Fine-structural characterization and phylogeny of *Sphaerodinium* (Suessiales, Dinophyceae), with the description of an unusual type of freshwater dinoflagellate cyst

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**Abstract**

Two strains of *Sphaerodinium* were established from two mountain areas in Portugal and examined by light microscopy, scanning and transmission electron microscopy, and sequence analyses of nuclear-encoded SSU, ITS1–5.8S–ITS2 and LSU rDNA. Both strains were identified as *S. polonicum* var. *tatricum* on the basis of comparison with the original taxonomic descriptions within the genus. The two strains were nearly identical in morphology and ultrastructure, except for the presence of pseudograna-like thylakoid stacks within more rounded chloroplast lobes in one of them. Sexual reproduction occurred in culture batches and resting cysts with single or grouped processes with wide bases and distal platforms with slightly recurved margins were seen to develop by sudden retraction of planozygote cytoplasm. Morphological, fine-structural and molecular characters were compared with previously available information from *S. cracoviene*, allowing for a more robust characterization of the genus. Important characters include a type F eyespot, a pusule canal linking the transverse flagellar canal to a collecting chamber connected to regular pusular tubes, a ventral fibre extending from the proximal-right side of the longitudinal basal body, and a membranous, lamellar body with a honeycomb pattern near the flagellar base area. The latter two features are shared with *Baldinia anauniensis*.

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**Introduction**

The genus *Sphaerodinium* Wołoszyńska includes freshwater, thecate dinoflagellates with relatively large plates, which are comparable in size to those of peridinioid and gonyaulacoid species (Moestrup and Calado 2018; Schiller 1935; Starmach 1974; Wołoszyńska 1916). The plate disposition in six latitudinal series is, according to Taylor (2004), of the peridinioid type. It differs, however, from what is common in peridinioids in having four intercalary plates (instead of zero to three) and six postcingular plates, rather than the usual five. However, it is now known that there is no close evolutionary relationship between the Peridiniales and *Sphaerodinium* (Craveiro et al. 2010; Moestrup and Calado 2018).
With the exception of *S. fimbriatum* R.H. Thompson, all species in the genus were described by Woloszyńska (1916, three species and one variety: 1930, one species; see also Schiller 1935). *Sphaerodinium fimbriatum* has fimbriate projections on sutures and plates of the hypothecone, which make it quite distinct (Carty 2014; Couté and Ittis 1984; Thompson 1951). However, the significance of characters used by Woloszyńska to distinguish taxa within *Sphaerodinium* has been the subject of divergent opinions and some monographic treatments of freshwater dinoflagellates lump her taxa into a single species with varieties (e.g. Huber-Pestalozzi 1950). Moestrup and Calado (2018) opted for presenting the original concepts of Woloszyńska’s *Sphaerodinium* taxa to allow their morphologies to be recognized in natural populations so that their taxonomic status may be established by further studies.

The detailed morpho-molecular study of a dinoflagellate population collected near Cracow, Poland, identified as *S. cracoviense* Woloszyńska, suggested a phylogenetic affinity with the Borghillaceae and Suesiaceae/Symbiodiniaceae sensu lato, placing *Sphaerodinium* in or near the order Suesiales; and serial section electron microscopy revealed several unusual features that may be useful for characterizing the parameters of this group of species (Craveiro et al. 2010). However, determining which of these characters are variable among species and which are stable, and therefore relevant characters at the generic level, requires comparative analysis of several species within the genus. In the present account, we examined two strains started from material collected in two mountain areas in Portugal. Their morphology was almost mutually identical, but both showed consistent differences from *S. cracoviense*. Comparisons between nuclear-encoded ribosomal operon sequences of *S. cracoviense* and the new strains confirmed the existence of species-level diversity within *Sphaerodinium*. Ultrastructural analysis of this new material revealed a number of potentially stable features that may be suggested as features characterizing the genus *Sphaerodinium* and possibly the family Sphaerodiniaceae.

In addition, the morphology and formation process of resting cysts that were produced in batches of the two strains is described and compared with previously reported information about freshwater dinoflagellate cysts.

**Material and methods**

**Biological material**

Two uni-algal cultures were used, both started from single cells isolated from plankton samples taken in two mountain sites in Portugal (both at 350–400 m alt.). Collection site 1 was a pond in Vila do Gerês, Peneda-Gerês National Park in Northern Portugal, sampled 1 September 2014 (41°43’49.97”N, 8°09’43.22”W). Collection site 2 was a water tank next to the Bucaco Palace Hotel, a 19th century royal retreat built in the Bucaco Forest, Central Portugal, sampled 7 July 2015 (40°22’33.74”N, 8°21’55.62”W). Cultures were grown in MBL culture medium (Nichols 1973) at 18 °C with 12:12 light:dark photoperiod and photon flux density of about 25 μmol m⁻² s⁻¹. Cells of both strains closely agreed in general characteristics and cell cover plate arrangement with the original description of *Sphaerodinium polonicum* var. *tarticum* Woloszyńska (Woloszyńska 1916) and this name is used herein. See further consideration on the organisms’ identity in Discussion.

**Light microscopy (LM)**

Swimming cells, stained cells, empty thecae and cysts were photographed with a DP70 Olympus camera (Olympus Corp., Tokyo, Japan) mounted on a Zeiss Axioplan 2 imaging light microscope (Carl Zeiss, Oberkochen, Germany). Encystment was recorded with a JVC TK-C1481BEG colour video camera (Nobrain SD Ltd, Reading, United Kingdom) coupled to a Leitz Labovert FS inverted light microscope (Leica Microsystems, Wetzlar, Germany). Nuclei were stained with acetocarmine added to the edge of a preparation coverslip and briefly heated over a flame.

**Scanning electron microscopy (SEM)**

Different fixation protocols were used for the visualization of different aspects of vegetative cells: 1) good preservation of cell shape and flagella was obtained with a 1-h fixation of a 2:1 mixture of cell suspension and a fixative made of 2% aqueous OsO₄ and saturated aqueous HgCl₂ (3:1, v/v); 2) somewhat swollen cells showing more distinct surface knobs were obtained with a 1-h fixation of a 1:1 mixture of cell suspension and 50% ethanol. Cyst preservation was similar for both fixation protocols. Fixed cells and cysts were retained on Nucleopore polycarbonate filters with 5-μm pore size (Whatman, GE Healthcare Life Sciences, Maidstone, United Kingdom). Filters from fixation 1 were washed with distilled water for 30–60 min. All material was dehydrated in a graded ethanol series (10–15 min in each concentration) and critical-point-dried in a Baltec CPD-030 (Balzers, Liechtenstein). Filters were glued onto stubs and sputter-coated with gold-palladium. Cells were observed with the scanning electron microscopes Hitachi S-4100 (Hitachi High-Technologies Corp., Tokyo, Japan) and JEOL JSM 6335F (Jeol Ltd, Tokyo, Japan).

**Transmission electron microscopy (TEM)**

The general steps for the preparation of cells of *Sphaerodinium polonicum* var. *tarticum* for TEM observations were similar to the ones used for *S. cracoviense* (Craveiro et al. 2010). In particular, swimming cells of both strains were picked up with a micropipette and immersed in full concentration fixative. Cells of both strains fixed for 20–30 min in a mixture of 1% glutaraldehyde and 0.5% OsO₄ (final concen-
trations) in phosphate buffer 0.1 M, pH 7.2, were examined. In addition, cells from the Buçaco isolate fixed for 1 h in 2% glutaraldehyde (final concentration) in the same buffer were also examined. Serial sections of two cells from Buçaco (both fixations) and one cell from Gerês were examined with a JEM 1010 electron microscope (JEOL Ltd., Tokyo, Japan), fitted with a Gatan Orius digital camera (Gatan, Inc., Pleasanton, California USA).

**DNA extraction and PCR amplification**

About 50–70 swimming cells from each strain of *Sphaerodinium polonicum* var. *tetricum* and *S. cracoviense* were pipette-washed in miliQ water before DNA extraction with QuickExtract™ FFPE DNA Extraction Kit (epicentre, Illumina company, San Diego, California USA), following instructions from the manufacturer. Two microliters of extracted DNA were used in each PCR amplification of SSU rDNA, ITS1-5.8S-ITS2 rDNA and LSU rDNA. Amplification of LSU rDNA followed Pandeirada et al. (2017) with two modifications: in the nested-PCR following the first PCR amplification the dinoflagellate specific Dino-ND primer was used instead of the terminal primer 28-1483R; and 2 µl of the first PCR product were added (instead of 1 µl) (see primer sequences in Hansen et al. 2007). Amplifications of SSU rDNA and ITS rDNA were based on Takano and Horiguchi (2005), skipping the first round of PCR amplification and going directly to the second round. The products from all PCR amplifications were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and sent to Macrogen Europe (Amsterdam, The Netherlands) for sequencing in both directions with the primers indicated in Hansen et al. (2007), Pandeirada et al. (2017) and Takano and Horiguchi (2005), except for 28-1483R, which was replaced by the Dino-ND.

To increase the number of taxa of Tovelliaceae for SSU rDNA and concatenated phylogenetic analyses we added previously unpublished SSU rDNA sequences of three species of *Tovellia* Moestrup, K. Lindberg & Daugbjerg. PCR amplification of SSU rDNA from *Tovellia aveirensis* Pandeirada, Craveiro, Daugbjerg, Moestrup & Calado, *T. rinoi* Pandeirada, Craveiro, Daugbjerg, Moestrup & Calado and *T. rubescens* Pandeirada, Craveiro, Daugbjerg, Moestrup & Calado was performed from groups of 2–10 swimming cells isolated from the original cultures to PCR tubes that were frozen for at least 24 h. After the addition of a bead of illustrate puReTak Ready-To-Go PCR (GE Healthcare, UK Ltd, Buckinghamshire, UK) in each tube, the procedure for PCR amplification was the same as in Takano and Horiguchi (2005) except for the use of the terminal primer 28-1483R instead of LSUR2 in the first PCR amplification round and using 1 µl (instead of 0.5 µl) from the first PCR product in the second PCR amplification round. Purification of PCR products was made with the same kit as for *Sphaerodinium polonicum* var. *tetricum*. Sequencing in both directions was made in Macrogen Europe (Amsterdam, The Netherlands) with the same primers used in the PCR amplification.

**Phylogeny**

Phylogeny based on LSU rDNA: partial sequences of the two *Sphaerodinium polonicum* var. *tetricum* strains were added to a data matrix previously compiled and used by Pandeirada et al. (2017). However, this data matrix was slightly modified prior to phylogenetic analyses as *Sphaerodinium cracoviense* (Craveiro et al. 2010) and *Dactyloplidum pterobellum* Kazuya Takahashi, Moestrup & Iwataki (Takahashi et al. 2021) were added and a few dinoflagellates of no importance to this study were deleted. The data matrix was edited in Jalview (ver. 14, Waterhouse et al. 2009) and sequences aligned with Mafft (default setting) as implemented in the software. A total of 48 genera and 71 species of dinoflagellates were considered and 1680 base pairs including introduced gaps were analysed using Bayesian inference and maximum likelihood. Three ciliates, four apicomplexans and Perkinus andrewsi Coss, Robledo, Ruiz & Vasta formed the outgroup. For Bayesian analyses we used the program MrBayes (ver. 3.2.6 × 64, Ronquist and Huelsenbeck 2003) with 5 × 10⁸ generations and a tree was sampled every 1000 generations. The burn-in was evaluated by plotting LnL scores as a function of generations and it was reached after 501,000 generations (conservative estimate). This left 4500 trees for construction of a 50% majority-rule consensus tree. jModeltest (ver. 2.1.7, Darriba et al. 2012) was used for selecting the best parameter settings for maximum likelihood among the 88 different models examined, and GTR + I+G was chosen as the best fit model (pinvar = 0.2 and shape = 0.61). The robustness of the tree topology in ML was evaluated with 1000 bootstrap replications using PhyML (Guindon et al. 2010) available at the Montpellier bioinformatics platform (http://www.atgc-montpellier.fr/phyml/).

Phylogeny based on SSU rDNA: nearly complete nuclear-encoded SSU rDNA sequences of *Sphaerodinium cracoviense* (1731 bp) and of both strains of *S. polonicum* var. *tetricum* (1736 bp of the Buçaco strain and 1747 bp of the Gerês strain) were added to a data matrix comparing 31 other dinoflagellate genera (68 strains). These included species assigned to Tovelliaceae (three genera, seven strains), Borghiellaceae (five genera, five strains) and Suessiaceae (10 genera and 32 strains). Gymnodinium catenatum H.W. Graham formed the outgroup taxon and was thus used to polarize the ingroup. The final data matrix comprised 1760 base pairs including introduced gaps and the sequences were manually edited in Jalview (ver. 14, Waterhouse et al. 2009) and aligned using Mafft with default settings as implemented in the software. Bayesian inference used 10 mill generations and a tree was sampled for each 1000’s generation. A burn-in was evaluated to occur after 501,000 generations, thus leaving 9500 trees for construction of a majority-rule consensus tree. Maximum likelihood bootstrap analyses used the parameter
settings suggested by jmodel test. The best fit model was GTR + I + G (proportion of invariable sites \( I = 0.48 \) and gamma shape \( G = 0.588 \)).

Phylogeny based on concatenation of SSU and LSU rDNA: we included as many overlapping dinoflagellate taxa for which both LSU and SSU rDNA sequences were available. Thus, a concatenated data matrix comprising 24 genera (45 strains) was compiled. The alignment included 2854 bp including introduced gaps. The approach for alignment and phylogenetic analyses were similar to those described for analyses of SSU rDNA except that in Bayesian analysis SSU and LSU rDNA sequences were divided into two partitions. This allowed each of these regions to evolve under different models of evolution using the ‘unlink’ option. The software jmodeltest suggested the GTR + I + G model as the best fit and the proportion of invariable sites \( (I = 0.373) \) and gamma shape \( (G = 0.518) \) were used for bootstrap analysis using PhyML. The aligned matrices are available upon request to n.daugbjerg@bio.ku.dk.

Sequence divergence

The sequences determined here (nuclear-encoded SSU and LSU rDNA and the internal transcribed spacers) were compared using PAUP* (ver. 4.0a build 161, Swofford 2002) and divergence estimates used both p-values and the Kimura-2-parameter model.

Results

Morphology and plate pattern

The external morphology of cells from the two strains analysed herein closely matched the original description of *Sphaerodinium polonicum var. tetricum*. The following description is primarily based on the strain from Gerês, in Northern Portugal, with reference here and there to slight variations observed in the strain from Buçaco, in Central Portugal. Vegetative, mobile cells are shown in Figs. 1 and 2. They were spherical to slightly oval, very little compressed dorsoventrally or not at all (Fig. 1A–C). Epi- and hypocone were of similar size. The slightly descending, transversely oriented cingulum was about 3 \( \mu \text{m} \) wide and had its distal end about 1–1.5 cingulum widths posterior to the proximal, left end (Fig. 1G, H; Fig. 2A). Cell length and width mostly fell within the range 22–32 \( \mu \text{m} \) (mean = 27.0 \( \mu \text{m} \); std. dev. = 2.6) and 20–27 \( \mu \text{m} \) (mean = 23.7 \( \mu \text{m} \); std. dev. = 2.1) (n = 43), respectively; exceptional cells, either small or large, extended the size limits to 19.5–47 \( \mu \text{m} \times 16–31 \mu \text{m} \). Abundant yellowish-brown chloroplast lobes ramified extensively from deep inside the cytoplasm to the periphery; a large, red eyespot, mostly up to \( 8 \times 8 \mu \text{m} \), typically with a concave anterior edge, was visible along the middle-anterior part of the sulcus; roundish bodies with an orange tinge, reminiscent of accumulation bodies were visible in the epicone (Fig. 1A–C). Immobile cells with the cingulum faintly marked were frequent on the bottom of wells, usually with a single prominent eyespot, sometimes with two (Fig. 1D). In most cells the nucleus was concealed by the numerous peripheral chloroplast lobes and its shape and position were not readily perceptible in LM. Cells stained with acetoarmine showed an ellipsoid nucleus, positioned in the mid-dorsal cytoplasm with its longer axis along the cingulum (Fig. 1E). A few cells revealed presumably dividing, elongated nuclei (Fig. 1F).

The amphiesma of vegetative, motile cells was relatively thin and details were only visible in LM in empty thecae. The tabulation had a nearly symmetrical plate arrangement in the epithea including, in Kofoidian notation, four apical plates centred around an apical complex and four intercalary plates (Figs. 1G, I, J, L; 2 A, C–E). The apical complex was roughly rectangular, with the long axis oriented from ventral-right to dorsal-left (Figs. 1I; 2 C), and displayed three platelets (Fig. 2D): a central, elongated platelet (marked 1), 1.3 \( \mu \text{m} \) long and 0.5 \( \mu \text{m} \) wide, with an axial row of small knobs; a larger platelet (marked 2) that surrounded the lateral and dorsal sides of the central platelet; and a smaller platelet (marked 3) that contacted platelets 1 and 2 on the ventral side. In the ventral area, a narrow rectangular plate, labelled Z in Figs. 1 and 2, separated the first apical plate from the sulcal plates (Figs. 1G; 2 A, C). Plate Z was 4–5 \( \mu \text{m} \) long and ca. 2 \( \mu \text{m} \) wide and contacted laterally precingular plates 1 and 7, thereby closing on the ventral side a ring of eight plates covering the epicone next to the cingulum. The number of cingular plates was difficult to ascertain, but a maximum of eight plates were counted in a few cells (Figs. 1M, N; 2 A, F); cingular plates appeared to have a similar length, except for the first two, which were slightly shorter. Empty thecae displayed two flap-like thickenings on the anterior-left part of the sulcus when viewed in LM (Fig. 1G, H, K; Fig. S1A). Six plates were identified in the sulcus (Figs. 1H, K; 2 A, B, F): four small plates (asterisks in Fig. 2A, B) occupied the anterior part of the sulcus below plate Z, filling the area between the proximal and distal ends of the cingulum and surrounding the flagellar pores; adjacent to the lowermost of these four plates was a single, larger plate (middle sulcal, ms) that ranged the full width of the sulcus and separated a small, squarish to rhombic posterior sulcal plate (ps) from the upper part of the sulcus (Fig. 2A, B, F). The hypotheca had six postcingular plates, and two antapical plates of similar size (Figs. 1K–N; 2 A, B, F).

Plate sutures were generally ornamented by rows of knobs, which were visible in LM observations of empty thecae and in SEM observations (Fig. 1G–N; Fig. 2A, B).

Vegetative, motile cells of the Buçaco isolate matched the features described above except for the morphology of the chloroplast (Figs. S1 and S2). Chloroplast lobes of the strain from Buçaco were usually less numerous and more rounded, and appeared less profusely interconnected than in the strain from Gerês (Fig. 3A–C). However, a minority of cells from
Fig. 1. *Sphaerodinium polonicum* var. *tatricum* from Gerês, LM. (A, B) Surface focus and optical section of vegetative cell in ventral view displaying dense arrangement of chloroplast lobes, the conspicuous eyespot (e) in the sulcal area, and roundish orange bodies, reminiscent of accumulation bodies, in the epicone and at cingulum level (arrows). (C) Lateral view of cell exiting the theca (arrow), which opened along the cingulum. (D) Cell with two eyespots (presumably dividing). (E, F) Cells stained with acetocarmine showing in (E) the ellipsoid nucleus (n), and in (F) an apparently dividing nucleus. (G–N) Empty thecae with plates labelled according to Kofoidian notation: ventral view (G, H); apical view (I, J) showing apical complex (arrow in I); antapical view (K); dorsal view (L); right view (M); left view (N). ms, middle sulcal plate; ps, posterior sulcal plate. (A–F) to the same scale. (G–N) to the same scale. Scale bars = 10 μm.

Fig. 2. *Sphaerodinium polonicum* var. *tatricum* from Gerês, SEM. Kofoidian notation. Swollen cells in A and B prepared with fixation schedule 2; cells with preserved furrows and flagella (C–F) prepared with fixation schedule 1. (A, B) Ventral view of whole, swollen cell (only hypotheca visible in B); arrows point to knobs over plate sutures. Sulcus with four small plates (asterisks) surrounding the flagellar pores, a middle (ms) and a posterior (ps) sulcal plates. (C) Apical-ventral view showing location of apical complex (arrow) and both the transverse (TF) and longitudinal flagellum (LF) in place. (D) Apical complex with elongated platelet (1) bearing a row of small knobs, surrounded by platelet 2 on the dorsal and lateral sides and by platelet 3 on the ventral side. (E, F) Apical and antapical views. Scale bars = 8 μm (A); 10 μm (B); 5 μm (C, E, F); 500 nm (D).
Buçaco displayed a chloroplast arrangement very similar to the one observed in cells from Gerês (Fig. 3D–F).

Cyst morphology and encystment

Cysts of both strains of *Sphaerodinium polonica* var. *tetricum* were similar in appearance and size range: 34–46 μm long (mean = 39.8 μm; std. dev. = 3.5; n = 26) and 28–39 μm wide (mean = 33.0 μm; std. dev. = 3.0; n = 22), not including processes. Cyst contents varied from light yellow-brown to darker orange-brown (Fig. 4A–F) and usually contained several round, orange to red bodies interpretable as accumulation bodies or perhaps lipid droplets (Fig. 4A, D). The eyespot was often visible in recently formed cysts (Fig. 4C). Cysts were subspherical with a more or less pronounced equatorial constriction (paracingulum) (Fig. 4H, J, thick arrow). Cysts were ornamented by simple or most often branched processes up to 10 μm long (n = 22). These wall processes tapered from broad and somewhat flattened bases and widened abruptly near the tips, which were somewhat flattened and showed recurved margins (Fig. 4). Most bases of processes were nearly aligned and apparently marked the position of some of the plate limits of the cell that originated the cyst (Fig. 4H, J), although it was not clear if they reflected a paratabulation. Numerous small knobs were scattered over the cyst surface (Fig. 4G–J) and formed short alignments here and there, perhaps also in the location of former plate sutures (Fig. 4J, double arrows). Cysts were often surrounded by an envelope, which looked like a colourless periphagm sensu Ellegaard et al. (2003) (Figs. 4A, E, thick arrow; 4 K, L). Recognizable plate limits, visible in SEM, established a link between these cyst covers and the former amphiesma of the encysting cell (Fig. 4K, L, double arrows). As shown in Fig. 4, the cyst type appeared to be proximochorate to chorate. The cysts were not treated by acetolysis.

The encystment process was similar in both strains of *Sphaerodinium polonica* var. *tetricum*. Most of the external morphological changes from motile cell to cyst occurred over the course of a few seconds following the transfer of the encysting cell to a new culture well or microscope slide. The process is outlined in Fig. 5. The encysting stages were large, dark cells with prominent accumulation bodies (Fig. 5A, wide arrows). Detailed observations of these cells were made difficult by their rapid transformation in response to being transferred to a slide and it was only possible to briefly observe two longitudinal flagella in few cases (Fig. 5B, arrows). Pairs of fusing cells that were occasionally seen swimming in the culture batches were the likely origin of planozygotes with paired longitudinal flagella matching the features of encysting cells. During the process of encystment the flagella were soon discarded after the cells stopped swimming (Fig. 5C) and the peripheral cytoplasm suddenly retracted from the amphiesma (Fig. 5D, E; see also Video S1 in Supplementary material). The retraction was not uniform and led to the formation of the process-bearing surface of
the cyst, which remained surrounded by a colourless cover, presumably derived from the amphiesma of the encysting cell (Fig. 5G, H). This outer, delicate cover was eventually lost and was partially or totally absent in presumably older cysts from aged culture batches. The observation of empty cyst walls in culture wells indicated that germination took place in the cultures, but the germination process was never observed and the archeopyle type could not be determined.

**General internal fine-structure**

Cells of *Sphaerodinium polonicum* var. *tatricum* displayed features typical of dinoflagellates (Figs. 6 and 7). In sectioned cells, the ellipsoid nucleus was in a mid-dorsal position, approximately at cingulum level (Fig. 6A). The periphery of the cytoplasm was mainly occupied by chloroplast lobes, large ellipsoid vesicles with irregular contents, and trichocysts (respectively marked ch, short black arrows and t in Figs. 6A, 7A). Starch grains and a few oil droplets were present in the inner portion of cytoplasm (Figs. 6A, 7A, st, o). Two other kinds of vesicles were seen: small, mainly peripheral vesicles with electron-dense, spherical or ellipsoidal granules (Figs. 6B, 7D arrows); and electron-translucent vesicles associated with electron-opaque microbodies with irregular shape, more common in the inner portion of the cytoplasm (Fig. 6A, D, short, white arrows). The eyespot, located along the sulcus, was extraplastidal and composed by two types of elements: a row of variously developed brick-
like or crystal-like elements apparently enclosed in a long, flat vesicle located underneath the longitudinal microtubular root (LMR, named r1 in Moestrup 2000) (Figs. 6C, 7 B, double arrow); and at least one layer of more or less fused oil globules underlying the brick-like elements (Figs. 6C, 7 B, o). The pusular system was found in the ventral area, between the sulcus and the nucleus. The most conspicuous part of the pusular system extended from the transverse flagellar canal in the format of a tube with an inner diameter of 230–290 nm, herein called pusule canal following Craveiro et al. (2010) (Fig. 6D–F, pc). The pusule canal was accompanied by a rather discrete layer of fibrous material, barely visible in Fig. 6E, F (arrowhead). About 5 μm into the cell, the pusule canal connected with a sac some 450 nm in diameter, the so-called collecting chamber, from which radiated 100-nm wide, typical pusular tubes (Fig. 6E, G).

Chloroplast lobes in cells from both strains were prominent at the cytoplasm periphery and extended inward into numerous, ramifying lobes (Figs. 6A, D, 7 A). However, the thylakoid arrangement showed variation: regular, three-thylakoid lamellae were visible in the chloroplasts of the strain isolated from Gerês (Fig. 6B), whereas in the strain from Buçaco thylakoid lamellae were more irregular, with stretches of 3–14 associated thylakoids, reminiscent of pseudogranum (Fig. 7E–G). Small areas without thylakoids were found in chloroplasts of both strains (Figs. 6A, 7 A, long, white arrows).

**Flagellar apparatus**

The organization of the flagellar bases and associated structures was similar in cells from both strains. The general organization of the flagellar base area is shown as viewed from the left in a series of sections taken from a cell of the Gerês isolate (Fig. 8). Different viewpoints from two series of sections from cells of the Buçaco strain are given in Fig. 9. As estimated from serial sections, the basal bodies were inserted at an angle of about 120°. Each single-membrane bounded region where the flagella emerged from the cytoplasm was externally limited by a ring of fibrous material that showed striations in at least some views (Figs. 8I, 9 A–C); these striated collars were not connected by any prominent fibres, but some electron-opaque material was present along the cell surface in the area between the collars (not shown). Replicated basal bodies were seen in one cell, located side by side near the anterior end of the transverse basal body (TB); their orientation was not ascertained (Fig. 8C–E, RB1 and RB2). Functional basal bodies, i.e. those associated with emergent flagella, were associated with three microtubule-containing roots. A single, multistranded microtubular root was associated with the left side of the longitudinal basal body (LB) near its proximal end. The number of microtubules in this longitudinal microtubular root (LMR; labelled r1 in Moestrup 2000) varied from about 10 near the LB to some 30–40 further down along the sulcus (Figs. 8B–E; 9 A, C). Two thin fibres connected the LB to the ventral face of the LMR/r1 some 250 nm from its proximal end (Fig. 9A, white arrow). An electron-opaque fibre about 70 nm wide contacted the dorsal-anterior side of the LMR/r1, surrounded the dorsal side of the proximal end of the LB and progressed toward the ventral-posterior side of the cell (Figs. 8F–I, 9 A, C). As seen in Fig. 9C, this ventral fibre (VF) seemed to extend from electron-opaque material lining the dorsal face of the LMR/r1 near the point of attachment of a clearly striated connective (named striated root connecting, SRC) linking this root with the proximal 500 nm or so of the main fibrous root of the TB, the transverse striated root (TSR). The TSR extended for about 1.5...
μm from the dorsal-posterior side of TB toward the left and ended near the left side of the transverse striated collar (TSC) (Fig. 9A–C). A microtubule ran along the TSR (Fig. 9C, short arrow), making up the microtubular component of the root (r4 in Moestrup 2000). The second root associated with the TB was a single microtubule that was seen parallel to one of the trip Iets of the anterior-dorsal side of the basal body (Fig. 8B). This transverse microtubular root (TMR/r3 in Moestrup 2000) continued around the transverse flagellar canal (TFC) and nucleated several rows of microtubules that extended dorsally, roughly along the right-hand side of the pusule canal (marked TMRE in Fig. 8A, which is about 1 μm to the right of Fig. 6D, F). At least two membranous bodies with a honeycomb pattern and an electron-opaque spot at the centre of the hexagonal units were visible near the flagellar bases (Fig. 8B–I). Approximately longitudinal sections along these membranous structures showed a lamellate appearance (Fig. 9A, D).

A row of some 15 microtubules, interpretable as a homologue of a microtubular strand of the peduncle (MSP) based on its location, was seen between the ventral surface and the TFC (Fig. 8A). The MSP extended near the anterior edge of the TSC (Fig. 9A) and continued past the pusule canal, curving in a dorsal direction, just anterior to the microtubular extensions of the TMR/r3 (not shown).

**Phylogeny based on LSU rDNA**

The tree topology from phylogenetic inferences based on nuclear-encoded LSU rDNA sequences is depicted in Fig. 10. The resolution of the deepest branches was not well supported.
as both posterior probabilities (pp) and bootstrap values were low (evolutionary lineages were not resolved). However, the clade with the genus *Sphaerodinium* received maximum support (pp = 1.0 and BS = 100%) and formed a sister taxon to a clade containing Borghiellaceae, Suessiaceae and Symbiodiniaceae s.s. The family Suessiaceae plus Symbiodiniaceae s.s. formed a well-supported clade (pp = 1.0 and BS = 91%) that grouped with Baldinia Gert Hansen & Daugbjerg and Borghiella spp. to form a statistically unsupported trichotomy (Fig. 10). Dactylodinium Kazuya Takahashi, Moestrup & Ivataki appeared as a sister group to the remaining Suessiaceae, although *D. pterobolotum* has a type-B eyespot.

**Phylogeny based on SSU rDNA**

The phylogenetic relationship between terminal lineages generally received high support in terms of posterior probabilities and bootstrap support values (Fig. 11). In contrast, the tree topology for many of the deepest branches was not well supported and the phylogeny of the monophyletic clade of Suessiaceae and Symbiodiniaceae s.s. was likewise unresolved. *Dactylodinium* formed a sister group, with maximum support, to a clade containing the Suessiaceae and Symbiodiniaceae s.s. The monophyletic *Sphaerodinium* (with two species, three strains) formed a sister group to a clade that grouped “*Glenodinium* sp.”, *Baldinia* and the two coccid forms *Cystodinium phaseolus* Pascher and *Phytodinium* sp.; and the *Sphaerodinium-Baldinia-Cystodinium* clade appeared as sister to a morphologically undefined species here labelled *Borghiella* sp., with moderate support (PP = 1.0 and BS = 75%). Hence, nuclear-encoded SSU rDNA did not support monophyly of the Borghiellaceae. Though the tree topology indicated a sister group relationship between Tovelliaceae and the clade with Sphaerodiniaceae-Borghiellaceae-Suessiaceae *s.l.* this branching had no statistical support (PP = 0.69, BS = <50%).

Fig. 7. *Sphaerodinium polonicum* var. *taticum* from Buçaco. TEM. (A) Longitudinal section of a cell seen from left-ventral side. The eyespot (e) is visible in the somewhat retracted ventral region. Chloroplast lobes (ch) are visible intercalated with large starch grains (st). Some thylakoid-free areas are visible inside some chloroplast lobes (long white arrow). Large ellipsoid vesicles at the periphery, between chloroplast lobes (short black arrows). (B) Section oriented obliquely relative to the surface, giving an almost ventral view of some crystal-like elements of the eyespot (double arrow). The oil components (o) appear fused into continuous layers. (C) Pusular tubes near the collection chamber (not visible). (D) Peripheral vesicle with electron-opaque, spherical or ellipsoidal granules of unknown significance (arrow). (E–G) Chloroplast lobes with different thylakoid arrangements, including three-thylakoid lamellae (black arrows) and stacks of up to 14 thylakoids in a pseudogranalike arrangement (white arrows). Scale bars = 2 μm (A); 200 nm (B–E); 100 nm (F, G).
Phylogeny based on concatenation of SSU and LSU rDNA

The concatenated data matrix comprised fewer dinoflagellates as the complete ribosomal operon has not been determined for many of the taxa included in the single gene analyses. Sphaerodiniaceae and Suessiaceae each formed highly supported monophyletic clades (PP = 1.0, BS = 100%), whereas Borghiellaceae received less support (PP = 0.76, BS = 71%) (Fig. 12). A clade with both species of Dactylo- lodinium appeared as a sister group to the Suessiaceae with maximum support. The clade comprising these three families was well supported (PP = 1.0, BS = 96%), but the relationship between them could not be established (PP = 0.59, BS = 75%). In this phylogenetic analysis the monophyletic Tovelliaceae formed a highly supported sister taxon to the three families mentioned above (PP = 1.0, BS = 94%). Similar to each of the single gene analyses, the deepest branches in the concatenated phylogeny received little or no support (Fig. 12) preventing a rewarding discussion of the evolutionary history of the dinoflagellates.
Sequence divergence

To further elucidate the relatedness of the two strains of *Sphaerodinium polonicum var. tatricum* we compared ITS1, 5.8S rDNA and ITS2 as single fragments and all of them combined (Table 1). Including all three fragments the divergence was 2.1%. For ITS1 and ITS2 fragments, the sequence divergence was 3.7 and 1.1%, respectively. Interestingly divergence values for 5.8S rDNA are marginally higher than ITS2 (1.3 versus 1.1%).
Fig. 10. Phylogeny of *Sphaerodinium polonicum* var. *tatricum* based on nuclear-encoded partial LSU rDNA sequences and inferred from Bayesian analysis (BA). The analysis was based on 1680 base pairs including introduced gaps and encompassed 48 genera and 71 species of dinoflagellates (i.e. the ingroup). The outgroup comprised 4 apicomplexans, 3 ciliates and *Perkinsus*. The robustness of the tree topology was evaluated by posterior probabilities (PP ≥ 0.5) from BA and 1000 bootstrap replications (BS ≥ 50%) in maximum likelihood (ML). These values were written at internodes. Filled circles were used to indicate the highest possible support in BA (1.0) and ML bootstrap (100%) whereas PP < 0.5 and BS < 50% were indicated by a dash (–). GenBank accession numbers were provided in parentheses following the species names. The character distribution of eyespot types was indicated by grey boxes. *Symbiodiniaceae* s.s., used in the sense of LaJeunesse et al. (2018), are represented in the tree only by two species of *Symbiodinium*, which appear nested within the *Suessiaceae*. The branch lengths are proportional to the number of character changes and a scale bar was provided below.

**Discussion**

**Morphological comparisons and identity of the organisms**

The genus *Sphaerodinium* is readily identified by the nearly symmetrical amphiesmal plate arrangement on the epicone, which includes four intercalary plates (Kofoidian notation), and the presence on the hypocone of six postcingular plates (Moestrup and Calado 2018). Comparison with the original descriptions of the five species and one variety described in *Sphaerodinium* shows a good match between our material and the original drawings of *S. polonicum* var. *tatricum*, both in general morphology and in plate arrangement (Woloszyńska 1916). Of particular importance is the papillate appearance of plates, and especially of plate sutures, that is clearly shown in light micrographs of empty thecae of both strains herein reported on, which matches what Woloszyńska described for both *S. polonicum* and its variety. The two flap-like thickenings on the left-anterior part of the
Fig. 11. Phylogeny of *Sphaerodinium polonicum* var. *tatricum* based on nearly complete nuclear-encoded SSU rDNA sequences and inferred from Bayesian analysis (BA). The analysis was based on 1760 base pairs including introduced gaps and encompassed 31 genera (68 strains) of dinoflagellates including the outgroup (i.e. *Gymnodinium catenatum*). The robustness of the tree topology was evaluated by posterior probabilities (PP ≥ 0.5) from BA and 1000 bootstrap replications (BS ≥ 50%) in maximum likelihood (ML). These values were written at internodes. Filled circles were used to indicate the highest possible support in BA (1.0) and ML bootstrap (100%) whereas PP < 0.5 and BS < 50% were indicated by a dash (–). GenBank accession numbers are provided in parentheses following the species names. The families Sphaerodiniaceae, Suessiaceae and Symbiodiniaceae s.s., and Tovelliaceae are indicated on the phylogenetic tree together with their type of eyespot. Symbiodiniaceae s.s., used in the sense of LaJeunesse et al. (2018), are represented in the tree only by species of *Symbiodinium*, which appear nested within the Suessiaceae. The branch lengths are proportional to the number of character changes, see scale bar.

sulcus, just below the proximal end of the cingulum, closely match Woloszyńska’s original illustrations of *S. polonicum* var. *tatricum* (Woloszyńska 1916, 1952); and the absence of corresponding thickenings on the right edge of the sulcus sets our material apart from typical *S. polonicum* (Moestrup and Calado 2018; Woloszyńska 1916). Another distinguishing feature mentioned in the original description of the variety was the deeper extension of the anterior part of the sulcus...
Fig. 12. Phylogeny of \textit{Sphaerodinium polonicum} var. \textit{tatricum} based on concatenation of 45 nuclear-encoded LSU and SSU rDNA sequences and including a total of 24 dinoflagellate genera. Bayesian analysis and maximum likelihood analyses were based on 2854 base pairs including introduced gaps. Robustness of tree topologies were evaluated by posterior probabilities (PP \( \geq 0.5 \)) in BA and 1000 bootstrap replications (BS \( \geq 50\% \)) in maximum likelihood (ML). Support values are written at internodes. Filled circles indicate the highest possible support in BA (PP = 1.0) and in ML bootstrap (BS = 100\%). PP < 0.5 and BS < 50\% are indicated by a dash (−). GenBank accession numbers are provided following all species names. The families Borghiellaceae, Sphaerodiniaceae, Suessiaceae and Tovelliaceae are indicated on the phylogenetic tree together with their type of eyespot. Branch lengths are proportional to the number of character changes, see scale bar.

...The plate composition of the furrows is often difficult to work out and sulcal plates were not usually described in detail, even by exceptional observers like Wołoszyńska. However, the shape of the sulcus of \textit{S. polonicum} var. \textit{tatricum} was described as wider near the posterior end and drawn as somewhat asymmetric (Wołoszyńska 1916, 1952). The slight deviation toward the right of the posterior sulcal plate of both strains described herein creates an irregular, asymmetric shape that appears compatible with the original illustrations. As shown in both the Gerês and the Buçaco strains, the sulcal plate arrangement is unusual in having a middle plate (ms) contacting postcingular plates on both sides of the sulcus, thereby separating the posterior sulcal from a set of...
Table 1. Sequence divergence estimates in percent between *Sphaerodinium polonicum* var. *tatricum* (Gerès) and *S. polonicum* var. *tatricum* (Buçaco) based on ITS1, 5.8 S rDNA, ITS2 and all three fragments combined (in total 533 base pairs). Uncorrected distances (P-values) and distance values calculated using the Kimura-2-parameter model were estimated using PAUP* (ver. 4.0a build 161). Genbank accession numbers: MT584215 (Gerès); MT584216 (Buçaco).

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<th>P-values (%)</th>
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<td>5.8 S rDNA</td>
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<td>All three fragments</td>
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four smaller plates on the anterior part of the sulcus, near the flagellar pores. This is quite different from what was found in *S. cracoviense*, in which the posterior sulcal plate extended along the right side of the sulcus and reached the right sulcal plate at the level of the cingulum (Craveiro et al. 2010).

Perhaps less significant but worth noting is the slight asymmetry of apical plate 3, which commonly shared a shorter suture with plate 3a than with 2a; the irregularly hexagonal shape of this central apical plate very closely matched the apical view of *S. polonicum* var. *tatricum* in Wołoszyńska (1952) and was noted as a feature of the variety by Moestrup and Calado (2018). The apical complex of *S. polonicum* var. *tatricum* was marked by Wołoszyńska (1952) as a small rectangle with its long axis oriented from ventral-right to dorsal-left; a similar orientation was here shown for both strains. Although little detail was visible in the apical complex by LM, SEM revealed an organization similar to that found in *S. cracoviense* (Craveiro et al. 2010).

The nucleus of *S. polonicum* was described as horseshoe-shaped and drawn with its long axis parallel to the cingulum (Wołoszyńska 1916). In the shorter description of the variety the horseshoe shape of the nucleus is again mentioned, this time without illustration (Wołoszyńska 1916). In contrast, non-dividing nuclei in both strains reported herein appeared ellipsoidal in aceticarmine-stained cells and in TEM sections. The significance of this discrepancy is not clear. In view of the overall agreement with the characters described above, and until a population with similar characters and a horseshoe-shaped nucleus is studied by modern methods, we opt to identify our material as *S. polonicum* var. *tatricum*.

The morphology of the larger cells that developed into resting cysts, as shown in Fig. 5A, deviated from the smoothly round shape of vegetative cells. The outline of these cells, presumed to be planozygotes, is more similar to typical *S. polonicum* than it is to *S. polonicum* var. *tatricum*. Wołoszyńska (1916) mentioned sexual reproduction in typical *S. polonicum* and the possibility that the two taxa are part of the same life cycle (and therefore represent a single taxon) comes to mind. However, due to the small number of fusing cells in culture batches and the rapid transformation of planozygotes into cysts, we were unable to examine in detail the thecae of these larger cells, leaving this hypothesis unsupported.

Cysts and encystment

In the original description of several *Sphaerodinium* species, Wołoszyńska (1916) mentioned resting cysts (‘Dauerzellen’) for *S. cracoviense* only, describing them simply as spherical to ovoid. However, resting or resistance cells were not illustrated, and it is uncertain whether division cysts or temporarily immobile cells were misinterpreted by Wołoszyńska. Neither sexual reproduction nor resting cysts were reported in cultured strains of *S. cracoviense* (Craveiro et al. 2010). The resting cysts reported here for *S. polonicum* var. *tatricum* show a complex morphology that has no obvious match among reported resting cysts produced by freshwater dinoflagellates (reviewed by Mertens et al. 2012; see also Craveiro et al. 2013; Li et al. 2015; Pandeirada et al. 2014, 2017; Takahashi et al. 2015). The morphology of the cysts shown in Fig. 4 bears some superficial resemblance to cysts produced by marine species of the Gonyaulax spinifera (Claparede & J. Lachmann) Diesing complex (Ellegaard et al. 2003). However, the phylogenetic and ecological distance between *Sphaerodinium* and the Gonyaulaceae suggests that cyst processes in the two groups may be the result of convergent evolution.

The encystment process reported herein appears unusual, both in the mechanism that leads to the formation of wall projections and in the speed with which it occurs. The sudden retraction of the cytoplasm of encysting cells of *S. polonicum* var. *tatricum* resulted in the processes being nearly formed within seconds, in contrast with the longer times involved in the commonly reported outward growth of processes from wall layers deposited on the cytoplasmic surface, under the more or less disrupted cell cover (Bravo and Figueroa 2014; Kokinos and Anderson 1995; Pandeirada et al. 2017; Stosch 1973). Cysts with a smooth surface often mature inside the cell cover (which presumably contains at least the outer part of the amphiesma), as observed in, e.g. *Tovellia apiculata* (Stosch) Moestrup, K. Lindberg & Daugbjerg, and in *Chimonodinium lomnickii* (Wołoszyńska) Craveiro, Calado, Daugbjerg, Gert Hansen & Moestrup, *Peridinium cinctum* (O.F. Müller) Ehrenberg and other peridinioids (Craveiro et al. 2011; Lefèvre 1932; Stosch 1973). However, when cyst processes are formed the cell cover is either released before process growth is completed (e.g., in *Tovellia rinoi*), or it remains around the process-bearing surface as seen in *Biecheleria pseudopalustris* (J. Schiller) Moestrup, K. Lindberg & Daugbjerg and *Lingulodinium polyedra* (F. Stein) J.D. Dodge, swelling to accommodate the growth of processes (Kokinos and Anderson 1995; Pandeirada et al. 2017; Stosch 1973). Although the outer amphiesma was visible around many cysts of *S. polonicum* var. *tatricum* no more than a
s slight swelling was ever detected and the detachment of the outer cell cover from the forming cyst surface seemed to result essentially from the cytoplasm retraction.

**Chloroplasts**

In contrast with the general similarity between the morphological features of the strains from Gerês and Bucaco, cells from the Bucaco culture batches were usually distinct by their less numerous and more rounded chloroplast lobes. Whereas the chloroplasts of cells from Gerês displayed the predominant association of thylakoids in groups of three, as commonly found in the peridinin-containing chloroplast type, chloroplasts of the Bucaco strain had lamellae with 3–14 associated thylakoids along part of their length. Unusual thylakoid arrangements in grana- or pseudogranula-like associations have been reported from a small number of apparently distantly related dinoflagellate species (Craveiro et al. 2013; Dodge 1975; Hansen et al. 1996; Jeong et al. 2014; Takahashi et al. 2015, 2017). However, the stability of pseudogranula-like thylakoid associations in dinoflagellate species is generally unknown. The recent finding in *Kirihwa asteri* Boutrup, Tillmann, Daugbjerg & Moestrup of a strain with up to 45-thylakoid stacks in the chloroplast, which had 100% identical nuclear-encoded LSU rDNA (>1400 base pairs) to another strain with only three-thylakoid lamellae, suggests that this feature has limited taxonomic value (Boutrup et al. 2017). Neither the function nor the mechanisms leading to the formation of pseudogranula-like structures in the Bucaco strain of *S. polonicum var. taticricum* are understood. The rounded chloroplast lobes seemed to be a stable, intrinsic (i.e. genetically determined) feature maintained through successive generations of cells of this strain, but they were not detected in the strain from Gerês, which was grown in the same medium and maintained in the same light and temperature conditions. However, in view of the general morphological similarity and the relatively small divergence found between compared ITS1, 5.8S and ITS 2 sequences of the two strains we prefer to refer them to the same taxon.

**Eyespot, pusule, MSP and flagellar apparatus**

The voluminous eyespot of *S. polonicum var. taticricum* was slightly less conspicuous in LM than the eyespot of *S. cracoviense*, but it is also possible that sections containing this area, which was relatively small in *S. cracoviense*, may have been missed. A similarity has been noted between the pusular system of *Sphaerodinium* species and that described from *Naia-dinium polonicum* (Wołoszyńska-Carty, namely the presence in this species of a pusule canal connected to an inner collecting chamber associated with long, regular pusular tubes (Craveiro et al. 2015). However, the pusule canal of *N. polonicum* opens on the ventral side into the LFC and differs structurally from that of *Sphaerodinium* by being partly enveloped by a vesicle (Craveiro et al. 2015). It appears unlikely that the two structures are evolutionarily closely related.

In *S. polonicum* var. *taticricum*, as in *S. cracoviense*, a single row of microtubules was found in a position, and with an orientation, that makes it likely homologous with microtubular strands extending into peduncles, particularly those used for feeding (Craveiro et al. 2010; Hansen and Calado 1999). However, in both species of *Sphaerodinium* this MSP lacked a definite area of association with the cell surface, and it was not accompanied by vesicles with electron-opaque contents, which are regularly present when the MSP is involved in feeding (Calado et al. 1998, 2006). No function is known at present for the MSP of *Sphaerodinium*. On the ventral surface of *S. cracoviense*, between the two flagellar collars, a relatively small ventral ridge was demonstrated (Craveiro et al. 2010). A smaller amount of fibrous material in a similar location of *S. polonicum* var. *taticricum* (mentioned above but not shown) is here interpreted as an even smaller ventral ridge. A well-developed ventral ridge is a common feature on the mid-ventral area of gymnodinioid and woloszynskioi dinoflagellate cell types (e.g., Calado et al. 1998; Lindberg et al. 2005) and the presence of a ventral ridge was among the features showing that the long tradi- tion of classifying *Sphaerodinium* among peridinioiids was unjustified.

A direct comparison of the flagellar apparatus of *S. polonicum var. taticricum* with that of *S. cracoviense* shows great similarity in the main features, although fibrous structures, such as collars and their extensions, appear less developed in the former. Of particular interest are the unusual structures previously found in *S. cracoviense*, of which those also found in the cultures described herein may stand as typical features of the genus. The presence of a distinct SRC linking electron-opaque material on the dorsal side of the LMR/r1 with the TSR near its connection with the TB is common to both species of *Sphaerodinium*, as is the absence in this area of any traces of the layered connective that is typical of peridinioiids (Calado et al. 1999; Craveiro et al. 2016). The extension of the TMR/r3, which occurs as several rows of microtubules that extend along the pusule canal in both species, is unusual and is perhaps involved in the orien- tation of the pusular system (Craveiro et al. 2010). Thin fibres connecting the ventral surface of the LMR/r1 with LB triplets are uncommon in peridinioiids but were reported in several species of Suessiales (Craveiro et al. 2010; Hansen...

Some of the most striking features of the flagellar base area that are common to *S. cracoviense* and *S. polonicum* var. *triticum* are the ventral fibre extending from the right-anterior side of the LB and the membranous bodies with hexagonal units in a honeycomb pattern, also referred to as lamellar bodies by Craveiro et al. (2010). The lamellar body was first described from *Baldinia anauniensis* Gert Hansen & Daugbjerg where membranous structures of this kind were found to fill a large area in sections near the base of the LB (Hansen et al. 2007). How these membranous structures interact with other components is unknown, but the repeating, patterned arrangement invites speculation about interaction with light and suggests a function in phototaxis (Craveiro et al. 2010; Hansen et al. 2007).

The ventral fibre has been documented from *B. anauniensis* and *Dactylodinium pterobolotum*, two species with a type B eyespot (sensu Daugbjerg and Moestrup, 2007) suggesting affinity with the Borghiellaceae, but was found lacking in *Borghiella anderseni* Daugbjerg, Andreasen, Happel, Pandeirada, Gert Hansen, Craveiro, Calado & Moestrup and *B. dodgei* Moestrup, Gert Hansen & Daugbjerg (Daugbjerg et al. 2014; Hansen et al. 2007; Moestrup et al. 2008; Takahashi et al. 2017). A lamellar body of the kind described above is currently known only from *Baldinia* and *Sphaerodinium*.

However, having only the difference in chloroplast morphology and thylakoid arrangement as distinctive features, which may be of little value (Boutrup et al. 2017), we prefer to treat them at present as two different strains of the same taxon.

In view of the shared unusual characteristics, particularly the ventral fibre and the lamellate body, and the species position in phylogenetic inferences, *Baldinia anauniensis* stands out as the closest known relative of *Sphaerodinium*. In our phylogenetic inferences, *Dactylodinium* appeared as closely related to the Suessiaceae and Symbiodinaceae s.s., in agreement with Knechtel et al. (2020). Although a type B eyespot was found in *D. pterobolotum* (Takahashi et al. 2017), no crystal-like units were observed in the eyespot of *D. arachnoides* W.M. Lum, Kazuya Takahashi, Takayama & Iwataki, which was therefore classified as a type A eyespot (Lum et al. 2019). The inclusion of *Dactylodinium* in the Borghiellaceae is therefore not supported. The presence in the flagellar apparatus of *D. pterobolotum* of a ventral fibre apparently homologous with that seen in *Sphaerodinium* also suggests a close evolutionary relationship. However, both *B. anauniensis* and *D. pterobolotum* show important structural differences from *Sphaerodinium*, both external and in cytoplasmic features, which appear compatible with their current classification outside the Sphaerodiniaceae.

**Phylogeny**

The three phylogenetic trees, one based on LSU, another on SSU rDNA and the other on concatenated LSU and SSU rDNA, display the two species of *Sphaerodinium* as members of the same clade with maximum support, and identify their closest relatives as species with eyespot type B (Borghiellaceae) or E (Suessiaceae and Symbiodinaceae s.s.), suggesting a common origin for the presence of crystal-like units in dinoflagellate eyespots (Takahashi et al. 2017). The presence of predominantly cocoid forms (*Cystodinium*, *Phytodinium*) among the sister groups to the *Sphaerodinium* clade in the SSU rDNA phylogeny highlights the need for further work on these predominantly immobile species, which were traditionally grouped in a separate order (Moestrup and Calado 2018). As noted by Craveiro et al. (2010), the *Sphaerodinium* clade appears to have no close relatives in the trees, suggesting its classification in a separate family. The position of the genus *Sphaerodinium* (Sphaerodiniaceae) as an early diverging branch of the Suessiidae agrees with other DNA-based phylogenies (Knechtel et al. 2020; Takahashi et al. 2017) and is compatible with the presence in *Sphaerodinium* of some apparently peridiniod characteristics, such as the arrangement of amphialgal plates. The distances calculated between the two strains of *S. polonicum* var. *triticum* in the ITS1-5.8S rDNA-ITS2 portion of the ribosomal operon would perhaps justify the recognition of two different taxa.

**Conclusions**

On the basis of detailed examination of *S. cracoviense* and *S. polonicum* var. *triticum* the following features can be listed as characteristic of *Sphaerodinium* in addition to the general arrangement of the main amphialgal plates: apical complex with three platelets, the middle one linear and with an axial row of small knobs; eyespot of type F, i.e. an extraplastidial eyespot combining a single, ventral row of crystal-like units with at least one layer of more or less fused oil globules; pusular system including a pusule canal linking the transverse flagellar canal with a diluted portion in the central cytoplasm, from which radiate numerous regular, 100-nm wide pusular tubes; flagellar apparatus with three microtubular roots, LMR/r1, TMR/r3 and TSRM/r4, and a ventral fibre extending ventroposteriorly from the proximal-right side of the longitudinal basal body; TMRE/r3-extension made of several rows of microtubules oriented roughly parallel to the pusule canal; one or several lamellar bodies in the flagellar base area, consisting of membranous structures with a honeycomb pattern in cross section and an electron-opaque spot in the middle of each hexagonal honeycomb unit; resting cyst with an equatorial constriction, ornamented overall with processes, some of which are branched, rapidly formed by cytoplasmic retraction (seen only in *S. polonicum* var. *triticum*); closest known relatives are the type B eyespot-bearing members of the Borghiellaceae, particularly *Baldinia anauniensis*, which shares with *Sphaerodinium* the ventral fibre and the lamellar body.
CRedit authorship contribution statement

Mariana S. Pandeirada: Investigation, Writing - original draft, Visualization. Sandra C. Craveiro: Resources, Writing - review & editing, Visualization. Niels Daugbjerg: Formal analysis, Writing - review & editing. Øjvind Moestrup: Resources, Writing - review & editing. António J. Calado: Conceptualization, Writing - review & editing, Supervision.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ejop.2021.125770.

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