Baldinia anauniensis gen. et sp. nov.: a 'new' dinoflagellate from Lake Tovel, N. Italy

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The green form of '*Glenodinium sanguineum* Marchesoni' *sensu* Baldi was investigated in detail by light microscopy, scanning electron microscopy, transmission electron microscopy and by analysis of the SSU- and LSU rDNA genes. These data demonstrated it to be different from the red form of '*G. sanguineum*' (now *Tovellia sanguinea*) and to represent a new species, *Baldinia anauniensis* gen. et sp. nov. It lacked thecal plates and an apical groove, and was characterised by the presence of a peculiar lamellar body similar to that described from certain dinoflagellates harbouring a diatom endosymbiont. However, *B. anauniensis* had a typical dinoflagellate chloroplast, and pigment analysis by high-performance liquid chromatography revealed peridinin as the major carotenoid pigment. Phylogenies based on either SSU or LSU rDNA revealed a relationship to suessialoid and woloszynskioid lineages rather than to the diatom-containing species, though morphological support for this relationship could only be argued by the absence of trichcyst and to some extent the intraplastidial eyespot type. A ventral fibre attached to the right side of the longitudinal basal body, an arrangement not observed in any other dinoflagellate before. Numerous food vacuoles with cryptophyte remnants demonstrated that *B. anauniensis* was mixotrophic.

KEY WORDS: Baldinia anauniensis, Dinoflagellates, Kryptoperidinium foliaceum, LSU rDNA, Phylogeny, Ultrastructure

INTRODUCTION

Lake Tovel, situated in the northern part of the Brenta Dolomites, Italy, is well known for its reoccurring reddening caused by blooms of the dinoflagellate 'Glenodinium sanguineum'; or rather was - as the phenomenon has essentially been absent since 1964. The causes for the disappearance of this tourist attraction have since been studied extensively (see Cavalca et al. 2001; Corradini et al. 2001, for reviews). Field studies made by Baldi (1938, 1941) represent an important reference work of the phenomenon. Particularly, the 1941 paper describes in details the 'polymorphism' of 'G. sanguineum'. According to Baldi 'G. sanguineum' occurred in two different but interchangeable forms, a red and green, respectively. The green form has been the dominating dinoflagellate in the lake during summer since 1964, but has apparently lost its ability to transform into the red form, by some ascribed to as lack of carotenogenesis (e.g. Cavalca et al. 2001). Recently, it has become evident that Baldi's green form encompasses a separate species different from 'G. sanguineum' (Flaim et al. 2004). Also, the red form, once considered to be synonymous with Woloszynskia coronata (Wolosz.) R.H. Thompson (Popovský & Pfiester 1990), has recently been shown to be a separate species, albeit closely related, and is now placed in the newly erected genus Tovellia (viz. T. sanguinea) (Lindberg et al. 2005, Moestrup et al. 2006). However, Baldi's green form has never been examined in detail using modern techniques. In this paper we demonstrate that the green form sensu Baldi is a new species, which we name Baldinia anauniensis gen. et sp. nov.

MATERIAL AND METHODS

Cells of the green form used in this study originated from field material collected in the 'Red Bay', Lake Tovel, 29 July, 30 July, 7 August 2003 and 6 July 2004. A clonal culture (strain greenGS clonal) was established from a sample collected 29 July 2003 and grown in a mixture of equal amounts of sterile filtered lake water, DYIV medium and 2xL16 (Andersen *et al.* 1997; Lindstrøm 1991), at 15°C, an illumination of *c*. 50 µmol m⁻² s⁻¹ and a 12:12 h L:D cycle.

The resting cyst (Fig. 9) originated from a sediment sample collected by G. Flaim during winter 2003.

Light microscopy (LM)

LM of live cells was made using either a Leitz Diaplan using $\times 63$ dry lens or an Olympus Provis AX 70 using a $\times 60$ oil immersion lens. Microphotography was made using either Leica DFC300FX or Zeiss Axiocam HR digital cameras.

Scanning electron microscopy (SEM)

Fixation for SEM was carried out by mixing one volume of sample with one volume of a fixation cocktail consisting of three volumes of 2% OsO₄ and one volume of saturated HgCl₂, a slightly modified version of Párducz's (1967) schedule. The field sample was fixed on location for about 4 h, before being brought back to the lab and concentrated onto an Isopore polycarbonate filter (Millipore) of 5 µm pore size. The clonal culture was only fixed for 15 min. and placed onto a poly-L-lysine (Sigma)-coated circular coverslip. After washing in dH₂O for 1 h, samples were dehydrated in an ethanol series: 30%, 50% for 10 min in each change. In 70% dehydration the field sample was stopped, and it was stored at 4°C

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Figs 1-9. Baldinia anauniensis, Light microscopy.

Figs 1–3. Field material. Note eyespot (e), part of the nucleus (N), orange food vacuoles and accumulation bodies (arrowhead). **Figs 4–8.** Clonal culture. Note radiating chloroplast, pyrenoid (py) and accumulation body (arrowhead).

Fig. 7. Cingular border pointing to the left (arrow).

Fig. 8. Temporary cyst with accumulation body (arrowhead). Fig. 9. Resting cyst from field sample, with outer wall (arrow) and inner wall (arrowhead). Note red accumulation body.

for about 1 mo, the clonal culture only overnight. Then dehydration was completed in 96% and 99.9%, 10 min in each change and finally in two changes of 100% ethanol, 30 min in each change. Critical-point drying was made using a BAL-TEC CPD-030. The filters or coverslips were mounted on stubs and coated with palladium–platinum, and examined using a JEOL JSM-6335F field emission scanning electron microscope.

The SEM stubs of the field material (Figs 11–13) and clonal culture (Fig. 10) have been deposited at the Botanical Museum, University of Copenhagen, under the accession no. CA59251 and CA59262, respectively.

Transmisson electron microscopy (TEM)

Several fixation schedules were applied but photographs used in the present paper originate from three slightly different schedules:

• Schedule 1. The clonal culture was initially fixed in a mixture of 1% glutaraldehyde and 0.02% OsO_4 in 0.1 M phosphate buffer, pH 7.5 (final concentrations) for 20 min; it was pelleted by centrifugation and washed in buffer for 5 min. Postfixation was in 1% OsO_4 in 0.15 M buffer for 1 h. After a brief rinse in buffer, the material was dehydrated in a graded alcohol series and embedded in Spurr's resin via propylene oxide, as described in Hansen (1989). This schedule provided the best preservation and most of the photographs originate from this fixation (Figs 16–26, 31, 32, 35–43, 45–58). The resin block has been been deposited at the Botanical Museum, University of Copenhagen, under the accession no. CAT2384. • Schedule 2. The clonal culture was initially fixed in 2% glutaraldehyde and 0.25% OsO₄ in 0.05 M phosphate buffer pH 7.5 (final concentrations) for 20 min; it was pelleted by centrifugation and washed in buffer for 5 min. Postfixation was in 2% OsO₄ in 0.1 M buffer for 1 h. Dehydration and embedding follow schedule 1. It was used for Fig. 44 and the resin block has been deposited at the Botanical Museum, University of Copenhagen, under the accession no. CA59254.

• Schedule 3. A field sample collected 29 July 2003 was brought back to the laboratory at Istituto Agrario and fixed in 2% glutaraldehyde and 0.5% OsO₄ in 0.1 M phosphate buffer, pH 7.5 (final concentrations) for 1 h; it was pelleted by centrifugation and washed in buffer for 5 min. Postfixation was in 1% OsO₄ in 0.1 M buffer for 1 h. Dehydration was partly as in schedule 1, but instead of finishing with two changes of propylene oxide, two changes of 100% acetone were used, 15 min in each change. Embedding in Spurr's resin was via acetone. This schedule was used for Figs 27–30, 33 and 34 and the resin block has been deposited at the Botanical Museum, University of Copenhagen, under the accession no. CA59253.

The material was sectioned on a LKB 2088 Ultrotome V ultramicrotome using a diamond knife and the sections were collected on a slot grid and placed on a Formvar film. After staining in uranyl acetate and lead citrate, sections were examined in a JEOL JEM-1010 electron microscope operated at 80 kV. Photographs were taken using a GATAN 792 digital camera. Three-dimensional (3D) reconstruction of the lamellar body and flagellar apparatus was made with MacStereology software (Ranfurly Microsystem).

Pigments

Thirty millilitres of the clonal culture were gently filtered through 25-mm Advantec GF 75 glass fibre filters (Toyo Roshi Kaisha, Japan), which were immediately rolled up in cryotubes and placed in liquid nitrogen. Filters were subsequently transferred to 2.5 ml of methanol, sonicated for 30 s, and filtered (0.2 μ m pore size). One millilitre of this filtrate was mixed with 250 μ l of water immediately before analysis. High-performance liquid chromatography (HPLC) analyses were performed on a Shimadzu LC 10A system with a Supelcosil C18 column (250 × 4.6 mm, 5 μ m) using the method of Wright *et al.* (1991). Pigments were identified by retention times and absorption spectra identical to those of authentic standards, and quantified against standards purchased from DHI Water & Environment, Hørsholm, Denmark.

DNA extraction and cysts of Baldinia

Live cells (10 ml) of *B. anauniensis* gen. et sp. nov. and *Kryptoperidinium foliaceum* [strain K-0638 from the Scandinavia Culture Centre for Algae and Protozoa but originally isolated from a water sample collected by Yolanda Pazos (Centro de Control da Calidade do Medio Mariño, Spain)] were harvested by centrifugation at 2500 rpm for 10 min. The resulting pellets were transferred to 1.5-ml Eppendorf tubes and stored at -20° C for at least 5 days until extraction of total genomic DNA. DNA was extracted using the cetyltrimethylammoniumbromide (CTAB) method but with a few modifications as outlined in Hansen *et al.* (2003).

Cysts from B. anauniensis were isolated from a Lake To-



Figs 10–13. Baldinia anauniensis, SEM.

- Fig. 10. Ventral view of a cell with smooth surface and intact flagella. Transverse flagellum (tf), longitudinal flagellum (lf).
- Fig. 11. Cell with partly broken flagella, but visible amphiesmal pattern. Putative ventral ridge (arrowheads). Cingulum (c).
- Fig. 12. Apical view of a cell. No apical structure is present.

Fig. 13. Cell seen at higher magnification. The amphiesmal vesicles are penetrated by pores (arrowheads). The cingulum (c) contains only one row of amphiesmal vesicles.

vel sediment sample using a capillary pipette. Under a dissecting microscope single cysts were fractured with a sterile needle and transferred to 0.5-ml Eppendorf tubes. Further processing of the cyst samples is described in Moestrup et al. (2006).

Polymerase chain reaction (PCR) amplification and sequence determination of SSU and LSU rDNA

To infer the phylogeny of *B. anauniensis* gen. et sp. nov. and *K. foliaceum* we amplified the small- and large-subunit ribo-



Fig. 14. Diagrammatic drawing of *Baldinia anauniensis*, based mainly on LM and SEM observations.

somal DNA genes of *B. anauniensis* and large subunit (LSU) of *K. foliaceum.* PCR fragments of the small subunit (SSU) rDNA gene from *B. anauniensis* were obtained using forward primer ND1 in combination with reverse primer ND6. For SSU rDNA sequence determination, we used the terminal primers ND1 and ND6 and the internal primers ND2, ND3, ND7 and ND8. PCR fragments of the LSU rDNA gene from *B. anauniensis* (flagellate and cyst stage, respectivley) were obtained using forward primer D1F and reverse primer ND28-1483R. For LSU rDNA sequence determination, we used terminal primers D1F and ND28-1483R and internal primers D3A, D2Cr and D3B.

In terms of genetic compartments, *K. foliaceum* represents a chimera with its diatom endosymbiont still containing the nucleus, chloroplasts and mitochondria (Jeffrey & Vesk 1976, Chesnik et al. 1997). Hence, this dinoflagellate has five separate sets of genomic DNA, two of which contain their own copy of the nuclear-encoded LSU rDNA gene. This represents a challenge to molecular systematists because the use of eu-



Fig. 15. HPLC chromatogram of the major pigments.

karyotic-specific primers designed to amplify the ribosomal LSU rDNA gene (or SSU rDNA) would result in amplification of the gene copy in both the dinoflagellate and diatom. Two approaches have been suggested to overcome this problem. One involves fine mechanical separation of the dinoflagellate nucleus from the cell under the microscope using a capillary pipette. The isolated dinoflagellate nucleus, easily identifiable because of the permanently condensed chromosomes, is then used in PCR amplification of nuclear-encoded ribosomal genes (Horiguchi & Takano 2006). The alternative approach involves the design of a dinoflagellate-specific ribosomal primer. Here we used the latter approach (see Hansen & Daugbjerg 2004). However, this approach has one pitfall when applied to environmental samples, as heterotrophic dinoflagellates might have preyed on other dinoflagellates, thus resulting in an additional ribosomal sequence.

A comparison of the K. foliaceum LSU rDNA sequence determined in this study using the dinoflagellate-specific primer with the K. foliaceum LSU rDNA sequence available in GenBank (accession number AY916545) shows a sequence divergence of 29.6% using uncorrected "p" in PAUP* and including introduced gaps. Population genetic differences cannot explain such a high sequence divergence. When performing a nucleotide-nucleotide BLAST (blastn) available at National Center for Biotechnology Information's (NCBI) website, the K. foliaceum (strain CS291, AY916545) sequence groups with the pennate diatoms Nitzschia, Cylindrotheca and Pseudonitzschia. It is therefore likely that the LSU rDNA gene of the endosymbiont was determined instead of that originating from the dinoflagellate. This example highlights potential problems with chimeras such as the dinoflagellates harbouring a eukaryote symbiont, if careful consideration of this complex molecular situation is not met.

Table 1 lists all primers (and their sequences) used in this study. PCR amplification conditions and automated sequencing in both directions have been described in Hansen *et al.* (2003) and Hansen & Daugbjerg (2004).

Alignment and phylogenetic analyses

SSU rDNA sequences from *B. anauniensis* gen. et sp. nov. were added to an alignment comprising a diverse assemblage of other dinoflagellates available in GenBank (see Table 2). The alignment was edited by eye using SeaView (Galtier et al. 1996) and comprised 1763 base pairs. The ciliate *Tetra*-



Figs 16-21. Baldinia anauniensis. TEM, general ultrastructure.

Fig. 16. Longitudinal section revealing the central pyrenoid (py) and associated chloroplast (chl), part of the nucleus (N) and peripheral mucocysts (mu). An accumulation body (ac) is located anteriorly. Tubules of the pusule are visible in the cell center (arrow). Vesicles with virus-like particles (arrowheads).

Fig. 17. Same structures as in Fig. 16 but cell seen in cross-view. Notice central cavity of the pyrenoid with tubules of the pusule (arrow). Longitudinal basal body (LB); flagellar root (R1), nucleolus (nu).

Figs 18 & 19. Three membranes (arrow) surround the chloroplast and the pyrenoid, respectively.

Fig. 20. Nuclear envelope with a pore (arrow).

Fig. 21. The amphiesma; plasma membrane (pm), amphiesmal vesicle (av), outer amphiesmal vesicle membrane (ovm), inner amphiesmal vesicle membrane (ivm). Arrowheads indicate at the cortical microtubules.

hymena pyriformis was used to polarize the ingroup of 42 dinoflagellates. The LSU rDNA sequences determined from *B. anauniensis* and *K. foliaceum* were added to an existing alignment previously used to infer the phylogeny of *Esoptro-dinium gemma* (Calado et al. 2006) and incorporated information from the secondary structure as suggested by de Rijk et al. (2000). The two rDNA data sets were analysed using identical approaches for the phylogenetic inference. Maximum parsimony (MP) and neighbor-joining (NJ) analyses were performed using PAUP* ver. 4b10 (Swofford 2003) whereas Bayesian analysis was performed using MrBayes vers. 3.1 (Ronquist & Huelsenbeck 2003). In MP 1000 random additions were done using a heuristic search option and a branch-swapping algorithm (TBR). All characters were unordered, weighted equally and gaps were treated as missing data. In

total, 1000 replications were performed in unweighted parsimony bootstrap analysis. To find the best-fit model for the SSU and LSU rDNA data sets, respectively, we used Modeltest ver. 3.6 (Posada and Crandell 1998). Modeltest suggested the TrN+I+G as the best-fit model for both ribosomal genes. Table 3 shows the parameter settings suggested by Modeltest and used in NJ to compute dissimilarity values. The distance matrices obtained were then used as input to build trees with NJ. One thousand replications were performed in NJ bootstrap analyses with maximum likelihood settings as suggested by Modeltest. For Bayesian analysis the General Time Reversible (GTR) substitution model was invoked with base frequencies and substitution rate matrix estimated from the data matrix. Four simultaneous Monte Carlo Markov chains (MCMC; Yang & Rannala 1997) were run from random trees for a total of 1,000,000 generations (Metropolis-coupled MCMC). A single tree was sampled every 50 generations and the "burn-in" was evaluated for stationarity by examining the plateau in loglikelihood over generations using a combination of Tracer (ver.1.2.1 by A. Rambaut & A. Drummond) and an Excel spreadsheet. In both SSU and LSU rDNA data sets the burnin of the chains occurred in fewer than 8000 generations so the first 160 trees were discarded, leaving 19,840 trees for estimating posterior probabilities (PP). Thus, PP values were obtained from the 50% majority rule consensus of the kept trees.

RESULTS

Description

Baldinia Gert Hansen et Daugbjerg gen. nov.

Cellulae mobiles liberviventes (or non sessiles). Chloroplasti peridinin capientes et membranis tribus circumcincti. Stigma intra chloroplastum corperibus lateriformis inter stigma et R1 radicem flagelli situm. Fibra ventralis corpore basali longitudinalique consociata. Corpus lamellare praesens. Sulcus apicalis et catilli thecae absentes.

Cells motile and free-living. Peridinin-containing chloroplasts surrounded by three membranes. Intraplastidial eyespot with bricklike bodies situated between the eyespot and the R1 flagellar root. Ventral fibre associated with the longitudinal basal body. Lamellar body present. Apical groove and thecal plates absent.

ETYMOLOGY: *Baldinia* refers to E. Baldi, who initially described it as the green form of '*Glenodinium sanguineum*'.

TYPE SPECIES: Baldinia anauniensis to be described below.

Baldinia anauniensis Gert Hansen et Daugbjerg sp. nov. Non-Glenodinium sanguineum sensu Baldi (1941) aut sensu Dodge et al. (1987)

Cellulae ovoideae et episomate et hyposomate hemisphaerico. 23– 28 μ m longae et 18–24 μ m latae. Cingulum medianum et cinguli unius latitudine dispositum. Sulcus angustus antapicem non attingens. Vesiculae amphiesmales numerosaeque praesentes. Chloroplastus unicus flavovirensque et ex pyrenoide centrali radians. Nucleus U-formis in centro cellulae. Collum longitudinale striatumque deminutum. Pusula tubularis in cavitate pyrenoidis sita. Trichocystae absentes. Pedunculus praesens. Cystae quiescentes pariete externo laevique inflatoque. Cellula circa 35 μ m longa et 28 μ m lata. Pars apicalis truncata pars antapicalis indentata. Paries interior tenuis et arte adpressus ad partem cytoplasmicam rotundatamque.

Cells ovoid with hemispherical epi- and hyposome. Length 23–28 μ m; width 18–24 μ m. Cingulum median and displaced one cingular width. Sulcus narrow, not reaching the antapex. Numerous amphiesmal vesicles present. One yellow-greenish chloroplast radiating from a central pyrenoid complex. U-shaped nucleus in the centre of the cell. Longitudinal striated collar reduced. Tubular pusule situated in pyrenoid cavity. Trichocysts absent. Peduncle present.

Resting cysts with smooth and inflated outer wall. Length c. 35 μ m; width c. 28 μ m. The apical part truncated, the antapical part indented. Inner wall thin and closely appressed to the rounded cytoplasmic part.

HOLOTYPUS: A resin block of the clonal culture established from material collected 29 July 2003 has been deposited at The Botanical Museum, University of Copenhagen, accession no. CAT2384.

Fig. 14 has been chosen to represent the type in accordance to fulfil article 39.1 of the International Code of Botanical Nomenclature (ICBN).

TYPE LOCALITY: Lake Tovel.

ETYMOLOGY: *anauniensis* refer to Anaunia used in the past for Val the Non, the valley where Lake Tovel is situated.

LM and SEM

Although cells of B. anauniensis typically measured 24-26 μ m in length, cells as small as c. 15 μ m and up to 34 μ m were occasionally observed (not shown). The length: width ratio was around 1.2. The cell was ovoid with a hemispherical epi- and hyposoma, though the episome might be somewhat conical and the antapex slightly truncated (Figs 1-3, 10, 11). The cingulum was displaced one cingular width, and the cingular border, demarcating the beginning of the cingulum, was clearly deflected to the left (Figs 7, 10-12). The sulcus was narrow and short, not reaching the antapex (Figs 10, 11, 14). The cell was only slightly dorsoventrally flattened (Fig. 12). The cell surface, in 'normally' prepared cells for SEM, was smooth, and penetrated by numerous scattered pores; however, prolonged dehydration revealed the pattern of the amphiesmal vesicles, consisting of about 100 vesicles (Figs 11, 12). Only one row of vesicles was present within the cingulum (Fig. 13). Up to five pores might be present within a single amphiesmal vesicle. These pores might be the exit points for vesicles, with dense contents, usually termed mucocysts (see later). An apical groove, usually present in unarmored dinoflagellates, was absent, irrespective of the preparation methods used for SEM (Figs 10, 12). The chloroplast was yellowish or greenish and radiated from a centrally located pyrenoid (Figs 1-6, 14), though the pyrenoid might be difficult to see in LM. The chloroplast was often restricted toward the central part of the cell, leaving a hyaline border around the cell (Figs 1, 2). This was particularly evident in field material. A red eyespot measuring c. 0.35 µm in length and c. 0.25 µm in width was situated within the sulcus (Fig. 1). The eyespot might sometimes be obscured in cells with orange accumulation bodies or in cells having a well-developed chloroplast (Figs 2, 4). The former might represent food vacuoles, as vacuoles with cryptophyte remnants were observed in TEM (see later). The nucleus was situated in the middorsal part of the cell. It was wider than long and U-shaped, as evidenced by the presence of one lobe in the left part of the cell in Fig. 2 and the right part of the cells in Figs 4 and 5.

Cells were relatively fast swimming and very delicate, making LM observation tedious; furthermore, cells readily transformed into temporary cysts (Fig. 8). Cells were mixotrophic as revealed by the presence of food vacuoles within the cell from field samples, but the food uptake behaviour was never seen either in field or cultured material.

The resting cyst had a distinctly shaped outer cyst wall, with an antapical indentation. The cyst most likely represented the result of sexual reproduction (see Flaim *et al.* 2004). Resting cysts were never observed in the clonal culture but occurred in a nonclonal culture, indicating that *B. anauniensis* is heterothallic.

Pigment profile

Pigment analysis by HPLC revealed the presence of peridinin as the major carotenoid (Fig. 15). Additional pigments identified in *B. anauniensis* were chlorophyll a and c. The different types of chlorophyll c could not be separated in the present



HPLC method. The accessory pigments dinoxanthin and diatoxanthin were also detected.

TEM

GENERAL ULTRASTRUCTURE: *Baldinia anauniensis* had a complement of typical dinoflagellate organelles but also some unusual features. The nucleus, or dinokaryon, with dense chromosomes and a nucleolus, was situated in the dorsal/central part of the cell (Figs 16, 17). The dinokaryon had a nuclear envelope penetrated by nuclear pores, similar to other eukaryotes and most other dinoflagellates (Fig. 20). The mitochondria had tubular cristae (e.g. Fig. 24). Trichocysts were absent but numerous large vesicles with a somewhat dense content, normally referred to as mucocysts, were situated along the cell periphery (Figs 16, 17).

The chloroplast consisted of numerous lobes that extended from a centrally located pyrenoid system to the peripheral part of the cell (Figs 16, 17). Both the chloroplast and the pyrenoid were surrounded by three membranes (Figs 18, 19). The pyrenoid delineated a central cavity that opened toward the ventral side of the cell. Tubules of the pusular system extended into this cavity (Figs 16, 17).

The amphiesma consisted of numerous amphiesmal vesicles subtending the plasma membrane (Fig. 21). The vesicles contained indistinct dark staining material that occasionally had a platelike appearance (Fig. 32), but typical thecal plates were absent. A very thin opaque layer was situated underneath the amphiesmal vesicles that might represent the pellicle *sensu* Höhfeld & Melkonian (1992). Microtubules making up the peripheral cytoskeleton were located underneath the amphiesmal vesicles and the opaque layer. They occured at regular intervals in groups of two or three (not shown) but in the cingulum only one by one (Fig. 21).

The pusule originated from a tubular invagination of the cell surface near the exit point of the longitudinal flagellum and extended in a convoluted manner into the aforementioned cavity of the pyrenoid. It was surrounded by three membranes. The inner membrane, which was lined with 'fuzzy' or fibrous material, was continuous with the plasma membrane. The two outer membranes, which extended along and closely associated with the pusular tubule, originated from an amphiesmal vesicle, which made a distinct constriction at the entry point of the pusular tubule (Figs 23, 24). Numerous electron-dense bodies were usually associated with the proximal part of the pusule (Figs 22, 25).

THE EYESPOT: An eyespot was located in the sulcus. It consisted of rows of electron-opaque globules arranged in a single layer within the chloroplast. A large narrow vesicle containing

a single layer of electron-translucent bricklike structures was situated close to the eyespot. The R1 flagellar root and to some extent a large microtubular strand was located in between the cell surface and the brick-containing vesicle (Figs 23, 26). The microtubular strand contained up to 40-50 microtubules, and extended from the posterior part of the sulcus to near or slightly beyond the exit point of the peduncle. Its precise starting and termination points were not determined, but it was always situated to the right of the ventral ridge (see later) (Figs 26, 29, 32, 48, 50). The microtubular strand and the R1 flagellar root formed two close but separate bands and extended along the midventral surface of the cell for a considerable distance before the R1 bent into the cell. It is possible that the two strands merged posteriorly, but this was not followed in detail. A pair of microtubules was located between the two strands (Fig. 26). Their origin was uncertain, but they were followed from the posterior portion of the sulcus and 46 sections toward the anterior part of the cell before vanishing into dense material that marked the starting point of the ventral ridge (not shown).

THE VENTRAL RIDGE: The ventral ridge was a layered structure consisting of two or more dense layers separated by one or more electron-translucent layers. It was located underneath the plasma membrane and spanned the distance between the exit points of the longitudinal and transverse flagellar canals, or rather slightly beyond these (Figs 23, 30-32, 50, 56). It seemed likely that the thickened flange visible in SEM between the cingular ends represented the ventral ridge (Fig. 11, arrowheads). Amphiesmal vesicles were partly absent from the ridge, but dense material from the ridge was associated with the amphiesmal vesicles flanking the ridge. The vesicle situated at the right side of the ridge was usually somewhat hook-shaped, at least in part of its course. In one cell it contained a dark-staining platelike structure, and numerous delicate fibres were located on the outer amphiesmal membrane (Figs 32, 50). The distance between the flanking vesicles was variable (compare Fig. 29, 32 with Fig. 48), indicating that the cell somehow was able to contract and expand the area devoid of amphiesmal vesicles.

Some cells fixed according to schedule 2 had an inflated dense and somewhat globular extension of the ridge, situated near the microtubular strand of the peduncle (see later) (Fig. 44). The origin of this material was not clear.

STRIATED COLLARS: Each of the flagellar canals had a striated collar. The collar of the transverse canal was well developed (Figs 39, 41, 42). An extension of the collar ran along the R4 flagellar root (Figs 41, 42) and a fibre connected it with the ventral ridge (Fig. 56). The collar of the longitudinal canal,

Figs 22-26. Baldinia anauniensis, TEM, pusule and eyespot.

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Fig. 22. Pusule (pu) in transverse section, surrounded by dense bodies (arrowhead).

Fig. 23. The pu invaginates from the flagellar canal. The ventral ridge (vr) and microtubular strand of the peduncle (msp) with associated dense bodies (arrow) are also visible, as are the lamellar body (lab), the eyespot (e) and the brick-containing vesicle (arrowhead). Longitudinal flagellum (lf), with paraxonemal rod (arrow).

Fig. 24. The outer two membranes of the pusule (arrows) originate from an amphiesmal vesicle. 'Fuzzy' material is present on the innermost membrane of the pusule (arrowhead). Mitochondria (mi).

Fig. 25. Longitudinal section of the pusule with associated dense bodies (arrowhead).

Fig. 26. The eyespot in cross-section. The bricklike structures (arrowhead) are located between the eyespot and the microtubular strand (ms) and R1 flagellar root. A pair of microtubules (arrow) separates the ms and R1.



Figs 27–32. Baldinia anauniensis, TEM, food vacuoles, peduncle and the ventral ridge.
Fig. 27. Food vacuole. Trichocysts (arrow) and chloroplast with starch bodies (arrowhead) originate from the prey organism.
Fig. 28. Food vacuole seen at higher magnification revealing numerous cryptophyte trichocysts (arrows) and a mitochondrion with flat cristae (arrowhead).

however, was reduced and only partly surrounded the flagellar canal. This collar was also associated with the ventral ridge via fibrillar material (Fig. 31).

THE PEDUNCLE: Baldinia anauniensis had a microtubular strand (msp) that represented a retracted peduncle. It originated within the cell and terminated in dense material at the tip of the ventral ridge (Figs 23, 29, 44, 48, 56). It was not clear if this material represented the peduncular collar seen in other dinoflagellates, (e.g. Calado et al. 1998) or it was merely the ventral ridge. Numerous electron-dense and somewhat elongated bodies were situated in the vicinity and along the msp, and cells from the field sample often had a cluster of elongated and narrow but less dense bodies terminating together with the msp (Figs 23, 29, 48). Cells with a protruded peduncle (feeding tube) were never observed, but cells from the field sample contained food vacuoles with cryptophyte remnants (Fig. 27). These were identified by the characteristic cryptophyte trichocysts and mitochondria with flat cristae (Fig. 28), as opposed to the tubular cristae of the dinoflagellate, and confirmed that B. anauniensis is mixotrophic.

VIRUS-LIKE PARTICLES: Many cells in the field sample contained virus-like particles. Two different types were observed. One type measured about 130 nm in diameter and had a distinct hexagonal shape (Fig. 33). The other type, only measuring about 60 nm or less, had a less distinct hexagonal shape and was congregated in large membrane-enclosed vesicles (Fig. 34). These vesicles were situated along the cell periphery and could represent some sort of excretory vesicles by means of which the cell excretes virus particles.

LAMELLAR BODY: The lamellar body was one of the most distinct and unusual structures of *B. anauniensis*. It might be regarded as a specialised part of the ER, and occupied a substantial part of the area around the proximal end of the lon-gitudinal basal body (LB) (Figs 23, 35, 36, 50, 51, 55, 56). The membrane cisternae of the ER formed a complex but highly ordered system of hexagonally shaped cavities, measuring about 60 nm in diameter. These contained a central electron-dense structure associated with indistinct fibrillar material (Figs 35, 37). Part of the LB and associated structures were completely surrounded by the lamellar body (Figs. 39, 50, 51).

FLAGELLAR APPARATUS: A diagrammatic 3D reconstruction of the flagellar apparatus (FA) is shown in Fig. 56.

The two basal bodies inserted at an angle of about 155° to each other (Fig. 40). A small connective (bbc) interconnected the two basal bodies (Fig. 46).

The R3 and R4 flagellar roots were attached to the right and left side of the TB, respectively (Figs 41, 42, 45, 47). The R3 (previously TMR) continued along the transverse flagellar canal, where it nucleated numerous microtubules, the trans-



Figs 33 & 34. Baldinia anauniensis, TEM, virus-like particles.
Fig. 33. Virus-like particles (arrow).
Fig. 34. Vesicle containing numerous small virus-like particles (ar-

verse microtubular extension (TMRE) that continued into the more central part of the cell (Figs 45, 48). The R4 (previously TSR+TSRM) consisted of a striated fibre and a single micro-tubule (Figs 42, 43). The R4 continued to the left along the transverse flagellar canal and the cingulum for a considerable distance (Figs 41, 42).

A large multimembered microtubular root, the R1 (previously LMR), was situated to the left of the LB (Figs 35, 38, 39, 50, 51, 53, 54). It consisted of about 30 microtubules and originated at the very proximal part of the LB. It continued in a posterior direction, making a bend toward the cell surface and passed the eyespot (Figs 26, 39, 44). A single microtubular root, the R2 (previously SMR), originated on the right side of the LB and continued posteriorly (Figs 51, 55).

A number of fibrous connectives or fibres were associated with the various roots. Thus, the R1 root was attached to the

row).

Fig. 29. Cell in cross-section. The microtubular strand of the peduncle (msp) is lined by numerous dense bodies (arrows). Narrow club-shaped bodies terminate together with the msp (arrowhead).

Fig. 30. The ventral ridge in longitudinal section.

Fig. 31. The longitudinal flagellum within the flagellar canal, and the incomplete longitudinal striated collar (LSC) associated with the vr. The ms is located on the right of the vr.

Fig. 32. Same cell as in Fig. 31 but 39 sections further toward the anterior part of the cell. The vr consists of alternating dense and translucent layers. The right adjoining amphiesmal vesicle contains platelike material (arrowhead) and has fine fibres (arrows) on its outer membrane.



Figs 35–37. Baldinia anauniensis, TEM, the lamellar body.

Fig. 35. Cross-section of the lamellar body. VF, ventral fibre; DF, dorsal fibre.

Fig. 36. The lamellar body is connected to the endoplasmic reticulum (ER).

Fig. 37. The lamellar body in both longitudinal and cross-view, respectively. Notice the central dense structure with associated 'fuzzy' material (double arrow).

LB by two fibrillar connectives, the LB/R1c₁ and LB/R1c₂, respectively (Figs 53, 54). The LB/R1c₁ was situated slightly posterior to LB/R1c₂ and attached to the two rightmost microtubules of the R1 and one of the LB triplets. It was relatively short and only visible for two consecutive sections, i.e. about 160 nm long. The LB/R1c₂ also attached to one LB triplet but to 10 microtubules or so of the R1 root. It was visible on four consecutive sections, i.e. about 320 nm long. No striation pattern was observed in either of these connectives. The R1 was also attached to the transverse basal body (TB) by a striated connective (R1/TBc) furcating into three

arms, each of which attached to one of the TB triplets (Fig. 47). Its attachment to the R1 was interesting, as it attached on the three rightmost microtubules of the R1, which seemed to be slightly longer than the other R1 microtubules (Fig. 49). Another striated fibre (src) interconnected the R1 and R4 flagellar roots (Fig. 47). A dorsal fibre (DF), with almost the same striation pattern as the striated fibre of R4, was located on the dorsal side of the R1 (Figs 44–46). It was attached to the 5–10 rightmost R1 microtubules (Figs 35, 51, 53, 55). A novel structure, which we term the ventral fibre (VF), associated with the proximal right ventral part of the LB (Figs 35, 51, 53, 55).

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- Fig. 38. Section at the level of the ventral fibre.
- Fig. 39. The transverse striated collar (TSC). Notice also part of the eyespot (e).
- **Fig. 40.** The angle between the LB and TB is obtuse. The LB/R1c₂ (arrowhead) and the LB/R1c₁ (arrow).
- Fig. 41. Extension of the TSC (arrow) close to the R4 flagellar root. The striated root connective (src), collared pit (cp).
- Fig. 42. The R3 root is associated with the TB. The R4 consists of a striated fibre and a single microtubule (arrow). Flagellar canal (FC).
- Fig. 43. Another cell showing the R4, with associated microtubule (arrow), in cross-section.

Figs 38-43. Baldinia anauniensis, TEM. The flagellar apparatus in oblique longitudinal view. The cell is seen from the ventral side and the nonadjacent serial sections are moving from ventral to dorsal. The encircled numbers are section numbers.





Figs 44–47. Baldinia anauniensis, TEM. The flagellar apparatus in longitudinal lateral view. Ventral is to the viewer's left.
Fig. 44. The striation pattern of the dorsal fibre (DF) is very distinct. Notice also the dense extension (arrowhead) of the ventral ridge (arrow).
Figs 45–47. Nonadjacent serial sections of the flagellar apparatus of another cell. The cell is seen from the outside, and the sections are moving from right to left. The encircled numbers are section numbers.

Fig. 45. The proximal parts of the ventral fibre and R4 root attached to the LB and TB, respectively. Notice also flagellar root R3 and its microtubular extension (TMRE). Dense bodies are present near the msp (arrow).

Fig. 46. Section that has been tilted to reveal the delicate connective (bbc) between the basal bodies.

Fig. 47. The connective between the R1 and TB (R1/TBc); notice its three branches (arrowheads). The striated root connective (src) is also visible.

38, 45, 51–54). It continued in a posterior direction and had not previously been observed in any other dinoflagellate. A diminutive extension or connective attached this fibre to the LB (Fig. 52).

Phylogeny based on ribosomal genes

The LSU rDNA sequence of the flagellate and cyst stage of *B. anauniensis* was 100% identical (data not shown), thus pro-

viding a positive link between the cyst stage (Fig. 9) isolated from an environmental sample, and the flagellate stage (Figs 1–7) from the clonal culture. The phylogenetic inference based on SSU rDNA sequence data showed *B. anauniensis* forming a sister taxon to *Polarella, Symbiodinium* and *Gymnodinium simplex* (Fig. 57). However, this relationship was not supported in terms of bootstrap values or posterior probabilities. Similarly, the topology for the deeper branches of the tree received less than 50% bootstrap values or posterior probabilities. There was no support in favor of a relationship between *Baldinia* and *Kryptoperidinium*.

The inference based on LSU rDNA also did not provide good bootstrap support values or posterior probabilities for the branching pattern of the deep nodes (Fig. 58). There was moderate bootstrap support (68% in MP) to strong support from posterior probabilities (100%) for a sister group relationship between Baldinia and the clade comprising Woloszynskia tenuissima, W. pseudopalustris and Polarella glacialis. LSU rDNA favoured an early divergence of K. foliaceum as it formed a sister taxon to all dinoflagellates except the three species belonging to Amphidinium (Fig. 58). Neither the SSU nor the LSU rDNA sequences therefore supported a relationship between B. anauniensis and K. foliaceum. A concatenated analysis combining SSU and LSU rDNA sequences into a single data matrix was not attempted because the two ribosomal gene sequences are not available from all dinoflagellates included in the separate phylogenetic analyses.

DISCUSSION

Identity of the species

Searching the literature for previously described freshwater dinoflagellates did not reveal any obvious candidates being similar to B. anauniensis, except perhaps Glenodinium bernardinense Chodat. This species was also found in a highaltitude alpine lake, Entre-deux Tours, near St. Bernard (Chodat 1923). It has about the same cell size as B. anauniensis and also possesses a radiating chloroplast and a red stigma within the sulcus. In addition, some of the resting stages depicted by Chodat might indeed resemble the resting cyst of B. anauniensis. However, the nucleus was described and illustrated as being eccentric rather than central and the colour of the chloroplasts 'pale brown not yellowish-green' (translated from the Latin diagnosis). However, the most important difference between B. anauniensis and G. bernardinense appears to be the different orientations of the oblique proximal borders of the cingulum; running from anterior right to left in B. anauniensis but from anterior left to right in G. bernardinense. We therefore prefer keeping them as separate species. Glenodinium bernardinense might be related to B. anauniensis, although this remains very speculative until a detailed analysis of G. bernardinense has been made, a species that to our knowledge has not been observed since its original description. It may be argued to use the older generic name Glenodinium rather than creating a new genus. However, Glenodinium was originally defined by the presence of a characteristic horseshoe-shaped stigma (Ehrenberg 1836, 1838). Some regard Glenodinium as a senior synonym of Sphaerodinium, (e.g. Loeblich 1980; Fensome et al. 1993), a genus originally

created by Woloszynska (1916) for species with a particular thecal plate tabulation, and indeed *Sphaerodinium* species have an eyespot similar to *Glenodinium cinctum* Ehrenberg. Others consider the genus *Glenodinium* too poorly defined and its use to be avoided (e.g. Bourrelly 1968). In any case, *B. anauniensis* lacks a horseshoe-shaped eyespot and thecal plates. It cannot be placed in *Glenodinium* whatever arguments are used to define this genus. The closest relative to *B. anauniensis*, on the basis of the current LSU phylogeny, are *Woloszynskia tenuissima, W. pseudopalustris* and *Polarella glacialis*, but all these species possess thecal plates and also differ from *B. anauniensis* in other features (see later). We therefore cannot place *B. anauniensis* in either *Woloszynskia* or *Polarella* but instead in a genus of its own.

Ultrastructure vs LSU, SSU phylogeny

Baldinia anauniensis possesses a number of typical dinoflagellate features such as the nucleus, mitochondria and chloroplast, but also some unusual characteristics to be discussed in the following.

The lack of an apical structure is rather unusual, as some kind of apical structure, i.e. the acrobase or apical groove, is present in most unarmoured and suessialean dinoflagellates, the latter characterised by seven or more latitudinal series of amphiesmal vesicles (Fensome et al. 1993). The apical groove structure, a tiny apical furrow, is now widely used in the classification of unarmoured dinoflagellates (e.g. Daugbjerg et al. 2000, de Salas et al. 2003), and the presence of a delicate apical line (ALP) of tiny plates has recently been used as one of the distinctive characters of the genus Tovellia (Lindberg et al. 2005). Even W. tenuissima, a close relative to B. anauniensis in the present LSU phylogeny, has an apical stripe with numerous small knobs along its side (G. Hansen, unpublished observations) very similar to that reported for Symbiodinium microadriaticum, W. halophila and 'Glenodinium sanguineum' sensu Dodge et al. (1987) (Loeblich & Sherley 1979; Flaim et al. 2004; Kremp et al. 2005). To our knowledge the only other thin-walled dinoflagellate that seems to lack an apical structure is P. glacialis (Montresor et al. 1999), but this species differs from B. anauniensis by the presence of thin plates, a different cyst type and a different eyespot structure.

The pusule of *B. anauniensis* is similar to that observed in Prosoaulax lacustris (F. Stein) Calado et Moestrup and 'G. sanguineum' sensu Dodge et al. 1987, and a similar pusule type seems also to be present in Woloszynskia halophila, W. tenuissima, and W. tylota (Mapletoft, M. Montgom., J. Waters et P. Wells) B.T. Bibby et J.D. Dodge (Crawford et al. 1970; Bibby & Dodge 1972; Dodge et al. 1987; Calado et al. 1998; Kremp et al. 2005). Its location in a 'cavity' of the pyrenoid is, however, unusual and has not been observed in other dinoflagellates. The pusules of the tovelliaceans is basically similar, but here the inner pusular membrane is often covered by electron-dense 'knobs' (Crawford & Dodge 1971, Lindberg et al. 2005). The outer pusular membranes have been suggested to be of vacuolar origin (Dodge 1972), but in B. anauniensis they clearly originated from an amphiesmal vesicle, and this may therfore also apply to other dinoflagellates. Several different pusular types occur in the dinoflagellates (see Dodge 1972), but their function(s) and phylogenetic significance are





Fig. 55. *Baldinia anauniensis*, TEM. Section that has been tilted to reveal the R2 root in longitudinal view.

still unknown. Generally a collection chamber is part of the pusule complex of gymnodinioid species, e.g. *Gymnodinium aureolum* (Hulburt) Gert Hansen, *G. catenatum* and *G. nolleri* (Rees & Hallegraeff 1991; Ellegaard & Moestrup 1999; Hansen 2001) and a somewhat similar arrangement, in form of a large sac pusule, is present in many thecate species (e.g. *Gonyaulax spinifera* Diesing, *Peridinium cinctum* (O. F. Müller) Ehrenberg and *Peridiniopsis borgii* Lemmermann (Hansen *et al.* 1996; Calado *et al.* 1999; Calado & Moestrup 2002).

The lack of trichocyst in B. anauniensis is unusual but not unique, as this structure is absent in such diverse organisms as Aureodinium pigmentosum J.D. Dodge, Gymnodinium fuscum, and some benthic prorocentroids e.g. Prorocentrum lima and P. maculosum Faust, indicating that trichocysts have been lost repeatedly (Dodge 1967; Zhou & Fritz 1993; Hansen et al. 2000). It is, however, striking that it is also missing in 'Glenodinium sanguineum' sensu Dodge, Polarella glacialis (based on published micrograps), Symbiodinium linucheae (Trench et Thin) LaJeunesse [note: this combination (La-Jeunesse 2001) is invalid according to ICBN art. 32.2, incorrect basionym citation], Woloszynskia halophila and W. tenuissima (Crawford et al. 1970; Trench & Thinh 1995; Montresor et al. 1999; Kremp et al. 2005), all representing genera that make up a major clade together with B. anauniensis in the present LSU or SSU phylogeny, indicating that trichocysts were lost in a common ancestor to these species.

Viruses have been reported from many algal species and



Fig. 56. Diagrammatic illustration of the flagellar apparatus of *Baldinia anauniensis* based on 3D reconstruction of serial sections.

might play a role in the decline of algal blooms (see e.g. Reisser 1993; Brussaard 2004). We did not observe any signs of degradation of the nucleus or other cell organelles, and cultured cells also contained virus-like particles. They did not

Figs 48–54. Baldinia anauniensis, TEM. The flagellar apparatus in transverse view. The cell is seen from the outside anterior and the nonadjacent serial sections are moving from anterior to posterior. The encircled numbers are section numbers.

Fig. 48. Section near the exit point of the peduncle. Notice that the area devoid of amphiesmal vesicles are wider (arrowhead) than in Fig. 29.

Fig. 49. The R1/TBc is attached to three longer microtubules of the R1.

Fig. 50. The lamellar body is very close to the LB and R1 root.

Fig. 51. Section close to the starting point of the DF and VF. Inset: same section tilted to reveal the R2 root.

Fig. 52. A narrow extension connects the more distal part of the VF to one of the LB triplets.

Figs 53, 54. The LB/R1c₂ and LB/R1c₁ connecting the R1 root with one of the LB triplets.



Prorocentrum micans Karenia brevis Karenia mikimotoi Karlodinium veneficum Heterocapsa triquetra Heterocapsa rotundata Akashiwo sanguinea Cochlodinium polvkrikoides Durinskia baltica Krvptoperidinium foliaceum Gymnodinium catenatum Lepidodinium viride Gymnodinium fuscum Pheopolykrikos hartmanii Gymnodinium impudicum Gymnodinium sp. (LaTo2) Gyrodinium spirale Gyrodinium rubrum Dinophysis norvegica Dinophysis acuta Dinophysis acuminata Scrippsiella trochoidea Polarella glacialis Gymnodinium simplex Symbiodinium microadriaticum Baldinia anauniensis Prorocentrum lima Amphidinium massartii Amphidinium carterae Peridinium willei Peridinium bipes Togula britannica Ceratium furca Ceratium fusus Ceratium hirundinella Alexandrium tamarense Alexandrium catenella Alexandrium affine Alexandrium margalefii Coolia monitis

Fig. 57. Phylogeny of Baldinia anauniensis on the basis of nuclearencoded SSU rDNA and inferred from unweighted maximum parsimony (MP) analysis. The alignment included 1763 characters and 613 of these were parsimony informative. The MP analysis produced four equally parsimonious trees, each 2961 steps long [confidence interval (CI) = 0.494 and (RI) = 0.532], and the tree shown is a strict consensus based on the four parsimonious trees. Bootstrap values or support from posterior probabilities of \geq 50% are written to the left of internal nodes. Numbers from MP bootstrap (1000 replications) are written first while bootstrap values from neighbor-joining analyses based on the maximum likelihood settings obtained using Modeltest (TrN+I+G model and with 1000 replications) are written next. The third number is the posterior probability from Bayesian analyses. The ciliate Tetrahymena pyriformis is used as an outgroup.

seem to affect the growth of the culture detrimentally. Some virus particles have been reported to leave the host cell without killing it. This occurs by a budding process and cell lysis is avoided (e.g. Alberts et al. 1994). We did not observe any signs of this process in B. anauniensis, but excretion could be a possibility for the small virus-like particles.

No other protist group displays so many different eyespot types as the dinoflagellates, and it has recently become evident that their fine structure is phylogenetically significant (Horiguchi 2003). Apart from the highly specialised ocelli present in certain unarmoured species (e.g. Greuet 1987), four different types might be distinguished, all situated in the sulcal area close to the R1 flagellar root. In the first type the osmiophilic globules are not bounded by any membrane but lie freely in the cytoplasm. This type corresponds to Dodge's (1984) type A. Species with this type form a natural group, the Tovelliaceae, that presently contains the genera Esoptrodinium, Jad-



50 changes

Fig. 58. Phylogeny of Baldinia anauniensis on the basis of nuclearencoded LSU rDNA and inferred from unweighted maximum parsimony (MP) analysis. The alignment included 1477 base pairs of which 1124 were considered unambiguous (546 characters were parsimony informative). The MP analysis produced one single most parsimonious tree, 2807 steps long (CI = 0.418 and RI = 0.537). The branch lengths are proportional to the number of changes. Bootstrap values or support from posterior probabilities of $\geq 50\%$ are written to the left of internal nodes. Numbers from MP bootstrap (1000 replications) are written first while bootstrap values from neighbor-joining analyses based on the maximum likelihood settings obtained using Modeltest (TrN+I+G model and with 1000 replications) are written next. The third numbers are posterior probabilities from Bayesian analyses. The ciliates Tetrahymena pyriformis and T. thermophila are used for outgroup rooting.

wigia, and Tovellia (Lindberg et al. 2005; Calado et al. 2006; Moestrup et al. 2006). In the second type the osmiophiphilic globules are enclosed by three membranes (Dodge's type B), considered to represent a reduced chloroplast. Species having this type comprise such diverse taxa as Durinskia baltica, Kryptoperidinium foliaceum, Peridinium quinquecorne Abé, Dinothrix paradoxa Pascher, Galeidinium rugatum Tamura et Horiguchi, and Gymnodinium heterolobatum Horiguchi et Pienaar (Tomas & Cox 1973; Jeffrey & Vesk 1976; Horiguchi & Pienaar 1991, 1994; Horiguchi 2003; Tamura et al. 2005). A shared feature of these species is that they contain a reduced diatom endosymbiont. Interestingly, at least the former three

Table	1. Primers	used to	determine	nuclear-encoded	SSU	and	LSU
rDNA	nucleotide	sequence	es and their	r sequences.			

rDNA primers	Sequence $(5'-3')$
SSU (terminal primers)	
ND1 (forward)	AACCTGGTTGATCCTGCCAG
ND6 (reverse)	GATCCTTCTGCAGGTTCACC
SSU (internal primers)	
ND2 (forward)	GATTCCGGAGAGGGAGCCTG
ND3 (forward)	GCCGCGGUAAUUCCAGCTCC
ND7 (reverse)	GAACGGCCATGCACCACC
ND8 (reverse)	TCTGAGAATTTCACCTCT
LSU (terminal primers)	
D1 (forward)	ACCCGCTGAATTTAAGCATA
ND28-1483 (reverse)	GCTACTACCACCAAGATCTGC
Dino-specific (reverse)	ACACCTCGGAAGACAAGGT
LSU (internal primers)	
D3A (forward)	GACCCGTCTTGAACACGGA
D3B (reverse)	TCGGAGGGAACCAGCTAC
D2C (reverse)	CCTTGGTCCGTGTTTCAAGA

species have a lamellar body very similar to that of B. anauniensis (see below). Quite surprisingly, molecular evidence has recently shown that these species, irrespective of their seemingly different morphotypes, form a natural group (Inagaki et al. 2000; Horiguchi 2003; Tamura et al. 2005; Horiguchi & Takano 2006). In the third type, the eyespot consists of numerous layers of crystalline bricklike bodies and this has been found in S. linucheae, P. glacialis, W. halophila, Gymnodinium natalense Horiguchi et Pienaar and Prosoaulax lacustris (Trench & Thinh 1995; Calado et al. 1998; Montresor et al. 1999; Calado & Moestrup 2005, Kremp et al. 2005). Molecular data on the first three species have shown these to be closely related, and it now seems reasonable to believe that this eyespot type is a characteristic of the suessialean lineage. In the last eyespot type the osmiophiphilic globules are situated within the chloroplast (Dodge's type C). This type has, besides B. anauniensis, been found in such diverse taxa as e.g. Peridinium cinctum and Peridiniopsis borgei, but is also present in Woloszynskia tenuissima and 'Glenodinium sanguineum' sensu Dodge et al. 1987, species that are related to B. anauniensis (Crawford et al. 1970; Dodge et al. 1987; Calado et al. 1999; Calado & Moestrup 2002). This type seems to be the most common type in the dinoflagellates and it is basically similar to the eyespot found in many other protist groups e.g. chrysophytes, cryptophytes and chlorophytes (e.g. Dodge 1969). In many species, but apparently not in P. cinctum (e.g. Calado et al. 1999), a flattened vesicle containing bricklike bodies is situated between the eyespot and the R1 root, indicating that the suessialean type has originated from this type by the loss of the osmiophilic globules and proliferation of the brick-containing vesicle.

One of the most unusual features of *B. anauniensis* is the lamellar body. A very similar structure, also originating from the ER and also situated close to the basal bodies, has only been found in *D. baltica, K. foliaceum,* and *P. quinquecorne,* all species containing a diatom endosymbiont and an eyespot most likely representing a reduced dinoflagellate chloroplast (Dodge & Crawford 1969; Tomas & Cox 1973; Jeffrey & Vesk 1976; Horiguchi & Pienaar 1991). This indicates a close phylogenetic relationship between these species and *B. an*-

auniensis, i.e. the eyespot of Baldinia being the predecessor to the triple-membrane-bound eyespot characteristic of this group. However, neither the LSU nor the SSU phylogenies revealed a close relationship between B. anauniensis and D. baltica or K. foliaceum. Dodge & Crawford (1969) suggested the lamellar body to be a photoreceptor, pointing out the striking similarity to the flattened 'disks' of the rod cells of the vertebrate eye. This is further supported by the presence of highly specialized piles of smooth endoplasmic reticulum (ER) cisternae in photoreceptor cells of some insect eyes. This structure, which has some resemblance to the lamellar body, has been shown to accumulate Ca2+ and suggested to control the levels of free Ca²⁺ in the cell (Walz 1982). It therefore seems likely that the lamellar body has a similar function. In certain green algae, Ca2+ fluxes have been shown to play a key role in phototaxis (Harz & Hegemann 1991). Thus, light stimulation of the photoreceptor, a rhodopsin-like protein, leads to rapid influx of Ca²⁺ into the flagella and likely change of the flagellar beat (Hegemann 1997). This further strengthens the idea that the lamellar body of dinoflagellates plays a role in phototaxis. It may function either as a source of Ca2+ to the flagellar bases or a sink preventing too high levels of free Ca²⁺ in the cytosol, which may cause improper functioning of for example the mitochondria (Walz 1982). It is difficult to imagine how such a highly specialised structure can occur in two apparently unrelated dinoflagellate groups. However, structures with a striking resemblance to the lamellar body of B. anauniensis have been observed in a number of vertebrate cell types. Thus, peculiar configurations of the ER referred to as lamellar bodies, tubular axoplasm, tubuloampular or honeycomb structures have been observed in certain cells of the diencephalon of fishes, neurons of cat cerebellum, pancreatic B cells of rat and man, cholinergic neurons of the nucleus basalis of ferrets and in a number of tumour cells (Morales & Duncan 1966; Lanzing & van Lennep 1970; Graf & Heitz 1980; Carstens 1984; Henderson 1989). The function of these structures is unknown, but since they often occur in e.g. tumour cells, it has been suggested that they might regulate the intracellular levels of Ca²⁺ in highly metabolically active cells (Henderson 1989).

The FA of B. anauniensis had the typical flagellar root complement found in most other dinoflagellates, i.e. a large multimembered microtubular root (R1) associated with the longitudinal basal body and a single-membered and compound root, the R3 and R4, respectively, associated with the TB. The presence of an R2 root is interesting, as this root is presently known only in certain gonyalacoid and peridinioid dinoflagellates (Calado & Moestrup 1997; Hansen et al. 1997; Hansen & Moestrup 1998; Calado et al. 1999). A putative R2 root was found in Gymnodinium chlorophorum (Hansen & Moestrup 2005), and its presence now also in B. anauniensis suggests that it may be more common in dinoflagellates than hitherto conceived. Often, the basal part of the R2 is partly embedded in dense fibrous material originating from the LB, e.g. Protoceratium reticulatum Bütschli (Hansen et al. 1997, figs 21, 34) and Alexandrium catenella (Hansen & Moestrup 1998, figs 9, 27). Hansen & Moestrup (1998) speculated that the fibrous material of Ceratium furcoides (Levander) Langhans might obscure the R2. This may also be the situation in the tovelliaceans, since Tovellia sp. and Jadwigia applanata Moestrup, Lindberg et Daugbjerg (see Lindberg et al. 2005)

Table 2. List of dinoflagellate	s (ingroup) and ciliates	(outgroup) included	in the phylogenetic	analyses of SSU	and LSU rDNA,	respectively.
GenBank accession numbers a	re also given.					

	GenBank accession	GenBank accession
	numbers for	numbers for
Species (in alphabetical order)	SSU rDNA	LSU rDNA
Akashiwo sanguinea (Hirasaka) Gert Hansen & Moestrup	U41085	AF260396
Alexandrium affine (Inouye & Fukuyo) Balech	AB088282	AY294612
Alexandrium catenalla (Whedon & Kofoid) Balech	AJ535392	
Alexandrium margalefii Balech	U27498	AY154957
Alexandrium tamarense (Lebour) Balech	AF022191	 AV455660
Amphidinium terdmanii Kofoid & Swezy	AI-274231	AY455675
Amphidinium massartii Biecheler	AF274255	AY455670
Baldinia anauniensis Gert Hansen & Daugbjerg	EF052682	EF052683
Cochlodinium polykrikoides Margalef	AY421782	_
Ceratium furca (Ehrenberg) Claparède & Lachmann	AJ276699	_
Ceratium fusus (Ehrenberg) Dujardin	AF022153	AF206390
Ceratium hirundinella (O.F. Müller) Dujardin	AY443014	
Ceratium lineatum (Ehrenberg) Cleve	 A 1415500	AF260391
Dinonhysis acuminata Claparède & Lachmann	AJ415509 A 1506972	
Dinophysis acuta Ehrenberg	A 1506973	
Dinophysis dedud Emenorg Dinophysis norvegica Claparède & Lachmann	AB073119	AY571375
Durinskia baltica (M. Levander) S. Carty & Eleanor R. Cox	AF231803	
Esoptrodinium gemma P. Javornický		DQ289020
Gonyaulax baltica Ellegaard, Lewis & Harding	_	AF260388
Gymnodinium catenatum L.W. Graham	AF022193	AF200672
Gymnodinium chlorophorum Elbrächter & Schnepf		AF200669
Gymnodinium fuscum (Ehrenberg) Stein	AF022194	AF200676
Gymnodinium impuaicum (Fraga & Bravo) Gert Hansen & Moestrup	AF022197	AF200674 AF200673
Gymnodinium simpler (Lohmann) Kofoid et Swezy	 U41086	AI 200073
<i>Gymnodinium</i> sp. (strain LaTo2)	AY829527	
Gyrodinium dominans Hulburt		AY571370
Gyrodinium rubrum (Kofoid & Swezy) Takano & Horiguchi	AB120003	AY571369
Gyrodinium spirale (Bergh) Kofoid & Swezy	AB120001	AY571371
Heterocapsa rotundata (Lohman) Gert Hansen	AF274267	AF260400
Heterocapsa triquetra (Ehrenberg) F. Stein	AY421787	AF260401
Jadwigia applanata Moestrup, Lindberg & Daugbjerg (strain CCAC 0021)		AY950447
Jaawigia appianata (strain Fw 145) Karania hravis (Davis) Gert Hansen & Moestrup	 A E274259	A 1950448 A E200677
Karenia mikimotoi (Miyake & Kominami ex Oda) Gert Hansen & Moestrup	AF022195	AF200681
Karlodinium veneficum (Ballantine) J. Larsen	AY245692	AF200675
Kryptoperidinium foliaceum (Stein) Lindemann	AF274268	EF052684
Lepidodinium viride Watanabe, Inouye, Sawaguchi & Chihara	AF022199	_
Oxyrrhis marina Dujardin	AF482425	
Peridiniella catenata (Levander) Balech	—	AF260398
Peridinium bipes F. Stein	AF231805	AF260385
Peridinium palatinum Lauterborn	 A E274280	AF260394
Peridinium willer Huitteid-Kaas	AF2/4280	AF260384
Polarella glacialis Montresor, Procaccini & Stoecker	AY179607	 AV571373
Prorocentrum lima (Ehrenberg) Dodge	Y16235	
Prorocentrum micans Ehrenberg	AY585526	AF260377
Prorocentrum minimum (Pavillard) Schiller	Y16238	AF260379
Scrippsiella trochoidea var. aciculifera Montresor	AF274277	AF260393
Symbiodinium microadriaticum Freudenthal	AY456111	
Togula britannica Flø Jørgensen, Murray & Daugbjerg	AY443010	AY455679
Togula jolla Flø Jørgensen, Murray & Daugbjerg		AY455680
Tovellia coronata (Woloszynska) Moestrup, Lindberg & Daugbjerg (strain KL B1)		AY950445
Toyellia coronata (Strain KL F1) Toyellia sanguinea Moestrup, Cert Hansen, Daughierg, Elaim & D'Andrea		A 1 950440 DO320627
Woloszynskia nseudonalustris (Schiller) Kisselev		AF260402
Woloszynskia tenuissima (Lauterborn) Thompson	_	AY571374
Outgroup (ciliates)		
Tetrahymena pyriformis (Ebranbero) I woff	¥56171	X54004
Tetrahymena pyrijornus (Eliciberg) Ewon Tetrahymena thermophila Nannev & McCov	AJ01/1	X54512

Table 3. Parameter settings as suggested by Modeltest (ver. 3.6) for the SSU and LSU rDNA sequences, respectively used to compute dissimilarity values in NJ analysis.

Gene/ parameter settings	Among- sites rate hetero- geneity α	Estimated proportion of invariable sites I	Substitution rate categories	Base frequencies
SSU rDNA	0.4829	0.2445	A-C: 1.4638 A-G: 3.9362 A-T: 1.3451 C-G: 0.8183 C-T: 8.384	A: 0.2662 C: 0.1784 G: 0.2561 T: 0.2993
LSU rDNA	0.6411	0.2627	A–G: 2.8187 C–T: 6.7128	A: 0.2859 C: 0.1722 G: 0.2714 T: 0.2705

possess 'an extending fibre' in exactly the same position as the R2 root (Roberts & Timpano 1989, as Woloszynskia sp.; Roberts et al. 1995a, as Woloszynskia limnetica Bursa). Flagella having two microtubular roots seem to be widespread in many protist groups and might have evolved early in eukaryote evolution (Moestrup 2000). Furthermore, Moestrup (2000) suggested the R1 flagellar root to be homologous with the R3 root, and root R4 with the R2 root, on the basis of flagellar transformation details. That means one flagellum being immature, the transverse in dinoflagellates, transforms into its mature state, the longitudinal flagellum, in the next generation (Heimann et al. 1995). Although the R4 root seems to be completely absorbed during cell division (Heimann et al. 1995), it is intriguing to consider the dark fibrous material of R2 as being homologous with the striated fibre of R4. The R2 root in dinoflagellates seems to be a plesiomorphic character that has been lost in some species.

The presence of a prominent VF in *B. anauniensis* has not been observed in other dinoflagellates. But less prominent fibre(s) associated with right side of the LB have also been observed in *C. furcoides, Jadwigia applanata, Tovellia* sp., and the highly divergent *Oxyrrhis marina* (Roberts 1985, 1989; Roberts & Timpano 1989; Roberts *et al.* 1995a). The latter species has been shown to be the most 'primitive' dinoflagellate in protein gene phylogenies (Harper *et al.* 2005), and it is also the most basal species in the present SSU phylogeny, although this is unsupported. Thus, if the VF of these species is homologous, it would imply that it has been lost several times, but this remains rather speculative until further investigations of this structure.

A peduncle has been observed in a number of dinoflagellates including dinophysoid, gymnodinioid, gonyaulacoid, peridinioid, prorocentroid, and suessioid lineages (Jacobson & Andersen 1994; Roberts *et al.* 1995b; Calado & Moestrup 1997; Calado *et al.* 1998; Hansen & Moestrup 1998; Hansen 2001). We did not observe the process of food ingestion in *B. anauniensis*, but the ubiquitous presence of food vacuoles in field material clearly demonstrated that mixotrophy is common. Cryptophytes might be the preferred prey organism, albeit further investigations are needed to confirm prey specificity.

The large dense protrusion observed in some cells is interesting (Fig. 44). It was originally observed in *Lepidodinium* *viride* M.M. Watanabe, S. suda, I. Inouye Sawaguchi et Chihara and suggested to be involved in attachment of the cell to the substrate (Watanabe *et al.* 1990). However, it was subsequently found also in *Protoceratium reticulatum* and *Gymnodinium aureolum* (Hansen *et al.* 1997; Hansen 2001). Hansen & Moestrup (2005) suggested it to be part of the peduncle. The present investigation has further demonstrated it to be a nonpermanent structure, and its presence seems to be influenced by fixation procedure or physiological state of the cells.

A ventral ridge has been found in a number of primarily unarmoured species, e.g. Amphidinium rhyncocephalum Anissimowa, Gymnodinium chlorophorum, Gyrodinium spirale (Bergh) Kofoid et Swezy and Prosoaulax lacustris (Calado et al. 1998; Farmer & Roberts 1989; Hansen & Daugbjerg 2004; Hansen & Moestrup 2005), but does also seem to be present in the tovelliaceans, which possess delicate thecal plates (Roberts & Timpano 1989; Lindberg et al. 2005). Often microtubules are located along or near the ridge, and their arrangements seem to be very similar in B. anauniensis and P. lacustris. Prosoaulax lacustris also possesses two separate bands of microtubules, the R1 flagellar root and a microtubular strand, respectively, but these are separated by two pairs of microtubules rather than one as in B. anauniensis (Calado et al. 1998). In P. lacustris, one pair seems to become embedded in the ventral ridge, but we were not able to confirm this in B. anauniensis. Amphidinium rhyncocephalum appears to have a somewhat similar system, which may perhaps be widespread among the dinoflagellates. In A. rhyncocephalum three microtubules run along the ventral ridge, and these seem eventually to continue into the cytoplasm, nucleating numerous microtubules (Farmer & Roberts 1989). As in B. anauniensis, P. lacustris possesses an incomplete longitudinal striated collar. This has not been observed in other dinoflagellates except for the putative incomplete 'collar' found in Gyrodinium spirale (Hansen & Daugbjerg 2004).

In conclusion, B. anauniensis looks superficially like a 'normal' gymnodinioid dinoflagellate, but the lack of an apical structure, presence of a ventral fibre and the peculiar lamellar body sets it apart from most other dinoflagellates. Particularly, the last feature is intriguing, as it has previously only been found in species with a diatom endosymbiont. However, neither LSU rDNA nor the more conservative SSU rDNA gene was able to prove a relationship between B. anauniensis and these species. Phylogenies based on these ribosomal genes indicated a relationship to suessioid and woloszynskioid species, with which B. anauniensis shares characters such as lack of trichocysts and type of pusule. Polarella glacialis also lacks an apical structure, but details of the flagellar apparatus are lacking for these groups. Nevertheless, molecular, morphological and ultrastructural evidence has revealed that B. anauniensis differs significantly from Tovellia sanguinea, two dinoflagellates that in the past were thought to represent forms of the same species.

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