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

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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. amphidinioides* (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

52 contrast to all these species, the marine *N. myriopyrenoides* (H. Yamaguchi, T.
53 Nakayama, A. Kai & I. Inouye) Y. Takano & H. Yamaguchi appears to be more
54 prey-specific because cells observed from natural population possess only blue-green
55 coloured chloroplasts (Yamaguchi *et al*. 2011). Thus, the specificity of this predator
56 genus for its cryptomonad prey is variable.
57 The freshwater species *Nusuttodinium acidotum* (Nygaard) Y. Takano & T.
58 Horiguchi and *N. aeruginosum* (F. Stein) Y. Takano & T. Horiguchi show a stricter
59 relationship with their cryptomonad prey than marine species, and are only known to
60 possess blue-green kleptochloroplasts (Wilcox & Wedemayer 1984; Schnepf *et al*.
61 1989; Farmer & Roberts 1990; Fields & Rhodes 1991). In culture, the cells of *N.*
62 *acidotum* were observed to ingest two species of the blue-green cryptomonad genus
63 *Chroomonas*, but not *Cryptomonas* sp. (Fields & Rhodes 1991). The
64 (klepto)chloroplasts of *N. acidotum* have been shown, by microscopic absorption
65 analysis, to contain the same profile (phycocyanin 645 responsible for the blue-green
66 colour) as that of wild *Chroomonas* sp., suggesting that *N. acidotum* ingests the
67 co-occurring *Chroomonas* sp. (Barsanti *et al*. 2009). Xia *et al*. (2013) used absorption
68 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

171 cells had a pointed antapex (Fig. 1J, L). The cingulum encircled the cell without
172 displacement (Fig. 1). All collected cells possessed an enlarged blue-green
173 kleptochloroplast that occupied most of the cell (Fig. 1). No obvious difference with
174 regard to the degree of enlargement, colouration or arrangement of the kleptochloroplast
175 was detectable at the LM level. No food vacuole or accumulation body was observed in
176 the cell.
177
178 **Phylogenetic analysis of ITS in the dinoflagellate host**
179 The ITS sequences of 15 individual dinoflagellates were determined, but the LM data of
180 only 13 were captured (Fig. 1); ITS sequencing was not successful for the cells depicted
181 in Fig. 1E, H and the LM data of the two cells for which sequences were obtained was
182 not captured (Table 1). The phylogenetic tree (Fig. 2) showed that *Nusuttodinium*
183 *aeruginosum*, including our sequences, was monophyletic although a reasonable
184 bootstrap (BS) value was not obtained [<50 %; Bayesian posterior probability (PP) =
185 0.81]. Within the *N. aeruginosum* clade, four subclades were recognised (Fig. 2). The
186 subclade D1, composed of two registered sequences of *N. aeruginosum* from Uto-ike in
187 Kagawa Pref., Japan (AB921311) and a pond in Aomori Pref., Japan (AB921313),

188 rooted basally with little BS or PP support. Each of the remaining subclades (D2 to D4)
189 was supported by moderate to high BS values. The subclade D2, consisting of the
190 sequence of an isolate of *N. aeruginosum* (AB921316) from Tatara-numa pond in
191 Gunma Pref., Japan and other sequences from cells isolated from Ainosato Park, has
192 moderate BS support (BS/PP = 71/0.98). Our sequences in this clade were very similar,
193 but differed from that of *N. aeruginosum* AB921216 by 10 sites. The subclade D3 and
194 the subclade D4 together formed a further robust clade (96/0.99). Each subclade was
195 highly-supported (subclade D3 = 86/0.99, subclade D4 = 100/0.99). The subclade D3
196 comprised three registered sequences of *N. aeruginosum* from Sapporo (AB921315),
197 Tokotan pond (AB921312) and Denmark (AB921317) and the two sequences of the
198 cells currently sampled from Tokotan pond. All these sequences were identical and our
199 material here was collected from the same locality as *N. aeruginosum* AB921312 and in
200 the subclade D3. The subclade D4 is populated by sequences from cells collected in this
201 study from four different ponds. *Nusuttodinium aeruginosum* collected at Docho and
202 used as material in other investigations (Onuma & Horiguchi 2013; 2015) falls in this
203 subclade. This phylogenetic analysis showed that the cells used in this study formed a
204 clade with sequences deposited as *N. aeruginosum*, and not with *N. acidotum* (Fig. 2).

205

206 **Phylogenetic analysis of 16S rDNA in kleptochloroplast**

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

208 dinoflagellate cells possessing kleptochloroplasts and 12 free-living *Chroomonas* and

209 *Hemiselmis*. Hoef-Emden (2008, 2012, 2014) showed that *Chroomonas* and *Hemiselmis*

210 formed a robust clade (the *Chroomonas*/*Hemiselmis* clade) comprising four subclades.

211 Although BS support for each subclade in the current study is not high, the composition

212 of the inclusive clade concurred with that in Hoef-Emden (2008, 2012, 2014), and we

213 have therefore followed the subclade terminology of Hoef-Emden (2008, 2012, 2014)

214 (Fig. 3). *Chroomonas caudata* Geitler (NIES-712) was the strain included to represent

215 the subclade 3 (Hoef-Emden 2008, 2012, 2014) and it rooted at the base of

216 *Chroomonas*/*Hemiselmis* clade in this study. A clade composed of *Chroomonas*

217 *nordstedtii* Hansgirg (NIES-706) and *Chroomonas* sp. HrL01 was the next to branch off,

218 with moderate BS support (77/0.99). The same strain of *C. nordstedtii* was positioned in

219 the subclade 3 of Hoef-Emden (2012). The remaining sequences of

220 *Chroomonas*/*Hemiselmis* formed a clade supported by 0.74 of PP. The subclade 2 was

221 composed of sequences of *Hemiselmis* with full BS/PP support. *Chroomonas* sp.

222 isolated from Ainosato Park was a sister to the subclade 2 with no deposited sequence
223 forming a clade with it. The PP of the subclade 4 was high (0.97), and all the sequences
224 recovered from the kleptochloroplasts in the dinoflagellate cells and almost all the
225 strains of free-living *Chroomonas* that we established were included in the subclade 4
226 (Fig. 3).
227 Two sister clades were recovered within the subclade 4 (Fig. 3). The clade 4-A,
228 supported by high PP (80/0.99), contained two sequences of dinoflagellate
229 kleptochloroplast, three of our sequences of *Chroomonas* spp. and *Chroomonas*
230 *coerulea* (Geitler) Skuja (NIES-713). The clade 4-B was composed of all other
231 sequences obtained from the dinoflagellate cells, one sequence of free-living
232 *Chroomona*s sp. isolated from the same sample as the dinoflagellates, two sequences of
233 *Chroomonas* spp. registered in Genbank/EMBL/DDBJ, two sequences of symbiont in
234 *Nusuttodinium myriopyrenoides* and five sequences of *Chroomonas* spp. that we
235 established (Table 1 and 2, Fig. 3). Sequences of kleptochloroplasts originating from
236 Wada pond (cell1 130330 and cell2 130330), Yurigahara Park (cell3 130607) and
237 Ainosato Park (cell1 130917 and cell2 130917) were identical. A further six
238 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 a*l. 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

antapex (Wilcox &Wedemayer 1984; Farmer & Roberts 1990; Fields & Rhodes 1991). 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

290 abundant in nature making the chances of a dinoflagellate encountering it more likely
291 than those of the subclade 1 – 3. To assess this, feeding experiments using culture
292 strains of various *Chroomonas* spp. is required.
293 This study also showed that the sequences of the kleptochloroplast were not
294 identical to each other and distributed throughout the subclade 4 in the phylogenetic tree.
295 At least three distinct sequences of *Chroomonas* were obtained from the dinoflagellate
296 subclades D2 and D4. This result strongly suggests that *N. aeruginosum* has an ability to
297 ingest different species of *Chroomonas* and subsequently use them as kleptochloroplasts.
298 However, in this study, we could not find any specificity in the interaction between the
299 dinoflagellate host subclade and the *Chroomonas* prey subclade and could not rule out
300 the possibility that the specificity of cryptomonad choice is variable among the
301 subclades of dinoflagellate host. In conclusion, this study suggests that *N. aeruginosum*
302 has some degree of the specificity to *Chroomonas* at least to subclade level, but does not
303 confine its cryptomonad prey to any particular species.
304 This level of specificity, i.e. not species-specific, but moderate specificity restricted
305 more or less to the generic level, has some parallels with the symbiont in other
306 kleptochloroplastidic dinoflagellates, as well as in *Hatena arenicola* Okamoto & Inouye

307 and *Mesodinium rubrum* Leegaard. The kleptochloroplast of the katablepharid, *H.*
308 *arenicola*, is obtained from the chloroplast of *Nephroselmis* (Nephroselmidophyceae
309 Cavalier-Smith 1993), and the host is unable to coordinate the division of its chloroplast
310 with its own cell division (Okamoto & Inouye 2006). The phylogenetic analysis of the
311 symbiont suggested that *H. arenicola* accepts as least three species of *Nephroselmis*
312 (Yamaguchi *et al*. 2014). A marine ciliate, *M. rubrum*, uses the cryptomonad nucleus for
313 maintenance of its kleptochloroplast (Johnson *et al*. 2007), and this phenomenon,
314 karyoklepty, is also seen in *N. aeruginosum* (Onuma & Horiguchi 2015). *Mesodinium*
315 *rubrum* possesses kleptochloroplasts obtained from the cryptomonad genera
316 *Geminigera*/*Teleaulax*, and can be cultured using either of them as a food source
317 (Johnson & Stoecker 2005; Johnson *et al*. 2006; Park *et al*. 2006). This shows that prey
318 specificity in *M. rubrum* is not restricted to the species level. Interestingly, almost all *M.*
319 *rubrum* cells investigated from several localities on the Japanese coast possessed
320 kleptochloroplasts originating from *T. amphioxeia* (W. Conrad) D. R. A. Hill, implying
321 *Teleaulax* is much more preferred as food source than *Geminigera* at least in Japanese
322 coasts (Nishitani *et al*. 2010). Various species of the armoured dinoflagellate *Dinophysis*
323 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 it 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 *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 *N. aeruginosum* can use any subclade 4 *Chroomonas* and establish the coordinated division and growth of the two compartments as has been confirmed for *Chroomonas* sp. Dc01, the strain used in the experiments of *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 *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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542 **Table 1**. List of *Nusuttodinium aeruginosum* isolates

Isolate No.	Sampling site	Latitude and longitude	Sampling date	Image	Subclade in Fig. 2	Subclade in Fig. 3	Accession number of ITS	Accession number of plastid 16S rDNA
Ainosato cell2 130607	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10’02”N: 141°24’31”E	7th Jun 2013	Fig. 1C	D2	4-B	LC082165	LC082181
Ainosato cell1 130917	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10’02”N: 141°24’31”E	17th Sep 2013	Fig. 1D	D4	4-B	LC082166	LC082182
Ainosato cell2 130917	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10’02”N: 141°24’31”E	17th Sep 2013	Fig. 1E	No data	4-B	-	LC082183
Ainosato cell1 140417	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10’02”N: 141°24’31”E	17th Apr 2014	Fig. 1I	D2	4-B	LC082167	LC082184
Ainosato cell2 140417	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10’02”N: 141°24’31”E	17th Apr 2014	Fig. 1J	D2	4-A	LC082168	LC082185

Ainosato cell3 140417	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10’02”N: 141°24’31”E	17th Apr 2014	Fig. 1K	D2	4-B	LC082169	LC082186
Ainosato cell4 140417	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10’02”N: 141°24’31”E	17th Apr 2014	Fig. 1L	D2	4-B	LC082170	LC082187
Docho cell1 140919	Docho South Pond, Sapporo City, Hokkaido Prefecture	43°03’48’’N: 141°20’56’’E	19th Sep 2014	Fig. 1M	D4	4-B	LC082171	LC082188
Docho cell2 140919	Docho South Pond, Sapporo City, Hokkaido Prefecture	43°03’48’’N: 141°20’56’’E	19th Sep 2014	Fig. 1N	D4	4-B	LC082172	LC082189
Docho cell3 140919	Docho South Pond, Sapporo City, Hokkaido Prefecture	43°03’48’’N: 141°20’56’’E	19th Sep 2014	Fig. 1O	D4	4-B	LC082173	LC082190
Tokotan cell1 131005	Tokotan pond, Akkeshi Town, Hokkaido Prefecture	43°0’08”N: 144°52’07”E	5th Oct 2013	Fig. 1F	No data	4-B	-	LC082191
Tokotan cell2 131005	Tokotan pond, Akkeshi Town, Hokkaido Prefecture	43°0’08”N: 144°52’07”E	5th Oct 2013	Fig. 1G	D3	4-B	LC082174	LC082192
Tokotan cell3	Tokotan pond, Akkeshi	43°0’08”N:	5th Oct	Fig. 1H	D3	4-B	LC082175	LC082193

131005	Town, Hokkaido Prefecture	144°52’07”E	2013					
Yurigahara	Yurigahara Park, Sapporo	43°07’39”N:	7th Jun	Fig. 1B	D4	4-B	LC082176	LC082194
cell3 130607	City, Hokkaido Prefecture	141°21’49”E	2013					
Yurigahara	Yurigahara Park, Sapporo	43°07’39”N:	7th Jun	No data	No data	4-A	-	LC082195
cell4 130607	City, Hokkaido Prefecture	141°21’49”E	2013					
Yurigahara	Yurigahara Park, Sapporo	43°07’39”N:	7th Jun	No data	D4	4-B	LC082177	LC082196
cell6 130607	City, Hokkaido Prefecture	141°21’49”E	2013					
Wada cell1	Wada pond, Kofu City,	35°41’23”N:	30th Mar	No data	D4	4-B	LC082178	LC082197
130330	Yamanashi Prefecture	138°33’44”E	2013					
Wada cell2	Wada pond, Kofu City,	35°41’23”N:	30th Mar	Fig. 1A	D4	4-B	LC082179	LC082198
130330	Yamanashi Prefecture	138°33’44”E	2013					

543

544

545

Table 2. List of the strains (or isolates) of *Chroomans* and *Hemiselmis*

Strain (or Isolate)	Habitat†	Sampling site	GPS	date	Accession

					number
***Chroomonas* sp. Ak01**	MA	Aininkappu Beach, Akkeshi Town, Hokkaido Prefecture	43°00’20’’N: 144°51’32’’E	28th Sep 2011	LC082199
***Chroomonas* sp. Dc01**	FW	Docho South Pond, Sapporo City, Hokkaido Prefecture	43°03’48’’N: 141°20’56’’E	24th Sep 2010	LC082200
***Chroomonas* sp. Hokudai**	MA	No data	No data	No data	LC082201
***Chroomonas* sp. HrL01**	FW	Hakuryu lake, Nanyo City, Yamagata Prefecture	38°03’20’’N: 140°10’43’’E	6th Nov 2009	LC082202
***Chroomonas* sp. Od06**	MA	a tide pool in Odo, Itoman City, Okinawa Prefecture	26°05’38”N: 127°42’52”E	23rd Apr 2013	LC082203
***Chroomonas* sp. OnL01**	FW	Onuma lake, Nanae Town,	41°59’02”N:	12th May 2012	LC082204

		Hokkaido Prefecture	140°40’16”E		
***Chroomonas* sp. Or05**	MA	Oshoro bay, Otaru City, Hokkaido Prefecture	43°12’34”N: 140°51’29”E	25th Mar 2012	LC082205
***Chroomonas* sp. Sd07**	MA	a sandy beach in Sado City, Niigata Prefecture	38°04’07”N: 138°27’36”E	15th Aug 2011	LC082206
***Chroomonas* sp. SkL01**	FW	Shikotsu lake, Chitose City, Hokkaido Prefecture	42°46’18”N: 141°24’09”E	12th May 2012	LC082207
***Hemiselmis* sp. Or06**	MA	Oshoro bay, Otaru City, Hokkaido Prefecture	43°12’34”N: 140°51’29”E	25th Mar 2012	LC082208
***Chroomonas* sp. Ainosato 140417 (Isolate)**	FW	Ainosato Park, Sapporo City, Hokkaido Prefecture	43°10’02”N: 141°24’31”E	17th Apr 2014	LC082209

***Chroomonas* sp. Docho 140919 (Isolate)**	FW	Docho South Pond, Sapporo City, Hokkaido Prefecture	43°03’48’’N: 141°20’56’’E	19th Sep 2014	LC082210

† MA; marine, FW; fresh water.

559 **FIGURE LEGENDS**

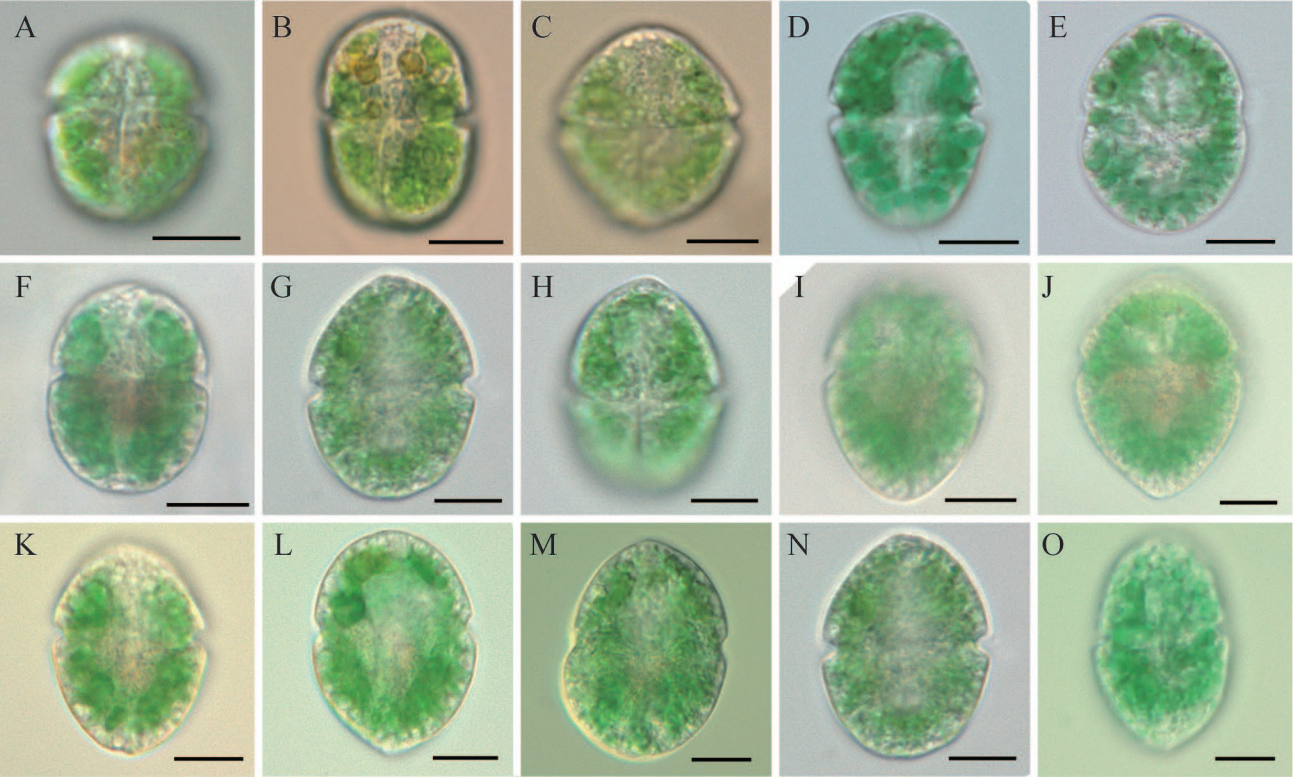
560 **Fig. 1**. Light micrographs of *Nusuttodinium aeruginosum* used for the phylogenetic
561 analyses. Note that the kleptochloroplasts are enlarged throughout the cell in all cells
562 and they look healthy and normal-looking. The names of the cells used in this
563 experiment are following: **A**. Wada cell2 130330. **B**. Yurigahara cell3 130607. **C**.
564 Ainosato cell2 130607. **D**. Ainosato cell1 130917. **E**. Ainosato cell2 130917. **F**. Tokotan
565 cell1 131005. **G**. Tokotan cell2 131005. **H**. Tokotan cell3 131005. **I**. Ainosato cell1
566 140417. **J**. Ainosato cell2 140417. **K**. Ainosato cell3 140417. **L**. Ainosato cell4 140417.
567 **M**. Docho cell1 140919. **N**. Docho cell2 140919. **O**. Docho cell3 140919. The sampling
568 site and date are shown in Table 1. Bar = 10 μm.

569

570 **Fig. 2.** Maximum likelihood (ML) tree inferred from dinoflagellate internal transcribed
571 spacer sequences. 558 sites were used in this analysis. The dinoflagellate cells collected
572 for this analysis are indicated in bold. The alphabet at the end of each OTU indicates
573 figure number in Fig. 1. The bootstrap and Bayesian posterior probability values are
574 provided at each node.

575

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.


A
B
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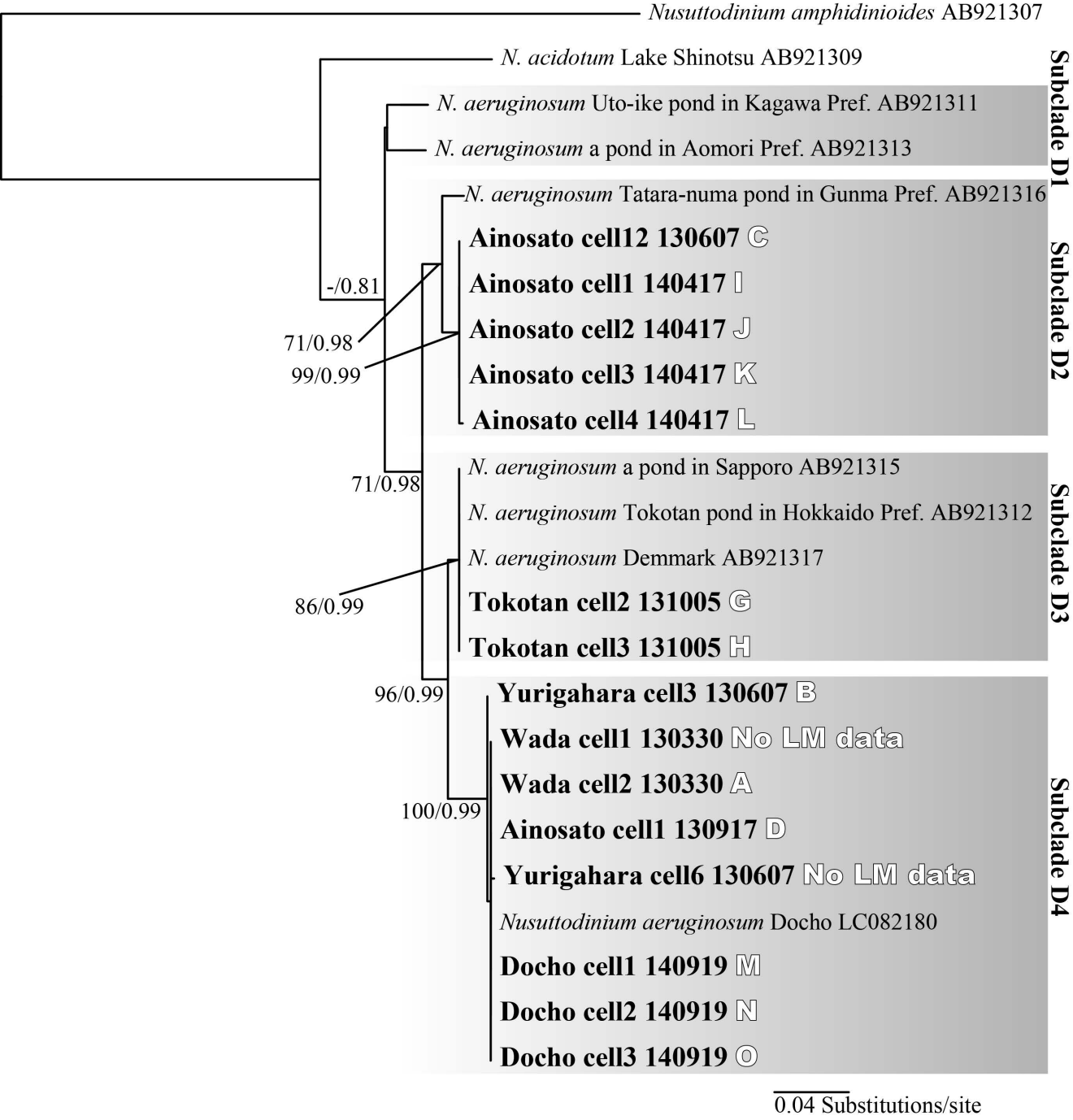
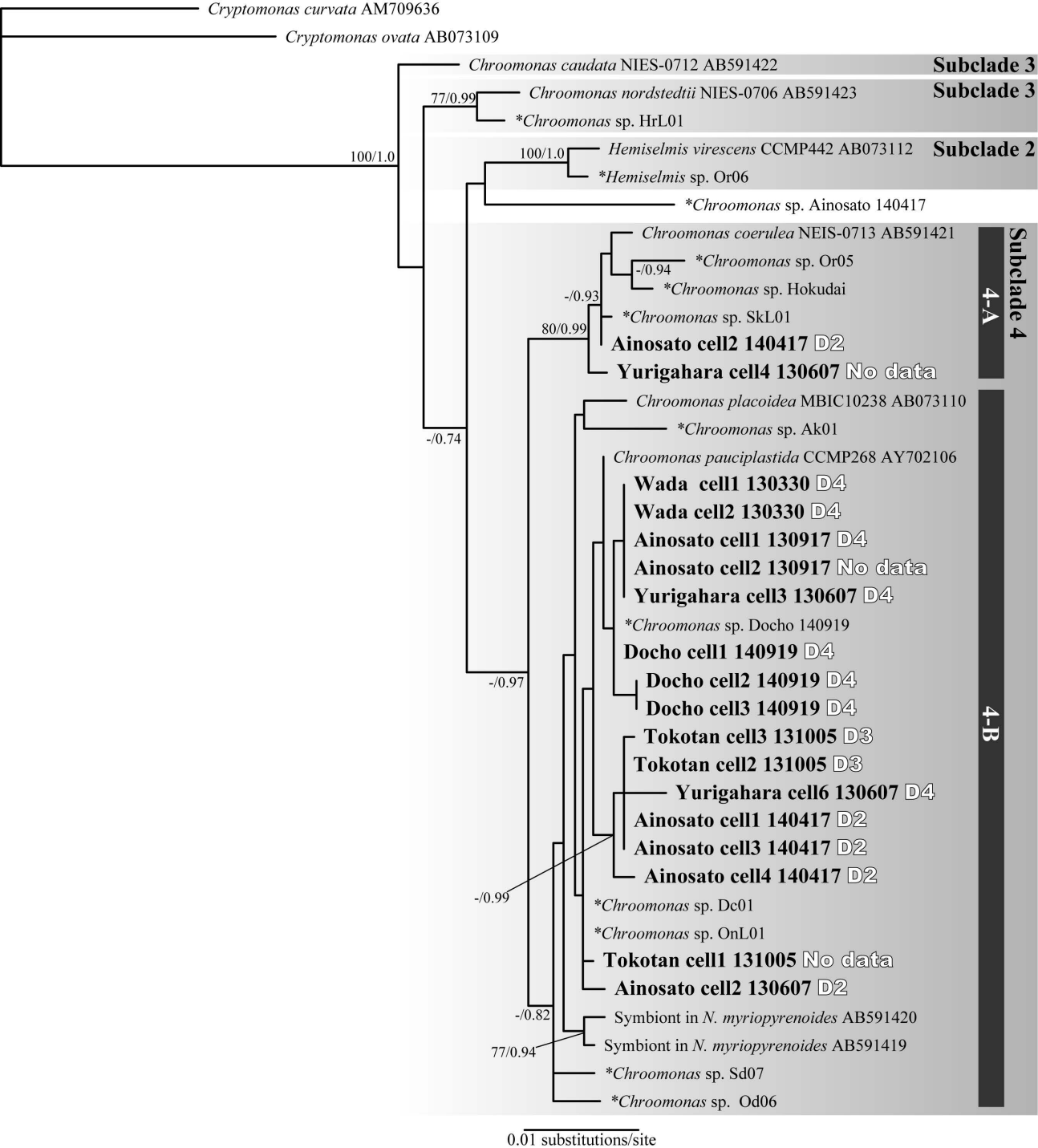

Nusuttodinium amphidinioides AB921307
N. acidotum Lake Shinotsu AB921309
N. aeruginosum Uto-ike pond in Kagawa Pref. AB921311
N. aeruginosum a pond in Aomori Pref. AB921313
N. aeruginosum Tatara-numa pond in Gunma Pref. AB921316
Ainosato cell12 130607 C
Ainosato cell1 140417 I
Ainosato cell2 140417 J
Ainosato cell3 140417 K
Ainosato cell4 140417 L
N. aeruginosum a pond in Sapporo AB921315
N. aeruginosum Tokotan pond in Hokkaido Pref. AB921312
N. aeruginosum Demmark AB921317
Tokotan cell2 131005 G
Tokotan cell3 131005 H
Yurigahara cell3 130607 B
Wada cell1 130330 No LM data
Wada cell2 130330 A
Ainosato cell1 130917 D
Yurigahara cell6 130607 No LM data
Nusuttodinium aeruginosum Docho LC082180
Docho cell1 140919 M
Docho cell2 140919 N
Docho cell3 140919 O
-/0.81
71/0.98
99/0.99
71/0.98
86/0.99
96/0.99
100/0.99
Subclade D1
Subclade D2
Subclade D3
Subclade D4
0.04 Substitutions/site


Cryptomonas curvata AM709636
Cryptomonas ovata AB073109
Chroomonas caudata NIES-0712 AB591422
Subclade 3
Chroomonas nordstedtii NIES-0706 AB591423
Subclade 3
77/0.99
*Chroomonas sp. HrL01
100/1.0
Hemiselmis virescens CCMP442 AB073112
Subclade 2
100/1.0
*Hemiselmis sp. Or06
*Chroomonas sp. Ainosato 140417
Chroomonas coerulea NEIS-0713 AB591421
Subclade 4
4-A
*Chroomonas sp. Or05
-/0.94
*Chroomonas sp. Hokudai
-/0.93
*Chroomonas sp. SkL01
80/0.99
Ainosato cell2 140417 D2
Yurigahara cell4 130607 No data
Chroomonas placoidea MBIC10238 AB073110
*Chroomonas sp. Ak01
-/0.74
Chroomonas pauciplastida CCMP268 AY702106
Wada cell1 130330 D4
Wada cell2 130330 D4
Ainosato cell1 130917 D4
Ainosato cell2 130917 No data
Yurigahara cell3 130607 D4
*Chroomonas sp. Docho 140919
Docho cell1 140919 D4
-/0.97
Docho cell2 140919 D4
Docho cell3 140919 D4
4-B
Tokotan cell3 131005 D3
Tokotan cell2 131005 D3
Yurigahara cell6 130607 D4
Ainosato cell1 140417 D2
Ainosato cell3 140417 D2
Ainosato cell4 140417 D2
-/0.99
*Chroomonas sp. Dc01
*Chroomonas sp. OnL01
Tokotan cell1 131005 No data
Ainosato cell2 130607 D2
-/0.82
Symbiont in N. myriopyrenoides AB591420
77/0.94
Symbiont in N. myriopyrenoides AB591419
*Chroomonas sp. Sd07
*Chroomonas sp. Od06
0.01 substitutions/site