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Norrisiella sphaerica gen. et sp. nov., a new coccoid chlorarachniophyte from Baja California, Mexico

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Abstract

A new chlorarachniophyte, *Norrisiella sphaerica* S. Ota et K. Ishida gen. et sp. nov., is described from the coast of Baja California, Mexico. We examined its morphology, ultrastructure and life cycle in detail, using light microscopy, transmission electron microscopy and time-lapse videomicroscopy. We found that this chlorarachniophyte possessed the following characteristics: (i) vegetative cells were coccoid and possessed a cell wall, (ii) a pyrenoid was slightly invaded by plate-like periplastidial compartment from the tip of the pyrenoid, (iii) a nucleomorph was located near the pyrenoid base in the periplastidial compartment, (iv) cells reproduced vegetatively via autospores, and (v) a flagellate stage was present in the life cycle. This combination of characteristics differs from any of the described chlorarachniophyte genera, and therefore a new genus is established. Fluorescent microscopic observations suggested that the alga formed multinucleate cells prior to forming autospores. Time-lapse observations during autospore formation showed that cytokinesis occurred simultaneously in the multinucleate cells. Zoospores were also produced, and video sequences captured the release of zoospores from coccoid cells.

Key words: alga, autospore, Chlorarachniophyceae, life cycle, taxonomy, time-lapse video microscopy.

Introduction

Chlorarachniophytes are amoeboid, coccoid or flagellate marine micro-algae in temperate to tropical coastal regions or open ocean waters. McFadden et al. (1994) showed that the chlorarachniophyte chloroplast originates from a secondary endosymbiosis, based on molecular data such as *in situ* hybridization analysis and northern blot analysis. This evidence is congruent with ultrastructural features, i.e., the chloroplast is surrounded by four membranes, and a vestigial endosymbiont nucleus called the nucleomorph is located in the periplastidial compartment (the space between inner two and outer two chloroplast membranes) (Hibberd and Norris 1984; Ludwig and Gibbs 1987). Recent molecular phylogenetic studies, such as SSU rDNA, alpha-tubulin, beta-tubulin and actin phylogenies, suggest that the chlorarachniophyte host component is related to cercozoans, a group of heterotrophic protists that includes abundant and ecologically significant soil, freshwater and marine amoeboflagellates. (e.g. *Cercomonas*, *Euglypha* and *Heteromita*) (Bhattacharya et al. 1995; Van de Peer et. al. 1996; Keeling et al. 1998; Ishida et al. 1999; Keeling 2001; Cavalier-Smith and Chao 2003; Bass et al. 2005). The cercozoans are recognized as a member of an eukaryotic super-group 'Rhizaria' (Adl et al. 2005) that is a large assemblage of morphologically

and ecologically diverse protists, including the Haplosporidia, Foraminifera and Radiolaria. On the other hand, molecular phylogenetic analyses of chloroplasts and nucleomorphs demonstrated a close relationship to green algae/streptophytes (McFadden et al. 1995; Van de Peer et. al. 1996; Ishida et al. 1997, 1999), suggesting that the origin of the endosymbiont is a green alga or a green algal-like organism.

Ishida et al. (1996) proposed a generic classification system for the phylum Chlorarachniophyta using pyrenoid ultrastructure and nucleomorph location. Three genera, *Chlorarachnion* Geitler (1930) emend. Ishida et Y. Hara (1996), *Lotharella* Ishida et Y. Hara (1996), and *Gymnochlora* Ishida et Y. Hara (1996), were recognized (Ishida et al. 1996). The pyrenoid of *Chlorarachnion* possesses a deep groove created by an invagination of the two inner chloroplast membranes, and the nucleomorph is located in the groove of the pyrenoid (Hibberd and Norris 1984; Ishida et al. 1996). The pyrenoid of *Lotharella* is divided longitudinally into two halves by a thin plate-like invagination of the two inner chloroplast membranes, and the nucleomorph is located near the pyrenoid base (Ishida et al. 1996). The pyrenoid of *Gymnochlora* is invaded by many tublar structures originating from the innermost chloroplast menbrane, and the nucleomorph is located near the pyrenoid base (Ishida et al. 1996). Moestrup and Sengco (2001) established the genus *Bigelowiella* Moestrup for an organism whose

vegetative cells were flagellate. They did not use the generic criteria proposed by Ishida et al. (1996), because (i) *B. natans*, the type species of *Bigelowiella*, was a first chlorarachniophyte flagellate to be discovered (Moestrup and Sengco 2001), and (ii) they thought the pyrenoid ultrastructure of *B. natans* was unstable character (Moestrup, personal communication). Another genus, *Cryptochlora*, was established based on the observation of light microscopic morphology and characterized by the amoeboid cells being solitary (Calderon-Saenz and Schnetter 1987).

Species descriptions are based mainly on life cycle patterns and vegetative morphology (Ishida et al. 1996; Ishida et al. 2000; Dietz et al. 2003; Ota et al. 2005, 2007). There are four described species of *Lotharella*: *L. globosa* (Ishida et Y. Hara) Ishida et Y. Hara (1996), *L. amoebiformis* Ishida et Y. Hara (2000), *L. polymorpha* Dietz, Ehlers, Wilhelm, Gil-Rodríguez et Schnetter (2003) and *L. vacuolata* S. Ota et Ishida (2005). Among these, *L. globosa, L. polymorpha* and *L. vacuolata* are vegetatively coccoid whereas *L. amoebiformis* forms solitary amoeboid cells. There are two described species of *Bigelowiella*, *B. natans* Moestrup (2001) and *B. longifila* S. Ota et Ishida (2007). *B. natans* is a flagellate from the open ocean water of the Sargasso Sea, and *B. longifila* has a dimorphic life cycle with alternating solitary amoeboid and flagellate vegetative stages.

From along the coast of Baja California, Mexico, another coccoid chlorarachniophyte was isolated and established as a clonal culture (BC52). Previously, Ishida et al. (1999) showed that strain BC52 was a sister to *Bigelowiella*, but it formed a distinct clade. In this study, we formally describe strain BC52 based on light and electron microscopic observations. We also report time-lapse video observations to document the life cycle of this alga.

Materials and methods

The sample was collected on May 22, 1992 from the coastal region near La Banqueta, Baja California Sur, Mexico. The samples were sent to Japan for isolation and detailed observation. A unialgal culture (strain BC52) was established using the micropipette method from an enrichment culture containing filaments of an unidentified siphonous green alga. For this study, strain BC52 was grown in 60-mm diameter tissue culture dishes (Asahi Technoglass, Tokyo, Japan) or glass tubes, using ESM (Kasai et al. 2004) or f/2-Si Medium (Andersen et al. 2005). Cultures were maintained at 20˚ C with 12: 12-h light: dark cycle under 80 - 100 μmol photons $m^{-2}{\cdot}s^{-1}$ cool-white illumination.

Cells were observed with Nomarski differential interference contrast optics using Leica DMLB or DMR microscopes (Leica, Wetzlar, Germany). Light micrographs were taken using a DP50 CCD camera (Olympus, Tokyo, Japan) or a VB6010 CCD camera (Keyence, Osaka, Japan). For fluorescence microscopy, cells were fixed with glutaraldehyde (0.3-0.5% final conc.) and stained with 5 μg/ml of 4', 6-diamidino-2-phenylindole (DAPI; Wako Pure Chemical Industries, Osaka, Japan) dissolved in filtered seawater containing a modified S buffer (Miyamura and Hori 1991).

For time-lapse video microscopy, cells were grown for several days on a small, sterile coverslip (e.g. 18 × 18 mm, Matsunami No.1, Osaka, Japan) placed in a culture dish. After incubation, the coverslip was removed and placed on a microscope slide. A large coverslip (e.g. 24 × 24 mm, Matsunami No.1, Osaka, Japan) was placed over the small coverslip. To reduce evaporation, the edges of the large coverslip were sealed with 'VALAP', a 1:1:1 mixture of paraffin wax, lanolin and Vaseline. The cells were examined with Nomarski differential interference contrast optics using the Leica DMLB microscope or a Nikon Optiphot microscope (Nikon, Tokyo, Japan). Video images were taken with a color video camera (model:HC-300Z, FujiFilm, Tokyo, Japan) or a color 3CCD camera (model:QIC-CLR-12, QImaging, British Columbia, Canada).

For thin sections, the fixation and dehydration procedures follow Ota et al. (2005). After dehydration, the cells were soaked in a 1:1 mixture of ethanol and Spurr's resin (Spurr 1969) for 3-9 h at room temperature, then immersed in 100% Spurr's resin at room temperature. The pellet was embedded with the fresh resin and polymerized overnight at 70˚C. Ultrathin sections were cut on a Reichert Ultracut S ultramicrotome (Leica, Wien, Austria) using a diamond knife, mounted on copper mesh or one-slot grids coated with polyvinyl Formvar films, and stained with uranyl acetate and lead citrate (Reynolds 1963). For whole mount preparations, cells were fixed for a few minutes with glutaraldehyde (1% final conc.) in 0.25 M sucrose/ 0.2 M cacodylate buffer (pH 7.2). Then the cells were mounted on copper mesh grids coated with polyvinyl Formvar films, and washed the Milli-Q water. The cells were stained with uranyl acetate for about 30 s. Observations were made with JEM-1210 or JEM-1010 transmission electron microscopes (JEOL, Tokyo, Japan) at 80 kV.

Description

Norrisiella S. Ota et K. Ishida gen. nov.

Cellulae vegetativae, solitariae, pariete. Chloroplastus bilobus, viridis, cum pyrenoide

projecta. Pyrenoidis invasionibus brevi spatio periplasti ex apice pyrenoidis. Nucleomorphus prope basim pyrenoidem. Status proximus zoosporae globosus.

Vegetative cells solitary, with a cell wall. Chloroplast bilobed, green, with a pyrenoid. Pyrenoid slightly invaded by periplastidial compartment at the tip of the pyrenoid. A nucleomorph located near the base of the pyrenoid. The next stage of zoospore is coccoid.

Type species Norrisiella sphaerica S. Ota et K. Ishida.

Etymology The genus name *Norrisiella* is dedicated to Dr. Richard E. Norris who re-discovered *Chlorarachnion reptans* and successfully established a unialgal culture. Hibberd and Norris (1984) established the Phylum Chlorarachniophyta by examining, in detail, the culture of *C. reptans*.

Norrisiella sphaerica S. Ota et K. Ishida sp. nov.

Figs 1-7.

Cellulae globosae nucleo uno (cellulae globosae typicae), pariete, 5-9 μm diam. Cellulae globosae multinucleis (cellulae globosae ante divisionem), pariete, 10-15 μm diam.; propagatione asexuali per divisionem cellularum in pariete materno. Autosporae 2-6 in cellula materna. Zoosporae sunt in orbis vitae; zoosporae pyriformes, ellipticae

vel ovatae, flagello uno. Cellulae globosae nucleo uno,1-2 chloroplastis ad peripheriam.

Single-nuclear coccoid cells (typical coccoid cells) with a cell wall, 5-9 μm in diameter. Multi-nuclear coccoid cells (pre-division coccoid cells) with a cell wall, 10-15 μm in diameter; asexual propagation by means of division of cells within the maternal cell wall. Two to six autospores per mother cell. Zoospore stage present in the life cycle; zoospores pyriform, ellipsoid or ovate, with a single flagellum. Single-nuclear coccoid cell with 1-2 chloroplast(s) at the cell periphery.

Holotype One microscope slide (TNS-AL-56303), deposited in the Department of Botany, the National Museum of Nature and Science, Tokyo (TNS). *Isotype*: One glass vial of EM blocks (TNS-AL-56304) in TNS.

Type locality La Banqueta, Baja California Sur, Mexico.

Distribution Known only from the type locality.

Habitat Costal region, littoral.

Authentic culture Strain BC52. The culture was deposited in the National Institute for Environmental Studies (NIES), Tsukuba, Japan.

Etymology The species epithet *sphaerica*, refers to the shape of vegetative cells.

Results

Light microscopic morphology

Vegetative cells were spherical, 5-9 μm (mean = 7μm, *n* = 30) in diameter, with cell walls (Fig. 1a). Each cell possessed a single nucleus (Fig. 1b) and one or two parietal chloroplast(s) (Fig. 1c). The chloroplasts were green, bilobed, and a single bulbous pyrenoid usually projected toward the center of the cell (Fig. 1c). Prior to cell division, cells reached 10-15 μm in diameter (Fig. 1d) and bore several (two to six) nuclei (Figs. 1e-g; arrowhead in Figs. 1h-j). The nuclei were located in the cell periphery or in the middle region of the cell (Fig. 1g). The cells usually divided into several daughter cells (autospores) within the mother cell walls (Figs. 1h, k). After cytokinesis, the daughter cells formed cell walls within parental cell wall (Figs. 1h, k), and each daughter cell possessed a single nucleus (Figs. 1h-j). The number of daughter cells per mother cell was two to six. Two daughter cells per mother cell were frequently observed (frequency = 43%, *n* = 70); 3-4 daughter cells were sometimes observed (frequencies =18% and 15% respectively, *n* = 70); 5-6 daughter cells were rare (frequencies = 2% and 5% respectively, *n* = 70).

Coccoid cells with reddish particles were present but very rare (Fig. 1l). In old cultures, cysts were formed that possessed a thickened cell wall and often had a granular cytoplasm (Fig. 1m). Naked cells, which were ovate, kidney-shaped or irregular in shape, were occasionally observed (Fig. 1n). No vacuoles were observed in the cells throughout the life cycle.

Zoospores were pyriform, ellipsoid or ovate, 6-15 μm in length and 4-7 μm in width (Figs. 2a, b). Each zoospore cell possessed 1-2 chloroplast(s). The anterior and posterior ends of the zoospore were often rounded (Fig. 2a). The cytoplasm of the anterior part of zoospores was often granular (Figs. 2a, b). A single flagellum, approximately 12 μm in length with a terminal hairpoint, emerged laterally from the mid region of the cell (Fig. 2c). The flagellum coiled helically around the cell body (Fig. 2d), and the cells rotated uniformly along their longitudinal axis during swimming.

Life cycle

The life cycle of *Norrisiella sphaerica* is summarized in Figure 3. It included typical coccoid cells, large multi-nuclear coccoid cells, cysts and zoospores. Vegetative coccoid cells (single-nuclear coccoid cells) were dominant in cultures, but amoeboid cells were never observed (Fig. 3 a-b-c). Zoospores originate from the coccoid cells (Fig.

3 a-d-e) and became coccoid (Fig. 3 e-a) [see supplementary movie (clip1.mov)]. Zoospore formation was observed in both fresh or old cultures (2-3 months old cultures) during light period. The ratio of zoospore/coccoid cells was usually very low, approximately 0.5%. Cysts were observed when the culture was deteriorating (Fig. 3f). The rarely observed naked cells (e.g., Fig. 1n) were probably derived from zoospores after settlement. Sexual reproduction was not observed.

Time-lapse video microscopic observation of autospore and zoospore formation

Normal coccoid cells increased their cell size quickly, within a few hours (Figs. 4a-b). In Figure 4b, c (cells are labeled by arrows), cell diameters were approximately 12 μm and the cells were presumably multi-nuclear. Thereafter, cytokinesis occurred synchronously and several autospores were formed (four and five cells, respectively). The daughter cells were released, presumably, by the rupture of the parental cell wall (Fig. 4f). In the present time-lapse video observation, the entire process took approximately 16 hours. [See supplementary movie (clip2.mov)]

The release of zoospores was also captured using time-lapse video microscopy. Unlike autosporulation, no increase in cell size was observed prior to cell division (Figs. 5a-b). After cytokinesis, the four zoospores were contained within the parental cell wall,

although the parental wall was not clearly seen in video images (Fig. 5b). One of the zoospores ('z_1' in Fig. 5c) was released from the pearental cell about 6 min after the cytokinesis. The release of zoospore z_2 occurred approximately 18 min after the first release (Figs. 5d-e). The third zoospore, z_3, was released about 1.5 h after the release of z_2 (Figs. 5f-h). Before the release of z_3, the two zoospores z_3 and z_4 rotated vigorously [See supplementary movie (clip3.mov)]. The last zoospore, z_4, left in the cell wall at about 50 min after the release of zoospore z_3. The entire process took approximately 5 hours in the present time-lapse video observation.

Transmission electron microscopic observation of ultrastructure

Coccoid cells possessed a thin cell wall (Fig. 6a). The nucleus was located near the cell center and contained an electron opaque droplet in the nucleolus (Fig. 6b). The droplet was mostly located near the rim of the nucleolus (Fig. 6b), or more rarely near the middle of the nucleolus (not shown).

The parietal bilobed chloroplast possessed a projecting pyrenoid (Fig. 6a). The chloroplast was surrounded by four membranes, i.e. an inner two of chloroplast envelope membranes (first and second membranes), a periplastidial membrane (third) and an outermost membrane (fourth) (Figs. 6c, d). The periplastidial compartment, i.e.,

the space between the second and the third membrane, was partially widened especially in the concave side of the chloroplast (Fig. 6a). The chloroplast lamellae consisted of one to three loosely stacked thylakoids (Fig. 6d).

The pyrenoid had an electron opaque matrix. It projected from the concave side of the chloroplast and was capped by a cytoplasmic vesicle (capping vesicle) that contained a slightly electron-opaque substance (Fig. 6a, Fig. 7a). A shallow invagination of the periplastidial compartment was present at the top of the pyrenoid (Figs. 7a, b). In a transverse section of the pyrenoid, the invagination was not observed (Fig. 7c). A nucleomorph was located in the periplastidial compartment near the base of the pyrenoid (Fig. 7a). Several mitochondrial profiles with tubular cristae (Fig. 7d) were always visible in the cytoplasm. Cells containing autospores were sometimes observed (Fig. 7e). There was a large opening in each maternal cell wall, through which the autospores were presumably released. Each autospore possessed its own cell wall (Fig. 7e). Several vesicles containing crystal-like material of unknown composition were often found in the cytoplasm (Fig. 7f). Several Golgi bodies were also seen in the cytoplasm, associated with the endoplasmic reticulum (Fig. 7g).

A few sections of zoospores were also observed, and the general ultrastructure of zoospore was basically identical to the vegetative coccoid cell. Major differences were:

(i) no cell wall was observed in the zoospores and (ii) several vesicles containing electron opaque material were often seen just beneath the plasmalemma of zoospores (Fig. 7h).

Discussion

Taxonomy

Ishida et al. (1996) emphasized pyrenoid ultrastructure and nucleomorph position for generic criteria. The pyrenoid of *Norrisiella sphaerica* has a shallow plate-like invagination of periplastidial compartment into the pyrenoid matrix, and the nucleomorph is located within the periplastidial compartment near the pyrenoid base. These ultrastructural characteristics differ in the three genera *Gymnochlora*, *Lotharella* and *Chlorarachnion*, however, they are identical to those of *Bigelowiella* (information unavailable for *Cryptochlora*) (Table 1). Nevertheless, *Cryptochlora* is clearly different from *N. sphaerica* in several light microscopic features (Table 1): (i) an amoeboid life stage is present, and (ii) amoeboid cells possess filopodia but not plasmodia (reticulopodia) (Calderon-Saenz and Schnetter 1987). Therefore, *N. sphaerica* could not be placed in the genus *Cryptochlora*.

The pyrenoid/nucleomorph ultrastructural characteristics observed in *N. sphaerica* have also been reported in *Bigelowiella* (Gilson and McFadden 1999; Moestrup and Sengco 2001; Ota et al. 2007). *Bigelowiella* is a planktonic alga and it is mainly characterized by the flagellate vegetative cells (Moestrup and Sengco 2001). Because *Norrisiella sphaerica* has coccoid vegetative cells, it could not be assigned to the genus *Bigelowiella*.

Therefore, this new chlorarachniophyte is distinguished from all the known chlorarachniophyte genera (Table 1), it has distinct characteristics (e.g., pyrenoid ultrastructure and habitat), and we believe that it is reasonable to refer it to a new genus. This conclusion is also supported by previous molecular phylogenetic analyses of nuclear and nucleomorph SSU rDNA (Ishida et al. 1999). In the trees, strain BC52 did not locate in any major clades represented by known chlorarachniophyte genera, and it formed a sister relationship with the planktonic chlorarachniophyte clade (= #242/#1408/#1239/#1258 clade), which appears to represent the genus *Bigelowiella* (Gilson and McFadden 1999; Ota et al. 2007). Although *N. sphaerica* takes a sister position to the *Bigelowiella* clade, the branch length between them is relatively deep, suggesting that *N. sphaerica* is genetically fairly different from the *Bigelowiella* species.

Formation of autospores and zoospores

Although most chlorarachniophytes multiply via binary cell division (Ishida et al. 2000; Dietz et al. 2003; Ota et al. 2005; Ota et al. 2007), *Norrisiella sphaerica* proliferates by autosporulation, a process that has been reported only for *Cryptochlora perforans*. In *Cr. perforans*, autospores are formed from ‘large coccoid cells’ (Beutlich and Schnetter 1993). In *Norrisiella sphaerica*, autospore- and zoospore-formation were observed simultaneously in the same culture. In *N. sphaerica*, the major difference between the autospore formation and the zoospore formation is the size of parent cell. During autosporulation, the parent cell size increases before cytokinesis, whereas no parent cell increase occurs prior to zoospore formation. However, it is difficult to distinguish morphologically between the autospore-forming cells and the zoospore-forming cells before cytokinesis.

Zoospores of *N. sphaerica* were observed in both fresh and old cultures, suggesting that nutrient conditions might not be significant for zoospore formation. In contrast zoospore formation in *Cryptochlora perforans* and *L. vacuolata* always occurred when the culture medium culture medium was replenished (Calderon-Saenz and Schnetter 1989; Beutlich and Schnetter 1993; Ota et al. 2005). This is a remarkable difference among the zoospore-forming chlorarachniophytes. In *N. sphaerica*, zoospore

formation seems to be more concerned with the LD cycle than with nutrient conditions.

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Table 1. Comparison of life cycle stages, morphology and ultrastructure among chlorarachniophyte genera

	Chlorarachnion[a]	*Cryptochlora*[b]	*Lotharella*[c]	*Gymnochlora*[d]	*Bigelowiella*[e]	*Norrisiella* gen. nov.[f]
Main vegetative stage	Amoeboid	Coccoid	Amoeboid/ coccoid[a]	Amoeboid	Flagellate/ amoeboid	Coccoid
Flagellate cell (zoospore)	Present	Present	Present	Absent	Present	Present
Next stage of flagellate cell	Amoeboid	Coccoid	Coccoid	-	flagellate	Coccoid
Reticulopodial colony	Present	Absent	Present or absent[g]	Absent	Absent	Absent
Pyrenoid ultrastructure	Nm-containing type[h]	Unknown	Deep slit type[i]	tubular invagination type[j]	Shallow slit type[k]	Shallow slit type[k]
Location of a nucleomorph	In the pyrenoid	Unknown	Near the pyrenoid base	Near the pyrenoid base	Near the pyrenoid base	Near the pyrenoid base
Autospore formation as vegetative reproduction	Absent	Present	Absent	Absent	Absent	Presemt

[a]Hibberd and Norris (1984); [b]Calderon-Saenz and Schnetter (1989), Beutlich and Schnetter (1993); [c]Ishida and Hara (1994), Ishida et al. (2000); Dietz et al. (2003); Ota et al. (2005); [d]Ishida et al. (1996); [e]Moestrup and Sengco (2001), Ota et al. (2007); [f]Present study; [g]*L. globosa* and *L. amoebiformis* do not from the reticulopodial colony; [h]Pyrenoid matrix is invaded deeply by periplastidial compartment, and a nucleomorph is located in the invagination of the pyrenoid; [i]Pyrenoid matrix is divided longitudinally into two halves by an invagination of inner two of four chloroplast membranes; [j]Pyrenoid matrix possesses many tubular invaginations of an innermost chloroplast membrane; [k]Pyrenoid matrix is invaded longitudinally by shallow plate like periplastidial compartment.

FIGURE LEGENDS

Fig. 1. Differential interference contrast (DIC) images and DAPI-stained florescence images of *Norrisiella sphaerica* gen. et sp. nov. **a** Overview of vegetative cells (DIC). **b** Superimposed image, showing DAPI-stained nucleus (arrows). **c** Details of the vegetative cells. Projecting pyrenoids (Py) are visible. **d** Large pre-division coccoid cell (arrow). **e-g** DIC image (e), DAPI stained nuclei (f) and superimposed image (g) of multi-nuclear cell. **h-j** DIC image (h), DAPI-stained nuclei (i), and superimposed image (j) of autospores (arrows) and two-nuclear stage cell (arrowhead). **k** Autospores within maternal cell walls (arrows) (DIC). **l** Coccoid cell containing a red droplet (arrow) (DIC). **m** Cyst with thick cell wall (DIC). **n** Kidney-shaped naked cell (DIC). *C* chloroplast.

Fig. 2. Differential interference images (a, b and d) and a whole-mount image (c) of zoospores in *Norrisiella sphaerica* gen. et sp. nov. **a** Pyriform zoospore. Granular appearance is visible (arrow). **b** Ellipsoid zoospore. Granular appearance is visible (arrow). **c** Whole-mount zoospore showing an emergent flagellum with a terminal hairpoint (arrow). **d** Ellipsoid zoospore with a flagellum (arrows). *C* chloroplast.

Fig. 3. Life cycle of *Norrisiella sphaerica* gen. et sp. nov. **a** Coccoid cell. **b** Large coccoid cell in whose the cytoplasm has divided. **c** Autospores. **d** Coccoid cells containing zoospores. **e** Zoospores. **f** Cyst.

Fig. 4. Time-lapse video sequence of autospore formation in *Norrisiella sphaerica* gen. et sp. nov. **a** Two coccoid cells (arrows) just prior to cytokinesis. **b, c** The two cells enter cytokinesis (arrows). **d, e** Four and five autospores are formed, respectively. **f** Release of autospores. [See supplementary movie (clip2.mov)]

Fig. 5. Time-lapse video sequence of the zoospore formation in *Norrisiella sphaerica* gen. et sp. nov. **a** Coccoid cell just prior to cytokinesis. **b** Cytokinesis has been completed and four daughter cells (z_1-z_4) are visible. **c** Release of zoospore z_1. **d** Release of zoospore z_2. **e-g** Daughter cells rotating around each other. **g, h** Zoospore z_3 swims away. **h** Zoospore z_4 is released. [See supplementary movie (clip3.mov)]

Fig. 6. Transmission electron micrographs of *Norrisiella sphaerica* gen. et sp. nov. **a** General ultrastructure of vegetative cell. The boxed area is enlarged in Fig. 8c. **b**.

Nucleus with electron-opaque droplet (arrow). **c** Higher magnification of chloroplast membranes showing two outer membranes (arrowheads) and two inner membranes (arrows). A periplastidal compartment is visible. **d** Transverse section of the chloroplast showing lamellae with 1-3 thylakoids. The outer two (arrowhead) and the inner two (arrow) chloroplast membranes are visible. *C* chloroplast, *Cv* capping vesicle, *M* mitochondrion, *N* nucleus, *Py* pyrenoid, * periplastidial compartment.

Fig. 7. Transmission electron micrographs of *Norrisiella sphaerica* gen. et sp. nov. **a** Longitudinal section of the pyrenoid, showing a shallow invagination (arrows) of the two inner membranes in the pyrenoid matrix from the top of the pyrenoid. Nucleomorph is located near the pyrenoid base in the periplastidial compartment. **b** Transverse section of the tip of the pyrenoid, showing the slit-like invagination in the pyrenoid (arrows). **c** Transverse section of the middle region of the pyrenoid, showing the lack of invagination of the periplastidial compartment. **d** Mitochondria with tubular cristae. **e** Releasing autospores showing mother cell wall (arrows) and daughter cell walls (arrowheads). **f** Vesicle containing crystal-like materials (arrows). **g** Golgi body and endoplasmic reticulum (arrow). **h** Longitudinal section of zoospore, showing general ultrastructure and vesicles containing electron opaque-material just beneath the

plasmalemma (arrows). *C* chloroplast, *Cv* capping vesicle, *G* Golgi body, *M* mitochondrion, *N* nucleus, *Nm* nucleomorph, *Py* pyrenoid.

LEGENDS FOR SUPPLEMENTARY ELECTRONIC MATERIALS

clip1.mov

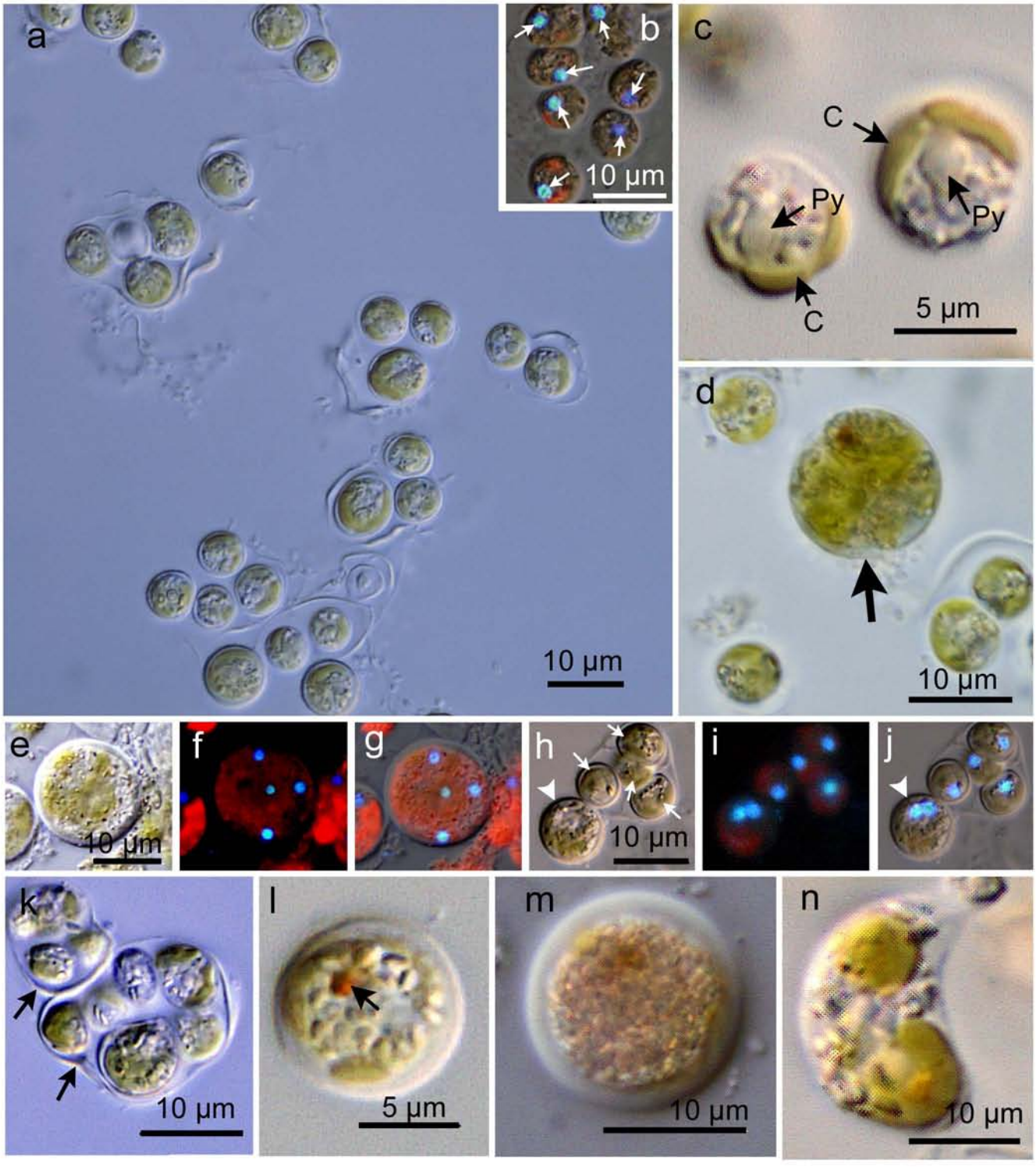
QuickTime Movie, settlement of zoospores. (2.27 MB)

clip2.mov

QuickTime Movie, autospore formation. (9.05 MB)

clip3.mov

QuickTime Movie, zoospore formation. (8.71 MB)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b
10 µm
c
C
Py
Py
C
5 µm
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10 µm
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e
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Fig. 1

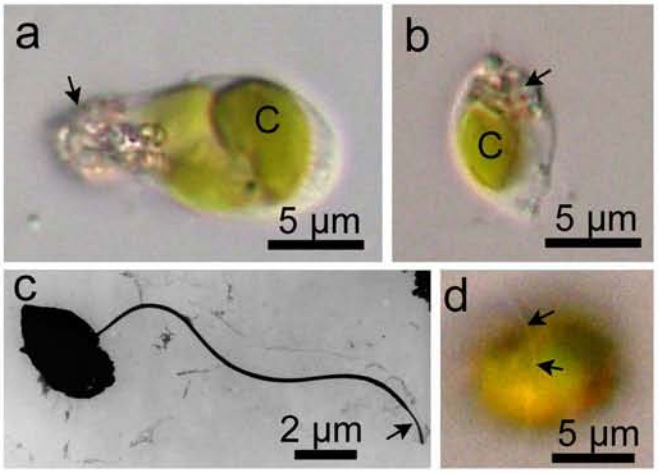
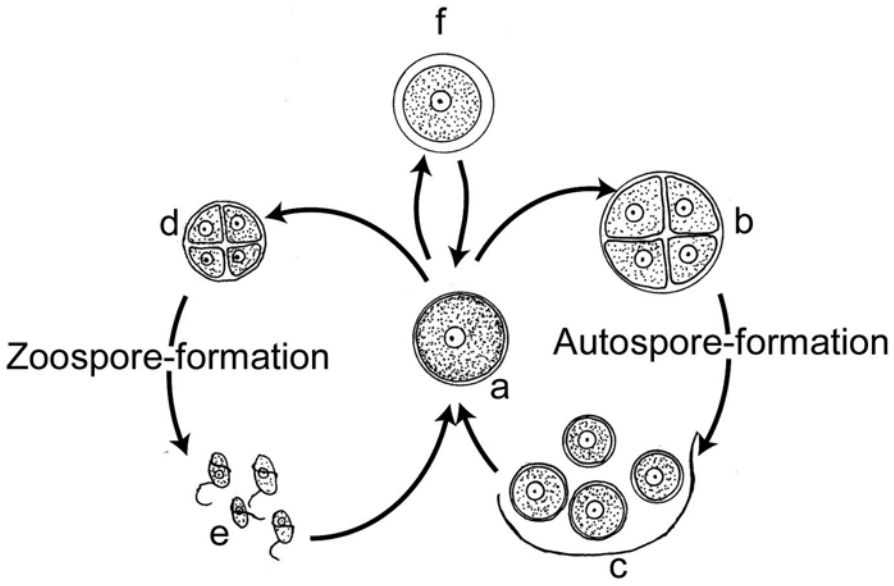


Fig. 2



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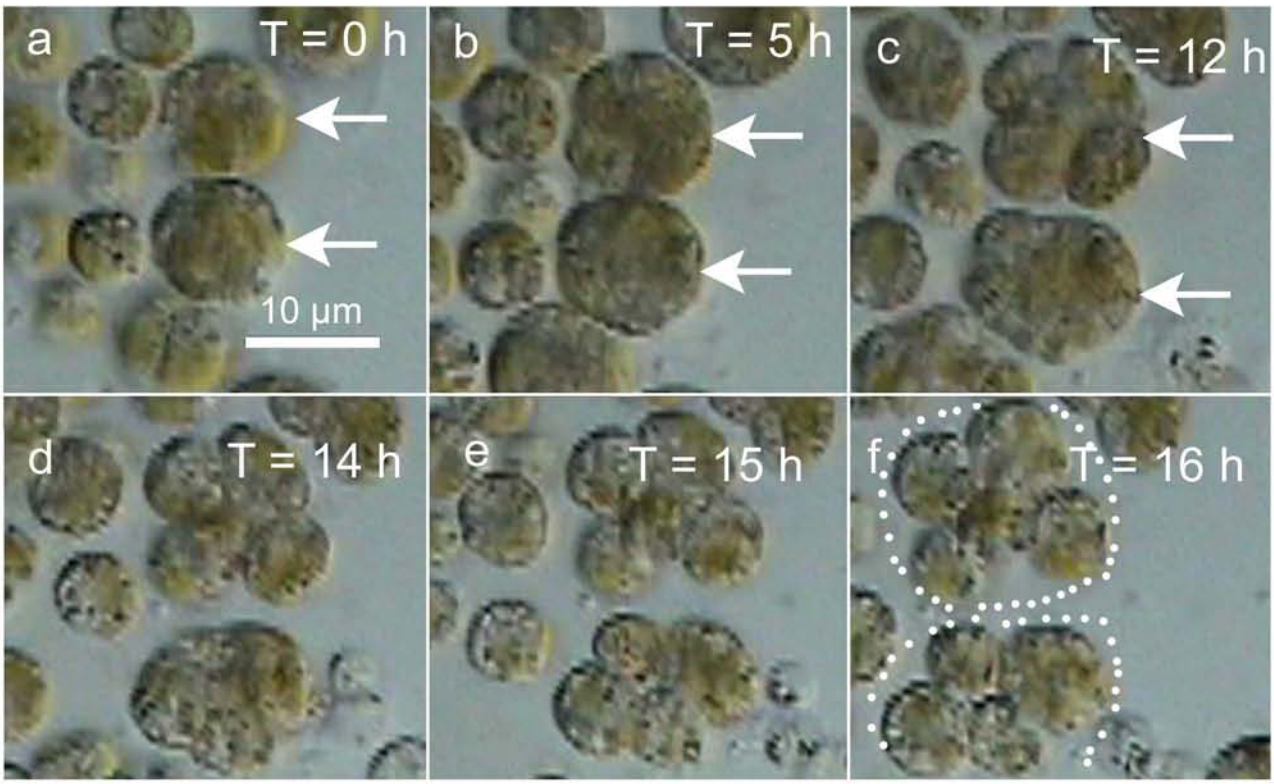
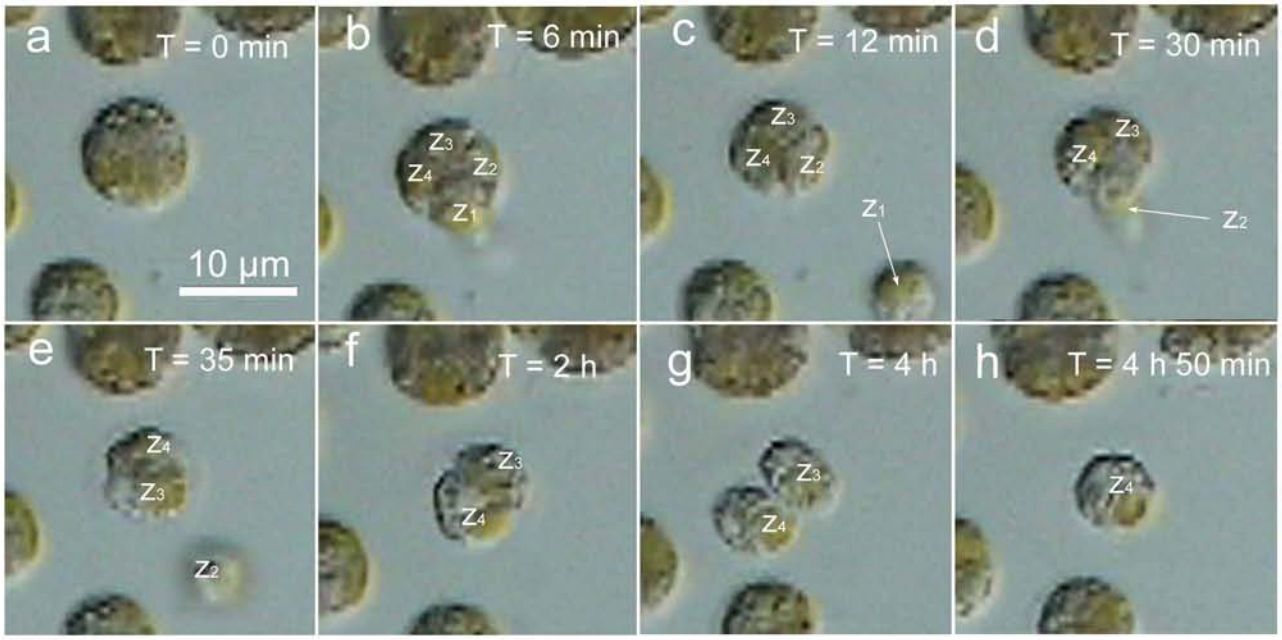


Fig. 4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10 µm
b
T = 6 min
Z3
Z4
Z2
Z1
c
T = 12 min
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Z4
Z2
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d
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Z3
Z4
Z2
e
T = 35 min
Z4
Z3
Z2
f
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Z3
Z4
g
T = 4 h
Z3
Z4
h
T = 4 h 50 min
Z4

Fig. 5

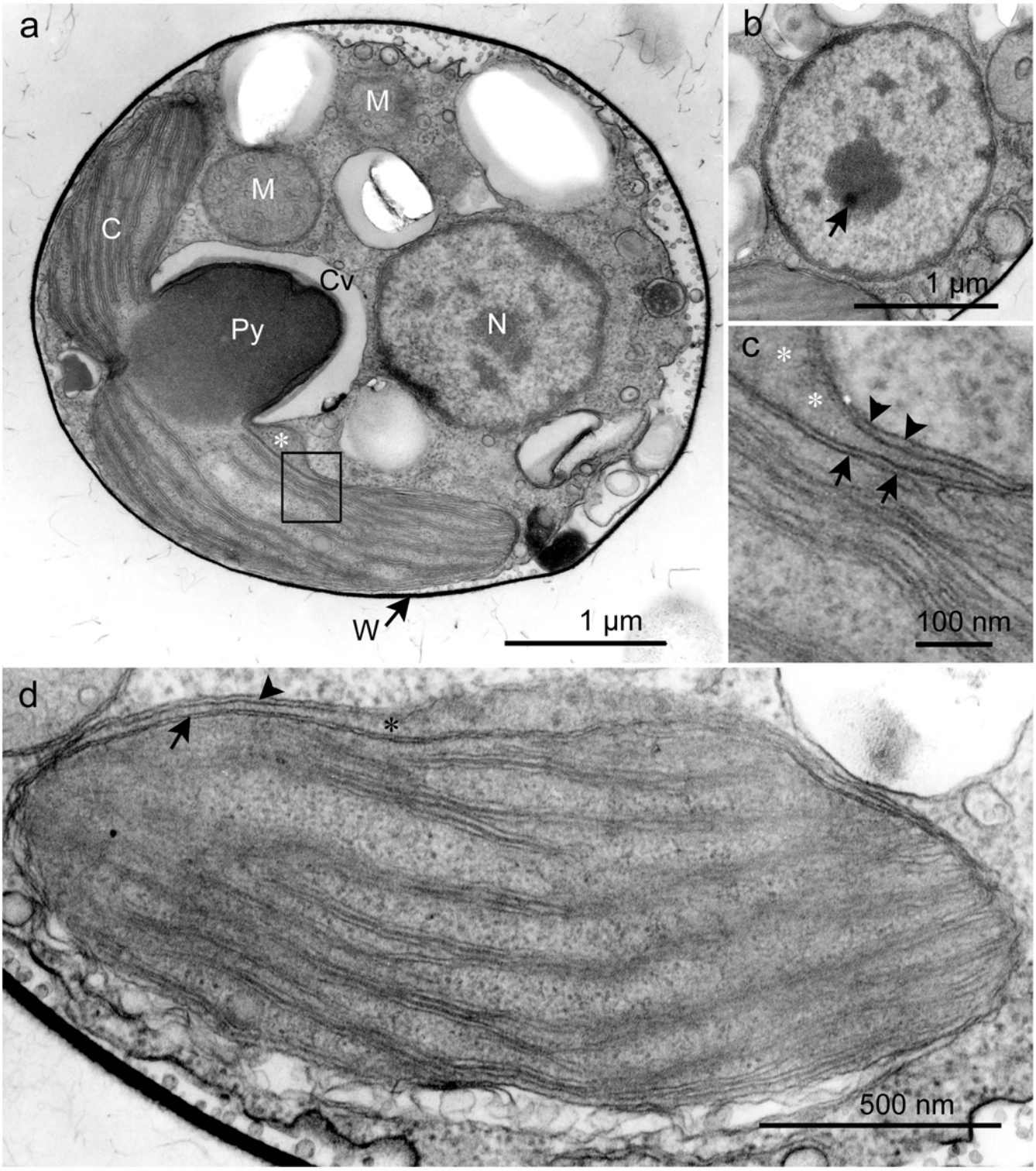

Fig. 6

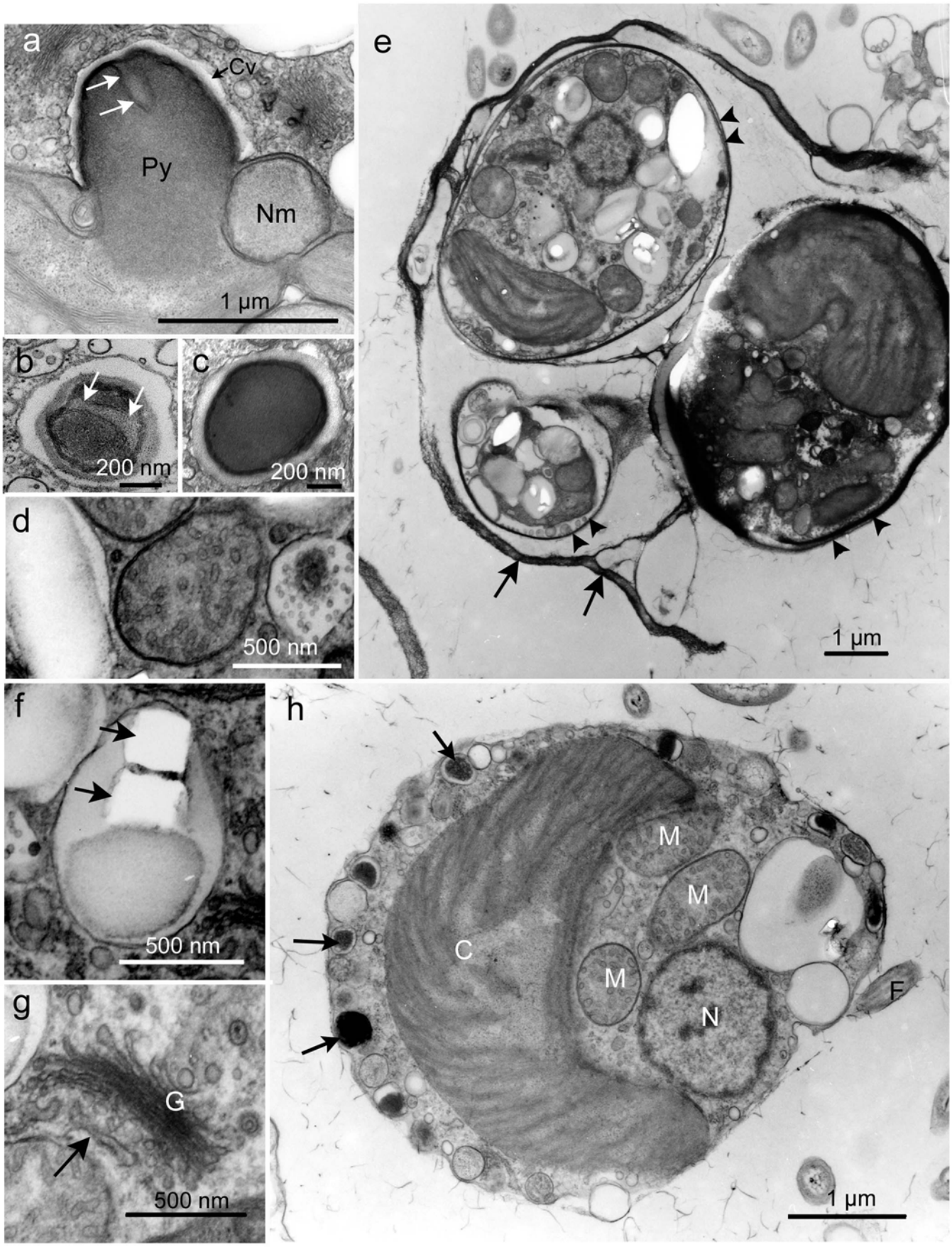
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Fig. 7