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Author(s)
Onuma, Ryo; Horiguchi, Takeo

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Specificity of *Chroomonas* (Cryptophyceae) as a source of kleptochloroplast for *Nusuttodinium aeruginosum* (Dinophyceae)

Ryo Onuma¹*, Takeo Horiguchi²

¹Department of Natural History Sciences, Graduate School of Science, Hokkaido University, North 10, West 8, Sapporo 060-0810 Japan

²Department of Biological Sciences, Faculty of Science, Hokkaido University, North 10, West 8, Sapporo, 060-0810 Japan

*To whom correspondence should be addressed.

Email: ronuma@nig.ac.jp

Present address: Division of Symbiosis and Cell Evolution, Department of Cell Genetics, National Institute of Genetics, Yata 1111, Mishima, Shizuoka, 411-8540, Japan

Running title: Specificity of kleptochloroplast source
SUMMARY

The unarmoured dinoflagellate *Nusuttodinium aeruginosum* retains a kleptochloroplast, which is a transient chloroplast stolen from members of the cryptomonad genus, *Chroomonas*. Both *N. aeruginosum* and the closely related *N. acidotum* have been shown to restrict their diet to a limited number of species of this blue-green genus of cryptophyte. However, it is still unclear how flexible the predators are with regard to the ingestion and utilisation of *Chroomonas* spp. as a source of kleptochloroplast. To address specificity of cryptomonad in *N. aeruginosum*, we collected the cells of *N. aeruginosum* from several ponds in Japan, and analysed the phylogeny of the kleptochloroplasts based on their plastidial 16S rDNA sequences. All sequences obtained in this study were restricted to only one (the subclade 4) of four subclades known to comprise the *Chroomonas/Hemiselmis* clade. Therefore, *N. aeruginosum* is specific in its dietary requirements, selecting their prey within the subclade level.

Key words: *Chroomonas*, kleptochloroplast, *Nusuttodinium aeruginosum*, prey specificity, single cell PCR
INTRODUCTION

Dinoflagellates possess chloroplasts of variable origins. Typical photosynthetic species possess chloroplasts derived from a red alga (the peridinin-type), while others obtain them from diatoms, haptophytes or green algae (e.g. Horiguchi 2006). In addition, some aplastidial dinoflagellates steal chloroplasts from other photosynthetic algae and utilise them for a limited period in a phenomenon called kleptochloroplastidy (e.g. Schnepf & Elbrächter 1992; Schnepf 1993). The genus *Nusuttodinium* is known to be an example of such kleptochloroplastidic dinoflagellates (Takano *et al.* 2014; Onuma *et al.* 2015) that shows considerable variation in its specificity of cryptomonad as a source of its kleptochloroplast. The marine species *N. latum* (Lebour) Y. Takano & T. Horiguchi and *N. poecilochroum* (J. Larsen) Y. Takano & T. Horiguchi can ingest multiple cryptophyte species, indicating that they show no or little specificity within the division/phylum (Larsen 1985, 1988; Horiguchi & Pienaar 1992). The freshwater species *N. amphiдинioides* (Geitler) Y. Takano & T. Horiguchi possesses yellowish-brown and blue-green coloured cryptomonads in natural population and is thus capable of ingesting at least two species of cryptomonads (Takano *et al.* 2014). In
contrast to all these species, the marine *N. myriopyrenoides* (H. Yamaguchi, T. Nakayama, A. Kai & I. Inouye) Y. Takano & H. Yamaguchi appears to be more prey-specific because cells observed from natural population possess only blue-green coloured chloroplasts (Yamaguchi *et al.* 2011). Thus, the specificity of this predator genus for its cryptomonad prey is variable.

The freshwater species *Nusuttodinium acidotum* (Nygaard) Y. Takano & T. Horiguchi and *N. aeruginosum* (F. Stein) Y. Takano & T. Horiguchi show a stricter relationship with their cryptomonad prey than marine species, and are only known to possess blue-green kleptochloroplasts (Wilcox & Wedemayer 1984; Schnepf *et al.* 1989; Farmer & Roberts 1990; Fields & Rhodes 1991). In culture, the cells of *N. acidotum* were observed to ingest two species of the blue-green cryptomonad genus *Chroomonas*, but not *Cryptomonas* sp. (Fields & Rhodes 1991). The (klepto)chloroplasts of *N. acidotum* have been shown, by microscopic absorption analysis, to contain the same profile (phycocyanin 645 responsible for the blue-green colour) as that of wild *Chroomonas* sp., suggesting that *N. acidotum* ingests the co-occurring *Chroomonas* sp. (Barsanti *et al.* 2009). Xia *et al.* (2013) used absorption spectrometry and phylogenetic analyses of nucleomorph SSU rDNA and chloroplast
23S rDNA to identify the source of the kleptochloroplast in *N. acidotum*, which was originally described as *Gymnodinium eucyaneum* but later synonymised with *N. acidotum* (Takano et al. 2014). One nucleomorph SSU rDNA sequence and one 23S rDNA sequence of *Chroomonas* were identified (Xia et al. 2013). Although the cryptomonad prey of *N. acidotum* is clearly restricted to the genus *Chroomonas*, its specific identity has never been addressed in either *N. acidotum* or *N. aeruginosum*. Therefore, it remains unclear how flexible these species are with regard to their cryptomonad diet for the acquisition of their kleptochloroplasts. This study aimed to address the specificity of the diet of individual cells of *N. aeruginosum* sampled from natural populations in various ponds or lakes by analysing portions of the genome of the predator and the prey.

**MATERIALS AND METHODS**

**Sampling and light microscopic (LM) observations**

Cells of *Nusuttodinium aeruginosum* were collected using a 10 µm mesh plankton net. The locations and dates of sampling are listed in Table 1. Individual cells of *N. aeruginosum* were identified using an inverted microscope (CKX-40, Olympus, Tokyo,
Japan) and extricated from the water sample by micropipette. The isolated cells were used for subsequent observation. For LM observations, cells were observed using the ZEISS Axioskop2 Plus (Carl Zeiss, Tokyo, Japan) and photographs were taken with a CCD camera DS-Fi1 (Nikon, Tokyo, Japan) or ZEISS AxioCam ERc 5s (Carl Zeiss). The number of the kleptochloroplast was counted by bright-field microscopic observation.

Strains of *Chroomonas* were established from marine sand samples and from freshwater samples collected at various sites (Table 2). Resultant isolates were cultured either in Daigo IMK medium (for marine isolates) (Wako, Osaka, Japan) or AF-6 medium (for freshwater isolates) (Kato 1982) under the same conditions as described in Onuma and Horiguchi (2013).

**Single cell PCR**

After securing a photographic record of each isolated dinoflagellate cell, the coverslip was removed and the cell was transferred by micropipette through a series of several sterile drops of fresh AF-6 medium on depression slides. The cell was then transferred into a 200 µL PCR tube containing 10 µL of Quick Extract 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 the DNA template for each PCR amplification.

In the first round of PCR for dinoflagellate internal transcribed spacer (ITS; including the ITS1, 5.8S rDNA and ITS2 regions), the primers SR12cF, 25D1R (Takano & Horiguchi 2006) and 1 μL of the DNA solution, as DNA template, were used. In the second round of PCR, 0.1 μL of 1/100-diluted first PCR product was used as DNA template, and the same primers were used. The conditions for both the first and second round of PCR were an 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 the cryptomonad plastidial 16S rDNA, 1 μL of the DNA solution, terminal primers pl16SF1 (5’-GAGCTCGCGCCTGATTAGCTAGTTGG-3’) and pl16SR1 (5’-CTTGTTACGACTTCACCCCAG-3’), designed in this study based on known cryptomonad sequences, were used. In the second round of PCR, 0.1 μL of 1/100-diluted first PCR product was used as DNA template, and two sets of primers...
were used; pl16SF1 together with pl16SR2 (5’- CTTGTTACGACTTCACCCCAG-3’), and pl16SF2 (5’-GAGACGACAGCTAGGGGAGCAAATGGG-3’) together with pl16SR1. The conditions for the first round of PCR were an 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 in the cycles.

The purified PCR products of the dinoflagellate ITS and the 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). Both forward and reverse strands were sequenced. To sequence the 16S rDNA of the cultured Chroomonas strains, approximately 100 cells were transferred from a culture of each strain established into a 200 µL PCR tube containing 10 µL of FFPE DNA Extraction Solution. The rest of treatment for PCR and sequencing followed the methods outlined above to obtain the kleptochloroplast 16S rDNA sequence extracted from the dinoflagellate cells.
Phylogenetic analyses

Both the dinoflagellate ITS and the chloroplast 16S rDNA sequences were aligned manually. *Nusuttodinium amphidinioides*, and *Cryptomonas curvata* Ehrenberg together with *Cry. ovata* Ehrenberg were used as an outgroup for the analyses of dinoflagellate ITS and chloroplast 16S rDNA, respectively (Hoef-Emden 2008; Takano et al. 2014).

The accession numbers of sequences are indicated in Table 1 and Table 2. To explore which model of sequence evolution for maximum likelihood (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 GTR + I + G for the dinoflagellate ITS and the 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 ML analyses with PAUP* version 4.0b10 (Swofford 2002). Bootstrap analysis for ML was calculated to require 100 replicates.

For Bayesian analysis, GTR + G and GTR + I + G model were selected by MrModeltest 2.2 (Nylander 2004) as a suitable evolutionary model for the
dinoflagellate ITS and for the 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 the dinoflagellate ITS phylogeny, while 12,000,000 generations were required for the 16S chloroplast rDNA phylogeny, when the average standard deviations of split frequencies fell below 0.01, indicating a convergence of the iterations. Trees were sampled every 100 generations. The first 125,000 generations were discarded as burn-in for the dinoflagellate ITS data set, whereas the first 2,500,000 generations were discarded for the 16S chloroplast rDNA data set. Posterior probabilities were calculated from all post burn-in trees.

RESULTS

LM observations

*Nusuttodinium aeruginosum* cells were collected from five ponds and lakes and, in the case of the pond at Ainosato Park, on three separate occasions (Table 1). The cells were noted to be ovoid or elliptic with hemispherical epicones, equal or slightly smaller in size to the hypocones in size (Fig. 1). The hypocone was rounded (Fig. 1) and in some
cells had a pointed antapex (Fig. 1J, L). The cingulum encircled the cell without displacement (Fig. 1). All collected cells possessed an enlarged blue-green kleptochloroplast that occupied most of the cell (Fig. 1). No obvious difference with regard to the degree of enlargement, colouration or arrangement of the kleptochloroplast was detectable at the LM level. No food vacuole or accumulation body was observed in the cell.

Phylogenetic analysis of ITS in the dinoflagellate host

The ITS sequences of 15 individual dinoflagellates were determined, but the LM data of only 13 were captured (Fig. 1); ITS sequencing was not successful for the cells depicted in Fig. 1E, H and the LM data of the two cells for which sequences were obtained was not captured (Table 1). The phylogenetic tree (Fig. 2) showed that *Nusuttodinium aeruginosum*, including our sequences, was monophyletic although a reasonable bootstrap (BS) value was not obtained [<50 %; Bayesian posterior probability (PP) = 0.81]. Within the *N. aeruginosum* clade, four subclades were recognised (Fig. 2). The subclade D1, composed of two registered sequences of *N. aeruginosum* from Uto-ike in Kagawa Pref., Japan (AB921311) and a pond in Aomori Pref., Japan (AB921313),
rooted basally with little BS or PP support. Each of the remaining subclades (D2 to D4) was supported by moderate to high BS values. The subclade D2, consisting of the sequence of an isolate of *N. aeruginosum* (AB921316) from Tatara-numa pond in Gunma Pref., Japan and other sequences from cells isolated from Ainosato Park, has moderate BS support (BS/PP = 71/0.98). Our sequences in this clade were very similar, but differed from that of *N. aeruginosum* AB921216 by 10 sites. The subclade D3 and the subclade D4 together formed a further robust clade (96/0.99). Each subclade was highly-supported (subclade D3 = 86/0.99, subclade D4 = 100/0.99). The subclade D3 comprised three registered sequences of *N. aeruginosum* from Sapporo (AB921315), Tokotan pond (AB921312) and Denmark (AB921317) and the two sequences of the cells currently sampled from Tokotan pond. All these sequences were identical and our material here was collected from the same locality as *N. aeruginosum* AB921312 and in the subclade D3. The subclade D4 is populated by sequences from cells collected in this study from four different ponds. *Nusuttodinium aeruginosum* collected at Docho and used as material in other investigations (Onuma & Horiguchi 2013; 2015) falls in this subclade. This phylogenetic analysis showed that the cells used in this study formed a clade with sequences deposited as *N. aeruginosum*, and not with *N. acidotum* (Fig. 2).
Phylogenetic analysis of 16S rDNA in kleptochloroplast

In this study, the sequences of chloroplast 16S rDNA were obtained from 18 dinoflagellate cells possessing kleptochloroplasts and 12 free-living *Chroomonas* and *Hemiselmis*. Hoef-Emden (2008, 2012, 2014) showed that *Chroomonas* and *Hemiselmis* formed a robust clade (the *Chroomonas/Hemiselmis* clade) comprising four subclades. Although BS support for each subclade in the current study is not high, the composition of the inclusive clade concurred with that in Hoef-Emden (2008, 2012, 2014), and we have therefore followed the subclade terminology of Hoef-Emden (2008, 2012, 2014) (Fig. 3). *Chroomonas caudata* Geitler (NIES-712) was the strain included to represent the subclade 3 (Hoef-Emden 2008, 2012, 2014) and it rooted at the base of *Chroomonas/Hemiselmis* clade in this study. A clade composed of *Chroomonas nordstedtii* Hansgirg (NIES-706) and *Chroomonas* sp. HrL01 was the next to branch off, with moderate BS support (77/0.99). The same strain of *C. nordstedtii* was positioned in the subclade 3 of Hoef-Emden (2012). The remaining sequences of *Chroomonas/Hemiselmis* formed a clade supported by 0.74 of PP. The subclade 2 was composed of sequences of *Hemiselmis* with full BS/PP support. *Chroomonas* sp.
isolated from Ainosato Park was a sister to the subclade 2 with no deposited sequence forming a clade with it. The PP of the subclade 4 was high (0.97), and all the sequences recovered from the kleptochloroplasts in the dinoflagellate cells and almost all the strains of free-living *Chroomonas* that we established were included in the subclade 4 (Fig. 3).

Two sister clades were recovered within the subclade 4 (Fig. 3). The clade 4-A, supported by high PP (80/0.99), contained two sequences of dinoflagellate kleptochloroplast, three of our sequences of *Chroomonas* spp. and *Chroomonas coerulea* (Geitler) Skuja (NIES-713). The clade 4-B was composed of all other sequences obtained from the dinoflagellate cells, one sequence of free-living *Chroomonas* sp. isolated from the same sample as the dinoflagellates, two sequences of *Chroomonas* spp. registered in Genbank/EMBL/DDBJ, two sequences of symbiont in *Nusuttodinium myriopyrenoides* and five sequences of *Chroomonas* spp. that we established (Table 1 and 2, Fig. 3). Sequences of kleptochloroplasts originating from Wada pond (cell1 130330 and cell2 130330), Yurigahara Park (cell3 130607) and Ainosato Park (cell1 130917 and cell2 130917) were identical. A further six kleptochloroplast sequences formed another clade with high PP values (0.99) within the
clade 4-B (Fig. 3). This phylogenetic tree indicated that at least three different
sequences of *Chroomonas* were detected in dinoflagellate hosts from Ainosato Park and
Yurigahara Park (Fig. 3). Interestingly, the kleptochloroplast signals from
dinoflagellates collected from Ainosato Park on the 17th Apr 2014, differed from that of
the free-living *Chroomonas* sp. (Ainosato 140417) isolated at the same time from this
locality. In contrast, the kleptochloroplast sequence of a cell collected from Docho pond
(Docho cell1 140919) was identical to that of a free-living *Chroomonas* sp.
(*Chroomonas* sp. Docho 140919) collected at the same time and locality (Fig. 3).

**DISCUSSION**

**Identification of the dinoflagellate host**

All the dinoflagellate cells collected in this study possessed a cingulum located almost
in the middle of the cell, resulting in a similar size of the epicone and the hypocone.
This character makes it different to *Nusuttodinium amphidinioides*, which has a smaller
epicone (Takano et al. 2014). Thus the cells collected in this study belong to either *N.*
*acidotum* or *N. aeruginosum*. *Nusuttodinium aeruginosum* was originally described as
possessing a rounded hypocone (Stein 1883), while *N. acidotum* possesses a pointed
However, it is known that the shape of the antapices of both species readily alters probably depending on water conditions or its stage in the cell cycle, so that either has the potential to possess either form of antapex (Takano et al. 2014). However, Takano et al. (2014) also showed that the shape of the antapex of vast majority of cells of each species was true to its original description, and these two forms can be distinguished from each other by their ITS sequences (Takano et al. 2014). Therefore, *N. acidotum* and *N. aeruginosum* remain as distinct species and the rounded antapex sometimes found in *N. acidotum* is regarded as simply as a precursor stage to cell division (Takano et al. 2014). Almost all the cells collected in this study possessed a rounded hypocone with only the occasional pointed antapex. The phylogenetic analysis showed that this method of identification is appropriate, as all the dinoflagellate cells were positioned within the *N. aeruginosum* clade in the ITS tree regardless of the occasional possession of a 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*
The present 16S rDNA analyses clearly indicated that all the kleptochloroplasts in *Nusuttodinium aeruginosum* cells were included in the subclade 4 of the *Chroomonas/Hemiselmis* clade as resolved in free-living cryptomonads (Hoef-Emden 2008, 2012, 2014). LM observations showed that the kleptochloroplasts were enlarged and occupied the bulk of the dinoflagellate interior as was described in the previous studies on *N. acidotum* and *N. aeruginosum* (Wilcox & Wedemayer 1984; Schnepf et al. 1989; Farmer & Roberts 1990; Fields & Rhodes 1991). It is clear that kleptochloroplasts originating from the subclade 4 cryptomonads remain healthy and normal-looking as to the shape and size of kleptochloroplast. No cryptomonads belonging to the subclades 1, 2 and 3 were recovered from any currently investigated dinoflagellate cells, making it likely that *N. aeruginosum* has specificity for its cryptomonad prey and that *Chroomonas* spp. belonging to the subclades 1, 2 and 3 are inappropriate sources of kleptochloroplasts. This is substantiated by our observation that *N. aeruginosum* cells from Ainosato Park on 17th April 2014 had not ingested a free-living and co-occurring the subclade 2 *Chroomonas* sp. (Ainosato 140417 in Fig. 3). This supports the preference of this dinoflagellate to ingest *Chroomonas* from the subclade 4. However, it is possible that the members of the subclade 4 are far more
abundant in nature making the chances of a dinoflagellate encountering it more likely than those of the subclade 1 – 3. To assess this, feeding experiments using culture strains of various *Chroomonas* spp. is required.

This study also showed that the sequences of the kleptochloroplast were not identical to each other and distributed throughout the subclade 4 in the phylogenetic tree. At least three distinct sequences of *Chroomonas* were obtained from the dinoflagellate subclades D2 and D4. This result strongly suggests that *N. aeruginosum* has an ability to ingest different species of *Chroomonas* and subsequently use them as kleptochloroplasts. However, in this study, we could not find any specificity in the interaction between the dinoflagellate host subclade and the *Chroomonas* prey subclade and could not rule out the possibility that the specificity of cryptomonad choice is variable among the subclades of dinoflagellate host. In conclusion, this study suggests that *N. aeruginosum* has some degree of the specificity to *Chroomonas* at least to subclade level, but does not confine its cryptomonad prey to any particular species.

This level of specificity, i.e. not species-specific, but moderate specificity restricted more or less to the generic level, has some parallels with the symbiont in other kleptochloroplastidic dinoflagellates, as well as in *Hatena arenicola* Okamoto & Inouye.
and *Mesodinium rubrum* Leegaard. The kleptochloroplast of the katablepharid, *H. arenicola*, is obtained from the chloroplast of *Nephroselmis* (Nephroselmidophyceae Cavalier-Smith 1993), and the host is unable to coordinate the division of its chloroplast with its own cell division (Okamoto & Inouye 2006). The phylogenetic analysis of the symbiont suggested that *H. arenicola* accepts as least three species of *Nephroselmis* (Yamaguchi et al. 2014). A marine ciliate, *M. rubrum*, uses the cryptomonad nucleus for maintenance of its kleptochloroplast (Johnson et al. 2007), and this phenomenon, karyoklepty, is also seen in *N. aeruginosum* (Onuma & Horiguchi 2015). *Mesodinium rubrum* possesses kleptochloroplasts obtained from the cryptomonad genera *Geminigera/Teleaulax*, and can be cultured using either of them as a food source (Johnson & Stoecker 2005; Johnson et al. 2006; Park et al. 2006). This shows that prey specificity in *M. rubrum* is not restricted to the species level. Interestingly, almost all *M. rubrum* cells investigated from several localities on the Japanese coast possessed kleptochloroplasts originating from *T. amphioxea* (W. Conrad) D. R. A. Hill, implying *Teleaulax* is much more preferred as food source than *Geminigera* at least in Japanese coasts (Nishitani et al. 2010). Various species of the armoured dinoflagellate *Dinophysis* ingest *M. rubrum* and in turn retain its cryptomonad kleptochloroplasts for up to 2
months (Park et al. 2006; Nagai et al. 2008; Nishitani et al. 2008; Park et al. 2008). The origins of the kleptochloroplast in *Dinophysis* spp. collected from natural environments have been identified as the chloroplast of *Teleaulax* or *Geminigera* based on phylogenetic analyses of chloroplast genes (Takishita et al. 2002; Hackett et al. 2003; Minnhagen & Janson 2006; Kim et al. 2012). In addition to these chloroplasts, Kim et al. (2012) detected green chloroplasts originating from *Chroomonas* sp. in field-collected cells of *D. acuminata* Claparède & Lachmann (Kim et al. 2012). A recent cultural study of *Dinophysis caudata* Saville-Kent showed that the dinoflagellate is capable of ingesting *Mesodinium coatsi* Nam, Shin, Kang, Yih, & Park whose kleptochloroplast is acquired from *Chroomonas* sp. (Kim et al. 2015). However, these green chloroplasts were digested within 26 h of ingestion by the dinoflagellate cell, and the ‘survival’ time of these chloroplasts is much shorter than that of the reddish-brown chloroplasts derived from *Teleaulax* (Kim et al. 2015). An unarmoured dinoflagellate (referred to as RSD) collected from the Ross Sea ingests the chloroplast from a haptophyte, *Phaeocystis antarctica* Karsten, and maintains it for 29.5 months (Gast et al. 2007; Sellers et al. 2014). However, the RSD never ingest other haptophytes, such as *Pseudohaptolina arctica* Edvardsen & Eikrem, indicating that RSD restricts their
symbiont at least to the generic level (Sellers et al. 2014). *Nusuttodinium aeruginosum*, like all these above-mentioned species, show advanced kleptochloroplastidy with regard to the enlargement, the longevity of the captive chloroplast or the phenomenon of karyoklepty (Okamoto & Inouye 2006; Gast et al. 2007; Johnson et al. 2007; Park et al. 2008; Sellers et al. 2014; Onuma & Horiguchi 2015). Species that possess such an advanced state of kleptochloroplastidy might well be expected to have a relatively restricted relationship with the symbiont. On the other hand, the marine species, *Nusuttodinium poecilochroum*, is able to ingest cryptomonads non-specifically (Larsen 1985, 1988). A morphological investigation of this organism showed that it digests the nucleus of its prey shortly after the ingestion and the kleptochloroplast does not become enlarged (Onuma & Horiguchi 2013). Such an approach to kleptochloroplastidy is considered ‘primitive’. Thus, at least for *Nusuttodinium* spp., a dinoflagellate host that shows relatively primitive traits of kleptochloroplastidy tends not to be restrictive in its choice of prey species, while those with enlarged kleptochloroplasts exhibit some prey preference, implying that the restriction of prey might be an essential step to acquiring advanced kleptochloroplastidy with a long-lived plastid, and ultimately leading to the establishment of a permanent or ‘true’ chloroplast.
Although some degree of cryptomonad prey specificity in \textit{Nusuttodinium aeruginosum} has been demonstrated, this study posits a further question. Because we have only observed the cells sampled from the field, it is still unclear whether \textit{N. aeruginosum} can use any subclade 4 \textit{Chroomonas} and establish the coordinated division and growth of the two compartments as has been confirmed for \textit{Chroomonas sp. Dc01}, the strain used in the experiments of \textit{N. aeruginosum} in Onuma & Horiguchi (2013, 2015). To address this, further laboratory-based experiments are needed using different combinations of cryptomonad prey with the dinoflagellate host in question. In addition, the difficulty in obtaining a well-resolved phylogenetic tree makes identification of the cryptomonad symbiont at the specific level difficult. One reason for this difficulty is that the cryptomonad nucleus is often lost during the division of the host cell in wild populations and thus it is not possible to use nuclear-encoded cryptomonad genes for the analyses (Schnepf \textit{et al.} 1989; Onuma & Horiguchi 2015). To identify the cryptomonad symbiont at the specific level, further phylogenetic analysis is required using multiple genes that specifically reside in the chloroplast genome.

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Table 1. List of *Nusuttodinium aeruginosum* isolates

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Sampling site</th>
<th>Latitude and longitude</th>
<th>Sampling date</th>
<th>Image</th>
<th>Subclade in Fig. 2</th>
<th>Subclade in Fig. 3</th>
<th>Accession number of ITS</th>
<th>Accession number of plastid 16S rDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ainosato cell2</td>
<td>Ainosato Park, Sapporo</td>
<td>43°10'02&quot;N: 141°24'31&quot;E</td>
<td>7th Jun</td>
<td>Fig. 1C</td>
<td>D2</td>
<td>4-B</td>
<td>LC082165</td>
<td>LC082181</td>
</tr>
<tr>
<td>130607</td>
<td>City, Hokkaido Prefecture</td>
<td>43°10'02&quot;N: 141°24'31&quot;E</td>
<td>17th Sep</td>
<td>Fig. 1D</td>
<td>D4</td>
<td>4-B</td>
<td>LC082166</td>
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**Table 2.** List of the strains (or isolates) of *Chroomans* and *Hemiselmis*

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<th>Strain (or Isolate)</th>
<th>Habitat†</th>
<th>Sampling site</th>
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<th>Accession</th>
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<tr>
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† MA; marine, FW; fresh water.
FIGURE LEGENDS

Fig. 1. Light micrographs of *Nusuttodinium aeruginosum* used for 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 1. Bar = 10 μm.

Fig. 2. Maximum likelihood (ML) tree inferred from dinoflagellate internal transcribed spacer sequences. 558 sites were used in this analysis. The dinoflagellate cells collected for this analysis are indicated in bold. The alphabet at the end of each OTU indicates figure number in Fig. 1. The bootstrap and Bayesian posterior probability values are provided at each node.
Fig. 3. ML tree inferred from chloroplast 16S rDNA sequences. The sequences obtained from the kleptochloroplast in the dinoflagellate cells are indicated in bold. 1178 sites were used in this analysis. The white text next to the OTU name indicates the corresponding subclade of the dinoflagellate that possesses kleptochloroplast. Asterisks indicate the strains of *Chroomonas* and *Hemiselmis* established in this study. Other information is the same as Fig. 2.