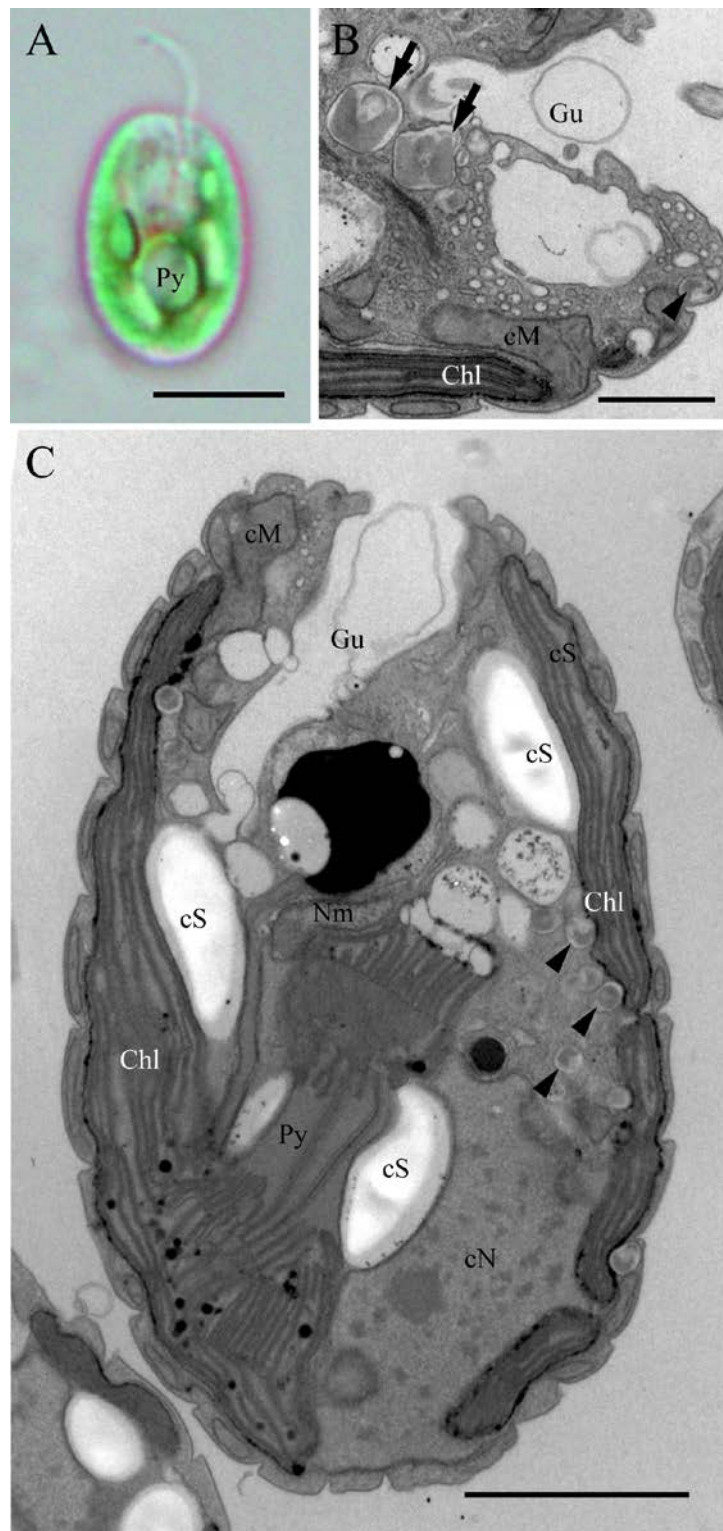


Fig. 36. light and TEM micrographs of *Chroomonas* sp. used as prey for *Nusuttodinium aeruginosum*.

A. light micrograph of *Chroomonas* sp. A pyrenoid (Py) is visible. Bar = 5 µm.

B. TEM micrograph of gullet-surrounding (arrow) and peripheral (arrowhead) ejectosomes. Chloroplast (Chl), mitochondria (cM) and gullet (Gu) are shown. Bar = 1 µm.

C. Longitudinal section of *Chroomonas* sp. showing a chloroplast (Chl), a nucleus (cN), a nucleomorph (Nm), mitochondria (cM), a gullet (G), a pyrenoid (Py), starch granules (cS), peripheral ejectosomes (arrowhead). Bar = 2 µm.

Chapter 2-2. An ultrastructural study on morphological transition of kleptochloroplast and the distribution of the cryptomonad nucleus, and implication of karyoklepty in *Nusuttodinium aeruginosum* (Dinophyceae)

INTRODUCTION

In the previous chapter 2-1, I have compared morphological transition of cryptomonad prey after ingestion among three *Nusuttodinium* species. The observations were limited to the cells before undergoing cell division. However, in this chapter, I would like to deal with the cell division accompanied with the synchronised kleptochloroplast division. Because synchronised kleptochloroplast division takes place only in freshwater species, I would like to concentrate on *N. aeruginosum* here. *Nusuttodinium acidotum* and *N. aeruginosum* retain not only the kleptochloroplast but also the nucleus, nucleomorph and mitochondria of the cryptomonad within their cytoplasm (Wilcox & Wedemayer 1984; Schnepf *et al.* 1989; Farmer & Roberts 1990; Fields & Rhodes 1991; Onuma & Horiguchi 2013). The membrane structure of the kleptochloroplast is invariable in any natural population, but the composition of other cryptomonad organelles is unstable, especially with respect to the presence or absence of the cryptomonad nucleus and nucleomorph. For example, only 10% of *N. aeruginosum* cells and 33-57% of *N. acidotum* cells are reported to retain the cryptomonad nucleus in natural populations (Schnepf *et al.* 1989; Farmer & Roberts 1990; Fields & Rhodes 1991). Fields and Rhodes (1991) reported that *Nusuttodinium acidotum* can retain the kleptochloroplast for at least 14 days even in unialgal culture where host cell division is active. This means that the host cell can divide its kleptochloroplast simultaneously with host cell division and apportion them to each of the daughter cells (Fields & Rhodes 1991). Although they successfully maintained the strain for at least 9 months by co-culturing them with *Chroomonas* sp. as prey (Fields & Rhodes 1991), they did not observe the mechanisms of inheritance of the cryptomonad organelles (nucleus and nucleomorph). LM observation of *N. acidotum* shows that,

when the host cell divides, the nuclear substance is randomly distributed into the daughter cells (Shi *et al.* 1983). However, this study was performed using cells from natural populations with no concomitant TEM observations being made, with the result that the precise pattern of inheritance of the cryptomonad nucleus remains unclear.

In addition to these questions regarding the enlargement of the kleptochloroplast and the loss/retention of the cryptomonad nucleus, another revolves around the functionality of the retained cryptomonad nucleus. This is prompted by the fact that cells which have apparently lost the cryptomonad nucleus continue to look healthy in nature (Schnepf *et al.* 1989; Farmer & Roberts 1990). In the kleptochloroplastidic ciliate, *Mesodinium rubrum*, the ingested cryptomonad nucleus, which is retained in the host cytoplasm in addition to cryptomonad chloroplasts, remains transcriptionally-active for 30 days. Such a phenomenon, where the host cell uses the stolen algal cell's nucleus, is known as karyoklepty (Johnson *et al.* 2007). It is therefore possible that the retained cryptomonad nucleus in *Nusuttodinium acidotum* and *N. aeruginosum* is similarly functional, but this still needs to be investigated.

Therefore, to observe morphological changes and the distribution of kleptochloroplasts and other organelles to each daughter cell, I observed both cells following the first division and all four cells after the second division. The role of the cryptomonad nucleus in maintaining the kleptochloroplast was determined by following the morphology of all the fifth generation daughter cells (32 in number) derived from a single cell. Each daughter was isolated after every cell division. The size of the kleptochloroplast was determined in each of the 32 cells at the end and the cell possessing the cryptomonad nucleus was identified by LM. My *a priori* assumption was that the dinoflagellate with the largest kleptochloroplast must have retained the cryptomonad nucleus. This was confirmed by isolating the cell possessing the largest kleptochloroplast, and checking it by TEM to confirm the presence of the cryptomonad nucleus.

MATERIAL AND METHODS

Culture for experiments

All observations undertaken in this study used a strain of *Nusuttodinium aeruginosum* that I established previously (Chapter 2-1; Onuma & Horiguchi 2013). I maintained the strain in culture with the prey cryptomonad, *Chroomonas* sp. (strain Dc01) grown in AF-6 medium as described in Chapter 2-1. For the various treatments, the starvation, feeding and culture methods followed the methods described in Chapter 2-1.

LM and TEM observation

All methods to observe the cell during kleptochloroplast enlargement followed the methods in Chapter 2-1, except that the LM photographs were taken with a CCD camera ZEISS AxioCam ERc 5s (Carl Zeiss Japan, Tokyo).

For LM observation of first two daughter cells following the division of the cell that ingested the cryptomonad, one daughter was isolated and observed with a ZEISS Axioskop2 Plus (Carl Zeiss Japan, Tokyo) and photographs were taken using a CCD camera ZEISS AxioCam ERc 5s. Observations on the other daughter subsequently followed. For TEM observation, isolated first daughter cells (2 cell stage), were individually transferred into a drop of AF-6 medium in a plastic petri dish (35 mm in diameter), and then fixed with half-strength Karnovsky fixative (2.5% glutaraldehyde and 2% paraformaldehyde, final concentration) in 0.1 M cacodylate buffer at pH 7.0 for 2 h at room temperature. After the pre-fixation both daughter cells were attached to the same poly-L-lysine pre-coated Thermanox plastic coverslip (Thermo Scientific, Kanagawa, Japan). Subsequent treatments for TEM observation followed those outlined in Chapter 1.

For LM observations of cells at the 4 cell stage, I separated the daughter cells at the 2 cell stage (after the first host cell division) and transferred the isolated cell into a separate well of the microplate to prevent confusion between the cell lineages. After the second host cell division in each well, I isolated and observed the four daughter cells one by one using the same method outlined above for the 2 cell stage. For TEM observations at the 4 cell stage, I separated the daughter cells after the first host cell

division, and after the second division, I picked up and processed the two daughter cells of each lineage together but separately from those of the other lineage. Each of the resultant pairs of each lineage were placed into a drop of AF-6 medium in two different plastic petri dishes. Subsequent treatment followed the same method as described above for the 2 cell stage.

For observation of cells resulting from 5 successive cell divisions (the 32 cell stage), I isolated each daughter cell after every cell division into separate wells of a microplate to facilitate the absolute identification of cell lineages. After the fifth cell division, each pair of daughter cells was transferred into a single drop of AF-6 medium in separate plastic petri dishes respectively and pre-fixed as described before. The cells were fixed at room temperature for 2 h. After fixation, the cells transferred into a drop of 0.1 M cacodylate buffer on slide glass and observed on LM. After LM observation, the cell possessing the cryptomonad nucleus was attached to poly-L-lysine pre-coated Thermanox plastic coverslip separately, and prepared for TEM observation as mentioned in Chapter 1.

RESULTS

Morphological observations after host cell division

LM and TEM observations of the cells after the first host cell division (2 cell stage):

The enlargement of kleptochloroplast continued for 5-6 days (120 – 148 h) following ingestion and during this period, cell division never took place. After the kleptochloroplast reached its maximum size, the first host cell division took place. At that time, the kleptochloroplast simultaneously divided and was inherited by both daughter cells. In this study, 3 pairs of daughter cells from the first division were observed by LM, while 3 other pairs were observed by TEM (Table 4). Each daughter cell had a cup-shaped, blue-green kleptochloroplast filling its volume (Fig. 37A-D), indicating that the kleptochloroplast seemingly was equally-inherited by each daughter cell. However, only one of the daughter cells inherited the cryptomonad nucleus, visible at the LM level in the hypocone of the cell (Fig. 37A, C). Observation using TEM

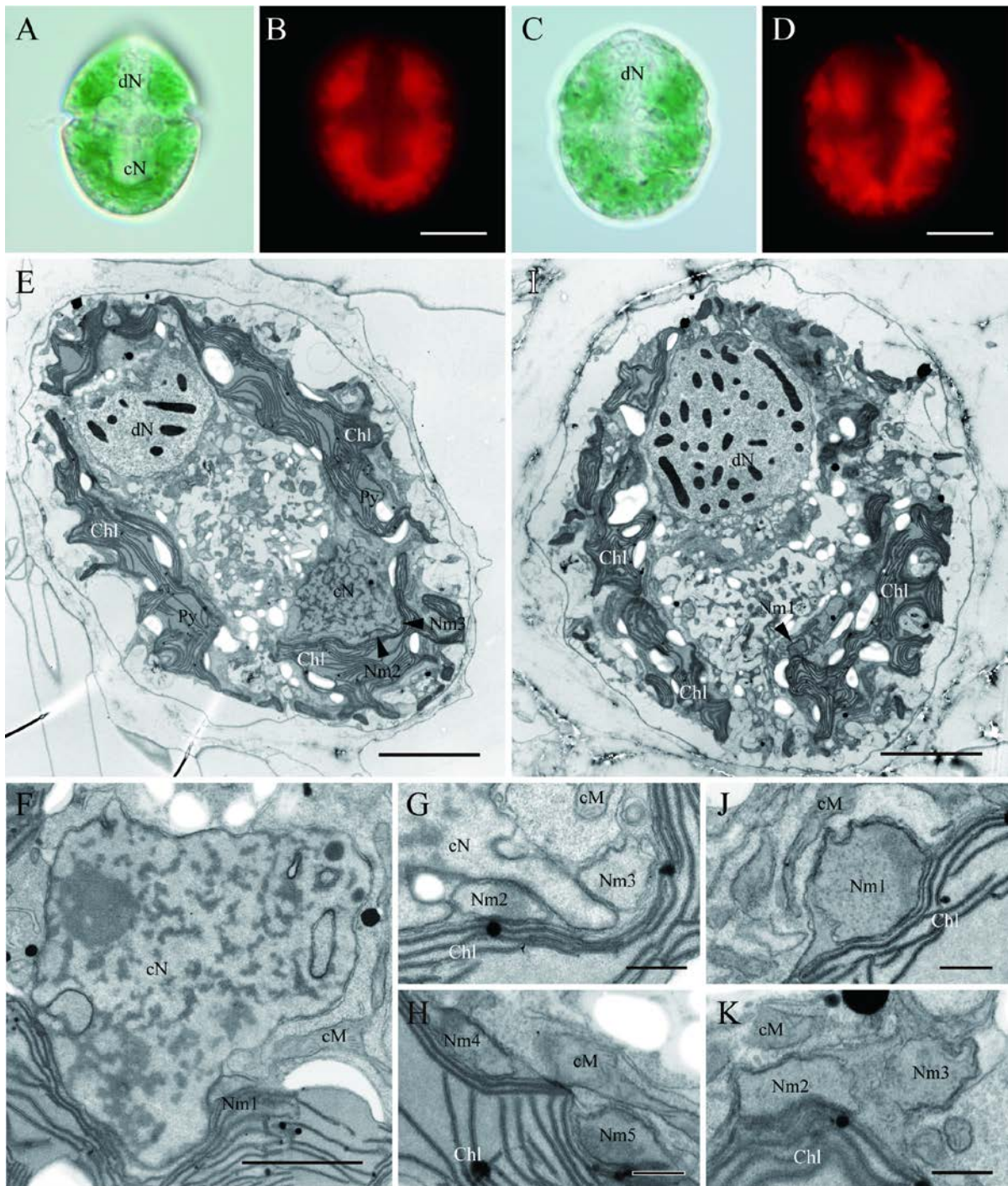


Fig. 37. Bright field (A, C), fluorescence (B, D) and TEM micrographs (E-K) of the two daughter cells of *Nusuttodinium aeruginosum* following the first division after the ingestion event.

A-B, C-D. Bright field and fluorescence micrographs of the daughter cell with, and without, the cryptomonad nucleus respectively, showing similar-sized and cup-shaped kleptochloroplasts that expand throughout the host cell. Bar = 10 μm.

E, I. TEM micrographs of the two daughter cells respectively, showing the equally-divided kleptochloroplast that ramifies throughout each daughter. The presence of the cryptomonad nucleus in the former only is confirmed. Arrowheads indicate nucleomorphs (Nm1-3). Bar = 5 μm.

F-H. TEM details of the daughter with the nucleated cryptomonad. **F.** The intact, double-membraned cryptomonad nucleus and a nucleomorph (Nm1). Bar = 2 μm.

G-H. Further nucleomorphs either near the cryptomonad nucleus (Nm2 and Nm3) or distanced from it (Nm4 and Nm5). Bar = 500 nm.

J-K. TEM details of intact nucleomorphs in the daughter lacking the cryptomonad nucleus. Bar = 500 nm.

Abbreviations: Chl, kleptochloroplast; cN, cryptomonad nucleus; Nm, nucleomorph; cM, cryptomonad mitochondria; Py, pyrenoid; dN, dinoflagellate nucleus.

confirmed that the kleptochloroplast occupied most of the host cell volume and had duplicated pyrenoids, just as is found in cells prior to division (Fig. 37E, I). It also confirmed that the cryptomonad nucleus was inherited by only one cell (Fig. 37E, I). The cryptomonad nucleus was situated in the hypocone, indicating the position was not changed by cell division (Fig. 37E). In contrast to the cryptomonad nucleus, plural nucleomorphs were inherited by both daughter cells (Fig. 37G, H, J, K). However, the nucleomorphs were not equally distributed between the two daughters, i.e. the cell possessing the cryptomonad nucleus inherited 4-8 nucleomorphs, while the other cell inherited 2-3 (see Table 5). Most of nucleomorphs in the former cell were located between the kleptochloroplast and the cryptomonad nucleus (Fig. 37F, G), while some of them were detected in the position where separated from the cryptomonad nucleus (Fig. 37H). In the cell lacking a cryptomonad nucleus, the nucleomorphs were positioned in the hypocone (Fig. 37I Nm1; The close-ups of the nucleomorphs were shown in Fig. 37J, K). Both daughters had cryptomonad mitochondria within the cryptomonad cytoplasm (Fig. 37F-H, J). The membrane structure surrounding the kleptochloroplast was composed of double chloroplast membranes and double chloroplast ER, and the cryptomonad cytoplasm was separated by a single membrane from the dinoflagellate cytoplasm, indicating that the membranes were unaltered after the first division (Fig. 40A).

LM and TEM observations of the cells after the second host cell division (4 cell stage): I observed 4 sets (set I – IV, 4 cells in each set) of daughter cells after the second host cell divisions using LM and 2 sets using TEM. All daughters of one set examined by TEM are shown in Figure 38 and 39. The two daughter cells derived from the cell that inherited the cryptomonad nucleus after the first cell division are shown in Figure 38 and the two daughter cells derived from the cell lacking the cryptomonad nucleus are shown in Figure 39. LM observation showed that all four daughters inherited a kleptochloroplast that retained its cup-shape and which expanded to fill the host cell (Fig. 38A-D, Fig. 39A-D). The cryptomonad nucleus was detected in only one daughter, indicating that it was not capable of division even through the second host cell division (Fig. 38A, C, Fig. 39A, C). TEM observation confirmed that the cryptomonad

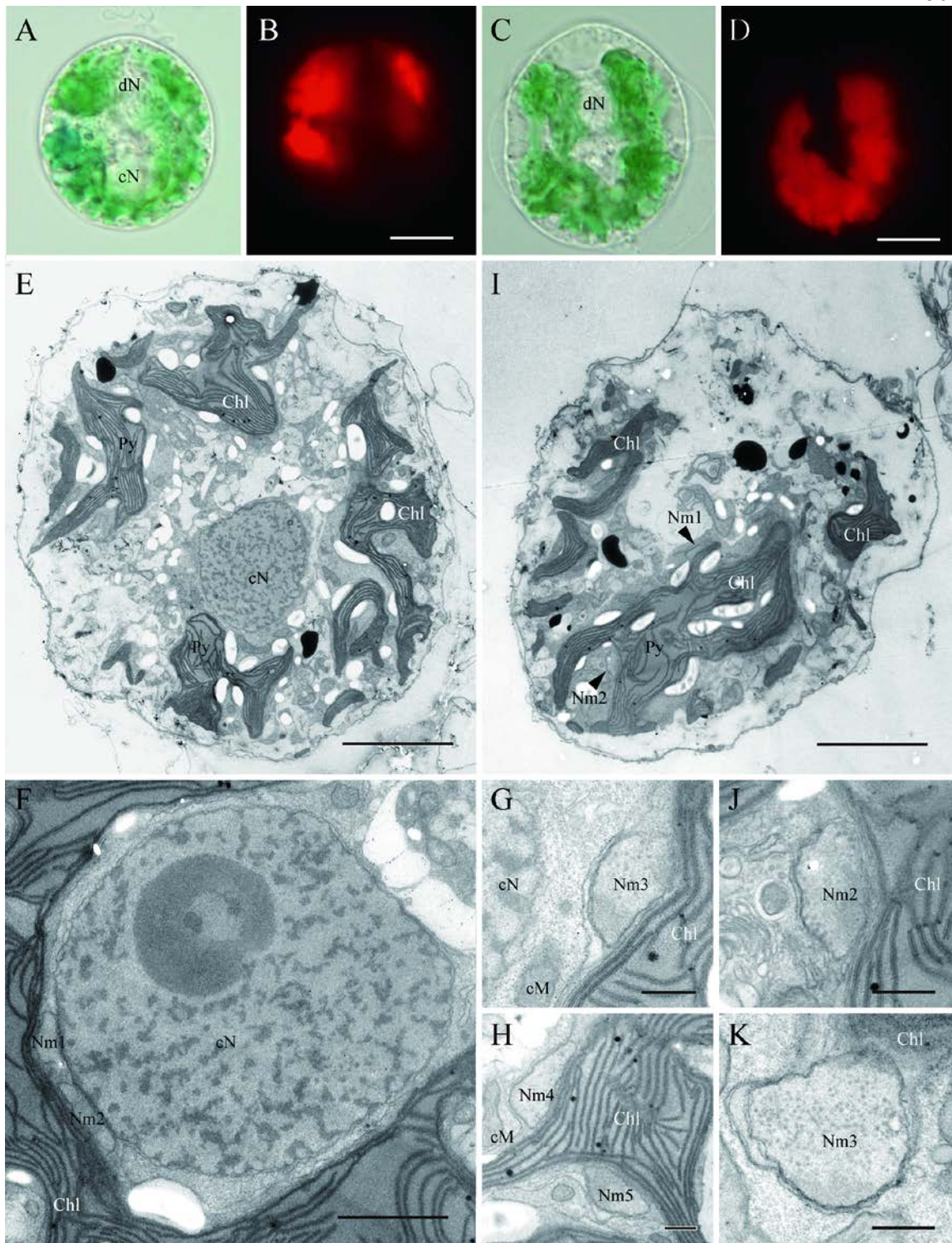


Fig. 38. Bright field (A, C), fluorescence (B, D) and TEM micrographs (E-K) of daughter cells of *Nusuttodinium aeruginosum* following the second division after the ingestion event of the line possessing the cryptomonad nucleus. **A-B, C-D.** Bright field and fluorescence micrographs of the daughter cell with and without the cryptomonad nucleus respectively, showing the cup-shaped kleptochloroplast pervading much of the host cell. Bar = 10 μm. **E, I.** TEM micrograph of the two daughter cells respectively, showing the equally-divided kleptochloroplast that ramifies throughout each daughter. Bar = 5 μm. **F-H.** TEM details of the daughter with the nucleated cryptomonad. **F.** The intact cryptomonad nucleus and two nucleomorphs located between the nucleus and the kleptochloroplast. Bar = 2 μm. **G-H.** Further nucleomorphs near the cryptomonad nucleus (Nm3) or in the middle of the cryptomonad cell (Nm4 and Nm5). Bar = 500 nm. **J-K.** TEM details of nucleomorphs (Nm2 and Nm3) in the cell lacking a cryptomonad nucleus. Bar = 500 nm. Abbreviations: Chl, kleptochloroplast; cN, cryptomonad nucleus; Nm, nucleomorph; cM, cryptomonad mitochondria; Py, pyrenoid; dN, dinoflagellate nucleus.

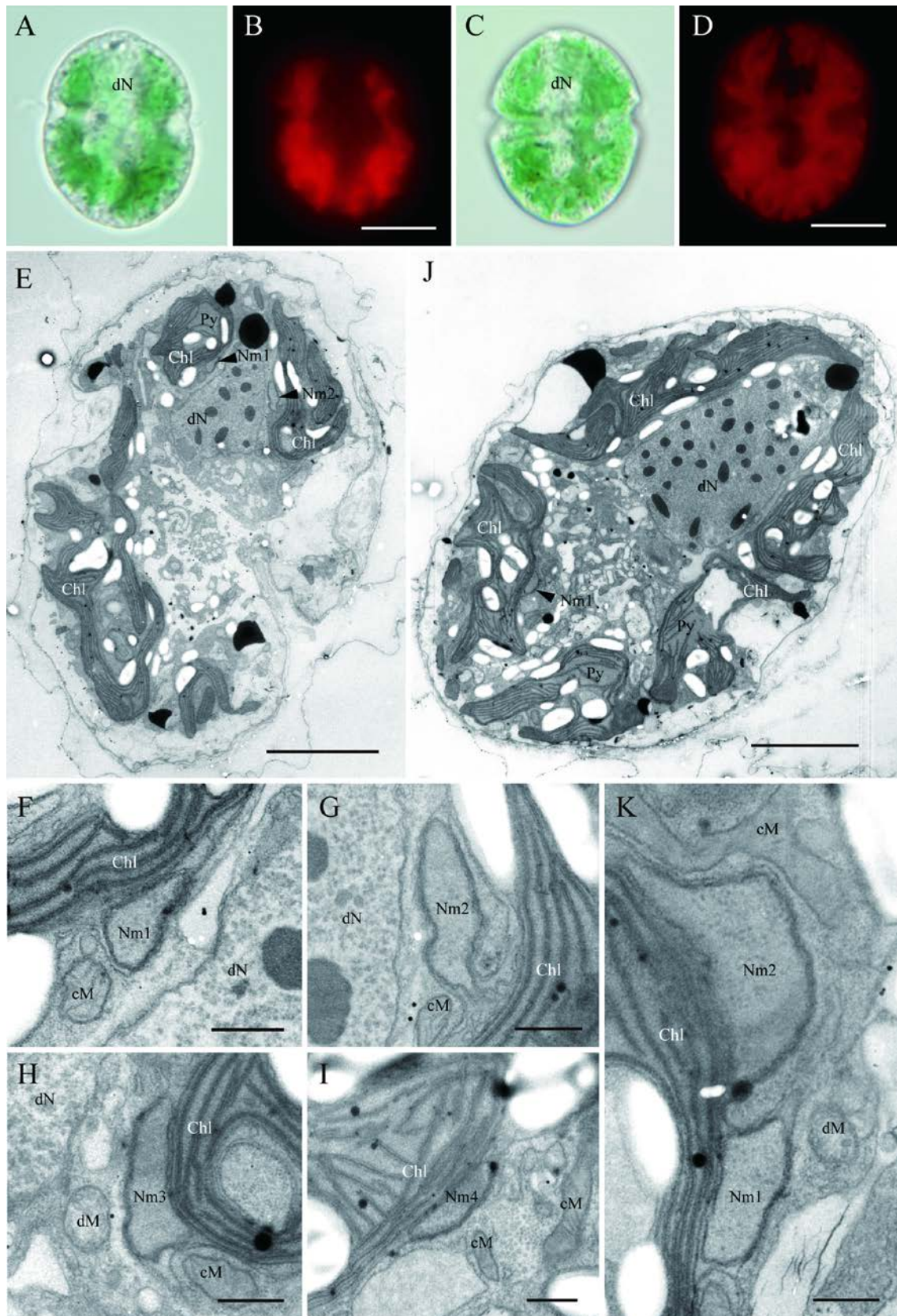


Fig. 39. Bright field (A,C), fluorescence (B, D) and TEM micrographs (E-K) of the daughter cells of *Nusuttodinium aeruginosum* following the second division after the ingestion event of the line lacking the cryptomonad nucleus.

A-B, C-D. Bright field and fluorescence micrographs respectively of each of the daughters, showing the cup-shaped kleptochloroplast still pervading the cell. Bar = 10 μ m.

E, J. TEM micrographs of each of the daughters. Note that the kleptochloroplast pervades the host cell Bar = 5 μ m.

F-I. TEM details of the nucleomorphs in one of the daughters. Nm1-Nm3 are situated near the dinoflagellate nucleus while Nm4 is located at the middle of the cryptomonad cell. Bar = 500 nm.

K. TEM details of intact nucleomorphs of the other daughter. Bar = 500 nm.

Abbreviations: Chl, kleptochloroplast; Nm, nucleomorph; cM, cryptomonad mitochondria; Py, pyrenoid; dN, dinoflagellate nucleus; dM, dinoflagellate mitochondria.

nucleus was only inherited by one of the four daughter cells (Fig. 38E). The cryptomonad nucleus retained its nuclear membranes and nucleolus (Fig. 38G). Nucleomorphs were distributed to all 4 cells (Fig. 38E-K, Fig. 39F-K). In the cell possessing the cryptomonad nucleus, nucleomorphs tended to be situated around the cryptomonad nucleus (Fig. 38G, H), but with some more-distantly positioned (Fig. 38I). In cells lacking the cryptomonad nucleus, almost all the nucleomorphs were positioned inside the cup-shaped kleptochloroplast, some of them near the dinoflagellate nucleus (Fig. 39G-I). Nucleomorphs were not equally-distributed among the four daughters (Table 6). TEM observations were suggestive of a random distribution of nucleomorphs between daughters and of a random number of putative nucleomorph division events, but there was a tendency for the cell possessing the cryptomonad nucleus to inherit the largest number of nucleomorphs (Table 5 and 6). The integrity of all membranes around the kleptochloroplast was not compromised in any of the four daughter cells (Fig. 40B).

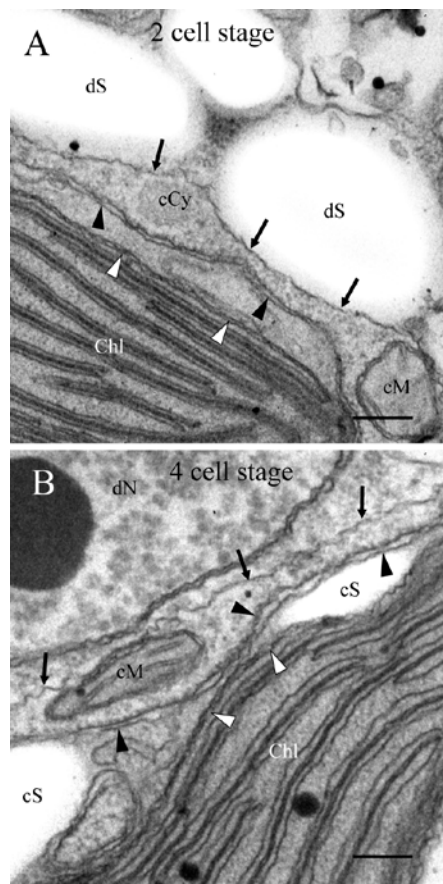


Fig. 40. Transmission electron micrographs of the surrounding membranes of the kleptochloroplast in *Nusuttodinium aeruginosum*.

A, B. Surrounding chloroplast membranes after one cell division (the 2 cell stage; A) and after two cycles of cell division (the 4 cell stage; B). The four membranes (two chloroplast membranes and two chloroplast ER membranes) and the cytoplasmic boundary membrane are still intact. Bar = 200 nm.

White arrowheads, arrowheads and arrows indicate chloroplast membranes, chloroplast ER membranes and the membrane between the cryptomonad and the dinoflagellate cytoplasm. Abbreviations: Chl, kleptochloroplast; cM, cryptomonad mitochondria; cS, cryptomonad starch; cCy, cryptomonad cytoplasm; dN, dinoflagellate nucleus; dS, dinoflagellate starch.

LM and TEM observations of the cells after the fifth host cell division (32 cell stage): The extent of the kleptochloroplast in each of the 32 daughter cells was observed by chloroplast autofluorescence. After LM observation, the cell thought to have the cryptomonad nucleus was selected and processed for TEM observation to confirm its presence. I repeated the five generation cell tracking on four different initial cells (i.e. in quadruplicate). The numbering system of the divided cells is shown in Figure 41. In one of the four replicates, I obtained LM micrographs of (almost; see below) all the final daughter cells (Fig. 42). It was clear that all cells inherited the kleptochloroplast even after the fifth cell division, although Cell 31 stopped cell division after the fourth cell division and digested its kleptochloroplast before fifth cell division (Fig. 42). Another observation was that Cell M (Fig. 41), the cell that inherited cryptomonad nucleus (see Fig. 43), possessed the largest kleptochloroplast (Fig. 42M) and that Cell N (Fig. 41), derived from the same parent cell (Cell 22) as Cell M, had a similarly-large kleptochloroplast to that of Cell M (Fig. 42N). Moreover, Cell M, N, O and P (=Fig. 42M-P) that were derived from the same mother cell (Cell 11, Fig. 41) had the largest

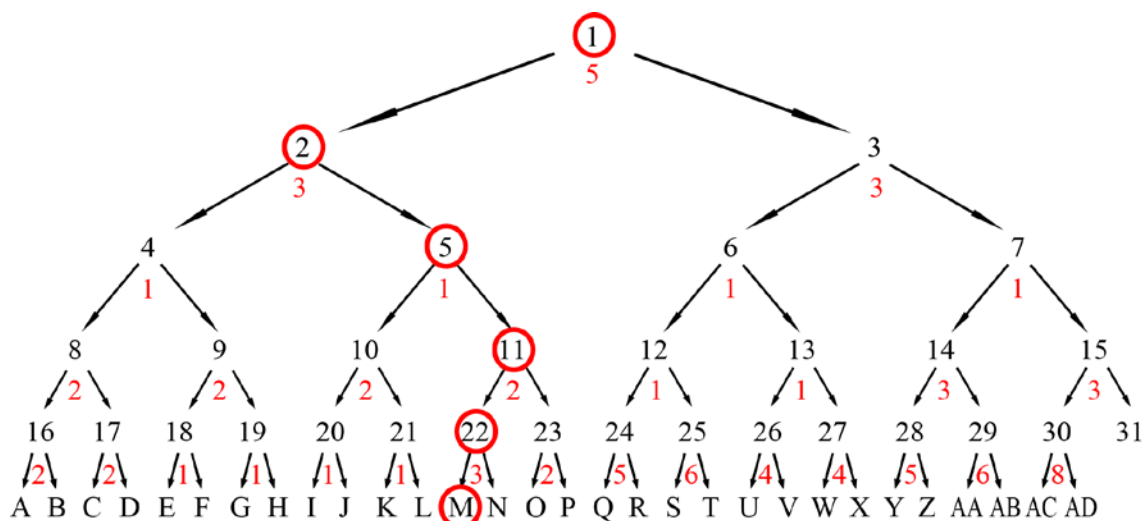


Fig. 41. The numbering system used in this study to unambiguously refer to cell lineages and individual cells through 5 successive division cycles following the ingestion of a single *Chroomonas* sp. cell by a single cell of *Nusuttodinium aeruginosum*. The number under Cell 1 indicates the number of days taken between the ingestion of a cryptomonad cell to the first cell division. The remaining numbers under the (bold) cell numbers indicate the number of days for that cell to next divide. The cells after the fifth division cycle are labeled alphabetically and correspond to the identifying characters in Figure 42. A circle around a cell number indicates cells that inherited the cryptomonad nucleus.

kleptochloroplasts of all final generation cells (= Fig. 42A-L, 42Q-AC). By contrast, Cells Q-AC, which were derived from Cell 3 (Fig. 41), that had lost the cryptomonad nucleus after the first cell division, had reduced kleptochloroplasts that failed to form a cup-shape (Fig. 42Q-AC). Unfortunately, Cell AD collapsed before a LM micrograph was captured, but the size of the kleptochloroplast was similar to that of Cell AC (Fig. 42AC). In other tracking events, cells with a cryptomonad nucleus and some cells

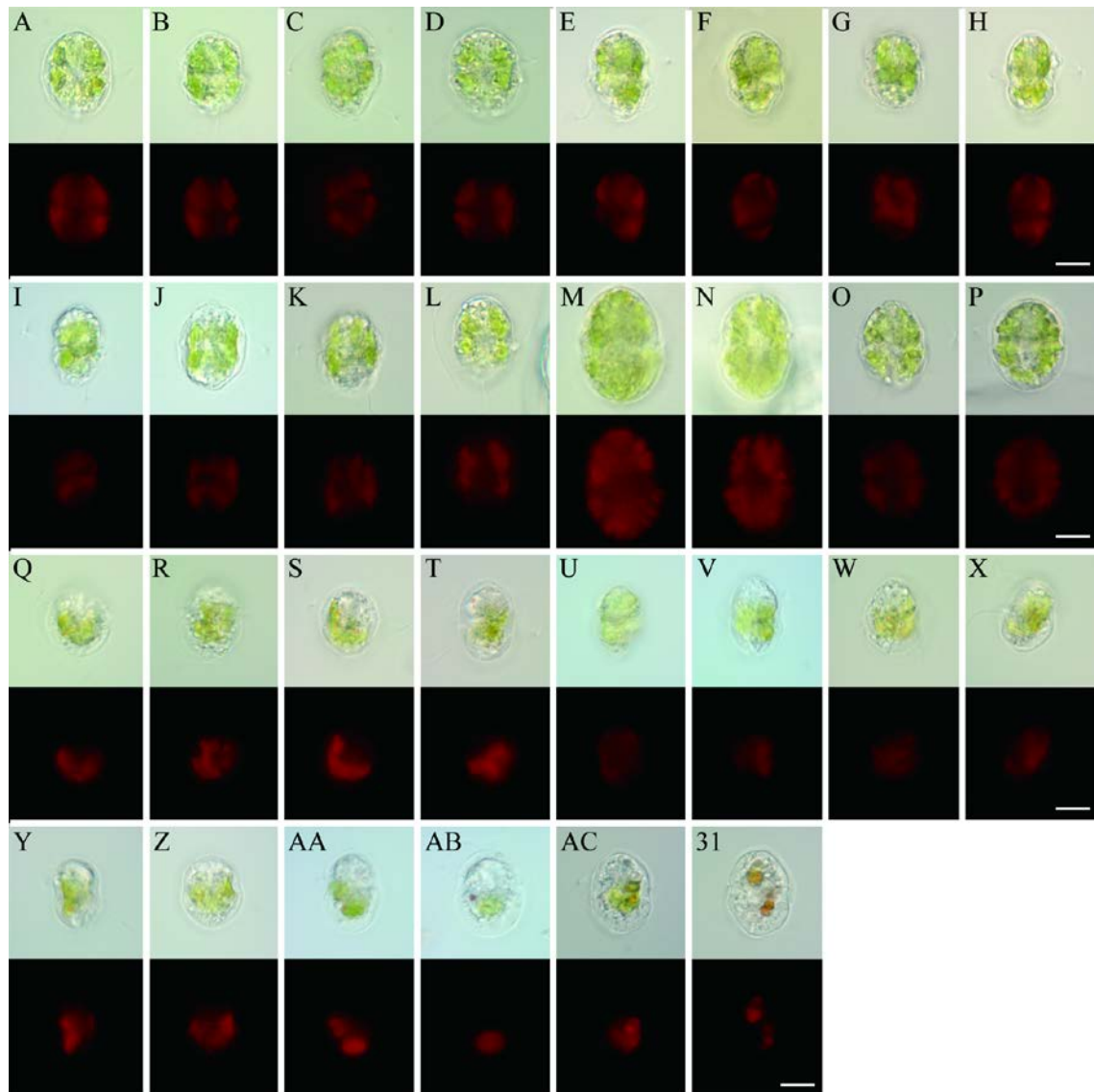


Fig. 42. Bright field and fluorescence micrographs of 29 of the anticipated 32 cells after the fifth cell division cycle following a single ingestion event in *Nusuttodinium aeruginosum*. Each fluorescence micrograph corresponds to bright field micrograph shown above. Note that Cell M (M), which has inherited the cryptomonad nucleus, possesses the largest kleptochloroplast and that Cells N-P (N-P) possess large kleptochloroplasts relative to the rest of this generation. The other kleptochloroplasts (in Cells A-H, I-L and Q-AC) are smaller than those of Cell M and Cells N-P. LM micrograph is lacking due to loss of Cell AD before photographing. Cell 31 had digested its kleptochloroplast before the fifth cell division. Bar = 10 μ m.

lacking a cryptomonad nucleus were observed after being isolated and this same tendency as described above was confirmed (data not shown). TEM observations on Cell M (Fig. 42), identified at LM as possessing a cryptomonad nucleus (Fig. 43), confirmed its presence (Fig. 43A, B). In addition, Cell M possessed 12 nucleomorphs (confirmed by serial sectioning and 5 of which are shown in Fig. 43A-D). In this preparation, no sign of digestion was detected in the cryptomonad nucleus, the kleptochloroplast or the cryptomonad cytoplasm with cryptomonad mitochondria because all these organelles retained intact membrane structures (Fig. 43D). The same results were recovered in all four replicate trackings up to the 32 cell stage. Thus cells that inherited the cryptomonad nucleus possessed the largest kleptochloroplast and cells with the longest history of having a cryptomonad nucleus tended to possess larger kleptochloroplasts than those which had lost it earlier on.

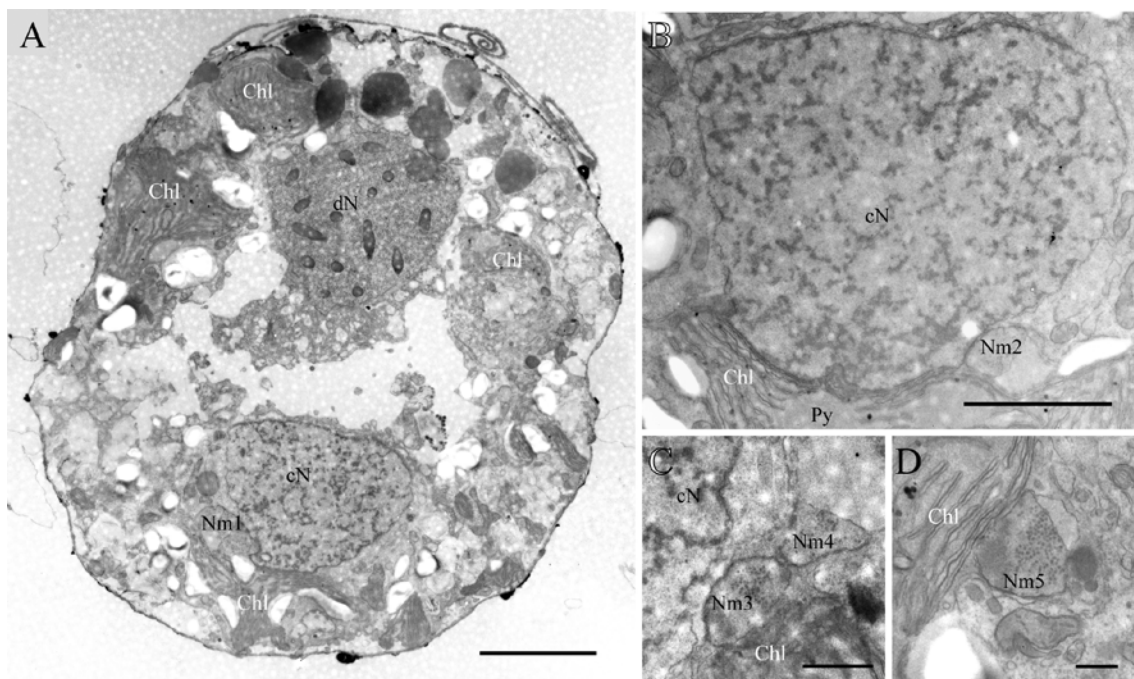


Fig. 43. TEM micrographs of the *Nusuttodinium aeruginosum* cell that inherited the cryptomonad nucleus five division cycles after the original ingestion event.

A. The cell has a similar extensive kleptochloroplast and intact cryptomonad nucleus (cN) as witnessed soon directly after ingestion. Bar = 5 μ m.

B. Detail of the intact nature of the cryptomonad nucleus and nucleomorphs located near the nucleus, showing no sign of digestion. Bar = 3 μ m.

C. Nucleomorph (Nm3 and Nm4) situated near the cryptomonad nucleus showing its intact structure. Bar = 1 μ m.

D. Nucleomorph (Nm5) positioned in the middle of the cell showing no effect of digestion. Bar = 500 nm. Abbreviations: Chl, kleptochloroplast; Nm, nucleomorph; dN, dinoflagellate nucleus.

DISCUSSION

This study follows morphological changes in daughter cells following 1, 2, and 5 division cycles after ingestion using the single-cell LM and TEM methods. In this study, I was able to observe, for the first time, all the daughter cells resulting from five successive division cycles from a single cell following an ingestion event. I was able to demonstrate that the nucleus of ingested cryptomonad plays a substantial role in maintaining the size of kleptochloroplast (= karyoklepty). Karyoklepty and the enlargement of the kleptochloroplast, seen in *Nusuttodinium aeruginosum* might provide insight into the evolutionary steps required for the acquisition of a true chloroplast, as discussed below.

Distribution of cryptomonad nucleus and nucleomorph

This study indicated that the earlier division of the kleptochloroplast allows both daughter cells of *Nusuttodinium aeruginosum* to inherit a kleptochloroplast, but the cryptomonad nucleus is randomly inherited by only one daughter cell due to the inability of the cryptomonad nucleus to divide. Previous studies report that 33-57% of *N. acidotum* cells in the natural population retain a cryptomonad nucleus (Farmer & Roberts 1990; Fields & Rhodes 1991). Another LM observation on cells of *N. acidotum* (= previously described as *Gymnodinium eucyaneum*) shows that cryptomonad nuclear substance is passed to one daughter cell by chance during host cell division (Shi *et al.* 1983). In *N. aeruginosum*, a cryptomonad nucleus was detected in only 10% of collected cells (Schnepf *et al.* 1989). The cryptomonad nucleus observed in some cells of *N. acidotum* is unlikely to be digested because its structure remains intact (Wilcox & Wedemayer 1984; Farmer & Roberts 1990). Therefore, cells of *N. aeruginosum* and *N. acidotum* that lack a cryptomonad nucleus have this state because the cryptomonad nucleus cannot divide in the host cell and can only be inherited by one of the daughter cells. It is not brought about by the digestion of the cryptomonad nucleus after the ingestion event, as was witnessed in *N. poecilochroum* (Onuma & Horiguchi 2013). In free-living cryptomonads, nuclear division occurs by mitosis involving microtubules

and a spindle. These studies indicate that the microtubules form at the anterior region of the cell near the replicated basal bodies and extend toward the nucleus in prophase (Oakley & Dodge 1976; McKerracher & Gibbs 1982). In metaphase and anaphase, the microtubules form the spindle and attach to the condensed chromosomes, functioning to separate them (Oakley & Dodge 1976; McKerracher & Gibbs 1982). In *N. aeruginosum* cell, the typical body plan of the cryptomonad cell is drastically changed due to ingestion and the enlargement of the chloroplast. Although basal bodies accompany the cryptomonad during ingestion by the *N. aeruginosum* cell (Onuma & Horiguchi 2013 Fig. 10B), cryptomonad basal bodies and microtubules are absent in the enlarged kleptochloroplast compartment of *N. aeruginosum* and *N. acidotum* cells (Wilcox & Wedemayer 1984; Schnepf *et al.* 1989; Farmer & Roberts 1990). Therefore, it is reasonable to assume that the cryptomonad nucleus cannot divide in the host cell because the cryptomonad, upon ingestion, has lost control of its machinery to maintain its shape and spindle (i.e. the microtubules). On the other hand, a previous study showed the presence of two cryptomonad nuclei in a single cell of *N. acidotum* collected from the natural population (Farmer & Roberts 1990). In addition, a light microscopic study on a cell of *N. acidotum* sampled from a natural population reported that the cryptomonad nucleus can divide, but involved the formation of a single cleavage on the nucleus, somewhat reminiscent of an amitotic division (Shi *et al.* 1983). These studies imply that a cryptomonad nucleus in *N. acidotum* hosts can divide. However, these studies relied on wild samples which are difficult subjects for tracing morphological change and, especially in the latter species, TEM confirmation of the division of the cryptomonad nucleus is lacking. A further study is required to pursue cryptomonad division, especially focused on whether chromosomes condense and microtubules form when the cell has been ingested by *N. acidotum*.

This study showed that duplicated nucleomorphs were inherited by the daughter cells through at least 5 division cycles following ingestion. However, the distribution of inherited nucleomorphs in daughter cells was not equal, indicating that the process is random. In the free-living cryptomonad, the division of both the nucleomorph and chloroplast takes place in preprophase. One product of the duplicated nucleomorph

moves to the opposite side of the dividing cell, and cell division subsequently occurs. In this way, a single nucleomorph is inherited by each daughter cell (Oakley & Dodge 1976; McKerracher & Gibbs 1982; Sato *et al.* 2014). In contrast, successive divisions of the nucleomorph in a *Nusuttodinium aeruginosum* host precede kleptochloroplast division. This suggests that the regulation of nucleomorph division, as seen in free-living cryptomonads, is lost following ingestion, but division of the kleptochloroplast must be controlled by the dinoflagellate host to ensure that it synchronises with host cell division. However, further study is required to elucidate whether duplicated nucleomorphs have a complete nucleomorph genome, what mechanisms are employed for the division of the nucleomorph and kleptochloroplast, and whether the inherited nucleomorph is functional.

Karyoklepty in *Nusuttodinium aeruginosum* and the evolutionary step toward acquiring a ‘true’ chloroplast

No significant difference was observed, either in LM or TEM, in the size of the kleptochloroplast in cells of the same generation following ingestion up to, but not including, the third division cycle. Thus the kleptochloroplast is divided and distributed between the daughter cells almost equally. LM and TEM observation of all products following 5 successive division cycles (32 cell stage) clearly showed that the cells derived from the first daughter that lacked a cryptomonad nucleus (Cell 3; Fig. 41), only retained small kleptochloroplasts, showing that the kleptochloroplast progressively reduced in size with each generation without any subsequent enlargement. On the other hand, cells in the lineage derived from the daughter that retained the cryptomonad nucleus (Cell 2; Fig. 41), tend to possess larger kleptochloroplasts; the longer they have the nucleus present, the greater the growth of the kleptochloroplast following division. Furthermore, the cell that ultimately inherits the cryptomonad nucleus possesses the largest kleptochloroplast for that particular generation. Because only host cells with a cryptomonad nucleus can continue enlarging their kleptochloroplast, while host cells lacking it can only divide and not grow the kleptochloroplast, the size of the kleptochloroplast in cells within any generation becomes more disparate with each

successive division, but only becomes really obvious after the fifth cell division. Moreover, it is possible that the dinoflagellate genome and cytological function lacks the information and ability to enlarge the kleptochloroplast. Therefore, to prolong the activity of the kleptochloroplast, *Nusuttodinium aeruginosum* needs to keep the cryptomonad nucleus transcriptionally active and uses it to enlarge the kleptochloroplast.

In the marine kleptochloroplastidic dinoflagellate *Nusuttodinium poecilochroum*, the dinoflagellate host digests the ingested cryptomonad nucleus at an early stage and its kleptochloroplast is never enlarged in the host cell (Chapter 2-1; Onuma & Horiguchi 2013). In contrast, *N. latum*, *N. myriopyrenoides* and *N. acidotum* tend to enlarge their kleptochloroplast as mentioned above and these species all retain the cryptomonad nucleus in the host cell (Chapter 2-1; Horiguchi & Pienaar 1992; Yamaguchi *et al.* 2011). In addition, *Hatena arenicola* possesses an enlarged chloroplast and also retains the stolen nucleus (Okamoto & Inouye 2006). The concurrences of enlarged chloroplast and stolen nucleus suggest that the presence or absence of stolen nucleus is directly-related to the ability of the host cell to enlarge its kleptochloroplast or not.

The kleptochloroplastidic ciliate *Mesodinium rubrum* is the first organism reported to maintain transcription activity in its captive cryptomonad nucleus to permit division of the cryptomonad chloroplast after ingestion (Johnson *et al.* 2007). *M. rubrum* ingests multiple cryptomonad nuclei and retains them in their original cryptomonad cytoplasm, keeping their function for up to 30 days (Gustafson *et al.* 2000; Johnson *et al.* 2007). However, once the host cell loses the cryptomonad nucleus, the rate of chloroplast division and the growth rate of the host cell decline significantly, suggesting that the cryptomonad nucleus has an effect on organelle concentration and biochemical potential (Johnson *et al.* 2007). Just as in *M. rubrum*, *Nusuttodinium aeruginosum* probably requires the cryptomonad nucleus to maintain the kleptochloroplast for a longer period. In my preliminary experiment, *N. aeruginosum* cells inheriting the cryptomonad nucleus can undergo further host cell divisions beyond the fifth generation. As mentioned above, in kleptochloroplastidy, as seen in *M. rubrum* and *N. aeruginosum*, the host organisms can increase the chloroplast volume or number in the host cell after

ingestion, and pass the chloroplast into the cells of next generation following its division. Such phenomena are interpreted as pre-requisites for the acquisition of a permanent chloroplast. However, the ingested nucleus is also required to maintain the kleptochloroplast for a longer period, so a critical step in acquiring true chloroplast would be to synchronise the division of the endosymbiont's nucleus with that of the host cell.

This is not too far-fetched, because the retention of an endosymbiotic nucleus is known in dinoflagellates possessing a permanent diatom endosymbiont and commonly referred to as dinotoms (Horiguchi 2006; Imanian *et al.* 2010). Although a dozen dinotom species are known, the well-studied ones include *Durinskia baltica* (Levander) Carty & Cox and *Kryptoperidinium foliaceum* (Stein) Lindemann. These dinotoms retain diatom cytoplasm separated by a single membrane from the dinoflagellate cytoplasm and the cytoplasm contains a diatom nucleus and mitochondria in addition to the chloroplast (Tomas & Cox 1973; Jeffrey & Vesk 1976). When the host cell divides, the diatom nucleus synchronously divides and so it is inherited by each of the daughter cells (Tippit & Pickett-Heaps 1976; Figueroa *et al.* 2009). During the diatom nuclear division, chromatin is not condensed and the organelle divides amitotically, by a simple constriction and without the aid of a spindle (Tippit & Pickett-Heaps 1976). Transcriptome analysis showed that dinotoms have two distinct sets of transcript for the tryptophan biosynthetic pathway (Imanian & Keeling 2014). One of these sets of proteins are derived from the diatom symbiont, and are suggested to be encoded in the diatom nucleus and expressed in the diatom endosymbiont (Imanian & Keeling 2014), which indicates that the diatom nucleus is more or less functional despite of its amitotic division. Therefore, it appears that one of the key events for the permanent establishment of an endosymbiont's nucleus (and thus endosymbiosis) would be the acquisition of a successful amitotic mechanism for nuclear division.

This study has shown that *Nusuttodinium aeruginosum* displays an evolutionarily-advanced form of kleptochloroplastidy with a significantly enlarged kleptochloroplast capable of division and karyoklepty. However, the number of generations over which the host cell can keep the cryptomonad nucleus and the

kleptochloroplast fully functional remains unclear. To address this, further morphological and cultural observations are required. In addition, it is still unknown how the cryptomonad nucleus affects the kleptochloroplast at the molecular level. *N. aeruginosum* would be an appropriate subject organism in studies that attempt to understand the process of endosymbiosis, and I intend in future to investigate this species at the genomic level.

Chapter 3. Specificity of *Chroomonas* prey as a source of kleptochloroplast in *Nusuttodinium aeruginosum*

INTRODUCTION

Among the genus *Nusuttodinium*, it is known that there are differences as to specificity of cryptomonad that dinoflagellates can ingest and use the ingested chloroplast as kleptochloroplast. Marine species *N. latum* and *N. poecilochroum* can ingest multiple species belonging to Cryptophyceae, indicating that they show no or little specificity to cryptomonad (Larsen 1988; Horiguchi & Pienaar 1992). Freshwater species *N. amphidinioides* is reported to possess yellowish-brown and blue-green coloured cryptomonad in natural population, i.e., *N. amphidinioides* can ingest at least two species of cryptomonads (Takano *et al.* 2014). By contrast to these species, marine representative *N. myriopyrenoides* seems to be more prey-specific because the cells collected from natural population possess only blue-green coloured chloroplast (Yamaguchi *et al.* 2011). Thus, the specificity of cryptomonad is variable within the genus.

Freshwater species *Nusuttodinium acidotum* and *N. aeruginosum* show stricter relationship with cryptomonads than marine species, and are known to possess only blue-green kleptochloroplast (Wilcox & Wedemayer 1984; Schnepf *et al.* 1989; Farmer & Roberts 1990; Fields & Rhodes 1991). In cultural study of *N. acidotum*, the cells of *N. acidotum* ingested two species of blue-green cryptomonad cells identified as member of the genus *Chroomonas* only, and never ingested *Cryptomonas* sp. (Fields & Rhodes 1991). Absorption microscopic observation for estimate of pigment composition showed that *N. acidotum* possessed chloroplast containing phycobilin, phycocyanin 645 responsible blue-green colouration, and that wild *Chroomonas* sp. cell had the same pigment composition, suggesting *N. acidotum* has ingested the co-occurring *Chroomonas* sp. (Barsanti *et al.* 2009). Xia *et al.* (2013) identified the species as source of kleptochloroplast in *N. acidotum* using chloroplast 23S rDNA phylogeny, and they obtained only one 23S rDNA sequence from *N. acidotum* (Xia *et al.* 2013). Although it

has been indicated that cryptomonad prey of *N. acidotum* is restricted to the genus *Chroomonas*, however, comprehensive study for identification of cryptomonad origin has never been conducted in any species of *Nusuttodinium*. Therefore, it is still unclear how much *N. acidotum* and *N. aeruginosum* can be flexible to ingest cryptomonad cells and use their chloroplasts as kleptochloroplasts in *Chroomonas* species level.

In this chapter, to reveal the specificity of cryptomonads in *Nusuttodinium aeruginosum*, I collected *N. aeruginosum* cells from natural population in various ponds or lakes, and obtained the sequences of the dinoflagellate internal transcribed spacer (ITS) region and (klepto)chloroplast 16S rDNA from the same cell. And then I performed phylogenetic analyses of the ITS and chloroplast 16S rDNA, and estimated the specificity based on the comparison between the host and the kleptochloroplast phylogeny.

MATERIAL AND METHODS

Sampling and light microscopical (LM) observations

The cells of *Nusuttodinium aeruginosum* were collected using 10 µm plankton net. The locations of sampling and date are listed in Table 7. The cells of *N. aeruginosum* were picked up from the sample under the inverted microscope (CKX-40, Olympus, Tokyo, Japan). The isolated cells were used for subsequent observation. For LM observations, the cells of *Nusuttodinium aeruginosum* were observed with the same methods described in Chapter 1.

To establish strains of *Chroomonas*, sand samples and freshwater samples were collected at various sites (Table 8). The isolated marine and freshwater *Chroomonas* cells were cultured in IMK and AF-6 media under the same conditions described in Chapter 2, respectively.

Single cell PCR

After photographic record of the dinoflagellate cell was made, the cell was picked up after removing the coverslip and rinsed by transferring it to a drop of sterilized fresh

AF-6 medium on depression slide glass several times. And then the cell was transferred into a 200 μ L PCR tube containing 10 μ L of Quick ExtractTM FFPE DNA Extraction Solution (Epicentre, Madison, WI, USA). The PCR tube containing the single cell was incubated at 56 °C for 60 min and then 94 °C for 3 min, and 1 μ L of the resulting extract was used as DNA template for each PCR amplification.

In the first round of PCR for dinoflagellate ITS including ITS1, 5.8S, ITS2, the primers SR12cF and 25D1R (Takano & Horiguchi 2006; Table 2) and 1 μ L of FFPE product as DNA template were used. In the second round of PCR, 0.1 μ L of 1/100-diluted first PCR product was used as DNA template, and the same primers were used. The conditions for both first and second round of PCR were one initial step of denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 45 sec. The final extension step was at 72 °C for 7 min.

In the first round of PCR for 16S rDNA, 1 μ L of FFPE product, terminal primers pl16SF1 and pl16SR1 that designed in this study were used (Table 2). In the second round of PCR, 0.1 μ L of 1/100-diluted first PCR product was used as DNA template, and two set of primers (pl16SF1 and pl16SR2, pl16SF2 and pl16SR1; Table 2) were used. The conditions for the first round of PCR were one initial step of denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 61 °C for 30 sec and extension at 72 °C for 1 min. The final extension step was at 72 °C for 7 min. The conditions for the second round of PCR were the same as those for the first PCR except extension for 30 sec.

The purified PCR products of dinoflagellate ITS and chloroplast 16S rDNA were directly sequenced using the ABI PRISM BigDye Terminator Cycle Sequence Kit (Applied Biosystem, Tokyo, Japan) and a DNA autosequencer ABI PRISM310 Genetic Analyzer (Applied Biosystem, Tokyo, Japan). Both forward and reverse strands were sequenced.

To sequence 16S rDNA of the cultured *Chroomonas* strains, approximately 100 cells were picked up from the strain, and put into a 200 μ L PCR tube containing 10 μ L of FFPE DNA Extraction Solution. The rest of treatment, PCR and sequencing followed

the methods for kleptochloroplast 16S rDNA in the dinoflagellate cells as mentioned above.

Phylogenetic analyses

Both dinoflagellate ITS and chloroplast 16S rDNA sequences were aligned manually. *Nusuttodinium amphidinioides*, and *Cryptomonas curvata* Ehrenberg and *Cry. ovata* Ehrenberg were used as an outgroup in the analyses of dinoflagellate ITS and chloroplast 16S rDNA, respectively. The accession numbers of sequences are indicated in each tree. To explore which model of sequence evolution for ML best fits the datasets, the program jModeltest version 2.1.4 (Darriba *et al.* 2012), which uses the Akaike information criterion (AIC) was used. The model selected for the ML analysis by AIC for dataset was GTR + G and HKY + I + G for dinoflagellate ITS and chloroplast 16S rDNA, respectively. In the ML analysis, a heuristic search was performed with a TBR branch-swapping algorithm, and the starting tree was obtained by the neighbor joining (NJ) method. The aligned sequences were examined using maximum likelihood (ML) analyses with PAUP* version 4.0b10 (Swofford 2003). Bootstrap analysis for ML was calculated to require 100 replicates.

For Bayesian analysis, GTR + G and HKY + I + G model was selected by MrModeltest 2.2 (Nylander *et al.* 2004) as a suitable evolutionary model dinoflagellate ITS and chloroplast 16S rDNA, respectively. Bayesian analyses were calculated with MrBayes 3.2.1 (Huelsenbeck & Ronquist 2001). Markov chain Monte Carlo iterations were carried out until 1,000,000 generations were attained for dinoflagellate ITS phylogeny, while until 2,000,000 generations were required for 16S chloroplast rDNA phylogeny, when the average standard deviations of split frequencies fell below 0.01, indicating a convergence of the iterations.

RESULTS

LM observations

I collected *Nusuttodinium aeruginosum* cells from five different ponds and lakes, and of

these ponds, I conducted the sampling at the pond in Ainosato Park three times. The collected cells were ovoid or elliptic. The epicone of the cells was hemispherical and equal with the hypocone in size or slightly smaller than the hypocone (Fig. 44). The hypocone was rounded (Fig. 44), and some cells had the pointed antapex at the posterior of the cell (Fig. 44J, L). The cingulum encircled the cell without displacement (Fig. 44). All collected cell possessed blue-green kleptochloroplast enlarged throughout the cell (Fig. 44). No obvious difference was detected as to the degree of enlargement, colouration and arrangement of kleptochloroplast in LM observation. The food vacuole and accumulation body were not observed in the cell.

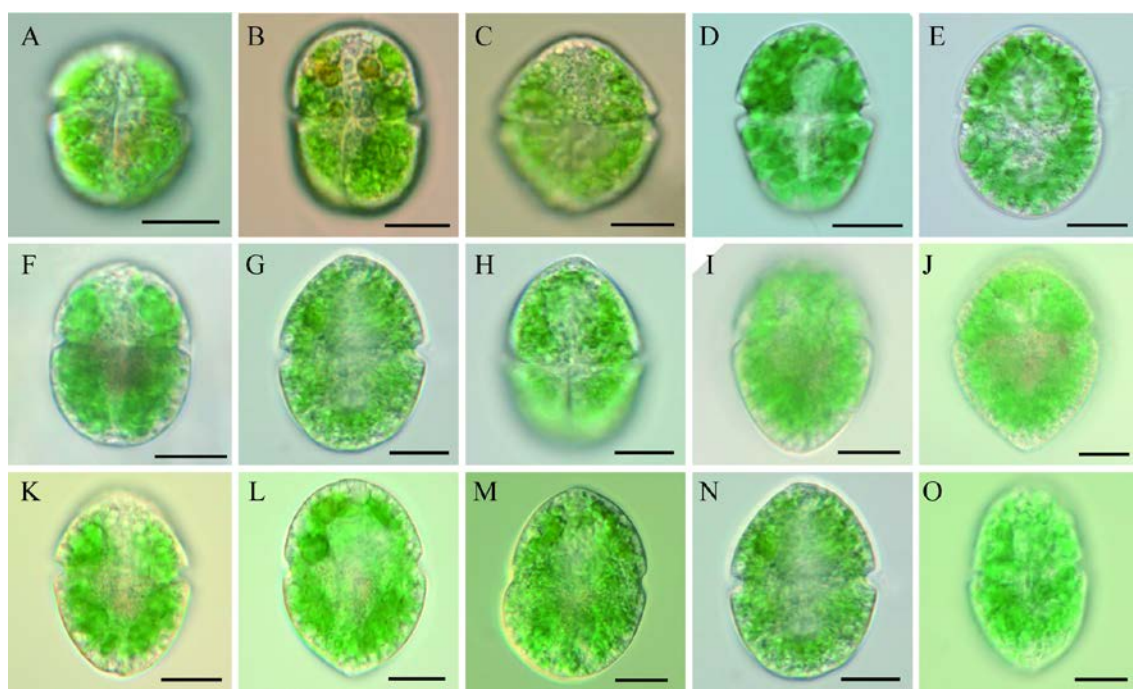


Fig. 44. Light micrographs of *Nusuttodinium aeruginosum* used for the sequencing and the phylogenetic analyses. Note that the kleptochloroplasts are enlarged throughout the cell in all cells and they look healthy and normal-looking. The names of the cells used in this experiment are following: **A**; Wada cell2 130330, **B**; Yurigahara cell3 130607, **C**; Ainosato cell2 130607, **D**; Ainosato cell1 130917, **E**; Ainosato cell2 130917, **F**; Tokotan cell1 131005, **G**; Tokotan cell2 131005, **H**; Tokotan cell3 131005, **I**; Ainosato cell1 140417, **J**; Ainosato cell2 140417, **K**; Ainosato cell3 140417, **L**; Ainosato cell4 140417, **M**; Docho cell1 140919, **N**; Docho cell2 140919, **O**; Docho cell3 140919. The sampling site and date are shown in Table 7. Bar = 10 μ m.

Phylogenetic analysis of ITS in the dinoflagellate host

In this study, 15 sequences of the dinoflagellate ITS were determined. Phylogenetic tree (Fig. 45) showed the monophyly of *Nusuttodinium aeruginosum* including my sequences although reasonable bootstrap value was not obtained (<50 %) (Bayesian posterior probability (PP = 0.81). Within the *N. aeruginosum* clade, four subclades were recognised (Fig. 45). Subclade D1 was located at the most basal position of *N. aeruginosum* clade, and no high support value was obtained for this clade. This clade was composed of two registered sequences of *N. aeruginosum* from Uto-ike in Kagawa Pref. (AB921311) and a pond in Aomori Pref. (AB921313). The subclade D2, D3 and D4 formed a clade that supported by moderate to high BS values. The subclade D2 is supported by moderate values (BS/PP = 71/0.98), and the subclade D3 and the subclade D4 formed a further robust clade (BS/PP = 96/0.99). The subclade D2 consisted of the sequence of *N. aeruginosum* (AB921316) from Tatara-numa pond in Gunma Pref., Japan and all other sequences were from Ainosato Park, and supported by reasonable values (BS/PP = 71/0.98). My sequences in this clade were almost identical and different from *N. aeruginosum* AB921216 by 10 sites. The subclades D3 and D4 were highly-supported, respectively (BS/PP of Subclade D3 = 86/0.99, BS/PP of Subclade D4 = 100/0.99). The subclade D3 was composed of three registered sequences of *N. aeruginosum* from Sapporo (AB921315), Tokotan pond (AB921312) and Denmark (AB921317) and two sequences of the cells sampled from Tokotan pond. These sequences were identical to each other, and *N. aeruginosum* AB921312 and my materials in the subclade D3 were collected from the same locality. The subclade D4 was assembled from sequences of the cells collected from four different ponds in this study. *Nusuttodinium aeruginosum* collected at Docho and used for the experiments in Chapter 1 and 2 was included in the subclade D4. This phylogenetic tree indicated that the cells collected in this study forms clades with the sequences deposited as *N. aeruginosum*, not with *N. acidotum* (Fig. 45).

Phylogenetic analysis of 16S rDNA in kleptochloroplast

In this chapter, the sequences of chloroplast 16S rDNA were obtained from 18



Fig. 45. Maximum likelihood tree inferred from dinoflagellate internal transcribed spacer (ITS) sequences. The dinoflagellate cells collected for this analysis are indicated in bold. The bootstrap (BS) and Bayesian posterior probability values (PP) are provided at each node (BS/PP).

dinoflagellate cells possessing kleptochloroplasts and 12 strains of *Chroomonas* and *Hemiselmis*. Hoef-Emden (2008; 2014) showed that *Chroomonas* and *Hemiselmis* form a robust clade (*Chroomonas/Hemiselmis* clade) and four subclades could be recognised in *Chroomonas/Hemiselmis* clade. Although BS supports for each subclade were not high, the four subclades in this study concurred with those in Hoef-Emden (2008; 2014), and I named each subclade according to Hoef-Emden (2008; 2014) (Fig. 46). In this study too, the monophyly of *Chroomonas* and *Hemiselmis* was confirmed (Fig. 46). The

subclade 3 consisted of one sequence, *Chroomonas caudata* Geitler (NIES-0712), was positioned at the base of *Chroomonas/Hemiselmis* clade. Subclade1 was the next branched group and supported by moderate BS value (BS/PP = 79/0.99). This clade contained two sequences, *Chroomonas nordstedtii* Hansgirg (NIES-0706) and *Chroomonas* sp. HrL01. The subclade 2 and subclade 4 formed a clade supported by moderate PP (0.84), and subclade 1 was a sister clade to this clade. The subclade 2 was composed of the sequences of *Hemiselmis* and the *Chroomonas* sp. isolated from Ainosato Park. The PP of the subclade 4 was relatively high (0.91), and all sequences obtained from kleptochloroplasts in the dinoflagellate cells and almost all strains of *Chroomonas* I established were included in the subclade 4 (Fig. 46).

Within the subclade 4, several further clades were recognised, but the branching order could not be resolved due to lack of supports at almost all nodes (Fig. 46). The clade 4-A contained two sequences of dinoflagellate kleptochloroplast and *Chroomonas coerulea* (Geitler) Skuja (NIES-0713) and was supported relatively-high BS and PP (BS/PP = 89/1.0). The clade 4-B was composed of the sequences of the dinoflagellate cells from Docho south pond, *Chroomonas* sp. isolated from the same sample as the dinoflagellates and *Chroomonas pauciplastida* Hansgirg (CCMP268) although high support could not be obtained for this clade. The clade 4-C consisted of sequences of chloroplasts in dinoflagellate cells collected from Wada pond, Yurigahara Park and Ainosato Park. These sequences were identical to each other, but support was not convincing. The clade 4-D also contained eight sequences of the kleptochloroplasts. Within the clade 4-D, six sequences of kleptochloroplast formed a further clade that supported by reasonable values (BS/PP = 75/0.99). This phylogenetic tree indicated that at least three different sequences of *Chroomonas* were detected in the dinoflagellate host from Ainosato Park and Yurigahara Park. The sequences included in the clade 4-D were originated from various subclades of the dinoflagellates, whereas the clades 4-B and 4-C were occupied by the dinoflagellates included in the subclade D4 (Fig. 46).

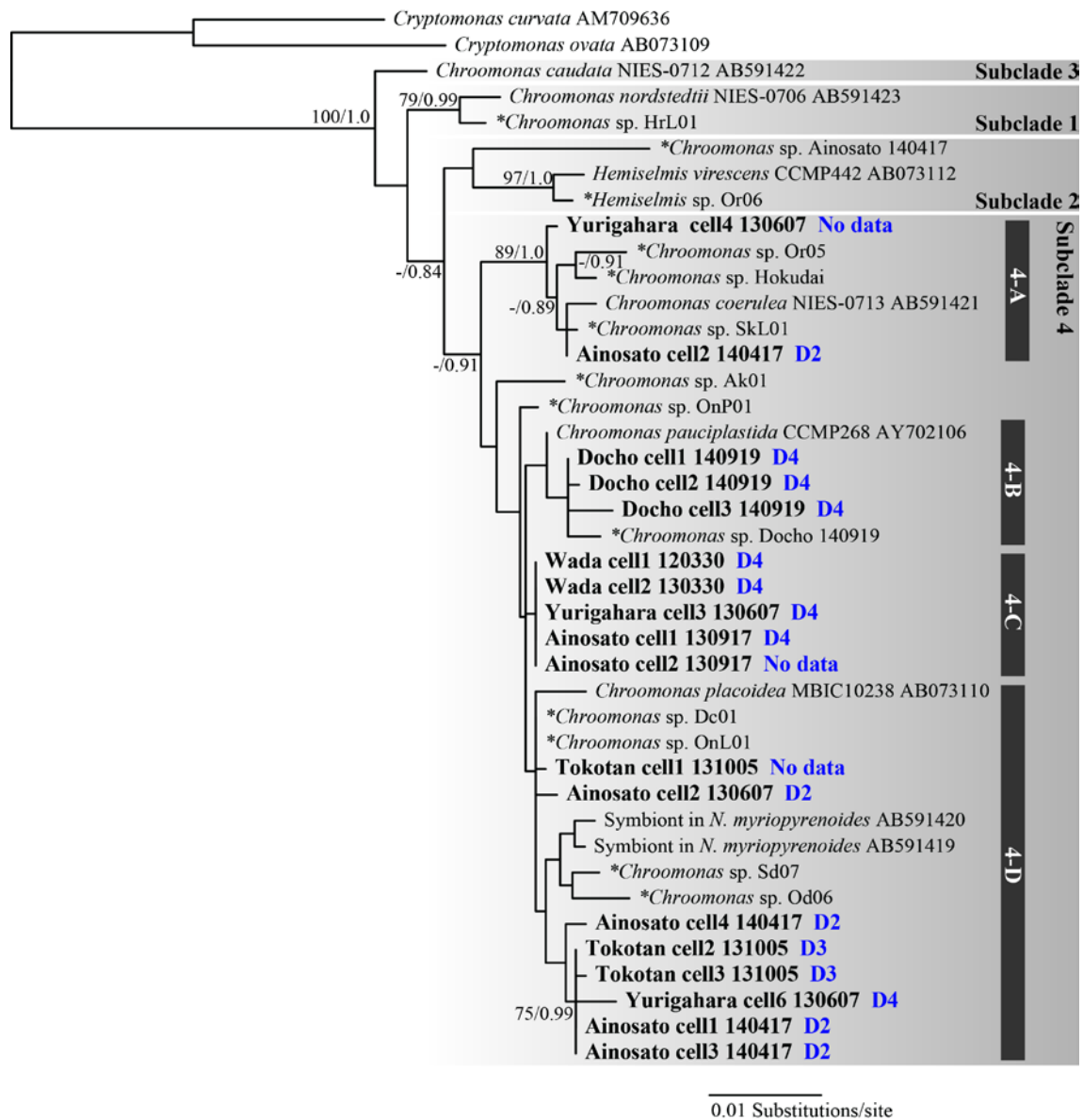


Fig. 46. Maximum likelihood tree inferred from chloroplast 16S rDNA sequences. The sequences obtained from the kleptochloroplast in the dinoflagellate cells are indicated in bold. The text in blue indicates the corresponding subclade of the dinoflagellate that possesses kleptochloroplast. Asterisks indicate the strains of *Chroomonas* and *Hemiselmis* established in this study. The bootstrap (BS) and Bayesian posterior probability values (PP) are provided at each node (BS/PP).

DISCUSSION

Identification of the dinoflagellate host

All dinoflagellate cells collected in this study possessed the cingulum located at almost middle part of the cell, and this position of the cingulum resulted in the similar size of the epicone with the hypocone. This character is different from that of *Nusuttodinium amphidinioides*, which possesses the smaller epicone than the hypocone (Takano *et al.* 2014), indicating that the cells collected in this study should be identified as *N. acidotum* or *N. aeruginosum*. *N. aeruginosum* was originally described as possessing a rounded hypocone (Stein 1883), while *N. acidotum* possesses a pointed antapex (Wilcox & Wedemayer 1984; Farmer & Roberts 1990; Fields & Rhodes 1991). However, it is known that the shape of the antapexes in both *N. aeruginosum* and *N. acidotum* easily alter depending probably on environmental condition and population density, and both species have a potential to possess either pointed or rounded antapex within the same species (Takano *et al.* 2014). However, at the same time, Takano *et al.* (2014) showed that typical forms of *N. acidotum* and *N. aeruginosum* are different from each other and the former species exhibits a pointed and the latter species shows a rounded antapex respectively and these two forms can be distinguished from each other by ITS sequences (Takano *et al.* 2014). Therefore, *N. acidotum* and *N. aeruginosum* are suggested to be distinct species and the rounded antapex sometimes found in *N. acidotum* are regarded as simply showing the beginning of the cell division (Takano *et al.* 2014). Almost all the cells collected in this study possessed the rounded hypocone although occasionally some cells possessed the pointed antapex. The phylogenetic analysis showed that all sequences of the dinoflagellate ITS were completely included in *N. aeruginosum* clade regardless of the possession of the slightly pointed antapex. This result indicates that the dinoflagellates collected in this study should be identified as *N. aeruginosum*.

Phylogeny of kleptochloroplasts and specificity of *Chroomonas*

This study clearly indicates that all sequences of kleptochloroplasts in *Nusuttodinium*

aeruginosum cells were included in Subclade 4, which is resolved in previous studies (Hoef-Emden 2008; 2014). LM observation shows that the kleptochloroplasts occupied throughout the dinoflagellate cells as is the case in *N. acidotum* and *N. aeruginosum* observed in the previous studies (Chapter 2-1; Wilcox and Wedemayer 1984; Schnepf *et al.* 1989; Farmer & Roberts 1990; Fields & Rhodes 1991), suggesting that the dinoflagellates having the kleptochloroplasts originated from cryptomonad included in the subclade 4 seem to be healthy and normal-looking and that the members of cryptomonads belonging to the clade 4 are appropriate as sources of the kleptochloroplasts. On the other hand, no sequences of cryptomonads included in subclades 1, 2 and 3 were recovered from the dinoflagellate cells, implying that *N. aeruginosum* has specificity for the selection of the cryptomonad prey and *Chroomonas* spp. belonging to the subclades 1, 2 and 3 are inappropriate species as sources of kleptochloroplasts. According to my culture study, *N. aeruginosum* cells collected at Ainosato Park on 17th April 2014 did not ingest *Chroomonas* sp. included in the subclade 2, even though the latter was isolated from the same sample with *N. aeruginosum*. This result supports the preference to ingest *Chroomonas* from the clade 4. In my preliminary observation, *N. aeruginosum* was able to ingest *Chroomonas* sp. HrL01 (subclade 1), but could not be cultured by feeding the latter species, suggesting that *N. aeruginosum* has no capacity to utilise chloroplast of *Chroomonas* belonging to the subclade 1 despite ability to ingest them. This study also showed that the sequences of the kleptochloroplast were not identical each other and distributed throughout the subclade 4 (4-A to 4-D) in the phylogenetic tree. At least three distinct sequences of *Chroomonas* were obtained from the dinoflagellate cells included in the subclade D2 and D4, respectively. This result strongly suggests that *N. aeruginosum* has an ability to ingest multiple species of *Chroomonas*, and can use as kleptochloroplast. However, because *Chroomonas* in clade 4-B and 4-C contained the sequence originated from dinoflagellate cells included in the subclade D4 only, it is unclear whether the dinoflagellate of the other clade can use the chloroplast of *Chroomonas* in clade 4-B or 4-C. In conclusion, this study suggests that *N. aeruginosum* has some degree of the specificity to *Chroomonas* in subclade level, but does not restrict cryptomonad

symbiont into species level.

This situation can be comparable with the specificities of the symbiont in *Hatena arenicola*, *Mesodinium rubrum* and kleptochloroplastidic dinoflagellates. The kleptochloroplast of katabrepharid *H. arenicola* is originated from the chloroplast of *Nephroselmis* (Prasinophyceae), and the host is unable to divide its kleptochloroplast simultaneously with the host cell division (Okamoto & Inouye 2006). The phylogenetic study of the symbiont suggested that *H. arenicola* accepts as least three species of *Nephroselmis* (Yamaguchi *et al.* 2014).

A marine ciliate, *Mesodinium rubrum* possesses kleptochloroplast obtained from cryptomonad *Geminigera/Teleaulax* (Johnson & Stoecker 2005; Johnson *et al.* 2006; Park *et al.* 2006), and uses the cryptomonad nucleus for maintenance of kleptochloroplast (Johnson *et al.* 2007). Interestingly, despite the fact that *M. rubrum* can be cultured with either *Geminigera* or *Teleaulax*, *M. rubrum* collected from several Japanese coasts have possessed the kleptochloroplast originated only from *T. amphioxeia* (W. Conrad) D. R. A. Hill, suggesting *M. rubra* has significant preference for the cryptomonad prey (Nishitani *et al.* 2010). The unnamed dinoflagellate collected from the Ross Sea (RSD) ingests the chloroplast from haptophyte *Phaeocystis antarctica* Karsten and retains the chloroplast for 29.5 months (Gast *et al.* 2007; Sellers *et al.* 2014). However, the RSD never ingest other haptophyte *Pseudohaptolina arctica* Edvardsen & Eikrem, indicating that RSD restricts their symbiont at least at the genus level (Sellers *et al.* 2014). These species mentioned above play advanced kleptochloroplastidy as to the enlargement, the longevity of the chloroplast or karyoklepty (Okamoto & Inouye 2006; Gast *et al.* 2007; Sellers *et al.* 2014) (See also Chapter 2). Therefore, these species including *Nusuttodinium aeruginosum* possess the advanced state of kleptochloroplast and they might tend to have relatively-restricted relationship with the symbiont. On the other hand, marine species *N. latum* can ingest multiple genera belonging to Cryptophyceae and enlarge at least three type of chloroplast (Horiguchi & Pienaar 1992). Morphological transition of kleptochloroplast indicates that *N. latum* retains the cryptomonad organelles such as cryptomonad nucleus and nucleomorph inside the chloroplast (Chapter 2-1), and this strategy might allow the

dinoflagellate to enlarge the chloroplasts originated from multiple species.

Although some degree of the specificity to cryptomonad in *Nusuttodinium aeruginosum* has been demonstrated, this study provides further question, i.e. because I have only observed the cells from nature, it is still unclear whether *N. aeruginosum* can use any species included in the subclade 4 and divide simultaneously with the host division and enlarge again as can be confirmed in *Chroomonas* sp. Dc01 (the strain used in the experiments in Chapter 2). To address this question, further cultural experiments are needed using another combinations of dinoflagellates and cryptomonad preys. In addition, difficulty of obtaining highly resolved phylogenetic tree makes identification of cryptomonad symbiont at species level difficult. One reason for this difficulty is that in the cells of natural population, the cryptomonad nucleus are often lost during host cell division and thus it is not possible to use nuclear-encoded cryptomonad genes for the analyses (Chapter 2-2, Schnepf *et al.* 1989). To identify the cryptomonad symbiont in species level, further phylogenetic analysis is required using multiple genes that encoded in chloroplast genome.

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Table 1. List of dinoflagellates used in this study

species	locality	GPS	date
<i>Pellucidodinium psammophilum</i> Aininkappu	Aininkappu Beach, Akkeshi Town, Hokkaido Prefecture	43°00'20"N: 144°51'32"E	28th Sep 2011, 22th Jun 2012
<i>P. psammophilum</i> Nosappu	Cape Nosappu, Nemuro City, Hokkaido Prefecture	43°23'07"N: 145°48'01"E	8th Jun 2013
<i>P. psammophilum</i> Senjoiwa	Senjoiwa, Esashi Town, Hokkaido Prefecture	44°57'36"N: 142°35'01"E	30th Apr 2014
<i>Nusuttodinium desymbiontum</i> Ishikari	Ishikari beach, Ishikari City, Hokkaido Prefecture	43°15'26"N: 141°21'26"E	2nd Mar 2012, 27th Nov 2012
<i>N. desymbiontum</i> Abuta	a sandy beach in Abuta Town, Hokkaido Prefecture	42°32'08"N: 140°46'27"E	11th May 2012
<i>N. desymbiontum</i> Fukaura	a sandy beach in Fukaura Town, Aomori Prefecture	40°35'03"N: 139°55'13"E	5th May 2012
<i>N. aeruginosum</i> Docho	Docho South Pond, Sapporo City, Hokkaido Prefecture	43°03'48"N: 141°20'56"E	24th Sep 2010
<i>N. aeruginosum</i> Ainosato	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10'02"N: 141°24'31"E	19th Apr 2011
<i>N. aeruginosum</i> Onuma	Lake Onuma, Nanae Town, Hokkaido Prefecture	41°59'02"N: 140°40'16"E	12th May 2012
<i>N. amphidinioides</i> Ainosato	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10'02"N: 141°24'31"E	19th Apr 2011

Table 1. (continued)

<i>N. amphidinioides</i> Ono	Ono Pond, Sapporo City, Hokkaido Prefecture	43°04'27"N: 141°20'28"E	16th Apr 2011
<i>N. latum</i> SA no28	South Africa: Port Edward, KwaZulu-Natal	31°03'S 30°13'E	28th Sep 2011
<i>N. latum</i> SA no43	South Africa: Palm Beach, KwaZulu-Natal	30°59'S 30°16'E	29th Sep 2011
<i>N. poecilochroum</i> Shibagaki Beach	Shibagaki Beach, Hakui City, Ishikawa Prefecture	36°57'03"N: 136°45'34"E	29th Mar 2010
<i>N. poecilochroum</i> Aininkappu	Aininkappu Beach, Akkeshi Town, Hokkaido Prefecture	43°00'20"N: 144°51'32"E	28th Jun 2011
<i>N. poecilochroum</i> Yamakoshi	Yamakoshi Harbour, Yakumo Town, Hokkaido Prefecture	42°13'38"N: 140°20'37"E	11th May 2012
<i>N. poecilochroum</i> Hokuto	A sandy beach in Hokuto City, Hokkaido Prefecture	41°49'05"N: 140°38'39"E	12th May 2012
<i>Spiniferodinium galeiforme</i>	Kenmin-no-hama Beach, Kere City, Hiroshima Prefecture	34°9'50"N: 132°44'34"E	22nd May 2002
<i>S. palauense</i>	Republic of Palau: Koror, Ongael Island, Uet era Ongael	7°15'27"N: 134°22'57"E	May 2003

Table 2. Oligonucleotide primers used for amplification and sequencing

Primer code	Synthesis direction	Sequence (5' - 3')	Reference
Small subunit rDNA			
SR1	Forward	TACCTGGTTGATCCTGCCAG	Nakayama <i>et al.</i> 1996
SR4	Forward	AGGGCAAGTCTGGTGCCAG	Nakayama <i>et al.</i> 1996
SR5	Reverse	ACTACGAGCTTTTTAACTGC	Nakayama <i>et al.</i> 1996
SR6	Forward	GTCAGAGGTGAAATTCTTGG	Nakayama <i>et al.</i> 1996
SR7	Reverse	TCCTTGGGCAAATGCTTTCGC	Nakayama <i>et al.</i> 1996
SR8	Forward	GGATTGACAGATTGAGAGCT	Nakayama <i>et al.</i> 1996
SR11	Reverse	CGCTTACTAGGAATTCCTCG	Nakayama <i>et al.</i> 1996
SR12b	Reverse	CGGAAACCTTGTTACGACTTCTCC	Takano & Horiguchi 2004
Internal transcribed spacer (ITS) region including ITS1, 5.8S rDNA and ITS2			
SR12cF	Forward	TAGAGGAAGGAGAAGTCGTAA	Takano & Horiguchi 2006
25F1R	Reverse	ATATGCTTAAATTCAGCGG	Takano & Horiguchi 2006
Large subunit rDNA			
D1R	Forward	ACCCGCTGAATTTAAGCATA	Hansen <i>et al.</i> 2000
D3B	Reverse	GACCCGTCTTGAAACACGGA	Hansen <i>et al.</i> 2000
D3A	Forward	GACCCGTCTTGAAACACGGA	Hansen <i>et al.</i> 2000
28-1483B	Reverse	GCTACTACCACCAAGATCTGC	Daugbjerg <i>et al.</i> 2000
Cytochrome oxidase 1 (cox1)			
Dinocox1F	Foward	AAAAATTGTAATCATAAACGCTTAGG	Lin <i>et al.</i> 2002
Dinocox1F2	Forward	GATTATATCTCCTGAAAACCAGAACTTC	Zhang <i>et al.</i> 2007
Dinocox1R	Reverse	TGTTGAGCCACCTATAGTAAACATTA	Zhang <i>et al.</i> 2007
Dinocox1R2	Reverse	AGTTATTCCTGATCCAATAGATGACAG	Zhang <i>et al.</i> 2007
Cytochrome b (cob)			
PMCOBF	Forward	ATGAAATCTCATTTACAAACATATCC	Lin <i>et al.</i> 2002
Dinocob4F	Forward	TCCAACATTGAAGAGGTTCTTT	Zhang <i>et al.</i> 2007
Dinocob5R	Reverse	ATTWGTAAATDACWGTWGCWCCCCA	Zhang <i>et al.</i> 2007
Dinocob6Rk	Reverse	ATTGGCATAGGAAATACCATTCTGG	This study

Table 2. *(Continued)*

Chloroplast 16S rDNA			
pl16SF1	Forward	GAGCTCGCGCCTGATTAGCTAGTTGG	This study
pl16SF2	Forward	GAGACGACAGCTAGGGGAGCAAATGGG	This study
pl16SR1	Reverse	CTTGTTACGACTTCACCCCAG	This study
pl16SR2	Reverse	GCCTTAGCTACGATACTGCACGG	This study

Table 3. Comparisons of some *Amphidinium* species *sensu lato*

	Cell length (µm)	Cell width (µm)	Shape of epicone	Notch by the sulcus in the hypocone end	Height of cingulum	Chloroplast	Habitat	Reference
<i>Pellucidodinium psammophilum</i>	21-30	18-24	Hemispherical, dome-shaped	Present	Right	Absent	Marine, sand-dwelling	This study
<i>Nusuttodinium desymbiontum</i>	16-21	11-17	Broadly rounded with a ‘beret’-like projection	Present (very slight)	Equal	Absent	Marine, sand-dwelling	This study
<i>Amphidinium dentatum</i>	40	32	Triangular	Present	Left	Blue-green	Marine	Kofoid & Swezy (1921)
<i>Amphidinium flexum</i>	42-60	28-40	Broadly rounded	Present	Left	Absent	Marine	Herdman (1924), Dodge (1982)
<i>Amphidinium globosum</i>	8-16	7-14	Broadly rounded	Absent	Almost equal	Present	Marine	Kofoid & Swezy (1921), Dodge (1982)
<i>Amphidinium lissae</i>	17-18	13-14	Broadly rounded	Absent	Almost equal	Blue-green	Marine, planktonic?	Schiller (1933)
<i>Gymnodinium venator</i>	50-60	35	Rounded	Preset	Left	Absent	Marine	Herdman (1924), Flø Jørgensen <i>et al.</i> (2004)
<i>Nusuttoinimum amphidinioides</i>	18-25	15-25	Truncated-conical	Absent	Equal	Kleptochloroplast from cryptomonad	Freshwater	Schiller (1933) Takano <i>et al.</i> (2014)
<i>Nusuttodinium latum</i>	16-22	16-22	Flattened, slightly rounded	Present	Slightly left	Kleptochloroplast from cryptomonad	Marine, sand-dwelling	Lebour (1925), Horiguchi & Pienaar (1992)
<i>Nusuttodinium poecilochroum</i>	13-18	10-13	Button-shaped	Absent	Equal	Kleptochloroplast from cryptomonad	Marine, sand-dwelling	Larsen (1985; 1988)

Table 4. The number of the cells used for observations

	<i>Nusuttodinium poecilochroum</i>			<i>Nusuttodinium latum</i>			<i>Nusuttodinium aeruginosum</i>		
	LM	TEM	CLSM	LM	TEM	CLSM	LM	TEM	CLSM
0 min	5	7	17	3	4	6	3	3	15
10 min	5	2	-	-	-	-	3	3	-
20 min	3	2	-	-	-	-	3	2	-
30 min	5	2	-	-	-	-	4	3	-
1 h	9	3	22	3	4	10	2	2	16
2 h	10	3	-	-	-	-	2	3	-
3 h	20	3	-	-	-	-	3	3	-
4 h	4	3	20	3	5	7	2	2	15
6 h	14	4	13	-	-	-	3	3	14
12 h	11	3	16	-	-	-	5	2	13
24 h	-	-	24	3	4	5	5	2	16
48 h	-	-	-	3	4	11	3	3	-
72 h	-	-	-	-	-	-	2	3	10
96 h	-	-	-	-	-	-	3	2	-
120 h	-	-	-	4	5	6	2	2	10
2 cell stage (pair)	-	-	-	-	-	-	3	3	-
4 cell stage (set)	-	-	-	-	-	-	5	2	-
32 cell stage (set)	-	-	-	-	-	-	4	4	-

LM, TEM, CLSM indicate light microscopy, transmission electron microscopy and confocal laser scanning microscopy, respectively.

Table 5. The number of nucleomorphs in each daughter cell of three different *Nusuttodinium aeruginosum* parent cells (Pair1-3).

	Cell possessing cN	Cell possessing no cN
Pair 1	7	3
Pair 2	4	2
Pair 3	8	3

Daughter cells are distinguished according to their possession or lack of a cryptomonad nucleus (cN).

Table 6. The number of the nucleomorphs inherited by daughter cells following subsequent two division cycles of two different *Nusuttodinium aeruginosum* parent cells that ingested a cryptomonad (Set1-2).

	Cell inheriting cN, derived from the cell possessing cN	Cell inheriting no cN, derived from the cell possessing cN	Cell inheriting no cN, derived from the cell possessing no cN	Cell inheriting no cN, derived from the cell possessing no cN
Set 1	9	4	2	6
Set 2	13	6	1	1

Daughter cells are distinguished both according to their possession or lack of a cryptomonad nucleus(cN) and to their most recent association with a cryptomonad nucleus.

Table 7. List of *Nusuttodinium aeruginosum* isolates

Isolate		Sampling site	GPS	date	LM	Subclade ITS	of Clade of <i>Chroomonas</i>
Ainosato 130607	cell2	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10'02"N: 141°24'31"E	7th Jun 2013	Fig. 44C	D2	D
Ainosato 130917	cell1	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10'02"N: 141°24'31"E	17th Sep 2013	Fig. 44D	D4	C
Ainosato 130917	cell2	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10'02"N: 141°24'31"E	17th Sep 2013	Fig. 44E	No data	C
Ainosato 140417	cell1	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10'02"N: 141°24'31"E	17th Apr 2014	Fig. 44I	D2	D
Ainosato 140417	cell2	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10'02"N: 141°24'31"E	17th Apr 2014	Fig. 44J	D2	A
Ainosato 140417	cell3	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10'02"N: 141°24'31"E	17th Apr 2014	Fig. 44K	D2	D
Ainosato 140417	cell4	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10'02"N: 141°24'31"E	17th Apr 2014	Fig. 44L	D2	D
Docho cell1 140919		Docho South Pond, Sapporo City, Hokkaido Prefecture	43°03'48"N: 141°20'56"E	19th Sep 2014	Fig. 44M	D4	B
Docho cell2 140919		Docho South Pond, Sapporo City, Hokkaido Prefecture	43°03'48"N: 141°20'56"E	19th Sep 2014	Fig. 44N	D4	B
Docho cell3 140919		Docho South Pond, Sapporo City, Hokkaido Prefecture	43°03'48"N: 141°20'56"E	19th Sep 2014	Fig. 44O	D4	B
Tokotan 131005	cell1	Tokotan pond, Akkeshi Town, Hokkaido Prefecture	43°0'08"N: 144°52'07"E	5th Oct 2013	Fig. 44F	D3	D
Tokotan 131005	cell1	Tokotan pond, Akkeshi Town, Hokkaido Prefecture	43°0'08"N: 144°52'07"E	5th Oct 2013	Fig. 44G	D3	D

Table 7. (Continued)

Tokotan 131005	cell3	Tokotan pond, Akkeshi Town, Hokkaido Prefecture	43°0'08"N: 144°52'07"E	5th Oct 2013	Fig. 44H	No data	D
Yurigahara 130607	cell3	Yurigahara Park, Sapporo City, Hokkaido Prefecture	43°07'39"N: 141°21'49"E	7th Jun 2013	Fig. 44B	D4	C
Yurigahara 130607	cell4	Yurigahara Park, Sapporo City, Hokkaido Prefecture	43°07'39"N: 141°21'49"E	7th Jun 2013	No data	No data	A
Yurigahara 130607	cell6	Yurigahara Park, Sapporo City, Hokkaido Prefecture	43°07'39"N: 141°21'49"E	7th Jun 2013	No data	D4	D
Wada cell1 130330		Wada pond, Kofu City, Yamanashi Prefecture	35°41'23"N: 138°33'44"E	30th Mar 2013	No data	D4	C
Wada cell2 130330		Wada pond, Kofu City, Yamanashi Prefecture	35°41'23"N: 138°33'44"E	30th Mar 2013	Fig. 44A	D4	C

The data of LM, Subclade of ITS and Clade of Chroomonas correspond to Fig. 44, Fig. 45 and Fig. 46, respectively.

Table 8. List of the strains of *Chroomonas* and *Hemiselmis*

Strain	Habitat	Sampling site	GPS	date
<i>Chroomonas</i> sp. Ak01	MA	Aininkappu Beach, Akkeshi Town, Hokkaido Prefecture	43°00'20"N: 144°51'32"E	28th Sep 2011
<i>Chroomonas</i> sp. Dc01	FW	Docho South Pond, Sapporo City, Hokkaido Prefecture	43°03'48"N: 141°20'56"E	24th Sep 2010
<i>Chroomonas</i> sp. Hokudai	MA	No data	No data	No data
<i>Chroomonas</i> sp. HrL01	FW	Hakuryu lake, Nanyo City, Yamagata Prefecture	38°03'20"N: 140°10'43"E	6th Nov 2009
<i>Chroomonas</i> sp. Od06	MA	a tide pool in Odo, Itoman City, Okinawa Prefecture	26°05'38"N: 127°42'52"E	23rd Apr 2013
<i>Chroomonas</i> sp. OnL01	FW	Onuma lake, Nanae Town, Hokkaido Prefecture	41°59'02"N: 140°40'16"E	12th May 2012
<i>Chroomonas</i> sp. OnP01	FW	Ono Pond, Sapporo City, Hokkaido Prefecture	43°04'27"N: 141°20'28"E	16th Apr 2011
<i>Chroomonas</i> sp. Or05	MA	Oshoro bay, Otaru City, Hokkaido Prefecture	43°12'34"N: 140°51'29"E	25th Mar 2012
<i>Chroomonas</i> sp. Sd07	MA	a sandy beach in Sado City, Niigata Prefecture	38°04'07"N: 138°27'36"E	15th Aug 2011
<i>Chroomonas</i> sp. SkL01	FW	Shikotsu lake, Chitose City, Hokkaido Prefecture	42°46'18"N: 141°24'09"E	12th May 2012
<i>Hemiselmis</i> sp. Or06	MA	Oshoro bay, Otaru City, Hokkaido Prefecture	43°12'34"N: 140°51'29"E	25th Mar 2012

MA and FW indicate marine and freshwater, respectively.