Kleptochloroplast enlargement, karyoklepty and the distribution of the cryptomonad nucleus in *Nusuttodinium* (= *Gymnodinium*) *aeruginosum* (Dinophyceae)

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The unarmoured freshwater dinoflagellate *Nusuttodinium* (= *Gymnodinium*) *aeruginosum* retains a cryptomonad-derived kleptochloroplast and nucleus, the former of which fills the bulk of its cell volume. The paucity of studies following morphological changes to the kleptochloroplast with time make it unclear how the kleptochloroplast enlarges and why the cell ultimately loses the cryptomonad nucleus. We observed, both at the light and electron microscope level, morphological changes to the kleptochloroplast incurred by the enlargement process under culture conditions. The distribution of the cryptomonad nucleus after host cell division was also investigated. The volume of the kleptochloroplast increased more than 20-fold, within 120 h of ingestion of the cryptomonad. 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 important cryptomonad nucleus division mechanism is lost during ingestion process, and that the cryptomonad nucleus carries important information for the enlargement of the kleptochloroplast.

Key words: cryptomonad nucleus; karyoklepty; kleptochloroplast; morphological transition; *Nusuttodinium aeruginosum*; ultrastructure

Introduction

Dinoflagellates have proved interesting subjects for the study of chloroplast endosymbiosis because some of them have replaced their original peridinin-containing chloroplasts with those of phylogenetically-distinct algae via serial secondary or tertiary endosymbiosis (e.g. Horiguchi 2006; Keeling 2013). In addition, some dinoflagellates possess unique chloroplasts, called kleptochloroplasts, which are transient chloroplasts retained in the host cell (Schnepf and Elbrächter 1992). These dinoflagellates originally are colourless and ingest photosynthetic algae, retaining their chloroplasts. However, these chloroplasts are eventually lost due to digestion or imperfect distribution to daughters of the host cell following division (Schnepf and Elbrächter 1992). Kleptochloroplastidy have been reported in many groups of organisms including sea slugs, ciliates, foraminifera and katablepharid in addition to dinoflagellates (e.g. Johnson 2011; Nowack and Melkonian 2010). Kleptochloroplastidy in dinoflagellates can be seen in both armoured and unarmoured species, and exhibit different types in terms of selection of prey, acquisition strategy and degree of retention of organelles of prey organisms other than chloroplast. The armoured dinoflagellates *Amylax* spp. and *Dinophysis* spp. retain cryptomonad chloroplasts and obtain them from kleptochloroplastidic ciliate *Mesodinium rubrum*, which retains the chloroplast derived from *Teleaulax* (Cryptophyceae), indicating that these two dinoflagellates obtain their kleptochloroplasts through the intermediate organisms (Koike and Takishita 2008;

Nagai et al. 2008; Nishitani et al. 2008; Park et al. 2006; Park et al. 2013). *Amylax* spp. ingest cryptomonad nucleus, nucleomorph and mitochondria in addition to the chloroplast and retain the chloroplast for about a month (Kim et al. 2014). By contrast, *Dinophysis* spp. retain the chloroplasts that elongated and arranged in group without any other cryptomonad organelles and keep them in the cell at least for two months (Park et al. 2008; Schnepf and Elbrächter 1988). *Cryptoperidiniopsis* sp. and *Pfiesteria piscicida* ingest cryptomonad cell directly and are suggested to use the chloroplast for carbon metabolisms (Eriksen et al. 2002; Lewitus et al. 1999). Two unnamed dinoflagellates (RS 24 and W5-1) obtain their kleptochloroplasts from the haptophyte *Phaeocystis* and retain the chloroplast for 29.5 months (Gast et al. 2007; Sellers et al. 2014). The unarmoured dinoflagellates *Amphidinium latum* (Horiguchi and Pienaar 1992), *A. poecilochroum* (Larsen 1988), *Gymnodinium acidotum* (Farmer and Roberts 1990; Fields and Rhodes 1991; Wilcox and Wedemayer 1984), *G. aeruginosum* (Schnepf et al.1989), *G. eucyaneum* (Xia et al. 2013), *G. gracilentum* (Skovgaard1998) and *G. myriopyrenoides* (Yamaguchi et al. 2011) are known to retain chloroplasts of cryptomonad origin. Recently, Takano et al. (2014) proposed a generic name, *Nusuttodinium* to accommodate *Amphidinium latum, A. poecilochroum, A. amphidinioides, Gymnodinium acidotum, G. aeruginosum* and *G. myriopyrenoides*. Therefore, hereafter we will use newly proposed combinations for these species.

Of these, the closely-related freshwater species, *Nusuttodinium acidotum* and *N. aeruginosum*, possess a single cup-shaped kleptochloroplast that occupies most of the

cell volume (Farmer and Roberts 1990; Fields and Rhodes 1991; Schnepf et al. 1989; Wilcox and Wedemayer 1984). These kleptochloroplasts were derived from the blue-green cryptomonad, *Chroomonas* spp. (Fields and Rhodes 1991; Onuma and Horiguchi 2013). The kleptochloroplast is surrounded by double chloroplast membranes and a double chloroplast endoplasmic reticulum (ER), and the cryptomonad cytoplasm containing the kleptochloroplast and additional cryptomonad organelles is separated from the dinoflagellate cytoplasm by a single membrane (referred as the 'perisymbiont' membrane in Schnepf et al. 1989) (Farmer and Roberts 1990; Fields and Rhodes 1991; Schnepf et al. 1989; Wilcox and Wedemayer 1984). These studies also showed that the kleptochloroplast is considerably enlarged within the host cell relative to the size of the original chloroplast in the free-living form of the cryptomonad prey. Previously, we followed the morphological transition of the kleptochloroplast in *N. aeruginosum* up to 24 h after the ingestion of *Chroomonas* sp. During this period, the kleptochloroplast was enlarged and deformed although not as enlarged as in field-sampled cells (Onuma and Horiguchi 2013). Using confocal microscopy, we demonstrated that the kleptochloroplast keeps growing up to 72 h after ingestion (Onuma and Horiguchi 2013). However, details of the morphological transition of a growing kleptochloroplast after 24 h have not been observed using a transmission electron microscope (TEM). Therefore, it remains unclear how the host dinoflagellate stimulates a single kleptochloroplast to occupy most of its cell from its original size in the free-living cryptomonad.

Another interesting question concerning *Nusuttodinium acidotum* and *N. aeruginosum* is the presence or absence of the cryptomonad nucleus. These dinoflagellates retain not only the kleptochloroplast but also the nucleus, nucleomorph and mitochondria of the cryptomonad within their cytoplasm (Farmer and Roberts 1990; Fields and Rhodes 1991; Onuma and Horiguchi 2013; Schnepf et al. 1989; Wilcox and Wedemayer 1984). 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, 10% of *N. aeruginosum* cells and 33-57% of *N. acidotum* cells are reported to retain the cryptomonad nucleus in natural populations (Farmer and Roberts 1990; Fields and Rhodes 1991; Schnepf et al. 1989). Fields and Rhodes (1991) reported that *N. 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 and Rhodes 1991). Although they successfully maintained the strain for at least 9 months by co-culturing them with *Chroomonas* sp. as prey (Fields and Rhodes 1991), they did not observe the mechanisms of inheritance of the cryptomonad organelles (nucleus and nucleomorph). In previous study on *Gymnodinium eucyaneum*, which has synonymised to *N. acidotum* (Takano et al. 2014), the kleptochloroplast, originated from *Chroomonas* sp., and nuclear substances (probably cryptomonad nuclei and nucleomorph) are retained in the host cell (Shi et al.

1983; Xia et al. 2013). Light microscopic (LM) observation 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 presence or absence 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 (Farmer and Roberts 1990; Schnepf et al. 1989). 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, the present study followed, at the LM and TEM level, the morphological transition of kleptochloroplasts in cells of *Nusuttodinium aeruginosum* during the enlargement process through 48, 72, 96 and 120 h after the ingestion of a single cryptomonad cell. Cell division only commenced once the kleptochloroplast enlargement process was completed. To observe morphological changes and the

distribution of kleptochloroplasts and other organelles to each daughter cell, we 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. Our *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.

Results

LM Observations during Kleptochloroplast Enlargement

The dinoflagellate cell has an enlarged, peripheral, cup-shaped kleptochloroplast within 48 h of ingestion of a single *Chroomonas* sp. cell. The periphery of the kleptochloroplast was ramified into small lobes (Fig. 1A, B) and the pyrenoid and cryptomonad nucleus were visible (Fig. 1A), but no cryptomonad eyespot was detected. After 72 h of ingestion, the kleptochloroplast almost filled the host and the lobes of the kleptochloroplast increased in size and number (Fig. 1C, D). At this stage, the pyrenoids

had also multiplied (Fig. 1C). By 96 h of ingestion, the lobes had extended to the cell periphery, filling the vast majority of the cell interior (Fig. 1E, F). By 120 h of ingestion, the kleptochloroplast further enlarged and its periphery became corrugated (Fig. 1G, H). The dinoflagellate cell grew with the progressive enlargement of the kleptochloroplast (Fig. 1). The cryptomonad nucleus was always located in the hypocone of the host cell. (Fig. 1A, C, E, G).

TEM Observations during Kleptochloroplast Enlargement

Membranes around cryptomonad cell and kleptochloroplast: By 48 h of ingestion, the kleptochloroplast was surrounded by two chloroplast membranes (Fig. 2A white arrowheads) and two chloroplast ER membranes (Fig. 2A black arrowheads), indicating that the membranes of the original cryptomonad chloroplast remained intact, and therefore identical to the structure of the kleptochloroplast observed 24 h after ingestion (Onuma and Horiguchi 2013). The cryptomonad cytoplasm containing mitochondria with flat cristae was separated from the dinoflagellate host by a single membrane (= the perisymbiont membrane) (Fig. 2A arrow). The thylakoid membranes were stacked in pairs (Fig. 2A). During kleptochloroplast enlargement, up to 120 h, the four kleptochloroplast membranes and the perisymbiont membrane were retained (Fig. 2B).

The kleptochloroplast and cryptomonad organelles: At the 48 h stage, the kleptochloroplast was enlarged and randomly ramified (Fig. 3A). The cryptomonad cytoplasm was contained within the cup-shaped kleptochloroplast, and contained the

cryptomonad nucleus, nucleomorph(s), and cryptomonad mitochondria (Fig. 3A). By 72 h of ingestion, the kleptochloroplast had almost reached the periphery of the host cell on all sides (Fig. 3B). The pyrenoids had multiplied and been distributed in both the epicone and the hypocone of the host cell (Fig 3B). The composition of the individual cryptomonad organelles was the same as in the previous stage (Fig. 3B). At 96 h, the number of pyrenoids had significantly increased and a longitudinal section of the cell showed at least 5 pyrenoids (Fig. 3C). By 120 h of ingestion, the host cell possessed an enlarged kleptochloroplast that pervaded the entire cell, the cryptomonad nucleus and the nucleomorph (Fig. 3D). TEM observations showed that the cryptomonad nucleus was always located in the 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. 3A-D). No digestive vacuole was observed.

The cryptomonad nucleus and nucleomorphs: We have observed three cells 48 h after ingesting a cryptomonad. One of these possessed a nucleomorph near the eyespot (Fig. 4A) while another had two nucleomorphs near the cryptomonad nucleus (Fig. 4B, 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. 4D, E). Serial sectioning confirmed that, the three cells we observed at the 72 h stage possessed 3, 3 and 2 nucleomorphs,

211 respectively, and it was suggested that the number of these nucleomorphs was increased
212 from a single nucleomorph in the original cryptomonad cell. We observed two cells at
213 the 96 h stage and detected 2 nucleomorphs in each cell. Of two cells observed at
214 the120 h stage, one had 9 nucleomorphs while the other had 8 (all nucleomorphs in the
215 latter cell are shown in Fig. 4F-J). Most of them were located near the cryptomonad
216 nucleus, but some were positioned in the middle of the host cell (Fig. 4J). During
217 kleptochloroplast enlargement, the cryptomonad nucleus retained the integrity of its
218 nuclear envelopes and no digestion of the nucleus was detected (Fig. 4B-F). No division
219 of cryptomonad nucleus was observed up to 120 h stage.
220
221 **Estimates of kleptochloroplast volumes**
222 The average kleptochloroplast volume (n = 10) was 1694.3 (± 519.6 SD) μm^3 (Fig. 5),
223 with a maximum and minimum volume of 2627.1 and 1064.4 μm^3, respectively. The
224 relatively large value of standard deviation indicated large variability of the volume
225 depending on the cells measured. These data, combined with those of Onuma and
226 Horiguchi (2013) (the volumes from 0 min stage (= right after ingestion) to 72 h stage),
227 are shown in Fig. 5. The volume at 120 h was more than 20-fold that at the 0 min stage
228 (Fig. 5). Serial autofluorescence images of the kleptochloroplast at the 120 h stage,
229 shows that the lobes of the kleptochloroplast had extended to reach the plasma
230 membrane on all sides, filling almost the entire host cytoplasm (Supplemental Movie
231 S1).

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 (see Supplemental Table S1). Each daughter cell had a cup-shaped, blue-green kleptochloroplast filling its volume (Fig. 6 A-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. 6A). Observation using TEM 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. 6E, I). It also confirmed that the cryptomonad nucleus was inherited by only one cell (Fig. 6E). The cryptomonad nucleus was situated in the hypocone, indicating the position was not changed by cell division (Fig. 6E). In contrast to the cryptomonad nucleus, nucleomorphs were inherited by both daughter cells (Fig. 6G, 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 1). Most of nucleomorphs in the former cell were located between the kleptochloroplast and the cryptomonad nucleus (Fig. 6F, G), while some of them were detected in the centre or the epicone of the host cell (Fig. 6H). In the cell lacking a cryptomonad nucleus, the nucleomorphs were positioned in the hypocone (Fig. 6I-K). Both daughters had cryptomonad mitochondria within the cryptomonad cytoplasm (Fig. 6F-H, J). The membrane structure surrounding the kleptochloroplast was unaltered after the first division (Fig. 2C).

LM and TEM observations of the cells after the second host cell division (4 cell stage): We 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 Figures 7 and 8. The two daughter cells derived from the cell that inherited the cryptomonad nucleus after the first cell division are shown in Figure 7 and the two daughter cells derived from the cell lacking the cryptomonad nucleus are shown in Figure 8. LM observation showed that all four daughters inherited a kleptochloroplast that retained its cup-shape and which enlarged to fill the host cell (Fig. 7A-D, Fig. 8A-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. 7A, C, Fig. 8A, C). TEM observation confirmed that the cryptomonad nucleus was only inherited by one of the four daughter cells (Fig. 7E). The cryptomonad nucleus retained its nuclear membranes and nucleolus (Fig. 7F). Nucleomorphs were distributed to all 4 cells (Fig. 7F-K, Fig. 8F-K). In the cell possessing the cryptomonad nucleus,

nucleomorphs tended to be situated around the cryptomonad nucleus (Fig. 7F, G), but with some more-distantly positioned (Fig. 7H). 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. 8G-H). Nucleomorphs were not equally-distributed among the four daughters (Table 2). 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 1 and 2). The integrity of all membranes around the kleptochloroplast was not compromised in any of the four daughter cells (Fig. 2D)

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. We 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 9. In one of the four replicates, we obtained LM micrographs of (almost; see below) all the final daughter cells (Fig. 10). 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. 10). Another observation was that Cell M (Fig. 9), the cell that inherited cryptomonad

295 nucleus (see Fig. 11), possessed the largest kleptochloroplast (Fig. 10M) and that Cell N
296 (Fig. 9), derived from the same parent cell (Cell 22) as Cell M, had a similarly-large
297 kleptochloroplast to that of Cell M (Fig. 10N). Moreover, Cell M, N, O and P (=Fig.
298 10M-P) that were derived from the same mother cell (Cell 11, Fig. 9) had the largest
299 kleptochloroplasts of all final generation cells (= Fig. 10A-L, 10Q-AC). By contrast,
300 Cells Q-AC, which were derived from Cell 3 (Fig. 9), that had lost the cryptomonad
301 nucleus after the first cell division, had reduced kleptochloroplasts that failed to form a
302 cup-shape (Fig. 10 Q-AC). Unfortunately, Cell AD collapsed before a LM micrograph
303 was captured, but the size of the kleptochloroplast was similar to that of Cell AC (Fig.
304 10AC). In other tracking experiments, cells with a cryptomonad nucleus and some cells
305 lacking a cryptomonad nucleus were observed after being isolated and this same
306 tendency as described above was confirmed (data not shown). TEM observations on
307 Cell M (Fig. 10), identified at LM as possessing a cryptomonad nucleus (Fig. 11),
308 confirmed its presence (Fig. 11A, B). In addition, Cell M possessed 12 nucleomorphs
309 (confirmed by serial sectioning and 5 of which are shown in Fig. 11A-D). In this
310 preparation, no sign of digestion was detected in the cryptomonad nucleus, the
311 kleptochloroplast or the cryptomonad cytoplasm with cryptomonad mitochondria, and
312 the thylakoid stacks were still retained (Fig. 11). The same results were recovered in all
313 four replicate trackings up to the 32 cell stage. Thus cells that inherited the cryptomonad
314 nucleus possessed the largest kleptochloroplast and cells with the longest history of
315 having a cryptomonad nucleus tended to possess larger kleptochloroplasts than those

316 which had lost it earlier on.

317

318 **Discussion**

319

320 **Enlargement of Kleptochloroplast**

321 This study revealed that the kleptochloroplast was significantly deformed and increased
322 its volume relative to the original cryptomonad chloroplast (Onuma and Horiguchi 2013
323 Supp. Fig. 2), and that the kleptochloroplast grew within the host cell, by developing
324 lobes, increasing the number of pyrenoids and extending the thylakoid membranes. The
325 cryptomonad nucleus, nucleomorphs, mitochondria and the cryptomonad cytoplasm
326 were retained in the dinoflagellate cell in addition to the kleptochloroplast. Such
327 enlargement and deformation parallel those found in kleptochloroplasts of other
328 dinoflagellates. In *Nusuttodinium latum*, the kleptochloroplast drastically deforms with
329 a concomitant multiplication of pyrenoids and nucleomorphs, and cryptomonad nucleus
330 and cytoplasm are retained with the kleptochloroplast like in *N. aeruginosum*. However,
331 the kleptochloroplast in *N. latum* never filled the bulk of the host cell and synchronous
332 division has never been observed (Horiguchi and Pienaar 1992). Contrary to *N. latum*, *N.*
333 *myriopyrenoides* enlarges kleptochloroplast throughout the host cell like in *N.*
334 *aeruginosum* (Yamaguchi et al. 2011). The cryptomonad pyrenoid in *N.*
335 *myriopyrenoides* can multiplicate to produce several bodies, each covered with a starch
336 sheath (Yamaguchi et al. 2011). Although *N. myriopyrenoides* retains the cryptomonad

337 nucleus and nucleomorph like in *N. aeruginosum*, there is still uncertainty about the
338 number of nucleomorphs and about whether synchronous kleptochloroplast/host
339 division occurs or not (Yamaguchi et al. 2011). Previous studies reported that *N.*
340 *acidotum* possesses a single kleptochloroplast that fills the bulk of the host cell, a
341 cryptomonad nucleus and several nucleomorphs (Farmer and Roberts 1990; Wilcox and
342 Wedemayer 1984). In unialgal culture, *N. acidotum* seems to be able to divide its single
343 kleptochloroplast and distribute it equally to the daughter cells, although the
344 morphology of the divided cell or the kleptochloroplast has never been investigated
345 (Fields and Rhodes 1991). It seems, then, that kleptochloroplastidy in *N. acidotum* is at
346 the same evolutionary stage as that in *N. aeruginosum*.
347 The katablepharid *Hatena arenicola* can also enlarge its chloroplast (Okamoto and
348 Inouye 2006). The kleptochloroplast here enlarges throughout the host cell, its volume
349 increasing more than ten-fold relative to that of the original *Nephroselmis* chloroplast.
350 The host cell can allow the symbiont cell to duplicate the pyrenoid and fill chloroplast
351 with thylakoid membrane, accompanied with enlargement of chloroplast (Okamoto and
352 Inouye 2006). Although *H. arenicola* is phylogenetically distanced from the
353 kleptochloroplastidic dinoflagellates mentioned above, the enlargement of the
354 chloroplast, including the generation of thylakoid membranes and the duplication of the
355 pyrenoid seem to be common features among kleptochloroplastidy.
356 Thus, organisms that enlarge the kleptochloroplast tend to elaborate on existing
357 thylakoid membrane and to duplicate the pyrenoid. Both have to be effected after

ingestion by the host cell. Because the kleptochloroplast is originally derived from a foreign photosynthetic alga, the host most probably does not have any chloroplast genes in its 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, *Nusuttodinium latum*, *N. myriopyrenoides*, *N. aeruginosum* and *Hatena arenicola* retain the ingested algal nucleus in addition to the kleptochloroplast (Farmer and Roberts 1990; Horiguchi and Pienaar 1992; Okamoto and Inouye 2006; Yamaguchi et al. 2011; Wilcox and Wedemayer 1984), implying the retained stolen nucleus plays a role for the enlargement of the kleptochloroplast.

Distribution of Cryptomonad Nucleus and Nucleomorph

This study indicated that both daughter cells of *Nusuttodinium aeruginosum* inherited a kleptochloroplast after each cell division, but the cryptomonad nucleus was inherited by only one of two daughter cells due to the inability of the cryptomonad nucleus to divide. Previous studies reported that 33-57% of *N. acidotum* and 10% of *N. aeruginosum* cells in the natural population retained a cryptomonad nucleus (Farmer and Roberts 1990; Fields and Rhodes 1991; Schnepf et al. 1989). LM observation on cells of *N. acidotum* (as *Gymnodinium eucyaneum*) shows that nuclear substance is passed to one daughter cell by chance during host cell division (Shi et al. 1983). Therefore, cells of these species that lack a cryptomonad nucleus must be brought about due to lack of synchronous division as observed in this study, not from digestion of the cryptomonad

nucleus, as was witnessed in *N. poecilochroum* (Onuma and Horiguchi 2013). In free-living cryptomonads, nuclear division occurs by mitosis involving microtubules and a spindle (Oakley and Dodge 1976; McKerracher and Gibbs 1982). However, cryptomonad basal bodies and microtubules are absent in the enlarged kleptochloroplast compartment of *N. aeruginosum* and *N. acidotum* cells (Farmer and Roberts 1990; Schnepf et al. 1989; Wilcox and Wedemayer 1984). 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 and Roberts 1990). In addition, a light microscopic study on a cell of *N. acidotum* (as *G. eucyaneum*) 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). 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*.

Previous studies on *Nusuttodinium acidotum* showed that its ingested cryptomonad possesses duplicated nucleomorphs, situated near the cryptomonad nucleus (Farmer and

Roberts 1990; Wilcox and Wedemayer 1984). The nucleomorph observed in this study was in a similar locality and duplicated up to 9 in the dinoflagellate cell before host cell division. Therefore, nucleomorph division was successfully demonstrated to occur in the host cell in culture as seen in *N. acidotum* in the natural population (Farmer and Roberts 1990; Wilcox and Wedemayer 1984). In free-living cryptomonads, nucleomorph division takes place in the absence of microtubules or a spindle (McKerracher and Gibbs 1982; Morrall and Greenwood 1982), implying that the nucleomorph in the dinoflagellate cell is divided with the same mechanism as that in free-living cryptomonad. This study also showed that duplicated nucleomorphs were inherited by the daughter cells although the distribution of inherited nucleomorphs in daughter cells was not equal, indicating that the process is random. In the free-living cryptomonad, one product of the duplicated nucleomorph moves to the opposite side of the dividing cell in preprophase, and cell division subsequently occurs (McKerracher and Gibbs 1982; Oakley and Dodge 1976; Sato et al. 2014). In contrast, successive divisions of the nucleomorph in a *N. aeruginosum* host precede kleptochloroplast division. This suggests that the control mechanism of number of nucleomorph per cell is lost following ingestion. However, further study is required to elucidate 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. 9), 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. 9), 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. 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 (Onuma and Horiguchi 2013). In contrast, the concurrences of enlarged chloroplast and stolen nucleus can be seen in *N. latum*, *N. myriopyrenoides*, *N. acidotum* and *Hatena arenicola* as mentioned above (Horiguchi and Pienaar 1992; Okamoto and Inouye 2006; Yamaguchi et al. 2011),

suggesting 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). Just as in *M. rubrum*, *Nusuttodinium aeruginosum* probably requires the cryptomonad nucleus to maintain the kleptochloroplast for a longer period although there are some differences between their kleptochloroplastidy as to the position of cryptomonad nucleus, i.e., *M. rubrum* retains cryptomonad nucleus separately from the chloroplast (Hibberd 1977; Lindholm 1985), while the cryptomonad nucleus in *N. aeruginosum* is consistently located in the proximity of the chloroplast. In our 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

463 endosymbiont’s nucleus with that of the host cell.

464 This is not too far-fetched, because the retention of an endosymbiotic nucleus is
465 known in dinoflagellates possessing a permanent diatom endosymbiont (e.g. Horiguchi
466 2006) and commonly referred to as dinotoms (Imanian et al. 2010). Although a dozen
467 dinotom species are known, the well-studied ones include *Durinskia baltica* and
468 *Kryptoperidinium foliaceum.* These dinotoms retain diatom cytoplasm separated by a
469 single membrane from the dinoflagellate cytoplasm and the cytoplasm contains a
470 diatom nucleus and mitochondria in addition to the chloroplast (Tomas and Cox 1973;
471 Jeffrey and Vesk 1976). When the host cell divides, the diatom nucleus synchronously
472 divides and so it is inherited by each of the daughter cells (Tippit and Pickett-Heaps
473 1976; Figueroa et al. 2009). During the diatom nuclear division, chromatin is not
474 condensed and the organelle divides amitotically, by a simple constriction and without
475 the aid of a spindle (Tippit and Pickett-Heaps 1976). Transcriptome analysis showed
476 that dinotoms have two distinct sets of transcript for the tryptophan biosynthetic
477 pathway (Imanian and Keeling 2014). One of these sets of proteins are derived from the
478 diatom symbiont, and are suggested to be encoded in the diatom nucleus and expressed
479 in the diatom endosymbiont (Imanian and Keeling 2014), which indicates that the
480 diatom nucleus is more or less functional despite of its amitotic division. Therefore, it
481 appears that one of the key events for the permanent establishment of an
482 endosymbiont’s nucleus (and thus endosymbiosis) would be the acquisition of a
483 successful amitotic mechanism for nuclear division.

484 This study has shown that *Nusuttodinium aeruginosum* displays an
485 evolutionarily-advanced form of kleptochloroplastidy with a significantly enlarged
486 kleptochloroplast capable of division and karyoklepty. However, the number of
487 generations over which the host cell can keep the cryptomonad nucleus and the
488 kleptochloroplast fully functional remains unclear. To address this, further
489 morphological observation is required. In addition, it is still unknown how the
490 cryptomonad nucleus affects the kleptochloroplast at the molecular level. *N.*
491 *aeruginosum* would be an appropriate subject organism in studies that attempt to
492 understand the process of endosymbiosis, and we intend in future to investigate this
493 species at the genomic level.
494
495 **Material and Methods**
496
497 **Culture for experiments:** All observations undertaken in this study used a strain of
498 *Nusuttodinium aeruginosum* that we established previously (Onuma and Horiguchi
499 2013). We maintained the strain in culture with the prey cryptomonad, *Chroomonas* sp.
500 (strain Dc01) grown in AF-6 medium as described in Onuma and Horiguchi 2013. For
501 the various treatments, the starvation, feeding and culture methods followed the
502 methods described in Onuma and Horiguchi (2013).
503 **LM and TEM observation and estimate of kleptochloroplast volume**: All methods
504 to observe the cell during kleptochloroplast enlargement followed the methods in

505 Onuma and Horiguchi (2013), except that the LM photographs were taken with a CCD
506 camera ZEISS AxioCam ERc 5s (Carl Zeiss Japan,Tokyo).
507 For LM observation of first two daughter cells following the division of the cell that
508 ingested the cryptomonad, one daughter was isolated and observed with a ZEISS
509 Axioskop2 Plus (Carl Zeiss Japan, Tokyo) and photographs were taken using a CCD
510 camera ZEISS AxioCam ERc 5s. Observations on the other daughter subsequently
511 followed. For TEM observation, isolated first daughter cells (2 cell stage), were
512 individually transferred into a drop of AF-6 medium in a plastic Petri dish (35 mm in
513 diameter), and then fixed with half-strength Karnovsky fixative (2.5% glutaraldehyde
514 and 2% paraformaldehyde, final concentration) in 0.1 M cacodylate buffer at pH 7.0 for
515 2 h at room temperature. After the pre-fixation both daughter cells were attached to the
516 same poly-L-lysine pre-coated Thermanox plastic coverslip (Thermo Scientific,
517 Kanagawa, Japan). Subsequent treatments for TEM observation followed those outlined
518 in Onuma and Horiguchi 2013.
519 For LM observations of cells at the 4 cell stage, we separated the daughter cells at
520 the 2 cell stage (after the first host cell division) and transferred the isolated cell into a
521 separate well of the microplate to prevent confusion between the cell lineages. After the
522 second host cell division in each well, we isolated and observed the four daughter cells
523 one by one using the same method outlined above for the 2 cell stage. For TEM
524 observations at the 4 cell stage, we separated the daughter cells after the first host cell
525 division, and after the second division, we 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), we 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 grass 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 Onuma and Horiguchi (2013).

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Tables and Figure Legends

Figure 1. Bright field and fluorescence micrographs following the morphological change in the 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 μm.

Figure 2. Transmission electron micrographs of the surrounding membranes of the kleptochloroplast in *Nusuttodinium aeruginosum*. **A.** The kleptochloroplast 48 h after ingestion is surrounded by four membranes, two chloroplast membranes (white arrowheads) and two chloroplast ER membranes (arrowheads). The cryptomonad cytoplasm is separated from that of the dinoflagellate by a single membrane (arrows). **B-D.** Surrounding **c**hloroplast membranes 120 h after ingestion (B), after one cell division (the 2 cell stage; C) and after two cycles of cell division (the 4 cell stage; D).

715 The four membranes and the cytoplasmic boundary membrane are still intact. Bar = 200
716 nm. Abbreviations: Chl, kleptochloroplast; cM, cryptomonad mitochondria; cS,
717 cryptomonad starch; cCy, cryptomonad cytoplasm; dN, dinoflagellate nucleus; dS,
718 dinoflagellate starch.
719
720 **Figure 3.** TEM micrographs following the morphological changes to the ingested
721 cryptomonad cells in *Nusuttodinium aeruginosum* over time since ingestion. **A.** TEM
722 micrograph 48 h after ingestion showing the early loss of normal cryptomonad shape
723 caused by peripheral lobing. Bar = 2 μm. **B-D.** Cells of *N. aeruginosum* 72, 96 and 120
724 h after ingestion showing the elaboration of the kleptochloroplast to fill much of the
725 host cell as well as the duplication of the pyrenoids. Note that a cryptomonad nucleus
726 and nucleomorphs (arrowheads) are located in the hypocone. Bar = 5 μm.
727 Abbreviations: Chl, kleptochloroplast; cN, cryptomonad nucleus; Nm, nucleomorph;
728 cM, cryptomonad mitochondria; Py, pyrenoid; cCy, cryptomonad cytoplasm; dN,
729 dinoflagellate nucleus.
730
731 **Figure 4.** TEM micrographs of cryptomonad nucleus and nucleomorphs in
732 *Nusuttodinium aeruginosum* cell. **A.** A nucleomorph 48 h after ingestion, near the
733 eyespot. **B, C.** Nucleomorphs in another cell 48 h after ingestion. The nucleomorph has
734 replicated and is near the cryptomonad nucleus. **D-E.** The cryptomonad nucleus and
735 nucleomorphs 72 h (D) and 96 h (E) after ingestion. Note that the nucleomorphs are

736 situated between the cryptomonad nucleus and the kleptochloroplast. **F-J.** The
737 cryptomonad nucleus and up to eight nucleomorphs (Nm1-Nm8) 120 h after ingestion.
738 Abbreviations: Chl, kleptochloroplast; cN, cryptomonad nucleus; Nm, nucleomorph;
739 cM, cryptomonad mitochondria; E, eyespot; cCy, cryptomonad cytoplasm; dM,
740 dinoflagellate mitochondria. Bar = 1 μm.
741
742 **Figure 5**. Change in volume of ingested kleptochloroplasts up to 120 h from the
743 ingestion of a cryptomonad by *Nusuttodinium aeruginosum*. Error bars indicate SD.
744
745 **Figure 6**. Bright field (**A, C**), fluorescence (**B, D**) and TEM micrographs (**E-K**) of the
746 two daughter cells of *Nusuttodinium aeruginosum* following the first division after the
747 ingestion event. **A-B, C-D**. Bright field and fluorescence micrographs of the daughter
748 cell with, and without, the cryptomonad nucleus respectively, showing similar-sized and
749 cup-shaped kleptochloroplasts that enlarge throughout the host cell. Bar = 10 μm. **E, I**.
750 TEM micrographs of the two daughter cells respectively, showing the equally-divided
751 kleptochloroplast that ramifies throughout each daughter. The presence of the
752 cryptomonad nucleus in the former only is confirmed. Arrowheads indicate
753 nucleomorphs (Nm1-3). Bar = 5 μm. **F-H**. TEM details of the daughter with the
754 nucleated cryptomonad. **F.** The intact, double-membraned cryptomonad nucleus and a
755 nucleomorph (Nm1). Bar = 2 μm. **G-H**. Further nucleomorphs either near the
756 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.

Figure 7. 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.

Figure 8. 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 μm. **E, J**. TEM micrographs of each of the daughters. Note that the kleptochloroplast pervades the host cell Bar = 5 μm. **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.

Figure 9. 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 10. A circle around a cell number indicates cells that inherited the

799 cryptomonad nucleus.

800

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

810

811 **Figure 11.** TEM micrographs of the *Nusuttodinium aeruginosum* cell that inherited the
812 cryptomonad nucleus five division cycles after the original ingestion event. **A.** The cell
813 has a similar extensive kleptochloroplast and intact cryptomonad nucleus (cN) as
814 witnessed soon directly after ingestion. Bar = 5 μm. **B.** Detail of the intact nature of the
815 cryptomonad nucleus and nucleomorphs located near the nucleus, showing no sign of
816 digestion. Bar = 3 μm. **C.** Nucleomorph (Nm3 and Nm4) situated near the cryptomonad
817 nucleus showing its intact structure. Bar = 1 μm. **D.** Nucleomorph (Nm5) positioned in
818 the middle of the cell showing no effect of digestion. Bar = 500 nm. Abbreviations: Chl,
819 kleptochloroplast; Nm, nucleomorph; dN, dinoflagellate nucleus.

820

821 **Table 1**. The number of nucleomorphs in each daughter cell of three different

822 *Nusuttodinium aeruginosum* parent cells (Pair1-3). Daughter cells are distinguished

823 according to their possession or lack of a cryptomonad nucleus (cN).

824

825 **Table 2**. The number of the nucleomorphs inherited by daughter cells following

826 subsequent two division cycles of two different *Nusuttodinium aeruginosum* parent cells

827 that ingested a cryptomonad (Set1-2). Daughter cells are distinguished both according

828 to their possession or lack of a cryptomonad nucleus(cN) and to their most recent

829 association with a cryptomonad nucleus.

830

831 **Supplemental Table 1**. The number of cells of *Nusuttodinium aeruginosum* used for

832 light microscopy (LM), transmission electron microscopy (TEM) and confocal laser

833 scanning microscopy (CLSM).

834

835 **Supplemental Movie 1**. Animation of an optically sectioned chloroplast of

836 *Nusuttodinium aeruginosum* under bright-field optics and autofluorescence, 120 h after

837 ingesting a cryptomonad. Bar = 10 μm.

Figure 1.

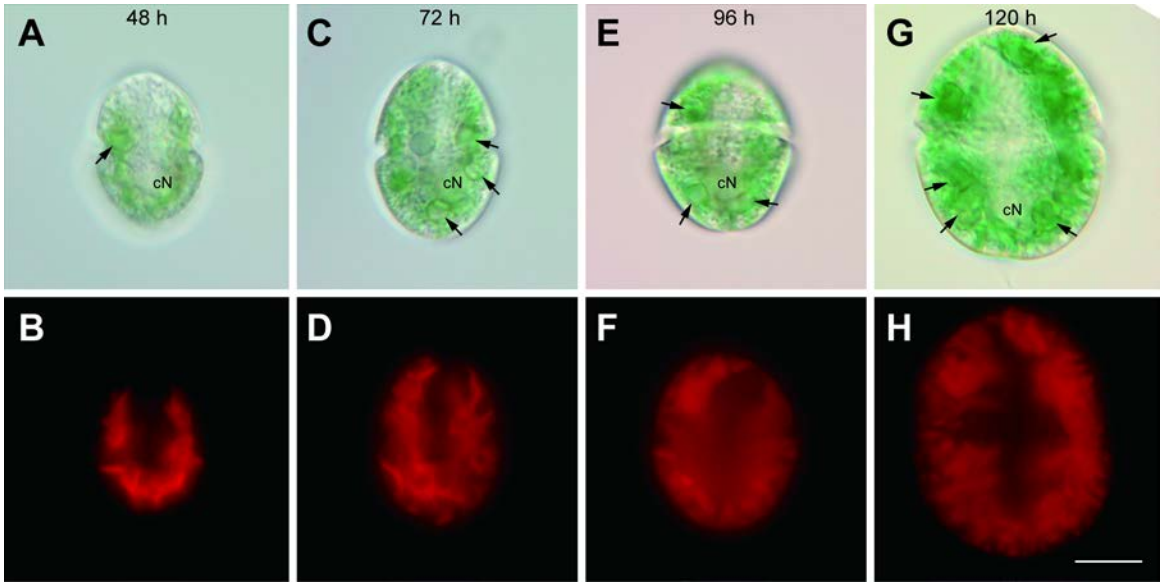

Figure 2.

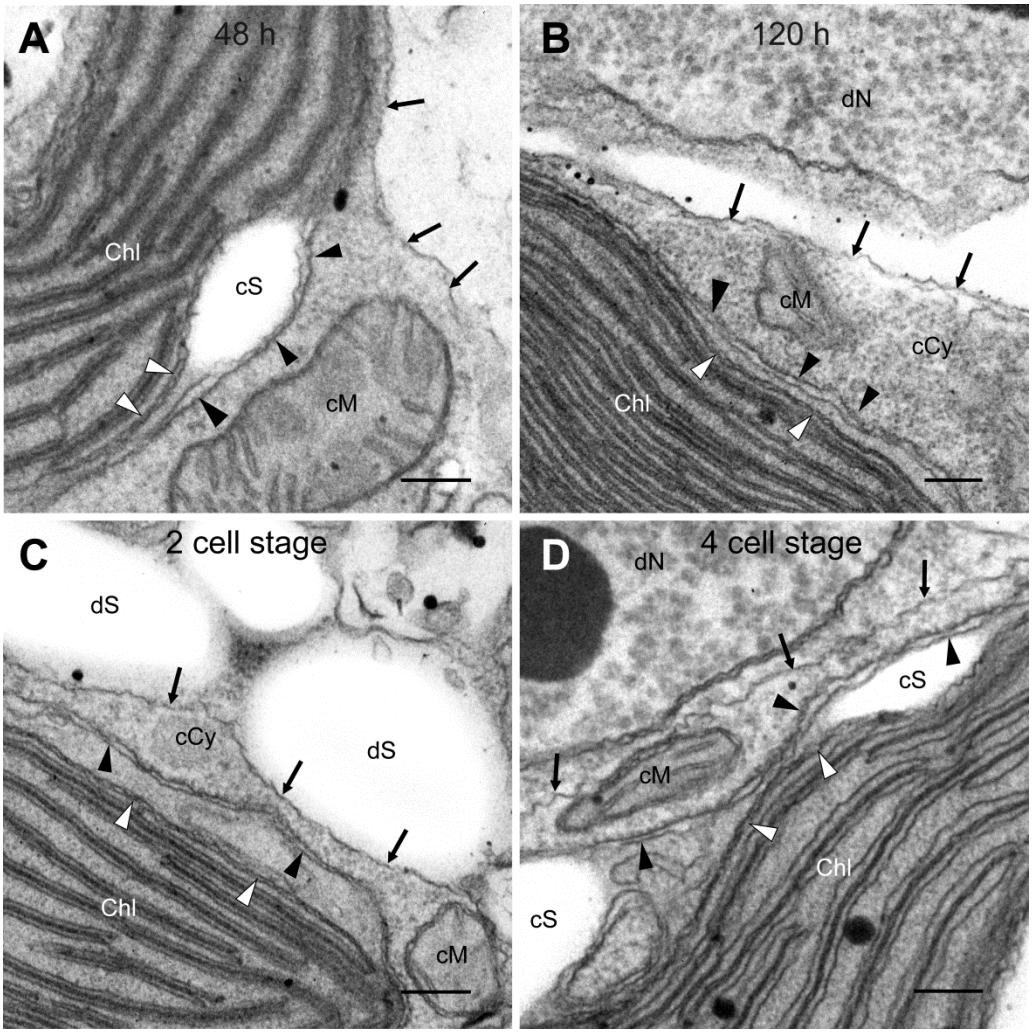
A
48 h
Chl
cS
cM
B
120 h
dN
cM
cCy
Chl
C
2 cell stage
dS
cCy
dS
Chl
cM
D
4 cell stage
dN
cS
cM
Chl
cS

Figure 3.

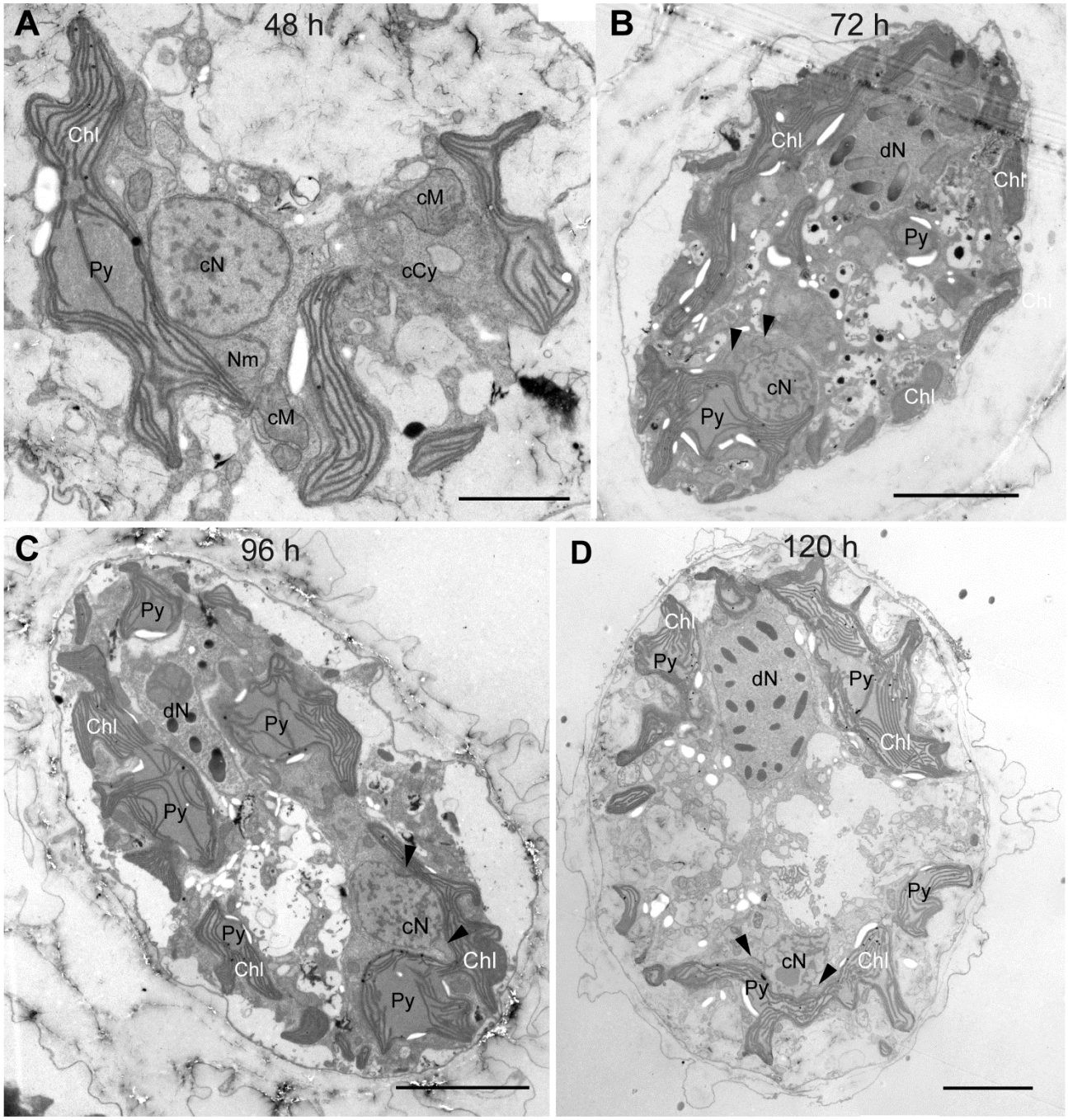
A
48 h
Chl
Py
cN
cM
cCy
Nm
cM
B
72 h
Chl
dN
Chl
Py
Chl
cN
Chl
Py
C
96 h
Py
Chl
dN
Py
Py
Py
Chl
cN
Chl
Py
D
120 h
Chl
Py
dN
Py
Chl
Py
cN
Chl
Py

Figure 4.

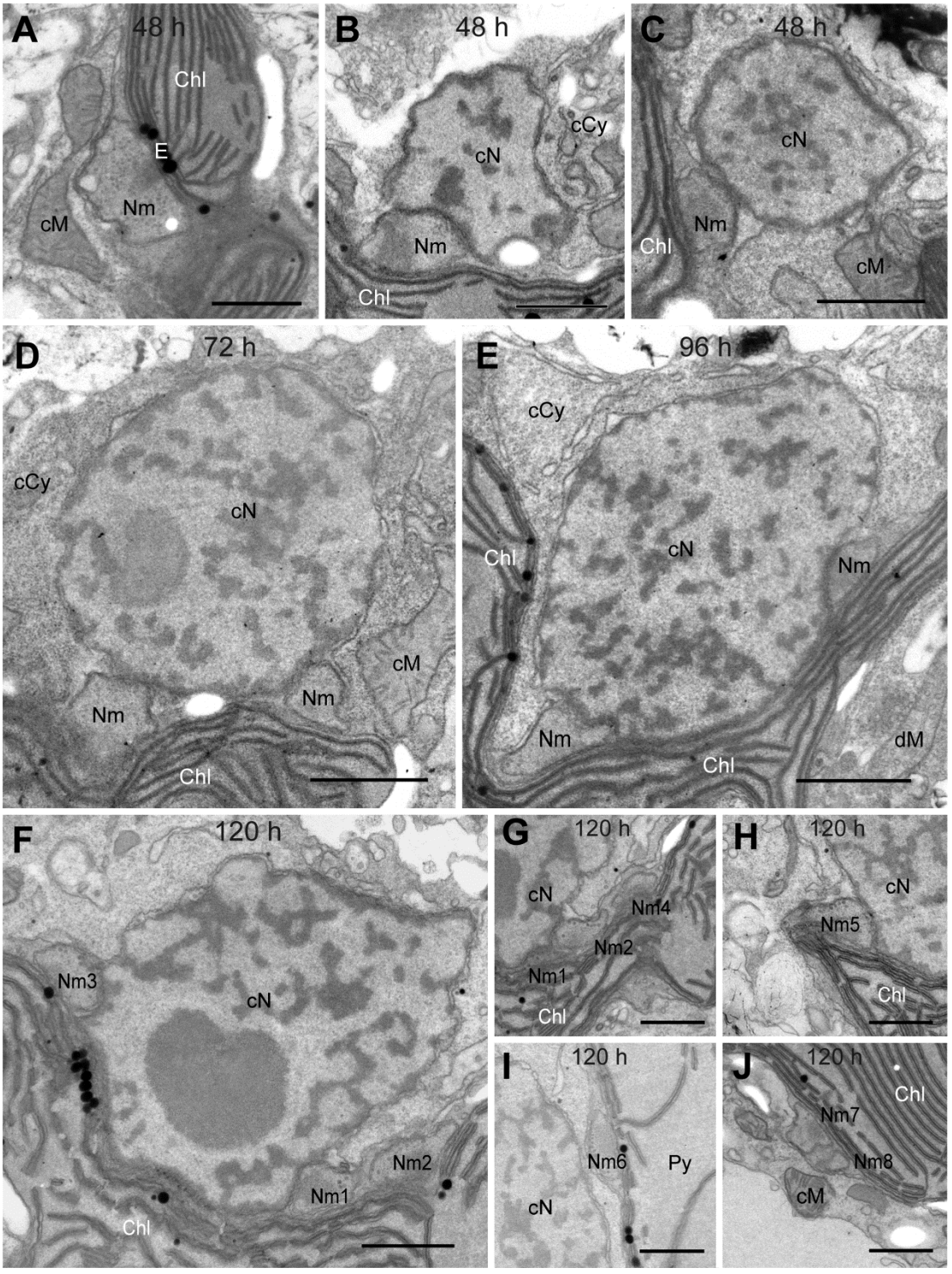
A
48 h
Chl
E
Nm
cM
B
48 h
cCy
cN
Nm
Chl
C
48 h
cN
Nm
Chl
cM
D
72 h
cCy
cN
cM
Nm
Nm
Chl
E
96 h
cCy
Chl
cN
Nm
Nm
dM
Chl
F
120 h
Nm3
cN
Nm2
Nm1
Chl
G
120 h
cN
Nm4
Nm2
Nm1
Chl
H
120 h
cN
Nm5
Chl
I
120 h
Nm6
Py
cN
J
120 h
Chl
Nm7
Nm8
cM

Figure 5.

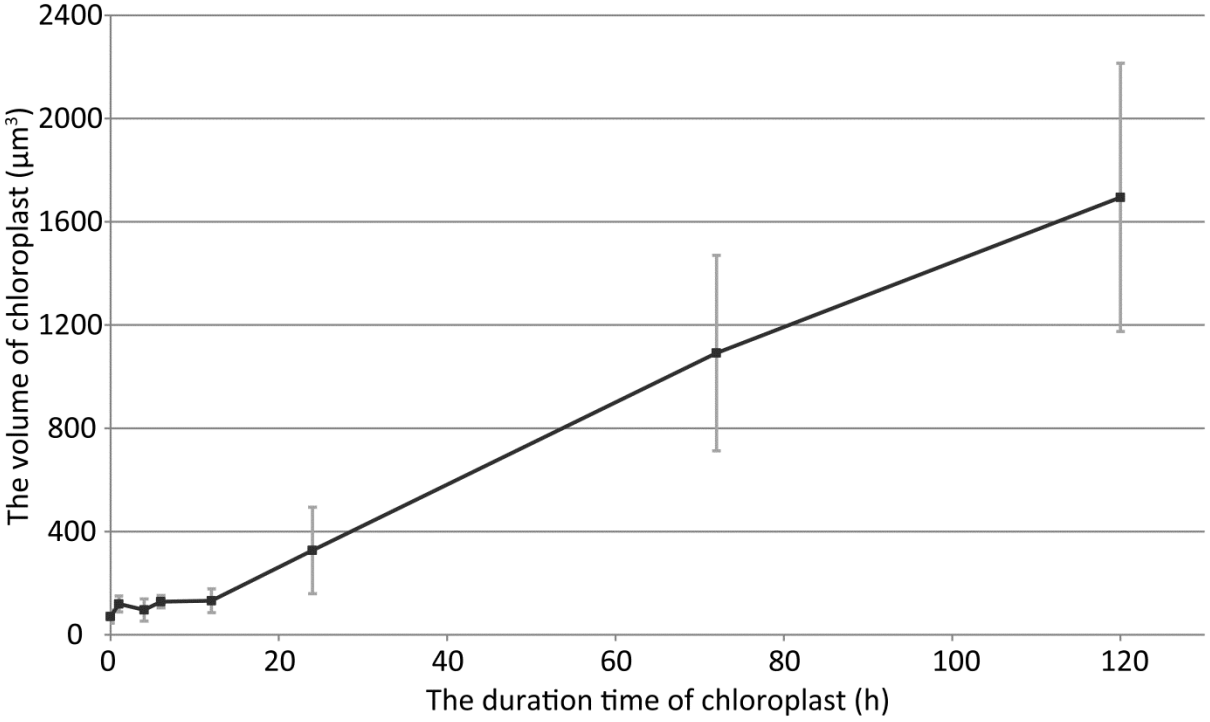
2400
2000
1600
1200
800
400
0
The volume of chloroplast (μm³)
0
20
40
60
80
100
120
The duration time of chloroplast (h)

Figure 6.

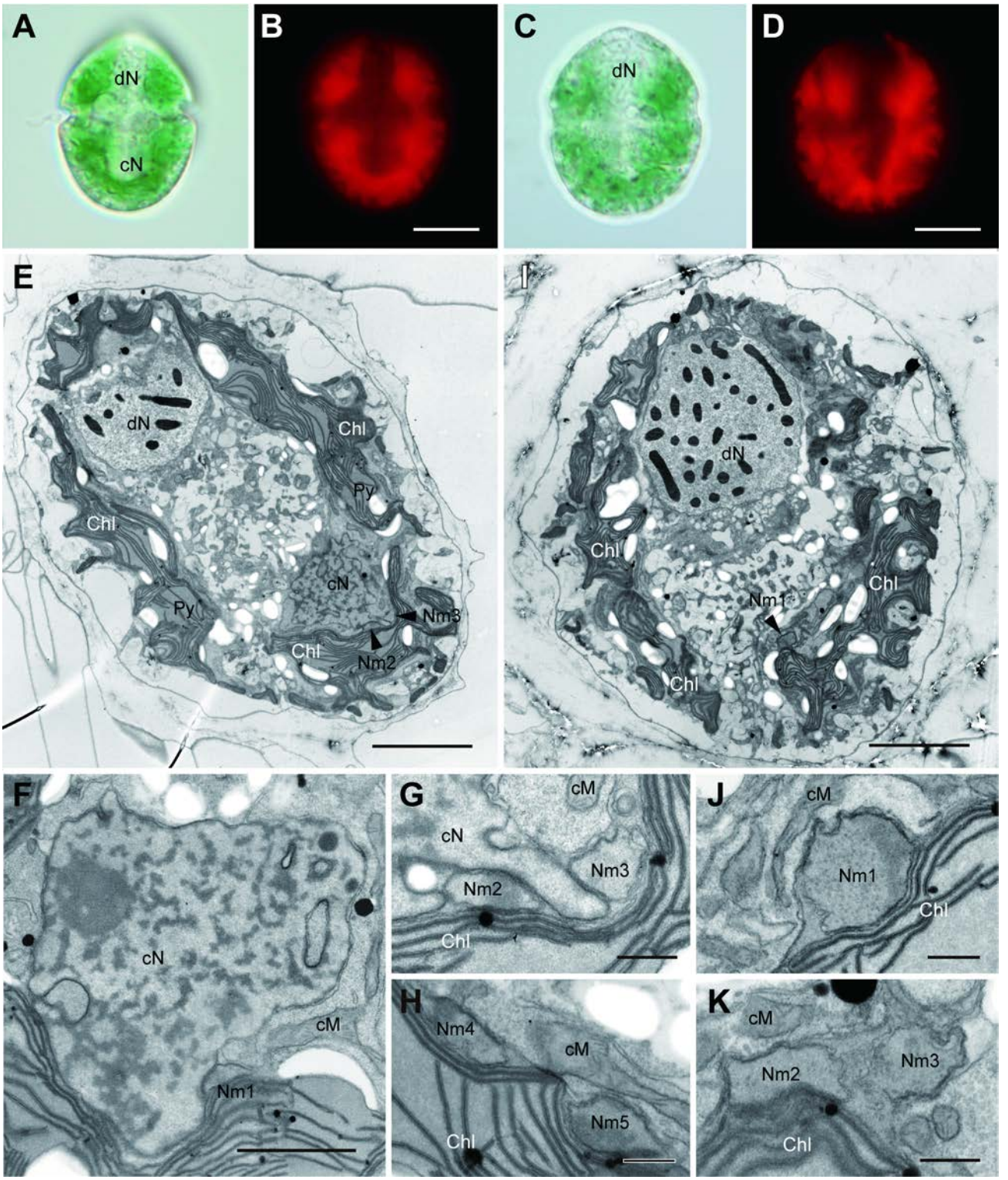
A
dN
cN
B
C
dN
D
E
dN
Chl
Py
Chl
cN
Py
Nm3
Chl
Nm2
I
dN
Chl
Chl
Nm1
Chl
F
cN
cM
Nm1
G
cM
cN
Nm3
Nm2
Chl
J
cM
Nm1
Chl
H
Nm4
cM
Nm5
Chl
K
cM
Nm3
Nm2
Chl

Figure 7.

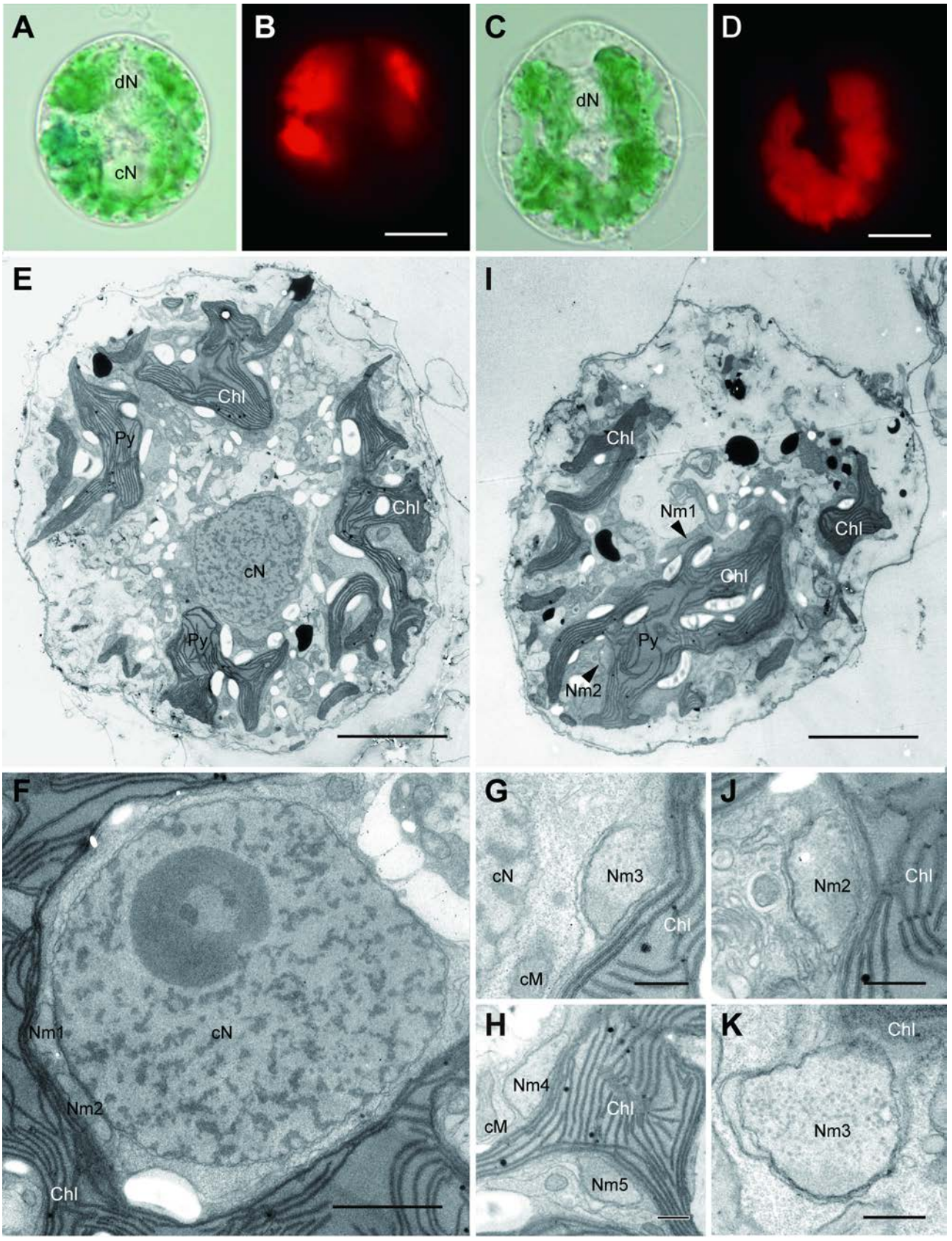
A
dN
cN
B
C
dN
D
E
Chl
Py
Chl
cN
Py
I
Chl
Nm1
Chl
Chl
Py
Nm2
F
Nm1
cN
Nm2
Chl
G
cN
Nm3
Chl
cM
J
Nm2
Chl
H
Nm4
Chl
cM
Nm5
K
Chl
Nm3

Figure 8.

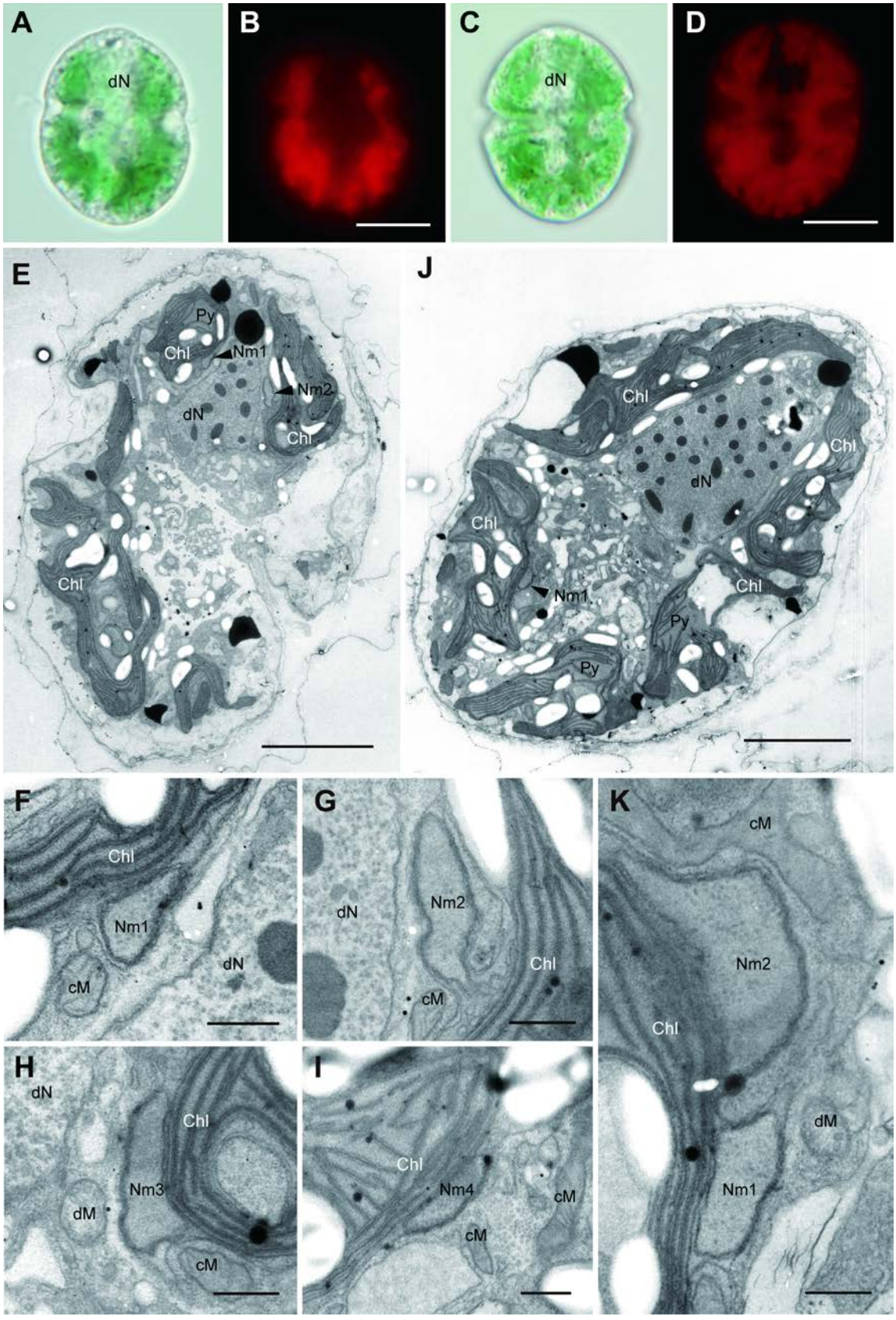
A
dN
B
C
dN
D
E
Py
Chl
Nm1
Nm2
dN
Chl
Chl
J
Chl
Chl
dN
Chl
Nm1
Chl
Py
Py
F
Chl
Nm1
dN
cM
G
dN
Nm2
Chl
cM
K
cM
Nm2
Chl
dM
Nm1
H
dN
Chl
Nm3
dM
cM
I
Chl
Nm4
cM
cM

Figure 9.

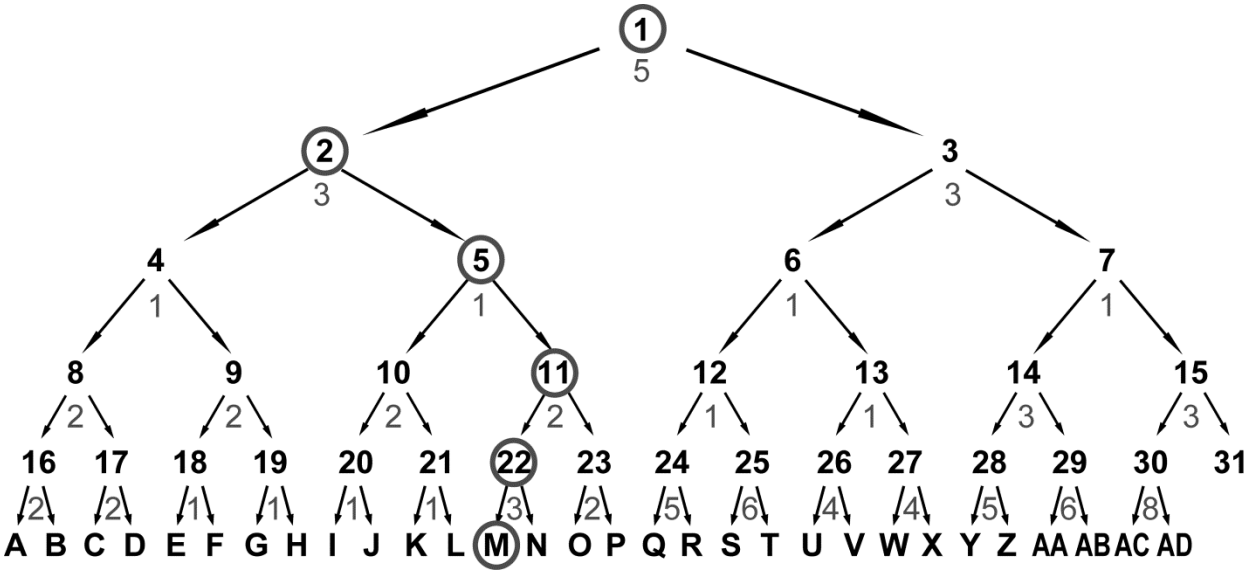
1
5
2
3
3
3
4
1
5
1
6
1
7
1
8
2
9
2
10
2
11
2
12
1
13
1
14
3
15
3
16
2
17
2
18
1
19
1
20
1
21
1
22
3
23
2
24
5
25
6
26
4
27
4
28
5
29
6
30
8
31
A B C D E F G H I J K L M N O P Q R S T U V W X Y Z AA AB AC AD

Figure 10.

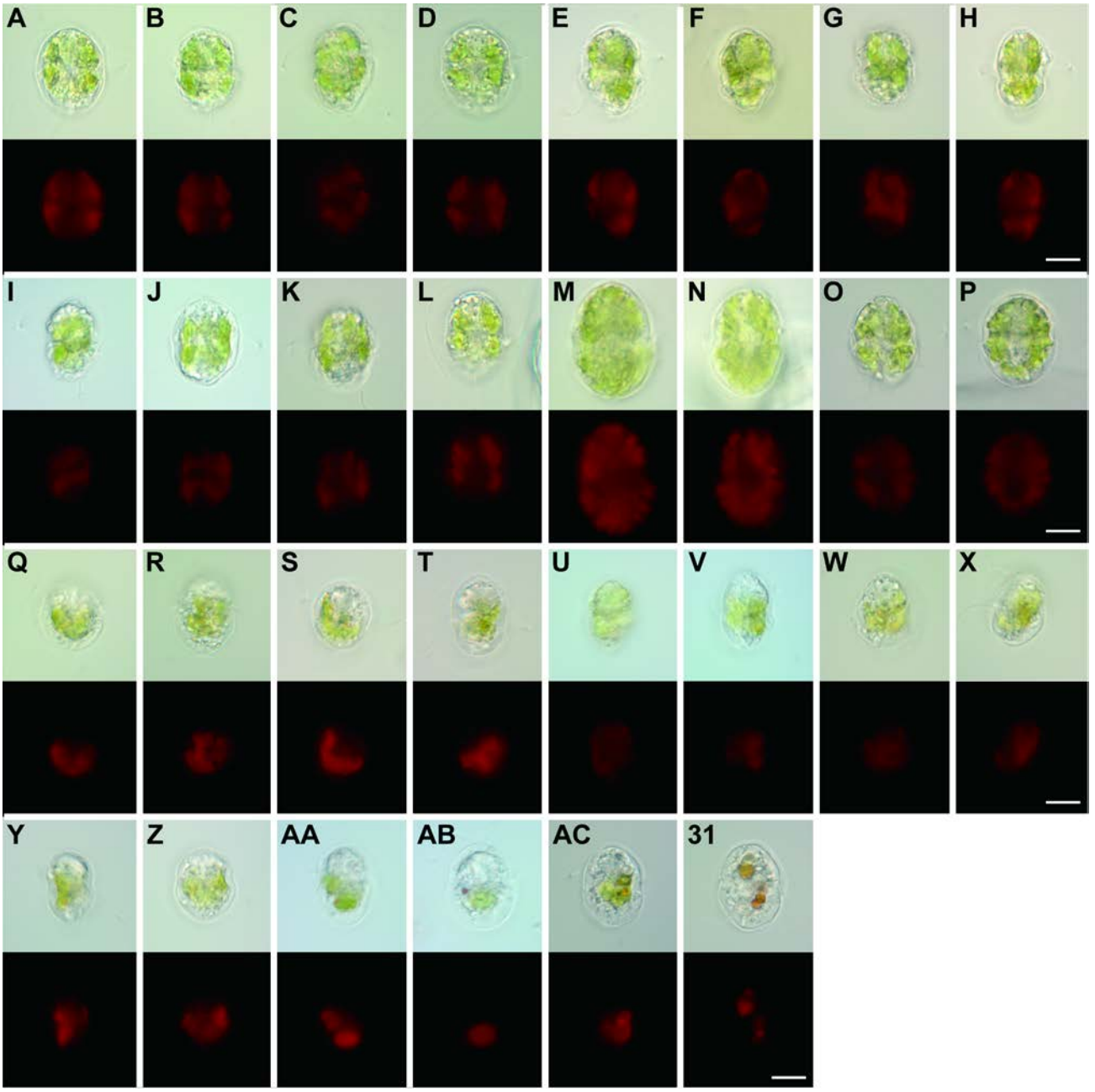

Figure 11.

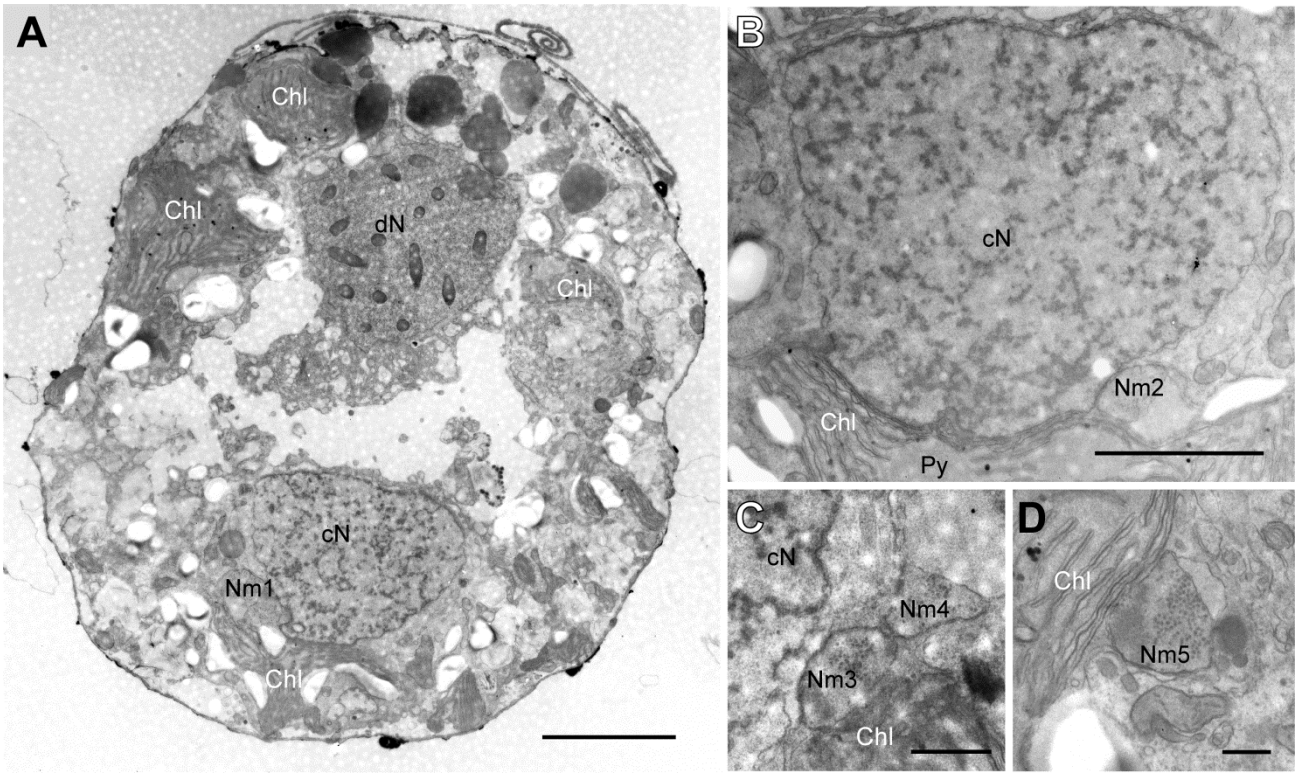
A
Chl
Chl
dN
Chl
cN
Nm1
Chl
B
cN
Nm2
Chl
Py
C
cN
Nm4
Nm3
Chl
D
Chl
Nm5

Table 1.

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

Table 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