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New pelagophytes show a novel mode of algal colony development and reveal a perforated theca that may define the class [1]

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ABSTRACT

Pelagophytes (Heterokonta) are a morphologically diverse class of marine algae historically united only by DNA sequences. We established clonal cultures of sand-dwelling pelagophytes collected from intertidal pools around Australia. Phylogenetic trees based on nuclear 18S rDNA and plastid *rbc*L, *psa*A, *psa*B, *psb*A and *psb*C sequences revealed two new genera, *Gazia* and *Glomerochrysis*, related to *Aureoumbra* in a distinct lineage within the Sarcinochrysidaceae (Pelagophyceae). The three new species (*Gazia saundersii*, *Gazia australica* and *Glomerochrysis psammophila*), along with an Australian strain of *Aureoumbra geitleri,* are characterized by dominant benthic stages that differ significantly from one another, while occasionally producing classic heterokont zoospores. The benthic stage of *Ga. saundersii* has a novel development not observed in any other colonial alga, consisting of large, spherical colonies (up to 140 μm in diameter) containing c. 2500 cells that eventually differentiate and segregate into a large number of daughter colonies that are subsequently liberated. Alternatively, colonies may differentiate into a mass of zoospores that escape and settle to develop into new colonies. In *Gl. psammophila*, cubic packets of cells form large, sticky clusters that bind sand together, while *Ga. australica* and *A. geitleri* are unicellular species. Using fixation by high pressure freezing, a distinctive perforated theca was observed by TEM in all genera of this lineage, and we hypothesize this unique covering may be the first morphological feature to characterize most, if not all, pelagophytes. This study substantiates the diverse nature of sand-dwelling pelagophytes as well as their mechanisms for thriving in a dynamic habitat.

KEY INDEX WORDS: *Aureoumbra*; *Gazia*; *Glomerochrysis;* Sarcinochrysidaceae; Pelagophyceae; molecular phylogeny; perforated theca; sand-dwelling

ABBREVIATIONS: HPF, high pressure freezing; ML, maximum likelihood; OTU, operational taxonomic unit

INTRODUCTION

The class Pelagophyceae was formed when DNA sequences revealed a highly unusual, truly uniflagellate organism not related to other algal classes (Andersen et al. 1993). A second study using DNA sequences showed that, in addition to the order Pelagomonadales, the order Sarcinochrysidales (Gayral and Billard 1977) also belonged in the Pelagophyceae (Saunders et al. 1997). There was some doubt that the taxon called "*Sarcinochrysis marina*" (e.g., West 1969, Gayral 1972) truly represented the organism described by Geitler (1930), and because *Sarcinochrysis marina* is the type species for the order Sarcinochrysidales, the transfer of the order to the Pelagophyceae was not completely certain. Recently, *Sarcinochrysis marina* was re-collected from the type locality and DNA sequences definitively proved that the Sarcinochrysidales belonged within the Pelagophyceae (Han et al. 2018).

The Pelagophyceae is unusual in two respects. First, all described species are marine, in contrast to other marine groups (e.g., Phaeophyceae, Rhodophyta) that have a few freshwater representatives. Furthermore, most species were found along coastlines, with *Pelagomonas* and *Pelagococcus* being the only true open ocean taxa. Second, every described species has been classified within the Pelagophyceae based solely on DNA sequences. In fact, no morphological feature uniting all the pelagophytes is known. Currently, the Pelagophyceae contains 14 known genera, while additional taxa may well be included once several suspect species have been isolated and DNA sequences obtained (for summary see Han et al. 2018).

Pelagophyte species range in size from 1.5 x 2-3 µm flagellates (*Pelagomonas*; Andersen et al. 1993), to macroscopic sheets 1 cm across (*Aureoscheda*; Wynne et al. 2014) and flowing colonies 3-5 cm long (*Chrysocystis*; Andersen et al. 1993, Lobban et al. 1995, Wynne et al. 2014). The planktonic forms are flagellate (*Pelagomonas, Ankylochrysis*; Andersen et al. 1993, Honda and Inouye 1995) or coccoid (*Aureococcus, Aureoumbra, Pelagococcus*; Lewin et al. 1977, Sieburth et al. 1988, DeYoe et al. 1997), whereas the attached species are predominantly capsoid organisms with vegetative cell division (*Arachnochrysis*, *Chrysocystis, Chrysoreinhardia, Pelagospilus*, *Sarcinochrysis, Sargassococcus* and *Sungminbooa*; see Han et al. 2018), with one filamentous form (*Andersenia*; Wetherbee et al. 2015). In addition, two genera, *Aureococcus* and *Aureoumbra*, produce harmful algal blooms (Cosper et al. 1989, DeYoe et al. 1997, Kang and Gobler 2017, Tang et al. 2019). Heterokont zoospores are produced by many genera, possessing an anterior flagellum bearing mastigonemes that beats as a sinusoidal wave and a short, stiff posterior flagellum that beats with a sculling motion.

During our exploration of sand-dwelling microalgae collected around Australia (Grant et al. 2011, 2013, Wetherbee et al. 2015, Wetherbee and Verbruggen 2016, Wetherbee et al. 2019a, 2019b) we discovered several unknown pelagophytes. The goal of this study was to describe the new pelagophyte taxa, *Gazia saundersii*, *Gazia australica* and *Glomerochrysis psammophila* and to infer their phylogenetic position among the pelagophytes. Our approach consists of light and electron-microscopic observations of culture strains, and phylogenetic inference based from multi-marker datasets. Our findings include the description of a novel mechanism of algal colony development in *Ga. saundersii.* In addition, we describe the presence throughout this lineage of an extracellular, perforated theca that we hypothesize may be the first morphological feature to characterize most, if not all, pelagophytes.

MATERIALS AND METHODS

Sampling, isolation and culture

The sample containing *Gazia saundersii* was collected in a shallow pool along the Merimbula boardwalk, Boggy Creek, Merimbula, NSW (-36.894497, 149.908072), in April 2015 (CSIRO strain CS-1320). The sample consisted of approximately 0.5 - 1.0 cm^3 of sand plus seawater that was placed into a 60 ml plastic jar and returned to the lab. A clonal culture (#22GB) was established by isolating large colonies of *Ga. saundersii* from a field sample by micro-pipetting into K enriched seawater medium (Keller et al. 1987). The culture was maintained in 60 ml plastic containers at 21ºC under Sylvania 58 Luxline Plus and Gro-Lux fluorescent lamps with a daily 10:14 h light:dark cycle; a stock culture was transferred into new K medium once a month. A sand sample containing *Gazia australica* was collected in a shallow pool along the Mill Bay Boardwalk, North Narooma, NSW (-36.208771, 150. 127383) in April 2015, and a clonal culture (#78) was established as described above (CSIRO strain CS-1321). A sand sample containing *Glomerochrysis psammophila* was collected from an intertidal pool at Five Finger Bay, West Australia (-23. 179307, 113.763776) in July 2016, and a clonal culture (WC1) was established as described above (CSIRO strain CS-1322). We also isolated a strain of *Aurioumbra geitleri* from an intertidal pool at Wye River, Victoria (38° 38’ 20”S; 143° 53’ 34”E) and established in a clonal culture as described above (CSIRO strain CS-1323).

Light microscopy

To observe the flagellate stage of *Gazia saundersii* and *Ga. australica*, a drop of cell culture was taken 2 h after the beginning of the light period when flagellates were most prevalent and mounted onto microscope slides with coverslips sealed with a 1:1:1 ratio of Vaseline, lanoline, and paraffin wax. For benthic cells, 5 mL of culture containing flagellates and benthic cells was poured over coverslips in small petri dishes and allowed to settle and grow for 3-14 days. Coverslips were then removed and inverted onto microscope slides and mounted as above. The benthic stage of *Glomerochrysis psammophila* was prepared as above. Flagellates and benthic cells were observed and recorded using a Zeiss AxioPlan 2 microscope (Carl Zeiss, Oberkochen, Germany) and photographs were taken using a Canon EOS 60D digital single-lens reflex camera (Canon USA, Melville, NY, USA).

Transmission electron microscopy

Cells were prepared by standard chemical fixation procedures and by high pressure freezing (HPF; Wetherbee et al. 2019a). The following procedure was most successful for the chemical fixation of *Gazia australica*, while the other species did not yield a satisfactory fixation with the several recipes tried. The benthic cells of all three species were collected and concentrated in 10 ml centrifuge tubes and chemically fixed using the following recipe; 2.5 mL of a 4% solution of glutaraldehyde mixed with 2.5 mL of a 4% solution of osmium tetroxide, both in K-seawater media on ice (a total of 5 mL). The fixative was mixed with 5 ml of the cell slurry and left for one hour before being washed 3x with K-media. Cells were then brought into distilled water in steps: 75:25, 50:50 and 25:70 K-media:distilled water. Cells were washed 3 times in distilled water and placed in a solution of 2% uranyl acetate in distilled water for one hour before gradual dehydration in EtOH (10%, 25%, 50%, 75%, 90% and 3x at 100%). Cells were then gradually embedded in either LR White (Merck KGaA, Darmstadt, Germany) or Spurr's medium (Spurr 1969). Thin (70 nm) sections were cut from either pellets of the flagellate preparation, or from plugs cut from the agarose films, using a Leica Ultracut S ultramicrotome (Leica Microsystems) and post-stained with 1% aqueous uranyl acetate and triple lead stain (Hanaichi et al. 1986). The sections were imaged using a Tecnai Spirit transmission electron microscope (ThermoFisher Scientific) equipped with an Eagle 2K CCD camera.

Alternatively, live cells were gently centrifuged and placed directly in Type B carriers without cryoprotectants before high pressure freezing using a Leica EM ICE high pressure freezer (Leica Microsystems, Austria; see Wetherbee et al. 2019a). The cells were freeze substituted in 2% osmium tetroxide and 8% 2,2-Dimethoxypropane (DMP) in acetone for 72 h at -85°C before being slowly warmed to room temperature over the next two days. The samples were then washed three times in acetone followed by gradual infiltration in Spurr's resin as above. Thin (70 nm) sections were stained and observed as above.

Molecular phylogenetics

Total genomic DNA was extracted using a modified CTAB protocol (Cremen et al. 2016). The 18S rRNA gene was amplified and sequenced using universal primers eukAF (5'-AAYCTGGTTGATCCTGCCAG-3') / eukBR (5'-GATCCWTCTGCAGGTTCACCTA-3'), and followed a thermocycling regime consisting of an initial temperature of 94°C for 2 min; 40 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 2 min; and a final elongation step of 72°C for 5 min. Forward and reverse reads were assembled and edited using Geneious Prime v.2019.2.3 (Kearse et al. 2012). For high-throughput sequencing, extracted DNA was sent to GENEWIZ (Suzhou, China), where libraries were generated using the Illumina VAHTS Universal DNA Library prep kit and protocols, and sequenced on the NovaSeq System (paired-end, 150 bp reads, ~4 Gb of data).

Recent phylogenies for pelagophytes have used six genes: the nuclear 18S rRNA, and the plastid genes *psa*A, *psa*B, *psb*A, *psb*C and *rbc*L (Han et al. 2018). To obtain these plastid genes, the Illumina data were assembled (Marcelino et al. 2016, Jackson et al. 2018) and chloroplast contigs annotated (Cremen et al. 2018, 2019) as previously described, and the relevant genes extracted from the assembly. Sequences of 18S rRNA and the five chloroplast markers were submitted to Genbank (Table S1 in the Supporting Information). We downloaded additional sequences of the same six genes from representatives across the pelagophyte tree of life as well as diatoms, bolidophytes and Dictyochophyceae as outgroups (Han et al. 2018; Table S1). Sequences were aligned for each gene with TranslatorX v.1.1 (Abascal et al. 2010), using Muscle v3.8.31 as the alignment algorithm (Edgar 2004). Following a visual check of the alignments, they were concatenated and the resulting supermatrix analysed with maximum likelihood (ML) searches in RAxML v.8.2.12 (Stamatakis 2014) using the GTR+Gamma model and using 25 randomized MP starting trees. Branch support was obtained by running 100 standard bootstrap replicates.

RESULTS

Gazia **Wetherbee & Bringloe, gen. nov.** (Sarcinochrysidaceae, Pelagophyceae)

Description – Marine, benthic; vegetative thalli unicellular or developing into large spherical colonies surrounded by a thick casing; cells rounded to irregular in shape, with 1-2 chloroplasts; stalked pyrenoids in chloroplast lobes; cells surrounded by a thin perforated theca with distinctive pores visible with electron microscopy; prior to cell division, cells enlarged, with two deeply lobed chloroplasts, each lobe with a stalked pyrenoid; reproduction by biflagellate zoospores with a single chloroplast; long anterior flagellum bearing mastigonemes, short smooth posterior flagellum; nuclear encoded 18S rDNA and plastid encoded *rbc*L, *psa*A, *psa*B, *psb*A and *psb*C sequences distinctive.

Type species – *Gazia saundersii* Wetherbee & Bringloe

Etymology – The genus is named in honour Dr Gary Saunders, or "Gaz" as he was affectionally known at the University of Melbourne where he undertook his postdoctoral studies with Dr Gerry Kraft. During the past three decades, Gaz has made many outstanding contributions to our knowledge of seaweeds from around the world, including Australia, and was also instrumental in the description of the class Pelagophyceae, of which *Gazia* is a new genus.

***Gazia saundersii* Wetherbee & Bringloe, sp. nov.**

Description – Colonial organism; initial benthic cell 12-14 μm in diameter prior to cell divisions giving rise to spherical colony, up to 140 μm in diameter (c. 2500 cells), with colony size even larger in culture; daughter colonies formed inside mother colony; mother colony (and daughter colonies) surrounded by multilayer casing; mother colony multilayer casing rupturing to release daughter colonies or zoospores; vegetative cells 6-8 μm in diameter following division, with a single lobed chloroplast; a stalked pyrenoid on each chloroplast lobe; prior to division cells enlarge to 10-12 μm with two lobed chloroplasts; cells surrounded by a perforated theca and a cell wall; zoospores biflagellate, oval to pyriform, 5.0-6.0 μm in width, 7.0-8.0 μm in length, with a single, deeply lobed, posterior chloroplast without an eyespot; flagella inserted subapically on the ventral surface; anterior flagellum long, with mastigonemes; posterior flagellum short, smooth; zoospores short lived, flattening on the substrate and forming a benthic cell; nuclear encoded 18S rDNA and plastid encoded *rbc*L, *psa*A, *psa*B, *psb*A and *psb*C gene sequences distinctive.

Holotype – MELU A #22GB, a mounted specimen derived from strain CS-1320, collected from sand in a shallow pool, by R. Wetherbee in April 2015.

Type locality – Merimbula, 100 M from the start of the Merimbula boardwalk, NSW, Australia (-36.894497, 149.908072).

Etymology –The specific epithet refers to Dr. Gary Saunders, for his many contributions to the study of algae.

Habitat – marine, sand-dwelling.

Culture lodgement – ANACC code: #22GB, New South Wales strain CS-1320; CSIRO, Hobart, Tasmania, Australia.

Gazia australica **Wetherbee & Bringloe, sp. nov.**

Description – Unicellular benthic organism; cells rounded to irregular in outline but flattened like a coin on the substratum; initially 10-12 µm in diameter, up to 14-16 µm in diameter prior to cell division; cells covered by a thin theca and cell wall; cell wall stains with toluidine blue; cells with two lobed, parietal chloroplasts; with pyrenoids; biflagellate zoospores produced directly from benthic cells; zoospores 5.0-6.0 in width, 8-10 µm in length; zoospore with one deeply lobed chloroplast, without eyespot; flagella inserted laterally; long anterior flagellum with mastigonemes, short, smooth posterior flagellum; nuclear encoded 18S rDNA and plastid encoded *rbc*L, *psa*A, *psa*B, *psb*A and *psb*C gene sequences distinctive.

Holotype – MELU A #78, a mounted specimen derived from strain CS-1321, collected from sand in a shallow pool, by R. Wetherbee in April, 2015.

Type locality - Shallow pool along the Mill Bay Boardwalk, North Narooma, NSW, Australia (-36.208771, 150. 127383).

Etymology –The specific epithet refers to the country it was collected in, Australia.

Habitat – marine, sand-dwelling.

Culture lodgement – ANACC code: #78, New South Wales strain CS-1321; CSIRO, Hobart, Tasmania, Australia.

Glomerochrysis psammophila **Wetherbee, gen. et sp. nov.**

Description – Colonial organism, with daughter colonies within the mother colony; daughter colonies tightly appressed, irregularly shaped, sarcinoid packets, typically containing 4, 8, 16 or 32 cells (larger colonies observed); vegetative colonial reproduction by cluster fragmentation; cells surrounded by one or more gelatinous sheaths derived from the mother, grandmother and great grandmother cell walls; cells 16-18 µm in diameter prior to division, oblong to spherical; cells two deeply lobed chloroplasts, each possessing a protruding pyrenoid; cells surrounded by a compact, perforated theca and cell wall; prior to cell division, cells enlarge considerably, undergo two rapid divisions to produce four daughter cells, 8-10 µm in diameter; immediately after formation, daughter cells with a single lobed chloroplast; four daughter cells encased in the mother cell wall; additional division cycles result in colonies of 8, 16 or 32 cells (or larger); colonies surrounded by multiple gel sheaths; zoospores very rare, biflagellate; appeared to have one lobed chloroplast; other details not observed; nuclear encoded 18S rDNA and plastid encoded *rbc*L, *psa*A, *psa*B, *psb*A and *psb*C sequences distinctive.

Holotype – MELU A #WC1, a mounted specimen derived from strain CS-1322, collected from sand in a deep tide pool, by S. Berin in May 2013.

Type locality – Deep tide pool, Five Finger Bay (strain WC1), West Australia (23°10’39” S, 113°45’48”E),

Etymology – *Glomerochrysis* refers to the “golden clusters” seen in field material, while the specific epithet is "sand-loving", where the species is found.

Habitat – marine, sand-dwelling.

Culture lodgement – ANACC code: WC1, West Australia strain CS-1322 CSIRO, Hobart, Tasmania, Australia.

Molecular phylogeny – The phylogenetic tree recovered the three main pelagophyte clades (Pelagomonadales, Chrysocystaceae, Sarcinochrysidaceae) with high support (Fig. 1). The new genera *Gazia* and *Glomerochrysis* were recovered as members of the Sarcinochrysidaceae, most closely related to *Aureoumbra*. *Gazia* was recovered as a sister genus to *Aureoumbra* (100% bootstrap support) whereas *Glomerochrysis* was sister to the lineage comprising *Aureoumbra* and *Gazia* (76% bootstrap support).

Light microscope observations for Gazia saundersii

Inhabiting sand in intertidal pools, *Gazia saundersii* was observed in field samples as spherical colonies of vastly different size, ranging from only 4 cells up to colonies c.140 μm in diameter, which would contain c.2500 tightly packed cells (Fig. 2, a and b). In culture, colonies grew to over 200 μm and were easily visible with the naked eye (Fig. 2b). Originally developed from a settled swarmer, the larger colonies were surrounded by a compact, multilayered casing that eventually ruptured, releasing daughter colonies that had differentiated within the mother casing (Fig. 2, c-k). Daughter colonies expelled from a single mother colony were almost all the same size (Fig. 2, f and g), although daughter colonies from smaller mother colonies were typically smaller in size (Fig. 2e). The size and shape of daughter colonies within an intact parental colony could not be discerned, as they were so tightly packed (Fig. 2, a and b). Emerging daughter colonies separated from one another, with spaces appearing between them as they escaped the surface of the mother colony and took on their spherical shape (Fig. 2, c-h). In culture, following release from the mother colony, the daughter colonies (now mother colonies) increased in size before releasing their own daughter colonies. Casings that surround all colonies readily stained with toluidine blue (Fig. 2, i-k), and were particularly obvious in large colonies that had released their contents (Fig. 2j).

Occasionally, mother colonies did not differentiate into daughter colonies, but instead released a massive quantity of small, oval, heterokont zoospores that settled and restarted the process of colony formation (Fig. 3, a-i). The parental colony casing was empty after zoospore release (Fig. 2k). Zoospores had heterokont flagella originating subapically in a small depression, a long flagellum with mastigonemes and a shorter, smooth flagellum. Zoospores contained a single, lobed chloroplast (Fig. 3b-d), were short lived (5-10 min) and either found a surface to attach to (e.g., Fig. 3, e-g), or quickly lost their flagella and became planktonic unicells. In culture, only settled zoospores seemed to form colonies. During settlement, zoospores circled the substratum and adhered first by the tip of their long, hairy flagellum (Fig. 3d), spun vigorously before becoming stationary, quickly adhered and flattened out over the coverslip before retracting the flagella axenemes (Fig. 3, e and f). Prior to division, the chloroplast divided and the benthic unicells had two, multilobed chloroplasts with pyrenoids, and were surrounded by a thin, compact theca and overlying cell wall that stained with toluidine blue (Fig. 3, f and g). Settled cells enlarged and divided twice to form 4-celled colonies surrounded by the mother cell wall (Fig. 3h). Colonies continued to divide, forming 8-celled colonies, then 16-celled colonies and so forth (Fig. 3i), with the cells becoming encased by an increasing number of former mother cell walls, now termed "gel sheaths" (see the development of *Glomerochrysis psammophila* clusters below). Overall, an individual colony was enclosed in a distinct casing derived from the original mother cell wall that expanded and thickened as the colonies increased in size (Fig. 2, i and j).

In cultures maintained for 3 y or more in full strength K medium, colonies decreased in size to the point where the species was virtually unicellular, replicating simply by division and the occasional production of zoospores that settled in the same manner as those describe above (Fig. 3, b-g). Cultures maintained in diluted K medium (e.g., K/10) maintained the wide range of colony sizes seen in newly collected field samples.

Light microscope observations for Gazia australica

This species remained unicellular, alternating between a dominant benthic stage where cell division occurred and the occasional formation of zoospores (Fig. 4, a-k). Solitary benthic cells of *Gazia australica* were rounded in shape and flattened out on the substratum like gold coins. Loosely arranged rafts often formed where zoospores settled and abutted one another, and therefore cells were irregular in shape (Fig. 4a). Two multilobed chloroplasts with stalked pyrenoids occupied the periphery of benthic cells (Fig. 4, a, b and e), which were covered by a thin theca and cell wall that stain with toluidine blue (Fig. 4, c and d). No casing or gel sheaths were observed. Zoospores of the sarcinochrysidalean-type were oval or tear-drop shaped, dorso-ventrally flattened where the two flagella were inserted sub-apically at an acute angle to the ventral cell surface (Fig. 4, g and h). The long flagellum was twice the length of the cell while the short flagellum was approximately the same length as the cell (Fig. 4h). Zoospores had a single, lobed chloroplast that occupied the posterior of the cell. Zoospores were short lived, quickly adhered to the substratum with the tip of their long flagellum (arrowhead, Fig. 4h), then spun constantly in one place for up to a minute. Zoospores subsequently became stationary and flipped over, adhering and flattening out on their dorsal surface before shedding the two flagella (Fig. 4, i-k). Occasionally, benthic cells divided and produced 2 zoospores rather than benthic daughter cells (Fig. 4f).

Light microscope observations for Glomerochrysis psammophila

Cells of *Glomerochrysis psammophila* were arranged in tightly appressed, irregular shaped clusters of sarcinoid-type colonies visible with the naked eye (Fig. 5, a and b). Cells were arranged in cubic packets, typically containing 4, 8, 16 or 32 cells, and were surrounded by one or more gelatinous sheaths, derived from the mother, grandmother, great grandmother etc. cell walls (Fig. 5, a-i). Larger colonies have been observed. The outermost gel sheaths were adhesive and bound colonies to one another and the substratum. Vegetative reproduction was mainly by cluster fragmentation of the larger colonies into smaller ones. Mature cells were oblong to spherical, surrounded by a compact theca and adjacent cell wall (see TEM images below), and contained two deeply lobed chloroplasts at the cell boundaries, each lobe possessing a protruding pyrenoid (Fig. 5c). Cells enlarge considerably prior to undergoing 2 divisions, the 4 resulting daughter cells each encased in a thin theca and containing a single lobed chloroplast. The packet of 4 cells were now surrounded by the mother cell wall, now termed a gel sheath (Fig. 5d). Further division cycles resulted in colonies of 8 or 16 cells (or larger) surrounded by multiple gel sheaths (Fig. 5, e-i). Heterokont zoospores were infrequent at best, at any stage in the cell cycle, and were too sparse to be photographed. Cell walls and gel sheaths were stained pink to purple with toluidine blue (Fig. 5, g-i).

Fine structural observations

The fine structure of both *Gazia* and *Glomerochrysis* was observed by both classical chemical fixation and high pressure freezing (HPF), although only *Gazia australica* cells yielded clear results using a chemical fix. HPF preservation varied between species for cytoplasmic features (e.g., organelles, Golgi, endoplasmic reticulum) but was consistently good for the preservation of the plasma membrane, perforated theca (see below) and most extracellular wall and mucilaginous layers. Preservation of the unicells of *Ga. saundersii* was good, but the large colonies did not fix well despite many attempts.

The fine structural features of all three species were similar and generally unremarkable, showing deeply lobed chloroplasts with prominent pyrenoids, nuclei, basal bodies, Golgi stacks and distinct regions of endoplasmic reticulum (Figs. 6, a-j, 7, a-h). Of most interest was a tight-fitting, perforated theca with distinctive pores. The theca formed adjacent to the plasma membrane (PM) and sandwiched a thin, electron opaque space that varied in thickness between species (Figs. 6, d,h and i, 7, f and g) and was often interspersed with thin, perpendicular fibres positioned between the PM and theca (e.g., Fig. 7, f and g). The theca encased the entire cell surface (Fig. 6, a and b) except at a small region on the ventral surface where the basal bodies were positioned (Fig. 6c). Cells doubled in size prior to division and daughter cells received a complete theca. The *Gazia australica* theca measured 60-80 nm in thickness (Fig. 6, d and e), covered by a fibrillar wall layer 100-120 nm thick (Fig. 6d) while the theca of *Ga. saundersii* is significantly thinner at 12-15 nm (Fig. 6, g-i), and the adjacent fibrillar wall 55-65 nm, though the fibrillar wall in this species did not stain well for TEM (e.g., Fig. 6, g-i). The theca of *Glomerochrysis psammophila* was 20-24 nm and the fibrillar wall 150-200 nm thick (Fig. 7, a and b). The opaque space between the plasma membrane and the theca was greater in this species at 45-55 nm with thin fibres connecting the two (Fig. 6, e-g).

Thecal pores perforated the entire thickness of thecae in all three species and appeared to be lined by a more electron dense coating (e.g., Figs. 6, f, i and j; 7, f and g). Pores were best seen in tangential or glancing section (Figs. 6, f and j; 7, f and g), and occasionally appeared to have material within the pores (e.g., Fig. 6j). *Gazia australica* had two types of pores, macropores and micropores. Macropores measured 20-25 nm in diameter in the chemical fix (Fig. 6, e and f) and 18-20 nm in HPF fixed cells and were observed to be either closely packed or irregularly scattered over the thecal surface. A network of tiny micropores (6-8 nm in diameter) also perforated the theca surface in this species, interspersed amongst the macropores (Fig. 6, e and f). Micropore density appeared to vary over the surface of a single cell and between different cells. Micropores were not observed in the relatively thin thecae of *Ga. saundersii* and *Glomerochrysis psammophila*, where the thecae appeared dominated by closely packed macropores (Figs. 6, i and j; 7g).

The cells of the compact packets of *Glomerochrysis psammophila* had the same general fine structure as the *Gazia* species described above (Fig. 7, a-h), including lobed chloroplasts with pyrenoids, Golgi stacks, regions of ER and a characteristic thin perforated theca sitting just off the PM Although zoospores were rarely observed in culture, basal bodies were seen in TEM sections (e.g., Fig. 7d). The thin theca measured 20-24 nm in thickness and contained tightly packed pores measuring 18-20 nm in diameter (Fig. 7, e-g). Following division, fibrillar wall layers formed adjacent to the theca (Fig. 7, a and b), but as cells grew prior to division a mucilaginous layer was secreted between the theca and fibrillar wall (Fig. 7c), the wall becoming the mother cell wall (or gel sheath) surrounding the new daughter cells. This process continued as colonies became larger, with cells at the core of larger colonies surrounded by up to 6 or more gel sheaths (Fig. 7h).

Observations on Aureoumbra

For comparison with the new taxa, we also investigated a representative of the related genus *Aureoumbra*. Our Australian strain of *A. geitleri* consisted of large masses of cells surrounded by a sticky mucilage that bound them to the substratum (Fig. 8a). Pre-division cells measured 10-12 µm in diameter, while following division daughter cells measure 6-8 µm. Parietal lobed chloroplasts, with stalked pyrenoids, dominated the cytoplasm (Fig. 8b). Small numbers of zoospores were occasionally observed, settled and flattened out on the substratum. The general fine structure of our strain of *A. geitleri* cells was virtually the same as for the *Gazia* and *Glomerochrysis* species described above, including chloroplast lobes, distinct pyrenoids, Golgi stacks, regions of ER, basal bodies and a characteristic thin, perforated theca sitting just off the PM (Fig. 8, c-e). The theca measured 35-45 nm in thickness and was separated from the PM by an opaque layer also observed in *Gazia* and *Glomerochrysis*. Thecal pores were observed in glancing section and measured 14-18 nm in diameter and were so tightly packed it was hard to discern any kind of matrix between the lined pores (Fig. 8f).

DISCUSSION

Our phylogenetic results based on five chloroplast genes and nuclear 18S rDNA sequences show that *Gazia, Glomerochrysis* and *Aureoumbra* form a clear lineage within the Sarcinochrysidaceae (Pelagophyceae). In addition to their affinities based on DNA sequences, the three new species described here, along with a strain of *A. geitleri* from Australia, are all sand-dwelling, sticky and with a dominant benthic stage where cell division occurs. The four species also occasionally produce heterokont zoospores that have two obvious roles, to extend the range of the species in their tide pool environment, and to re-establish the benthic stages within favourable light conditions if covered by sand. There is one key fine structural feature, a perforated theca, that species of this lineage as well as most other pelagophytes share (see below), although their general habit differs considerably from one another and there is no defining morphological feature that is localized to this lineage alone.

The habit of the two unicellular species studied here, *Gazia australica* and the Australian strain of *Aureoumbra geitleri,* differs significantly, the former a solitary, sand-dwelling benthic cell while the latter was observed in field samples as a mucilaginous, sticky mass of cells (a palmelloid colony) that adheres to and binds sand. Surprisingly, the Australian strain described here appears to differ from the type of *A. geitleri*, which was not benthic but described as "often free" or as a "sheet-like growth of cells found floating on the water surface in culture vessels, but not in nature" (Han et al. 2018). Despite the differences in habit, their DNA sequences are nearly identical (at most 1 bp difference between the Australian and published strain BEA0312B in 18S rRNA, *psa*A, *psa*B, *psb*A, *psb*C and *rbc*L; sequences available on Genbank: Table S1). This suggests that the differences may be explained by variations in the culturing conditions and/or the age of the respective cultures since their establishment. We cannot say whether the Australian strain was also planktonic in the field, as we only isolated and assessed species that adhered to surfaces.

The clusters of sarcinoid colonies in cubic packets observed in *Glomerochrysis psammophila* differ from other species in their lineage, but this morphology is common throughout the Pelagophyceae, particularly in the benthic species of *Chrysoreinhardia, Sarcinochrysis, Sargassococcus* and *Sungminbooa* (Han et al. 2018). Although zoospores are occasionally observed, colonies only reach a certain size before fragmenting into smaller colonies as the outer gel sheaths appear to break down. Fragmentation would be seemingly enhanced with clusters subjected to the movement of tide pool sand. Once again, the relationships in the lineage are only defined by the DNA sequences and the presence of a perforated theca.

The novel colony within a colony life stage and developmental cycle found in *Gazia saundersii* is not similar to any stage in the pelagophytes, heterokonts or algae in general. The development of a vegetative colony derived from a single cell is common throughout the algae, but the precise differentiation, segregation and release of a large number of smaller daughter colonies packed within a mother colony is unique. Superficially, *Volvox* gonidia are reminiscent of *Ga. saundersii* colonies within a mother colony, but the formation process is different, as there is no inversion and no flagellate cells (Starr 1984). *Phaeobotrys solitaria* also appears superficially like *Ga. saundersii,* but only daughter cells within a distinct colonial wall are present, i.e. no daughter colonies form within the parental wall (Ettl 1966). There are several benthic pelagophyte genera, including *Glomerochrysis psammophila* described here, with daughter colonies inside a mother colony (e.g., *Sarcinochrysis* and *Sungminbooa*; Han et al. 2018). However, these daughter colonies (like many colonial algae) are only held within a gelatinous matrix and they lack a well-defined multilayered casing as found with *Ga. saundersii*. Only *Ga. saundersii* has a colony of colonies with a strong, multi-layered casing that is maintained during mother colony formation and expansion, and during the differentiation of daughter colonies.

In *Gazia saundersii* the great majority of mother colonies generate daughter colonies that are virtually all the same size (e.g., Fig. 2, f and g), although interspersed with a few smaller daughter colonies that may arise from the few cells that are left behind following the initial segregation, but nonetheless used in colony formation. It is not known what triggers the sudden differentiation, segregation and discharge of daughter colonies, nor the alternative coordinated differentiation and uniform release of an enormous mass of motile zoospores (between 2000-3000 in large mother colonies). The release of zoospores coincides with the simultaneous rupture of the mother cell casing as well as any internal cell walls/gel sheaths/mucilage layers that would hinder the escape and distribution of the short-lived zoospores. Daughter colonies and zoospores are rarely left behind in spent mother cell casings, having been all quickly expelled out of mother cells as if an internal pressure is released, while zoospores are also motile.

An interesting question is when and how are the thick, multi-layered casings of daughter colonies differentiated within mother colonies of *Gazia saundersii*, seemingly from one or more gel sheaths/mucilaginous layers? Likewise, how are the internal gel sheaths/mucilage layers eliminated so that zoospores can be released unencumbered from disintegrating mother colonies? Mother cell casings appear to rupture in one or more places during daughter colony release, but casings often appear to disintegrate during zoospore release resulting in a quick dissemination of the zoospores. In other sand-dwelling pelagophytes we have observed in culture, including *Ga. australica, Glomerochrysis psammophila* and *Aureoumbra geitleri*, zoospores are only occasionally differentiated from dividing benthic cells under unknown conditions, though prevalent at the beginning of the light cycle (also in *Andersenia;* Wetherbee et al. 2015). This slow but continuous release of zoospores is one long term strategy for survival in sand, whereas in *Ga. saundersii* zoospore release is instantaneous and synchronized for maximum dissemination, presumably initiated under specific conditions that would best suit dispersal in a dynamic tide pool habitat.

One feature in this lineage that has not been described for any other pelagophyte is the presence of a perforated theca, defined here as a dense outer covering or sheath penetrated by distinctive pores. Thecae totally enclose benthic cells and zoospores except where basal bodies are positioned, and where flagella emerge in zoospores. Thecae are distinctive from cell walls/gel sheaths that are fibrillar, considerably less dense and totally encase benthic cells. Zoospores are surrounded only by a theca, no cell wall. In addition to the species described in this paper, we have observed a theca with distinct pores in pelagophytes from other lineages where material was fixed by HPF (Wetherbee, unpublished). The fixation protocols utilized for TEM can greatly alter the results, particularly for extracellular walls and mucilaginous layers that can be heavily distorted by chemical fixatives. HPF can also produce artefacts, mainly through ice damage, but can give consistent results for smaller cells, including the pelagophytes studied here and other small heterokonts such as *Chrysoparadoxa australica* (Wetherbee et al. 2019a). The size of thecal pores makes them difficult to observe, as they are only 14-24 nm in diameter (less than a microtubule). Likewise, as our thin sections are generally 70-80 nm thick, pores are largely masked in cross-section by the electron dense nature of thecae. However, pores can be readily observed in glancing sections at high magnification.

A theca has been previously described for a number of species now recognized as belonging to the Pelagophyceae, though structural details vary significantly. A theca was first described in what has become known as *Ankylochrysis lutea* (van der Veer 1970) and was comprised of three parallel electron dense layers, two smooth layers with an outer layer comprised of "tiny bodies". The thecal layers were very electron dense, and pores cannot be discerned in their figures. However, a subsequent fine structural study of this same species revealed a "bilayered external covering", or theca (Honda and Inouye 1995). There is no mention of pores, but they describe scale-like structures within the cells and on the cell surfaces. The authors also claimed a similar structure in *Sarcinochrysis* sp. and *Chrysonephos* sp., though no results were published. A thick, three-layered cell wall was described for *Pelagococcus* (Vesk and Jeffrey 1987), and though the authors did not mention a theca, several glancing sections seem to show a perforated theca amongst cell wall and mucilage layers (figs, 8, 11, 12 in Vesk and Jeffrey 1987). Additional reports of pelagophytes that could possess a perforated theca if fixed by HPF include the "exocellular polysaccharide-like layer" in *Aureococcus* (Sieburth et al. 1988), a thin, non-descript theca in *Pelagomonas* (Andersen et al. 1993) and a thin layer of "extracellular material" that runs parallel to the PM in *Aureoumbra lagunensis* (DeYoe et al. 1997).

Thecae vary considerably in thickness between the species studied (12-80 nm) but were always observed to encase benthic cells and zoospores except where basal bodies were positioned. The pores varied in size and distribution between species, ranging between 14-24 nm in diameter, appeared lined by an electron dense layer and were either randomly scattered over the surface or packed so closely there was difficulty discerning the thecal matrix. In *Gazia australica*, there was a second type of micropore not seen thus far in other species.

Based on morphology and position, we suggest that the perforated thecae functions in two ways. First is to give structure and strength to the cells (e.g., protection against salinity fluctuations), as do surface structures in other protists (e.g., cell walls, periplasts, frustules, etc.). Secondly, the theca appears to provide a platform for the synthesis of extracellular walls/gel sheaths and mucilage layers, which can be extensive in some species. Precursors for extracellular layers would quickly migrate from the cells through the thecal pores prior to the sequential formation of complex extracellular layers. In unicells like *Gazia australica* and *Aureoumbra geitleri*, a single cell wall layer forms on the outer surface of the theca, though cells may synthesize substantial quantities of adhesive mucilage that is so highly hydrated that it is not electron dense nor does it take up cytological stains well. In the more complex, multicellular colonies and clusters like seen in *Ga. saundersii* and *Glomerochrysis psammophila*, the theca provides a surface for the sequential formation of several thick cell walls and mucilage layers (Fig. 7, a-c and h). Although all cell walls are necessarily porous to a degree, the passage of precursor materials through the theca would seemingly be enhanced by the large pores. In addition, almost all sand-dwelling pelagophytes, regardless of size, possess a surface adhesive that is synthesized within the cells, secreted and maintained as the colonies enlarge over time. In addition, pores could also function in the quick mobilization of glycosidases for the breakdown of cell walls/gel sheaths and mucilage layers prior to zoospore release in *Ga. saundersii.*

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Fig. 1. Maximum likelihood tree showing the phylogenetic position of *Gazia* and *Glomerochrysis* among pelagophytes. Numbers on branches indicate bootstrap support and scale is in estimated substitutions per site.

Fig. 2. Vegetative colonies of *Gazia saundersii* gen. et sp. nov. (a) Vegetative colonies of various size, each surrounded by an overall casing (arrows). The outline of individual cells can be observed tightly packed within the colonies. (b) Group of large colonies that were too dense to make out daughter colonies or cells. (c - g) The casing of large colonies ruptured in several places and released numerous daughter colonies. The smaller colonies were irregularly shaped until separated from the surface of the mother colony where they became spherical. (h) Daughter colonies started to separate prior to release from the mother colony, as observed by the formation of spaces between them. (i - k) Colonies stained with toluidine blue. (i) small colony with distinct casing (arrows). (j)

The multilayered casing, 2.0 – 3.0 µm thick, with a distinctive outer layer (arrows) and a thinner inner layer (arrowheads) surrounding a layer that does not stain. Casings on baby colonies can be observed (white arrow). (k) A large colony has ruptured at a single point (asterisk) releasing a large quantity of zoospores. Scale bars = 20 µm (a,d,e,h), 100 µm (b,f), 50 µm (c,g), 10 µm (i), 5 µm (j), 40 µm (k).

Fig. 3. Stages in the vegetative cycle of *Gazia saundersia* gen. et sp. nov. (a) The casing of a medium sized colony has disintegrated releasing a large quantity of zoospores. In the video from which the image was taken, approximately half the zoospores were motile while the other half were still in stages of becoming motile, or had already retracted their flagella and were planktonic. At the same time, the slide and overlying coverslip were covered with settling zoospores. (b-d) Zoospores (5.0 - 6.0 µm in width, 7.0 – 8.0 µm in length) were pear-shaped, with two heterokont flagellar inserted subapically in a slight depression (arrows). A long, immature flagellum (lf) was directed anteriorly and c. 1.5 – 2.0 times the length of the cell, with the shorter, mature flagellum (sf) appressed against the cell surface and approximately the same length as the cell. Settlement was initiated when the tip of the long flagellum strongly attached to the slide or coverslip surface (arrow in d). (e) Zoospores settled on their dorsal side away from the flagellar insertion site (arrow), flattened out on the slide/coverslip and withdrew their flagellar axonemes, leaving the remaining membranes floating in the media. (f, g) Benthic cells enlarged prior to division (16 - 18 µm in diameter), had thin, compact thecae/cell walls that stained with toluidine blue (arrows). (h) Settled cells enlarged and divided twice in quick succession to produce 4 celled colonies (arrows) that became spherical, surrounded by their mother cell walls, now colony casings. Some colonies had already started to divide to produce 8-celled colonies. (i) Colonies continued to divide, each time doubling their number, to 8 and then 16 cells etc. The colony casing became thicker and more obvious as colonies enlarged (arrows). Scale bars = 25 µm (a) 5.0 µm (b-d) 10 µm (e-i)

Fig. 4. Light microscope images of *Gazia australica* gen. et sp. nov. (a) Loosely arranged raft of rounded to irregularly shaped cells flattened onto the slide surface, initially 8 -10 µm in diameter, but enlarged to 14 -16 µm prior to division. Two lobed, parietal chloroplasts are at the cell edges. Central cytoplasm is frothy with chrysolaminarin vacuoles that makes the opaque pyrenoids difficult to discern. (b) Recently settled cell showing the two lobes of the two chloroplasts (arrowheads), pyrenoids are observed in two lobes (arrows). (c, d) Cells stained with Toluidine Blue, in bright field (c) and DIC (d). Note the dense thecae/cell walls (arrows). No other outer covering was observed in this unicellular species. (e) Pre-division cell with multiple lobes of the two chloroplasts duplicated and aligned on the cell margins. (f) Daughter cells following division

were developing into zoospores with one flagellum showing (arrow). (g, h) Heterokont zoospores (5.0 – 6.0 µm in width and 6.5 - 7.5 µm in length), had a lateral insertion (arrow) of both the long, immature flagellum (lf) and short, mature flagellum (sf) in a ventral, narrow cavity (see also i and j). Two lobes of the chloroplast were in a posterior position and peripheral granules in an anterior position. Zoospore settlement was initiated when the tip of the long flagellum adhered strongly to the slide (arrowhead - tip not totally in focus). (i – k) Zoospore attachment occurred at the distal surface of the cell, exposing the ventral cavity where the flagella emerged (arrows). Cells flattened out to form the rounded benthic stage, the flagella were shed during the process (k). Note the two lobes of the chloroplast on the periphery of the flattened cell and the disappearing ventral cavity (k). Scale bars = 15 µm (a, c), 10 µm (b, d), 5.0 µm (e – k.0).

Fig. 5. Light microscope images of *Glomerochrysis psammophila* gen. et sp. nov. (a, b). Large, solid clusters of tightly adhered colonies of cells were visible with the naked eye. Individual colonies typically had packets of 4, 8, 16 cells (or larger) surrounded by one or more gelatinous sheaths depending of their size. (c) Cells were spheroidal, daughter cells 8-10 µm in diameter growing to 16-18 µm pre-division. Two deeply lobed chloroplasts (arrowheads) dominated the cytoplasm, each chloroplast with a stalked pyrenoid (arrows) that were hard to see in the compact cells. (d) Large cells (arrows) eventually divided twice to produce 4 celled colonies (asterisks) surrounded by the mother cell wall (now a gel sheath), each daughter cell contained one lobed chloroplast. (e) Cells of a 4 celled colony were dividing to form an 8 celled colony. One cell was yet to divide (asterisk) so there were 7 cells, only 3 in focus. The grandmother gel sheath is indicated by an arrow. (f) An 8 celled colony was in the process of enlarging to a 16 celled colony, with only 6 cells in focus. The upper 2 cells (asterisks) were yet to divide while 8 cells were out of focus. Note the great grandmother gel sheath (arrow). (g-i). Cells and colonies were lightly stained with toluidine blue to show the cell walls and gel sheaths. Cell walls (arrowheads), mother gel sheath (black arrow) and grandmother gel sheath (white arrow) were observed. (h) Packets of 8 cells in a cluster with walls and gel sheaths stained. (i) A great, great grandmother gel sheath (arrowhead) surrounded a 16 celled colony where cells were dividing again. Note the stained, closely fitting thecae/cell walls. Scale bars = 50 µm (a), 20 µm (b), 10 µm (c – i)

Fig. 6. Fine structural images of the benthic cells of *Gazia australica* gen. et sp. nov. and *Gazia saundersii* gen. et sp. nov. (a - f) *Ga. australica* cells chemically fixed except for d, which was HPF fixed and preserved the cell wall (cw), the theca (t) and PM (arrowheads). (a, b) Low magnification images of a benthic cell showed sections through chloroplasts lobes (c) including one with a pyrenoid (p). The electron dense, thin theca and cell wall was only just visible (arrowheads). (c)

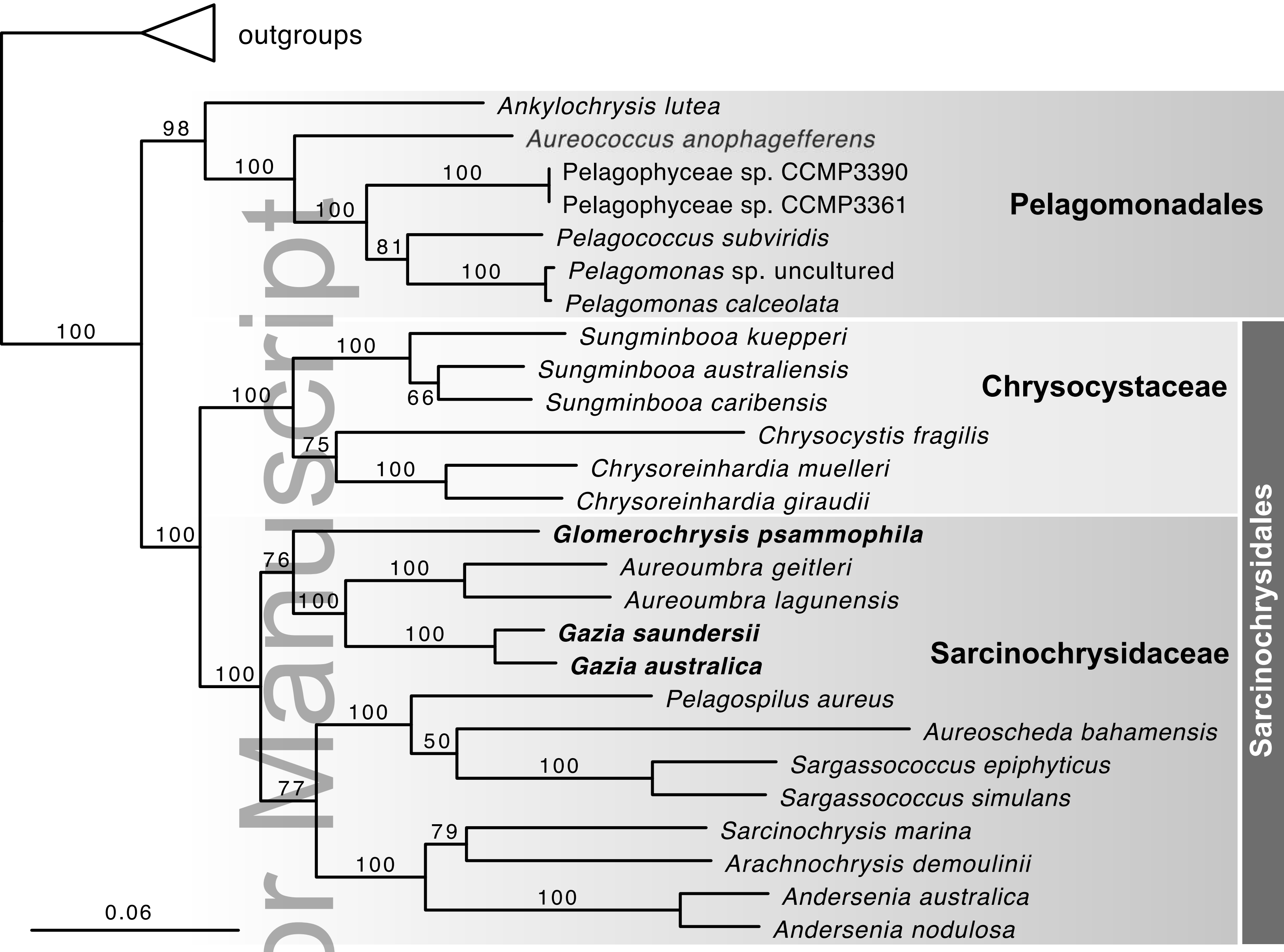
The theca encased the entire cell except in the ventral cavity where the theca ends (arrowheads) adjacent to where the basal bodies (bb) were located. (d). The cell wall (cw) was obvious in HPF fixed cells, adjacent to the theca (t), and separated by a narrow, opaque layer from the PM (arrowheads). (e, f) Macropores and micropores (arrowheads) are shown in high magnification. In glancing section, macropores were always the same diameter and were observed to penetrate the entire theca. A network of micropores was pervasive throughout the theca (t) and can be seen in both cross section and glancing section. Both pore types appeared to be lined with an electron denser layer against the thecal matrix. As the section*s* are 4-5 times the thickness of macropores, their structure and penetration was hard to make out in cross section alone. (g – j). *G. saundersii* cells had large Golgi stacks (G), well organized regions of endoplasmic reticulum (ER) and a thin theca (arrows) lying adjacent to the PM (arrowheads). (i) Pores were seen in glancing section (boxed) and in cross section (arrows) just above the PM (arrowhead). Note thin opaque layer between the PM and theca. (j) High magnification of area boxed in (i). Note that the pores were lined with an electron dense layer relative to the matrix of the theca. Pores appeared filled with material (arrowheads). Scale bars = 5 µm (a), 0.5 µm (b), 100 nm (c – h), 50 nm (i)

Fig. 7. Fine structural images of the benthic cells of *Glomerochrysis psammophila* gen. et sp. nov. (a – d) Low magnification, general fine structural features, including chloroplasts (c), nuclei (n), Golgi (G), endoplasmic reticulum (ER), basal body (bb), the theca (t), cell walls (cw), gel sheaths (gs) and intervening mucilaginous layers (mu). (a) Cells separated by multiple gel sheaths. (b) Cell walls were not stained and formed on the surface of the theca, adjacent to the PM (arrowheads), following division. Cell walls then separated from the theca with the appearance of a mucilage layer (arrows). (c) A mucilage layer was formed and the former cell wall is now a gel sheath. (d). A basal body in a benthic cell, though zoospores were rarely observed. (e – g) The thin theca and PM sandwiched an opaque layer that often had thin fibres connecting the two (arrowheads). Flattened vesicles (v) were common beneath the PM. Glancing sections revealed the packed pores in this species (arrows in g, boxed in f). (h) In large clusters, cells could be surrounded by many gel sheaths, in this case 6, with mucilage layers positioned between each. Scale bars = 2.0 µm (a), 250 nm (b - d), 100 nm (e - g), 0.5 µm (h)

Fig. 8. Light microscope and TEM images (HPF fixation) of an Australian strain of *Aureoumbra geitleri* cells. (a) Cells formed a gelatinous mass that sticks to sand grains. (b) Cells were irregular in shape, pre-division cells 10-12 µm in diameter, parietal lobed chloroplasts dominated the cytoplasm with pyrenoids that were best observed in TEM. (c, d). Low magnification, general fine structural features, including chloroplast lobes (c), pyrenoid (p), nuclei (n), mitochondrion (m), Golgi (G), basal body (bb), theca (arrows), plasma membrane (arrowheads) and cell walls (cw). (e)

Electron dense, perforated theca (arrows) was adjacent to the plasma membrane (arrowheads) and sandwiched a thin layer that does not stain. The cell wall (cw) likewise does not stain well in the HPF fixation. (f) Glancing section through the perforated theca. Pores (arrowheads) pack the theca and measure between 18-20 nm in diameter. Scale bars = 10 µm (a, b), 1.0 µm (c), 200 nm (d), 100 nm (e, f)

Table S1: GenBank accession information for sequences used in phylogenetic analyses, including new accession information for *Gazia australis*, *Gazia saundersii*, *Glomerochrysis psammophila*, and Australian *Aureoumbra geitleri.*



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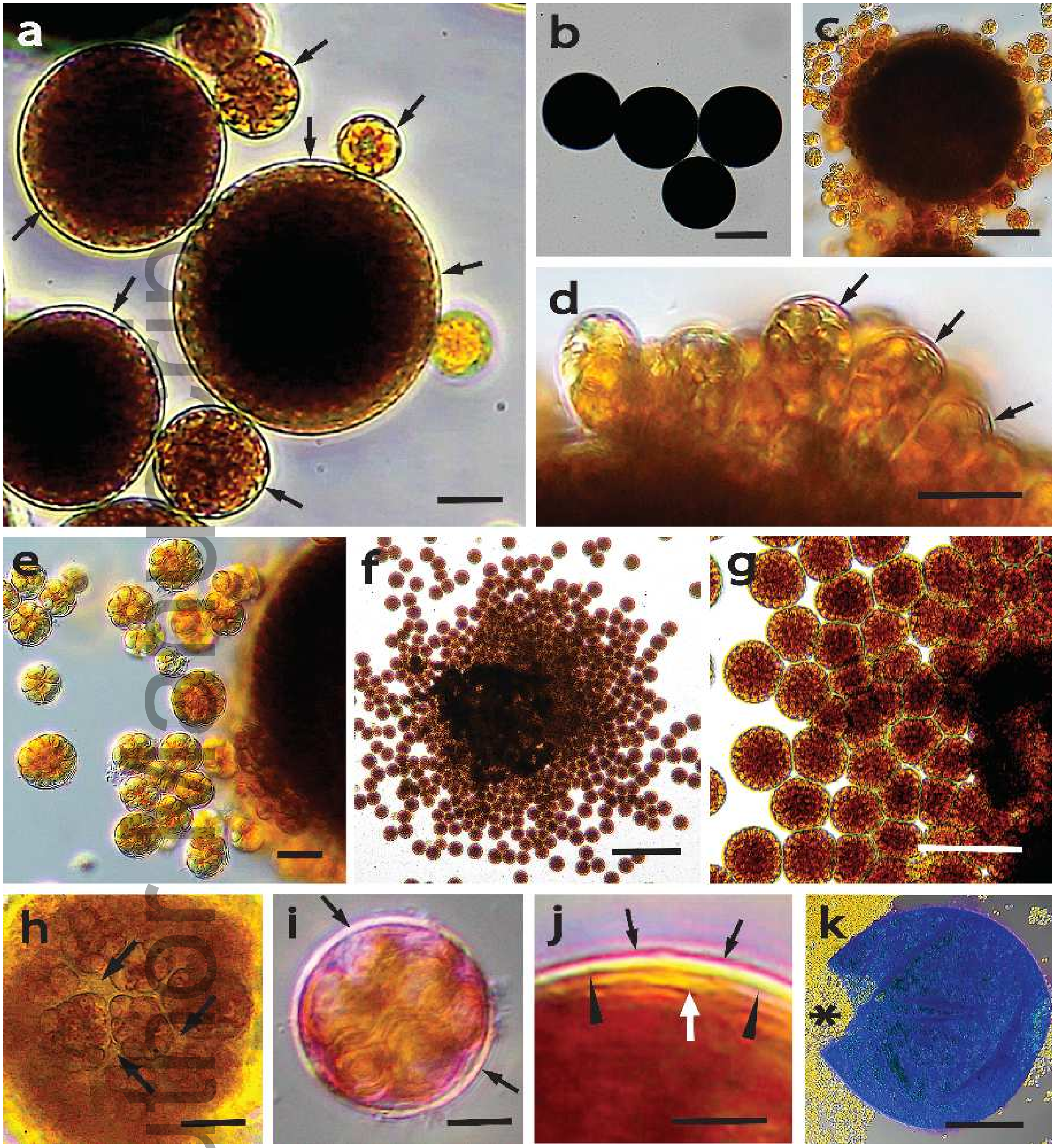

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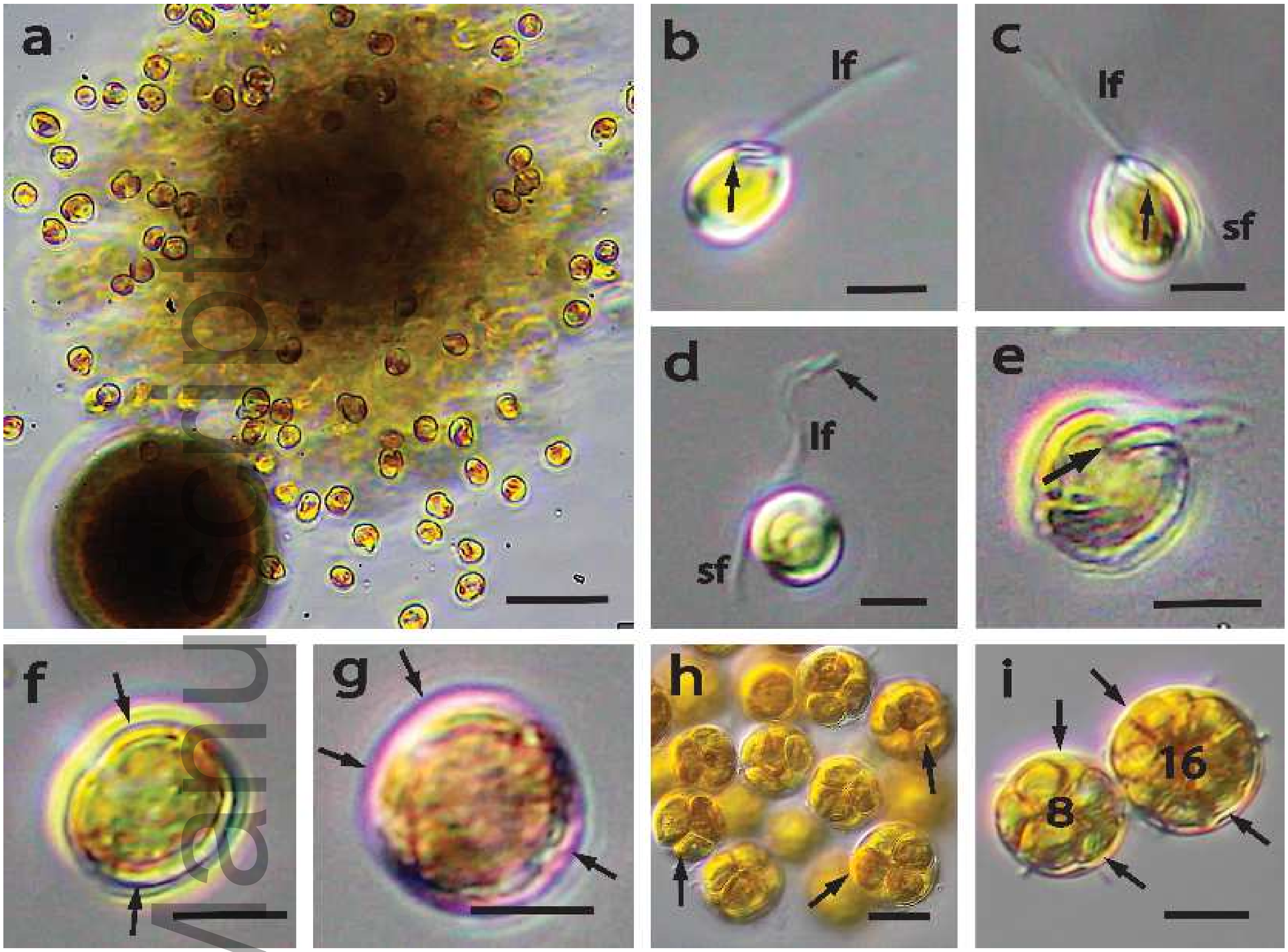

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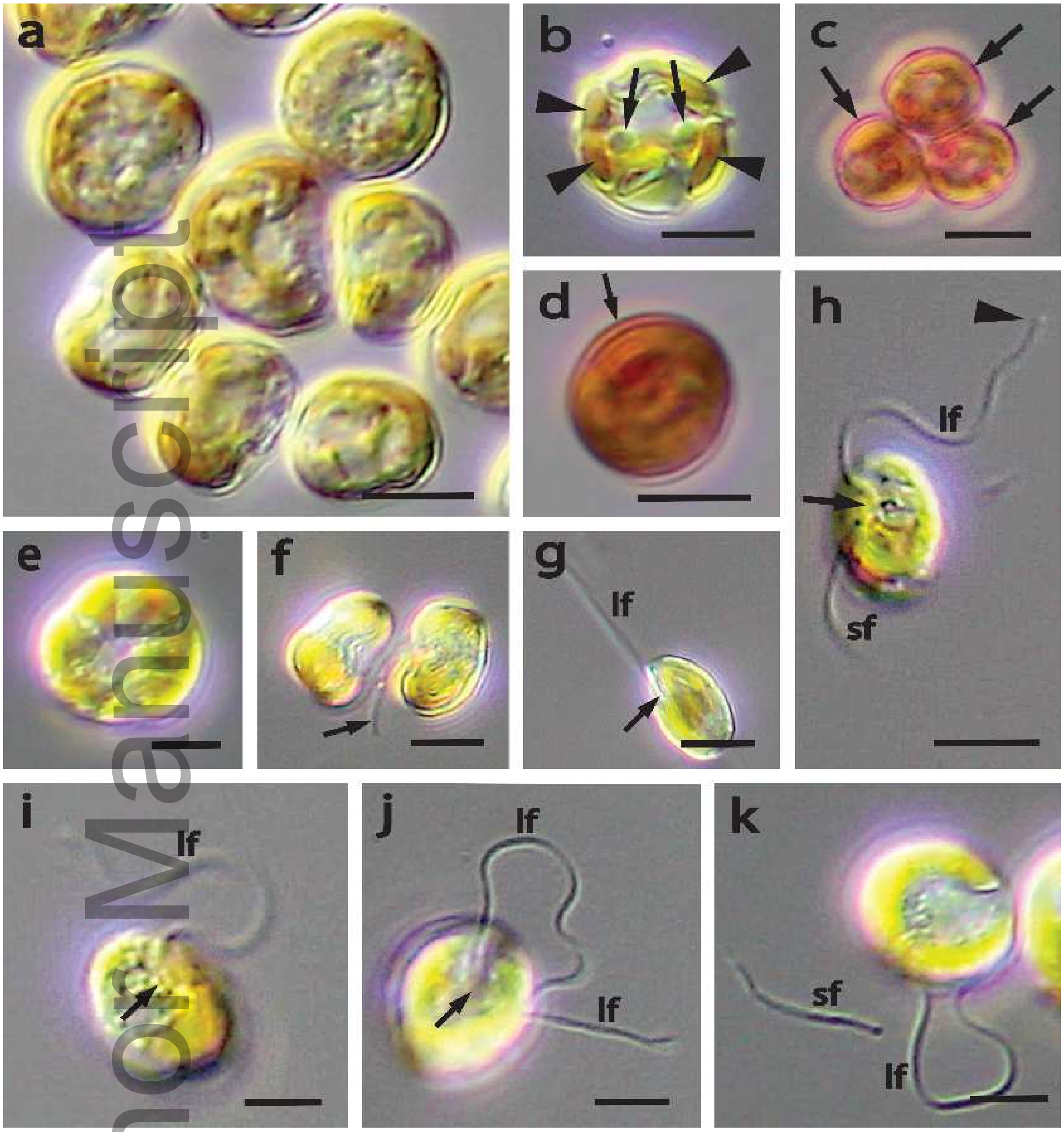

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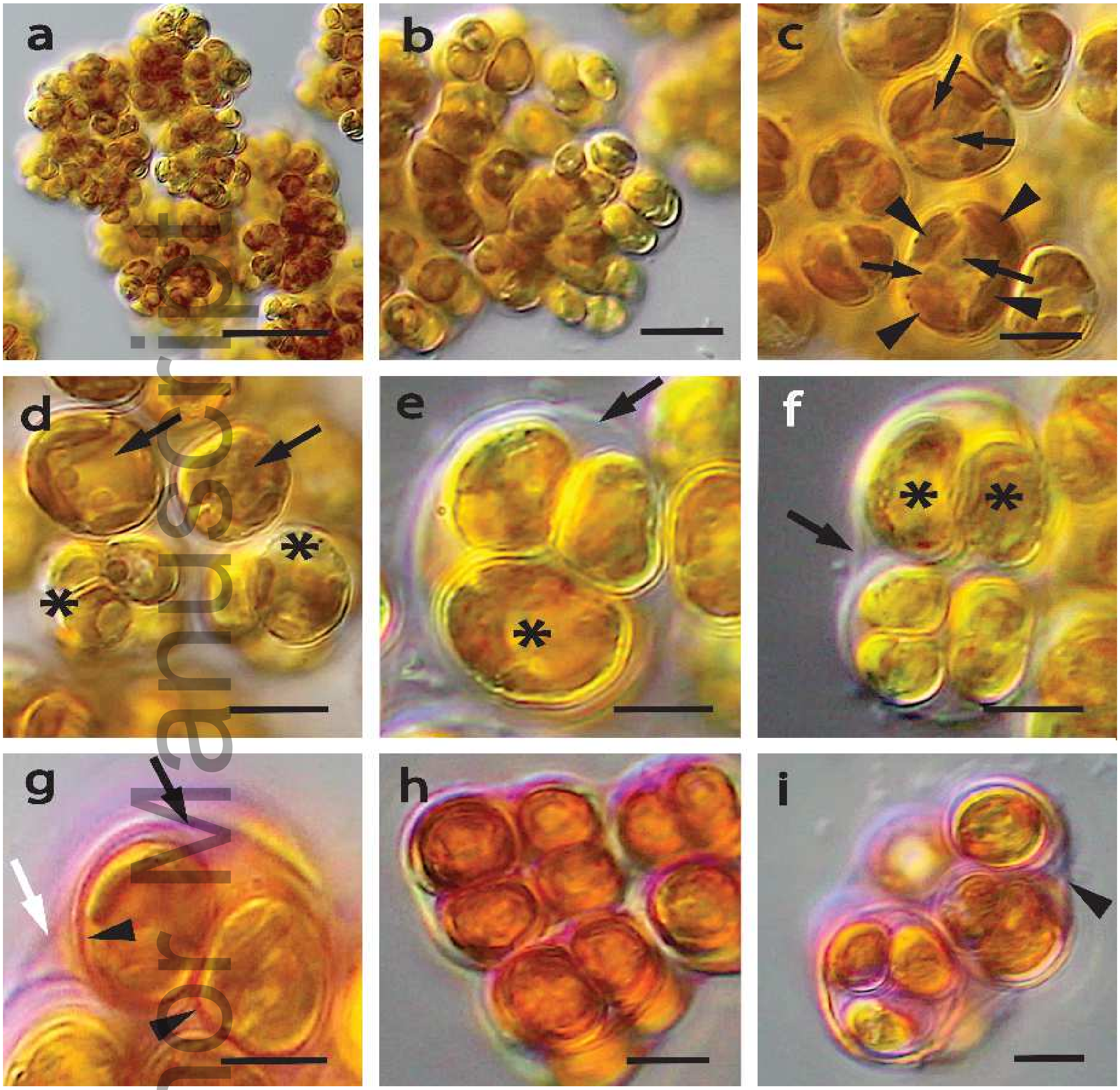

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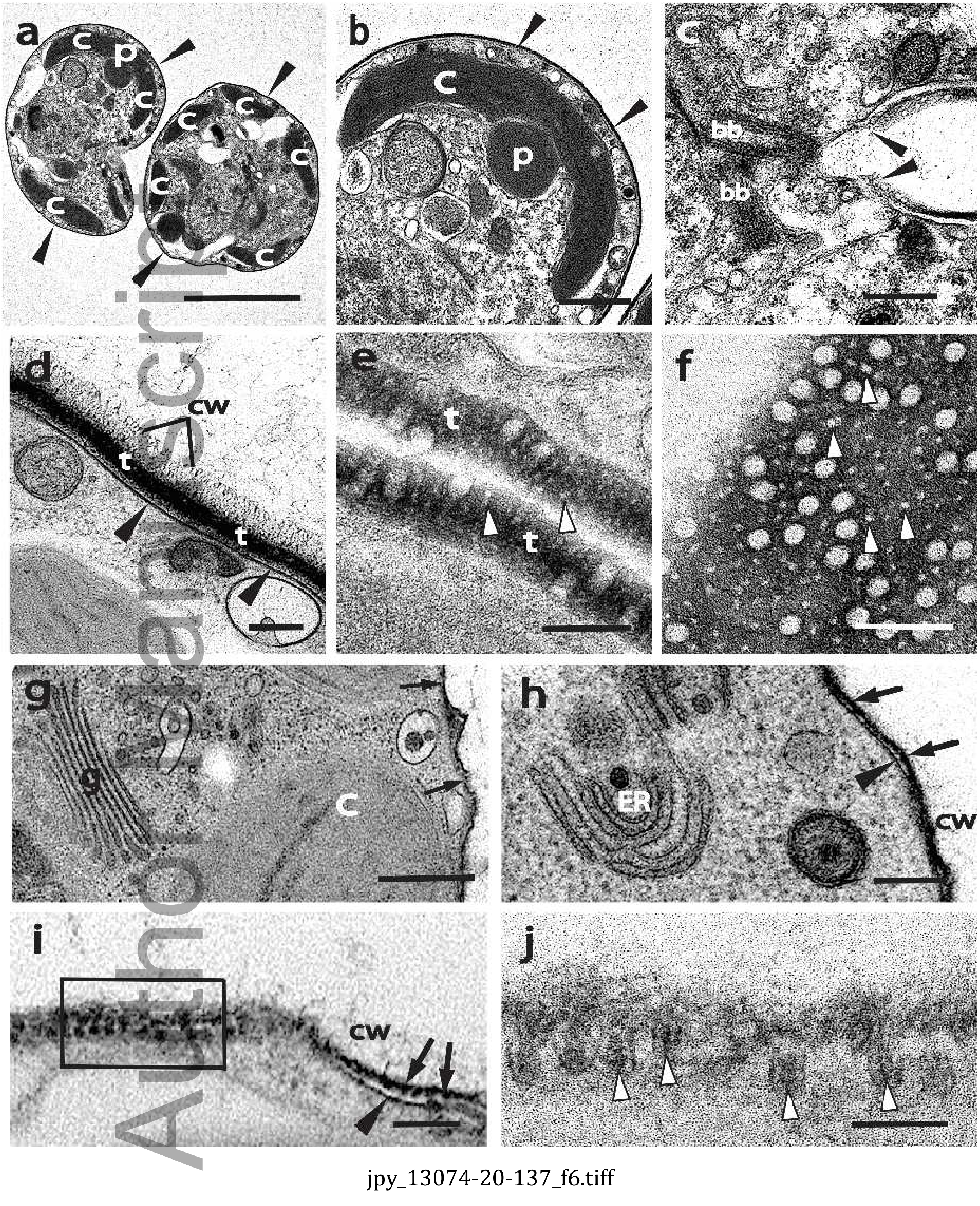

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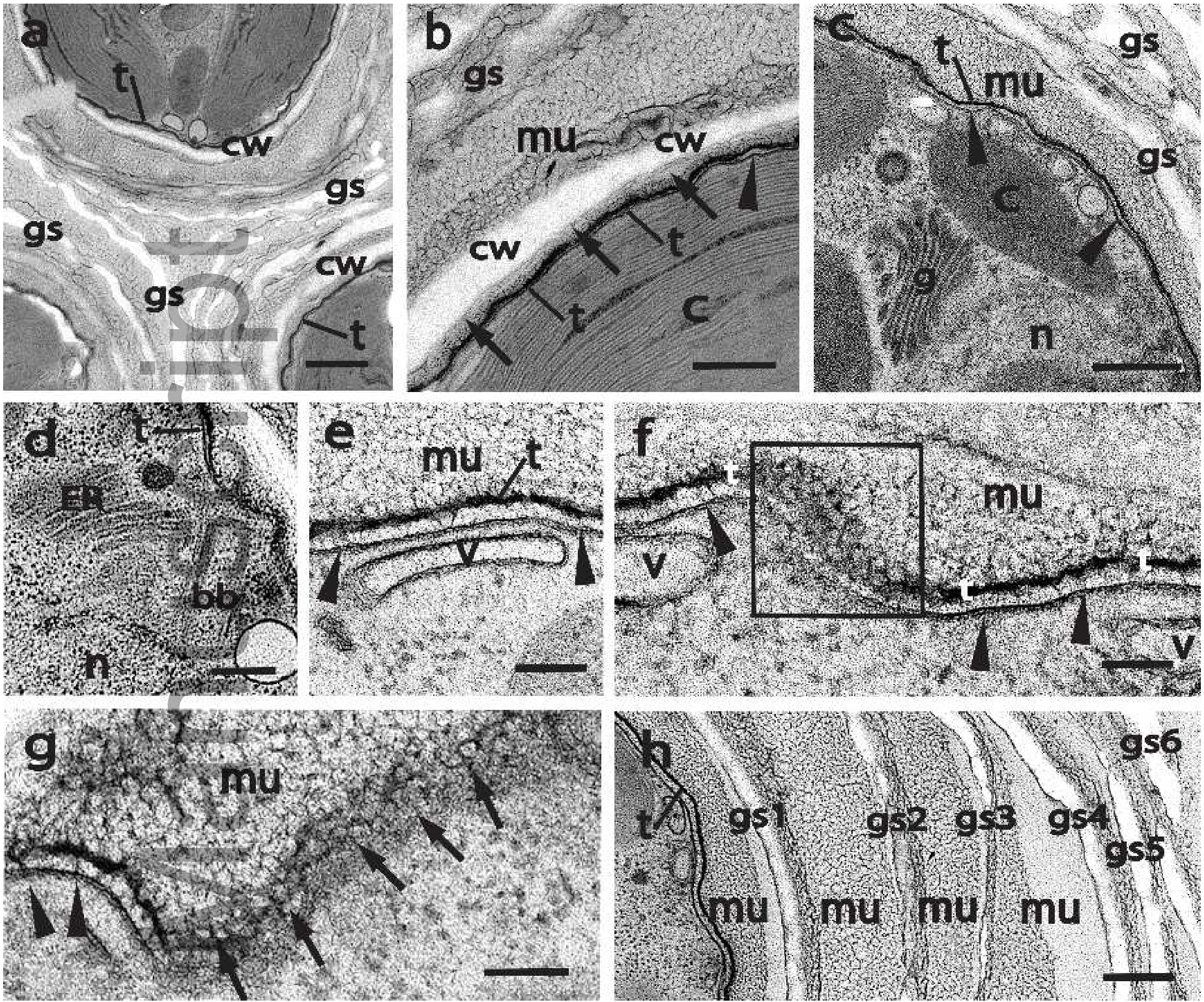

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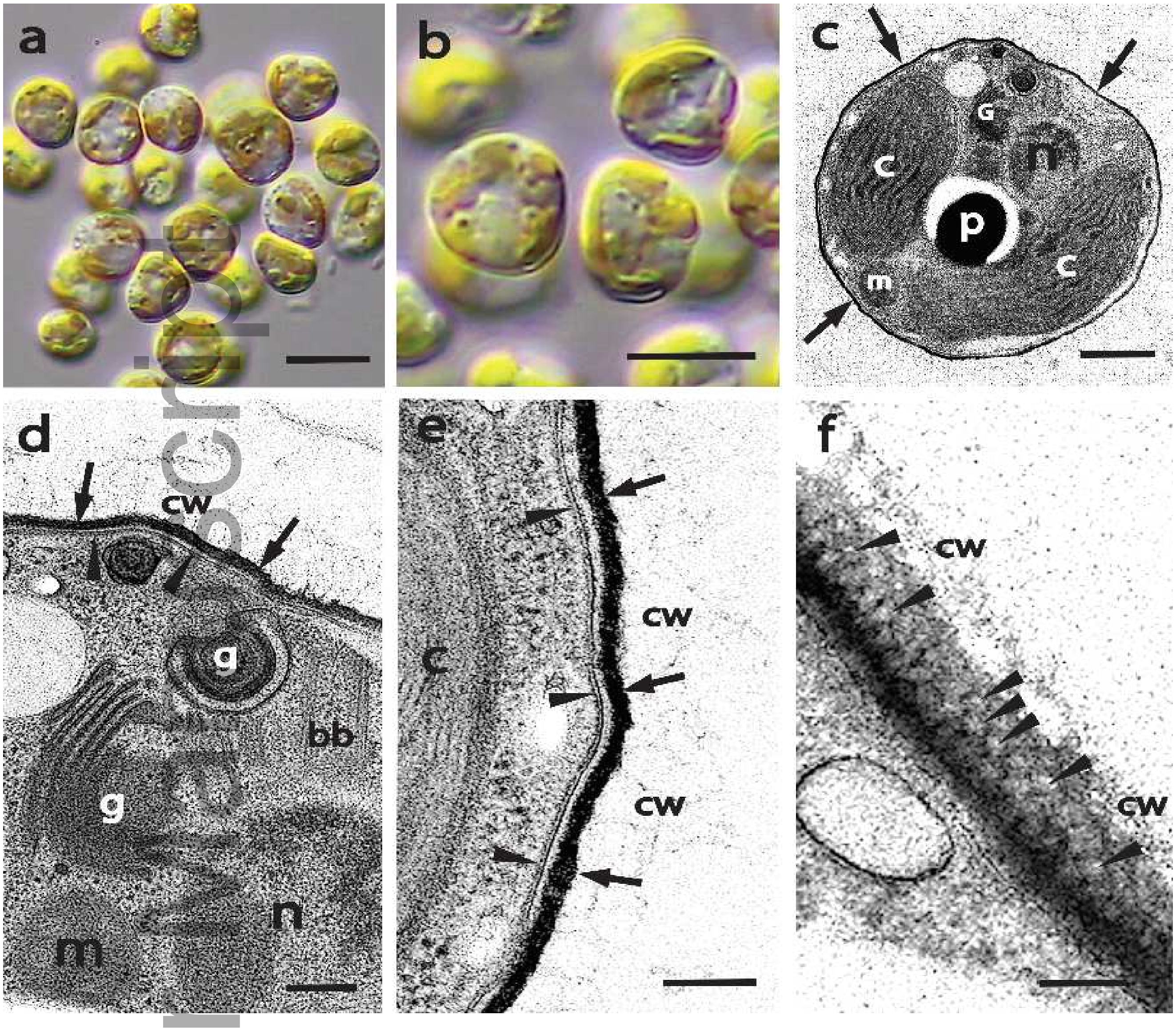

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