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***Viridiuvalis adhaerens* gen. et sp. nov., a novel colony-forming chlorarachniophyte**

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Abstract: A new chlorarachniophyte, *Viridiuvalis adhaerens* gen. et sp. nov. was isolated from the mucus on a coral reef from Zanpa Beach, Okinawa, Japan. The main vegetative stage of *V. adhaerens* consisted of unicellular coccoid cells with cell walls, although sarcinoid colonies and uniflagellate zoospores were also observed. *V. adhaerens* had chloroplasts with nucleomorphs and pyrenoids that were completely embedded in the chloroplast. A deep plate-like invagination of the periplastidal compartment (PPC) almost partitioned the pyrenoid and chloroplast components, which were surrounded by two membranes. The nucleomorph was positioned in the base of the invagination of the PPC. Molecular phylogenetic analyses using rRNA genes showed that *V. adhaerens* branched as a sister lineage of the *Amorphochlora* clade. The sarcinoid colony, pyrenoid embedded in the chloroplast, and nucleomorph located at the base of the deep invagination of the PPC have not been reported in other chlorarachniophytes. Based on these morphological and ultrastructural characteristics and the results of the molecular phylogenetic analyses, we propose *V. adhaerens* as a new genus and species of chlorarachniophyte.

Key words: Chlorarachniophyceae, Chlorarachniophycota, phylogeny, taxonomy, *Viridiuvalis adhaerens,* ultrastructure

Introduction

Archaeplastida (land plants, green algae, red algae, and glaucophytes) are occurred by single endosymbiosis (primary endosymbiosis) between its eukaryotic ancestor and a cyanobacterium that probably close to *Gloeomargarita lithophora* (Ponce-Toledo et al. 2017). Multiple secondary endosymbiosis subsequently occurred between heterotrophic protists in various eukaryotic lineages and red or green algae, which gave raise to diverse secondary algae (e.g. euglenids, haptophytes, and ochrophytes) (Keeling 2010). Chlorarachniophytes are the one of the secondary algae occurred by secondary endosymbiosis between a heterotrophic cercozoan and a green alga (Ishida et al. 1997; McFadden et al. 1995; Van de Peer et al. 1996). Recent phylogenetic study suggested that the green algal symbiont of chlorarachniophytes is closely related to Bryopsidales (Suzuki et al. 2016). The chlorarachniophyte chloroplast is surrounded by four membranes. The two outermost membranes are derived from host phagosome and green algal cytoplasmic membrane respectively, whereas two innermost membranes are derived from green algal chloroplast membrane (Cavalier-Smith 2000). The chlorarachniophyte chloroplast contains a remnant of green algal nucleus called nucleomorph in periplastidal compartment (PPC), a space between outer two and inner two chloroplast membrane (Ishida et al. 2007).

Geitler (1930) discovered the first chlorarachniophyte in the Canary Islands and described it as *Chlorarachnion reptans* Geitler. *C. reptans* was originally classified in the Xanthophyceae (Heterokontophyta) based on its amoeboid cell morphology, green chloroplasts, and lack of starch (Geitler 1930). A half century after the original description, Hibberd and Norris (1984) re-examined *C. reptans* by light and transmission electron microscopy and pigment analysis, and revealed its unique characteristics, namely, uniflagellate zoospores and chloroplasts surrounded by four membranes and containing a nucleomorph in the PPC, a projecting pyrenoid, and both chlorophyll *a* and *b*. Based on these characteristics, Hibberd and Norris (1984) established the division Chlorarachniophycota (= Chlorarachniophyta) and class Chlorarachniophyceae.

Chlorarachniophytes have been reported from various temperate to tropical coastal environments and from the surface water of open oceans, and eight genera and 13 species have been described so far (Calderon-Saenz and Schnetter 1987; Dietz et al. 2003; Hibberd and Norris 1984; Ishida and Hara 1994; Ishida et al. 1996, 2000; Moestrup and Sengco 2001; Ota et al. 2005, 2007a, 2007b, 2009a, 2009b, 2011; Ota and Vaulot 2012). Each genus and species of chlorarachniophytes has been well characterized by morphology and ultrastructure. Most chlorarachniophytes can dynamically change their morphology among amoeboid, coccoid, and flagellate cells (Ishida et al. 2007), and the lifecycle patterns of these three morphologies are one of the main traits used for the generic and specific classification of chlorarachniophytes (Ishida et al. 2007; Moestrup and Sengco 2001; Ota et al. 2007b). The ultrastructure of the pyrenoid and the position of the nucleomorph are also important for generic classification (Ishida et al. 1996, 2007). Except for *Partenskyella glossopodia* Ota, Vaulot & Ishida, which has no pyrenoid (Ota

et al. 2009b), chlorarachniophytes have a projecting pyrenoid with an invagination of the chloroplast membranes that occurs in several forms: a deep slit-like invagination of the PPC (*Amorphochlora*, *Lotharella*) (Dietz et al. 2003; Ishida and Hara 1994; Ishida et al. 2000; Ota et al. 2005, 2009a; Ota and Vaulot 2012); a deep slit-like invagination of the PPC that is filled with the nucleomorph (*Chlorarachnion*) (Hibberd and Norris 1984); a shallow slit-like invagination of the PPC (*Bigelowiella*, *Norrisiera*) (Moestrup and Sengco 2001; Ota et al. 2007a, 2007b); or a tubular invagination of the innermost chloroplast membrane (*Gymnochlora*) (Ishida et al. 1996; Ota et al. 2011).

Despite their high diversity in morphology, lifecycle, and ultrastructure, the chlorarachniophytes are a relatively small algal group (Ishida et al. 2007). On the other hand, about 50 unnamed strains including undescribed species are deposited in culture collections around the world (Gile et al. 2010). Moreover, several non-public strains show phylogenetic positions that are distinct from the described species (Ota et al. 2009b; Ota and Vaulot 2011). Environmental DNA surveys also suggest the existence of many undescribed species of chlorarachniophytes (Ota and Vaulot 2011). These studies suggest that chlorarachniophytes potentially have considerable hidden diversity, and it is important to perform taxonomic studies on chlorarachniophytes to understand their diversity.

In this study, we report a new chlorarachniophyte isolated from Okinawa, Japan in 2010. We performed light and electron microscopic observations and molecular phylogenetic analyses using rRNA genes on the new chlorarachniophyte, which revealed its unique morphological and ultrastructural characteristics including first-reported sarcinoid colony and pyrenoid that embedded in chloroplast in chlorarachniophytes. . Based on these results we described it as a new genus and species of chlorarachniophyte and discussed function and evolution of its unique morphologies and ultrastructural characteristics.

Materials and Methods

Sample collection and culture establishment

We collected the mucus covering a coral at Zanpa Beach, Okinawa, Japan (26.4355 N, 127.7152 E) on November 25, 2010. The mucus was suspended in ESM medium (Kasai et al. 2009) and incubated at 20 °C under a 14-h/10-h light/dark cycle for initial cultivation. A clonal culture of *Viridiuvalis adhaerens* (strain SRT040) was established from a cell in the incubated sample by the micropipetting isolation method. Strain SRT040 was maintained in ESM medium at 20 °C under a 14-h/10-h light/dark cycle. The SRT040 strain was deposited at the National Institute for the Environmental Sciences, Tsukuba, as NIES-4109.

Light microscopy

Living cells of the strain SRT040 were observed with Nomarski differential interference contrast (DIC) optics using either a Zeiss Axio imager A2 microscope (Zeiss, Oberkochen, Germany) equipped with an

Olympus DP71 CCD camera (Olympus, Tokyo, Japan), or a Nikon Optiphot microscope (Nikon, Tokyo, Japan) equipped with a Keyence VB6010 CCD camera (Keyence, Osaka, Japan). For time-lapse micrographs, cells were placed in a glass-bottomed dish and observed using an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP73 CCD camera (Olympus, Tokyo, Japan).

Transmission electron microscopy

For transmission electron microscopy, vegetative cells of the strain SRT040 were collected by centrifugation. The pelleted cells were fixed with glutaraldehyde and osmium tetroxide, and dehydrated as described by Ota et al. (2009a). For observation of zoospores, supernatant of the culture medium was fixed with an equivalent amount of a mixture of 0.16 M sodium cacodylate buffer, 4.5 % glutaraldehyde, and 0.23 M sucrose overnight, and then the zoospores were collected by centrifugation. The pelleted zoospores were then washed with sodium cacodylate buffer, postfixed in osmium tetroxide, and dehydrated as described by Ota et al. (2009a). Dehydrated cell pellets were embedded in Agar Low Viscosity Resin R1078 (Agar Scientific, Stansted, England). The resin was polymerized by heating at 60 °C for 12 h. For holotype specimen, resin-embedded vegetative cells were mounted on glass slide and polymerized at the same condition. Ultrathin sections were prepared on a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria), double stained with 2 % (w/v) uranyl acetate and lead citrate (Hanaichi et al. 1986; Sato 1968), and observed using a Hitachi H-7650 electron microscope (Hitachi High-Technologies Corp., Tokyo, Japan) equipped with a Veleta TEM CCD camera (Olympus, Tokyo, Japan).

DNA extraction and polymerase chain reaction (PCR)

Cells of the strain SRT040 were centrifuged and total DNA was extracted from the pellet using a DNeasy Plant Mini Kit (Qiagen Science, Valencia, CA) according to the manufacturer's instructions. SSU and LSU rRNA genes of the strain SRT040 were amplified by polymerase chain reaction (PCR) with 18F-18R and L1-L12 primers, respectively (Yabuki et al. 2010). Amplifications consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 min, and extension at 72 °C for 2 or 3 min. An additional extension for 4 min at 72 °C was performed at the end of the reaction. Amplified DNA fragments were purified after gel electrophoresis with a QIAquick Gel Extraction Kit (Qiagen Science, Valencia, CA) and then cloned into the p-GEM® T-easy Vector (Promega, Tokyo, Japan). The inserted DNA fragments were completely sequenced with a 3130 Genetic Analyzer (Applied Biosystems, Monza, Italy). The sequences of the SSU and LSU rRNA genes of the strain SRT040 were deposited as LC229571 and LC229572 in DDBJ database respectively.

Sequence alignments and phylogenetic analysis

We created new sequence datasets for molecular phylogenetic analyses of the SSU rRNA gene and the concatenated SSU and LSU rRNA genes. The sequences of the SSU and LSU rRNA genes of the strain SRT040 were added to each dataset. The sequences in the datasets were automatically aligned with MAFFT (Katoh and Standley 2013) and then edited manually with SeaView (Gouy et al. 2010). Ambiguously aligned regions were manually deleted from each alignment. The final alignment of SSU rRNA gene sequences comprised 67 taxa and 1618 positions, and the concatenated SSU and LSU rRNA alignment comprised 17 taxa and 4685 positions. The alignment files used in the analysis are available at TreeBASE (accession No. TB2:S21110 and TB2:S21112).

The maximum likelihood (ML) trees of the SSU rRNA genes and concatenated SSU and LSU rRNA genes were heuristically searched using RAxML v. 8.1.15 (Stamatakis 2014) under the GTR+Γ model. Tree searches started with 20 randomized maximum-parsimony trees, and the highest log likelihood (lnL) was selected as the ML tree. A non-parametric bootstrap analysis with 1000 replicates was conducted under the GTR+Γ model. Bayesian analyses were run using MrBayes v. 3.2.2 (Ronquist et al. 2012) with the GTR+Γ model for both SSU rDNA and concatenated SSU and LSU rDNA alignments. Two simultaneous MCMCMC, each consisting of one cold chain and three heated chains, were run for 1.5×10^5 and 1×10^5 generations, respectively. The lnL values and trees were sampled at 100-generation intervals. Convergence was assessed by the average standard deviation of split frequencies (ASDSF), and the first 5×10^4 (SSU rDNA) and 1×10^4 (concatenated SSU and LSU rDNA) generations for which ASDSF values were above 0.01 were discarded as "burn-in." Bayesian posterior probabilities and branch lengths were calculated from the remaining trees.

Results

Light microscopy

Morphology

Coccoid cells, sarcinoid colonies, and zoospores were observed in the culture of *Viridiuvalis adhaerens* gen. et sp. nov. (Fig. 1). The unicellular coccoid cells were spherical, surrounded by a cell wall, and 4–15 μm in diameter (Fig. 1a–d). The coccoid cells were immotile and were tightly attached on the bottom of the culture flask. The cells had a nucleus and highly reticulated greenish chloroplast (Fig. 1a–e). The number of chloroplast in coccoid cells was not clear because of its reticulated profile. One or two pyrenoids were observed in the chloroplast (Fig. 1c). Many colorless oval granules were observed in the cells (Fig. 1a–d). The granules almost filled the cytoplasm in larger coccoid cells (Fig. 1d). Cells occasionally had reddish particles (Fig. 1d, e). The number and size of the reddish particles in the cells increased as the cultures aged (Fig. 1e). The thickness of the cell wall also increased as the cultures aged (Fig. 1e). Empty cell walls with several pores, which were probably empty zoosporangia (see below), were observed on the bottom of the culture flask (Fig. 1f).

Sarcinoid colonies composed of two to many cells were occasionally observed (Fig. 1g–k). The

cells in the sarcinoid colonies were hemispherical to angular in shape and closely attached to each other (Fig. 1g, h). The sarcinoid colonies were also tightly attached to the substrate as the coccoid cells were. In aged cultures, the cell wall of each cell thickened and the number and size of the reddish particles increased as in the unicellular coccoid cells (Fig. 1i). The number of cells constituting a sarcinoid colony also increased in aged cultures. The sarcinoid colonies occasionally formed compound structures consisting of several sarcinoid colonial units (Fig. 1j, k).

Oval uniflagellated zoospores were observed in both fresh and aged cultures (Fig. 1l–o). Zoospores were 4–8 μm in length and had a chloroplast, colorless granules, and a subapically inserted flagellum extending posteriorly (Fig. 1m–o). Zoospores lacked reddish particles (Fig. 1m–o). Cells slowly swam in the culture medium with rotating around the longitudinal axis. In apical view, the flagellum wrapped counterclockwise around the cell during the swimming movement (Fig. 1m). The zoospores sometimes showed a slow gliding movement on the substrate and the flagellum trailed behind the cell (Fig. 1n, o). The gliding zoospores occasionally extended lobose pseudopodia (Fig. 1o). Cell division was not observed in the zoospores.

Lifecycle

The dominant form of the vegetative stage of *V. adhaerens* was the unicellular coccoid cell. However, sarcinoid colonies were occasionally more frequent than coccoid cells in aged cultures. The release of zoospores was observed in both coccoid cells (Fig. 2, 3) and sarcinoid colonies. Multiple zoospores were released from both stages, whereas the release of a single zoospore from a coccoid cell was also observed (Fig. 3). Large sarcinoid colonies were occasionally divided into several clusters (Fig. 4). Swimming zoospores occasionally attached to the substrate and showed slow gliding movement. Gliding zoospores also occasionally detached from the substrate and reverted to swimming cells. Zoospores became rounded off and lost the flagellum, and then changed to coccoid cells (Fig. 5). The lifecycle of *V. adhaerens* as predicted from this study is illustrated in Figure 6.

Transmission electron microscopy

The coccoid cells of *V. adhaerens* had a nucleus with a conspicuous nucleolus, and an electron-dense globule was located near the nucleolus (Fig. 7a, b). Mitochondrial profiles with tubular cristae and vesicles containing storage-product-like material were scattered in the cell (Fig. 7a, c, d). Several Golgi apparatuses were observed around the nucleus (Fig. 7e). Chloroplast profiles that surrounded by four membranes were arranged in the peripheral region of the cell (Fig. 7a d, 8). The chloroplast profiles had lamellae composed of one to three loosely stacked thylakoids and plastoglobuli (Fig. 7e–g). One or two pyrenoids were observed in chloroplast profiles (Fig. 1a, d) Pyrenoids were completely embedded in chloroplast profiles and did not project outside of them (Fig. 7a, e, f, 8). A plate-like deep invagination of the periplastidal compartment (PPC) was observed in the chloroplast profiles (Fig. 7a, e, f, 8). The

pyrenoid with the surrounding chloroplast profile was almost partitioned by the invagination (Fig. 7a, e, f, 8). A nucleomorph was located in the PPC near the base of the plate-like invagination (Fig. 7a, f, g, 8). The nucleomorph had a double-membrane envelope and dense globules located in its peripheral region (Fig. 7f, g). Vesicles containing electron-dense materials were observed just beneath the plasma membrane (Fig. 9a). The cytoplasm occasionally contained electron-dense globules that were probably the reddish particles observed under light microscopy (Fig. 8d–h, 9d). The cells were covered by laminate fibrous cell walls (Fig. 7a, 9). The thickness of the cell walls was not uniform (Fig. 7a, 9c, d). The ultrastructure of the cells forming sarcinoid colonies was generally consistent with that of the unicellular coccoid cells (Fig. 9b–d). In the sarcinoid colonies, however, the cell walls of mother and daughter cells were tightly adhered (Fig. 9b–d).

Zoospores lacked cell walls and were covered only by a plasma membrane (Fig. 10). The zoospore had a chloroplast, nucleus, Golgi apparatus, vesicles containing storage-product-like material, and mitochondrial profiles (Fig. 10). In addition to the vesicles containing dense materials, other vesicles containing eyeball-like materials that consist of fibrillar less dense peripheral region and electron-dense central part were located in the peripheral region of the zoospore (Fig. 10). Large vacuoles containing fibrous materials were also occasionally observed (Fig. 10).

Molecular phylogenetic analyses

The molecular phylogenetic tree based on the SSU rRNA genes recovered the monophyly of the chlorarachniophytes and of each genus in the group (Fig. 11). *Viridiuvalis adhaerens* branched as the sister lineage of the *Amorphochlora* clade that comprises *A*. *amoebiformis* (Ishida & Hara) Ishida, Yabuki & Ota and six unnamed strains with strong statistical support (bootstrap percentage: BP = 100 %; Bayesian posterior probability: BPP = 1; Fig. 11). The *V. adhaerens–Amorphochlora* clade formed a clade with the *Gymnochlora* clade with moderate statistical support (BP = 71 %, BPP = 1.00; Fig. 11). The *Bigelowiella* clade and *Norrisiella* clade also showed strong monophyly (BP = 100 %, BPP = 1; Fig. 11), although the phylogenetic relationships among the other clades were not resolved.

The phylogenetic tree using the concatenated SSU and LSU rRNA genes showed somewhat different topology from SSU rRNA gene tree and each branches in chlorarachniophytes were mostly well supported (Fig. 12). In the SSU and LSU rRNA gene tree, the *Chlorarachnion* clade and the *Bigelowiella-Norrisiella* clade form a monophyly and the *Partenskyella* clade positioned as their sister lineage. The *Lotharella* clade branched as a most basal lineage of chlorarachniophytes but the position was not strongly supported (BP = 74 %, BPP = 0.90; Fig. 12). The monophyly of the clade comprising *V. adhaerens*, the *Amorphochlora* clade, and the *Gymnochlora* clade was strongly supported in the SSU and LSU rRNA gene tree (BP = 98 %, BPP = 1; Fig. 12).

Discussion

Our morphological and ultrastructural observations show that *Viridiuvalis adhaerens* gen. et sp. nov. has the characteristics of the chlorarachniophytes such as chloroplasts surrounded by four membranes, a nucleomorph in the PPC, zoospores with a single flagellum, and mitochondrial profiles with tubular cristae. Reddish particles are also reported most of chlorarachniophyte species (e.g. Ishida et al. 2000; Ota et al. 2007a; Ota and Vaulot 2012). Our molecular phylogenetic analyses also strongly support the inclusion of *V. adhaerens* within the chlorarachniophytes. Based on these results, this species is evidently a member of chlorarachniophytes. Based on previous taxonomic studies on chlorarachniophytes, the following characteristics are recognized as generic criteria: 1) ultrastructure of the pyrenoid, 2) position of the nucleomorph, and 3) morphology of the main vegetative stage (Ishida et al. 1996, 2011; Ota et al. 2007b, 2009b). With the exception of *Cryptochlora*, for which ultrastructural information is lacking, all genera of chlorarachniophytes are well distinguished by combinations of these criteria. The pyrenoid of *V. adhaerens* is embedded in the chloroplast, and a plate-like wide invagination of the PPC was observed in the pyrenoid. These ultrastructural features are clearly different from those of other chlorarachniophytes that have a projecting pyrenoid (except for the pyrenoid-lacking *Partenskyella*) (Dietz et al. 2003; Hibberd and Norris 1984; Ishida and Hara 1994; Ishida et al. 1996, 2000; Moestrup and Sengco 2001; Ota et al. 2005, 2007a, b, 2009a, b, 2011; Ota and Vaulot 2012). Although *Cryptochlora perforans* lacks any ultrastructural information, light microscopic observation indicated that it also has projecting pyrenoid (Fig. 4e in Calderon-Saenz and Schnetter 1989). In most chlorarachniophytes, a slit-like invagination of the PPC is present in the pyrenoid as it is in *V. adhaerens*. However, the invagination of the PPC terminates within the pyrenoid in other chlorarachniophytes (Dietz et al. 2003; Hibberd and Norris 1984; Ishida and Hara 1994; Ishida et al. 2000; Moestrup and Sengco 2001; Ota et al. 2005, 2007a, b, 2009a; Ota and Vaulot 2011). The invagination of the PPC in *V. adhaerens*, by contrast, is very wide and nearly partitions the chloroplast into two components surrounded by the inner two membranes. This unique feature of *V. adhaerens* is probably related to the presence of the unusual embedded pyrenoid in this species. The nucleomorph of *V. adhaerens* is located in the PPC near the base of the plate-like invagination, whereas the nucleomorphs of other chlorarachniophytes are located either near the base of the pyrenoid (*Lotharella*, *Amorphochlora*, *Bigelowiella*, *Gymnochlora*, and *Norrisiella*) (Dietz et al. 2003; Ishida and Hara 1994; Ishida et al. 1996, 2000; Moestrup and Sengco 2001; Ota et al. 2005, 2007a, b, 2009a, 2011; Ota and Vaulot 2011), in the invagination in the pyrenoid (*Chlorarachnion*) (Hibberd and Norris 1984), or on the inner face of the cup-shaped chloroplast (*Partenskyella*) (Ota et al. 2009b). The ultrastructure of the pyrenoid and the position of the nucleomorph of *V. adhaerens* are not congruent with any other genera of chlorarachniophytes.

Phylogenetic analyses using rRNA genes show that *V. adhaerens* branches as the sister lineage of the *Amorphochlora* clade. However, the vegetative stage of *V. adhaerens* is an immotile cell covered with a cell wall and no amoeboid stage was observed, which contrasts with *A. amoebiformis*, the only described species of *Amorphochlora*, which has amoeboid cells as its main vegetative stage (Ishida et al.

2000). *Amorphochlora amoebiformis* has the projecting pyrenoid, the slit-like deep invagination of the PPC only in the pyrenoid, and the nucleomorph situated at the base of pyrenoid that are typical of chlorarachniophytes (Ishida et al. 2000; see above). The close relationship between *A. amoebiformis* and *V. adhaerens* suggests that the unique ultrastructural features of *V. adhaerens* (embedded pyrenoid, nucleomorph situated near the base of the wide plate-like invagination of the PPC) are secondarily derived from the typical condition mentioned above.

The vegetative stage of *V. adhaerens* is the unicellular coccoid cell or sarcinoid colony, and the cells are covered with a cell wall. In chlorarachniophytes, walled vegetative cells have also been reported in *Cryptochlora*, *Norrisiella*, and *Lotharella* (Calderon-Saenz and Schnetter 1987; Dietz et al. 2003; Ishida and Hara 1994; Ota et al. 2005, 2007b; Ota and Vaulot 2011), and their cell walls have unique ultrastructural features (information is lacking for *Cryptochlora*). *Norrisiella sphaerica* Ota & Ishida has a thin and electron-dense cell wall (Ota et al. 2007b), whereas *L. polymorpha* Dietz, Ehlers, Wilhelm, Gil-Rodríguez & Schnetter has a thick but homogeneous electron-dense cell wall (Dietz et al. 2003). *Lotharella reticulosa* Ota has a thin and less dense cell wall (Ota and Vaulot 2011). Although *L. vacuolata* Ota & Ishida and *L. globosa* Ishida & Hara have multilayered thick cell walls as in *V. adhaerens*, the walls of *L. vacuolata* consist of dense layers (Ota et al. 2005) whereas those of *L. globosa* consist of laminated dense and less dense layers (Ishida and Hara 1994). The cell wall of *V. adhaerens* consists of less dense laminated fibrous materials that can be distinguished from those of other chlorarachniophytes.

Zoospores of *V. adhaerens* have large vacuoles containing fibrous materials. Similar structures were also reported in zoospores of *L. globosa* (Ishida and Hara 1994), however, the vacuoles in *V. adhaerens* are larger than that of *L. globosa*. The vacuoles are probably precursors of cell wall as assumed *L. globosa* (Ishida and Hara 1994).Sarcinoid colonies are common in *V. adhaerens*, at least under culture conditions, and this feature is one of the main characteristics of this species, along with the ultrastructural features mentioned above. *Viridiuvalis adhaerens* is the first example of a chlorarachniophyte possessing sarcinoid colonies. Masses of two to six walled cells have also been reported in *Norrisiella* (Ota et al. 2007b), but these structures are considered to be autosporangia because they soon release daughter cells (autospores) from within the mother-cell walls. The cells of the sarcinoid colonies of *V. adhaerens* are not released from mother-cell walls, and each cell sometimes forms a sarcinoid subcolony of its own, leading to the formation of compound colonies. This unique organization of *V. adhaerens* probably occurs through the retention of daughter cells within the mother-cell wall after cell division. The cell walls of the sarcinoid colonies of *V. adhaerens* generally fuse, and this may prevent the release of the daughter cells and so form the unique colonial organization. Since no other chlorarachniophytes as well as heterotrophic filoseans form sarcinoid colony, this characteristic considered to be independently acquired in *V. adhaerens*. It is suggested that life cycles of chlorarachniophytes probably is the results of adaptation to environments (Ishida et al. 1999). Although many chlorarachniophytes have been found from coral reef environments, their isolation source were sand, seaweed, or seawater and have not been isolated from the

surface of a coral. The coral considered to be rather rigid substrate than sand and seaweed and therefore locomotive ability is probably less necessary for coral-adhering algae. The formation of sarcinoid colonies may prevent spreading of daughter cells in water column and retain them on the coral that probably suitable environment for *V. adhaerens*.

As described above, *V. adhaerens* is distinguished from other chlorarachniophytes in morphology and ultrastructure as well as the phylogenetic position. Although *V. adhaerens* branches as a sister lineage of *Amorphochlora* clade, differences of vegetative stages and ultrastructures are enough to separate them as different genera. *Cryptochlora perforans* lacks both ultrastructural and molecular information, and its main vegetative stage is coccoid cell. However, *C. perforans* is distinguished from *V. adhaerens* in having projecting pyrenoid. Therefore, we propose a new genus and species, *Viridiuvalis adhaerens*, that has 1) a pyrenoid that is completely embedded in the chloroplast, 2) a nucleomorph that is located in the PPC near the base of the plate-like wide invagination, and 3) a vegetative stage composed of unicellular coccoid cells and sarcinoid colonies.

Taxonomic treatment

Viridiuvalis Shiratori, Fujita, Shimizu, Ishida gen. nov. (ICN: International Code of Nomenclature for algae, fungi, and plants, ICZN: International Code of Zoological Nomenclature)

Main vegetative stage a unicellular walled coccoid but sarcinoid colonies also formed. Zoospores released from vegetative cells. Pyrenoid embedded in the chloroplast, with a deep plate-like invagination of the periplastidal compartment almost separating the pyrenoid and chloroplast. Nucleomorph located at the base of the invagination.

Type species: *Viridiuvalis adhaerens* Shiratori, Fujita, Shimizu, Ishida sp. nov.

Etymology: The generic name "*Viridiuvalis*" derived from Latin *viridi* (green), *uva* (bunch of grapes), and *-alis* (feminine suffix), referring to the greenish sarcinoid colony of the type species. *Viridiuvalis* considered to be of feminine gender.

Viridiuvalis adhaerens Shiratori, Fujita, Shimizu, Ishida sp. nov. (ICN, ICZN)

Coccoid cells, spherical and 4–15 μm in diameter, each cell with one to several chloroplasts, many oval colorless granules, and occasionally reddish particles. Coccoid cells covered by a laminate fibrous cell wall. Sarcinoid colonies frequently observed, sometimes with subcolonies. Zoospores oval, uniflagellate, and 4–8 μm in length.

Holotype: One microscope slide (TNS-AL-58932s), deposited in the herbarium of the National Museum of Nature and Science, Tsukuba (TNS).

Isotype: One EM block (TNS-AL-58932b), deposited in TNS. These cells are derived from the same sample as the holotype.

DNA sequence: Small subunit ribosomal RNA gene, LC229571. Large subunit ribosomal RNA gene,

LC229572.

Type locality: Mucus covering a coral from Zanpa Beach, Okinawa, Japan (26.4355 N, 127.7152 E).

Collection date: November 25, 2010.

Authentic culture: The strain SRT040 used for describing this species is deposited in and maintained by the National Institute for Environmental Sciences, Tsukuba, as NIES-4109.

Etymology: Specific epithet "*adhaerens*" (adhering) refers to the coccoid cells tightly attaching to the bottom of the culture flask.

Acknowledgments

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Figure captions

Fig. 1 DIC and fluorescent micrographs of *Viridiuvalis adhaerens* gen. et sp. nov. Arrows indicate pores on empty cell walls. Arrowheads indicate mother-cell walls. Double arrowheads indicate cell walls shared by several daughter cells within the mother-cell wall. C, chloroplast; F, flagellum; N, nucleus; Ps, pseudopodium; Py, pyrenoid; R, reddish particle. **a–d.** Coccoid cells. **e.** Autofluorescence of chloroplast(s) in a coccoid cell. **f**. Coccoid cell in aged culture. **g**. Empty cell walls. **h, i.** Sarcinoid colonies. **j.** Sarcinoid colony in aged culture. **k, l**. Compound sarcinoid colonies. **m–p.** Zoospores. Scale bars: **a, m–p**, 5 μm; **b–k**, 10 μm; **l**, 20 μm.

Fig. 2 Time-lapse observation of the release of multiple zoospores from a coccoid cell of *Viridiuvalis adhaerens* gen. et sp. nov. Scale bar: 10 μm.

Fig. 3 Time-lapse observation on the release of a single zoospore from a coccoid cell of *Viridiuvalis adhaerens* gen. et sp. nov. Scale bar: 10 μm.

Fig. 4 Time-lapse observation of the fragmentation of a large sarcinoid colony of *Viridiuvalis adhaerens* gen. et sp. nov. Scale bar: 20 μm.

Fig. 5 Time-lapse observation of the change of a zoospore to a coccoid cell in *Viridiuvalis adhaerens* gen. et sp. nov. F, flagellum. Scale bar: 10 μm.

Fig. 6 Estimated lifecycle of *Viridiuvalis adhaerens* gen. et sp. nov. Solid arrows indicate processes observed in this study. Dotted arrows indicate predicted processes.

Fig. 7 Transmission electron micrographs of *Viridiuvalis adhaerens* gen. et sp. nov. White arrowheads indicate chloroplast membranes. The white arrowheads are numbered from the outermost chloroplast membrane to the innermost membrane. White double arrowheads indicate electron-dense droplets near the nucleolus. Arrows indicate plastoglobuli. C, chloroplast; Dg, dense globule; G, Golgi apparatus; M, mitochondrial profile; N, nucleus; n, nucleolus; Nm, nucleomorph; PPC, periplastidal compartment; Py, pyrenoid; S, vesicle containing storage-product-like material. **a**. Coccoid cell. **b.** High magnification view of the nucleus. **c.** High magnification view of a vesicle containing storage-product-like material. **d.** Coccoid cell with two pyrenoids. **e.** High magnification view of the nucleus, mitochondrial profiles, and Golgi apparatuses. **f.** High magnification view of the periplastidal compartment and chloroplast membranes. **g.** High magnification view of the nucleomorph and pyrenoid. **h.** High magnification view of the nucleomorph. Scale bars: **a**, 2 μm; **b, 1** μm; **c, d,** 500 nm; **e, 1** μm; **f**, 500 nm; **g**, **h**, 1 μm.

Fig. 8 Transmission electron micrographs of selected serial sections of a chloroplast of *Viridiuvalis adhaerens* gen. et sp. nov. Triple arrowheads indicate electron-dense globules in the cytoplasm. C, chloroplast; Nm, nucleomorph; Py, pyrenoid. The invagination of the PPC in the pyrenoid appears in section **c**, and the chloroplast enclosed by two membranes is completely bisected in section **f**. Scale bar: 5 μm.

Fig. 9 Transmission electron micrographs of cell wall structures of *Viridiuvalis adhaerens* gen. et sp. nov. Double arrows indicate vesicles containing electron-dense materials. Triple arrowheads indicate electron-dense globules in the cytoplasm. **a.** High magnification view of the cell wall and vesicles containing electron-dense materials. **b.** High magnification view of the cell wall between two daughter cells. **c**. Sarcinoid colony. **d.** High magnification view of **c**. Scale bars: **a, b**, 1 μm; **c, d**, 5 μm.

Fig. 10 Transmission electron micrograph of a zoospore of *Viridiuvalis adhaerens* gen. et sp. nov. Double arrows indicate vesicles containing electron-dense material. Arrowheads indicate vesicles containing dense material surrounded by fibrous material. Asterisks indicate large vesicles containing fibrous material. B, basal body; C, chloroplast; M, mitochondrial profile; N, nucleus; Nm, nucleomorph. Scale bar: 1 μm.

Fig. 11 Maximum likelihood tree of chlorarachniophytes using 1618 positions from the SSU rRNA gene. Values on each node are bootstrap percentages (BP, left) and Bayesian posterior probabilities (BPP, right). Values are shown when BP ≥ 50 % and BPP ≥ 0.5.

Fig. 12 Maximum likelihood tree of chlorarachniophytes using 1622 positions from the SSU rRNA gene and 3063 positions from the LSU rRNA gene. Values on each node are bootstrap percentages (BP, left) and Bayesian posterior probabilities (BPP, right). Values are shown when BP ≥ 50 % and BPP ≥ 0.5.

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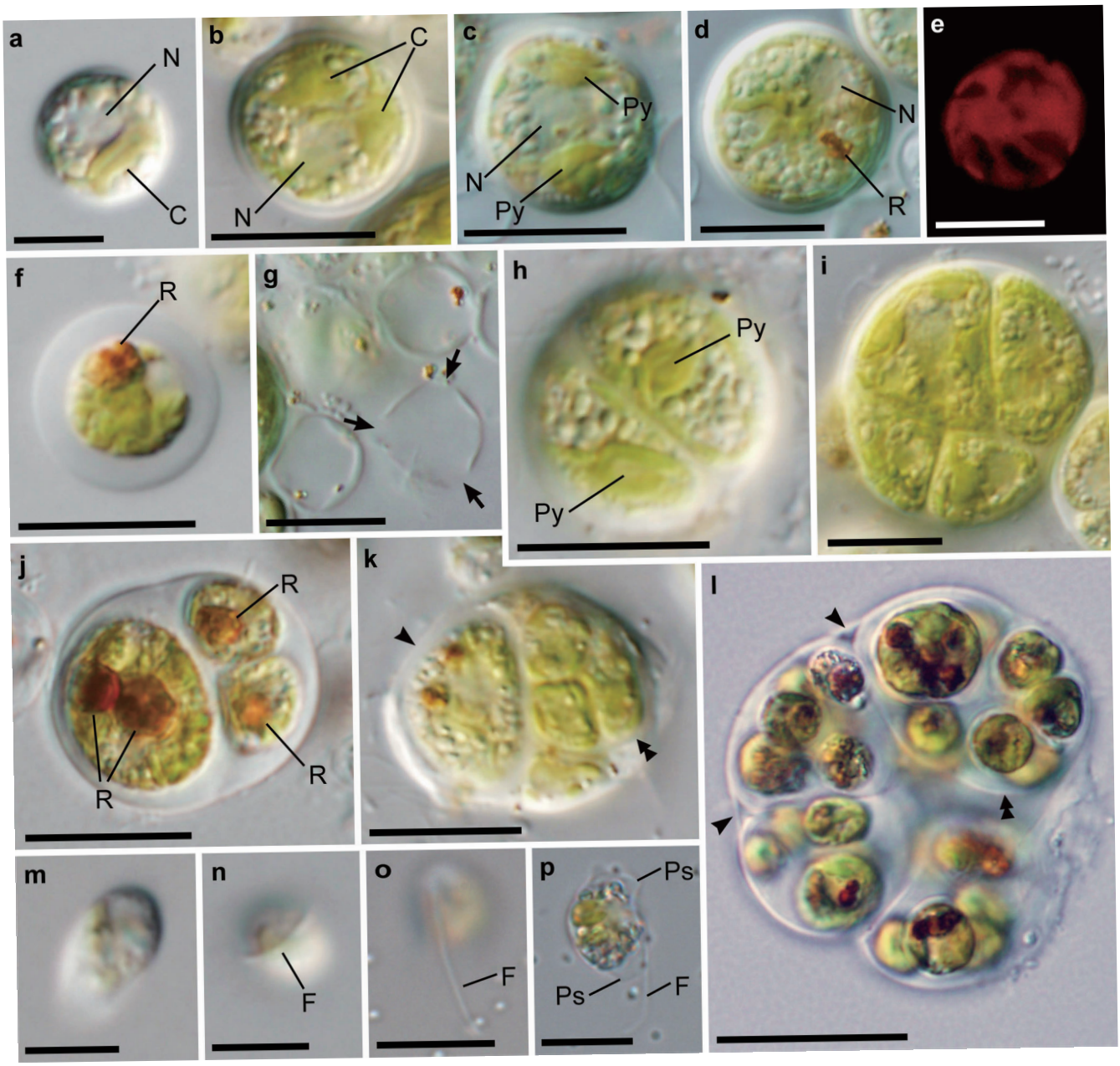
Fig. 1



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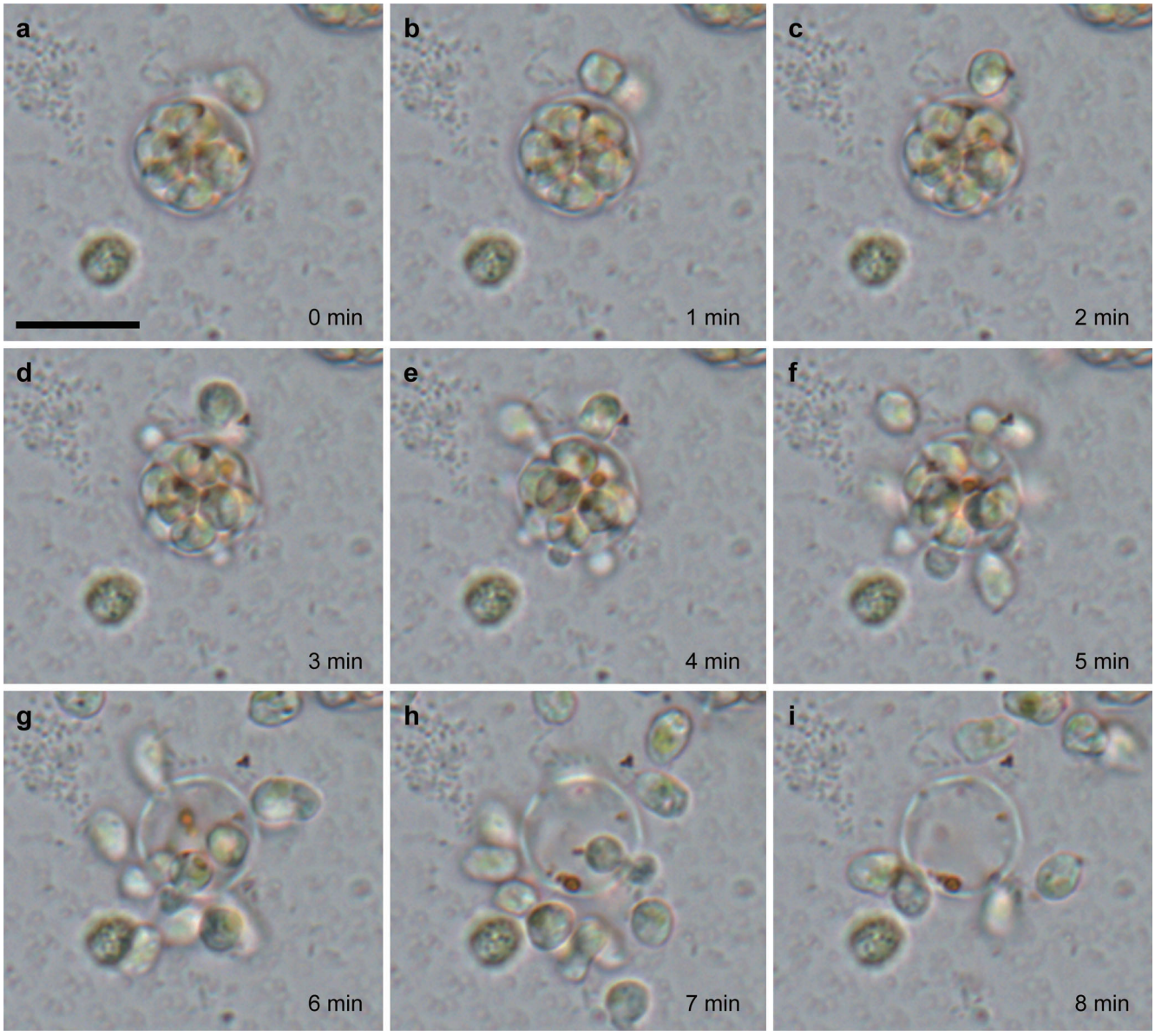

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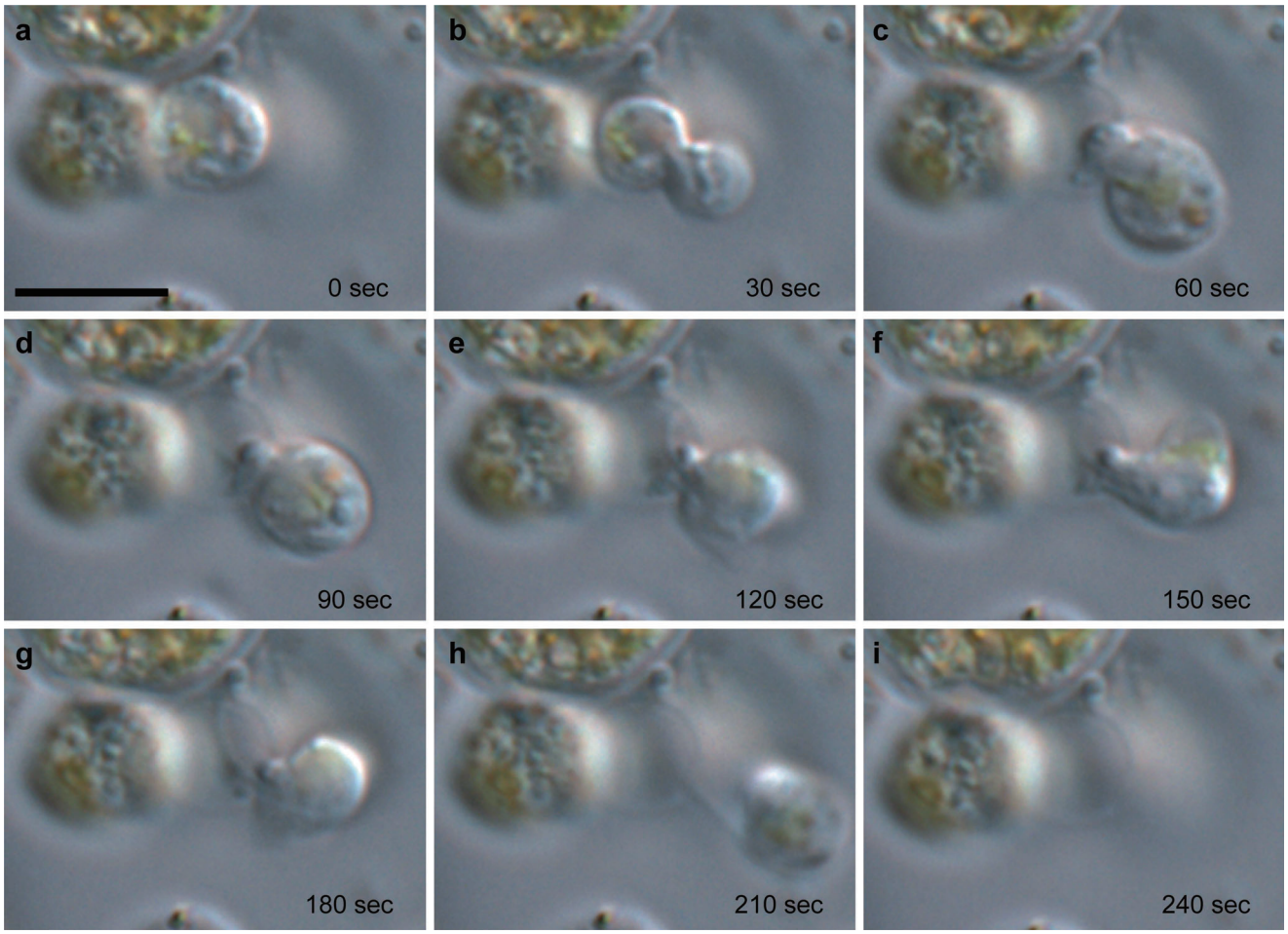
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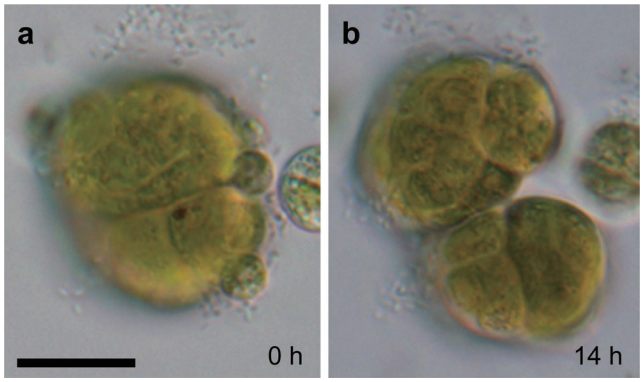

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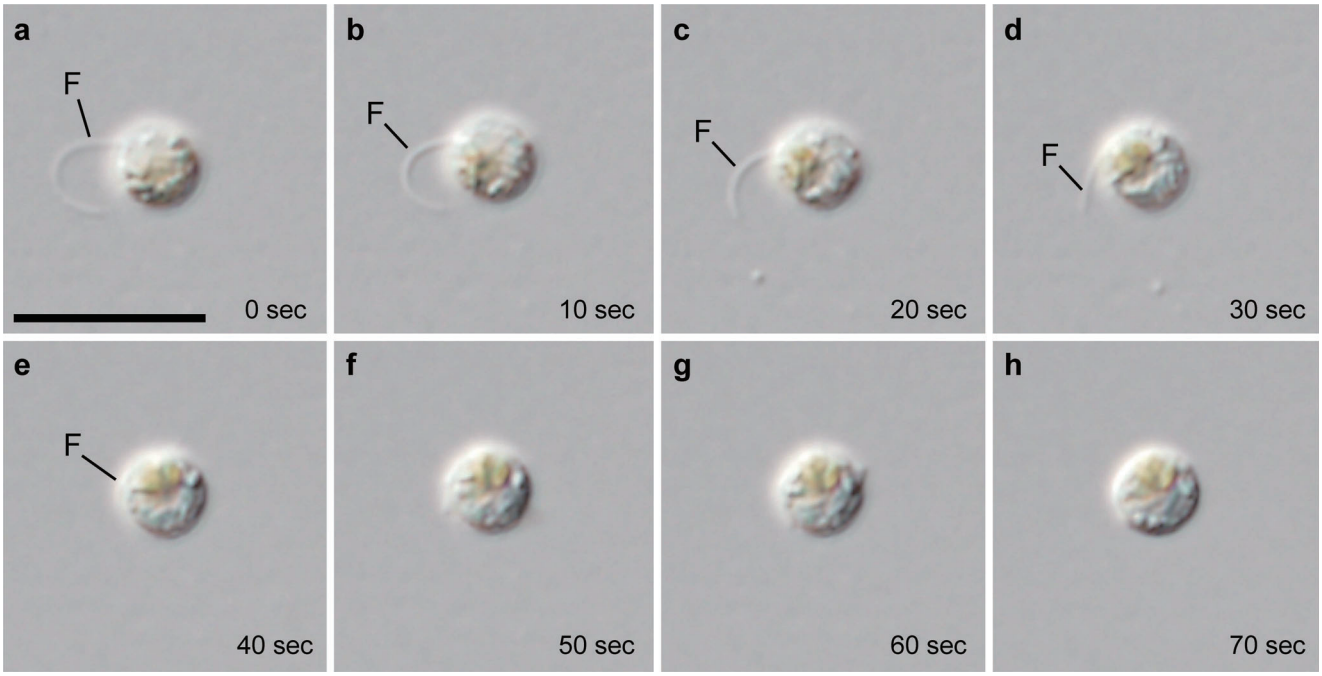
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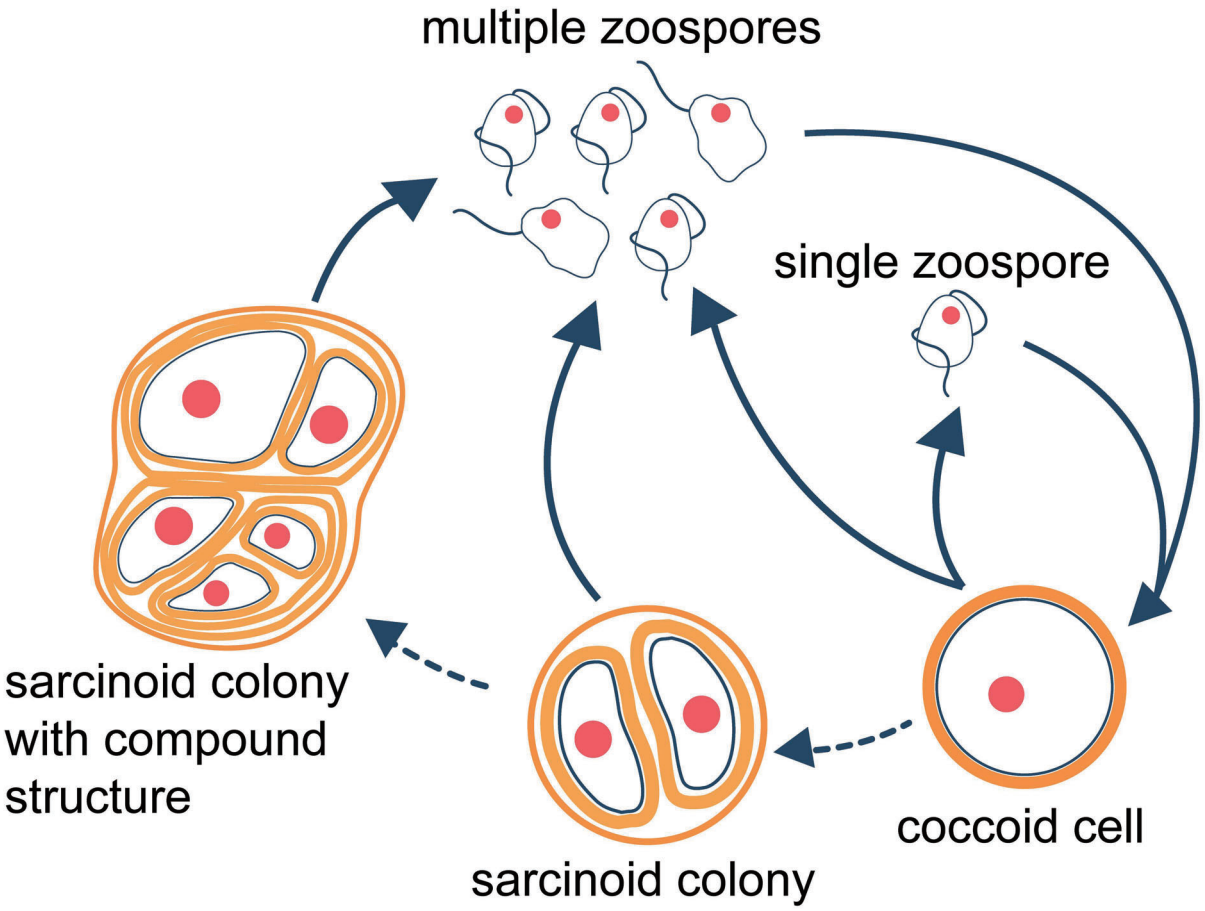
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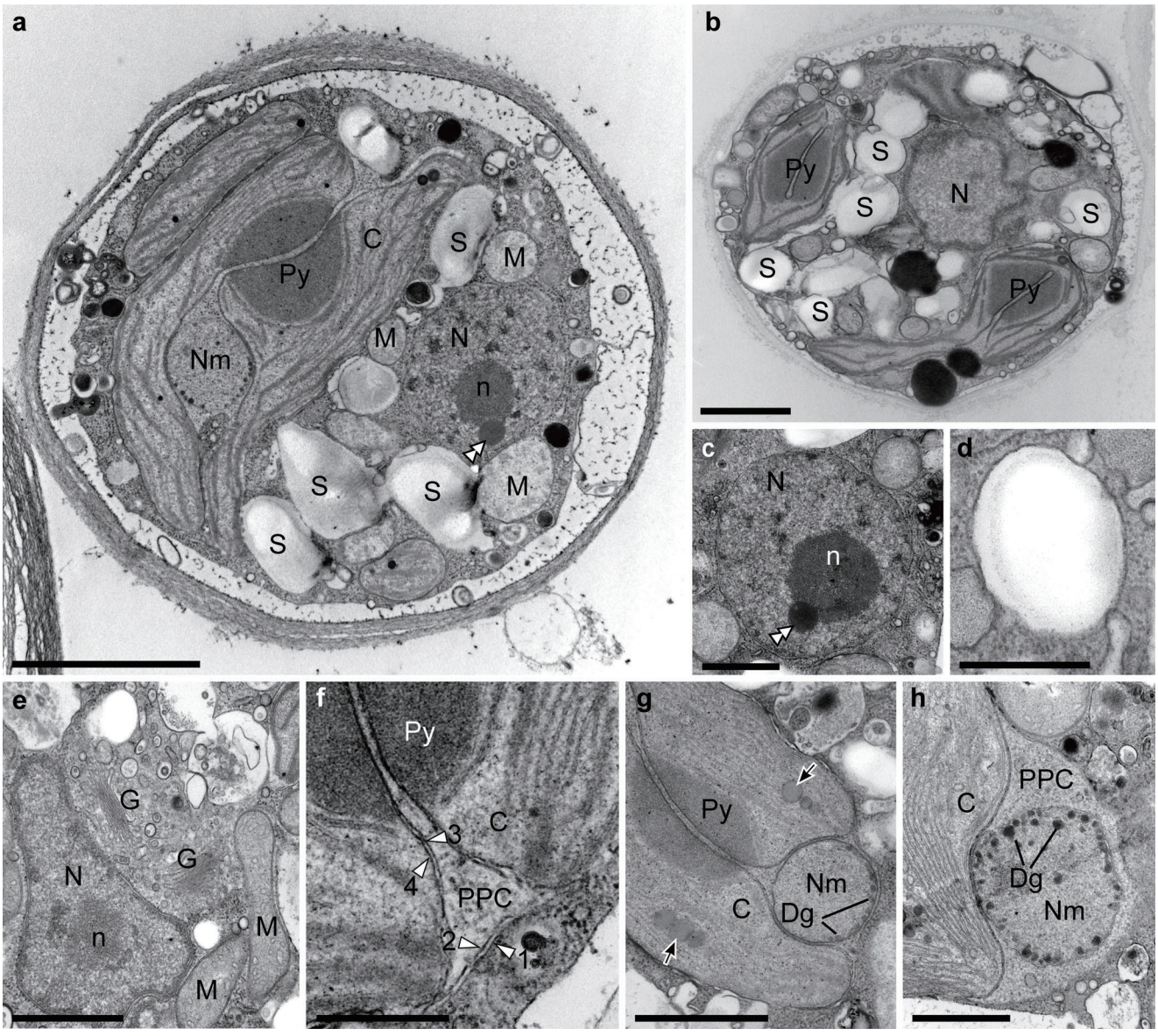
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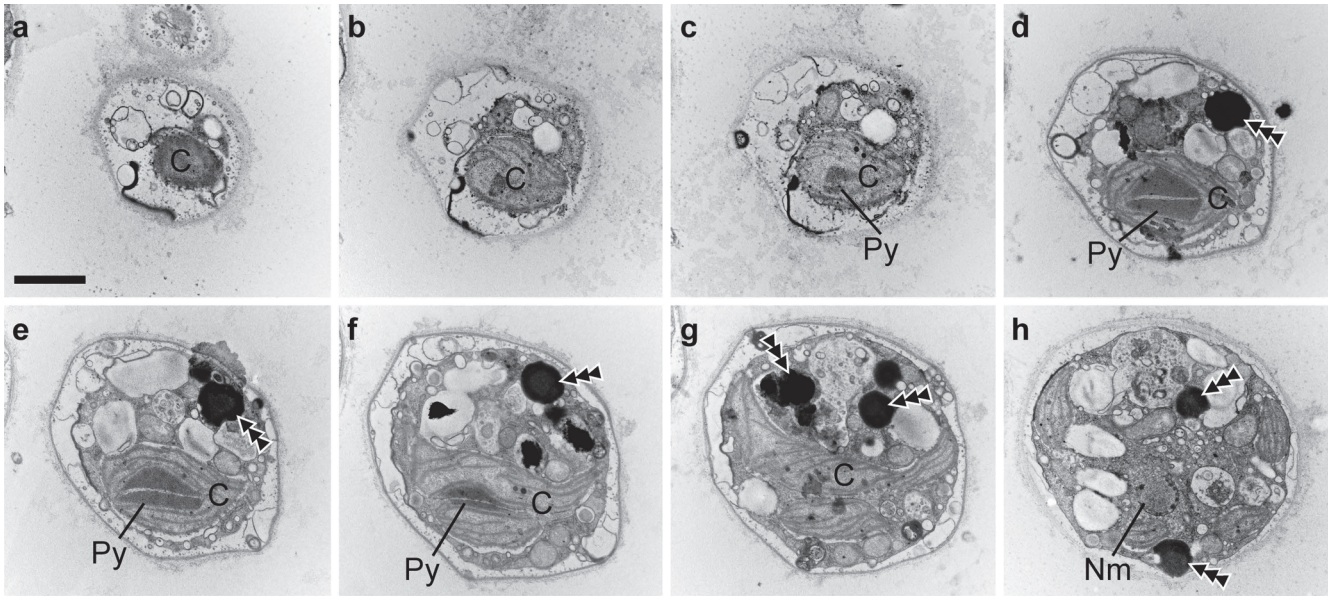
Fig. 8



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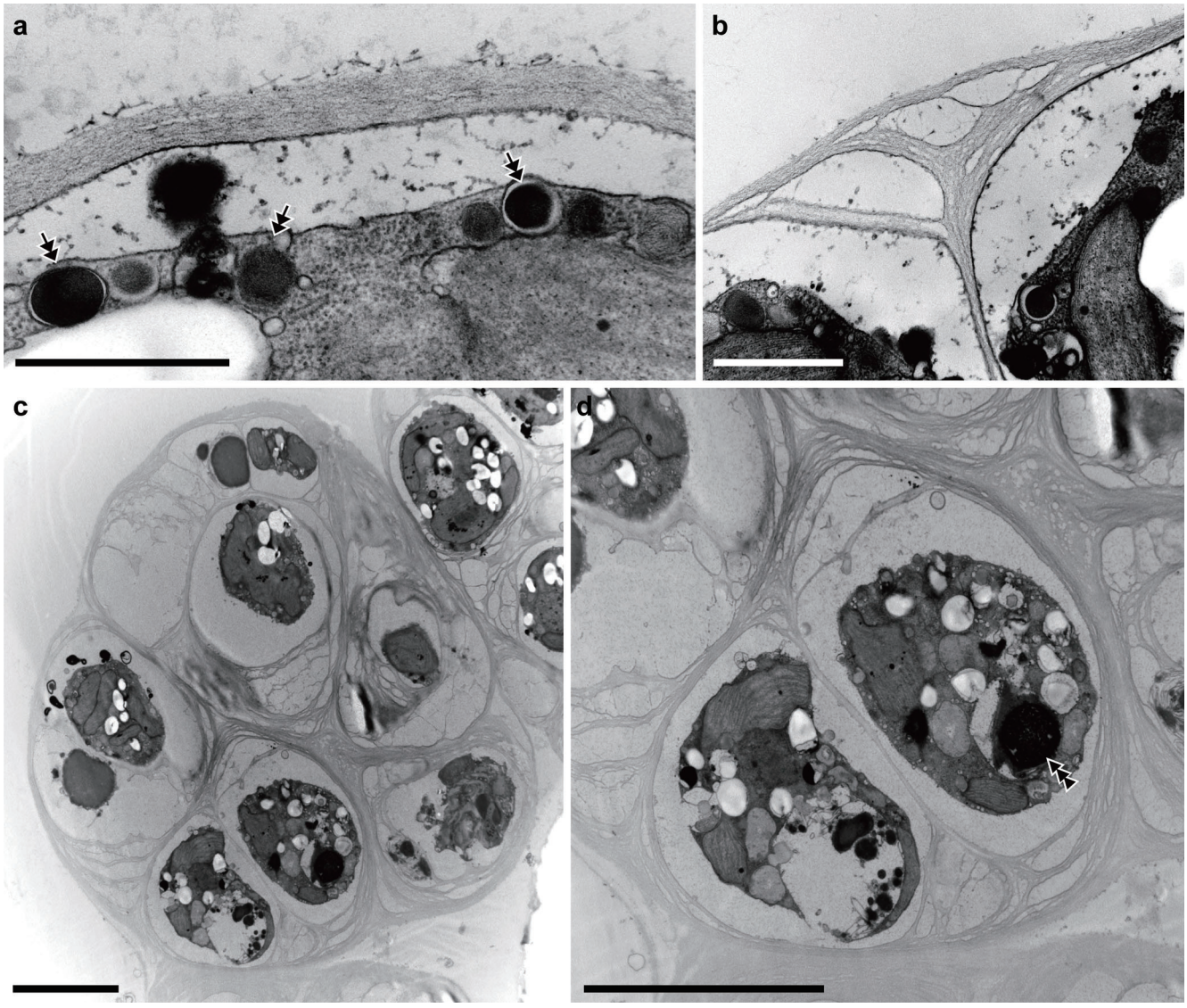

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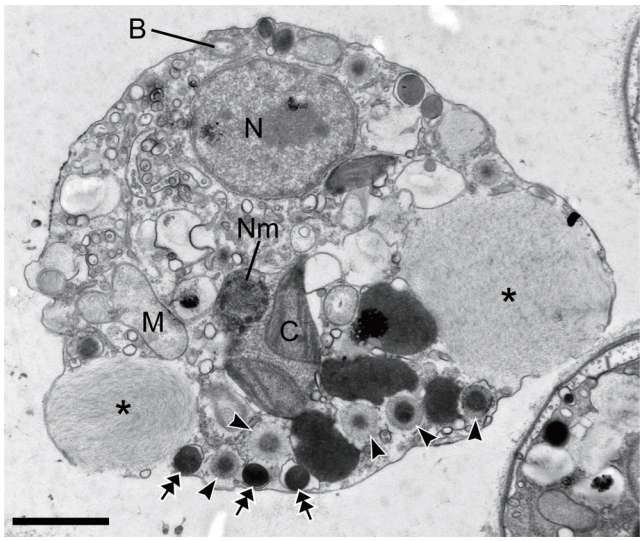

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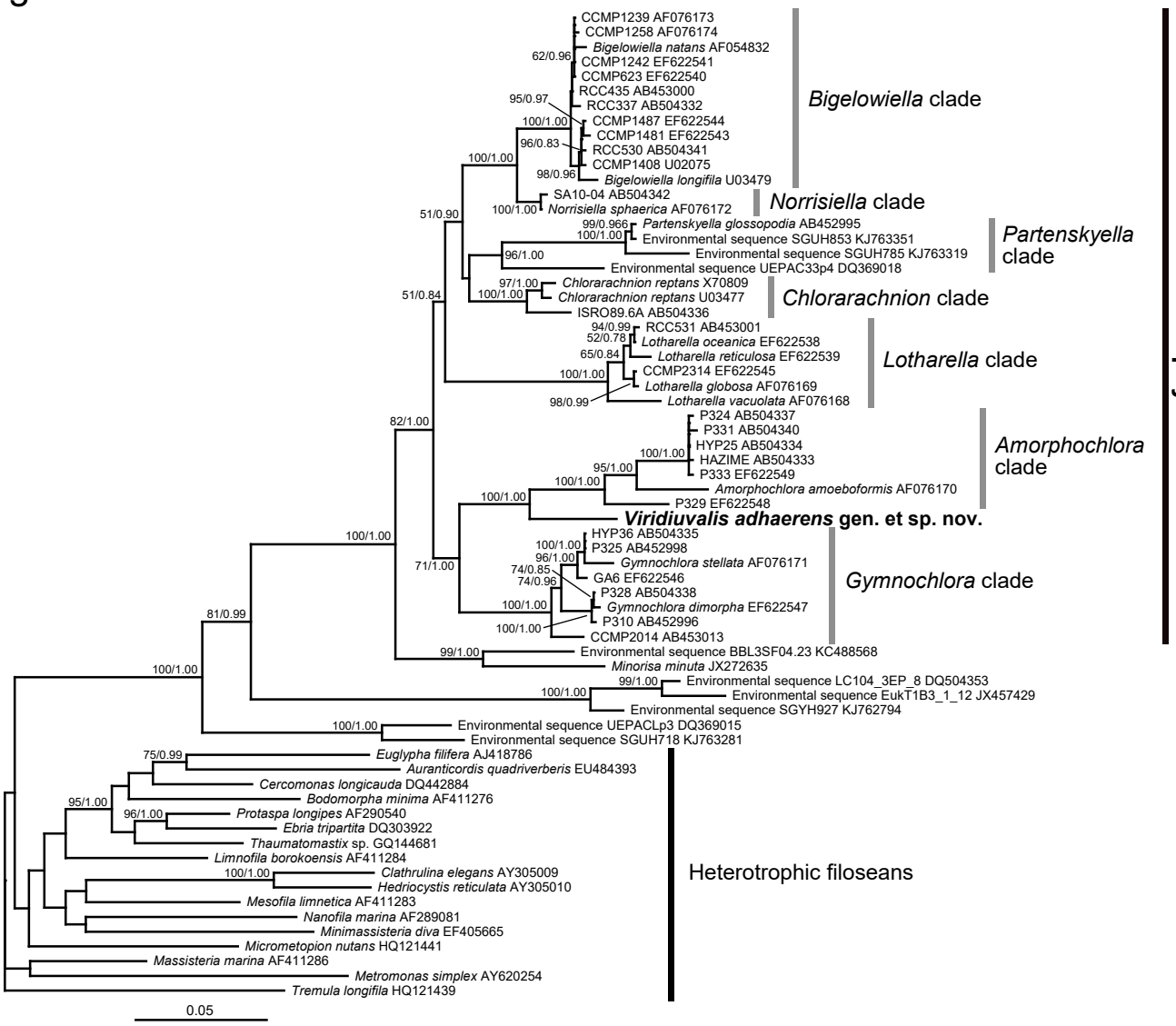
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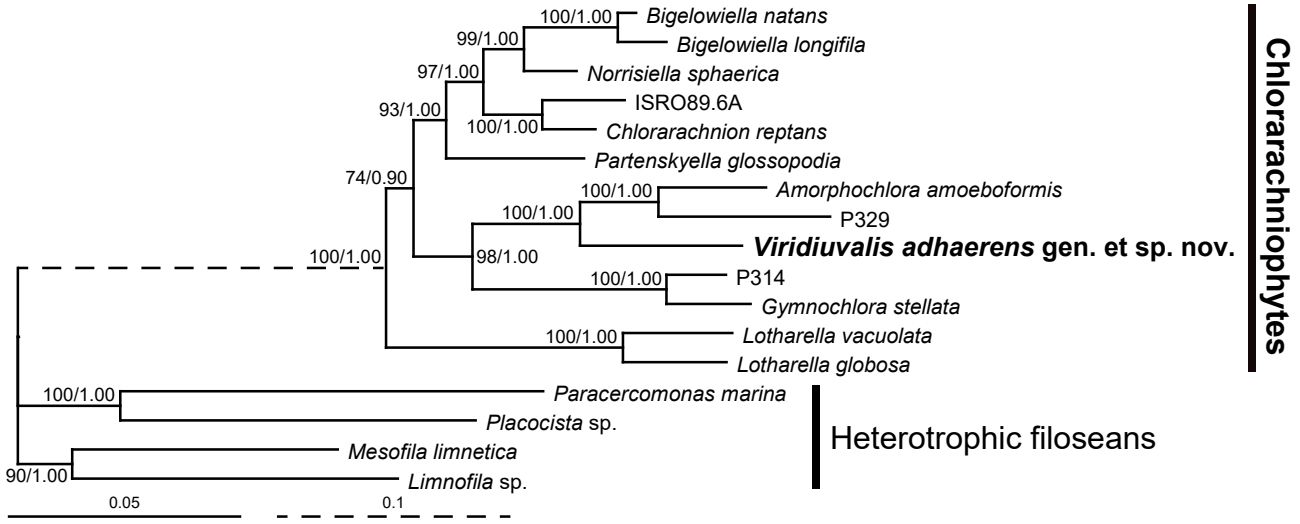
Fig. 12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