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Studies on woloszynskioid dinoflagellates IV: The genus *Biecheleria* gen. nov.

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SUMMARY

The well known freshwater dinoflagellate *Woloszynskia pseudopalustris* is transferred to the new genus *Biecheleria*, based on the very unusual structure of the eyespot (comprising a stack of cisternae), the apical apparatus of a single elongate amphiesma vesicle, the structure of the resting cyst, and molecular data. *Biecheleria* is phylogenetically related to *Symbiodinium* and *Polarella* of the family Suessiaceae. This family, which extends back to the Jurassic, is redefined with the eyespot (Type E *sensu* Moestrup and Daugbjerg) and apical apparatus as diagnostic features, unknown elsewhere in the dinoflagellates. *Biecheleria* also comprises the brackish water species *Biecheleria baltica* sp. nov. (presently identified as *Woloszynskia halophila*) and the marine species *Biecheleria natalensis* (syn. *Gymnodinium natalense*). *Gymnodinium halophilum* described in 1952 by B. Biecheler but apparently not subsequently refound, is transferred to *Biecheleria*. The Suessiaceae further includes the marine species *Protodinium simplex*, described by Lohmann in 1908 but shortly afterwards (1921) transferred to *Gymnodinium* by Kofoid and Swezy and subsequently known as *Gymnodinium simplex*. It only distantly related to *Gymnodinium*. A new family, the Borghiellaceae, is proposed for the sister group to the Suessiaceae, based on eyespot structure (Type B of Moestrup and Daugbjerg), the morphology of the apical apparatus (if present), and molecular data. It presently comprises the genera *Baldinia* and *Borghiella*. Cells of *Biecheleria pseudopalustris* and *B. baltica* contain a microtubular strand (msp) associated with vesicles containing opaque material. Such structures are known in other dinoflagellates to serve as a peduncle, indicating that the two species may be mixotrophic.

Key words: *Baldinia*, *Biecheleria*, *Borghiella*, Borghiellaceae, freshwater dinoflagellates, molecular phylogeny, ultrastructure.

INTRODUCTION

In previous articles we have reported on a number of thin-walled dinoflagellate species, known as woloszynskioids, whose cells are characterized by being covered by small, thin amphiesmal plates, too numerous to be described using the Kofoidean system of plate terminology. Our studies have shown that the woloszynskioid dinoflagellates are polyphyletic, and fall into several taxonomic groups. Four new genera have presently been created, *Tovellia* and *Jadwigia* of the new family Tovelliaceae (Lindberg *et al.* 2005), and the two genera *Borghiella* (Moestrup *et al.* 2008) and *Baldinia* (Hansen *et al.* 2007), whose relationships at the family level will be discussed below. Morphological features separating the groups include the ultrastructure of the eyespot as seen in transmission electron microscopy (TEM) thin sections (Types B, C or E *sensu* Moestrup & Daugbjerg 2007), the structure of the apical furrow apparatus (also known as 'apical furrow', acrobase' or 'carina'), and the type of resting cyst produced.

The first group of woloszynskioids, the Tovelliaceae, occupies an isolated position within the dinoflagellates both morphologically and in molecular trees based on nuclear-encoded rRNA. The family presently comprises only freshwater species and, in addition to *Tovellia* and *Jadwigia*, includes the very peculiar *Bernardinium* (probably synonymous with *Esoptrodinium*) whose cells possess only half a cingulum (on the cell's left side only). It also includes at least one, and probably many of the species presently included in *Katodinium*, but lack of knowledge about the type species of *Katodinium* hampers the establishing of a phylogenetically satisfactory taxonomy for species of this genus. The eyespot structure of the Tovelliaceae is unique within dinoflagellates and was designated type C by Moestrup and Daugbjerg (2007).

The second group presently contains two genera, *Borghiella* and *Baldinia*. Cells of *Borghiella* are superficially similar to species of the Tovelliaceae, but are phylogenetically unrelated. The eyespot belongs to type B.

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Baldinia anauniensis is an unusual, naked freshwater species that lacks an apical furrow apparatus (Hansen *et al.* 2007) but contains very unusual structures associated with the flagellar apparatus. Together with *Borghiella* it occupies a sister relationship to a rather diverse assemblage, which constitutes the third group and comprises *Woloszynskia pseudopalustris* (J. Schiller) Kisselev ex Elbrächter, the organism identified as *W. halophila* (Biecheler) Elbrächter et Kremp, *Polarella glacialis* Montresor, Procaccini et Stoecker, and species of *Symbiodinium*, endosymbionts of coral and other marine invertebrates. *Polarella* may be related to the Suessiaceae (Montresor *et al.* 1999), a family of otherwise extinct species extending back into the Mesozoic (Fensome *et al.* 1993). These species possess an eyespot of type E, which is restricted to this group of protists.

Over the last 10 years we have at regular intervals found the woloszynskioid *Woloszynskia pseudopalustris* at several localities in Denmark, on one occasion in bloom conditions. This is the largest known woloszynskioid dinoflagellate, described 90 years ago in present-day Ukraine (Wołoszyńska 1917) as *Gymnodinium palustre* Schilling forma, and it attained prominence as the dinoflagellate species in which the most detailed study of reproduction has been completed so far, including mitosis, sexual reproduction and meiosis (von Stosch 1973). Several years ago Calado and Craveiro, working in our lab, succeeded at culturing *W. pseudopalustris* by isolating motile cells, but the culture was subsequently lost. However, it provided the first published molecular data on woloszynskioids based on nuclear-encoded large subunit (LSU) rRNA sequences (Daugbjerg *et al.* 2000). Further attempts at isolating single cells have failed, including germination of resting cysts from the sediment. Many cysts germinated but the cultures died after a few cell divisions. Recently, Kremp *et al.* (2005) published a study of a brackish water species, identified as *Woloszynskia halophila,* found to be closely related to *W. pseudopalustris*. Kremp has kindly supplied a sample of her culture and below we present new information on these and related species, which we transfer to a new genus, *Biecheleria* gen. nov. The new genus also includes *Gymnodinium natalense* Horiguchi and Pienaar, a marine species from South Africa, which corresponds in the structure of the eyespot. Molecular data (e.g. LSU rRNA) are not available for *G. natalense*.

The Suessiaceae differs from the first two groups of woloszynskioids in all three features mentioned above: structure of the eyespot, structure of the 'apical furrow apparatus' and structure of the resting cysts. The fourth group will be described in the last paper of this series (Moestrup *et al.* 2009).

MATERIALS AND METHODS

Sampling sites and culture conditions

Woloszynskia pseudopalustris was collected over several consecutive years at Lake Vejlesø, Holte, and at Kalvemosen near Søllerød, both localities situated in the northern suburbs of Copenhagen, Denmark. The material illustrated in Figures 1, 2 and 11–19 is from Kalvemosen, collected 3 October and 10 October 2006. Cysts were isolated from sediments collected at Vejlesø 2 November 2005 and concentrated as described by Lindberg *et al.* (2005) (Figs 3–5 and 20–21). Fifty single cysts were isolated into the culture media DY IV, L16, four-strength L16 as well as in water from Lake Vejlesø filtered through 0.25-μm or 3-μm filters. Several cysts germinated with planozygote-like cells in early March 2006. Some of these divided to approximately 8–10 cells, but then died.

Figures 6–8 are from a culture established from Lake Vejlesø October 1995. The culture was maintained in four-strength L16 medium at 15°C and a 16 : 8 h LD (light : dark) regime.

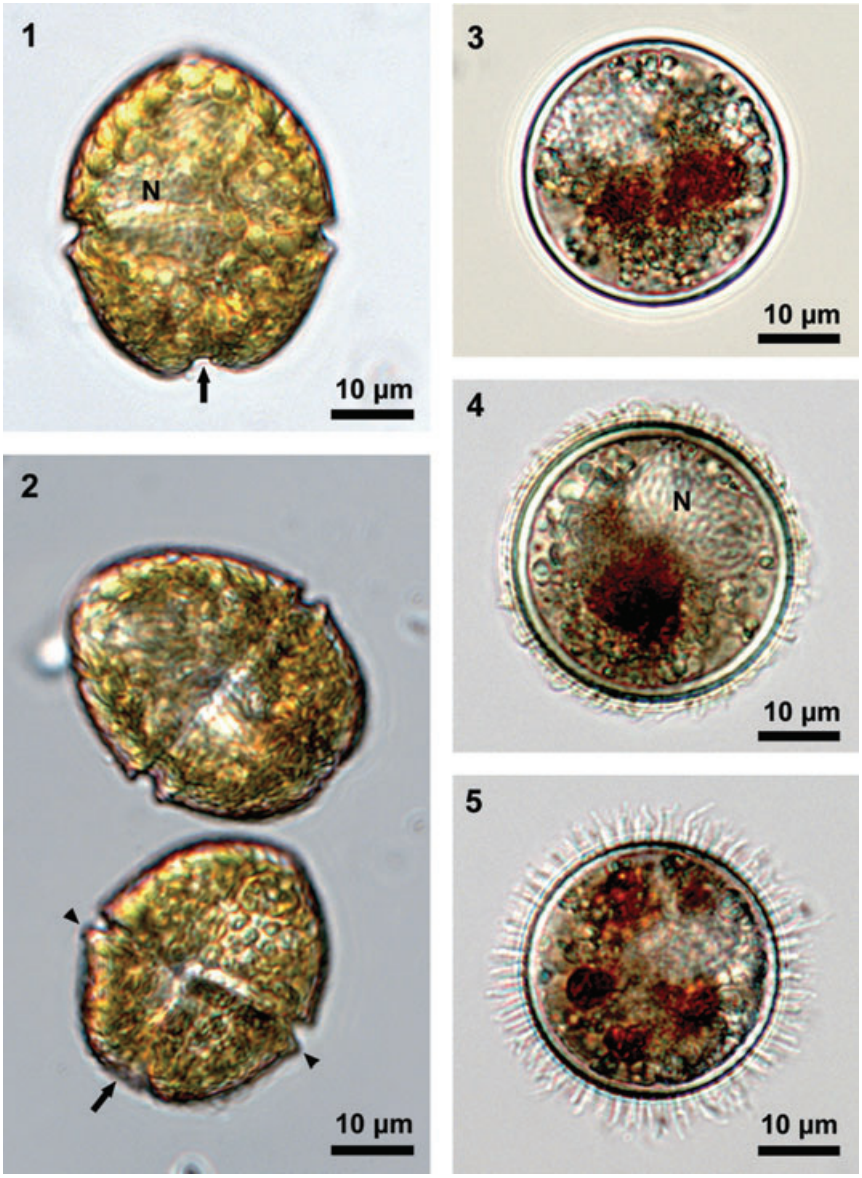
A subculture identified as *Woloszynskia halophila* was kindly provided by Anke Kremp. It originates from the Baltic Sea, close to the field station at Tvärminne, Finland. Cells are maintained in f/2 medium at a salinity of 7 psu in a 16 : 8 h LD regime and 4°C.

Light microscopy

For light microscopy we used a Zeiss (Oberkochen, Germany) Axiophot microscope fitted with Nomarski interference contrast and epifluorescence attachment. Cells were photographed on a Zeiss AxioCam HRc digital camera.

Scanning electron microscopy

A net plankton sample (pore size = 20 μm) was fixed (volume = 1.6 mL) in a pyrex glass for 15 min at room temperature in a mixture of 600 μL 2% OsO_4 and 200 μL saturated $HgCl_2$ solution. The cells were concentrated over a Millipore (Billerica, MA, USA) filter (pore size 8 μm) placed in a Millipore Swinnex holder tightened with an o-ring and screwed on to a disposable plastic syringe without the piston. The material was transferred from the pyrex glass to the open syringe, and distilled water was added regularly over 60 min. Dehydration followed in a graded ethanol series in the same setup, approximately 20 min in each change. The Swinnex holder was then detached from the syringe and critical point dried in CO_2 in a Baltec (Liechtenstein) CPD 030 critical point drier. The filter was mounted on a stub, coated for 90 s with platinum/palladium, and



Figs 1–5. *Biecheleria pseudopalustris* comb. nov., bright field light microscopy of live cells. 1,2. Motile cells, isolated from Lake Kalvemosen, Søllerød. 3–5. Resting cysts, isolated from sediments of Lake Vejlesø, Holte. 1. Cell in ventral view showing golden chloroplasts and large nucleus (N). The arrow indicates the ventral-antapical excavation, which is very distinct in the present cell as the cell is seen at a slightly oblique angle. 2. Two cells, the upper cell in dorsal view, the excavation hardly visible. The lower cell in ventral view shows the slightly displaced cingulum and the very distinct antapical border of the cingulum, which forms a discrete collar-like process (arrowheads). The antapical excavation (arrow) is masked by the underlying dorsal part of the hypocone, which is not excavated. 3–5. Resting cysts at different stages of development, all showing the clear thick walls and dark reddish pigment spots. Figure 3 shows a newly formed cyst with a smooth wall. Figure 4 is a cyst in an intermediate stage with short bristles on the surface (N, nucleus). Figure 5 shows a third cyst in the final stage of development, with long bristles.

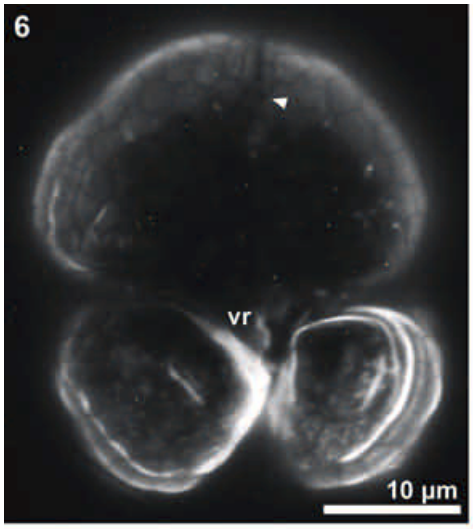
examined in a Japan Electron Optics Ltd (JEOL) (Tokyo. Japan) field emission scanning electron microscope JSM 6335F.

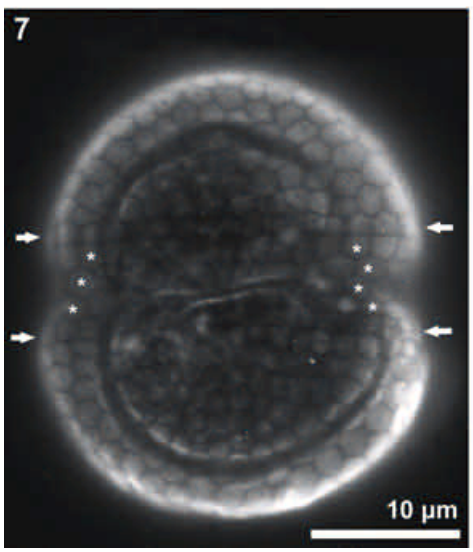
Transmission electron microscopy

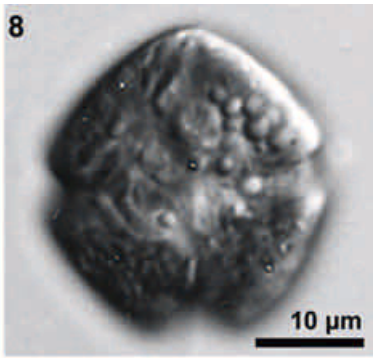
The best, but not entirely satisfactory fixation of *W. pseudopalustris*, was obtained as follows. (i) A mixed sample from Kalvemosen dominated by a bloom of *Woloszynskia pseudopalustris* was mixed 1:1 with 6% glutaraldehyde in 0.1 M phosphate buffer at 15°C and fixed for 2.5 h. It was concentrated by centrifugation and the pellet was rinsed in three changes of phosphate buffer, followed by post-osmication for 2 h in 1% osmium tetroxide at 4°C; and (ii) 10–15 swimming cells from germinated cysts were fixed for 2 h in 2% glutaraldehyde in 0.2 M phosphate buffer. They were transferred singly to 1.5% agar dissolved in buffer. The agar was cooled to solidify, and small blocks containing single cells were cut out from the agar. The blocks were subsequently rinsed in buffer (a total of three changes over 2 h) and post-osmicated in cold 1% osmium tetroxide in distilled water.

The Baltic material, identified as *Woloszynskia halophila* was somewhat better preserved. It was fixed for 1.5 h in a 1:1 mixture of culture and cold 2% glutaraldehyde in f/2 medium at 7 psu. It was rinsed in the growth medium for 2 h (three changes) and post-osmicated overnight in cold 1% osmium tetroxide in 7 psu seawater.

Following post-osmication, cells of all fixations were rinsed briefly in phosphate buffer (freshwater material) or in distilled water (marine material) before dehydration in an alcohol series: 20 min in each change of cold 30,







Figs 6–8. *Biecheleria pseudopalustris* comb. nov. Live cells from culture. 6,7. Fluorescence microscopy, cells stained with CalcoFluor White to show the amphiesmal plates and vesicles. Courtesy António J. Calado. 6. Ventral view, the antapical concavity very clear, the elongate apical vesicle (EAV) is seen as a dark line, bordered on each side by a row of elongate amphiesmal vesicles (arrowhead). The finger-like ventral ridge (vr) may also be distinguished. 7. Dorsal view. The arrows indicate the pre- and postcingular row of plates. The pentagonal plates in the precingular row create a straight epicone-cingular border (two upper arrows). The plates of the postcingular row are usually hexagonal (two lower arrows). Asterisks mark the horizontal cingular rows of plates, here four rows on the right and three on the left. Compare with Figure 19. 8. Cell consistent with the '*excavatum*-type' *sensu* Nygaard (1945).

50, 70 and 96% alcohol. Dehydration was completed at room temperature in two changes of absolute alcohol (approximately 1 h in total, but overnight for the agar-embedded cells), followed by two changes of propylene oxide (5 min in each change). The material was then transferred to a 1:1 mixture of propylene oxide and Spurr's resin mixture. It was left for 5 h overnight and subsequently transferred to 100% resin. It was left in another change of resin for 1 h to overnight and polymerized in a final change of resin for a minimum of 8 h at 70°C.

The cells were sectioned on a LKB 8800 Ultramicrotome (LKB, Bromma, Sweden) and sections contrasted with uranyl acetate and lead citrate before viewing in a JEM-1010 transmission electron microscope. They were photographed using a Gatan digital camera 792.

Alignment of nuclear-encoded LSU rRNA

In total, 43 dinoflagellate LSU rRNA sequences representing 25 genera and 42 species were retrieved from GenBank. Our group has previously determined these sequences in a series of studies addressing the phylogeny and evolutionary history of dinoflagellates. A diverse assemblage of ciliates (four species), apicomplexans (five species) and the genus *Perkinsus* formed the outgroup. A list comprising all species included in the phylogenetic analyses and their GenBank accession numbers is presented in Table 1. The LSU rRNA data matrix was aligned following the secondary structure as suggested by De Rijk *et al.* (2000) and finally edited manually using MacClade (ver. 3.08, Maddison & Maddison 2003). Compared with the secondary structure model for *Prorocentrum micans* (Lenaers *et al.* 1989) the ribosomal fragments included here started 27 base pairs downstream of domain D1 and ended six base pairs up the stem that forms domain D6. Due to unambiguous alignment of the highly divergent domain D2 (*sensu* Lenaers *et al.* 1989), this domain was excluded, thus leaving 1152 base pairs (including introduced gaps) for phylogenetic inference.

Table 1. List of dinoflagellates and outgroup species included in the phylogenetic analyses based on partial large subunit (LSU) rRNA

Species (in alphabetic order)	Genbank accession numbers
Akashiwo sanguinea (Hirasaka) Gert Hansen et Moestrup	AF260396
Alexandrium affine (Inouye et Fukuyo) Balech	AY294612
Alexandrium margalefii Balech	AY154957
Amphidinium carterae Hulburt	AY455669
Amphidinium herdmanii Kofoid et Swezy	AY455675
Amphidinium massartii Biecheler	AY455670
Baldinia anauniensis Gert Hansen et Daugbjerg	EF052683
Bernardinium bernardinense Chodat	DQ289020
Biecheleria baltica Moestrup, Lindberg et Daugbjerg	EF205019
Biecheleria pseudopalustris (J. Schiller) Moestrup, Lindberg et Daugbjerg	AF260402
Borghiella dodgei Moestrup, Gert Hansen et Daugbjerg	EU126801
Borghiella tenuissima (Lauterborn) Moestrup, Gert Hansen et Daugbjerg	AY571374
Ceratium fusus (Ehrenberg) Dujardin	AF206390
Ceratium lineatum (Ehrenberg) Cleve	AF260391
Dinophysis norvegica Claparède et Lachmann	AY571375
Gonyaulax baltica Ellegaard, Lewis et Harding	AF260388
Gymnodinium catenatum L.W. Graham	AF200672
Gymnodinium fuscum (Ehrenberg) Stein	AF200676
Gymnodinium nolleri Ellegaard et Moestrup	AF200673
Gyrodinium dominans Hulburt	AY571370
Gyrodinium rubrum (Kofoid et Swezy) Takano et Horiguchi	AY571369
Gyrodinium spirale (Bergh) Kofoid et Swezy	AY571371
Heterocapsa rotundata (Lohman) Gert Hansen	AF260400
Heterocapsa triquetra (Ehrenberg) F. Stein	AF260401
Jadwigia applanata Moestrup, Lindberg et Daugbjerg	AY950448
Karenia brevis (Davis) Gert Hansen et Moestrup	AF200677
Karenia mikimotoi (Miyake et Kominani ex Oda) Gert Hansen et Moestrup	AF200681
Karlodinium veneficum (Ballantine) J. Larsen	AF200675
Lepidodinium chlorophorum (Elbrächter et Schnepf) Gert Hansen, Botes et de Salas	AF200669
Peridiniella catenata (Levander) Balech	AF260398
Peridinium cinctum Ehrenberg	EF205011
Peridinium palatinum Lauterborn	AF260394
Peridinium willei Huitfeld-Kaas	AF260384
Polarella glacialis Montresor, Procaccini et Stoecker	AY571373
Prorocentrum micans Ehrenberg	AF260377
Prorocentrum minimum (Pavillard) Schiller	AF260379
Protodinium simplex Lohmann	AF260379
Scrippsiella trochoidea var. *aciculifera* Montresor	AF260393
Symbiodinium natans Gert Hansen et Daugbjerg	EU315917
Symbiodinium sp.	EF205014
Tovellia coronata (Wołoszyńska) Moestrup, Lindberg et Daugbjerg	AY950445
Tovellia sanguinea Moestrup, Gert Hansen, Daugbjerg, Flaim et D'Andrea	DQ320627
Outgroup taxa	
Ciliates	
Tetrahymena pyriformis (Ehrenberg) Lwoff	X54004
Tetrahymena thermophila Nanney et McCoy	X54512
Euplotes aediculatus Pierson	AF223571
Spathidium amphoriforme Greeff	AF223570
Apicomplexa	
Toxoplasma gondii Nicolle et Manceaux	X75429
Hammondia hammondi Frenkel	AF101077
Neospora canium Dubey, Carpenter, Speer, Topper et Uggla	AF001946
Sarcocystis neurona Dubey, Davis, Speer, Bowman, de Lahunta, Granstrom, Topper, Hamir, Cummings et Suter	AF092927
Theileria parva strain Muguga	AF013419
Perkinsea	
Perkinsus chesapeaki McLaughlin, Tall, Shaheen, El Sayed et Faisal	AY305326

GenBank accession numbers are also included.

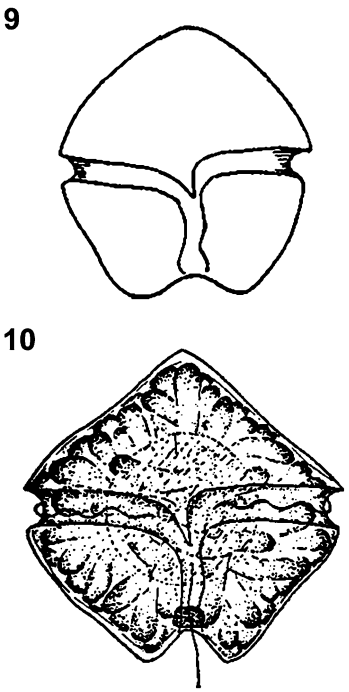
Phylogenetic analyses

The LSU rRNA data matrix was analyzed with Bayesian analysis (BA) using MrBayes (ver. 3.12, Ronquist & Huelsenbeck 2003) and maximum likelihood (ML) using PhyML (ver. 3.0, Guindon & Gascuel 2003). BA was done using a general time reversible (GTR) substitution model with base frequencies and substitution rate matrix estimated from the data. Two million Markov Chain Monte Carlo (MCMC) generations with four parallel chains (one cold and three heated) were conducted using the freely available Bioportal (www.bioportal.uio.no). A tree was sampled every 50 generations. We used 'Are We There Yet' (AWTY) by Wilgenbusch *et al.* (2004) to examine whether the BA had been running long enough. Plots of posterior probabilities of all splits for paired MCMC had converged after 2×10^6 generations. A graph was constructed by plotting log likelihood values as a function of generations. The lnL values reached a stationary level after 20.050 generations. All 401 trees below this level were discarded and the 50% majority rule consensus tree thus comprised 39.600 trees. The majority rule consensus tree was calculated using PAUP* (ver. 4b10, Swofford 2003). ML analysis was carried out using the online version of PhyMl available at the Montepellier bioinformatics platform. MrModeltest (Nylander 2004) gave information on the best model of nucleotide substitutions, and we used the parameter settings found for the gamma shape (a = 0.6453) and the proportion of invariable sites (I = 0.2056) in ML analysis. One hundred bootstrap replicates were run to obtain support values for the branching pattern. Consensus from the Phylip package (ver. 3.68, Felsenstein 2008) was used to obtain a 50% majority rule consensus tree. Posterior probabilities from BA and bootstrap values from ML analyses were mapped onto the Bayesian tree.

RESULTS

Light microscopy of *Woloszynskia pseudopalustris*

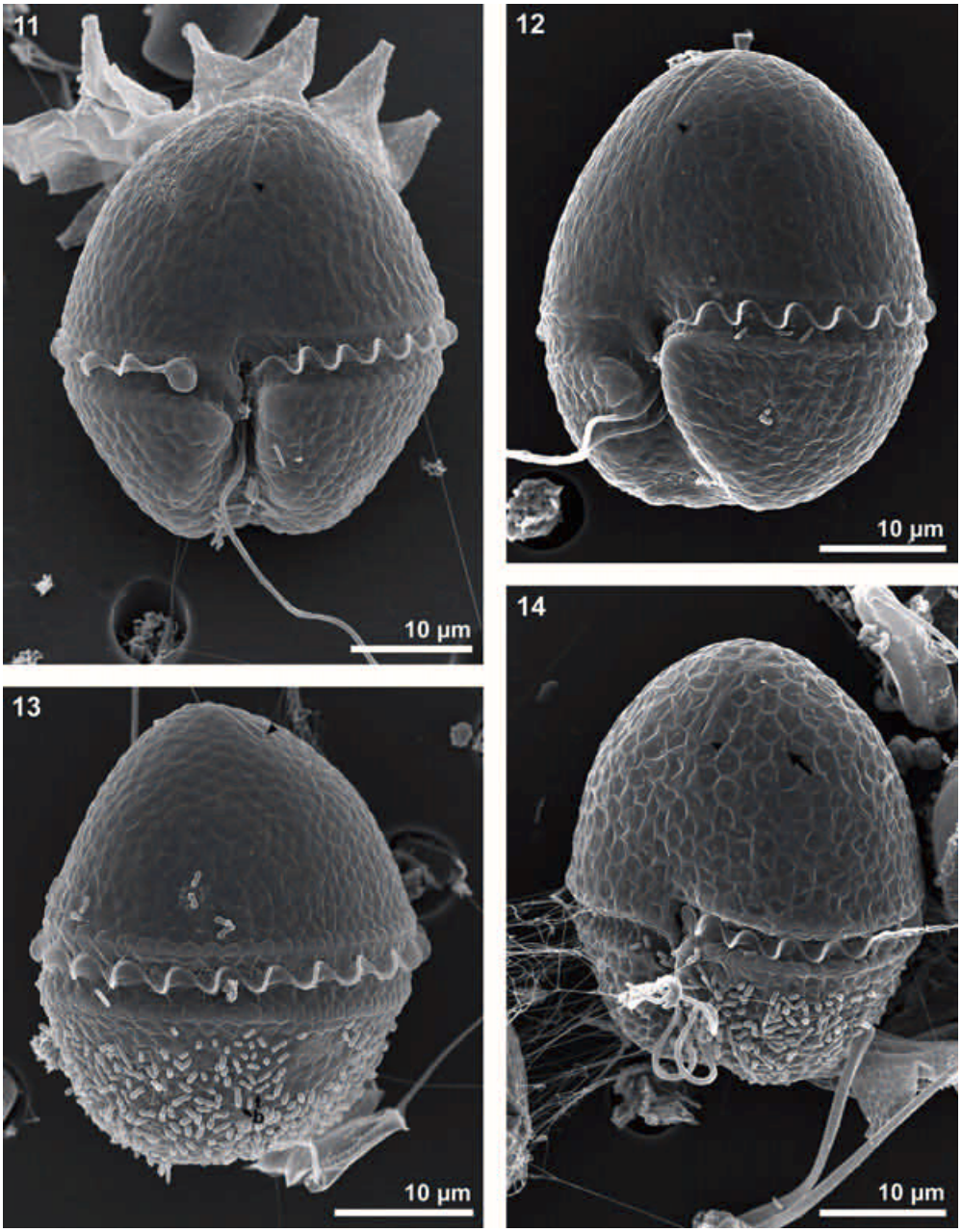
Live motile cells are illustrated in Figures 1, 2 and 8. Figures 1 and 8 show specimens with a pronounced antapical invagination,Figure 2 (top) a cell with a less pronounced invagination and another (bottom) in which the invagination is only visible from the ventral side (arrow). The large, centrally positioned nucleus is also visible (Fig. 1). A particular feature of this species is the pronounced posterior margin of the cingulum (Fig. 2, arrowheads). Figures 3–5 illustrate cysts collected from the sediment in Lake Vejlesø, and show the variation in spine development. Cysts were very numerous in the sediment, and all stages of apparent cyst maturation were found, from spineless, apparently immature cysts (Fig. 3) to cysts with short, curved spines (Fig. 4) to probably fully mature cysts covered by numerous long spines or bristles (Fig. 5). Figures 6 and 7 illustrate cells stained with calcofluor white, observed under the fluorescence microscope. The many amphiesmal vesicles were visible for a few seconds only and then disappeared. The anterior furrow apparatus was also visible (Fig. 6, arrowhead). The finger-like ventral ridge is visible in Figure 6. Illustrations of the similar species *Gymnodinium acuminatum* Christen are reproduced in Figures 9 and 10 (Christen 1959).



Figs 9,10. Original illustrations of *Gymnodinium acuminatum* nom. nud. by Christen (1959). Both cells show the deeply excavated hypocone and the finger-like process in the flagellar pore area. In Figure 9 the cell has been left for a while and the shape has changed slightly. No scale available.

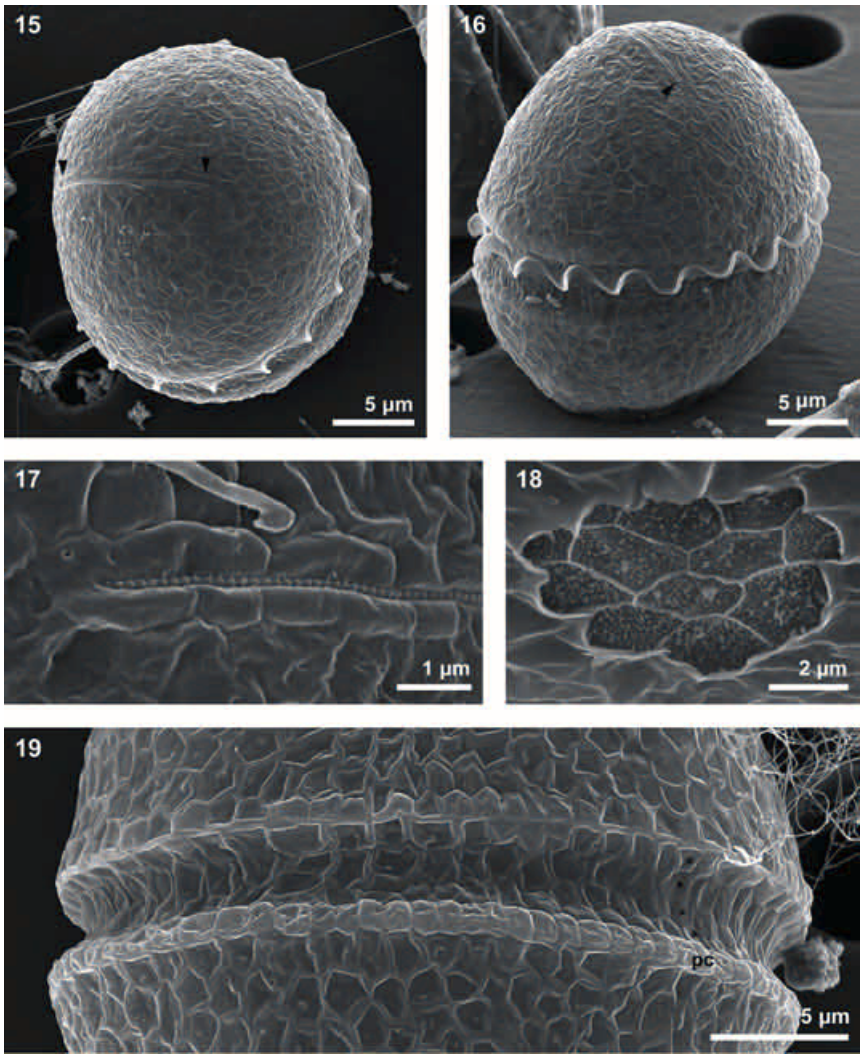
Scanning electron microscopy of *Woloszynskia pseudopalustris*

The general shape of the cell is shown in Figures 11–16. The figures also illustrate the many amphiesmal vesicles, the antapical invagination, the apical furrow apparatus, and the ventral ridge, which extends downwards as a finger-like projection. A special feature of this material, which was taken directly from Lake Kalvemosen and processed for SEM, was the presence of high numbers of bacteria on the hypocone of many cells (Figs 13,14). Such bacteria were nearly absent on the epicone (Fig. 13). In Figure 14, most amphiesmal vesicles show a central, pore-like structure perhaps reflecting the spines present on the resting cyst. The



Figs 11–14. *Biecheleria pseudopalustris*. Scanning electron microscopy (SEM) of wild material from Kalvemosen, Søllerød. 11. Cell in ventral view showing the elongate apical vesicle (EAV) (arrowhead), the antapical excavation and the ventral ridge area that extends downwards as a finger-like process towards the sulcus. 12. Planozygote seen in a ventral-lateral angle. On the epicone the outer amphiesmal membrane is well preserved and closely attached, and the plate pattern is clearly distinguishable. On the hypocone the membrane is more loose, and the plate pattern therefore less obvious. The arrowhead indicates the EAV. 13,14. Cells (Fig. 14a planozygote) showing the infection or symbiosis with rod-shaped bacteria (b), almost entirely restricted to the hypocone, both on the dorsal (Fig. 13) and the ventral (Fig. 14) side. Figure 14 also shows trichocyst pores in the amphiesmal vesicles of the epicone, and one has been indicated by an arrow. The arrowhead indicates the EAV.

apical furrow apparatus extended over the cell apex but reached only a short distance down the ventral and dorsal sides of the cell (Figures 15,16). It comprised a single, elongate, very narrow vesicle (EAV, elongate apical vesicle) bordered on each side by approximately seven narrow vesicles (Figs 15–17). The EAV may be distinguished as an opaque line in Figure 6. Figure 17 is a higher magnification of the apical furrow apparatus, showing the EAV ornamented with a row of approximately 60 knobs. When the external membranes of the cell have disappeared, the contents of the amphiesmal vesicles become very distinct (Fig. 18). Figure 19 is a



Figs 15–19. *Biecheleria pseudopalustris*. Scanning electron microscopy (SEM) of wild material from Lakes Kalvemosen and Søllerød. 15. Cell in apical view showing the whole elongate apical vesicle (EAV) (lengths indicated by the arrowheads) running directly over the apex. 16. Same cell, tilted to show that the EAV (arrowhead) extends for only a short distance down the dorsal side of the epicone. 17. The ventral part of the EAV showing the long, very narrow central plate ornamented with knobs. The plate is bordered on both sides by elongate narrow vesicles. 18. Surface area lacking the outer amphiesmal membrane, allowing visualization of the thin thecal plates and the sutures between the plates. 19. The cingulum is covered by three to four horizontal rows of vesicles (asterisks). The vesicles of the precingular row are pentagular, those in the postcingular row (pc) project slightly; compare with Figure 2.

higher magnification to show the horizontal rows of plates in the cingular region. The cingulum proper is covered by three to four rows of small amphiesmal vesicles (asterisks). The postcingular rim (pc in Fig. 19), and sometimes also the precingular rim (Fig. 13), are formed by a single row of very distinct, slightly protruding vesicles. The central pore-like structure is visible on several amphiesmal vesicles (Fig. 19). The mature resting cyst is illustrated in Figure 20 and at higher magnification in Figure 21. The pattern of hexagonal amphiesmal vesicles can be distinguished in Figure 21, most vesicles with one, rarely two, central, more or less curved spines.

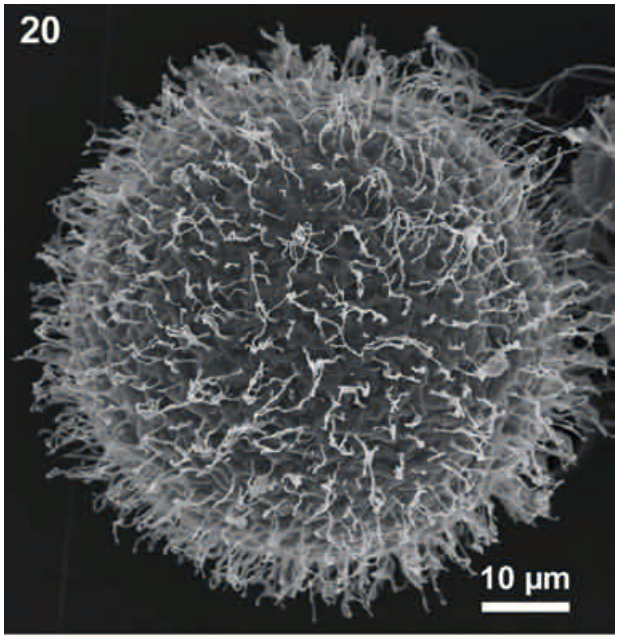
Transmission electron microscopy of *Woloszynskia pseudopalustris*

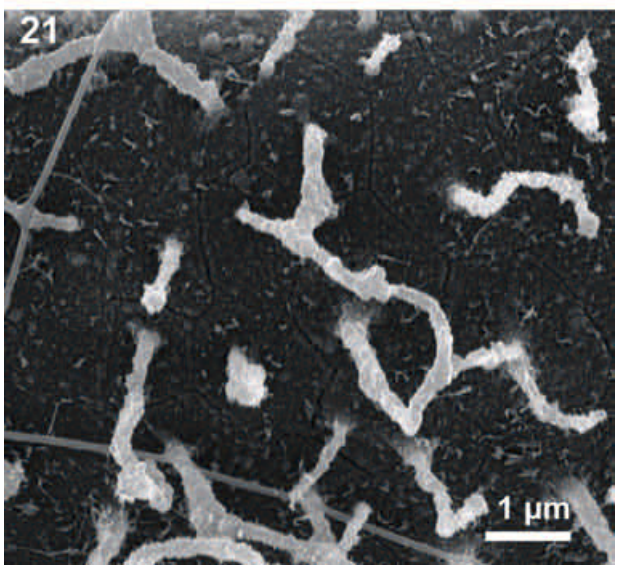
Since a well-preserved fixation of *W. pseudopalustris* was not obtained, only a few ultrastructural features will be provided (Figs 22–29).

The amphiesma vesicles contain very thin plates, only slightly thicker than the vesicle membrane (approximately 15–20 nm) (Fig. 27). The chloroplast is complex and probably forms a peripheral network that comprises several pyrenoids. Each pyrenoid is surrounded on the outside by a hemispherical starch grain (Figs 22,23). The chloroplasts contain thylakoids in stacks of three, while the pyrenoid matrix is penetrated by pairs of thylakoids (Fig. 22).

The eyespot comprises a stack of cisternae, each cisterna containing brick-like material (Fig. 23).

The flagellar apparatus was not examined in detail and Figure 25 will suffice to show the orientation of the two basal bodies to each other: the longitudinal flagellum basal body inserting on the side of the transverse flagellum basal body. The dorsal side of the multi-membered r_1 flagellar root at one end possesses a conspicuous opaque plate dorsally, as illustrated in the planozygote in Figure 26. A band of microtubules resembling peduncle microtubules was associated





Figs 20,21. *Biecheleria pseudopalustris*. Cysts isolated from sediment from Lake Vejlesø, Holte. Scanning electron microscopy (SEM). 20. Spherical cyst covered with thin hair-like bristles; compare with Figures 4,5. The cyst has shrunk during preparation, making the surface irregular. 21. Paraplates, each with one, rarely two, bristles.

with small vacuoles containing opaque material (Fig. 24).

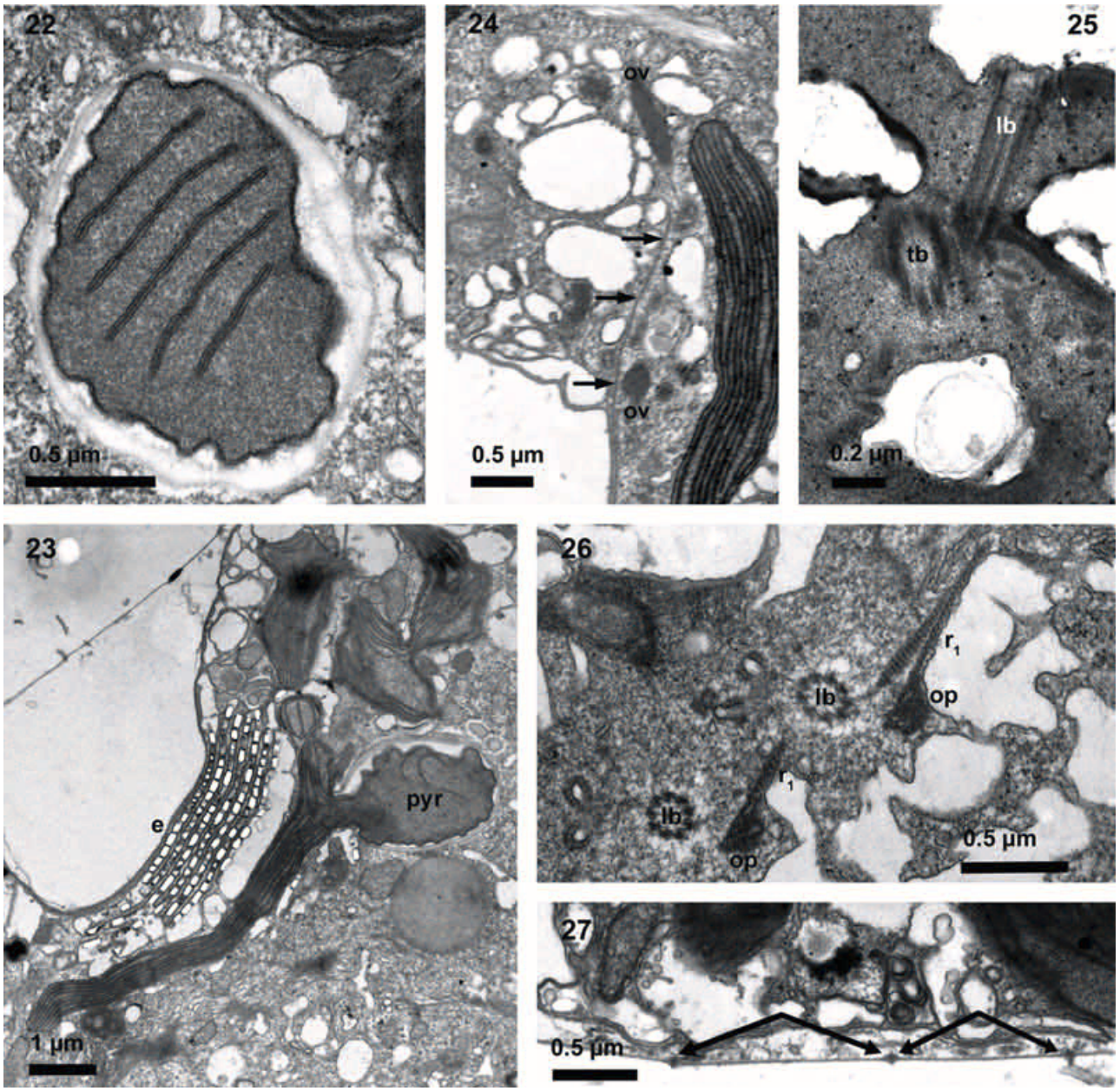
The planozygotes arising after germination of the hypnozygotes contained numerous bacteria in the cytoplasm (Fig. 28). In some cases bacteria were visible also in the nucleoplasm (Fig. 29). The bacteria were never located in vesicles; rather each bacterium cell was surrounded by a translucent halo, perhaps indicating a mucilaginous layer separating the bacteria from the host nucleoplasm and cytoplasm.

Further observations on the material identified as *Woloszynskia halophila*

The culture was examined by Kremp *et al.* (2005) and only selected features will be illustrated here. In contrast to what was stated by Kremp *et al.* (2005), the amphiesma vesicles were found to contain distinct amphiesma plates, equal in thickness to the amphiesma membranes (approximately 10 nm). The plates were characteristically ornamented, as shown in transverse and tangential sections of the plates (Figs 30–33). The chloroplast is similar to that of *W. pseudopalustre* and comprises a peripheral network containing several pyrenoids (Figs 34,35), each surrounded by a hollow starch grain (Fig. 35). The thylakoid lamellae in Figures 30, 31, 33 and 34 are swollen and poorly fixed but in other cells, typical three-thylakoid lamella were present. The matrix of each pyrenoid is penetrated by pairs of thylakoids (Fig. 36). The eyespot comprises as many as nine cisternae with brick-like contents (Figs 37,38). The flagellar basal bodies are located close to the eyespot (Fig. 37) and inserted as in *W. pseudopalustris*, that is, the longitudinal flagellum basal body inserts on the side of the transverse flagellum basal body (Fig. 39). The r_1 flagellar root possesses a similar opaque plate dorsally near the basal bodies (Fig. 42). Most unexpected was the finding of a band of microtubules, which from the cell interior proceeds towards the cell surface, terminating in the region of the ventral ridge (Figs 40,41 and at lower magnification in Fig. 39). Figure 37 is a lower magnification of Figure 39 and includes the ventral ridge region. The band is associated with numerous vesicles containing opaque material (Figs 39,40, also Fig. 37, top) and it undoubtedly represents a peduncle.

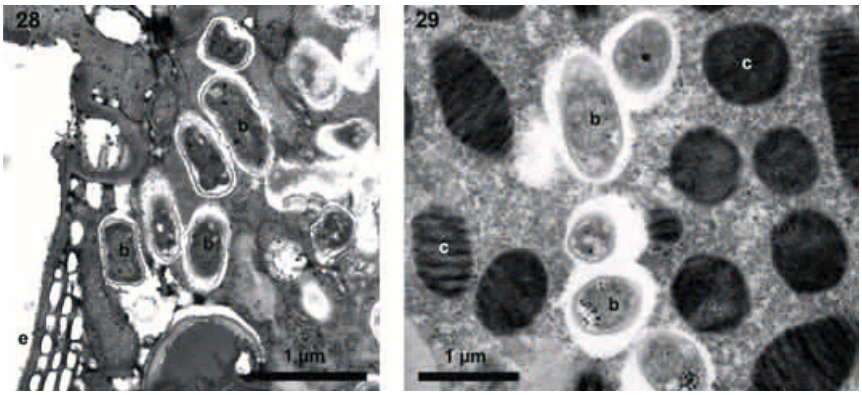
Phylogeny of *Biecheleria*

The phylogenetic inference based on Bayesian Analysis showed that *W. pseudopalustris* and *W. halophila sensu* Kremp *et al.* formed a highly supported monophyletic clade with three other genera of dinoflagellates also possessing type E eyespots (Fig. 43). Support values were BA = 1.0 and ML = 100%. The two species are transferred below to *Biecheleria* gen. nov. as *B. pseudopalustris* comb. nov. and *B. baltica* sp. nov. (syn. *Woloszynskia halophila sensu* Kremp *et al.*). That *Biecheleria pseudopalustris* and *B. baltica* are closely related was also indicated by a sequence divergence estimate based on 1221 base pairs. The LSU rRNA sequences only differed by 0.7%. Dinoflagellates with eyespots assigned to type E formed a sister group rela-

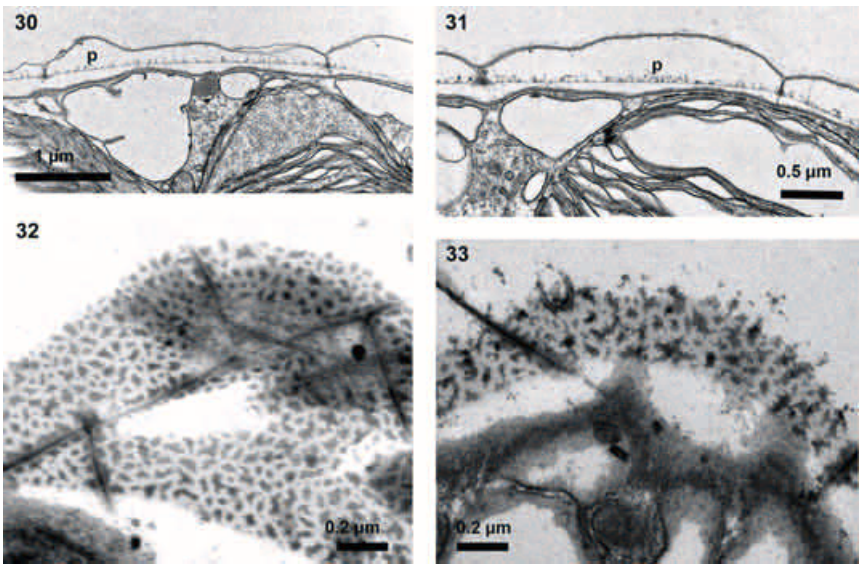


Figs 22–27. *Biecheleria pseudopalustris*, transmission electron microscopy (TEM). 22. The pyrenoid matrix penetrated by paired thylakoids. 23. Eyespot (e) and stalked pyrenoid (pyr). 24. The microtubular strand (arrows) and the opaque vesicles (ov) indicating the presence of a peduncle. 25. The longitudinal flagellum basal body (lb) inserts on the side of the transverse flagellum basal body (tb). 26. Planozygote with two parallel sets of basal bodies. The figure illustrates the longitudinal flagellum basal bodies (lb) and the associated r_1 roots, both associated with an opaque band of material (op) on the dorsal side of the root. 27. Section through the cell surface illustrating the very thin amphiesma plates (two plates indicated by angled arrows). The cell exterior is downwards. The cell membrane and the outer amphiesma membrane have disappeared.

tionship to the recently proposed genus *Borghiella* (Moestrup *et al.* 2008). *Borghiella* and *Baldinia* (Hansen *et al.* 2007) both possess type B eyespots *sensu* Moestrup and Daugbjerg (2007) but they appeared polyphyletic in this analysis. The branching pattern as suggested by BA showed *Bicheleria* spp. forming a sister group to *Symbiodinium* spp. and *Protodinium simplex*. Except for the early divergence of *Polarella glacialis*, the topology for the remaining genera possessing type E eyespots was well (PP = 0.95) to moderately well (PP = 0.65) resolved in terms of support values in BA (Fig. 43). In ML bootstrap analysis the relationship between *Symbiodinium* spp., *Protodinium* and *Biecheleria* spp. was not resolved (not shown).



Figs 28,29. Bacteria in cells of *Biecheleria pseudopalustris*. 28. Group of bacteria (b) in the cytoplasm. e, eyespot. 29. Bacteria (b) in the nucleoplasm; c, chromosomes.



Figs 30–33. The amphiesma of *Biecheleria baltica* sp. nov. 30,31. The amphiesma plates (p) are extremely thin and appear somewhat irregular in outline when observed in transverse sections. 32,33. Tangential views show the irregular outline of the amphiesma plates to be caused by ornamentation on the amphiesma plate surface. The ornamentation is made of very irregular projections.

DISCUSSION

Biecheleria Moestrup, Lindberg et Daugbjerg gen. nov.

Biecheleria ad Dinophyta pertinens. Cellulae periplasto tenui inclusae. Angustum vesiculae amphiesmatis elongatae (EAV) trans apicem cellulae e latere dorsali ad ventrale extensum. Chloroplasti et stigma praesens. Stigma cisternarum materiam latteratam continentium acervo formatum (typus E *sensu* Moestrup et Daugbjerg 2007). Divisio cellulae fissione binaria. Cystae quiescentes sphaericae, spinis nonnullis tectae.

Dinoflagellates surrounded by thin periplast. An apical furrow apparatus formed by single elongate narrow vesicle (EAV) extends over the apex from the ventral to the dorsal side of the cell. Chloroplasts and eyespot present, the eyespot formed by a stack of cisternae containing brick-like material (Type E *sensu* Moestrup & Daugbjerg 2007). Cell division by binary fission. The resting cyst spherical, covered with numerous spines or bristles.

Type species:

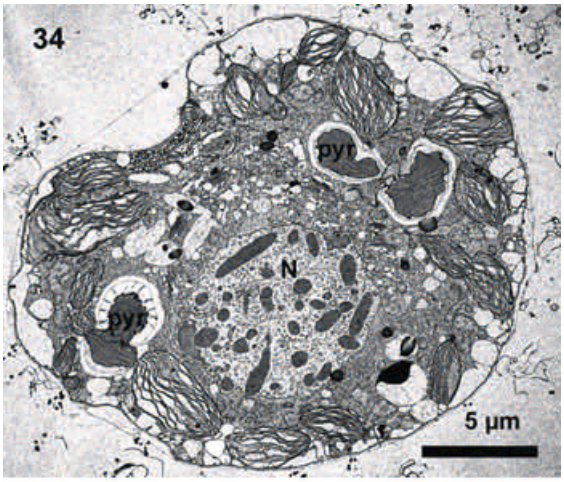
Biecheleria pseudopalustris (J. Schiller) Moestrup, Lindberg and Daugbjerg

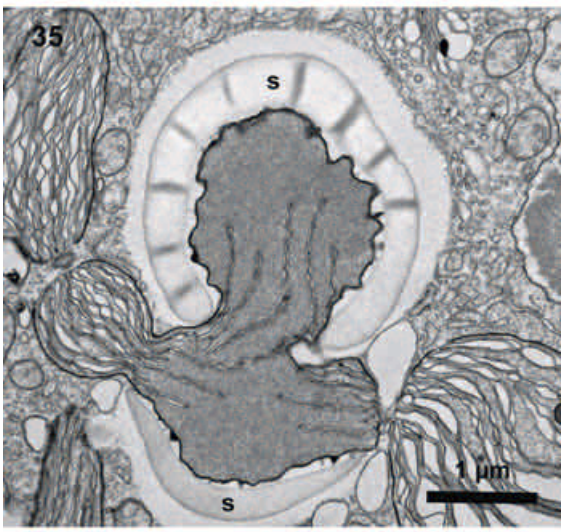
Basionym: *Gymnodinium pseudopalustre* J. Schiller (1933, 400, fig. 418a,b).

Synonyms: *Gymnodinium palustre* Schilling, forma (Wołoszyńska 1917); *Gymnodinium excavatum* (Nygaard 1945); *Woloszynskia pseudopalustris* (J. Schiller) Kisselev (1954) (invalid); *Woloszynskia pseudopalustris* (J. Schiller) Kisselev ex Elbrächter in Kremp *et al.* (2005, 635).

Etymology: The generic name commemorates Berthe Biecheler, a pioneer in light microscopical studies of dinoflagellates. Her unfinished PhD thesis was published in 1952, 13 years after her premature death.

Arguments for retaining only the type species of *Woloszynskia*, *W. reticulata* R.H. Thompson, in *Woloszynskia* have been given on several occasions; see for example Moestrup *et al.* (2008).





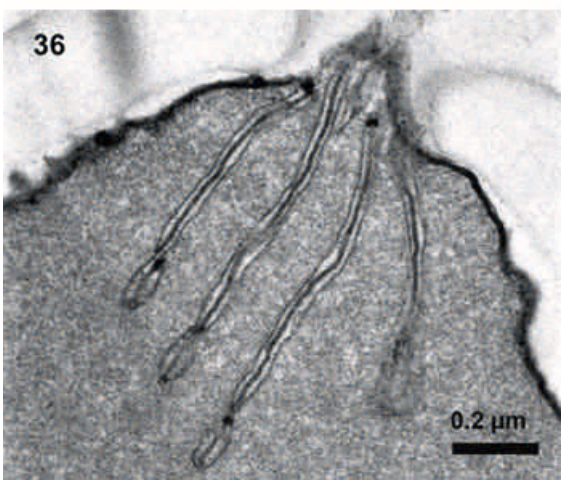


Fig 34–36. Some details of *Biecheleria baltica* sp. nov., supplementing the illustrations provided by Kremp *et al.* (2005), (as *Woloszynskia halophila*). 34. Transverse section at the level of the nucleus (N), to show profiles of pyrenoids (pyr); the pyrenoid on the lower left is double (compare with Fig. 35). 35. Each pyrenoid is lined by a convex starch grain (s). In this case, two opposite pyrenoids each show their respective starch grain. 36. The pyrenoid matrix is penetrated by pairs of thylakoids.

Identification of our material

A consistent feature of *Biecheleria pseudopalustris* is the posterior invagination of the cell (Fig. 1), although the size of the invagination varies in different cells. It also agrees with *Gymnodinium excavatum* Nygaard (Nygaard 1945, 1949), and the two taxa must be considered to represent the same species. Von Stosch (1973) failed to see amphiesmal plates even after staining, and this induced him to retain the species in *Gymnodinium*.

Biecheleria baltica sp. nov.

= *Woloszynskia halophila sensu* Kremp *et al.* (2005, 632, figs 1–4), non *Gymnodinium halophilum* Biecheler (1952, 33, figs X–XIV).

Cellulae ellipsoides aut sphaericae, epiconus et hypoconus paene eiusdem magnitudinis aut hypoconus paulo major. Cingulum descendens, circiter latitudine unius cinguli dimotum. Extensio brevis, forma digiti, ab latere dextrali epiconi, ad confluentem cinguli et sulci protrudens. Sulcus ad antapicem extendens, in hac regione distincte concavus. Cellulae paulum dorsiventraliter compressae, 17–35 µm longae et 12–32 µm latae. Nucleus centraliter positus, saepe paulum apicalis apparens. Cellulae tenuibus, ornatis laminis tectae, in 11–12 ordinibus latitudinalibus, 5–6 in epicono, 2 in cingulo, 3–4 in hypocono. EAV minus quam 2 µm longa, utrinque 2–3 tenuibus laminis delimitata. Lamina minor ad terminum ventralem in EAV. Chloroplasti aureo-brunnei. Cystae quiescentes sphaericae. In aqua frigida viventes, normaliter in aqua amara 0–6°C.

Cells ellipsoid or spherical, the epi- and the hypocone of almost equal size or the hypocone slightly larger. The cingulum descending, displaced approximately one cingulum width. A short, finger-like extension projects from the right hand side of the epicone at the confluence of the cingulum and the sulcus. The sulcus extends to the antapex, which is markedly concave in this region. Cells slightly dorso-ventrally compressed, 17–35 µm long and 12–32 µm wide. Nucleus located centrally, often appearing slightly anterior. Cells covered by thin, ornamented plates in 11–12 latitudinal rows, five to six in the epicone, two in the cingulum and three to four in the hypocone. The EAV is less than 2 µm long and bordered on each side by two to three narrow plates. A smaller plate is present at the ventral termination of the EAV. Chloroplasts golden-brown. Cysts spherical. Cold-water species, typically found in brackish water at 0–6°C.

Type material: A fixed and embedded sample has been deposited at the Botanical Museum, Copenhagen as number CAT-2411.

Kremp *et al.* (2005) studied a dinoflagellate from the Baltic Sea, which they identified as *Gymnodinium*

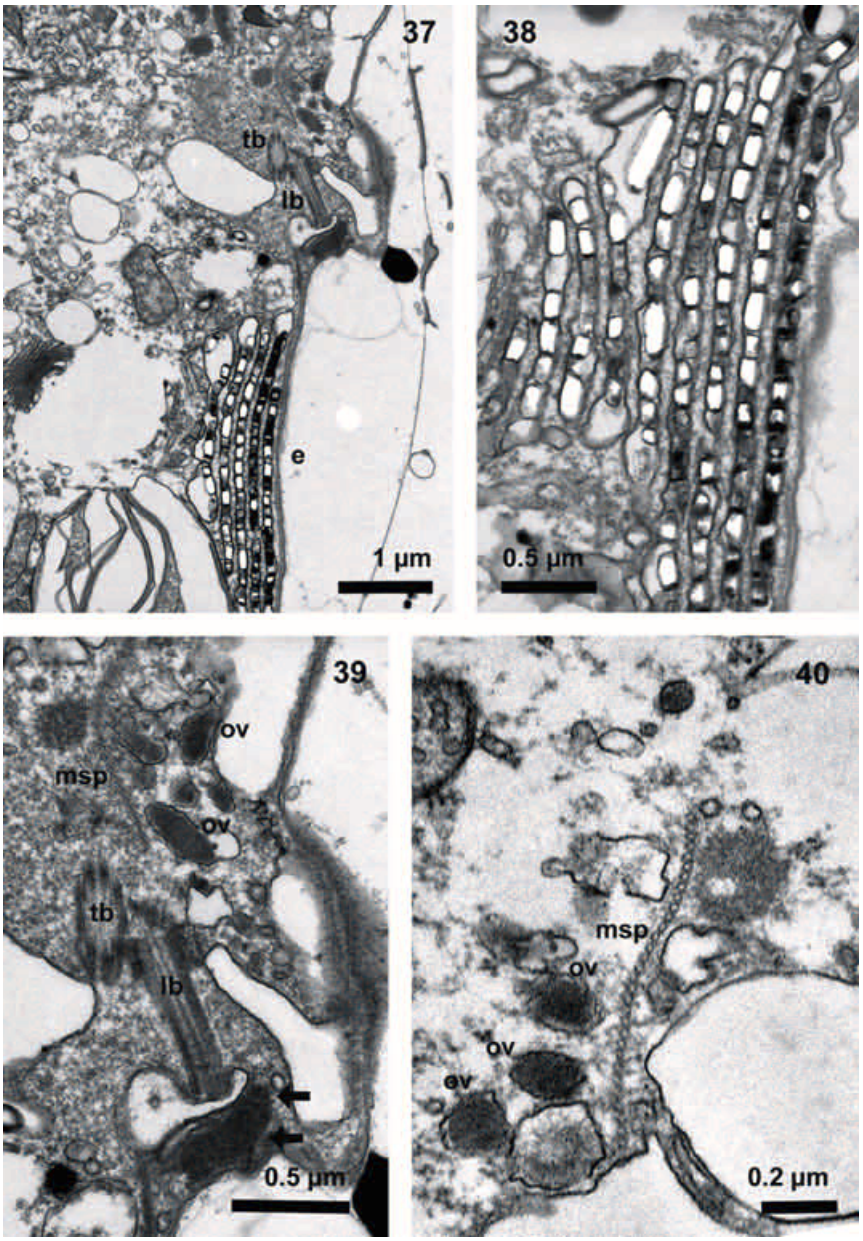
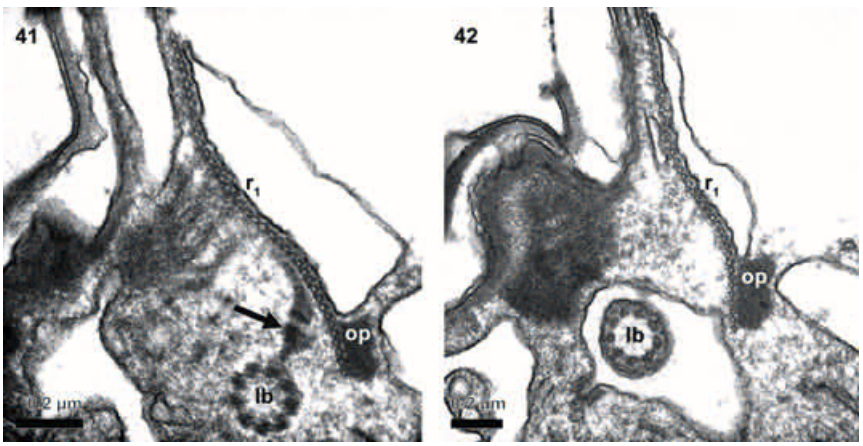


Fig 37–40. *Biecheleria baltica* sp. nov. 37. Longitudinal section of the cell showing the basal bodies (lb, tb) and the eyespot (e). 38. The eyespot with brick-like material in the cisternae. 39. The basal bodies (lb, tb) and the microtubular strand (msp) associated with opaque vesicles (ov), indicating that *B. baltica* is probably mixotrophic. Arrows indicate the collar around the longitudinal flagellum canal. 40. The microtubular strand (msp) and the opaque vesicles (ov) at higher magnification.



Figs 41,42. *Biecheleria baltica*. Details of the r_1 root, which is lined by an opaque strand (op) along the dorsal edge. The arrow in Figure 41 indicates a connecting fibre between the longitudinal flagellum basal body (lb) and the root microtubules.

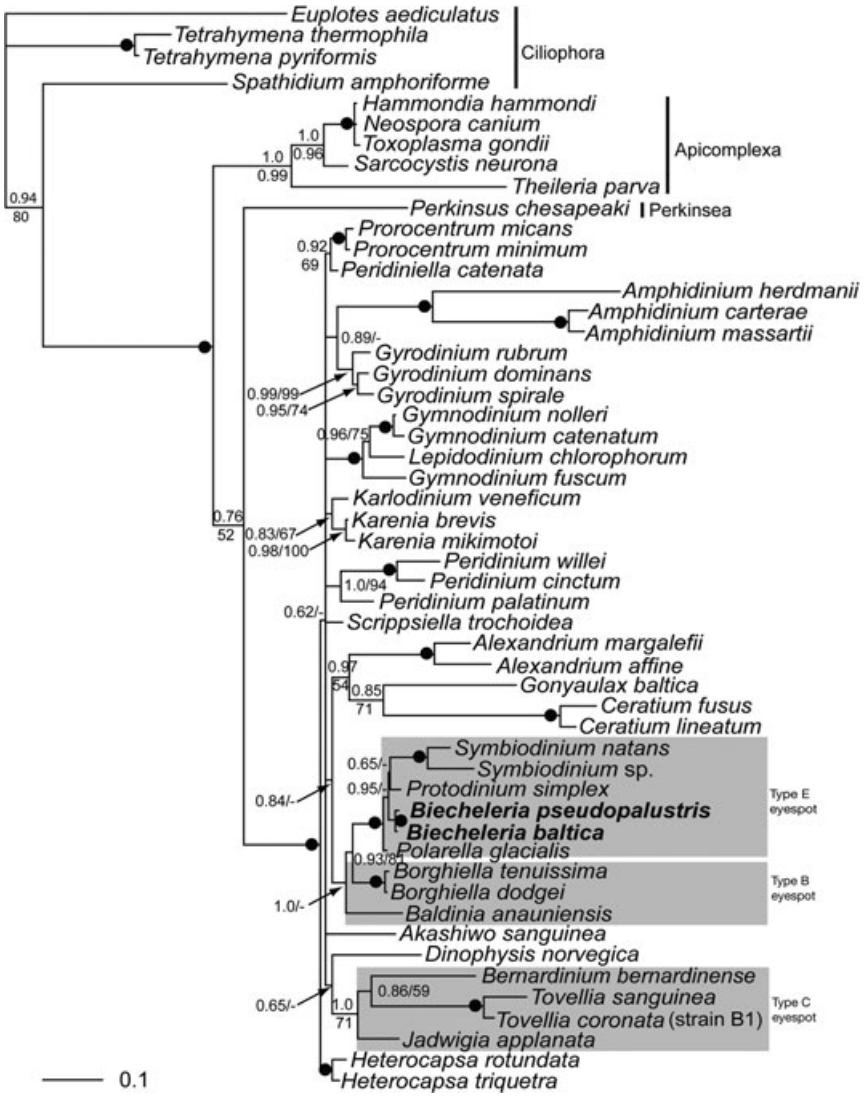


Fig. 43. Phylogeny of *Biecheleria baltica* (syn. *Woloszynskia halophila*) and *Biecheleria pseudopalustris* (syn. *Woloszynskia pseudopalustre sensu* Kremp *et al.)* based on nuclear-encoded large subunit (LSU) rRNA sequences (1152 base pairs, excluding the highly divergent domain D2) and inferred from Bayesian analyses. Branch lengths are proportional to the number of base changes. A diverse assemblage comprising four ciliates, five apicomplexa and *Perkinsus* was used to polarize the dinoflagellate ingroup. Posterior probabilities (≤1.0) and bootstrap values (≥50%) from maximum likelihood (ML) analyses using PhyML are listed at internodes. Numbers written first are posterior probabilities, followed by bootstrap values (100 replications). A filled black circle was used to indicate the highest possible support value in Bayesian Analysis (BA) and ML. The woloszynskioid dinoflagellates have been divided into three groups (grey boxes) based on their type of eyespot *sensu* Moestrup and Daugbjerg (2007).

halophilum Biecheler (1952) and transferred to *Woloszynskia* as *W. halophila*. Though clearly a woloszynskioid, we consider it unlikely that the material studied by Kremp *et al.* (2005) represents Biecheler's organism, due to both ecological and morphological differences.

Ecology: Biecheler's *G. halophilum* is fully marine and grew at temperatures between 7 and 14°C in high salinity water ('eaux trés salées' or 'eaux sur-salées' Biecheler (1952, 33)). We interpret this to mean fully marine. Single individuals were found in warmer and less saline waters, but never in high numbers. The material examined by Kremp *et al.* (2005) forms blooms in the brackish waters of the Baltic Sea. Growth experiments showed a wide salinity tolerance (6.5–30 psu), with optimal growth at salinities between 6 and 20 psu, at temperatures of 2–4°C (Kremp *et al.* 2005, as *W. halophila*). The two organisms thus occupy different ecological niches, both regarding salinity and temperature.

Morphology: Biecheler's *Gymnodinium halophilum* (reprinted here as Fig. 44) is covered with small amphiesmal vesicles in an apparently very irregular pattern. It is difficult to distinguish horizontal rows outside the cingular area. Biecheler illustrates (fig. X2, p. 34, reprinted here as Fig. 44b) approximately 17 rows of plates between apex and antapex. Eight rows of plates are present between apex and cingulum, two rows in the cingulum and at least seven rows on the hypocone. More importantly, Biecheler shows an EAV bordered on each side by at least five to six vesicles (fig. XIV, p. 36, reprinted here as Fig. 44c), as in *B. pseudopalustris* (length not indicated). In the Baltic material the periplast comprises fewer amphiesmal vesicles, arranged more regularly, typically five (to six) epiconal, two cingular and four hyposomal horizontal series, in some cases only three series of very large plates on the hypocone (Kremp *et al.* 2005, fig. 2C). The EAV is less than 2 μm long and lined by two (to three) narrow plates on each

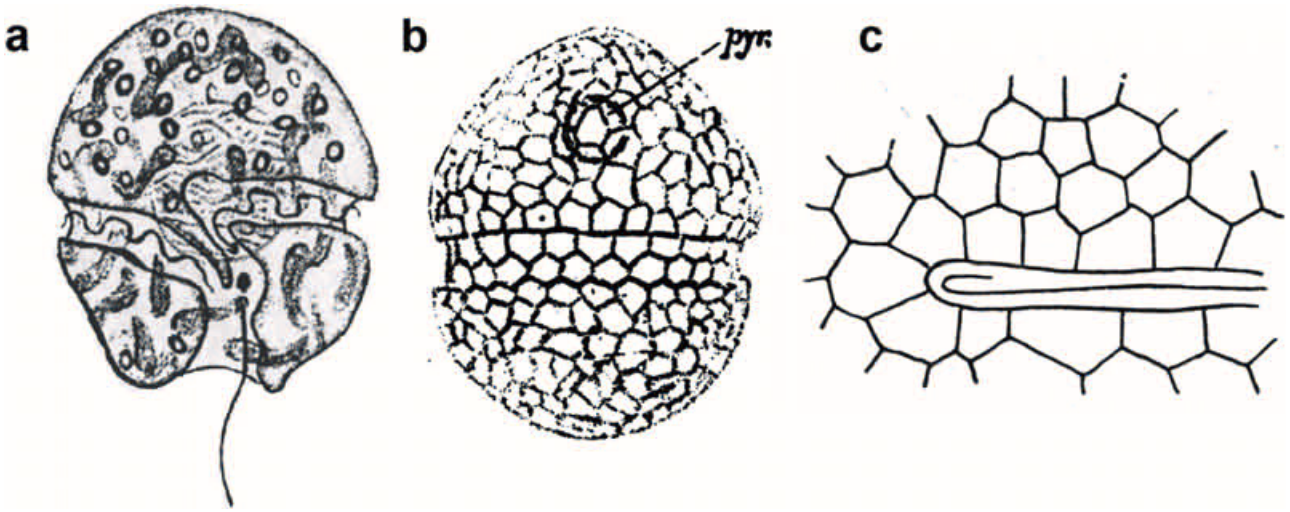


Fig. 44. (a,b) *Gymnodinium halophilum*, reprinted from Biecheler (1952), figures X1,X2, respectively. (c) *Gymnodinium halophilum*, acrobase, reprinted from Biecheler (1952), figure XIV.

side, with a smaller plate at the ventral end (Kremp *et al.* 2005, fig. 2G–I).

The number of amphiesmal plates varies somewhat between different individuals of woloszynskioids but it is unlikely that the number can vary to the extent mentioned above, and we conclude that these belong to different species.

Also, we have found the construction of the EAV complex to be very conservative. In some species it is short and bordered on each side by one to two (rarely three) elongate vesicles with a smaller vesicle at the ventral end as in *B. pseudopalustris,* the Baltic isolate and in *Symbiodinium* (Loeblich III & Sherley 1979; Hansen & Daugbjerg 2009). In other species the EAV is longer, bordered on each side by several (five or more) elongate vesicles as in Biecheler's drawing of *Gymnodinium halophilum* (reprinted in Fig. 44c).

It is not likely that the two different types occur within the same species. Finally, Biecheler described *G. halophilum* as having yellow-green ('jaune-vert') chloroplasts, whereas the Baltic isolate has golden-brown chloroplasts. The differences warrant the description of the organism studied by Kremp *et al.* (2005) as a separate, new species, *Biecheleria baltica* sp. nov.

Material corresponding to Biecheler's (1952) description has not been found since the original description. However, Biecheler's drawings of the cell (including the finger-like ventral ridge: Fig. 44a) and the EAV (Fig. 44c) justify transfer of the species to *Biecheleria* and we include an emended description of this species:

Biecheleria halophila (Biecheler) Moestrup, Lindberg et Daugbjerg. comb. nov.

Basionym: *Gymnodinium halophilum* Biecheler (1952, 33, figs X–XIV).

non *Woloszynskia halophila* Kremp *et al.* (2005, 632, figs 1–4).

Cells ellipsoidal or globular, the epi- and the hypocone of the same size or the epicone slightly larger, rarely the epicone smaller than the hypocone. A long finger-like projection extends from the right hand side of the epicone at the junction of the cingulum and the sulcus. Cells 10–27 μm long and 13–22 μm wide. Nucleus central, dorsal. Cells covered by thin plates in approximately 17 latitudinal rows, approximately eight in the epicone, two in the cingulum and approximately seven in the hypocone. The EAV is bordered on each side by five to six plates. A cold-water species typically present in full-strength seawater at 7–14°C.

Related species

Gymnodinium acuminatum

Gymnodinium acuminatum was described by Christen (1959) from several water bodies in the Lake Hausersee area in Switzerland. It has approximately the same size as *Biecheleria pseudopalustris* (27–30 μm long and 30–34 μm wide), but the epicone was described as almost triangular (Fig. 10). The cells contained a red carotenoid globule in the posterior part of the sulcus (Fig. 10), but a true stigma was said to be absent. Cells were lined by a hyaline, delicate but conspicuous membrane without visible structure. This species differs from *B. pseudopalustris* mainly in the shape of the epicone. However, in our culture of *B. pseudopalustris* we saw both cells with rounded and a more acuminate epicone, and an example of the latter is illustrated in Figure 8. Figure 9 is a cell of *Gymnodinium acuminatum* 'nach längeren Stehenlassen' (Christen 1959, 75). Christen also mentions that the acuminate shape of the

epicone often disappears after centrifugation, and that it greatly resembles Nygaard's *G. excavatum*. However, he lacked material to determine whether the two taxa were conspecific. It should be noted that Christen's description of the new species is not legitimate, since no type was indicated. In any case, we believe his material to belong to *B. pseudopalustris.*

Biecheleria natalensis (Horiguchi et Pienaar) Moestrup comb. nov.
Basionym: *Gymnodinium natalense* Horiguchi and Pienaar (1994, 22, figs 1–13).

This interesting species was described to form dense blooms in a tide pool on the Natal coast of Southern Africa (Horiguchi & Pienaar 1994). It is the first dinoflagellate in which the Type E eyespot was described (*sensu* Moestrup & Daugbjerg 2007). Scrutiny of the SEM provided by Horiguchi and Pienaar (their fig. 3) shows an apical furrow apparatus to be present. The structure of the pyrenoid differs from that found in *B. baltica* and *B. pseudopalustris*. The species does not belong in *Gymnodinium* as the genus was defined by Daugbjerg *et al.* (2000), *Gymnodinium* has a different type of apical furrow apparatus, and the pores in the nuclear envelope are restricted to special nuclear chambers. Horiguchi and Pienaar's figure 5 illustrates a very well-preserved cell, in which the nuclear pores are not associated with nuclear chambers. The special type of eyespot and the apical furrow justifies transfer to *Biecheleria*.

Phylogeny

The unique eyespot comprising a stack of Golgi body-like cisternae with brick-like contents characterizes *Biecheleria pseudopalustris*, *B. baltica*, *B. natalense, Polarella glacialis* and *Symbiodinium natans* (Hansen & Daugbjerg 2009). The sister group to this clade (Fig. 43), the *Borghiella* and *Baldinia* group, is characterized by an eyespot of carotenoid globules located within a chloroplast. The eyespot in these species is accompanied by brick-like material located in cisternae on the chloroplast surface (type B *sensu* Moestrup & Daugbjerg 2007). These features are so marked as to warrant separation at the family level. The second potentially important phylogenetic character is the structure of the apical furrow apparatus, which shows a characteristic variation. In *Baldinia* and *Polarella* an apical furrow seems to be absent, while in *Borghiella* it comprises a pair of parallel elongate, very narrow vesicles, one with poroids. In *Biecheleria* there is a single elongate, very narrow vesicle (EAV), lined on each side by either one to two (rarely three) narrow vesicles, with a smaller plate ventrally or, as in *B. pseudopalustris* and *B. halophila*, five or more narrow vesicles on each side of the EAV.

Symbiodinium is similar to the former group. Loeblich III and Sherley (1979) illustrated an elongate vesicle, surrounded on one side by two, on the other side by one large amphiesmal vesicle (loc. cit., pl. 4C), and Hansen and Daugbjerg (2009) have shown a similar feature in *S. natans*, including the small ventral plate. A third feature, which our recent studies have indicated to be phylogenetically important, is the structure of the resting cyst. Cysts of *Borghiella* and *Baldinia* are smooth, while those of *Biecheleria* are covered with spines of various lengths depending on the maturity of the cyst. Cysts of *Polarella* are covered with very heavy spines, while cysts appear to be unknown in *Symbiodinium*.

The chloroplast pigments of *Biecheleria* have not been examined (see Moestrup & Daugbjerg 2007 for a discussion of the eight different types of chloroplast presently known in dinoflagellates).

The available data are best expressed by considering the species discussed in this article as belonging to two families, which may be circumscribed as follows:

Family 1. Borghiellaceae fam. nov. Moestrup, Lindberg et Daugbjerg
Dinophyta stigma typi B *sensu* Moestrup et Daugbjerg 2007 habentia, intraplastidica, et in facie chloroplasti materia latterata quae in cisternis locata est, concommitata. Par angustum vesicularum amphiesmatis elongatarum parallelarum (*Borghiella*) aut pare absente (*Baldinia*). Stadium principale cycli vitae monadoideum. Cystae quiescentes laeves, sphaericae aut ovoideae in *Borghiella*, in *Baldinia* irregulariter cum invaginatione axiali elongatae.

Cum duo generibus *Borghiella* et *Baldinia*.

Dinoflagellates with eyespot of Type B *sensu* Moestrup and Daugbjerg (2007), that is, intraplastidic and accompanied on the chloroplast surface by brick-like material located in cisternae. Apical furrow apparatus comprising two parallel, elongate vesicles (*Borghiella*) or furrow system absent (*Baldinia*). Principal stage in the life cycle monadoid. Cysts smooth with a clear wall, spherical to oval in *Borghiella*, more irregularly elongate with an axial invagination in *Baldinia*.

With two genera: *Borghiella* and *Baldinia*.

Borghiella presently comprises two species: *B. dodgei* Moestrup, G. Hansen et Daugbjerg, and *B. tenuissima* (Lauterborn) Moestrup, G. Hansen et Daugbjerg.

Baldinia presently comprises only one species: *B. anauniensis* G. Hansen and Daugbjerg, However, the species described as *Glenodinium bernardinense* Chodat and Zender is very similar and undoubtedly belongs in the same genus. We therefore transfer it here as *Baldinia bernardinensis* (Chodat et Zender) **comb. nov.** (Basionym: *Glenodinium bernardinense* Chodat et Zender *in* Chodat (1924), Bull. Soc. Biol. Genève 15, p. 37).

Table 2. Selected morphological features of the three families Tovelliaceae, Borghiellaceae and Suessiaceae

	Group 1	Group 2	Group 3
Families Genera	Tovelliaceae *Tovellia*, *Jadwigia*, *Bernardinium* (*Esoptrodinium*)	Borghiellaceae *Borghiella*, *Baldinia*	Suessiaceae *Symbiodinium*, *Polarella*, *Protodinium Biecheleria*
Eyespot type† (if present)	Type C	Type B	Type E
Apical 'furrow'	Apical line of narrow plates (ALP) or vesicles	Pair of elongate anterior vesicles (PEV) (absent in *Baldinia*)	Single elongate amphiesmal vesicle (EAV) (absent in *Polarella*)
Horizontal rows of vesicles in cingulum	1+ (*Tovellia*, *Jadwigia*)	2+ (*Borghiella*) 1+ (*Baldinia*)	1+ (2?) (*Symbiodinium*) 2+ (*Polarella*) 3 (?) *Protodinium* 2+, 3–4 (*Biecheleria*)
Cysts (when known)	Two bipolar horns and central constriction (*Tovellia*); Smooth, rounded (*Jadwigia*, *Bernardinium*)	Smooth; Spherical-ovoid (*Borghiella*); Inflated, excavate posteriorly (*Baldinia*)	With spines (*Polarella*, *Biecheleria*)

†sensu Moestrup and Daugbjerg (2007). The number of horizontal rows of amphiesmal plates in the cingulum cannot always be readily determined as the first postcingular plate often extends over the posterior rim of the cingulum and into the cingulum proper. This is indicated with a +.

Family 2. Suessiaceae Fensome *et al. (1993) emend.*

Eyespot, if present, of type E *sensu* Moestrup and Daugbjerg (2007), that is, a stack of cisternae containing brick-like material. Apical furrow apparatus comprising a single elongate very narrow vesicle (EAV) surrounded by usually one to two narrow amphiesmal vesicles on each side and a smaller vesicle at the ventral end. In *B. pseudopalustris* and *B. halophila* the EAV is surrounded by a row of several narrow vesicles at each side.

Amphiesmal vesicles typically in 7–15 latitudinal series or more irregular, in *B. pseudopalustris* with more than 20 latitudinal series.

The principal stage of the life cycle monadoid or coccoid.

With spine-bearing resting cysts, in *Polarella* with paracingulum. Resting cysts unknown in *Symbiodinium.*

Extant genera: *Biecheleria* with four species: *B. pseudopalustris*, *B. halophila, B. baltica* and *B. natalensis*; *Polarella* with one species, *P. glacialis* Montresor *et al.* (1999), species concept in *Symbiodinium* somewhat uncertain.

Comment: *Symbiodinium* was classified as a separate family by Fensome *et al.* (1993) based on its main occurrence as endosymbionts in corals, jellyfish, etc. and the predominantly coccoid life stage. However the few comprehensive studies of *Symbiodinium*, which include ultrastructure and morphology (Loeblich III & Sherley 1979, Hansen & Daugbjerg 2009) have shown a flagellate stage with an eyespot of type E, and an EAV similar to some *Biecheleria*. A phylogenetic relationship between *Biecheleria* and *Symbiodinium* is further supported by the molecular studies and we therefore suggest including *Symbiodinium* with *Biecheleria* in the Suessiaceae. *Protodinium simplex* was described by Lohmann (1908) but transferred to *Gymnodinium* by Kofoid and Swezy (1921). In the phylogenetic tree (Fig. 43), the isolate CCAP 418 groups in the Suessiaceae and therefore does not belong in *Gymnodinium*. Dodge (1974) gave a brief account of its ultrastructure but there is no information about apical structure and eyespot (an eyespot was not reported by Lohmann).

Morphological features of the three families Tovelliaceae (Lindberg *et al.* 2005), Borghiellaceae (this study) and Suessiaceae (Fensome *et al.* 1993) have been assembled in Table 2.

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