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Baldinia droopii sp. nov. (Suessiales, Dinophyceae), a new species from a small rainwater rock pool near Tvärminne, south-western Finland

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ABSTRACT

A dinoflagellate isolated from a small rainwater rockpool in Finland was found to be a new species of *Baldinia*, here described as *B. droopii sp. nov*. This is only the third formally described *Baldinia* species and the second described by modern methods. The new species had a complement of generic characters that clearly affiliated it to *Baldinia*: presence of an internal honeycomb structure, termed a lamellar body, a ventral fibre associated with the longitudinal basal body and a pentagonal resting cyst; and the absence of an apical structure, thecal plates and trichocysts. The most distinctive species-specific characters were the presence of a resting cyst with tubiform processes, a paratabulation matching the pattern of the motile cell, and a life cycle exhibiting a diel rhythm, alternating between motile and non-motile coccoid cells. Motile cells occurred during the light period, peaking after 3–4 h of light period, while practically no motile cells occurred during the dark period. LSU rDNA sequences confirmed *B. droopii* as a new species, showing *c*. 14% sequence difference compared to *B. anauniensis*. Asexual reproduction occurred primarily by binary fission or via a so-called division cyst. Sexual reproduction occurred in the culture indicating that *D. droopii* is homothallic. The zygote may form a resistant cyst with tubiform processes, but there were indications that this stage may be by-passed. Different stages of the asexual and putative sexual reproduction were observed and documented by video recordings.

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Circadian rhythm; Cyst; Life cycle; LSU rDNA; Sexual reproduction

INTRODUCTION

In the period 2001–2004 a large multi-disciplinary project, SALTO (Studio sul mancato Arrossamento del Lago di Tovel) took place, whose primary aim was to shed light on the cause(s) of the sudden stop of red-coloured algal blooms in Lago di Tovel, Trentino, Italy (Borghi *et al.* 2006). This phenomenon, caused by a dinoflagellate then known as *Glenodinium sanguineum* Marchesoni *nom. illeg.*, was a large tourist attraction until 1964, when the blooming events stopped (see e.g. Flaim *et al.* 2004 for more details).

A significant element of the project was a thorough characterization of the organism involved. Earlier studies by Baldi (1938, 1941) suggested peculiar life cycle stages with an interchangeable red and green stage, and various cysts. The new studies revealed that three species in the lake resembled the organism considered to be the cause of the red colour. The species actually causing the reddening was named *Tovellia sanguinea* Moestrup, Gert Hansen, Daugbjerg, Flaim & d'Andrea, and a second species was named *Borghiella dodgei* Moestrup, Gert Hansen & Daugbjerg (= '*Glenodinium sanguineum*' *sensu* Dodge *et al.* 1987). The third species was named *Baldinia anauniensis* Gert Hansen & Daugbjerg (Moestrup *et al.* 2006, 2008; Hansen *et al.* 2007). The latter represented the green stage in Baldi's life cycle studies. All species are so-called woloszynskioid dinoflagellates, that is, covered by numerous polygonal amphiesmal vesicles that may contain relatively thin plates. This triggered a number of studies on woloszynskioid dinoflagellates (e.g. Moestrup *et al.* 2009, 2018; Daugbjerg *et al.* 2014; Pandeirada *et al.* 2019, and references therein). Presently *Tovellia* Moestrup, K. Lindberg & Daugbjerg comprises 13 species and *Borghiella* Moestrup, Gert Hansen & Daugbjerg 7 species. However, *Baldinia* Gert Hansen & Daugbjerg has until now only contained two species, *B. anauniensis* and *B. bernardinensis* (Chodat & Zender) Moestrup, K. Lindberg & Daugbjerg. The latter has not been observed since its description by Chodat (1924), but was transferred to *Baldinia* by Moestrup *et al.* (2009). The transfer was based on similarities in the shape of the resting cyst, but other generic characters, for example, lack of an apical structure (apical furrow) on the cell, and the presence of a lamellar body and a ventral fibre in the cell are unknown.

During a visit to Tvärminne Zoological field station near Hangö (Hanko), Finland in 2011, we sampled several small rock pools on the small islands in the vicinity of the field station. In a small rainwater pool an unidentified small woloszynskioid dinoflagellate was present. In the present paper it is demonstrated, based on phylogenetic analyses of partial LSU rDNA, combined with scanning and transmission electron microscopy, that it represents an unknown species of *Baldinia*, characterized in particular by a distinct cyst with tubiform processes and an LSU rDNA sequence that differs by 13.9% from *B. anauniensis* (estimate based on 1,333 base pairs including domains D1–D5).

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MATERIAL AND METHODS

Sampling and culturing

The material (net sample 20 µm mesh size) was collected 19 June 2011 from a freshwater rock-pool at Brännskär, Tvärminne, south-western Finland. The pool was elongate-ovoid and without vegetation on the sides and bottom. Its size, *c.* 4 × 2 m, suggested it belonged to Levander's (1900) type 6a pool (i.e. a permanent rainwater pond with smooth walls) but the shallow depth of the pool suggested that it may be susceptible to periodic desiccation. It may therefore also be classified as a type 5 pool: an ephemeral rainwater puddle in a shallow rock depression. The dinoflagellates *Bernardinium bernardinense* Chodat and *Parvodinium inconspicuum* (Lemmermann) Carty were the most abundant species in the pool. A species of *Baldinia* was also found, and a culture (K-1803) was established by transferring a single cell into modified WC+Se (MWC+Se) medium (Hansen 2018). Cultures were maintained at 15°C at an illumination of *c.* 50 µmol m^{-2} s^{-1} and a 18:6 h light:dark cycle.

Light microscopy and cell measurements

Observations were made using an Olympus BX-51 microscope equipped with an Olympus DP-72 digital camera (Olympus, Tokyo, Japan) or an Olympus BHS microscope with a Nikon 1 J2 digital camera (Nikon, Tokyo, Japan). The latter was also used for video recording at 1080p/60fps. The video footage was subsequently edited using iMovie (Apple Inc., Cupertino, California, USA) and saved in mp4 format. A small amount of culture was kept in a microchamber as a 'hanging drop' culture. The microchamber was a modified slide with a 11-mm circular hole, and the bottom was a coverslip glued to the slide with SuperGlue while the top was a coverslip sealed to the slide with vaseline. The 'hanging drop culture' was kept at ambient light and temperature for up to two weeks or more.

Morphometric measurements were made using the Fiji (Image J) open-source software (Schindelin *et al.* 2012). Histograms and normal distributions, based on the normal probability density function, were made using MS Excel.

Scanning electron microscopy (SEM)

Culture of *Baldinia droopii sp. nov.* was fixed for SEM by adding either 500 µl of culture to a fixative cocktail comprising 150 µl of 4% OsO_4 and 100 µl of saturated $HgCl_2$, or 400 µl of culture to a fixative cocktail comprising 300 µl of 2% OsO_4 and 200 µl of saturated $HgCl_2$. For resting cysts, a portion of culture was fixed in 1% Lugol's solution. Material was placed on poly-L-lysine-coated circular coverslips and fixed for 30 min or 1 h (Lugol-fixed). After fixation, cells were washed with distilled water for 30 min and dehydrated in an ethanol series: 10 min in each concentration of 30%, 50%, 70%, 90%, 96% and 99.9%, followed by two changes in absolute ethanol of 30 min each. Cells were critical-point dried using a Bal-Tec CPD 030 (Bal-Tec, Balzers, Liechtenstein) and sputter-coated with platinum-palladium using a JEOL JFC-2300 HR sputter coater (Jeol, Tokyo, Japan) before examination in a JEOL JSM-6333 F scanning electron microscope (Jeol), operated at 7 kV.

Transmission electron microscopy (TEM)

For TEM, the clonal culture was fixed in a mixture of 1% glutaraldehyde and 0.02% OsO_4 in 0.05 or 0.08 M phosphate buffer, pH 7.8 (final concentrations) for 25 min; it was pelleted by centrifugation and washed in buffer for 15 min. Post-fixation was in 1% OsO_4 in 0.075 M buffer for 1 h. After a brief rinse in buffer, the material was dehydrated in a graded ethanol series and embedded in Spurr's resin via propylene oxide, as described in Hansen (1989).

The material was sectioned on an LKB 2088 Ultrotome V ultramicrotome (LKB, Bromma, Sweden) using a diamond knife, and the sections were collected on slot grids and placed on Formvar film. After staining in uranyl acetate and lead citrate, sections were examined in a JEOL JEM-1010 electron microscope (Jeol), operated at 80 kV. Micrographs were taken using a GATAN Orius SC1000 digital camera (Pleasanton, California, USA).

DNA extraction and PCR amplification of partial LSU rDNA

Ten ml of a newly inoculated culture batch of *Baldinia droopii* was centrifuged for 10 min at 1,174× *g*. Prior to extraction of total genomic DNA, the cell pellet was transferred to an Eppendorf tube and frozen at –18°C. For DNA extraction the PowerPlant Pro DNA Isolation Kit was used, and the manufacturer's recommendations were followed (MO BIO Laboratories Inc., Carlsbad, California, USA). The nuclear-encoded LSU rDNA was amplified using forward primer D1R-F (Scholin *et al.* 1994) and the reverse primer 28–1483 R (Daugbjerg *et al.* 2000). The 5X Hot FIREPol Bend Master Mix (Solis BioDyne, Tartu, Estonia) was used as the amplification kit. The PCR conditions comprised one initial cycle of denaturation at 95°C for 12 min, followed by 35 cycles, each consisting of denaturation at 95°C for 15s, annealing at 55°C for 30s and extension at 72°C for 45s. A last step included extension for 5 min at 72°C. PCR products were confirmed by electrophoresis in an agarose gel with a final concentration of 1.5%. Amplified fragments were stained with GelRed and documented using the XR system from Bio-Rad (Hercules, California, USA). A molecular marker (100-base pair RAIN-BOW extended DNA ladder; BIORON GmbH, Ludwigshafen, Germany) was used to verify the expected length of amplified DNA fragments. These were then purified by ultrafiltration using the Nucleofast 96 PCR kit from Macherey-Nagel (GmbH & Co. KG, Düren, Germany). The purified product (approximately 1,480 base pairs) was determined in both directions using the two amplification primers in addition to three internal primers: D3A, D3B (Nunn *et al.* 1996) and D2R (Scholin *et al.* 1994). For sequence determination, we used the service provided by Macrogen. The contig, which comprised all five DNA fragments, was made using Geneious v2022.0.2.

Alignment and phylogenetic analysis

An alignment that focused on members of the Suessiales was compiled by retrieving species available from GenBank (13 July 2022) for which the LSU rRNA gene had been determined (520+ base pairs in length). The data matrix also included sequences of *Moestrupia* Gert Hansen & Daugbjerg, Tovelliaceae (4 genera) and *Heterocapsa* F. Stein (3 species), and these together with the suessialean taxa (57 sequences) were aligned using the Muscle software as implemented in Jalview v14 (Waterhouse *et al.* 2009). The aligned LSU rDNA data matrix comprised 65 taxa and 1,278 base pairs, including introduced gaps, and was analysed using Bayesian Analysis (BA) and RaXML as implemented in Geneious v2022.1.1. For BA 5 million generations were run with a tree sampled every 1,000 generations. The burn-in was set at 10% of the saved trees and thus resulted in 4,501 trees for estimating the consensus tree. For RAxML (v8; Stamatakis 2014) the GTR GAMMA option was used, and 5,000 bootstrap replications were included to evaluate the robustness of the tree topology. The percentage values were mapped onto the BA tree.

Genetic distances

The genetic distances between *Baldinia droopii* and *B. anauniensis* and between *Tovellia sanguinea* and *T. coronata* (Wołoszyńska) Moestrup, K. Lindberg & Daugbjerg (included for comparison) were calculated from the K2P model using PAUP* (v4.0a, build 169; Swofford 2002).

RESULTS

Baldinia droopii Gert Hansen, Daugbjerg & Moestrup *sp. nov.*

Figs 1–24

DESCRIPTION: Motile cells ovoid with hemispherical episome and hemispherical or truncated hyposome. Not dorsoventrally flattened. Length 19–35 µm (mean 26 µm), width 16–29 µm (mean 23 µm). Cingulum median and displaced one cingular width. Sulcus widening towards the antapex. Distinct orange type B eyespot *sensu* Moestrup & Daugbjerg (2007) located in the sulcus. Numerous pentagonal or hexagonal amphiesmal vesicles present. Apical structure absent. Two rows of amphiesmal vesicles within the cingulum. One greenish-yellow chloroplast radiates from a central pyrenoid complex. Nucleus hemispherical, situated in the episome. Lamellar body present. Ventral fibre present. Pusular tubules situated in pyrenoid cavity. Peduncle present. Trichocysts absent. Ovoid coccoid cells, surrounded by a smooth wall, form the dominant cell type. Length 23–37 µm (mean 29 µm), width 16–30 µm (mean 23 µm). Hypnozygotes (resting cysts) with a pentagonal outline; the apical part pointed, the antapical truncated or hemispherical. The outer cyst wall with distinct para-tabulation reflecting the amphiesmal vesicles of the motile stage. Each vesicle with a distinct tubiform process. Inner wall thin and appressed to the cytoplasmic part. Length 40–47 µm (mean 44 µm), width 30–41 µm (mean 34 µm).

HOLOTYPE: The hypnozygote (resting cyst) forms the holotype, as it is the most distinctive feature of *Baldinia droopii*. A SEM stub (SEM264) with hypnozygotes has been deposited at the Natural History Museum of Denmark, University of Copenhagen, accession number C-A-96298, and serves as type material. Figures 16, 17 are from this material.

OTHER ORIGINAL MATERIAL: Cells imbedded in resin blocks, Fix133 and Fix135 have been deposited at the Natural History Museum of Denmark, University of Copenhagen, accession number C-A-96299 and C-A-96300, respectively. SEM stubs of motile cells were unfortunately lost.

TYPE LOCALITY: Rainwater rockpool on Brännskär, a small island near Tvärminne Zoological Station, Finland (59°50.622′N, 23°16.397′E), sampled on 19 June 2011.

ETYMOLOGY: The epithet *droopii* commemorates Dr. Michael Droop (1918–2011) a prominent algal physiologist and culturist. Droop (1953) made comprehensive ecological studies of the algal flora of the many rock pools in the Tvärminne area, including the pools on Brännskär.

GENBANK ACCESSION: OP459417 (partial nuclear-encoded LSU rDNA).

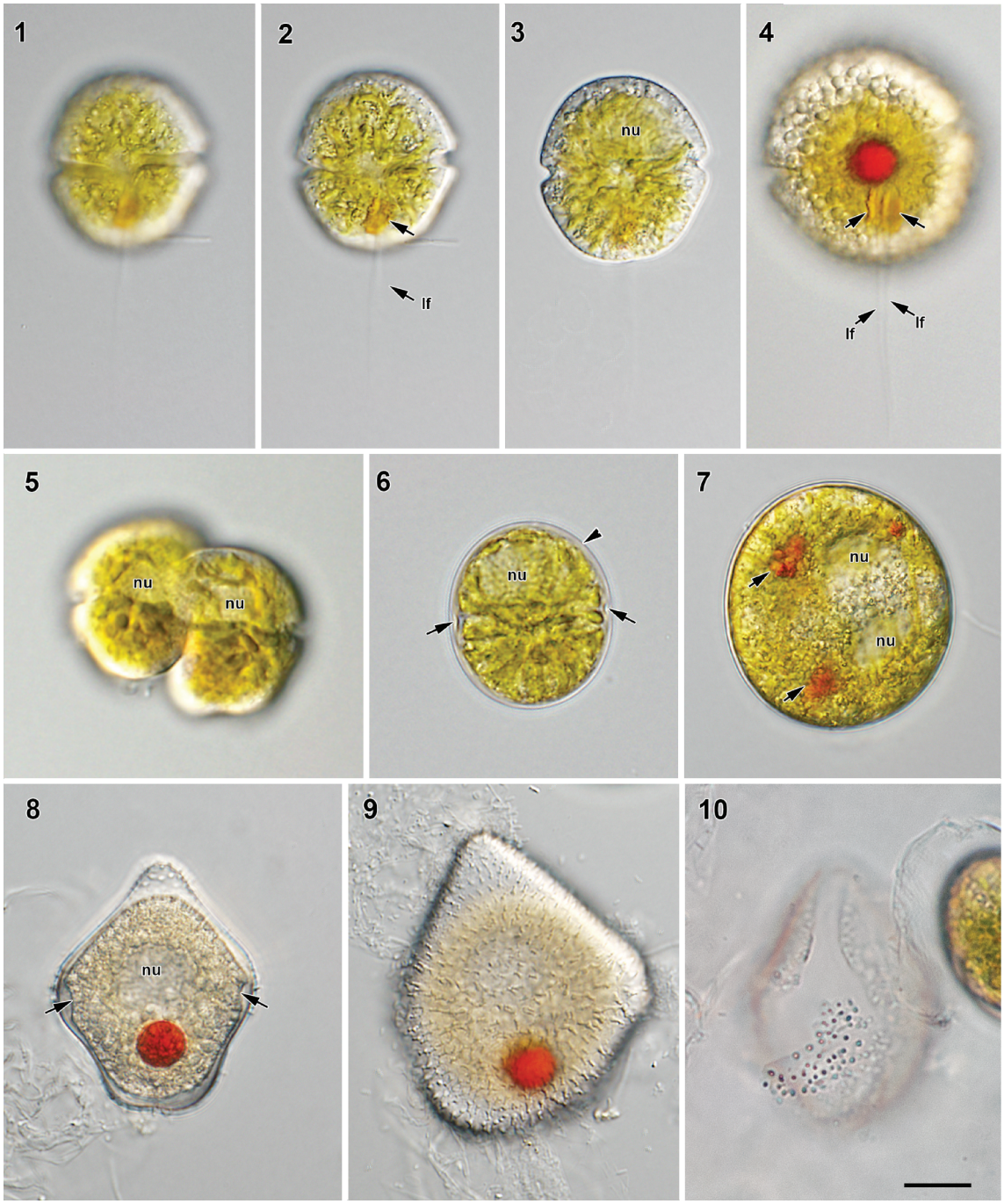
LM & SEM

The life cycle appeared complex and has not been not fully elucidated, but it included both a motile and a non-motile stage. Light seemed to control the transformation from one stage to the other. The highest number of motile cells occurred 3–4 h into the light period, then gradually decreased and there were essentially no motile cells during the dark period. Sexual reproduction occurred in the culture indicating that *B. droopii* is homothallic. Below we describe our observations of the different cell types and their putative roles in the life cycle.

MOTILE CELLS

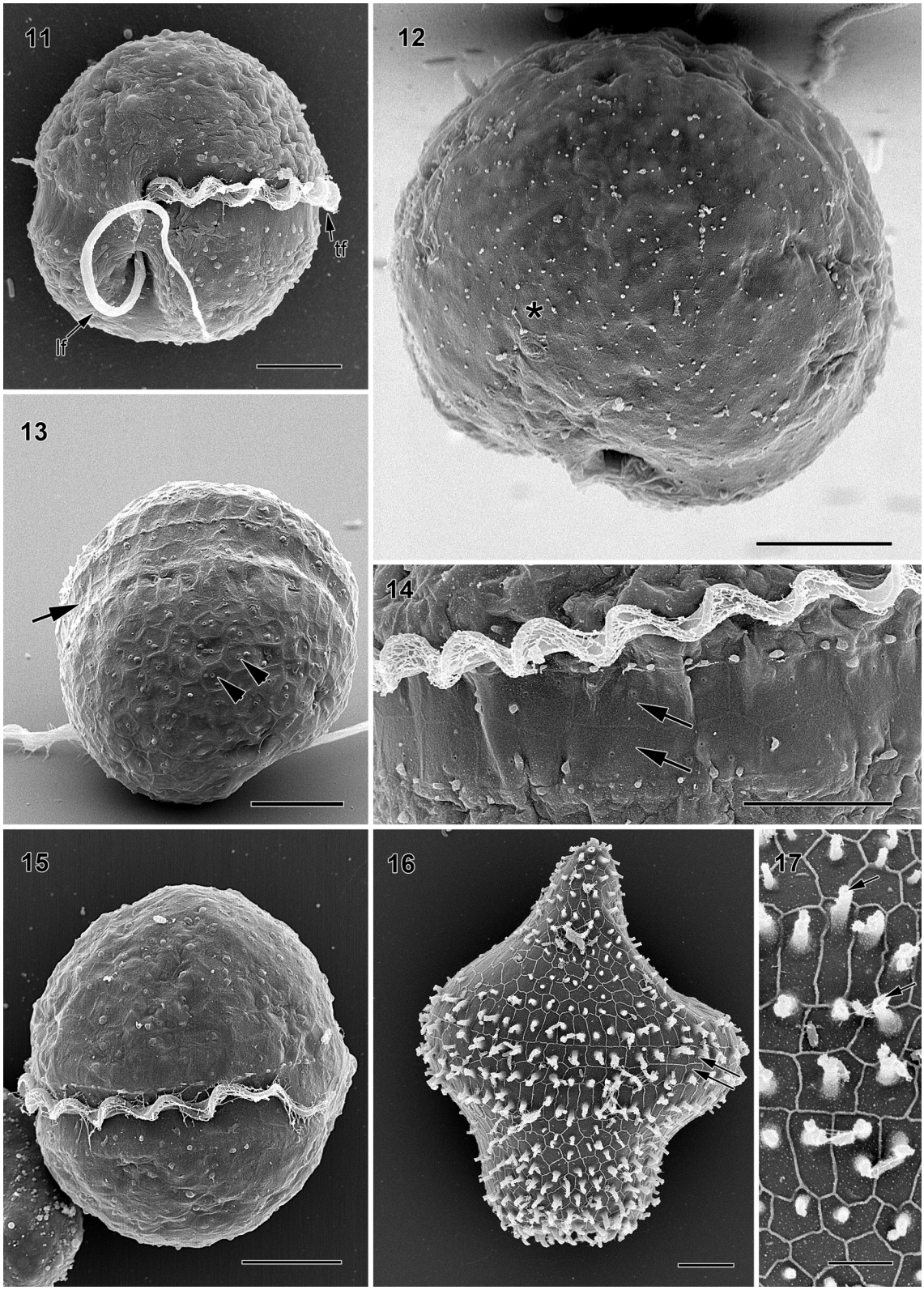
Motile cells of *B. droopii sp. nov.* displayed a moderate to fast swimming speed with the rotating movement typical of most dinoflagellates. However, prior to 'encystment' (see below) cells swam in a slow circling movement with the sulcus facing the substrate. The average cell length was 26 µm (s = 3.2 µm, range 19.1–35.4 µm; n = 100) and the average width 23.1 µm (s = 2.8 µm, range 16.0–24.0 µm; n = 100). For size distribution of cell length see Fig. S1. Cells were slightly longer than wide (average length-to-width ratio 1.1; s = 0.1; range 1.0–1.4; n = 100), and not dorsoventrally flattened (width-to-depth ratio 1.0, s = 0.1, range 1.0–1.1; n = 9). The cell was typically ovoid with a hemispherical episome and a truncated or hemispherical hyposome. The latter was often slightly indented due to the sulcus reaching the antapex, but some variation in cell shape was observed (Figs 1–3, S2–S25). The episome was typically slightly longer than the hyposome (average epi- to hyposome ratio 1.1, s = 0.1; range 0.7–1.4; n = 100). The cingulum was descending and displaced one cingular width. The right border marking the end of the cingulum was deflected to the left (Fig. 11). The nucleus was generally located in the left part of the episome (Fig. 3), but a more central location was also observed (e.g. Figs S4, S7, S10, S20, S23).

A few cells measured over 32 µm (see Fig. S1) and represented perhaps planozygotes or planomeiozygotes, that is, cells germinated from a resting cyst. The cell depicted in Fig. 4 measures 33.4 × 31.2 µm, has two longitudinal flagella, two eyespots and a large brick-red accumulation body, and most likely represents a planomeiozygote because of the large accumulation of storage globules. A planozygote in an early stage of formation, still with perpendicularly oriented cingula



Figs 1–10. LM of living cells of *Baldinia droopii sp. nov*. Scale bar = 10 µm.
Figs 1–3. Show different focus of a vegetative cell. lf: longitudinal flagellum, nu: nucleus. Notice also conspicuous, orange eyespot (arrow).
Fig. 4. Putative planomeiozygote with two longitudinal flagella, two eyespots (arrows), a large central red accumulation body and numerous storage granules.
Fig. 5. Dividing vegetative cells.
Fig. 6. Non-motile cell ('temporary cyst') bounded by a thin wall (arrowhead). Notice the cingulum still visible (arrows).
Fig. 7. Large non-motile cell ('cyst') with two nuclei and two accumulation bodies (arrows).
Fig. 8. Fully formed resting cyst (hypnozygote). The cingulum is apparent (arrows).
Fig. 9. High focus of a resting cyst. The numerous tubiform processes are evident.
Fig. 10. An empty resting cyst, with irregular opening.

but with the two nuclei in the process of fusion, is shown in Fig. S32. Smaller cells less than 23 µm long may represent microgametes, but we have no evidence for this. Fusion of cells, recognized by the perpendicularly oriented cingula, was observed once between a small cell and a 'normal'-sized cell (not shown), but fusion between equal-sized cells was more common. Vegetative cell division was generally by binary fission (Fig. 5; Video S1), and elimination of the accumulation body during cell division did sometimes occur (Video S1). However, cell division occurred occasionally within a division cyst. A sudden expansion of the two cells broke the cyst wall and released the cells. The newly germinated cells were



Figs 11–17. SEM of *Baldinia droopii sp. nov*.

Fig. 11. Ventral view. Longitudinal flagellum (lf), transverse flagellum (tf). Scale bar = 5 µm.

Fig. 12. Apical view. The amphiesmal pattern is faintly visible. One vesicle is marked by an asterisk. Notice that no apical structure is present. Scale bar = 5 µm.

Fig. 13. Antapical view. The cingulum consists of two rows of amphiesmal vesicles (arrow). Notice each amphiesmal vesicle has a pore (arrowheads). Scale bar = 5 µm.

Fig. 14. Details of the cingulum showing the two rows of amphiesmal vesicles (arrows). Scale bar = 5 µm.

Fig. 15. Dorsal view. Scale bar = 5 µm.

Fig. 16. Resting cyst (hypnozygote) with distinct paratabulation and a tubiform process within most of the amphiesmal vesicles. The two cingular rows of 'vesicles' (arrows). Scale bar = 5 µm.

Fig. 17. Details of the cingular- and adjacent 'vesicles'. Some processes have a small opening (arrows). Scale bar = 2 µm.

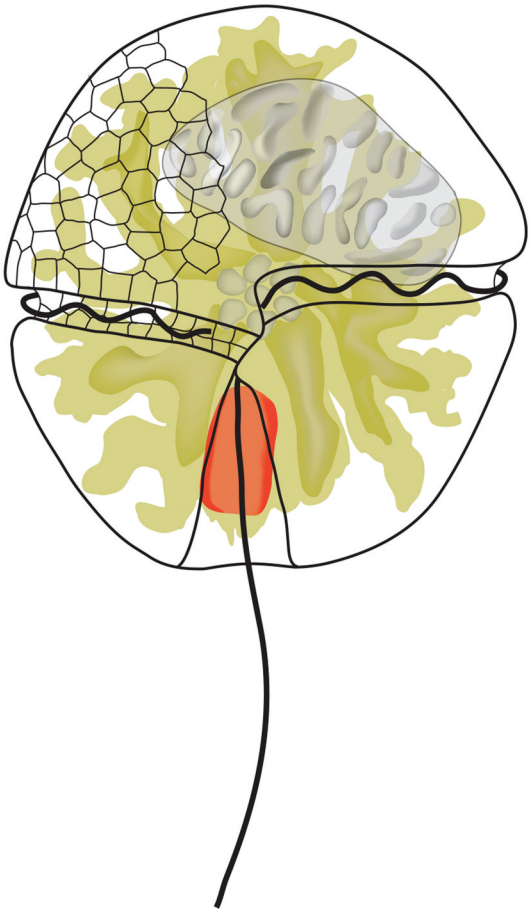


Fig. 18. Drawing of *Baldinia droopii sp. nov.*, ventral view (not to scale). Only part of the amphiesmal pattern is shown.

distinctly dorsoventrally flattened with a flat ventral side and a hemispherical dorsal side. The transverse flagellum at first undulated outside the cingulum, but as the cell gradually gained the typical shape the transverse flagellum became positioned within the cingulum (Fig. S36; Video S2).

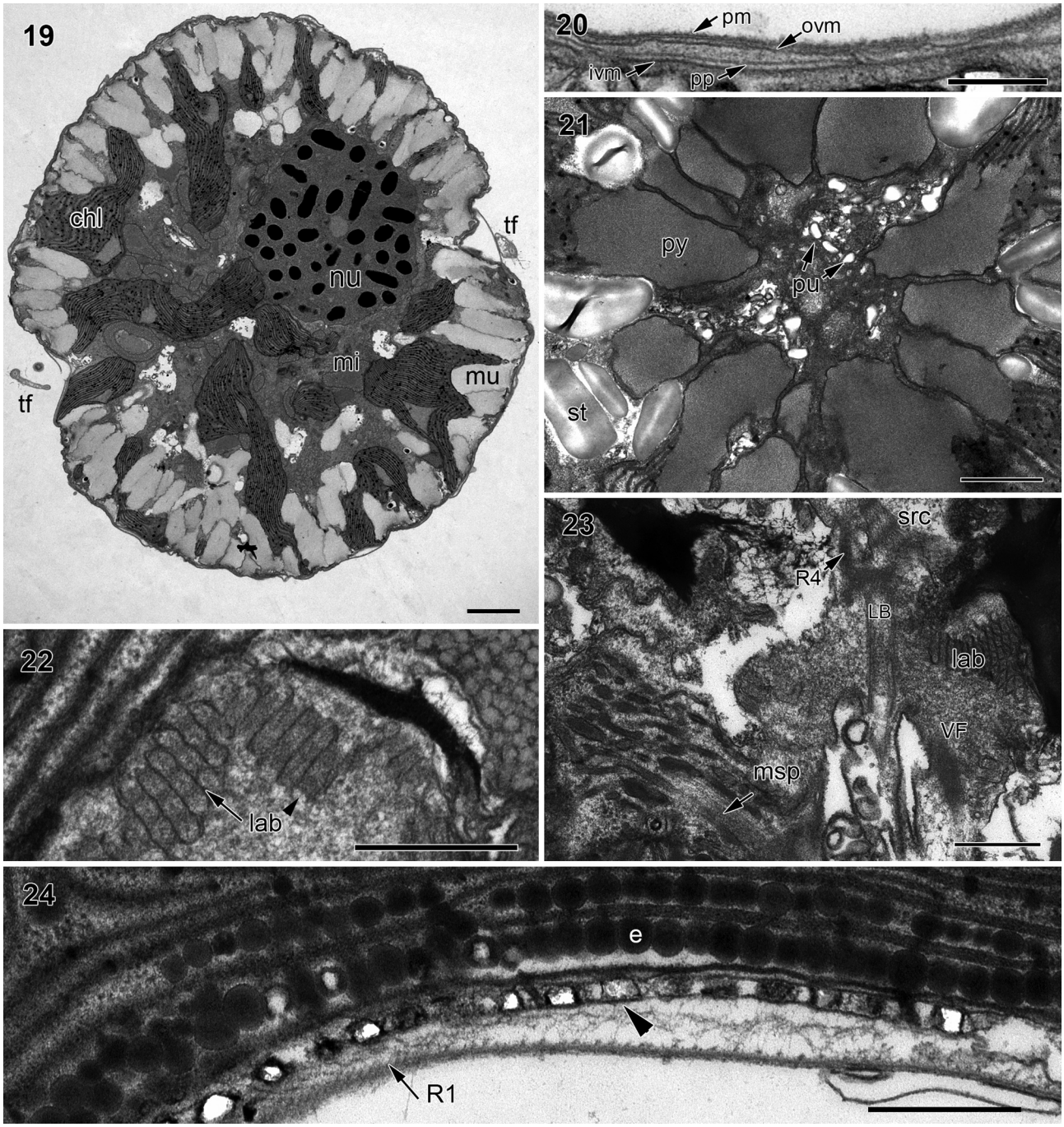
In SEM, the pattern of the amphiesmal vesicles was evident (Figs 11–15). The cells were covered with numerous pentagonal or hexagonal vesicles measuring *c.* 1.5–1.7 µm (longest dimension) (Fig. 13). Each vesicle had 1–3 more or less centrally located pore(s), maybe the exit points for the peripherally located 'mucocysts' (see below). The number of amphiesmal vesicles is roughly estimated to be over 400. The cingulum contained two rows of vesicles, and the shape of the cingular vesicles was generally more rectangular compared to the vesicles of the epi- and hyposome, except for the vesicles bordering the cingulum (Figs 13, 14). These latter vesicles were also markedly longer than wide. No apical structure or apparatus was present (Fig. 12), i.e. a particular apical differentiation of the amphiesmal vesicles such as one or two elongated apical vesicle(s) with fibrillary projections, the EAV or PEV in *Biecheleria* Moestrup, K. Lindberg & Daugbjerg and *Borghiella*, respectively (e.g. Moestrup *et al.* 2008, 2009). A diagrammatic drawing of the motile cell, including indication of the amphiesmal pattern, is depicted in Fig. 18.

NON-MOTILE COCCOID CELLS ('TEMPORARY CYSTS')

A substantial proportion of the cells were in a non-motile coccoid stage. Most of these attached to the glass wall of the test tube, notably in the upper part towards the light source, indicating positive phototaxis. Such cells, usually referred to as 'temporary cysts', were ovoid and surrounded by a smooth wall (Fig. 6). Their average length was 28.7 µm (s = 2.9, range 22.7–36.8 µm, n = 100) and the average width 23.0 µm (s = 2.6, range 16.1–29.7 µm, n = 100). For size distribution see Fig. S1. Usually the cingulum, nucleus and an orange or red accumulation body were visible inside these cells. We were able to follow their encystment and excystment using a hanging drop culture. Cells prior to encystment initiate a slow circling movement, the sulcus facing the bottom of the chamber. When a suitable place had been found the cell stopped its movement, attached to the substrate, discarded the flagella and transformed into a non-motile cell ('temporary cyst') by ecdysis, that is, shedding of the plasmalemma and outer amphiesmal membranes (Video S3). The process of ecdysis was very difficult to see, but the discarded outer membranes could sometimes be identified at the cell margin as a small-rounded sphere (Video S3). The process since the cell stopped movement, changed shape, and transformed into a 'cyst', that is, the coccoid stage, took only a few minutes. Excystment was also rapid and lasted less than a minute. The cell broke out of the cyst wall by a sudden amoeboid movement, regained the shape of the vegetative cell, and swam away leaving the cyst wall behind (Video S4). The culture contained numerous cyst walls from germinating cells (not shown). Interestingly, the change from motile to non-motile stages followed a light-induced diel rhythmicity. Thus, the first motile cells were seen 1–2 h into the light period, peaking the next 3–4 h. Only a few cells were seen to be moving during late afternoon, and essentially no cells were motile during the dark period. A large number of cells remained in the non-motile coccoid stage during the entire light period. Thus, 'temporary cyst' is somewhat misleading, as they constitute the main part of the cell cycle, the motile stage being the temporary part. The time period between encystment and excystment was very variable. Thus in a group of 12 newly settled cells, three cells germinated the day after settling (day 1), another six cells on day 2 and the last three cells on day 3. However, one cell was followed for 10 days before it excysted.

Cells continued their motility pattern when the hanging drop culture was placed in constant darkness, indicating an endogenous circadian rhythm. However, the peak of motile cells was 2–3 hours longer into the 'light period', compared to the normal light:dark cycle, and a substantial number of cells remained motile several hours into to the dark period. It was noted that a high number of non-motile cells underwent lysis during incubation in constant darkness, and after 5 d essentially all cells were dead. Probably the poor gas exchange, and respiratory processes during constant darkness led to suboptimal conditions within the hanging drop.

We regularly observed larger ovoid to rounded non-motile cells ('cysts') containing one or two nuclei (Figs. 7, S34). Typically, they also contained two accumulation bodies. Their average dimensions, length × width was 37.2 µm



Figs 19–24. TEM of *Baldinia droopii sp. nov*.

Fig. 19. Longitudinal section showing the nucleus (nu), chloroplast profiles (chl), mitochondria (mi) and putative mucocysts (mu); tf = transverse flagellum. Scale bar = 2 µm.

Fig. 20. Details of the amphiesmal vesicle: ovm and ivm: outer- and inner vesicle membrane, respectively. The amphiesmal vesicle contains trilaminar pellicle precursor (pp); pm = plasma membrane. Scale bar = 0.25 µm.

Fig. 21. The pyrenoid system (py). Tubules of the pusule (pu) are located in the central area of the cell. Starch granule (st). Scale bar = 1 µm.

Fig. 22. The lamellar body (lab) in cross- (arrow) and longitudinal view (arrowhead). Scale bar = 0.5 µm.

Fig. 23. Section showing the longitudinal basal body (LB), the ventral fibre (VF), transverse striated flagellar root (R4) and the striated root connective (src). The microtubular strand of the peduncle (msp) with associated dense vesicles and part of the lamellar body (lab) are also visible. Scale bar = 0.5 µm.

Fig. 24. Transverse section of the eyespot. Numerous dense eyespot globules are located inside the chloroplast (e). Brick-like structures (arrowhead) are situated inside an elongated vesicle that is located close to the eyespot Microtubules of the flagellar root R1 (arrow). Scale bar = 0.5 µm.

(s = 5.0, range 29.2–52.6 µm, n = 40) × 30.5 µm (s = 5.1, range 23.1–44.1 µm, n = 40). For size distribution see Fig. S1.

We were, on a few occasions, fortunate to follow the germination of these cells. A large cell with two nuclei was released, bearing two longitudinal flagella (Video S5). At the time of release the two nuclei appeared to be fusing. After about two hours the cell settled and transformed into a seemingly uninucleated non-motile cell. It was not followed further.

We also observed a bi-nucleate motile cell containing two accumulation bodies during transformation into a bi-nucleate

non-motile cell (Video S6). Large motile cells with two accumulation bodies (Fig. S33) are considered candidates for large non-motile uni-nucleate cells (Fig. S34).

Some non-motile cells, usually in the upper size range (>40 μm), had a dark-brownish colour similar to that of a planozygote (Fig. S37). They sometimes formed division cysts (Fig. S38). Daughter cells with two rather than one nucleus, that is, a four-nucleated division cyst (Fig. S39), and a division cyst containing three cells was observed on one occasion (Fig. S40).

The significance of these cells remains uncertain, but they were likely the result of sexual reproduction.

Non-motile cells that appeared to be 'dividing' directly were rarely observed (Fig. S35). Whether they were cells in fusion or division is uncertain. Thus, a motile fusing/dividing pair was observed in the hanging drop culture, settling on the bottom to become enclosed in a common wall. However, it disintegrated after a few days (not shown), but it is possible that such cells would have produced motile cells under optimal conditions.

HYPNOZYGOTES

Another type of non-motile cells were resting cysts or hypnozygotes, which appeared to be the result of sexual mating. Such cysts had a pentagonal outline with a pointed apex and a hemispherical or truncated antapex (Figs 8, 16). Their average length was 43.5 μm (s = 2.3 μm, range 40.3–47.2 μm, n = 18) and their average width 34.3 (s = 2.8 μm, range 29.7–40.5 μm, n = 18). The outer cyst wall was covered with numerous rod-shaped projections (Fig. 9). However, SEM revealed that some had small openings, indicating they were hollow (Figs 16, 17). In cyst terminology they are termed 'intratabular hollow tubiform processes' (e.g. Williams *et al.* 1978), but they are here referred to as tubiform processes. The inner wall or membrane was closely appressed to the cytoplasm, which in fully mature cysts was colourless or flesh-coloured, and had a depression reflecting the cingulum (Figs. 8, S30, S31). Numerous storage granules and a distinct red accumulation body were present in the cyst, and the nucleus was also visible. Cysts in different degrees of maturation were observed. Early or immature stages had a visible chloroplast, and the cytoplasm was appressed to the outer cyst wall. Mature stages lacked a recognizable chloroplast (Figs S26–S31). Formation of resting cysts was never observed within the hanging drop culture.

SEM of mature cysts revealed a para-tabulation very precisely reflecting the arrangement of the amphiesmal vesicles (Figs 16, 17). Each 'vesicle' had one or two tubiform processes that most likely corresponded to the site of the amphiesmal pores in the motile vegetative cell. Germination of the cysts was not observed, but empty cysts were occasionally found that had ruptured somewhat irregularly (Fig. 10). The cyst may have a chasmic archeopyle ('slit-like') *sensu* Matsuoka (1985), but this is somewhat uncertain.

TEM

Baldinia droopii sp. nov. had an internal cellular complement typical of dinoflagellates, that is, a dinokaryon with condensed chromosomes (Fig. 19) and an amphiesma consisting of flattened vesicles subtending the plasma membrane (Fig. 20). The amphiesmal vesicles contained a thin dark-staining plate-like or membrane-like structure. It had a trilaminar appearance and corresponded to the pellicle precursor found in a number of other dinoflagellates. However, cells also contained two rather unusual structures which have been found only in *Baldinia* and a few other dinoflagellates: (i) a so-called lamellar body situated near the flagellar base area (Figs 22, 23) and (ii) a ventral fibre associated with the longitudinal basal body (Fig. 23).

Numerous chloroplast lobes radiated from a centrally located pyrenoid system towards the cell periphery (Figs 19, 21). Pusular tubules were situated in the central area or cavity delineated by the pyrenoid system (Fig. 21). Numerous vesicles with electron-opaque content, putative 'mucocysts', were situated along the cell periphery (Fig. 19), but trichocysts were absent. A microtubular strand with numerous associated osmiophilic bodies was identified as a microtubular strand of the peduncle. The striated flagellar root, R4 (or transverse striated root, tsr) and the striated root connective (src) were also observed (Fig. 23). The eyespot was located in the sulcal region and comprised rows of osmiophilic globules within the chloroplast. A narrow vesicle containing brick-like structures was situated close to the eyespot, and the R1 flagellar root was situated in between the cell surface and the brick-containing vesicle (Fig. 24). This type of eyespot has been designated as a type B eyespot (Moestrup & Daugbjerg 2007).

Molecular phylogeny

The genetic distance between *B. droopii* and *B. anauniensis* was 0.139. This estimate was based on 1,333 base pairs of nuclear-encoded LSU rDNA and included the highly variable domain D2. The value is similar to the genetic distance between *T. sanguinea* and *T. coronata* of 0.128, which was based on 1,353 base pairs.

The tree topology based on LSU rDNA and inferred from BA is shown in Fig. 25. Using *Moestrupia oblonga* (J. Larsen & D.J. Patterson) Gert Hansen & Daugbjerg to polarize the remaining dinoflagellate taxa, the Tovelliaceae branched off as the earliest group, followed by *Heterocapsa* spp. Each of these monophyletic clades received high branch support (PP = 1.0, bootstrap = 97% and PP = 1.0, bootstrap = 100%, respectively). The monophyletic *Sphaerodinium* Wołoszyńska, with two species, formed the earliest diverging lineage within the Suessiales and this topology was supported by PP = 1.0, bootstrap = 97%. The species of particular interest for this study, *Baldinia droopii*, formed a sister taxon to *B. anauniensis* (PP = 1.0, bootstrap = 100%). However, the *Baldinia* lineage itself formed a trichotomy with the monophyletic *Borghiella* spp. clade (PP = 1.0, bootstrap = 100%) and the *Dactylodinium*/Suessiaceae clade (PP = 1.0, bootstrap = 95%). *Dactylodinium* Kazuya Takahashi, Moestrup & Iwataki was more closely related to Suessiaceae than to Borghiellaceae despite the presence of a type B eyespot. Sometimes it is included in Borghiellaceae, leaving this family paraphyletic (Takahashi *et al.* 2017). Some of the many

evolutionary lineages within the Suessiaceae were well resolved while others were not. In general, the clade containing the genus *Symbiodinium* Gert Hansen & Daugbjerg and the newly erected genera (e.g. *Effrenium* LaJeunesse & H.J. Jeong, *Durusdinium* LaJeunesse, *Cladocopium* LaJeunesse & H.J. Jeong and *Breviolum* J.E. Parkinson & LaJeunesse) previously belonging to *Symbiodinium* was well resolved (i.e. received high branch support) (Fig. 25). The deepest branches

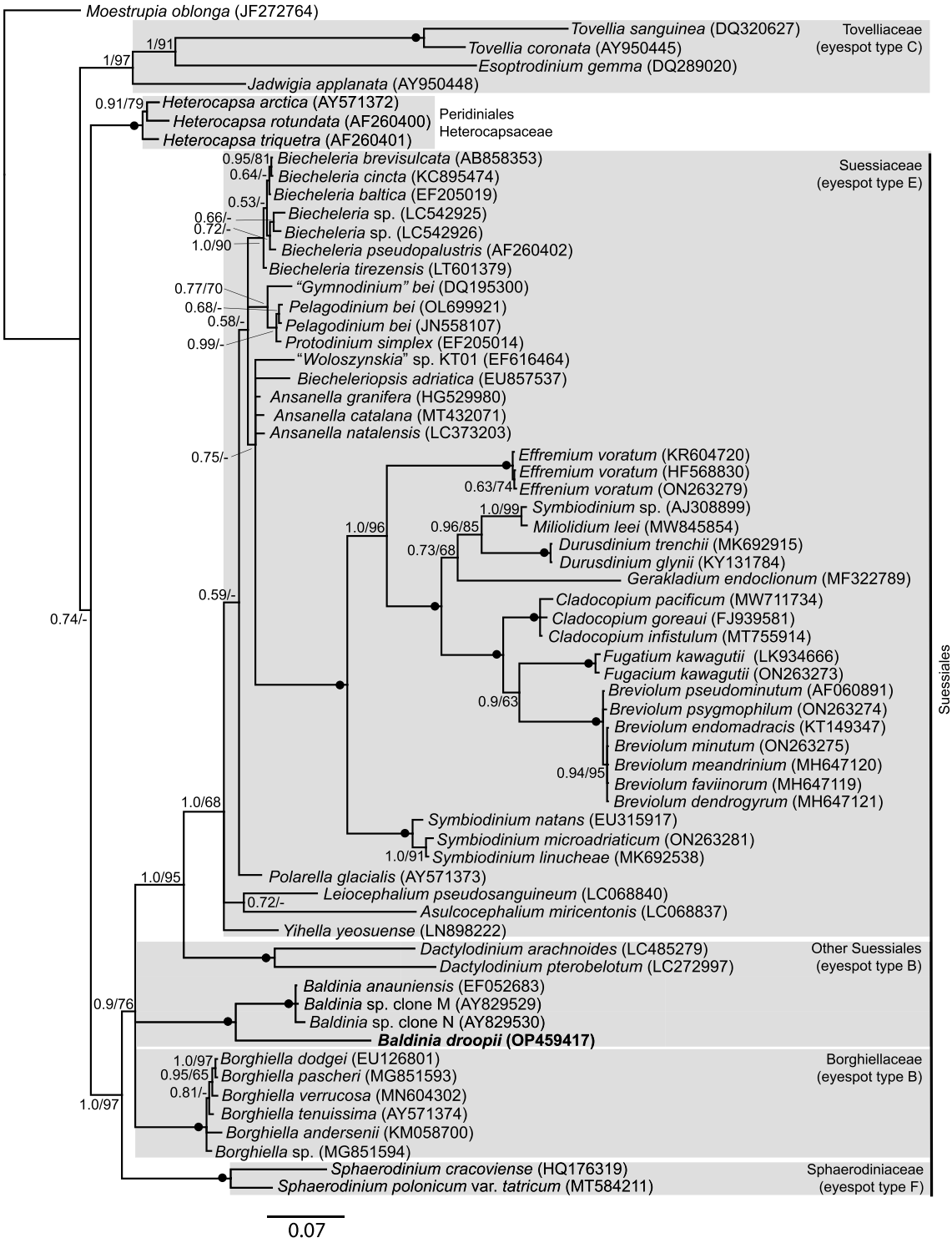


Fig. 25. Phylogeny of *Baldinia droopii sp. nov.* based on Bayesian analysis of nuclear-encoded partial LSU rDNA sequences. The analysis included 1,278 base pairs including introduced gaps. A total of 64 other dinoflagellates sequences were included and represented 26 genera and 52 identified species. *Moestrupia oblonga* formed the outgroup taxon. The robustness of the tree topology was evaluated by posterior probabilities (PP ≥ 0.5) from BA with 5 million generations and 5,000 bootstrap replications (BS ≥ 50%) from RaXML. These values were written at internodes and filled circles indicated the highest possible support in BA (1.0) and RaXML bootstrap (100%). PP values < 0.5 and BS < 50% were indicated by a dash (–). GenBank accession numbers were provided in parentheses following the species names. The families Sphaerodiniaceae, Borghiellaceae, Suessiaceae, Heterocapsaceae and Tovelliaceae were also indicated on the phylogenetic tree as well as eyespot types when present. The branch lengths are proportional to the number of character changes, see scale bar.

within Suessiaceae were not resolved, making it impossible to assess their evolutionary history. The three species of *Ansanella* H.J. Jeong, S.H. Jang, Moestrup & Nam Seon Kang included in this analysis did not form a monophyletic genus but rather an unresolved clade with *Biecheleriopsis adriatica* Moestrup, K. Lindberg & Daugbjerg, '*Woloszynskia*' sp. and *Symbiodinium sensu lato*.

DISCUSSION

Generic identity

Baldinia droopii sp. nov. displayed a combination of phenotypic characters that suggested it to belong to the genus *Baldinia*. This included the presence of a lamellar body, a ventral fibre, a type B eyespot, the lack of trichocysts, and the lack of an apical structure. The molecular phylogeny also supported that it belongs to *Baldinia*. The relatively high genetic distance (*c*. 14% based on partial LSU rDNA) between *B. anauniensis* and *B. droopii* was similar to distance between *Tovellia sanguinea* and *T. coronata* and thus not atypical for dinoflagellates.

None of the morphological structures present or lacking are unique to *Baldinia*. A lamellar body has also been found in *Sphaerodinium cracoviense* Wołoszyńska and *S. polonicum* var. *tatricum* Wołoszyńska, and suggested to be homologous with the lamellar body of *Baldinia* (Craveiro *et al*. 2010; Pandeirada *et al*. 2021). Dinoflagellates containing a diatom endosymbiont, so-called dinotoms, have a somewhat similar structure but lack the honeycomb pattern of *Baldinia* and *Sphaerodinium*, and the structures are perhaps not homologous, as discussed by Craveiro *et al*. (2010). A ventral fibre has also been observed in *S. cracoviense* and *S. polonicum* var. *tatricum*, suggesting that *Baldinia* is related to *Sphaerodinium*, though the latter has thecal plates with a somewhat 'peridinioid' arrangement, trichocysts and a different eyespot (type F; Craveiro *et al*. 2010; Pandeirada *et al*. 2021). The lack of an apical structure is rather unusual but the suessialeans *Asulcocephalium* Kazuya Takahashi, Moestrup & Iwataki, *Leiocephalium* Kazuya Takahashi, Moestrup & Iwataki and *Polarella* Montresor, Procaccini & Stoecker also lack an apical structure (Montresor *et al*. 1999; Takahashi *et al*. 2015). The lack of trichocysts is a common feature of species in the Borghiellaceae and Suessiaceae, apart from *Dactylodinium* (Takahashi *et al*. 2017; Lum *et al*. 2019). However, the pentagonal resting cyst of *Baldinia* has, to the best of our knowledge, not been observed in other dinoflagellates, and presently makes it the only autapomorphic character at the morphological level.

Species identity

Until now only two other species of *Baldinia* have been described, *B. anauniensis* and *B. bernardinense*. *Baldinia droopii* differs from *B. anauniensis* by the presence of a higher number of amphiesmal vesicles, *c*. 400 compared to the *c*. 100 in *B. anauniensis*. In addition, the cingulum in *B. anauniensis* comprises only one row of vesicles, compared to two in *B. droopii* (Hansen *et al*. 2007). Also, the plates adjacent to the cingulum differ, making the cingular area of *B. droopii* structurally more complex compared to *B. anauniensis*. This is rather unusual as the arrangement and number of cingular plates are typically more conservative and considered a generic character, particular within the peridinioid taxa (e.g. Balech 1974; Indelicato & Loeblich 1986).

A difference in the number of cingular vesicles has also been noted in the suessialean genus *Biecheleria*. In the freshwater type species *B. pseudopalustris* (J. Schiller) Moestrup, K. Lindberg & Daugbjerg *c*. 3–4 rows were present (Moestrup et al. 2009; Moestrup & Calado 2018) whereas only 2–3 rows were present in the five marine species *B. baltica* Moestrup, K. Lindberg & Daugbjerg, *B. brevisulcata* Kazuya Takahashi & Iwataki, *B. cincta* (Siano, Montresor & Zingone) Siano, *B. halophila* (Biecheler) Moestrup, K. Lindberg & Daugbjerg and *B. tirezensis* S. Fraga, Raho, J.P. Abad & I. Marín (Biecheler 1952; Lerch *et al*. 2005; Siano *et al*. 2009; Takahashi *et al*. 2014; Raho *et al*. 2018). The amphiesmal vesicles were reported to be empty in *B. anaunensis*, but contain pellicle precursor in *B. droopii*. Such material occurs in many dinoflagellates and is usually said to be plate-like or a darkly staining layer (see Morrill & Loeblich 1983 for a review). The process of pellicle formation was particularly well illustrated in *Borghiella pascheri* (Suchlandt) Moestrup (Moestrup *et al*. 2018), a species that forms 'red snow' and primarily occurs as non-motile cells.

The most distinctive difference between the two species is the cyst of *B. droopii* having tubiform processes, compared to the smooth-walled cyst of *B. anauniensis*. A distinct paratabulation corresponding to the amphiesmal pattern of the motile stage was apparent in *B. droopii*. Whether a paratabulation is present on the cyst wall of *B. anauniensis* is unknown, as no SEM analyses have been made. Resting cysts were readily produced in the culture of *B. droopii*, suggesting it to be homothallic, while resting cysts were never observed in the culture of *B. anauniensis* (Hansen, personal observations), which may therefore be heterothallic. However, it cannot be ruled out that the culture of *B. droopii* was initiated from a planozygote, that is, not being strictly monoclonal in such a case. Re-isolation of *B. droopii* with the aim of clarifying this question has been unsuccessful so far.

The presence of cysts with tubiform processes in *B. droopii* is interesting from a phylogenetic point of view. Thus, the family Borghiellaceae, in which *Baldinia* is sometimes placed (e.g. Moestrup & Calado 2018), has smooth-walled cysts as one of its diagnostic characters, as opposed to spiny cysts in the family Suessiaceae (Moestrup *et al*. 2009), that is, Borghiellaceae needs to be emendated to accommodate this character. However, as the phylogenetic analysis conducted here left the Borghiellaceae unresolved, and the family therefore not monophyletic, we find it premature to make such a change.

The other described species, *Baldinia bernardinensis*, has never been studied since its discovery in a small alpine lake 'Entre-deux Tours', near St. Bernard, Praz de Fort, Switzerland (Chodat 1924). It differs from *B. droopii* by having pale-brown rather than yellow-greenish chloroplast and in being distinctly dorsoventrally flattened. The resting cyst appears to be similar to *B. droopii* but with two notable

exceptions: (i) the outer cyst wall is smooth with a distinct depression corresponding to the paracingulum, and (ii) the early cyst stages may have small spines or processes that later disappear. Chodat (1924, fig. V) depicted one cyst with a few spines, but the significance of this drawing is not clear; does it represent a maturing cyst? The resting cyst of *B. bernardinensis* is smaller, 26–33 µm in length, compared 40.3–47.2 µm in *B. droopii*.

There can be little doubt that *B. droopii* represents a new species.

Remarks on the life cycle

We were able to gain some insight on the life cycle by following a small amount of culture in a hanging drop for several days. The large number of non-motile coccoid cells was striking in the culture tube, but their part in the cell cycle induced by the light period first became clear within the hanging drop culture. Should they be considered temporary cysts? Formation of temporary cysts has traditionally been associated with adverse conditions (e.g. Anderson *et al.* 1995), but it has now been shown that temporary cysts may sometimes be an integrated part of the life cycle (Garcés 2002). Thus, *Alexandrium taylori* Balech forms temporary cysts in the early evening, which settle to the bottom and, following cell division, give rise to motile cells the next morning, that is, they act as 'division cysts' (Garcés 2002; Garcés *et al.* 2002). A diel rhythmicity of motile vs non-motile cells controlled by the light and/or tidal period has also been observed in a number of tide pool dinoflagellates. Thus, *Scrippsiella hexapraecingula* T. Horiguichi & Chihara forms motile cells during the morning and they transform into the non-motile stage during the evening, attaching to the substrate by a gelatinous stalk. Cytokinesis takes place in the non-motile stage around midnight (Horiguchi & Chihara 1983). The diel cycle of motile vs non-motile stages of *Bysmatrum arenicola* T. Horiguchi & Pienaar and *Gymnodinium pyrenoidosum* T. Horiguchi & Chihara followed a similar pattern, and at high tide they returned and attached to the bottom *c.* 1–2 h before the tide pool became flooded, even when the light intensity was high. One or two motile cells (zoospores) were released *c.* one hour after the beginning of the light period (Horiguchi & Chihara 1988; Horiguchi & Pienaar 1988). The cells thereby avoid being flushed out during high tide. The diel rhythm with respect to light and motility is less obvious, but conditions within a tide pool, for example, light, nutrient gradients, water levels, etc., may be quite fluctuating and a period of motility gives the alga a possibility to reposition itself to more favourable areas within the pool. The tide pool of *B. droopii* was not susceptible to diurnal tidal flooding as the tidal amplitude in this region of the Baltic Sea is less than 1 cm (Medvedev *et al.* 2013). However, considerable fluctuation in the water levels due to evaporation may occur, particularly during the warm season. Other species in the ephemeral rainwater puddles on the small islands of the Tvärminne archipelago rockpools can survive complete drying out for a prolonged period of time. This applies to the chlorophyte *Haematococcus lacustris* (Girod-Chantrans) Rostafinski (=*H. pluvialis* Flotow) and the dinoflagellate *Nottbeckia ochracea* Gert Hansen, Daugbjerg & Moestrup, which are common in the same area. *Baldinia droopii* does not seem to tolerate a complete drying out, however, since attempts to 'revive' dried cells were unsuccessful. Nevertheless, it seems likely that the non-motile stage of *B. droopii* may provide some protection against short-term exsiccation of the pool.

Interestingly, the symbiodinians (dinoflagellates within the order Suessiales and endosymbionts in various invertebrates, notably corals) commonly produce motile cells when isolated into culture and show a diel pattern of non-motile vs motile cells, similar to *B. droopii* (e.g. Freudenthal 1962). Some studies have shown this to be a circadian rhythm, as the light:dark pattern continued 5 d in continuous darkness (e.g. Lerch & Cook 1984). Other studies have suggested that the rhythm is caused by light cues *per se* and do not represent an endogenous circadian rhythm (Yacobovitch *et al.*). The presence of free-living motile cells in symbiodinians may serve as a transfer mechanism for infecting the young stages of corals and other hosts (Trench 1987; Coffroth *et al.* 2006), but the significance of a diel rhythm of motility with respect to light appears to be unknown. A circadian rhythm with respect to motility vs non-motility seemed to be present in *B. droopii*, as motile cells occurred during constant darkness, albeit more irregularly.

Both vegetative and sexual reproduction was observed within the culture of *Baldinia droopii*. The occurrence of vegetative division by binary fission as well as within a division cyst is rather unusual. However, it has also been observed in *Alexandrium taylori* and *Borghiella andersenii* Daugbjerg, Andreasen, Happel, Pandeirada, Gert Hansen, Craveiro, Calado & Moestrup (Garcés *et al.* 1998; Daugbjerg *et al.* 2014). It also seems to occur in *Gymnodinium pseudomirabile* Gert Hansen & Flaim (Hansen & Flaim 2007). The ecological significance is not clear, but it has been speculated that binary fission is a faster method for rapid cell proliferation, while division within a cyst is slower but offers greater protection (Montresor 2002). Division cysts of *B. droopii* were quite rare in culture, but this could be different for *in situ* populations.

The presence of resting cysts in the culture suggested that sexual processes took place. However, details of the sexual life cycle remain speculative before more comprehensive studies of single-cell isolations of the different cell types are made. For example, are the large one- or two nuclei non-motile cells ('cysts') the result of cell fusion, that is, sexual reproduction, or arrested mitotic cell divisions? The presence of two accumulation bodies suggests sexual reproduction, as it seems unlikely that duplication of the accumulation body is under genetic control. This argument was used to distinguish 2C DNA content cells of the symbiodinian *Cladocopium latosorum* Turnham, Sampayo & LaJeunesse as being mitotic or meiotic, meiotic cells having two accumulation bodies (Figueroa *et al.* 2021). Dividing cells in which each daughter cell received one accumulation body were observed (Video S1) and could be explained as the first meiotic division although each daughter cell looked very similar to a normal vegetative cell in size and morphology. Resting cysts were never formed in the hanging drop culture, but fusing cell-

pairs recognized by their perpendicular cingula were observed. It suggests that the sexual reproduction may bypass the formation of a resistant resting cyst with its obligate dormancy period, similar to what has been observed in an increasingly number of dinoflagellates (e.g. Uchida *et al.* 1996; Figueroa *et al.* 2008, 2015). Interestingly, conversion of planozygotes into divisions cysts that produced motile cells was particular well documented in *Tovellia rinoi* Pandeirada, Craveiro, Daugbjerg, Moestrup & Calado (Pandeirada *et al.* 2017). Nuclear cyclosis, that is, rotation of the chromosomes, indicative of the zygotene to post-zygotene phase of meiosis (von Stosch 1973), was never observed in *Baldinia droopii*. On the other hand, it has never been observed in *Alexandrium minutum* Halim either although the life cycle of this species is very well studied (Figueroa *et al.* 2015). The presence of a three-cell, four-nuclei division cyst in *B. droopii* resembles the observations in *C. latosorum* and suggests a 'normal' two-step meiotic type (Figueroa *et al.* 2021). An asynchronous second meiotic division which resulted in three rather than four cells has also been observed in *Parvodinium inconspicuum* (Pfiester *et al.* 1984). The observation of a putative planomeiozygote may suggests that meiosis may occur after and not before germination of the resting cyst.

In summary, *B. droopii* displays a life cycle comprising vegetative cell division by either binary fission or a division cyst, and sexual reproduction which may lead to a resting cyst with distinct tubiform processes. The zygote may skip a dormancy period and transform into a 'temporary cyst' or, more correctly, a coccoid stage. The zygote may divide within this 'cyst', but direct division of the zygote cannot be excluded. Thus, the life cycle of *B. droopii* may involve multiple routes, and in this respect appears to be similar to what has been observed in other dinoflagellates (Figueroa *et al.* 2006; Kremp 2013, and references therein). A complicating factor is the alternating phases of a motile and a non-motile stage, with an apparently rather short window of the motile stage. Thus, cells may enter the non-motile or the coccoid stage at different stages of the life cycle, leading to one- or two-nuclei non-motile cells that may be vegetative (mitotic) or sexual (meioitic). Non-motile cells that appeared to be in the progress of division *per se*, may be due to incomplete cytokinesis or cell fusion within the 'window' of motility, that is, before the formation of the 'temporary cyst'.

Phylogeny and character evolution

The overall phylogeny showed high support for monophyly of the three families within the order Suessiales: Sphaerodiniaceae, Borghiellaceae and Suessiaceae, characterized by the presence of a type F, type B and type E eyespot, respectively. *Dactylodinium* and *Baldinia* have been placed in Borghiellaceae (Daugbjerg *et al.* 2014; Takahashi *et al.* 2017; Moestrup & Calado 2018; Lum *et al.* 2019), but this make Borghiellaceae paraphyletic. *Dactylodinium* is more closely related to Suessiaceae, but was placed in Borghiellaceae by Takahashi *et al.* (2017) due to the presence of a type B eyespot. They should probably be placed in a family of their own, and the same applies to *Baldinia*, but their phylogenetic position are presently unclear. Previous analyses based on LSU rDNA sequences have placed *B. anauniensis* as basal, sister to Borghiellaceae and Suessiaceae (Moestrup *et al.* 2009; Daugbjerg *et al.* 2014) or closer to Suessiaceae (Moestrup *et al.* 2018), although this was weakly supported. A recent phylogeny also based on LSU rDNA placed *B. anauniensis* as sister to Borghiellaceae with moderate support, but higher support was obtained in a concatenated phylogeny of LSU and SSU rDNA (Knechtel *et al.* 2020). The addition of *B. droopii* did not resolve the phylogenetic position of *Baldinia* within the Suessiales, and a multigene approach seems to be necessary to clarify its relation to other suessialean taxa.

The basal position of *Sphaerodinium* is interesting, as it shares two distinct characters with *Baldinia*, the lamellar body and the ventral fibre. Apart from *Baldinia*, these characters were apparently lost in other suessialean taxa. Otherwise, *Sphaerodinium* differs from other suessialean taxa by having fewer amphiesmal vesicles containing thicker thecal plates (*c.* 24, excluding cingular and sulcal vesicles), a different eyespot (type F) and the presence of trichocysts (Craveiro *et al.* 2010). Trichocysts re-evolved in the genus *Dactylodinium*, but they differ from other dinoflagellates in the presence of lateral hairs (Takahasi *et al.* 2017; Lum *et al.* 2019).

Most members of the Suessiales, including *Baldinia*, have many (>100) amphiesmal vesicles, but considerable variation may occur even within a genus. For example, *Dactylodinium arachnoides* W.M. Lum, Kazuya Takahashi, Takayama & Iwataki has *c.* 40 amphiesmal vesicles, but *D. pterobelotum* Kazuya Takahashi, Moestrup & Iwataki only *c.* 20 vesicles (excluding cingular and sulcal vesicles). The symbiodinians also have relatively few amphiesmal vesicles (*c.* 25). Thus, loss and gain of amphiesmal vesicles have occurred several times within the Suessiales. Also, the presence of thecal plates within the vesicles varies, from relatively thick plates in *Sphaerodinium*, to their absence in *Baldinia*. They may also be absent in *Asulcocephalium* and *Leiocephalium*. The amphismal vesicles in these genera contains two thin layers, a dark-staining inner layer, most likely the pellicle precursor, and a less dense outer layer (Takahashi *et al.* 2015). It is possible that the latter constitutes a very thin thecal plate. Thus, loss of thecal plates within the Suessiales has occurred at least once (*Baldinia*), but perhaps several times (*Asulcocephalium, Leiocephalium*).

Multiprotein phylogeny of dinoflagellates suggests a single origin of thecate dinoflagellates, from ancestors having a gonyaulacoid-peridinioid tabulation. The extant members of Suessiales were found to be late-branching within the thecates, that is, had a derived position, which does not support their position as early intermediates in the evolution of the thecate dinoflagellates (Janouškovec *et al.* 2016). Following the above authors, the modern gonyaulacoid-peridinioid tabulation types are thought to have originated in Early Jurassic, while fossil suessioids originated in Late Triassic, and could represent an intermediate stage between gymnodinioid and gonyaulacoid-peridinioid tabulation types (Janouškovec *et al.* 2016). Thus, placing extant 'suessialean' taxa in the fossil order and family Suessiales and Suessiaceae, respectively, may turn out to be misleading.

A dominant coccoid or immotile life-cycle stage within the Suessiales has primarily been restricted to the symbiodinians. However, the recent discovery that the coccoid *Dinastridium verrucosum* Baumeister belongs to *Borghiella* [*B. verrucosa* (Baumeister) Knechtel & Gottschling] was unexpected. In culture the motile stage is dominating (Knechtel *et al.* 2020), but the non-motile stage dominates in the type species *Dinastridium sexangulare* Pascher (Pascher 1927). Yet, it remains to be determined by molecular methods if Pascher's species is also a *Borghiella*, as the name *Dinastridium* has priority (Knechtel *et al.* 2020). The recent SSU-LSU rDNA-based phylogeny of Knechtel *et al.* (2020) suggests the coccoid dinoflagellates *Cystodinium phaseolus* Pascher and *Phytodinium* sp. as the closest sister group to *Baldinia*. The detailed fine structure of the zoospores in these species are not known but our preliminary studies on a culture of *Cystodinium* G.A. Klebs indicated an eyespot of type B, while a lamellar body was not observed (Moestrup, unpublished observations). Thus, non-motile life-stages occur in separate lineages within the Suessiales. A substantial number of cells also appear to be non-motile in *Sphaerodinium cracoviense* (personal observations, but also apparent in Craveiro *et al.* 2010, fig. 2) but their role in the life-cycle is not known. In contrast to other coccoid suessialeans, *Baldinia droopii* does not form aplanospores. That is, cell proliferation needs to proceed via the motile stage. Division cysts may be formed, but motile, not coccoid cells are released. In conclusion, we believe that this study has expanded our general understanding of the life cycle in dinoflagellates and further illustrates the multitude of ways these protists proliferate.

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