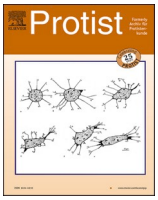
Protist 179 (2025) 126132

Contents lists available at ScienceDirect

Protist

journal homepage: www.elsevier.de/protis



Original article

Notable morphologies, molecular phylogenetics and taxonomy of the dinophyte *Borghiella tenuissima* (Borghiellaceae, Phytodiniales)

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ARTICLE INFO

Keywords:
development
dinoflagellate
freshwater
morphology
replication
reproduction

ABSTRACT

Mitotic division in dinophytes takes place either as desmoschisis or eleutheroschisis and usually, only one type is found in a species or group. Due to its big size, *Borghiella tenuissima* (Borghiellaceae) is a morphologically distinctive winter species. We collected plankton material in Germany and Poland, established unialgal strains and studied the morphology using light as well as scanning electron microscopy. We further obtained rRNA gene sequence data to embed them in molecular phylogenetics. Sequence data identified the strains as elements of the Borghiellaceae and showed no divergence among each other and to those of already published strains. The large flagellated cells had the characteristic, dorso-ventral flattened morphology of *B. tenuissima*, but many of them showed an undulated right margin of the hyposome. Contemporary material of *B. tenuissima* is remarkably smaller than stated in the protologue and other historical reports, which is why we have provisionally determined the studied material as *B.* cf. *tenuissima*. Moreover, *B.* cf. *tenuissima* exhibit different types of mitotic division (i.e., in the flagellated stage known as desmoschisis as well as in a deflagellated stage known as eleutheroschisis) at the same time, which might be more abundant among species of *Borghiella* than recognised before.

1. Introduction

The taxonomy and nomenclature of unarmoured (or gymnodinioid) dinophytes have seen major rearrangements since the dawn of molecular phylogenetics. As a result, a number of species has been assigned to lineages such as the Ptychodiscales or Tovelliales rather than to the Gymnodiniales. The Borghiellaceae have likewise been assigned as an element of the Phytodiniales (formerly classified as †Suessiales: Janouškovec et al., 2017; Moestrup and Calado, 2018; Müller et al., 2024) and from an evolutionary perspective, they may have lost the plate pattern characteristic of armoured (or thecate) dinophytes. Instead, Borghiellaceae have the amphiesmal vesicles covering the flagellated cell and containing very thin, plate-like structures of penta- or hexagonal shape in a honeycomb pattern (Moestrup and Calado, 2018). Moreover, a row of pores (visible as knobs) extends from a single, linear amphiesmal vesicle at the apex of the flagellated cell in the linear apical complex (LAC) of unknown function. An intraplastidic type B eyespot (Moestrup and Daugbjerg, 2007) has been further assigned to the ultrastructural traits of the group, but its presence is not shown yet for all species of *Borghiella* Moestrup, Gert Hansen & Daugbjerg.

Borghiella tenuissima Moestrup, Gert Hansen & Daugbjerg ex Moestrup & Calado is one of the few morphologically distinctive species of *Borghiella* due to the extreme dorso-ventral compression and the characteristic swimming behaviour. It was described from the German region of Ludwigshafen by Robert Lauterborn (1869–1952), who did not publish any image that could serve as original material (Lauterborn, 1894) but provided an illustration of the species only in a later publication (Lauterborn, 1899; here reproduced as Fig. 1a). According to Lange (1990), all (unpublished) research material of R. Lauterborn was destroyed during World War II.

Borghiella tenuissima is a stenotherm cold-water species, only occurring in the winter months (Steinberg, 1981) and preferring low light intensities (Höll, 1928; Schiller and Stefan, 1935). Cell size may change with the temperature of the water, from ca 36 µm in length in November (when the species starts occurring) to ca 65 µm under the ice in February (Schönhagen-Becker and Hegewald, 1979). Like in land plants,

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https://doi.org/10.1016/j.protis.2025.126132
Received 6 June 2025; Accepted 5 October 2025
Available online 9 October 2025

photoperiodism has been also considered for microalgae (Longobardi et al., 2022; Bruhn et al., 2024) such as *Borghiella ovum* A.Müll.bis & Gottschling (Müller et al., 2024), but the effect of the changing day length around seasons on microscopic organisms has not been studied in detail yet. Wołoszyńska (1918) was first to grow *B. tenuissima* in a jar on the windowsill at the Hydrobiological Station of Wigry (Poland).

In addition to light microscopic studies, information on morphology is available from scanning (Moestrup et al., 2008) and transmission electron microscopy (Crawford et al., 1971), and rRNA sequences have been obtained from two strains (GeoM*542: Gottschling et al., 2020; NORCCA K-0666: Hansen and Daugbjerg, 2004). Aside from the predominant flagellated cells, there is some confusion regarding the appearance of coccoid cells (of which R. Lauterborn himself was silent). It is illustrated as pentagonal in outline, with blunt edges (Wołoszyńska, 1917; reproduced as Fig. 1c), or reported as spherical (Stosch, 1973), whereas Popovský and Pfiester (1990) wrongly assigned the coccoid cell

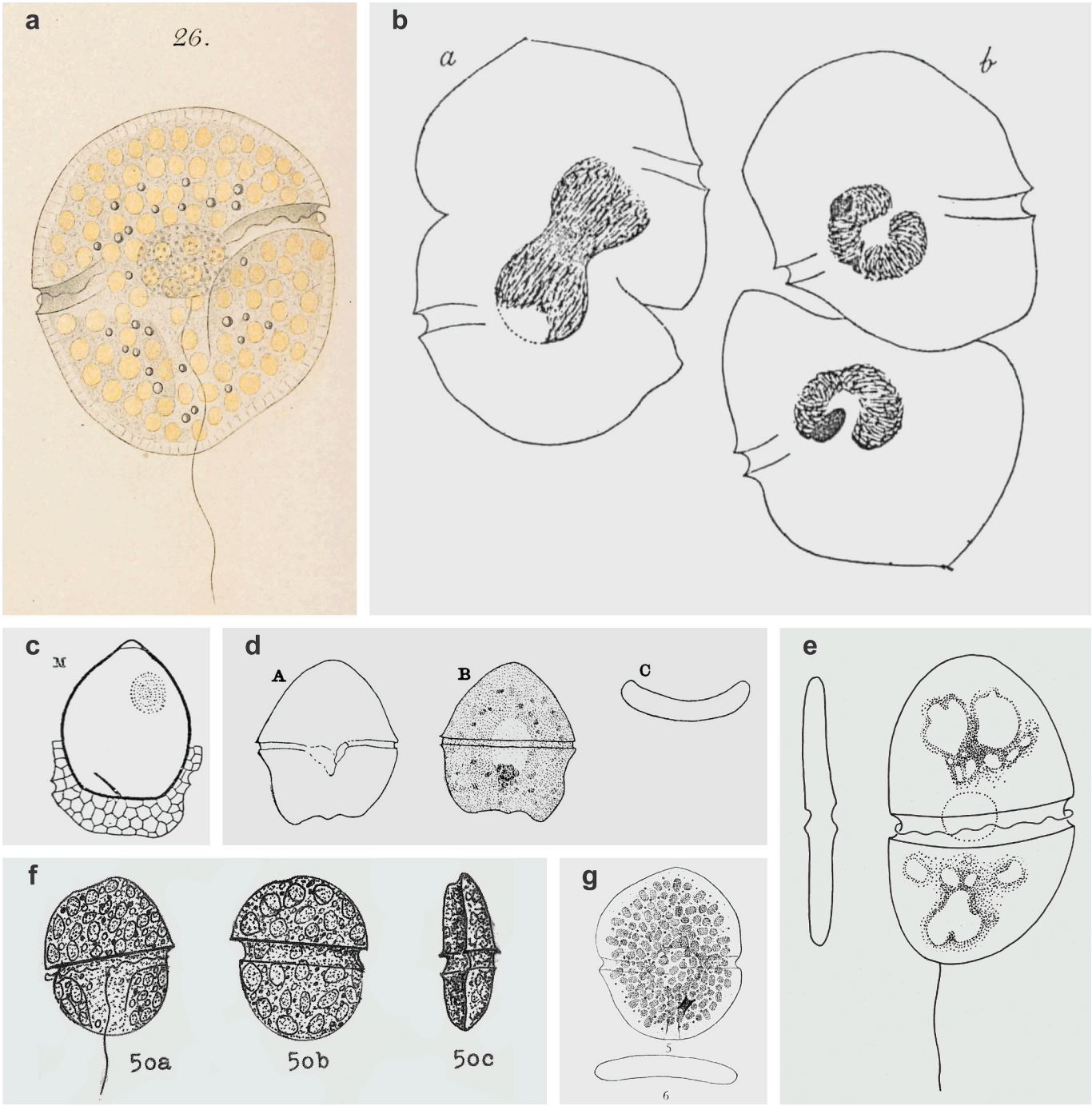

Fig. 1. Historical drawings with relevance to the study. a: Drawing of *Gymnodinium tenuissimum* (reproduced from Lauterborn, 1899; note that it does not necessarily represent original material, as it has been published later than the protologue; further note that the cell does not have an undulated right hyposome). **b:** Dividing cells of *B. tenuissima* (Wołoszyńska, 1918; note the unusual c-shaped nucleus in some of the cells). **c:** Coccoid cell assigned to *B. tenuissima* (Wołoszyńska, 1917; note the pentagonal shape in outline and the acute apex). **d:** *Glenodinium limos* (reproduced from Harris, 1940; note the undulated margin of the hyposome). **e:** *Cystodinium grabenseei* (reproduced with permission from a drawing published in Baumeister, 1969; and deposited at the Centre of Excellence for Dinophyte Taxonomy, CEDiT). **f:** *Glenodinium fungiforme* (reproduced with permission from Schiller, 1955; note the strong dorso-ventral compression). **g:** *Gymnodinium lens* (reproduced with permission from Fott, 1957; note the round outline of the cell's poles).

of *Tovellia leopoliensis* (Wołosz.) Moestrup, K.Lindb. & Daugbjerg (Tovelliales) to *B. tenuissima*.

Two fundamentally different types of mitotic (or asexual or vegetative) division are recognised in the dinophytes (Kwok et al., 2023). During desmoschisis, each daughter cell retains half the parent cover and newly generates the other half (leading to duplication of the parental cell, usually in the flagellated stage) whereas during eleutheroschisis, the parent cover is discharged (leading to complete regeneration of daughter cells, usually in the deflagellated stage). The terminology goes back to Groover and Bold (1969), who aimed at explaining differences in mitotic divisions of green algae and since Pfiester and Anderson (1987), it is also applied for dinophytes. With some phylogenetic correlation, most of them perform either desmoschisis (predominant in, e.g., gymnodinioid dinophytes and Gonyaulacales) or eleutheroschisis (predominant in, e.g., Peridiniales). However, particular dinophyte species exhibiting both types of mitotic division have been anecdotally noted in the younger literature (Figueroa et al., 2006, 2009; Tillmann and Elbrächter, 2013; Daugbjerg et al., 2014; Tillmann et al., 2020).

In this study, we present the morphologies of dinophyte strains from Germany and Poland that are identified as *B.* cf. *tenuissima*. They are consistent, but present a decent diversity of motile and immotile cells of various shapes, including cells interacting with each other. A considerable portion of the motile cells shows an undulated right side of the hyposome – these cells are buoyant, so teratology because of, for example, starvation is thus unlikely to assume (similar to *Gymnodinium undulatum* Wołosz.: Schiller, 1954). We report two different types of mitotic replication in *Borghiella* and in an experiment, the impact of temperature on cell size and shape is tested. We aim at increasing the knowledge of a supposedly widely distributed species of dinophytes with an ecological preference for the cold months of the year.

2. Materials and methods

Eight (seven new) strains were established by micropipetting from field material collected in Germany and Poland (details are provided in Table S1). Cultivation using freshwater WC growth medium (Woods Hole Combo, modified after Guillard and Lorenzen, 1972, without silicate) took place in climate chambers at 12 °C and a 12:12 h light:dark cycles. Four strains of living cells are still held in the culture collection at the Institute of Systematics, Biodiversity and Evolution of Plants (University of Munich) and are available upon request. To study the impact of temperature and photoperiod to cell size and morphology, strains GeoK*046, GeoK*065, GeoK*307 and GeoK*309 were cultivated in copy at 4 °C for four weeks. These copies were left in the chamber at 6:18 h light:dark cycles for further four weeks, and four new copies (from the original 12 °C and 12:12 h light:dark cycles) were also held under these conditions. After each experiment, cells were observed and measured in length and width.

Cells were observed and documented with a CKX41 inverted microscope (Olympus; Hamburg, Germany) equipped with a phase-contrast option. Images and videos were taken with a DP73 digital camera (Olympus) and if applicable, samples were covered with a droplet of Protogel (Protist Motility Inhibitor, C340; Manchester, UK). For nuclear staining, cells were treated with 4′,6-diamidino-2-phenylindole (DAPI, 10 μg ml^{-1} final concentration) for 10 min. For visualising of the nuclei, and also for observing chloroplasts applying autofluorescence, a DM1000 light microscope (Leica; Wetzler, Germany) equipped with a DAPI filter (Leica; excitation: 350/50, dichroic mirror: 400, emission BP 460/50) and an I3 filter (Leica; excitation: 450/490, dichroic mirror: 510, emission LP 515) was used as described previously (Romeikat et al., 2020). Methyl green (Roth; Karlsruhe, Germany) was also used for staining the nuclei, applying a concentration of 0.01 mg powder in 10 ml of WC medium. This 0.1 % solution was directly exposed to the slide already supporting the algal material. After an incubation time of 10 min, the nuclei were stained. Measurements of cells were made using the programs 'cellSens Entry' (Olympus) and 'Fiji' (https://imagej.net/software/fiji/).

For the preparation of permanent slides, cells of the strains were fixed with a 10 % formaldehyde (Roth), 5 % acetic acid (AppliChem; Darmstadt, Germany) and 50 % ethanol (Roth) formalin-aceto-alcohol solution in cacodylate buffer. Double-staining was performed using 0.5 % (water-based) astra blue in 2 % tartaric acid (Fluka; Buchs, Switzerland) in cacodylate buffer and 0.1 % (ethanol-based) eosin (Merck; Darmstadt, Germany) during a graded ethanol (Roth) series. Ethanol-based Technovit 7100 (Heraeus; Wehrheim, Germany) was used for embedding, following the manufacturer's instructions. For the final specimens, 40 ml aliquots of the Technovit mixture including the embedded samples were transferred to three microscope slides. The specimens are deposited at the Centre of Excellence for Dinophyte Taxonomy (CEDiT; Wilhelmshaven, Germany), and duplicates are held in the herbaria of Berlin, B and Munich, M (https://sweetgum.nybg.org/science/ih/).

The preparative techniques for scanning electron microscopy (SEM) were performed at room temperature and basically followed Romeikat et al. (2020) and Knechtel et al. (2020). As the dinophytes under study have thin and small amphiesmal vesicles, 1 ml of cells in WC medium were fixed with 500 μl of 1.5 % OsO_4 (Science Services; Munich, Germany) and incubated for 1 h. Afterwards, the cells were washed twice in cacodylate buffer in 10 min and 20 min intervals and with distilled water for 30 min, respectively. Cells were dehydrated using a graded acetone series (Roth) in 15 min intervals (10 %, 20 %, 40 %, 60 %, 80 %). For the last dehydration step, 100 % acetone was used, and this was repeated once for 15 min and then overnight. Each washing and dehydration step was followed by centrifugation (Eppendorf; Hamburg, Germany) at 300 rpm for 3 min. After critical point drying and mounting on aluminium stubs, cells were sputter-coated (BAL-TEC SCD 050; Schalksmühle, Germany) with platinum and supplied with Planocarbon (Plano; Wetzlar, Germany). The material was observed with a LEO438VP SEM (LEO; Cambridge, UK–England). All images were adjusted in Photoshop CS6 (Adobe; San José, USA–CA) and arranged with QuarkXPress 10.5 (Quark; Denver, USA–CO). The statistical calculations were made using Excel (Microsoft; Redmond, USA–WA).

Harvest of DNA and isolation, as well as PCR amplification and sequencing, are already described previously (Knechtel et al., 2020). To build the alignment, we defined three regions of the rRNA complex: SSU, ITS, LSU and studied a systematically representative set of Phytodiniales (Table S1, including information of the outgroup comprising Gymnodiniales and Dinophysales). We also performed NCBI Blast Searches (Altschul et al., 1990) and included a systematically representative set of *Borghiella* as well. Phylogenetic analyses were the same standards as applied in Knechtel et al. (2020).

3. Results

3.1. Light and electron microscopy

The morphologies of all strains were indistinguishable and therefore, they are described collectively. They exhibited motile (Figs. 2–3, 4d–j, S4a–b, Video S1) and immotile cells (Fig. 4a–c, k–m, S4c, Video S1), with an approximate ratio of 10:1. Motile, flagellated cells were circular in outline, dorso-ventrally strongly flattened and disc-shaped (Figs. 2, 3a–e, 4g–j). They swam in a coin-spin manner (Video S1). The episome was generally larger than the hyposome, and its apex was either round (Figs. 2a, c, g, k, 3c, e) or asymmetrically acute (Figs. 2f, 3a, d). The nearly complete cingulum was slightly descending, and the displacement was maximally half the cingulum width (Figs. 2a, 3a–e). The sulcus was rather shallow and extended neither to the antapex nor onto the episome (Fig. 3a, c–e). Sometimes, a protrusion at the intersection between cingulum and sulcus was noted (Fig. 3c–d).

Cell size was varying in a continuum, and no distinct classes could be observed, ranging from 24.3 to 45.1 μm (mean: 33.5 μm; SD: 4.3 μm; n =

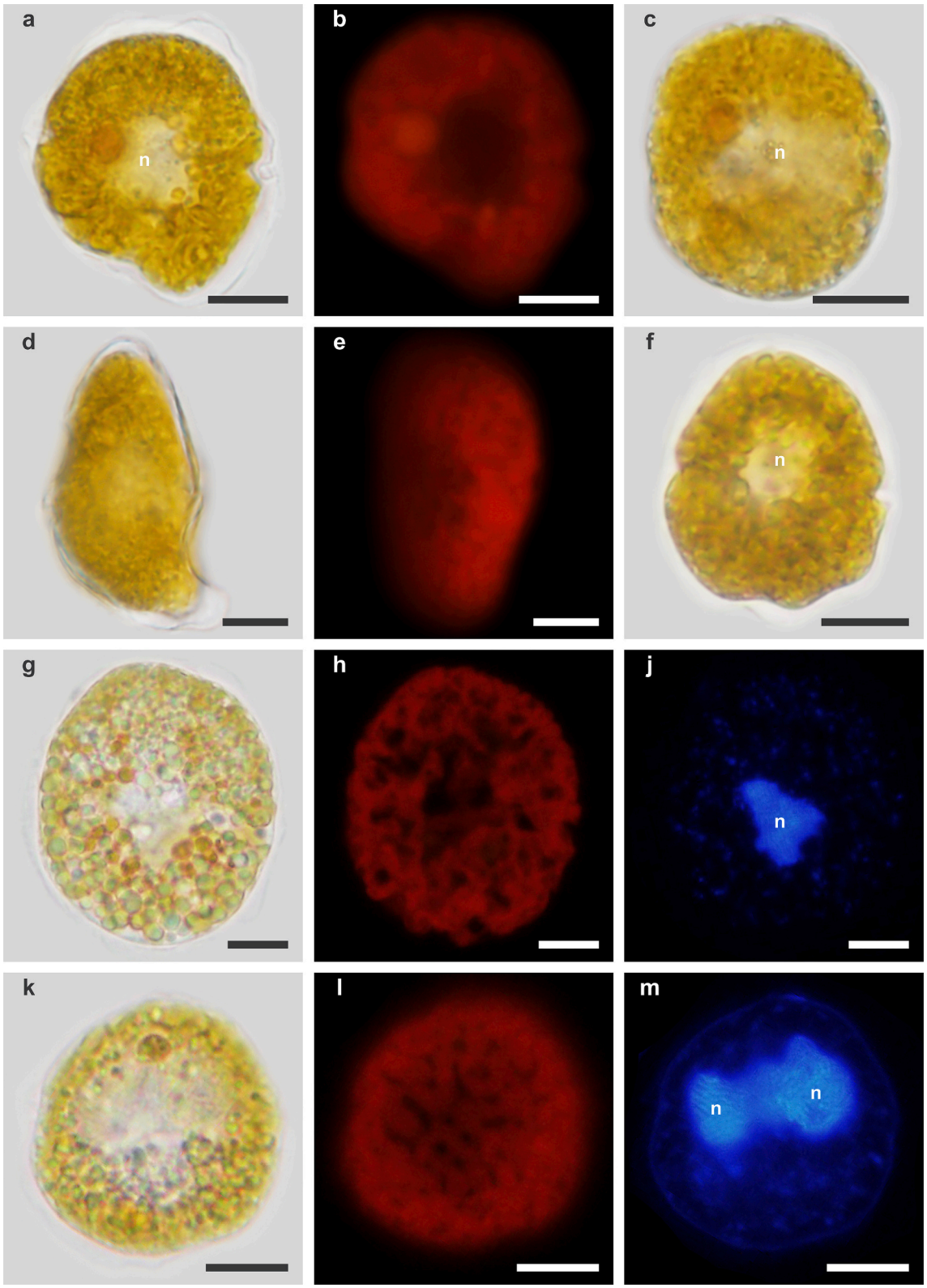

Fig. 2. Morphology of strains GeoK*046 (g–j), GeoK*065 (k–m), GeoK*307 (a–e) and GeoK*309 (f) in LM (a, c–d, f–g, k) and fluorescence LM (b, e, h–j, l–m). **a:** Ventral view (note undulated right margin of hyposome and orange cell component at the cingular level in the right-lateral side). **b:** Same cell as in a. **c:** Dorso-ventral view (note smooth right margin of hyposome and orange cell component at the cingular level in the right-lateral side). **d:** Lateral view. **e:** Same cell as in d. **f:** Dorsal view (note undulated right margin of hyposome). **g:** Dorso-ventral view (note smooth right margin of hyposome). **h:** Same cell as in g (note reticulate chloroplast). **j:** Same cell as in g (note central position of nucleus). **k:** Dorso-ventral view (note smooth right margin of hyposome). **l:** Same cell as in k (note reticulate chloroplast). **m:** Same cell as in k (note two connected nuclei). Abbreviations: n, nucleus. Scale bars: 10 μm.

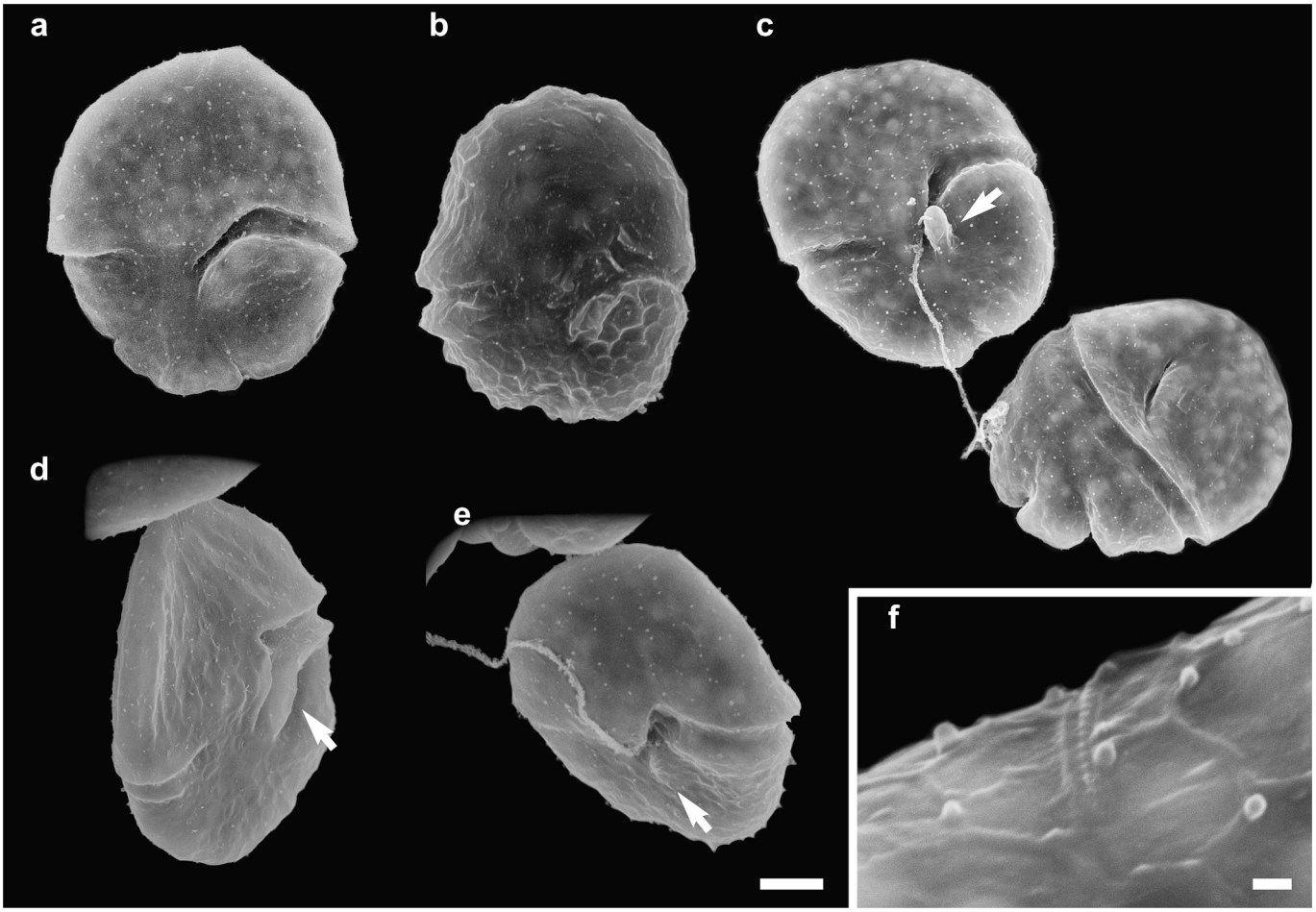

Fig. 3. Morphology of strain GeoK*065 in SEM. **a:** Ventral view (note slightly undulated right hyposome, descending cingulum, indistinct sulcus and monomorphous surface exhibiting small polygonal vesicles and knobs). **b:** Ventral view (note undulated right margin of hyposome and honeycomb pattern of vesicles more pronounced in hyposome than in episome). **c:** Ventral (top) and dorsal view (bottom) of two cells connected by the longitudinal flagellum of one cell (unclear whether this has biological meaning; note undulated right margin of hyposome, descending cingulum, indistinct sulcus and monomorphous surface exhibiting small polygonal vesicles and knobs; further note protrusion at intersection between cingulum and sulcus). **d:** Right ventral view (note smooth right margin of hyposome, descending cingulum, indistinct sulcus, monomorphous surface and protrusion at intersection between cingulum and sulcus). **e:** Ventral view, with the longitudinal flagellum reaching out to another cell (unclear whether this has biological meaning; note indistinct sulcus and honeycomb pattern of vesicles more pronounced in hyposome than in episome). **f:** Apical view (note linear apical complex). Arrows indicate protrusion at intersection between cingulum and sulcus, scale bars: 10 μm, except f: 1 μm.

208) in length and from 20.6 to 40.3 μm (mean: 28.9 μm; SD: 3.7 μm; n = 208) in width (Fig. S1). Decreased temperatures to 4 °C had a significant impact ($p < 0.05$) on cell size, and it became smaller rather than larger (e.g., mean length: 29.2 μm in Fig. S2). The parallel change of day length (to 6:18 h light:dark cycles) and temperature (to 4 °C) led to a significant increase ($p < 0.05$) in cell length up to 55 μm (mean length: 37.9 μm in Fig. S3). However, the protoplast of such large cells contracted and shrivelled remarkably within 10–15 min under the light microscope (Fig. 6d–e). Unusual morphologies were occasionally observed under these conditions in all strains (Figs. 6, S5), some of which with an acuminate apex or even with a stiff, stipe-like structure. More strikingly, smaller, much faster cells with a deviating morphology emerged (Fig. 6a–c, g–h), which always had a smooth margin of the hyposome. They accounted for 30–50 % of the motile cells only in the material maintained at 4 °C and 6:18 h light:dark cycles.

Back to the material cultivated under standard conditions, the right margin of the hyposome was undulated (Figs. 2a, f, 3a–c, S4a) in ca one third of cells (irrespectively of the geographic origin). The nucleus had a central position above the cingulum (Figs. 2j, m, 5a–f, S4a–b). The single reticulate chloroplast (or maybe two) was large and filled the cell (Fig. 2b, e, h, l). A larger number of (many) chloroplasts could be ruled out based on the observation of ruptured cells, whereby the lobes remained interconnected to each other and were not separated into numerous organelles. An eyespot was not observed. Cells were surrounded by a periplast of many pentagonal or hexagonal vesicles, and some knob-like structures were scattered over the surface in an arbitrary pattern (Fig. 3a–f). The sutures between the vesicles were more or less distinct, of varying width and if differentiated, then more pronounced on the hyposome than on the episome. On the episome, a furrow with small knobs as part of the LAC was observed (Fig. 3f). It was ca 3 μm long and extended diagonally over the apex.

Cell pairs in different shapes and different arrangements were observed on multiple occasions (Fig. 4a–j). An immotile type, deposited on the bottom of the cultivation flasks, comprised two cells (of equal or unequal size) included in a pellicle (Fig. 4a–c). It occurred in less than 10 %, and the fate of such cells (e.g., hatching) could not be determined. However, many empty pellicles were observed on the bottom of the cultivation flasks as well. Another type was represented by two connected cells (Video S1), which jointly swam together for 30 min and more. The pairs occurred in ca 10 % and rarely, the detachment of such cells could be observed (Video S1). These cells exhibited the shape of the regular motile cells (Fig. 4e) or were more or less rounded in outline

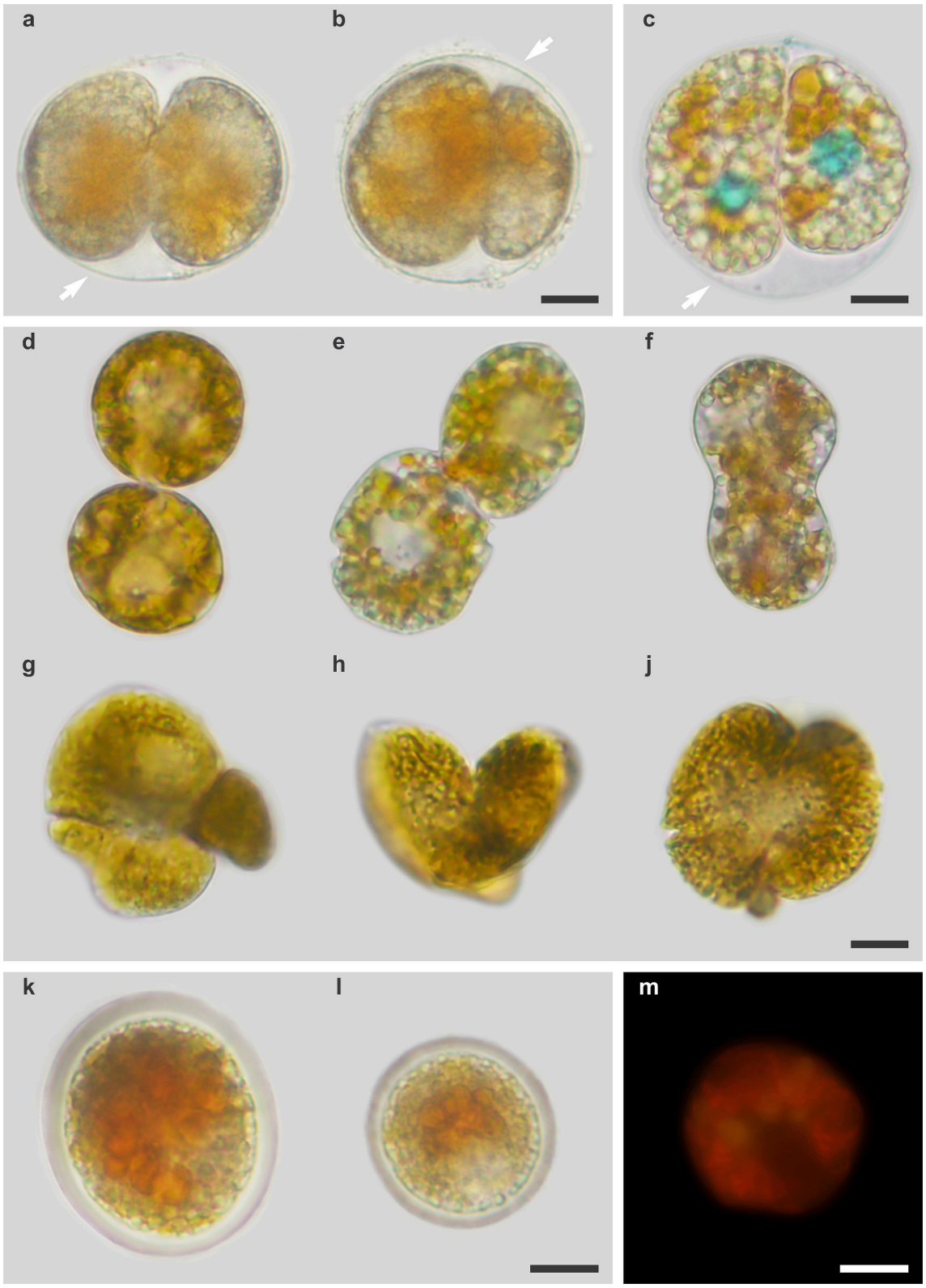

Fig. 4. Developmental stages of strains GeoK*307 (d, f–k, m) and GeoK*309 (a–c, e, l) in LM. a: Two cells included in a pellicle (eleutheroschisis; note same size of cells). **b:** Two cells included in a pellicle (eleutheroschisis; note unequal size of cells). **c:** Two cells included in a pellicle (eleutheroschisis; note nuclei after methyl green staining). **d:** Interconnected immotile cells (note serial orientation). **e:** Interconnected flagellated cells (note serial orientation). **f:** Motile, peanut-shaped cell. **g–j:** Two interconnected motile cells (all images are from the same cells, note perpendicular orientation). **k:** Coccoid cell (note ovate shape in outline). **l:** Coccoid cell (note circular shape in outline). **m:** Coccoid cell (note chloroplast indicating photosynthetic activity). Arrows indicate the pellicle, scale bars: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

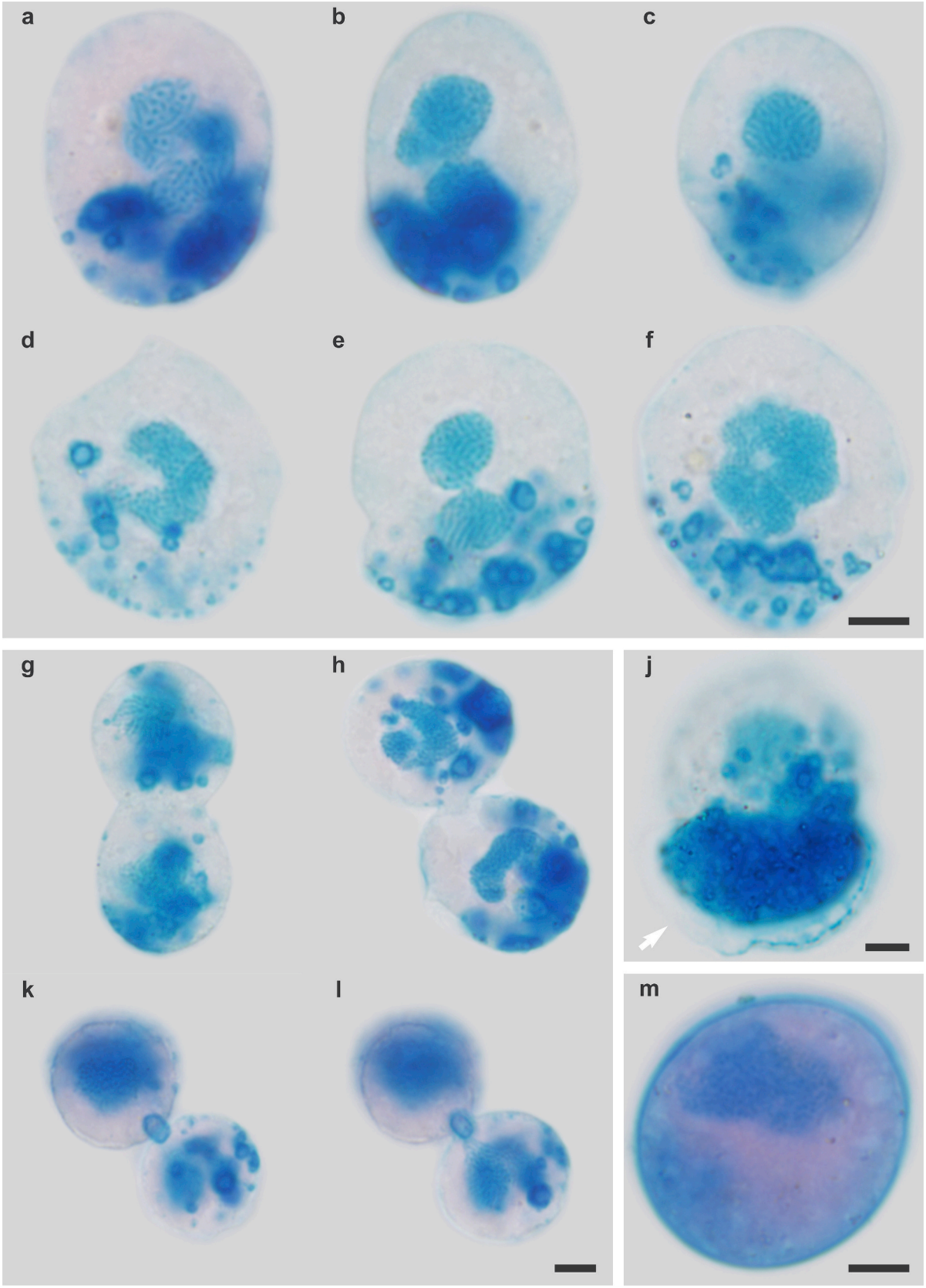

Fig. 5. Astra-blue stained permanent slides of strain GeoK*307 (note polar cytoplasmic staining). **a–f:** Dorso-ventral views with different shapes of nuclei, namely two interconnected (a–b, e), small circular (c), c-shaped (d), big circular (f). **g–h:** Interconnected cells with separate c-shaped nuclei. **j:** Ventral view with undulated right margin of hyposome (indicated by white arrow; reference image for orientation of cells, in which the posterior cytoplasm is more intensely stained than in the anterior half of the cell). **k–l:** Interconnected cells with a stained tube (both images show the same pair of cells at different focal levels). **m:** Coccoid cell. Scale bars: 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4d). The connection between the two cells was serially between the episome of one cell and the hyposome of the other cell and in a single observation, the two cells were ventrally connected in a perpendicular orientation (Fig. 4g–j, Video S1). Some morphologically similar cell pairs appeared peanut-shaped (Fig. 4f) and were motile (Video S1).

Single coccoid cells were either of ovate or circular shape in outline (Figs. 4k–m, S4c). Cell size ranged from 25.5 to 37.8 µm in length (mean: 31.9 µm; SD: 2.9 µm; n = 76) and 22.6–36.0 µm in width (mean: 29.8 µm; SD: 3.1 µm; n = 76). Coloration was similar in all cells varying from yellow-brown (in the periphery) to more orange-red (in the centre). As inferred from autofluorescence, coccoid cells exhibited functionally active chloroplasts (Fig. 4m). Despite multiple attempts, no nucleus could be stained using DAPI in coccoid cells, but was successfully demonstrated with astra blue staining in permanent preparations (Fig. 5m). Occasionally, coccoid cells changed their shape when cultivated at 4 °C and at 6:18 h light:dark cycles and became more elongated

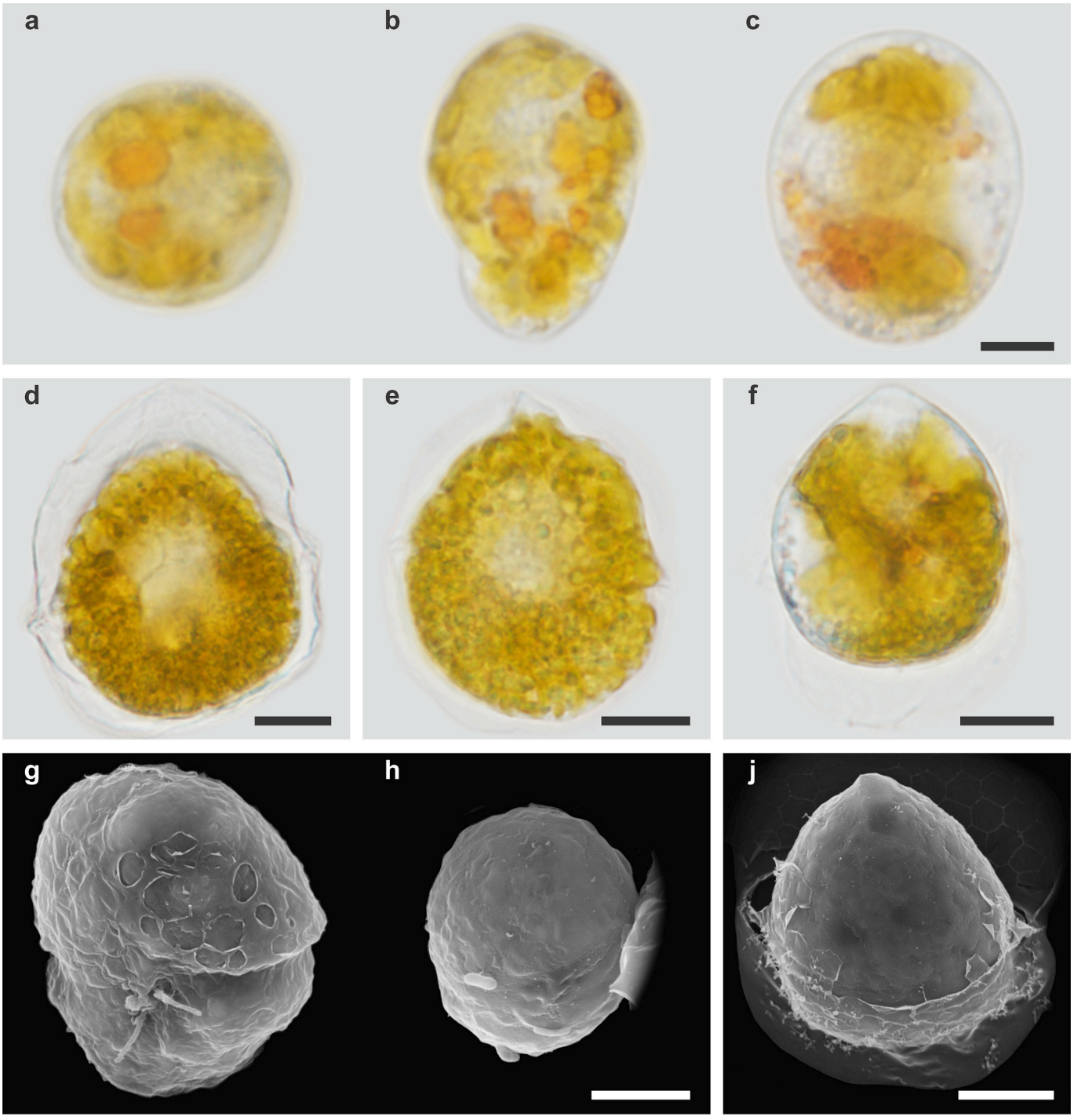

Fig. 6. Morphology under cultivation at 4 °C and under 6:18 h light:dark cycles of strains GeoK*046 (a–b), GeoK*065 (c) and GeoK*307 (d–j) in LM (a–f) and SEM (g–j). a–c: Small flagellated cells (note reduced size and lighter colouration). **d–e:** Large flagellated cells (note acute apex and shrinkage of protoplast). **f:** Coccoid cell (note acute apex and remnants of the former periplast exhibiting pentagonal or hexagonal vesicles, rendering a very similar appearance to Fig. 1c). **g–h:** Small flagellated cells. **j:** Coccoid cell (note acute apex and remnants of the former periplast exhibiting pentagonal or hexagonal vesicles, rendering a very similar appearance as in Fig. 1c). Scale bars: a–c, g–h: 5 µm, except d–f, j: 10 µm.

or ovoid with an acute apex (Figs. 6f, j, S5c).

3.2. Permanent slides

The cells on the permanent slides (Fig. 5) showed an internal polarity regarding the staining by astra blue: One half of the cell (supposedly the episome) was almost clear, whereas the other half (supposedly the hyposome) showed dark blue accumulations and clumps. The polarity was distinct also in cell pairs (Fig. 5g–h, k–l) as well as in coccoid cells (Fig. 5m but here, less obvious). In some cell pairs, a tube-like structure was discernible (Fig. 5k–l). The polygonal vesicles of the periplast were not visible but sometimes, multiple knobs on the cell surface were stained.

Almost all cells showed a very clear, strong staining of the nucleus. The nuclei varied in shape, ranging from small and globular (rare: Fig. 5c) to large and c-shaped (frequent: Fig. 5d, f). Each pairing cell also had an own c-shaped nucleus (Fig. 5g–h, k–l). The condensed chromosomes were stained by astra blue as well, allowing for a gross estimation of chromosome number in each nucleus ranging between 50 (globular nucleus) and 110 (c-shaped nucleus).

3.3. Molecular phylogenetics

The SSU + ITS+LSU alignment was 1818 + 790 + 3491 bp long and comprised 369 + 546 + 933 parsimony informative sites (30.3 %, mean of 26.0 per terminal taxon) and 2993 distinct RAxML alignment patterns. The internal topology of the best-scoring ML tree (−ln = 56,000.25; Fig. S6) showed high if not maximal statistical support for many crucial nodes. Phytodiniales (99 LBS, 1.00 BPP) were monophyletic with respect to the outgroup (Gymnodiniales: 100 LBS, 1.00 BPP and Dinophysales: 100 LBS, 1.00 BPP) and segregated into three lineages, namely Glenodiniaceae (67 LBS, 0.98 BPP), Borghiellaceae (100 LBS, 1.00 BPP) and Symbiodiniaceae (100 LBS, 1.00 BPP).

All species of *Borghiella* with DNA sequence information available [i.e., *B. andersenii* Daugbjerg, Andreasen, Happel, Pandeir., Gert Hansen, Craveiro, Calado & Moestrup, *B. dodgei* Moestrup, Gert Hansen & Daugbjerg, *B. pascheri* (Suchl.) Moestrup, *B. ovum*, *B.* cf. *tenuissima*, *B. verrucosa* (Baumeister) Knechtel & Gottschling] were distinct from each other, but clear relationships within Borghiellaceae could not be inferred. Irrespectively of geographic origin, all sequences gained from cultivated material of *B.* cf. *tenuissima* were indistinguishable, but the SSU sequence of a single cell from Lake Baikal (ZAV2011) showed considerable divergence.

4. Discussion

4.1. Taxonomic identity of Borghiella tenuissima

The species of *Borghiella* are usually difficult to delimitate due to the poorness of morphological traits (Daugbjerg et al., 2014; Knechtel et al., 2020; Moestrup et al., 2008; Müller et al., 2024). However, *B. tenuissima* appears very distinct because of its large size, the strong dorso-ventral flattening and last not least the swimming like a silver dollar or a copper penny rolls across the knuckles, or even like an amber leaf falling from a tree. The species has been repeatedly though not frequently reported from European freshwater habitats during the winter season (Lauterborn, 1894; Wołoszyńska, 1918; Höll, 1928; Schiller and Stefan, 1935; Nygaard, 1949; Crawford et al., 1971; Schönhagen-Becker and Hegewald, 1979; Steinberg, 1981; Tsarenko et al., 2006) and beyond (Hansen and Daugbjerg, 2004; Moestrup et al., 2008).

On more thorough scrutiny, differences between individual details of these reports become also clear, with seeming variation particularly in size and shape of the organism. Based on Schiller (1926), but different from the original description (Lauterborn, 1894) and a later illustration (Lauterborn, 1899), the sulcus of *B. tenuissima* extends well onto the episome, but also this has never been confirmed and is likely a wrong though variously reproduced interpretation (Moestrup et al., 2008). It remains unclear at present whether such differences refer to morphological variability within a species, artificial observations or diagnostic traits as expression of several distinct (though closely related) species. Taxonomic clarification of *B. tenuissima*, preferably based on the study of material collected at the type locality, would be an important first step of disentanglement, but we were yet unable to find the species during a first field campaign to Ludwigshafen in January 2024.

Prior to the present study, there was little knowledge of *B. tenuissima* based on the combination of both molecular and morphological data. In fact, the micrographs in Moestrup et al. (2008) originate from Greenlandic strain NORCCA K-0666 (*pers. comm.* G. Hansen), from which a LSU sequence is also available. It is identical to European sequences gained in the course of the present study. Furthermore, the ITS sequences of the strains investigated here, collected at three different localities and at different points in time, are not divergent. They all show great consistency regarding size and shape, as long as the environmental conditions do not change. However, size and shape change with decrease of temperature and/or day length, and the effect is the same for all strains indicating a high degree of intraspecific variability.

Like supposedly all species of *Borghiella* (Moestrup and Calado, 2018), also the material studied here shows the amphiesmal vesicles arranged in a honeycomb pattern. This characteristic surface is also seen in Symbiodiniaceae and (only distantly related) Tovelliales (Moestrup and Calado, 2018) and was firstly documented by Wołoszyńska (1916). In some cells of *B. tenuissima*, it is more pronounced on the hyposome than on the episome (which goes hand in hand with the polar staining in permanent slides), somewhat resembling *Woloszynskia reticulata* R.H. Thomps. (Thompson, 1950). The latter species currently is assigned to Tovelliaceae, but there is no DNA sequence data available at present supporting this classification. Anyway, previous illustrations and reports of *B. tenuissima* may vary in some details (see above) but even more noteworthy, an undulated right margin of the hyposome has only barely mentioned (also in Lauterborn, 1899: 388), if remarked on at all. It is not present in all cells, but in a considerable number of cells varying between strains and irrespectively of the geographic origin, and there is no indication that such cells were less vivacious than cells with a smooth margin.

In dinophytes, size may matter in the delimitation of species and subsequently, the question arises whether the material studied here is correctly identified as *B. tenuissima*. None of the cells, of which both micrographs and DNA sequence data are available now, reaches the size of 66 µm length specified in the protologue (Lauterborn, 1894) or even of up to 80 µm length in a later, frequently copied report (Schiller, 1931). Either the species in fact is even more variable in shape and size (rather supported based on the treatment in a colder environment and with a shorter day length), or *B. tenuissima* is a truly giant (and rare) dinophyte species with an exclusively smooth right margin, of which DNA sequence data are not yet available. We express this taxonomic reservation by preliminarily identifying the studied material as *B.* cf. *tenuissima*.

The treatment of the strains under colder temperatures and shorter day length indeed had an impact on size and on the morphology of some cells. Particularly, the development of long, stiff structures providing the shape of a unicorn is remarkable. To the best of our knowledge, this has never been documented before, but is reminiscent of *Stylodinium* G.A. Klebs (Moestrup and Calado, 2018), although *B.* cf. *tenuissima* has not attached to any surface in the present experiments. Stipe formation of such epiphytic algae, arising from the apical pore complex widely present among thecate dinophytes, has been observed only rarely (Baumeister, 1943; Horiguchi et al., 2000). Whether there is a shared underlying process also in *B.* cf. *tenuissima* remains elusive at present, also as the phylogenetic relationships of *Stylodinium* have not been worked out at present (Gottschling and Tillmann, 2024).

4.2. Two modes of vegetative replication

Vegetative replication during the flagellated stage (indicative for the desmoschisis type of mitosis) is documented for most of the *Borghiella* species (Moestrup et al., 2008; Daugbjerg et al., 2014; Knechtel et al., 2020), including *B. tenuissima* (Wołoszyńska, 1918), and the material of the present study. Dividing cells covered by the pellicle during the deflagellated stage (indicative for the eleutheroschisis type of mitosis (and known in the literature also as 'temporary cyst' or 'division cyst') is additionally known from *B. andersenii* (Daugbjerg et al., 2014), *B. pascheri* (Wołoszyńska, 1936; Nicholls, 2017; Moestrup et al., 2018), *B. ovum* (Müller et al., 2024), *B. verrucosa* (unpublished observations) as well as from the material presented here. Therefore, mitotic divisions under two fundamentally different mechanisms (Kwok et al., 2023) appear as consistent and presumably apomorphic trait of entire *Borghiella*, which would argue for the monophyly of the group. As evolutionary advantage, it may allow the algae to replicate in two different habitats, namely in the water column (applying desmoschisis) and in the sediment (applying eleutheroschisis).

Outside *Borghiella*, reports of two different types of mitotic division in the same species are only sporadic. They include phytodinialean *Baldinia droopii* Gert Hansen, Daugbjerg & Moestrup (Hansen et al., 2023) and *Biecheleria cestocoetes* (R.H.Thomps.) Moestrup, but already Moestrup and Calado (2018) raised doubts whether the latter is a true species of *Biecheleria* Moestrup, K.Lindb. & Daugbjerg. Other examples are peridinialean *Kryptoperidinium triquetrum* (Ehrenb.) Tillmann, Gottschling, Elbr., Kusber & Hoppenrath (Figueroa et al., 2009) and gonyaulacalean *Lingulaulax polyedra* (F.Stein) M.J.Head, K.N.Mert. & Fensome (Figueroa and Bravo, 2005). Notably, there are several reports in *Alexandrium* subgen. *Gessnerium* (Halim) Balech (Lim et al., 2005; Figueroa et al., 2006; Tillmann et al., 2020), which might identify another group consistently showing two different types of mitotic division like *Borghiella*.

Lindemann (1929) was probably first, who recognised this exciting phenomenon (i.e., desmoschisis and eleutheroschisis simultaneously) in *B. pascheri* (= *Gyrodinium nivale* Er.Lindem.), although he never observed the daughter cells hatching from the pellicle. Schiller (1954) confirmed and continued such research, although Moestrup and Calado (2018) did not want to exclude that he studied a mixture of species. In the more recent past, Daugbjerg et al. (2014) were the ones, who discerned different replications of gonyaulacalean and peridinialean dinophytes, respectively, at the same time in *B. andersenii*. If there are two different types of mitotic division in *Borghiella*, then reshaping of the flagella must be different as well, but corresponding knowledge is scarce. During eleutheroschisis, both longitudinal and transverse flagella are regenerated de novo whereas during desmoschisis, the two existing flagella are duplicated (Kwok et al., 2023). As first step during the latter development, the former transverse becomes a longitudinal flagellum (Heimann et al., 1995; Moestrup et al., 2008). As a consequence, the presence of two longitudinal flagella are not necessarily indicative for (plano-)zygote formation (as assumed by Daugbjerg et al., 2014) and can also be interpreted as early stage of desmoschisis.

4.3. Questionable sexual processes

From the perspective of metagenesis, the different shapes, connections and numbers of nuclei found in single cells of *B.* cf. *tenuissima* are more confusing than enlightening. This includes also the observation of temporarily c-shaped nuclei during mitotic division already noted by Wołoszyńska (1918; reproduced as Fig. 1b). This trait is reminiscent of *Peridinium* Ehrenb. (Dürr, 1979; Calado et al., 1999) or *Lingulaulax* M.J. Head, K.N.Mert. & Fensome (Tillmann et al., 2021), with which *Borghiella* is only distantly related. It remains unclear whether all present observations refer to regular mitotic division only, or are also an expression of sexual reproduction, which is so far only reported for *B. andersenii* (Daugbjerg et al., 2014) and *B. pascheri* (Moestrup et al., 2018) based on the observation of two longitudinal flagella. Cell fusion as sexual process is rarely documented for any species of *Borghiella* and has also been observed only once in the course of the present study for *B.* cf. *tenuissima*.

Confusion persists regarding shapes, functions or origins of coccoid cells in *Borghiella* and *B.* cf. *tenuissima* in particular. The morphology of some coccoid cells presented here, with their ovate shape and acute apex and even with the surrounding former periplast exhibiting pentagonal or hexagonal vesicles, is strikingly similar to the observations of Wołoszyńska (1917; Fig. 1c). However, such cells occur only at low temperatures and short days, which may correspond closer to the natural preferences of *B. tenuissima*. Other observations (cultivated at higher temperatures) rather agree with Stosch (1973) exhibiting the ovate or circular outline of the cells without an acute apex (it remains unclear at which temperatures he cultivated the material, but supposedly at higher values like other organisms presented in his study).

In other species of *Borghiella*, coccoid cells have been interpreted as hypnozygotes (i.e., dormant or resting stages: Moestrup et al., 2008; Flaim et al., 2010; Daugbjerg et al., 2014), but the absence of unequivocal sexual traits in the present material may indicate that they develop vegetatively, similarly to the observations of *B. pascheri* (Wołoszyńska, 1936; Nicholls, 2017). Furthermore, coccoid cells of at least *B. ovum* (Müller et al., 2024) and the organisms studied here are photosynthetically active (as inferred from autofluorescence of chloroplasts) and thus not dormant, again arguing for their vegetative origin. More research is necessary to entangle vegetative replication and sexual reproduction in species of *Borghiella*.

4.4. Taxonomic treatment

Borghiella tenuissima (Lauterborn) Moestrup, Gert Hansen & Daugbjerg ex Moestrup & Calado, Dinophyceae (Freshwater Flora of Central Europe 6): 329 (2018). *Gymnodinium tenuissimum* Lauterborn, Biologisches Centralblatt. Erlangen 14: 396 (1894). *Woloszynskia tenuissima* (Lauterborn) R.H.Thomps., Lloydia 13: 290 (1950). **Neotype** [illustration], **designated here**: Germany. Rhineland-Palatinate, Ludwigshafen, Maudach, winter 1893–94 [non-fossil]: R. Lauterborn, Zeitschrift für wissenschaftliche Zoologie 65: pl. XVIII 26! (1899) [http://phycobank.org/105429].

= *Glenodinium limos* T.M.Harris, Proceedings of the Linnean Society of London 152: 25, fig. 8a–c (1940).

= *Glenodinium fungiforme* J.Schiller, Wissenschaftliche Arbeiten aus dem Burgenland 9: 50, pl. X 50a–c (1955), ***syn. nov.***.

= *Gymnodinium lens* Fott, Preslia 29: 286–287, Figs. 5–6 (1957).

Remarks: All of R. Lauterborn's original material has presumably been destroyed (Lange, 1990), and there is no depiction of the species associated with the protologue. The informative illustration from a publication five years later by the same author shall serve as a neotype as long as no original material is allocated. For the taxonomic transfer of *G. tenuissima* to *Borghiella*, Moestrup et al. (2008) did not cite the correct basionym (Lauterborn, 1894) but a later publication (Lauterborn, 1899), which is not in accordance with ICN Arts 41.1 and 41.5. Moreover, Lauterborn (1899: 369–370) includes a reference to the protologue (Lauterborn, 1894) and the name itself and therefore, ICN Art. 41.8(a) does not apply in this case. In conclusion, the combination of Moestrup et al. (2008) was not validly published. Moestrup and Calado (2018) cite the correct basionym for the name, but without a '*comb. nov.*' (despite ICN Rec. 32 A.1). *Borghiella tenuissima* also does not appear in their list of taxonomic and nomenclatural novelties, making it doubtful whether the authors intended to validate it in their book. Nevertheless, there is no reason to assume that Art. 41.5 is not fulfilled in Moestrup and Calado (2018) and that the taxonomic combination is validly introduced there.

A number of species can be considered synonyms of *B. tenuissima* due

to the strong dorso-ventral flattening, and reported sizes are frequently much closer to our than to R. Lauterborn's observations. Synonymy between German *B. tenuissima* and English *Gl. limos* (Fig. 1d) was already assumed earlier (Moestrup and Calado, 2018), and we agree until further notice. Austrian *Gl. fungiforme* (Fig. 1f) was considered a synonym of thecate *N. polonicum* (Moestrup and Calado, 2018), but the latter species is by far not so dorso-ventrally compressed than J. Schiller's (being more similar to R. Lauterborn's) taxon. Czech *Gy. lens* (Fig. 1g) was considered a synonym of *T. leopoliensis* (Moestrup and Calado, 2018), but the latter has acuminate (not obtuse or round) poles and a u-shaped eyespot differing from B. Fott's and R. Lauterborn's taxa. Therefore, we agree with КрахМальный (2011) that *Gy. lens* is a synonym rather of *B. tenuissima*.

Baumeister (1951) described *Cystodinium grabenseei* Baumeister (with predominant coccoid cells) and provided also images from the supposed motile cells of this species (Fig. 1e; Baumeister, 1957, 1969). Particularly because of the strong dorso-ventral compression, they are also similar to *B. tenuissima*. Strong dorso-ventral compression is further known from *Nusuttodinium aeruginosum* (F.Stein) Y.Takano & T.Horig., but this can be easily delimited from *B. tenuissima* because of the blue-green kleptoplastids and the sulcus extending onto the episome (Stein, 1883).

CRediT authorship contribution statement

Büşra Şahin: Writing – original draft, Investigation, Formal analysis, Data curation. **Anna Müller:** Writing – review & editing, Investigation, Data curation. **Paweł M. Owsianny:** Writing – review & editing, Resources, Data curation. **Corinna Romeikat:** Writing – review & editing, Resources. **Danail P. Krastev:** Writing – review & editing, Investigation, Data curation. **Juliana Chacón:** Writing – review & editing, Investigation. **Marc Gottschling:** Writing – original draft, Validation, Supervision, Resources, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: We declare that the work described has not been published previously; the article is not under consideration for publication elsewhere; the article's publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out; and if accepted, the article will not be published elsewhere in the same form, in English or in any other language, including electronically, without the written consent of the copyright-holder.

Acknowledgements

Juliane Kretschmann (Murnau) established and studied strain GeoM*542, Walter König (Munich) set up equipment for temperature experiments, and Jennifer Grünert and Andreas Klingl (Munich) provided the opportunity of OsO_4 fixation – all this is gratefully acknowledged here. We thank the Czech Botanical Society (Petr Pyšek, Pruhonice), the Landesmuseum Burgenland (Kerstin Laditsch, Eisenstadt) and Heide Baumeister (Munich) for permissions to reproduce original material, HaJo Esser (Munich), Wolf-Hening Kusber (Berlin) and Juan Carlos Zamora (Geneva) for taxonomic discussion, Anina Neumann (Munich) for DNA sequencing and Rafael Matysiuk (Munich) for providing the computational power for the phylogenetic analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.protis.2025.126132.

Data availability

Data will be made available on request.

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