Studies on woloszynskioid dinoflagellates I: *Woloszynskia coronata* re-examined using light and electron microscopy and partial LSU rDNA sequences, with description of *Tovellia* gen. nov. and *Jadwigia* gen. nov. (Tovelliaceae fam. nov.)

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Sediment samples were collected from a small pond in southern Sweden. Several cysts from the samples germinated into clonal cultures, identified as *Woloszynskia coronata* (Wolosz.) R.H. Thompson 1951. They were compared with other species of *Woloszynskia* established in culture, using scanning electron microscopy, transmission electron microscopy, partial large subunit ribosomal DNA (LSU rDNA) and morphology of the resting cysts. Significant differences were found, and we conclude that the genus *Woloszynskia* as presently circumscribed is artificial, and comprises at least four genera. In this first paper we transfer *W. coronata* to a new genus; *Tovellia* gen. nov., type species: *Tovellia coronata* (Wolosz.) comb. nov. Previous studies on ultrastructure and DNA sequencing referring to *Woloszynskia coronata* are based on *W. coronata* var. *glabra,* which is raised to species level as *Tovellia glabra* sp. nov. Other species included in the new genus are *Tovellia apiculata* (basionym *Woloszynskia apiculata* Stosch) and *Tovellia stoschii* (basionym *Woloszynskia stoschii* R. Shyam & Sarma). Two identical cultures presently identified as *Woloszynskia limnetica* Bursa (from University of Washington Culture Collection, Seattle) and *W. pseudopalustris* (J. Schiller) Kiselev [from Culture Collection of Algae at the University of Cologne, Cologne] differ from *Tovellia* in LSU rDNA sequences and in cyst type and are transferred to *Jadwigia* gen. nov., as *J. applanata* sp. nov. The most striking feature of *Tovellia* and *Jadwigia* is the anatomy of the eyespot, which is extraplastidial, and composed of nonmembrane bound lipid globules. This type of eyespot is also present in *Katodinium campylops* (T.M. Harris) A.R. Loebl., a species undoubtedly related to *Tovellia,* and in '*Glenodinium* sp.' *sensu* Kreimer 1999, and together they form a distinct family, Tovelliaceae fam. nov.

INTRODUCTION

Woloszynskia is a genus of freshwater dinoflagellates established by R.H. Thompson (1951) to include *Gymnodinium*-like species whose cells are covered with many thin plates. Thompson based his new genus on Jadwiga Woloszynska's very careful studies from the early part of the 20th century, in which she demonstrated thecal plates in several species that at the time were included in *Gymnodinium* (Woloszynska 1917). *Gymnodinium* had been erected by Stein (1878) to hold athecate (naked) dinoflagellates, this feature justifying its separation from the thecate *Peridinium* Ehrenb. (1830). The plates in Woloszynska's species were not arranged in a plate pattern consistent with the Kofoidean tabulation system used to describe the arrangement of plates in other thecate dinoflagellates.

Thompson (1951) transferred nine *Gymnodinium* species to *Woloszynskia,* including *Woloszynskia coronata* (Wolosz.) R.H. Thompson and its variety *W. coronata* var. *glabra* (Wolosz.) R.H. Thompson. He did not designate a type for the genus but A.R. Loeblich Jr. & A.R. Loeblich III (1966) selected *W. reticulata* R.H. Thompson as the generic type. Today the genus holds 17 described species (Table 1) (Popovský & Pfiester 1990 recognised only 12 species). Judging from the

drawings supplied by Biecheler (1952), the marine species *Gymnodinium halophilum* Biecheler may also be a woloszynskioid.

Features characterizing species of *Woloszynskia* are cell size and morphology, and number and arrangement of the thecal plates. In all species except *W. reticulata* the thecal plates are thin, often hexagonal and arranged in latitudinal series. Epi- and hyposomal series may be interrupted by intercalary plates, which in some cases disrupt the series. The cingulum is covered by one or two series of plates. The estimated total number of plates ranges from c. 47 in *Woloszynskia coronata* (probably too low, see below) to c. 360 in *W. tenuissima* (Lauterborn) R.H. Thompson (Netzel & Dürr 1984). The choice of *W. reticulata* as type species was unfortunate because this species differs markedly from all others in having thick and concave plates on the hyposome. Some species of *Woloszynskia* have been described with an anterior so-called carina (Thompson 1951; von Stosch 1973; Shyam & Sarma 1975; Popovský & Pfiester 1990) or acrobase (Roberts *et al.* 1995). This is a straight or slightly curved apical 'line' on the episomal surface, extending over the anterior end from the ventral to the dorsal side of the cell. The ultrastructure of the apical line is described below.

Several species of *Woloszynskia* produce resting cysts (e.g. Woloszynska 1917; Thompson 1951; von Stosch 1973) and, in addition, some may produce temporary division cysts

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(Suchlandt 1916; von Stosch 1973; K. Lindberg, personal observations). The resting cysts belong to three different morphotypes: (1) round to oval and smooth-walled; (2) round to oval with numerous spines; and (3) slightly elongate with paracingulum and two axial horns, in addition to lateral protuberances or scattered, short, thick spines.

Woloszynskia species inhabit freshwater habitats such as lakes, ponds and pools. They are known from both temperate and tropical regions, and in temperate waters both summer and winter species are known. All species are photosynthetic, and mixotrophy is unknown. The genus has a wide geographical distribution [Europe (e.g. Woloszynska 1917), India (Shyam & Sarma 1975), USA (Thompson 1951) and Japan (Takano & Horiguchi 2003)].

The taxonomy of the genus remained untouched until von Stosch (1973), based on the morphology of the cysts, suggested that the genus possibly comprises three subgroups. This idea was not further pursued.

Based on morphological, ultrastructural and molecular data, and on differences in resting cyst morphology, we suggest that the genus *Woloszynskia* is artificial and comprises four or more genera, belonging to what we provisionally designate groups I–III. The three groups belong to three different families of dinoflagellates and the present paper deals with species of group I. We provide information on ultrastructure, including scanning electron microscopy (SEM), of vegetative cells and cysts of *W. coronata,* from cultures based on isolates from a small lake in southern Sweden. We also include a phylogenetic analysis, based on partial large subunit ribosomal DNA (LSU rDNA) sequences. We transfer *W. coronata* and related species to a new genus, *Tovellia* gen. nov., and material similar to *W. neglecta sensu* Woloszynska non *sensu* Schilling to a new genus, *Jadwigia* gen. nov. All members of group I are transferred to the new family Tovelliaceae fam. nov.

A preliminary report of our results was presented at DINO7 (Moestrup *et al.* 2003).

MATERIAL AND METHODS

Cultures

A surface sediment sample was collected in October 2001 from a small pond at Ugglehult near Aneboda, Smaaland, southern Sweden. To concentrate cysts from the sediment we applied a slightly modified version of the method described by Ellegaard *et al.* (2003). Subsamples were rinsed with tap water on 100 and 25 μm sieves. The 25–100 μm fraction was cleaned by ultrasonication for 2 min and rinsed again on the 25 μm sieve. The samples were then density separated using sodium polytungstate (Bolch 1997), using a specific gravity of 1.3.

The sediment and the concentrated material were stored at 4℃ in the dark. Cysts were isolated from the concentrated material, washed with several drops of DY IV medium (http://ccmp.bigelow.org) and placed in separate microwells in tissue plates (volume 350 μl) containing DY IV medium.

The tissue plates were kept at 4℃ in the dark for c. 1 mo, during which time isolation of single cysts took place. The plates were subsequently placed for 1 mo at 10℃ under a

Table 1. List of species presently included in the genus *Woloszynskia*, after date of first description.

W. neglecta (A.J. Schill. 1891) R.H. Thompson 1951
W. tenuissima (Lauterborn 1894) R.H. Thompson 1951
W. polonica (Wolosz. 1916) R.H. Thompson 1951
W. pascheri (Suchl. 1916) Stosch 1973
W. coronata (Wolosz. 1917) R.H. Thompson 1951
W. hiemale (Wolosz. 1917) R.H. Thompson 1951
W. leopoliensis (Wolosz. 1917) R.H. Thompson 1951
W. veris (Er. Lindemann 1925) R.H. Thompson 1951
W. pseudopalustris (J. Schiller 1933) Kiselev 1954
W. ordinata (Skuja 1939) R.H. Thompson 1951
W. cestocoetes (R.H. Thompson 1947) R.H. Thompson 1951
W. reticulata R.H. Thompson 1951
W. nygaardii (Christen 1958) A.R. Loebl. 1970
W. limnetica Bursa 1958
W. tylota (Mapletoft, M. Montgom., J. Waters et P. Wells 1966) B.T. Bibby & J.D. Dodge 1972
W. apiculata Stosch 1973
W. stoschii R. Shyam & Sarma 1975

16 : 8 h light : dark cycle, and then transferred to 15℃ under the same light conditions.

The cysts at 15℃ started germinating during the following weeks. Clonal cultures were transferred to tissue plates with larger micro wells (3.6 ml) and later to Nunclon flasks at 15℃ and a 16 : 8 h L : D regime. Of the 43 cysts isolated, 22 germinated, and 7 of these grew into stable cultures. Two clonal cultures labelled F1 and B1 form the basis of the studies reported below.

A culture labelled *Woloszynskia limnetica* Bursa was obtained from Ellen Duffield, University of Washington Culture Collection [FW 145, isolated in 1965 by Rita Horner, then University of British Columbia. The isolate was submitted in 1982 to Collection of Algae at the University of Texas at Austin (UTEX) and was known here as LB 2319. It later died]. The origin of the isolate is somewhat uncertain – probably British Columbia. It was maintained at 15℃.

A culture labelled *Woloszynskia pseudopalustris* (J. Schiller) Kiselev was obtained from Barbara Surek, University of Cologne, as CCAC 0021, and maintained at 15℃. It originates from Forschungsinstitut Senckenberg and was isolated in 1991 by A. Schilke from a small pond near Lochmühle (Germany).

Light microscopy

Live cells and cysts were examined using an Olympus Provis AX 70 microscope equipped with Differential Interference Contrast optics. Photographs and video recordings were taken on an Axio Cam digital camera (Zeiss, Germany) and a SVCam 085 digital video camera (SVS-Vistek Cameras Inc, USA), respectively. Images from video sequences were grabbed using Video Savant Pro version 4.0 (IO Industries, Canada).

Digital recordings were used for cell measurements.

Preparation for SEM

Woloszynskia coronata proved difficult to prepare for SEM, and several different fixatives and procedures were attempted before satisfactory results were achieved. Only a mixture of aqueous OsO_4 and a saturated aqueous $HgCl_2$ solution preserved the cell shape and the cell covering, and retained the

flagella intact. This fixation method is known as Párducz's instantaneous fixation technique (Párducz 1967). The method has been adapted to preservation of thin-walled dinoflagellates by Greg Strout, University of Oklahoma (personal communication). Different species require different mixtures of aqueous OsO_4 and saturated aqueous $HgCl_2$ in the fixative, and different ratios of fixative and culture. The optimal fixative for the Swedish isolate of *W. coronata* was a mixture of 2% aqueous OsO_4 and a saturated aqueous $HgCl_2$ solution in a 3 : 1 v/v ratio. The fixative was mixed with culture in a 1 : 2 v/v ratio. The cells were fixed for 15 min at room temperature, washed with distilled water for 30 min and dehydrated through a graded ethanol series. They were critical point dried in CO_2 in a Baltec CPD 030 critical point drier. The filters were mounted on stubs, coated with platinum–palladium and examined in a field emission scanning electron microscope JEOL JSM 6335F.

Preparation for TEM

Cells were fixed in 1.5% glutaraldehyde in phosphate buffer for 2 h, and then concentrated by centrifugation into a pellet. The pellet was subsequently washed in three changes of 0.1 M phosphate buffer, pH 7.2, for 3 × 30 min. It was postfixed in 2% OsO_4 for 2 h, and dehydrated through a graded ethanol series followed by propylene oxide. The pellet was embedded in Spurr's resin and serial sectioned with a diamond knife on a LKB Ultrotome V. Sections were stained with uranyl acetate and lead citrate and examined in a JEOL JEM 1010 transmission electron microscope.

DNA extraction and sequence determination of partial LSU rDNA

Ten millilitres of exponentially growing cultures of each of strains F1, B1, FW 145 and CAAC 0021 were centrifuged at 1500 rpm for 10 min. The pellet was transferred to a 1.5 ml Eppendorf tube and frozen at −18°C until DNA extraction. Extraction of total genomic DNA followed J.J. Doyle & J.L. Doyle (1987), with modifications as outlined in Hansen *et al.* (2003). Polymerase chain reaction conditions and sequencing in both directions of partial LSU rDNA using external primers D1R (Scholin *et al.* 1994) and 28–1483R (Daugbjerg *et al.* 2000) in addition to internal primers (D3A-F, D3B-R and D2C-R) have been described previously (Hansen *et al.* 2003).

Alignment and phylogenetic analyses

Partial LSU rDNA sequences of strains B1, F1, CCAC 0021 and FW 145 were aligned with 36 other dinoflagellate sequences obtained from GenBank (Table 2). Three ciliates [*Tetrahymena pyriformis* (Ehrenb.) A. Lwoff, *T. thermophila* Nanney & McCoy and *Spathidium amphoriforme* Greeff] served as the outgroup taxa (Table 2). The alignment incorporated information from the secondary structure of large subunit ribosomal RNA as suggested by de Rijk *et al.* (2000). The nucleotide data matrix consisted of 1477 base pairs (bp) including introduced gaps and covered domains D1–D6 (Lenaers *et al.* 1989). Due to ambiguous alignment we excluded the highly variable domain D2 from the phylogenetic analyses. Hence, a total of 1116 bp were analysed using maximum likelihood (ML), maximum parsimony (MP) and neighbour-

joining (NJ) methods using PAUP* version 4.0b10 (Swofford 2003). In order to find the best model for the LSU rDNA sequences we applied Modeltest (version 3.06, Posada & Crandall 1998). The best-fit model was TrN+I+G (Tamura & Nei 1993) with among-sites rate heterogeneity α = 0.6403, an estimated proportion of invariable sites I = 0.2373 and two substitution rate categories (A-G = 2.7976 and C-T = 6.5238). Base frequencies were set at A = 0.2858, C = 0.1711, G = 0.2707 and T = 0.2724). ML analyses were performed with 10 random additions of sequences. Because of computational constraints only 100 replicates were performed in ML bootstrap analysis using the 'fast' stepwise-addition in PAUP*. In MP 1000 random additions were done using the heuristic search option and a branch-swapping algorithm (TBR). Characters were unordered, weighted equally and gaps were treated as missing data. One thousand replicates were performed in unweighted parsimony bootstrap analysis. The best-fit model suggested by Modeltest (see above) was also used to compute dissimilarity values. The distance matrix obtained was used to build a tree with NJ. A total of 1000 replicates were performed in NJ bootstrap analysis.

Pigment analyses

Strain F1 (30 ml) was filtered onto 25 mm Advantec GF 75 glass fibre filters (Toyo Roshi Kaisha, Japan) and immediately stored at −80°C. The rest of the procedure follows Henriksen *et al.* (2002). The strain B1 was not analysed because the LSU rDNA sequence was identical to that determined for strain F1.

RESULTS AND DISCUSSION

Strains B1 and F1 Light microscopy

MORPHOLOGY: Cells are ovoid or nearly spherical (Figs 1–4) and slightly dorsoventrally compressed. The cingulum is left-handed and displaced about one cingulum width (Fig. 1). The episome is slightly longer than the hyposome. The proximal end of the cingulum is bordered by a ventral ridge, which extends over the area where the transverse flagellum emerges from the cell (Fig. 1). A bright-red eyespot is located ventrally along the proximal part of the sulcus, just below the ventral ridge (Figs 1, 2). The cell periphery contains numerous small sausage-shaped chloroplasts (Fig. 4). The colour of the chloroplasts is greenish but it is difficult to ascertain as the cytoplasm is packed with numerous small carotenoid droplets (Figs 1–4). The bright red colour of the droplets masks the other pigments and gives the cell a characteristic red colour. The intensity of the colour varies with the number of droplets, thus some cells appear bright red and others red with a greenish tinge.

The nucleus is located in the hyposome (Fig. 3). The longitudinal flagellum is notably long (Figs 1, 2).

CELL MOTILITY: Cells are positively phototactic and in the cultures rapidly accumulate towards an external light source. During swimming, the cells rotate around the longitudinal axis. The cells swim continuously, at moderate speed, sometimes slowing down for a short time before continuing movement.

Table 2. Dinoflagellates included in the phylogenetic analyses. Strain numbers and GenBank accession numbers are also provided (— = information not available). AJC, cultures of Antonio J. Calado; CCAC, Culture Collection of Algae at the University of Cologne; CCMP, Provasoli-Guillard National Center for Marine Phytoplankton; FW, University of Washington Culture Collection; JL, cultures of Jacob Larsen; K, Scandinavian Culture Collection of Algae and Protozoa; KL, cultures of Karin Lindberg; UTEX, Collection of Algae at the University of Texas at Austin; UW, cultures of Jane Lewis.

| Species	Strain no.	GenBank accession numbers
Dinophyceae		
Akashiwo sanguinea (Hirasaka) Gert Hansen & Moestrup	JL 36	AF260396
Alexandrium affine (H. Inoue & Fukuyo) Balech	—	AY294612
Alexandrium margalefii Balech	—	AY154957
Amphidinium carterae Hulburt	K-0654	AY455669
Amphidinium herdmanii Kof. & Swezy	K-0655	AY455675
Amphidinium massartii Biecheler	CCMP 1821	AY455670
Ceratium fusus (Ehrenb.) Dujard.	—	AF260390
Ceratium lineatum (Ehrenb.) Cleve	—	AF260391
Dinophysis norvegica Clap. & Lachm.	—	AY571375
Gonyaulax baltica Ellegaard, J. Lewis & I. Harding	UW 394	AY154962
Gonyaulax membranacea (Rossignol) Ellegaard, Daugbjerg, Rochon, J. Lewis & I. Harding	UW 398	AY154965
Gymnodinium catenatum H.W. Graham	—	AF200672
Gymnodinium chlorophorum Elbr. & Schnepf	K-0539	AF200669
Gymnodinium fuscum (Ehrenb.) F. Stein	CCMP 1677	AF200676
Gymnodinium impudicum (S. Fraga & I Bravo) Gert Hansen & Moestrup	JL 30	AF200674
Gymnodinium nolleri Ellegaard & Moestrup	K-0602	AF200673
Gyrodinium dominans Hulburt	—	AY571370
Gyrodinium rubrum (Kof. & Swezy) Takano & T. Horig.	—	AY571369
Gyrodinium spirale (Bergh) Kof. & Swezy	—	AY571371
Heterocapsa rotundata (Lohmann) Gert Hansen	K-0479	AF260400
Heterocapsa triquetra (Ehrenb.) F. Stein	K-0447	AF260401
Jadwigia applanata Moestrup, Lindberg & Daugbjerg	CCAC 0021	AY950447
Jadwigia applanata	FW 145	AY950448
Karenia brevis (C.C. Davis) Gert Hansen & Moestrup	JL 32	AF200677
Karenia mikimotoi (Miyake & Komin. *ex* M. Oda) Gert Hansen & Moestrup (J)	—	AF200681
Karlodinium micrum (B. Leadb. & J.D. Dodge) J. Larsen	K-0522	AF200675
Peridiniella catenata (Levander) Balech	K-0543	AF260398
Peridinium bipes F. Stein	AJC 8-847	AF260385
Peridinium cinctum (O.F. Müll.) Ehrenb.	AJC 4cl-a	AF260394
Peridinium willei Huitfeld-Kaas	AJC 2-675	AF260384
Polarella glacialis Montresor, Procaccini & Stoecker	—	AY571373
Prorocentrum micans Ehrenb.	K-0335	AF260377
Prorocentrum minimum (Pavill.) J. Schiller	K-0010	AF260379
Scrippsiella trochoidea var. *aciculifera* Montresor	K-0500	AF260393
Togula britannica (Herdman) Flø Jørgensen, Murray & Daugbjerg	K-0658	AY455679
Togula jolla Flø Jørgensen, Murray & Daugbjerg	UTEX 1562	AY455680
Tovellia coronata Moestrup, Lindberg & Daugbjerg	KL B1	AY950445
Tovellia coronata	KL F1	AY950446
Woloszynskia tenuissima (Lauterborn) R.H. Thompson	K-0666	AY571374
Woloszynskia pseudopalustris (Wolosz.) R.H. Thompson	—	AF260402
Ciliophora		
Spathidium amphoriforme Greeff	—	AF223570
Tetrahymena thermophila Nanney & McCoy	B1868VII	X54512
Tetrahymena pyriformis (Ehrenb.) A. Lwoff	GL-C	X54004

SEXUAL REPRODUCTION: Planozygotes with two longitudinal flagella have been observed in the culture on several occasions (not shown). 'Dancing groups' which according to von Stosch (1973) are also a sign of sexual reproduction, were also seen. The cyst is bright red (Figs 5–8). It has a distinct paracingulum, and both ends of the cyst wall extend into a blunt horn of somewhat variable size (Figs 5, 6). Two rows of short protuberances are present on the left and right hand side of the cyst, one above and the other below the paracingulum (Figs 5–7). In ventral or dorsal view the cyst is somewhat star-shaped (Figs 5, 6). In end view it appears oval (Figs 7, 8), the ventral side slightly concave, the dorsal side slightly convex (Fig. 16) and the protuberances on the left and right hand side are clearly visible (Fig. 7). As the culture seems to re-produce sexually, and we have observed different stages in the development from planozygote to cyst, we conclude that the cysts are hypnozygotes, but for simplicity we use the word cyst throughout this study.

Scanning electron microscopy

The cells lack the outer amphiesmal membrane, and details of the thecal plates with trichocyst pores are therefore clearly visible (Figs 9–13); compare with the transmission electron microscopy (TEM) thin sections (e.g. Fig. 22).

AMPHIESMAL PLATES: The cell is covered by mostly hexagonal or pentagonal amphiesmal plates (Figs 9, 10) often arranged in nine rows; four on the episome, four on the hypo-
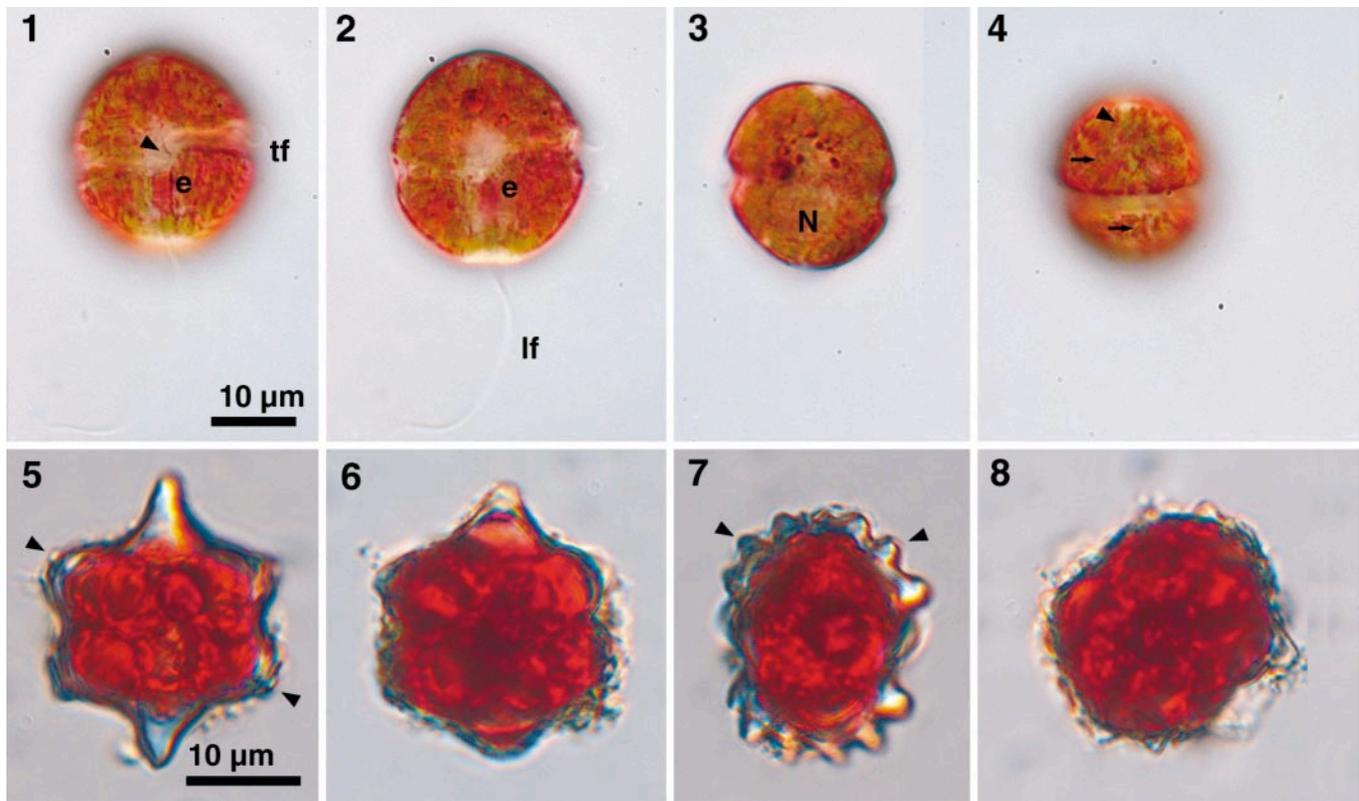
Figs 1–8. Differential Interference Contrast microscopy of *Tovellia coronata* comb. nov.
   Figs 1–4. *Tovellia coronata,* video-frame images. The episome is marginally larger than the hyposome. The ventral ridge is visible in Fig. 1 (arrowhead), just above the eyespot (e), which extends along the proximal part of the sulcus. Fig. 2 shows the extraordinary long posterior flagellum. The nucleus (N) is located in the hyposome (Fig. 3). The apical line of plates (ALP) is just visible in Fig. 4 (arrowhead), which also shows some of the peripherally located sausage-shaped chloroplasts (arrows). lf: longitudinal flagellum; tf: transverse flagellum.
   Figs 5–8. Resting cysts of *Tovellia coronata,* light microscopy. The cyst is extended into two opposite axial horns (Fig. 5) but the horns vary somewhat in length (compare Figs 5, 6). Both the episome and the hyposome bear a row of lateral knobs (Fig. 5, arrowheads, compare with Fig. 38d). The lateral knobs (arrowheads) are seen more clearly in end view of the cysts, which also shows the slightly flattened shape of the cyst (Figs 7, 8). Figs 5, 7: same cell; Figs 6, 8: same cell.

some and one in the cingulum. The number of rows varies slightly and the rows are sometimes interrupted by intercalary plates that disrupt the regular arrangement in series. Precingular and cingular plates are pentagonal. The precingular plates do not extend into the cingulum, and the episomal-cingular boundary is sharply edged (Figs 10, 12). Postcingular plates are hexagonal and extend for a short distance into the cingulum (Fig. 10). The cingular-hyposomal boundary is therefore smoothly rounded.

   Plates on the hyposome are arranged around a centrally placed hexagonal antapical plate (Fig. 12). Both strain F1 and B1 possess this plate, and in B1 it appears ornamented (Fig. 13).

THE APICAL LINE OF NARROW PLATES (ALP): On the episome is located what has been referred to as a carina (Thompson 1951; von Stosch 1973; Shyam & Sarma 1975; Popovský & Pfiester 1990). SEM showed this structure to be made of a row of several very narrow elongate thecal plates (acronym ALP, see below) (Fig. 11). Each plate is c. 0.3 µm wide and ornamented with small, centrally located thickenings. On each side of the line is a row of narrow thecal plates (Fig. 11), c. 1 µm wide. Other thecal plates are often c. 3 µm wide, but the size varies significantly.

   The ALP is nearly straight and extends from the ventral side of the epicone over the apex to the dorsal side. Its length is slightly variable, and this also applies to the number of narrow plates on each side (we observed three to seven).

   In our material the total number of plates on the cell is at least 60, including the sulcal plates and the ALP, but some variation was noted.

VENTRAL RIDGE: There is a distinct ventral ridge in the upper part of the sulcal area (Figs 9, 12). In Fig. 9 it appears as a distinct shoulder over the right side of the canal between the two ends of the cingulum.

SULCUS: SEM shows the sulcus extending to the antapical plate (Fig. 12), although in the light microscope it often appears to be shorter.

CYSTS: SEM (Figs 14–17) shows the cysts to possess a scabrate surface that lacks any sign of paratabulation. Thecal plates still attached to the surface are sometimes observed. The paracingulum is visible in dorsal and ventral view in Figs 14, 15, respectively, and the two rows of pre- and postcingular protuberances are visible in Figs 16 (end view), 17 (lateral view). An archaeopyle was not observed.

Transmission electron microscopy

GENERAL STRUCTURE OF THE CELL: The disposition of the cell organelles is visible in Figs 18–21. The chloroplasts are lo-

cated in the periphery of the cell and the centrally located nucleus takes up a substantial part of the hyposome (Figs 18–20). The slightly flattened shape of the cell is visible in Fig. 20. Fig. 21 shows a cell with a large number of carotenoid droplets in the cytoplasm, and these droplets are responsible for the red colour that characterises this species. Other organelles visible at the low magnification in Figs 18–21 are trichocysts (a 5 μm long trichocyst is visible in Fig. 18, left), and part of the pusule system (Fig. 19), discussed in more detail below. The eyespot is visible in the transverse section of the cell (Fig. 20), just below the sulcus (top of figure), and in the longitudinal section of the cell in Fig. 19.

THE AMPHIESMAL PLATES: The cell is covered with plates that lack a covering membrane (Fig. 22). The plates are generally thin, usually 40–100 nm thick, but distinctly thicker on the ventral side of the cell where the flagellar canals open to the exterior (Fig. 25 and perhaps Fig. 28, to be shown in more detail in a subsequent publication). A section through the anterior end of the cell illustrates one of the narrow plates of the ALP (Fig. 22), closely adjoining the neighbouring plates (compare with Fig. 11). In thin section the narrow apical plates of the ALP sometimes project slightly from the cell (Fig. 23) and this is probably caused by the line of small knobs seen in SEM (Fig. 11). Thecal plates are subtended by two distinct three-layered membranes, of which the inner obviously serves as a plasmalemma. A thin opaque layer is sometimes visible immediately beneath the plasmalemma (Fig. 22).

THE EYESPOT: The eyespot has a very unusual construction. It is composed of a layer of globules situated in the sulcal area (Figs 25–27), beneath the longitudinal microtubular root r_1 (*sensu* Moestrup 2000) (Fig. 27). The globules lack surrounding membranes and are not a part of a chloroplast. Sometimes the arrangement of the eyespot globules appears more irregular, thus in Fig. 26 some of the globules have fused into a large, more irregular body. A few globules may sometimes be seen to form a less developed second layer (Fig. 27). The eyespot globules are situated around the sides and the base of the sulcus and therefore form an elongate curved sheet (Figs 25, 26), which flattens distally (Fig. 27). It extends for approximately half the distance between the cingulum and the antapex (3–4 μm in Fig. 1). In Fig. 25 the eyespot has been sectioned for almost its entire length, and the globules on the two sides of the sulcus are associated with numerous microtubules.

Fig. 26 also shows a very conspicuous bundle of microtubules in oblique section, located on the right side of the sulcal area (on the cell's left). The microtubules are shown in transverse section in Fig. 24, where a total of c. 40 microtubules disposed in four layers are visible.

THE PUSULE SYSTEM: The pusule system of *W. coronata* is conspicuous and in Fig. 28 measures c. 5 μm in length. It comprises a tube that opens into the canal of the longitudinal flagellum, and more distally of a very complex system of tubules and sacs, the latter in close contact with what Crawford & Dodge (1971) termed a peripheral vesicular reticulum (Figs 28, 30). Many parts of the peripheral reticulum are filled with 'crystal'-like material (Figs 28, 32). On the lumen side, the inner tubules are often coated with rod-shaped bodies (Figs 29, 30) arranged in a regular pattern (Fig. 29). Sometimes the tubules contain both a layer of rod-shaped bodies and a membranous component (Fig. 31). The rod-shaped bodies measure c. 30 nm in length and c. 15 nm in width.

THE FLAGELLAR APPARATUS, PEDUNCLE: The cytoskeleton will be illustrated and discussed separately (Ø. Moestrup, unpublished observations).

SEXUAL REPRODUCTION: We have on a single occasion obtained sections through a cell with two separate sets of flagellar bases, each set with attached roots (Figs 33–35). The flagellar bases and roots were arranged in parallel and we know from our studies on the genera *Karlodinium* and *Esoptrodinium* (manuscripts in preparation) that this arrangement is characteristic of a fusion cell. The double set of flagellar bases may at first be confused with a cell in which the flagellar bases have replicated in preparation for cytokinesis. However, in such cells the orientation of the replicated flagellar bases and roots is different (cf. Heimann *et al*. 1995). The cell in Figs 33–35 confirms that sexual fusion takes place in the culture.

Pigment analysis

High-performance liquid chromatography (HPLC) analysis demonstrated the presence of the following lipid-soluble pigments in strain F1 of *Woloszynskia coronata*: chlorophyll *a* and *c,* peridinin, diadinoxanthin, dinoxanthin and β-carotene (Fig. 36).

LSU rDNA sequences

The 1429 bp included in a comparison of strains B1 and F1 revealed no differences. Hence, these strains are conspecific and part of the same population (Table 3). The sequence divergence between the two morphologically identical strains FW 145 and CCAC 0021 to be discussed below (compare Figs 39–43 with Figs 44–48) was 10.4% using 'uncorrected p' or 11.3% using the Kimura-2-parameter model (Table 3). In pair-wise comparisons between F1 and B1 and FW 145 and CCAP 0021 (all species assigned to group I in the tree in Fig. 37, see below) the sequence divergence was 22% or 26%, depending on substitution model. The sequence divergence among species assigned to group II (*W. pseudopalustris* and *Polarella glacialis*) was 4%. A sequence comparison of group I and group II wolozynskioids ranged from 14% to 30%.

Phylogeny of woloszynskioids

The phylogenetic inference of the woloszynskioids using NJ is shown in Fig. 37. The branching order of some of the deeper dinoflagellate lineages differed between this topology and those obtained by ML and MP analyses. However, because the topology of the deepest branches is not supported in terms of bootstrap values ($<$ 50%) the noted differences are considered nonsignificant. The woloszynskioids included in the tree assigned to group I and group II form two monophyletic groups supported by fairly high bootstrap values in most of the methods applied (Fig. 37). The branch lengths separating strains F1 and B1 and strains FW 145 and CCAC 0021 are relatively long, and similar in length to different species within Gonyaulacales (Ellegaard *et al*. 2003). On the other hand,
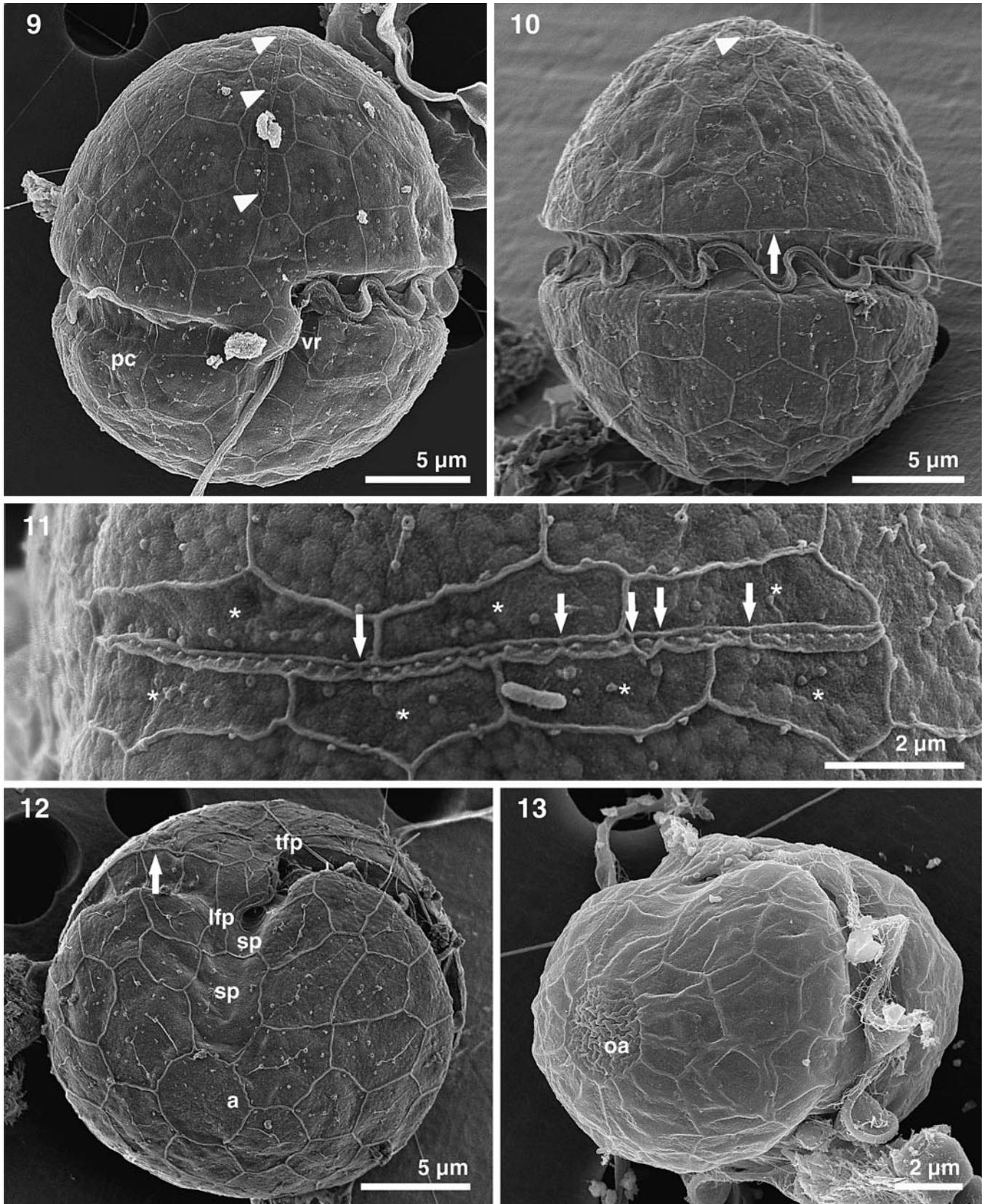
Figs 9–13. *Tovellia coronata,* field emission scanning electron microscope.
   Fig. 9. Ventral view showing flagella, amphiesmal plates, including the apical line of narrow plates ALP (arrowheads), the ventral ridge (vr) and the postcingular plates (pc) that extend into the cingulum.
   Fig. 10. Dorsal view. The cingulum is sharply delineated anteriorly by the posterior end of the precingular plates (arrow). The ALP is just visible (arrowhead).
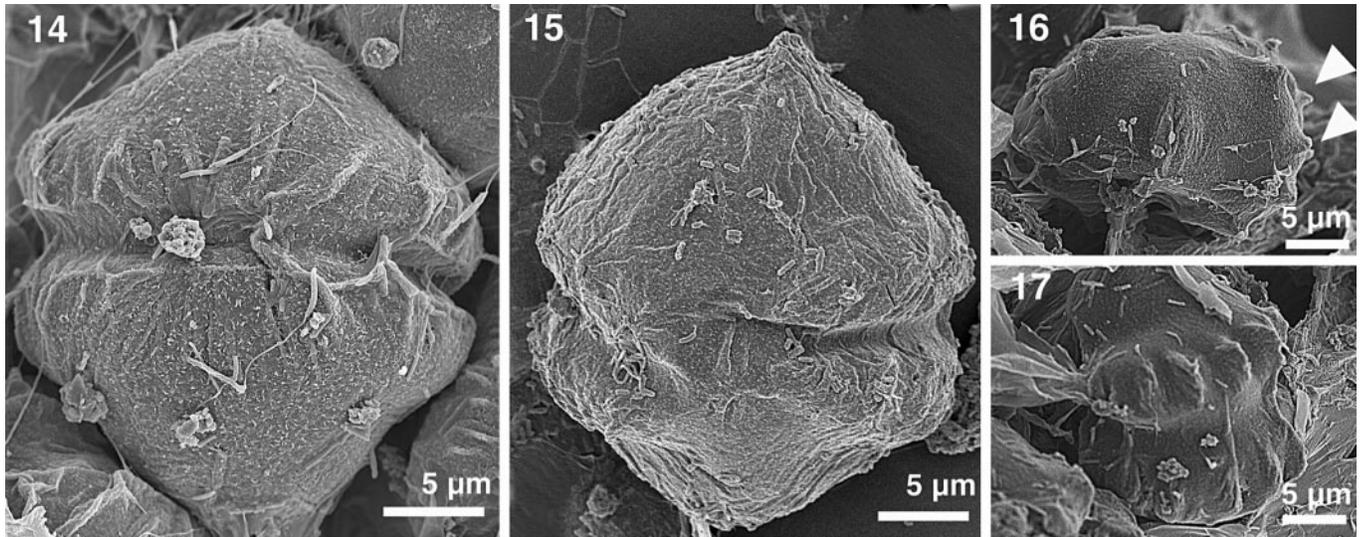
Figs 14–17. Cysts of *Tovellia coronata,* field emission scanning electron microscope. Figs 14, 15, cells in dorsal and ventral view, respectively. The lateral rows of projections (arrowheads) are more clearly seen in Fig. 16 (end view) and in the lateral view in Fig. 17.

the branch lengths of the two groups of strains are similar to those of *Togula jolla* and *T. britannica* and to species of *Amphidinium* (Flø Jørgensen *et al.* 2004a, b).

Identity of the Swedish isolates

Woloszynskia coronata was described in detail by Woloszynska (1917, as *Gymnodinium coronatum*), working from Lemberg (Lwow), then part of Austria-Hungary, now L'vov in Ukraine. Some of Woloszynska's almost unbelievably detailed drawings are reproduced here as Fig. 38a–f. Woloszynska characterised her new species as (1) possessing a plate pattern with relatively few rows of plates compared to other species currently placed in *Woloszynskia* (Fig. 38a–c, e–f); (2) having a distinct anterior line (Fig. 38a, b, e); (3) possessing a centrally placed, usually hexagonal, antapical plate with pearl-like ornamentations (Fig. 38c); and (4) having a red hypnozygote with an apical and an antapical horn, and small pre- and postcingular protuberances on each side (Fig. 38d) (Woloszynska 1917). Both of our strains F1 and B1 agree with this description, although the horns of the cysts produced by our strains are slightly shorter than in Woloszynska's material. Surprisingly, however, the antapical plate appears ornamented only in our strain B1 (Fig. 13, compare with Fig. 12). In our analyses of partial LSU rDNA (c. 1500 bp), the sequences were identical. We therefore conclude that the two strains are conspecific and that both belong to *Woloszynskia coronata*.

Identity of culture FW 145 from UWCC, Seattle ('*W. limnetica*') and CCAC 0021 from Cologne ('*W. pseudopalustris*')

The two cultures, which proved to be identical in terms of morphology agree well with Woloszynska's (1917) description

of *Glenodinium neglectum* J.A. Schill. However, Woloszynska's description differs markedly from Schilling's (1891) original description. Thus, in Woloszynska's material the two ends of the cingulum are displaced approximately one cingular width, whereas Schilling's drawing shows no displacement. Material resembling Schilling's illustration was found and illustrated by Javornický (1967). Javornický also showed a sausage-shaped nucleus in the left part of the cell. Lindemann (1929, as *Gymnodinium neglectum*) illustrated a cell that agreed well with Woloszynska's material in the displacement of the two ends of the cingulum and the dorsoventral compression, but possessed a sausage-shaped nucleus in the right anterior part of the cell. The latter feature was subsequently seen by Nygaard (1945, 1949, as *Gymnodinium neglectum*) and agrees with both the Seattle and the Cologne culture (Figs 39–48).

We conclude that two species have been mixed up under the name *Glenodinium neglectum* J.A. Schill. and its nomenclatural derivatives [*Gymnodinium neglectum* (J.A. Schill.) Er. Lindemann and *Woloszynskia neglecta* (A.J. Schilling) R.H. Thompson]. The true *W. neglecta* illustrated by Schilling, should be isolated in culture and examined further to determine its phylogenetic relationship. The species illustrated by Woloszynska and Lindemann is identical to the Seattle and the Cologne cultures and needs to be given a new species name.

Roberts *et al.* (1995) reported in detail on the Seattle culture, identified as *Woloszynskia limnetica* Bursa. The species possesses an eyespot of *coronata* type, i.e. the pigment globules are located outside the chloroplast. The authors failed to include morphological evidence for the identification of the

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Fig. 11. Anterior view of the cell to show the ALP, which comprises a row of very narrow plates; the arrows indicate the suture between individual plates. The plates carry a row of short centrally located knobs. The ALP is bordered on each side by a row of elongate plates (asterisks).

Figs 12, 13. The antapical end of the cell is characterised by a hexagonal plate, which may be smooth (a) or appear ornamented (oa). sp: sulcal plates; lfp: flagellar pore of longitudinal flagellum; tfp: flagellar pore of transverse flagellum (the flagella have been shed in the cell in Fig. 12); arrow indicates anterior ridge of the cingulum.
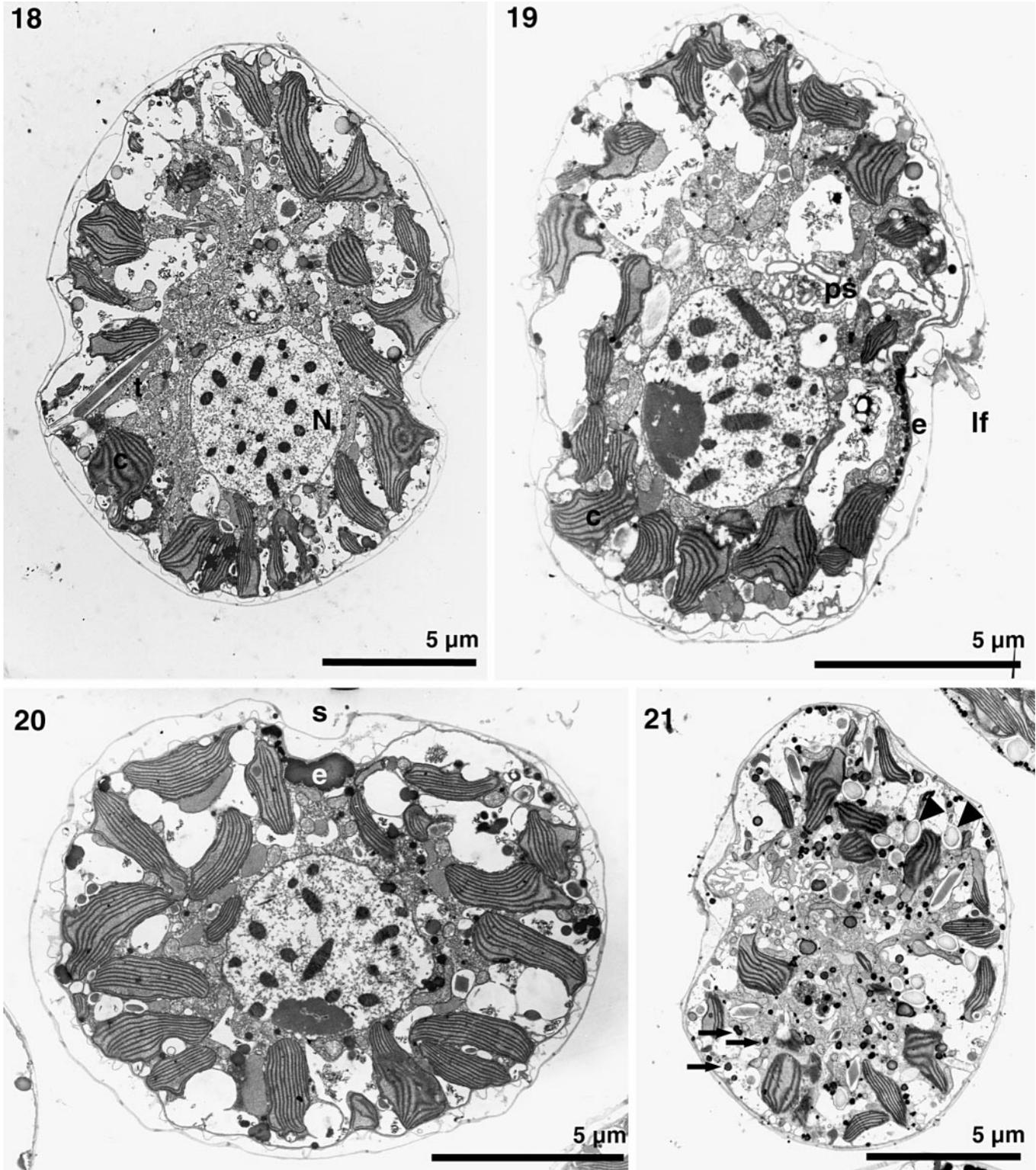
Figs 18–21. Ultrastructure of *Tovellia coronata*.
  Figs 18, 19. Longitudinal sections of the cell, showing cell shape and cingulum (Fig. 18), the nucleus (N) in the hyposome, a trichocyst (Fig. 18), the longitudinal flagellum (lf) close to the eyespot (e) (Fig. 19) and numerous chloroplasts (c) in the periphery of the cell. Part of the pusule system (ps) is visible close to the nucleus in Fig. 19.
  Fig. 20. Transverse section through the cell illustrating the flattened shape and the sulcus (s) with the eyespot (e).
  Fig. 21. Cell containing a large number of pigment globules (arrows) and starch grains (arrowheads).

culture as *W. limnetica* and the culture is no longer at the Texas Culture Collection. However, thanks to information from Rita Horner, who originally established the culture, we were able to secure a subculture of the strain from the University of Washington Culture Collection. This allowed us to perform a DNA study and to examine its taxonomy. We have by TEM confirmed the identity of the culture as UTEX LB 2319. The culture proved to be morphologically identical to CCAC 0021 from Cologne.

Taxonomy and phylogeny of *Woloszynskia*

As illustrated in the phylogenetic analysis (Fig. 37), the species of *Woloszynskia* included in the tree are widely separated (groups I and II, respectively), indicating that they are not as closely related as presently thought. This is supported by ultrastructural data and by cyst data. One of the most striking ultrastructural differences is the construction of the eyespot.

In members of group I, the eyespot comprises a group of pigment globules located outside the chloroplast. The globules are not surrounded by membranes, neither individually nor as a group. As in other dinoflagellates, the eyespot is located ventrally along flagellar root r_1, near the proximal end of the sulcus. This type of eyespot is restricted to a few dinoflagellates and it is not known in other algae although the lack of membrane somewhat recalls the eyespot of the Eustigmatophyceae (Hibberd & Leedale 1972).

Group I includes *Woloszynskia coronata* and the organisms studied by Roberts & Timpano (1989) and Roberts *et al.* (1995) under the names *Woloszynskia* sp. and *W. limnetica*. It also includes 'Glenodinium sp.' studied by Kreimer (1999). We have recently found that *Esoptrodinium* Javornický is a member of this group (A.J. Calado, unpublished observations). A micrograph published by Wilcox (1989) illustrates the same type of eyespot in *Katodinium campylops*, indicating that this species also belongs in group I.

In group II, eyespot-containing species examined possess another very unusual type of eyespot, which instead of pigment globules comprises a system of cisternae containing crystal-like material. In the light microscope the eyespot resembles a normal eyespot, and it is located on the ventral side of the cell along flagellar root r_1. It is not part of a chloroplast. This particular type of eyespot is unknown in other groups of algae.

The DNA sequence data show that group II includes *Woloszynskia pseudopalustris* (J. Schiller) Kiselev, not yet examined ultrastructurally. Eyespots of the crystal-containing type have been found in the marine cold-water plankton alga *P. glacialis* Montresor, Procaccini & Stoecker, the jellyfish symbiont *Gymnodinium linucheae* Trench & Thinh (Trench & Thinh 1995), the benthic marine autotroph *Gymnodinium natalense* T. Horig. & Pienaar (Horiguchi & Pienaar 1994) and the heterotrophic freshwater species *Amphidinium lacustre* F. Stein (Calado *et al.* 1998). DNA data have indicated that this ecologically exceptionally diverse group further includes species of *Symbiodinium,* endosymbionts of corals and the marine plankton species *Protodinium* (*Gymnodinium*) *simplex* Lohmann (Gast & Caron 1996; Montresor *et al.* 2003). In the arrangement of thecal plates, members of group II show resemblance to the Suessiales (Montresor *et al.* 1999), an order of dinoflagellates which until recently was thought to comprise extinct forms only. The Sues-

siales may eventually prove to comprise all the above-mentioned species, in addition to the extinct forms, in which case circumscription of the order will need to be emended to account for the many rows of plates in *W. pseudopalustris* (A. Calado, personal communication).

A group III (to be treated in detail in a separate publication) contains species in which the eyespot globules are located within the chloroplast. This is a common type of eyespot in algae, and it occurs in many heterokonts (e.g. brown algae and chrysophytes), green algae, etc. Group III includes e.g. *Woloszynskia tenuissima* (Lauterborn) R.H. Thompson (Crawford *et al.* 1970; Crawford & Dodge 1971), a species with smooth, spherical or subspherical cysts. Thus, species of *Woloszynskia* fall into at least three groups. This obviously raises the question about the taxonomic position of the type species, *W. reticulata*.

The type species of *Woloszynskia*

Woloszynskia reticulata was described from lakes and ponds in Kansas, USA (Thompson 1951), and it was subsequently refound in Crystal Lake, Norman, Oklahoma (Pfiester *et al.* 1980). An SEM image was included in the new North American flora (Carty 2003, fig. 8I). We have over the last 2 yr visited and collected in the lakes where Thompson found *W. reticulata*. Unfortunately many of the lakes have become polluted during the 50 yr since he made his collections (and some have changed name). Crystal Lake, Norman, visited by ØM in 2003, was also found to be polluted, and our attempts to find the organism from plankton or from cysts in the sediments have failed so far. Thompson provided some information about the thecal plates, which are unique within the genus in being rather thin on the episome but notably thick and concave on the hypocone. The cell also has a large crest or 'carina' (Thompson 1951; Pfiester *et al.* 1980) extending across the apical end from the ventral to the dorsal side of the cingulum, and the theca readily splits along this line (as in Carty 2003, fig. 8I). The cyst was described as spherical or slightly rhomboid, 'spinescent looking', and the spines were thought to be thecal plates that remained attached to the cyst along one of their margins, and opened outward to stand at right angles to the cyst wall. This explanation is not entirely convincing, however, it seems more likely that the 'plates' are spines protruding from the cyst, perhaps reflecting the tabulation pattern.

We have recently become aware that *Woloszynskia reticulata* was included in the Flora of Ukraine (Matvienko & Litvinenko 1977), and the distribution in Ukraine was given as estuaries and other water bodies near the Danube River. *Woloszynskia reticulata* therefore appears to be more widely distributed than indicated by the few published reports. The lack of reports is surprising considering the characteristic morphology of this species.

Although the information about *W. reticulata* is scarce, we conclude that the difference in thecal construction and the cyst type separates this species from group I as well as from groups II and III. None of the described species of *Woloszynskia* resemble *W. reticulata* to any great extent and the indications are presently that the genus is monotypic. Below we describe the two new genera of group I that need to be erected, *Tovellia* gen. nov. and *Jadwigia* gen. nov.
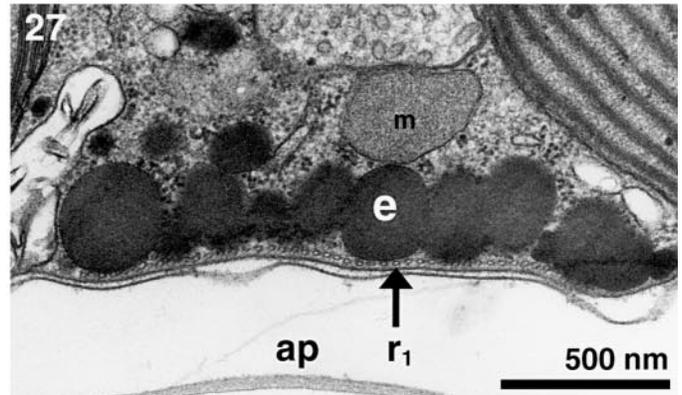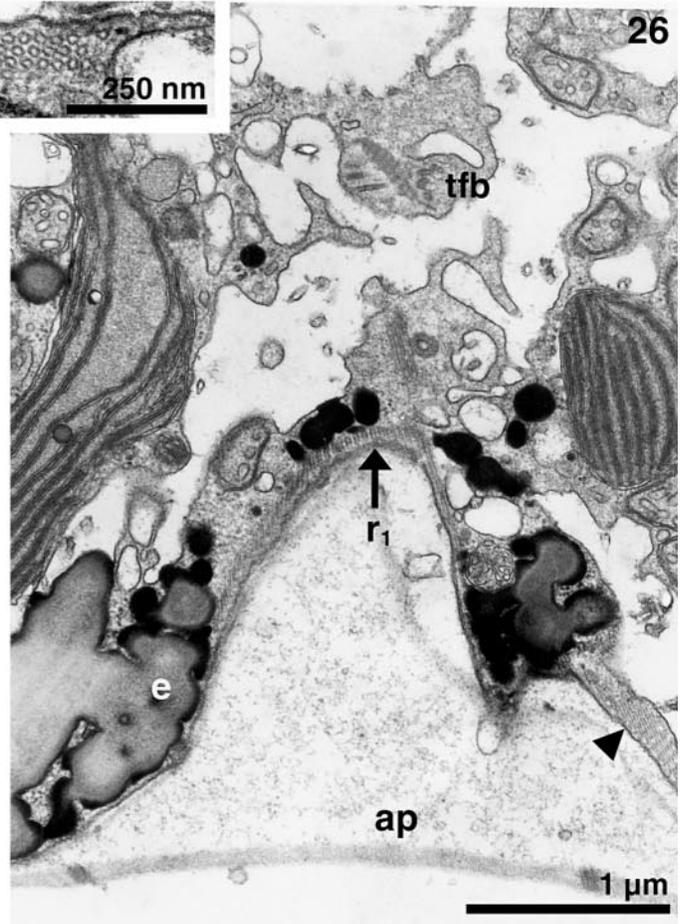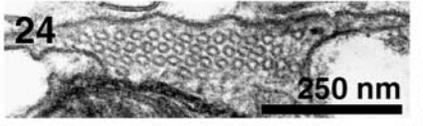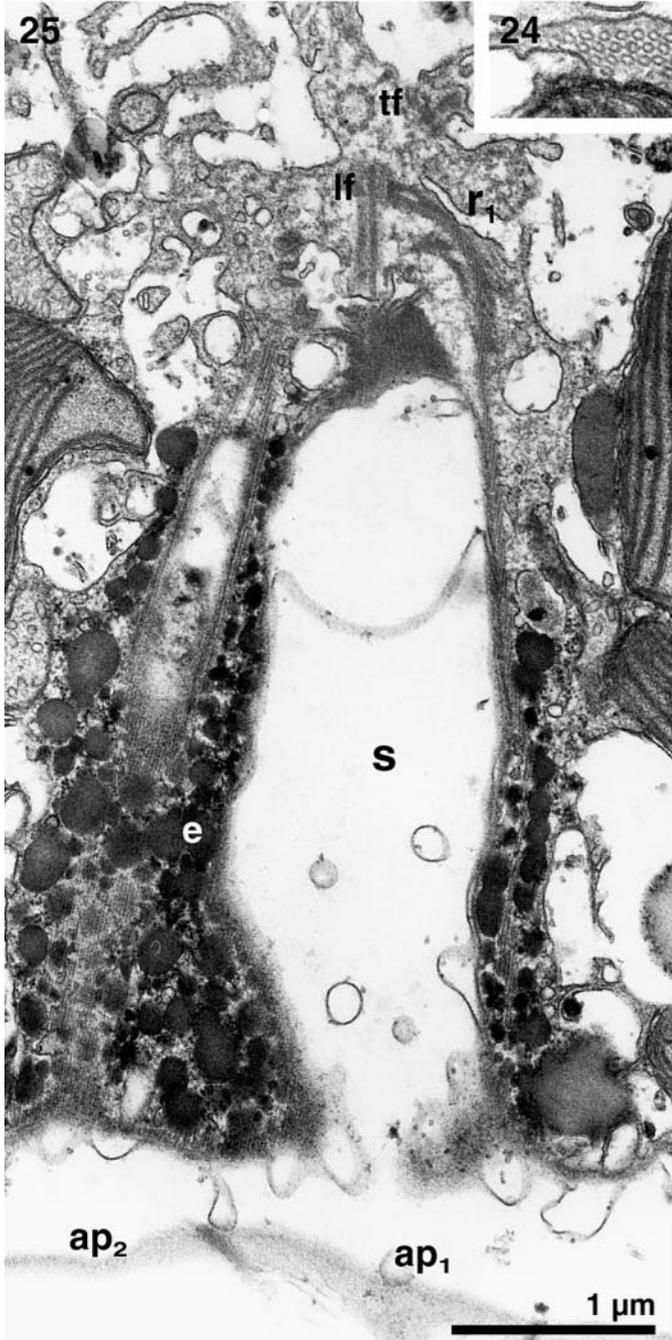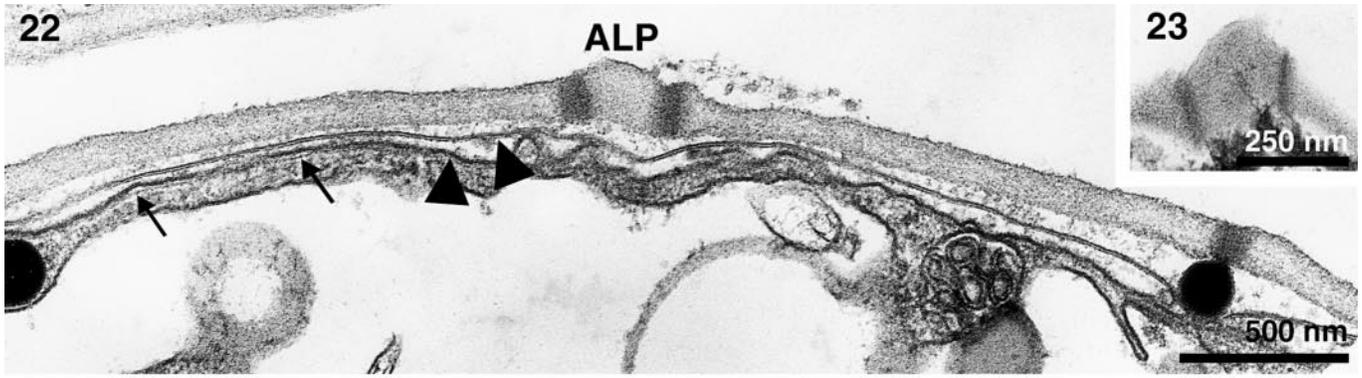
Tovellia Moestrup, Lindberg & Daugbjerg *gen. nov.*

Dinoflagellata cellulas plerumque pentagonalibus aut hexagonalibus laminis obtectas habentia. Linea laminarum recta aut paulum curvata trans partem anteriorem cellulae currit, ab parte ventrali ad partem dorsalem cellulae. Linea laminarum serie laminarum angustarum utrinque circumcincta. Stigma extra chloroplastum, globuli stigmatis nudi. Hypnozygotae paracingulum, cornua opposita et axialia, protuberationes praecingulares et postcingulares aut spinas breves disperses habent.

Dinoflagellates in which the cells are covered with many usually pentagonal or hexagonal plates. A straight or slightly curved line of plates extends across the anterior part of the cell, from the ventral to the dorsal side of the cell. The line is surrounded on each side by a row of narrow plates. The eyespot is extraplastidial, and the eyespot globules are not surrounded by membranes. The hypnozygote possesses a paracingulum, two opposite axial horns and pre- and postcingular protuberances or scattered short spines.

Tovellia coronata (Wolosz.) Moestrup, Lindberg & Daugbjerg *comb. nov.*

(Figs 1–35)

BASIONYM: *Gymnodinium coronatum* Woloszynska (1917, 115, 120, pls. 11, 13).

EMENDED DIAGNOSIS: Cells nearly spherical, very slightly dorsoventrally compressed. Epicone marginally larger than the hypocone. Cingulum displaced about one cingulum width. Cells 24.6 µm long (s = 3.1 µm) and 20.7 µm wide (s = 3.2 µm) (n = 63). Theca composed of approximately nine latitudinal rows of thecal plates, four on the epitheca, four on the hypotheca and one in the cingulum. A small number of intercalary plates sometimes interrupt the regular arrangement in rows. On the epicone, a nearly straight anterior line of narrow plates extends anteriorly from the ventral to the dorsal side. The hypothecal plates are radially arranged around a single centrally placed, usually hexagonal plate, which may be ornamented with low projections. The periphery of the cell contains large trichocysts and many sausage-shaped chloroplasts. The nucleus is located in the hyposome. The eyespot is located beneath the upper part of the sulcus. Both coated and smooth pusular canals are present. Numerous cytoplasmic carotenoid droplets commonly mask the other pigments, imparting a red colour to the cell, sometimes with a greenish undertone. Cyst with pre- and postcingular protuberances.

ETYMOLOGY: The name of the new genus is derived from Lake Tovel in the Italian Alps, famous for the reddening of its waters during summer. The reddening, which took place up till 1964, was referred to a dinoflagellate named *Glenodinium sanguineum* Marchesoni 1941, but recent studies have indicated that it was caused by a species of *Tovellia*. A preliminary report is being published by Flaim *et al.* (2004) and the organism will be described in a separate publication.

REMARKS: The two strains F1 and B1 were established from single cysts isolated at the same sampling site near Aneboda in southern Sweden and it is therefore not surprising that they are identical morphologically and genetically. Gene sequences are deposited at GenBank as numbers AY950446(F1) and AY950445(B1).

THE APICAL LINE OF NARROW PLATES (ALP): In *T. coronata* Wolosz. (1917, as *Gymnodinium coronatum*) termed this structure a 'schiefe Leiste' (oblique list), and Thompson (1951) used the name median girdle. Terms such as 'apical groove' *sensu* Takayama (1981, 1985) or 'acrobase' *sensu* Chatton & Hovasse (1934), used for a very similar structure in the motile cells of *Symbiodinium* (Loeblich & Sherley 1979, fig. 3A, and in particular fig. 4C), are not appropriate. It is not a groove, nor is it an acrobase, i.e. a more or less circular structure delineating the apical region of the cell. We prefer to use the term 'anterior line of narrow plates'. The ALP simply comprises a small number of narrow thecal plates arranged in a row. The plates are at level with the cell surface, and lined on each side by another row of wider but also elongate, quadrangular or pentagonal plates.

THE EXTERNAL CELL WALL: The amphiesma plates of dinoflagellates are typically enclosed in cisternae. Fixations in which the outer membranes are incomplete are not uncommon, however, and often give the impression of being fixation artefacts. However, in the case of *T. coronata* this is probably not the case. In one of our fixations (e.g. Figs 22, 26) both the cells and the area next to the amphiesma were well preserved and showed two distinct membranes beneath the plates. There was no membrane outside the plates. On a single occasion a cell was found in which very thin plates were present in typical amphiesmal vesicles, and this may have been a recently divided cell (data not shown). We conclude that the disappearance of the outer membranes in mature cells, i.e. cells with fully formed amphiesmal plates, is a normal phenomenon. The material examined by SEM also generally lacked an outer membrane, a possible exception being the cell in Fig. 13. The origin of the two membranes beneath the amphiesma plates is less clear, however. Dürr (1979) in *Peridinium cinctum* reported that two membranes developed underneath the amphiesmal vesicles prior to cell division, the innermost developing into a new plasmalemma. Three membranes were also seen in *Peridiniopsis borgei* by Calado and Moestrup (2002). In *T. coronata,* only two membranes were observed. The outermost of the two membranes is likely to represent the inner membrane of the amphiesma vesicle, and the inner membrane perhaps the new plasmalemma. However, we have no information about the ontogeny of the two membranes.

THE PUSULE: The pusule system of *W. coronata* var. *glabra* (described below as *Tovellia glabra* sp. nov.) was described


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Figs 22–27. Ultrastructure of *Tovellia coronata*.
  Figs 22, 23. The cell is bordered by amphiesmal plates that lack the outer membrane. The plates are underlain by two distinct membranes (arrowheads). The innermost membrane is underlain by a very thin electron-opaque layer (arrows). The ALP has been sectioned in Fig. 22. It sometimes projects slightly from the cell (Fig. 23).
  Fig. 24. Bundle of microtubules bordering the eyespot region of the cell (compare with Fig. 26).
  Fig. 25. Sulcal region in longitudinal section, showing the basal bodies of the transverse (tf) and longitudinal flagellum (lf), microtubules of the r_1 root and the large number of eyespot globules (e). The amphiesma plates of the sulcal region (ap_1) are thicker than the plates elsewhere on the cell (ap_2). s: sulcus.
  Figs 26, 27. The sulcus region in transverse section, showing the eyespot (e, some of the eyespot globules have fused in Fig. 26) and the r_1 flagellar root in the narrow space between the eyespot globules and the two external membranes. Arrowhead: bundle of microtubules on the cell's left hand side (cf. Fig. 24), ap: amphiesma plate; m: microbody; tfb: oblique section of the transverse flagellar base.
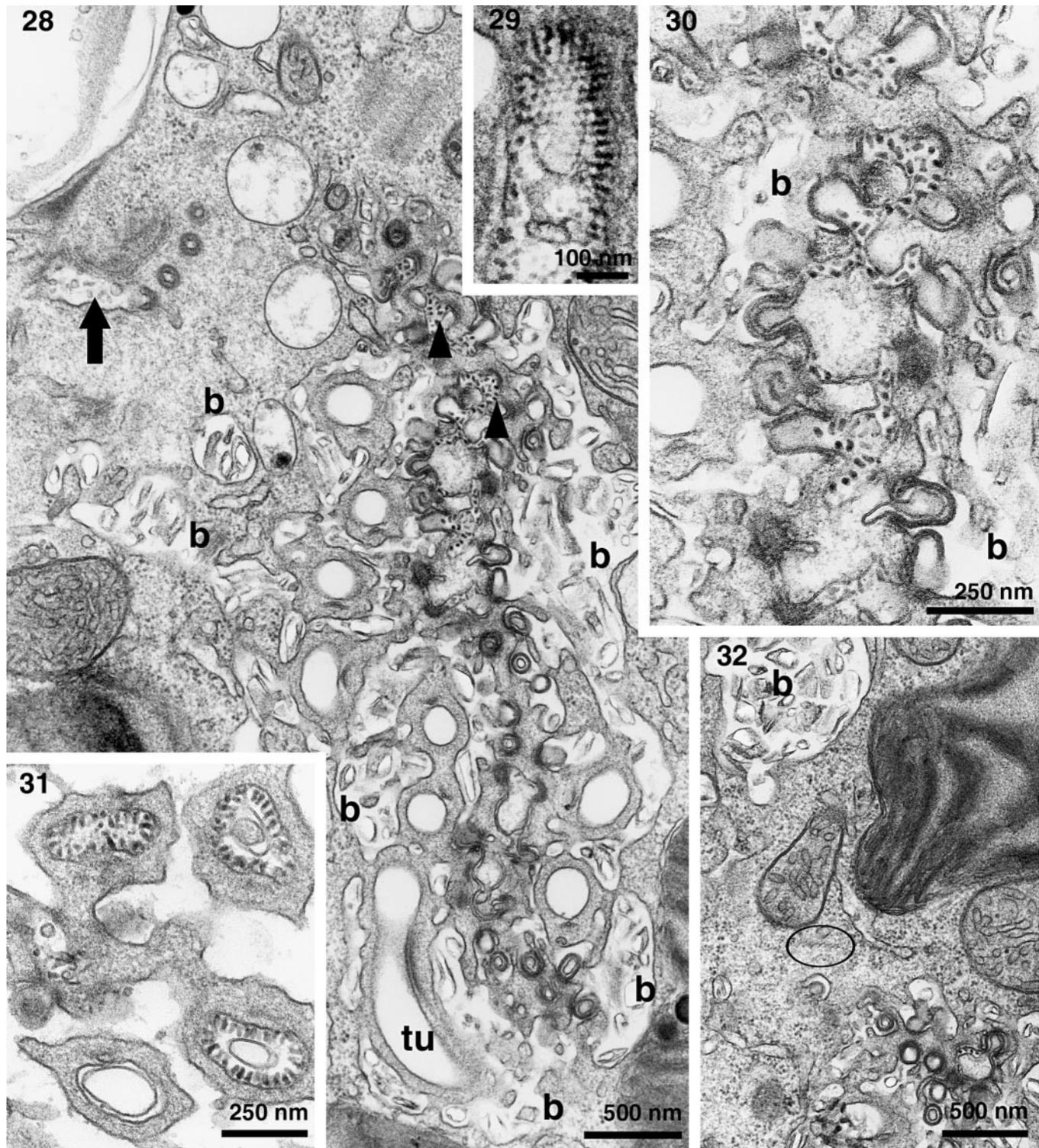
**Figs 28–32.** Details of the pusule system in *Tovellia coronata*.

Fig. 28. Overview showing the pusule system located next to the flagellar apparatus (the arrow indicates a flagellar canal). The pusule system comprises two parts, a tube (tu) that opens into the canal of the longitudinal flagellum, and a very complex inner part of tubules and sacs, some of which are coated on the inside by electron-opaque knobs (arrowheads). The elements of the inner part of the pusule systems are lined by an extensive peripheral vesicular reticulum that contains large amounts of crystal-like material (b).

Fig. 29. Inner elements of the pusule system, lined by closely arranged electron-opaque knobs.

Fig. 30. The inner part of the pusule system at higher magnification. b: crystal-containing cisternae of the peripheral vesicular reticulum.

Fig. 31. The cisternae lined by electron-opaque knobs often contain membranous material centrally.

Fig. 32. Cisterna with crystal-like material (b). The group of microtubules (encircled) is the bundle of microtubules nucleated by flagellar root r_3.
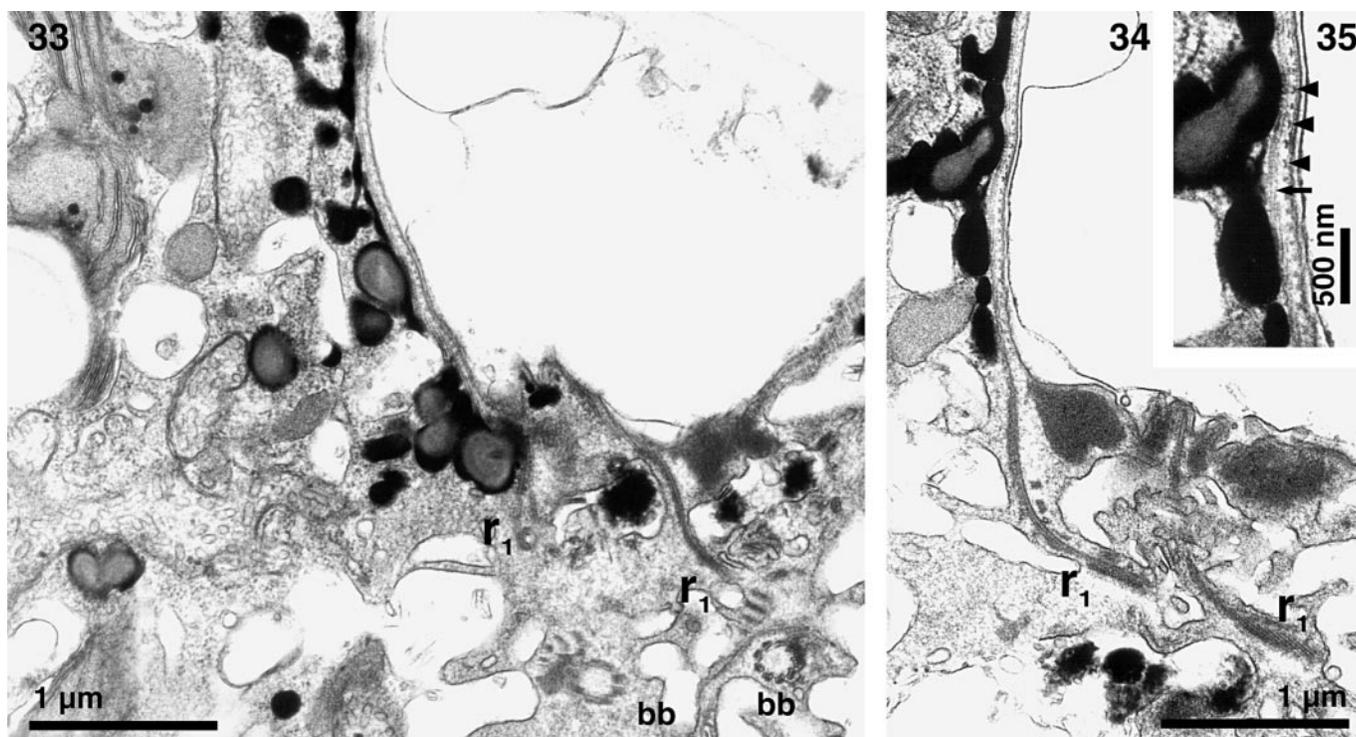
Figs 33–35. Ultrastructural details of planozygotes of *Tovellia coronata*. Two parallel sets of basal bodies (bb) are present, each accompanied by flagellar root r_1 (arrow in Fig. 35). Fig. 35 illustrates the very thin fibres (arrowheads) located at right angles to the r_1 microtubules and the two membranes lining the cell.

in detail by Crawford & Dodge (1971). Although basically similar to *T. coronata,* it differs in some of the details. Thus, Crawford & Dodge found the vesicular reticulum around the pusule to be empty in their material, whereas in *T. coronata* we found it to be filled with crystal-like material. Electron-opaque bodies were also present in Crawford & Dodge's material but apparently mainly in the large tubules of the pusule system (Crawford & Dodge 1971). In *T. coronata* the electron-opaque bodies occurred mainly in the smaller inner canals of the pusule. These differences are minor and may be due to different conditions in the cultures.

PIGMENTS: The pigments identified in *Tovellia coronata* are typical of peridinin-containing dinoflagellates (Fig. 36). The pigment responsible for the red colour of the cells remains
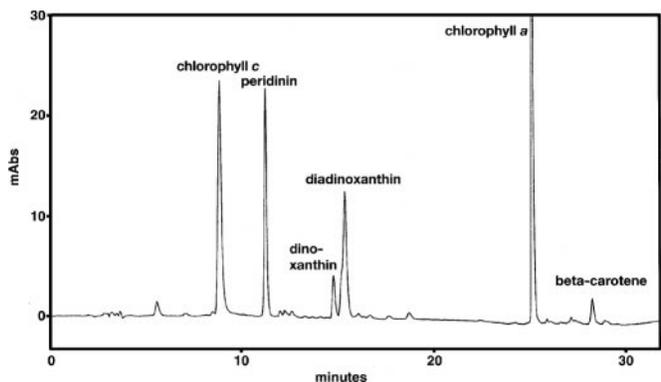
Fig. 36. Major lipid-soluble pigments of *Tovellia coronata* as revealed by HPLC.

uncertain. Astaxanthin was not identified in the HPLC analysis.

Other species of *Tovellia*

Tovellia glabra Moestrup, Lindberg & Daugbjerg *sp. nov.*

Fig. 38f

BASIONYM: *Gymnodinium coronatum* var. *glabrum* Woloszynska 1917: 121, Tafel 11, figs 20, 21 ('*glabra*').

Ab *Tovellia coronata,* lamina hexagonali antapicali carente et cellulas dorsoventraliter plus complanatiores habente differt.

Differs from *Tovellia coronata* in lacking the usually hexagonal antapical plate and in the cells being more flattened dorsoventrally.

REMARKS: There is no information about cyst type or apical line of narrow plates in this species. Woloszynska (1917) apparently mentioned only features distinguishing this taxon from *W. coronata.*

Several investigations have been published on an organism identified as *Woloszynskia coronata,* based on culture LB 1117/2 from CCAP (Dodge & Crawford 1970, 1971; Crawford & Dodge 1971; Dodge 1984; Lenaers *et al.* 1991). Crawford & Dodge (1971) mention, however, that the organism lacks the antapical plate and that it therefore represents var. *glabra.* The culture has subsequently been lost, both at the culture collection in Oban and with the authors of the above-mentioned articles. We are therefore unable to make a comparison between var. *glabra* and the nominal variety of *W. coronata,* based on LSU rDNA sequences. However, Lenaers *et al.* (1991) published a short DNA sequence from var *glabra* (LB 1117/2), comprising 352 bases, LSU rDNA, Domain D1.

Table 3. Sequence divergence (in percentage) of *Tovellia coronata*, *Jadwigia applanata* and *Polarella glacialis* based on 1429 bp of LSU rDNA. Strain numbers are provided in parentheses. Estimates of sequence divergence are based on 'uncorrected p' (above diagonal) and Kimura-2-parameter (below diagonal) using PAUP*.

	T. coronata (B1)	T. coronata (F1)	J. applanata (CCAC 0021)	J. applanata (FW 145)	W. tenuissima	W. pseudopalustris	Polarella glacialis
T. coronata (B1)	—	0	21.9	21.6	22.3	21.7	24.2
T. coronata (F1)	0	—	21.9	21.6	22.3	21.7	24.2
J. applanata (CCAC 0021)	26.2	26.1	—	10.4	17.6	16.3	19.3
J. applanata (FW 145)	26	25.9	11.3	—	16.6	14.0	21.5
W. tenuissima	26.8	26.7	20.2	18.9	—	10.7	13.6
W. pseudopalustris	26.0	26.0	18.5	15.7	11.6	—	4.0
Polarella glacialis	29.8	29.7	22.6	21.5	13.6	4.0	—

When compared with the equivalent sequence from the Swedish isolate, believed to represent the nominal variety, a difference of 25.7% using uncorrected p or 32.9% using the Kimura-2-parameter model was found between the two sequences of D1 (Table 4).

In addition to the difference in plate tabulation and the genetic differences, the illustrations of Crawford & Dodge (1971) show the nucleus of var. *glabra* to be located in the episome, whereas in the nominal variety it is located in the hyposome (present paper). Finally, the amphiesmal plates of var. *glabra* are thicker than in *W. coronata* (loc. cit. figs 8–10), 60–140 vs 40–100 nm in *W. coronata,* and in the published illustrations are located in amphiesmal vesicles.

We conclude that var. *glabra* should be considered a separate species.

Tovellia apiculata (Stosch) Moestrup, Lindberg & Daugbjerg *comb. nov.*

BASIONYM: *Woloszynskia apiculata* von Stosch (1973, 129, figs 33–41, 43–87).

We transfer *W. apiculata* to *Tovellia* because of the bipolar cyst type. von Stosch (1973) also illustrated an ALP in this species, termed by him a carina.

Tovellia leopoliensis (Wolosz.) Moestrup, Lindberg & Daugbjerg *comb. nov.*

BASIONYM: *Gymnodinium leopoliense* Woloszynska 1917: 119, Taf. (pl.) 11, fig. 6, Taf. (pl.) 13, figs C–E.

We transfer this species to *Tovellia* because of its bipolar cyst type. Woloszynska (1917) illustrated an ALP.

Tovellia nygaardii (Christen) Moestrup, Lindberg & Daugbjerg *comb. nov.*

BASIONYM: *Woloszynskia nygaardii* Christen 1958: 47, figs a–i ('*Nygaardi*').

SYNONYM: *Woloszynskia nygaardii* (Christen) A.R. Loebl. 1970.

Christen (1958) described this species in some detail from a number of freshwater localities in Kanton Zürich and Luzern in Switzerland. It was considered by Popovský & Pfiester (1990) to be a synonym of *W. coronata*. However, it differs in the arrangement of the chloroplasts, which in *T. nygaardii* typically diverge from a common centre in the cell, whereas the chloroplasts of *T. coronata* are parietal. Christen illustrated only the dorsal part of the plate covering but mentions in the text that the plate pattern is 'wie sie von Woloszynska für *Gymnodinium coronatum* beschrieben wurde. Die Platten der Hypovalva sind radial um die kleinen Antapikalplatte angeordnet' (Christen 1958, p. 46). We interpret this to mean that the cell has the usually hexangular antapical plate of *T. coronata* and *T. stoschii* (described below). The resting cysts possess two horns as in *T. coronata.*

### *Tovellia stoschii* (R. Shyam & Sarma) Moestrup, Lindberg & Daugbjerg *comb. nov.*

BASIONYM: *Woloszynskia stoschii* R. Shyam & Sarma 1975: 206, figs 1–9, 16–20.

This species resembles *T. coronata* in having a hexagonal antapical plate. It appears to be closely related to *T. coronata,* differing mainly in its larger cell dimensions, the much larger number and the different arrangement of thecal plates. An ALP (carina) was illustrated by Shyam & Sarma (1975). The cyst is unknown.

Tovellia sp.

The culture isolated by L. Pfiester in 1986 as *Woloszynskia* sp. and examined by Roberts & Timpano (1989) shows many similarities to *T. coronata,* including details of the eyespot, which appears to be identical. This taxon belongs in *Tovellia*. It has never been formally named but was described briefly by Timpano & Pfiester (1987). Its specific identity is difficult to ascertain.

→

Fig. 37. Phylogenetic relationships of woloszynskioids inferred by neighbour-joining and based on the maximum likelihood settings obtained using Model test (see Material and Methods). The analysis was based on partial LSU rDNA sequences (1116 bp). In ML analyses the best ln likelihood score was −13,384.630 using the TrN+I+G model. Parsimony analyses resulted in one single most parsimonious tree, 2761 steps long (CI = 0.433, RI = 0.543; tree topology not shown). Of the 1116 bp included 548 were parsimony informative. Only bootstrap values of 50% or higher are shown to the left of internal nodes. The first numbers are from neighbour-joining analyses (1000 replications), the second numbers are from maximum likelihood analyses (100 replications and using fast stepwise-addition) and the last numbers are from Parsimony analyses (1000 replications; characters were unweighted). Three ciliates were chosen as outgroup taxa. Woloszynskioids belonging to groups I, II and III (see text) are marked. Branch lengths are proportional to the number of substitutions per site.
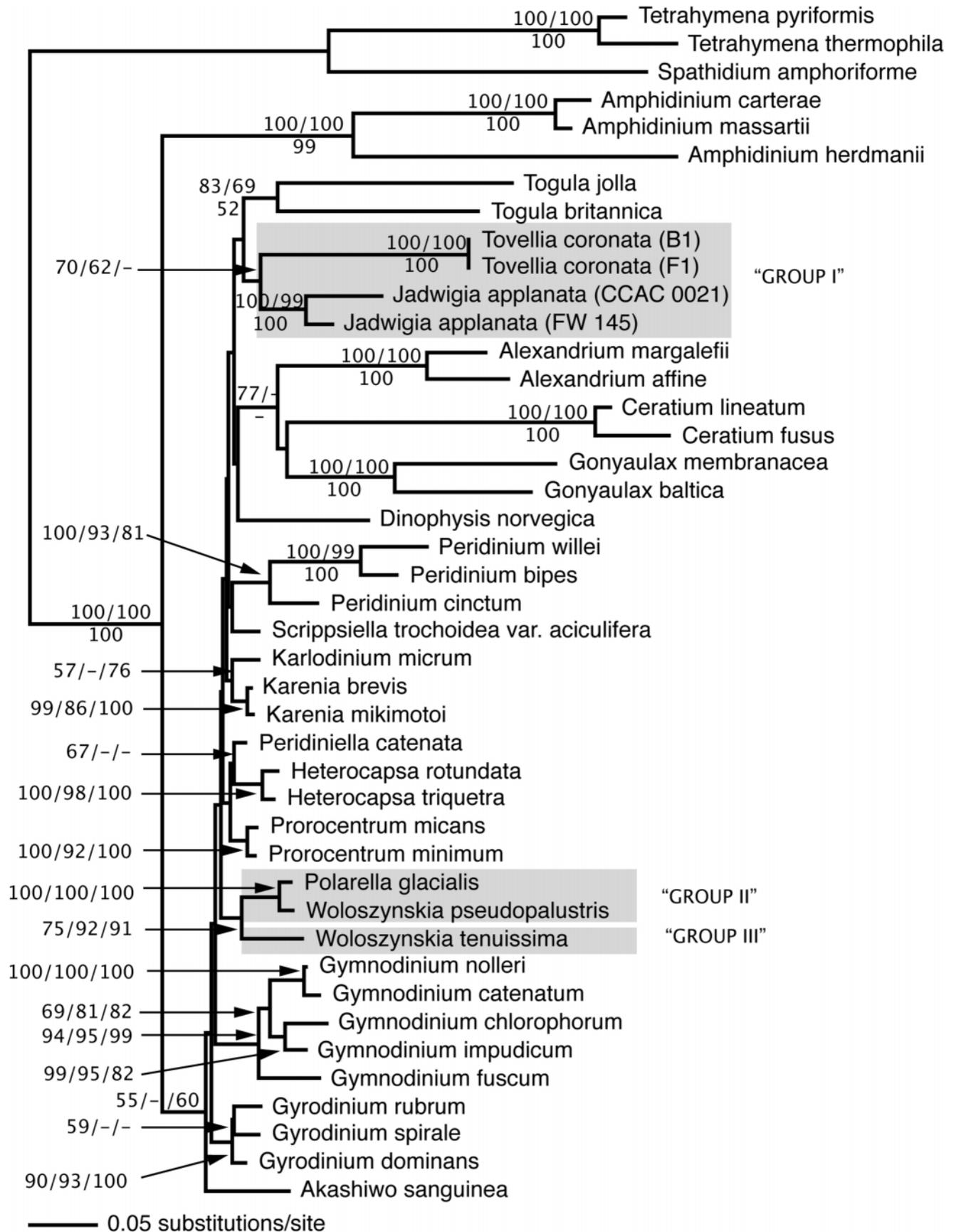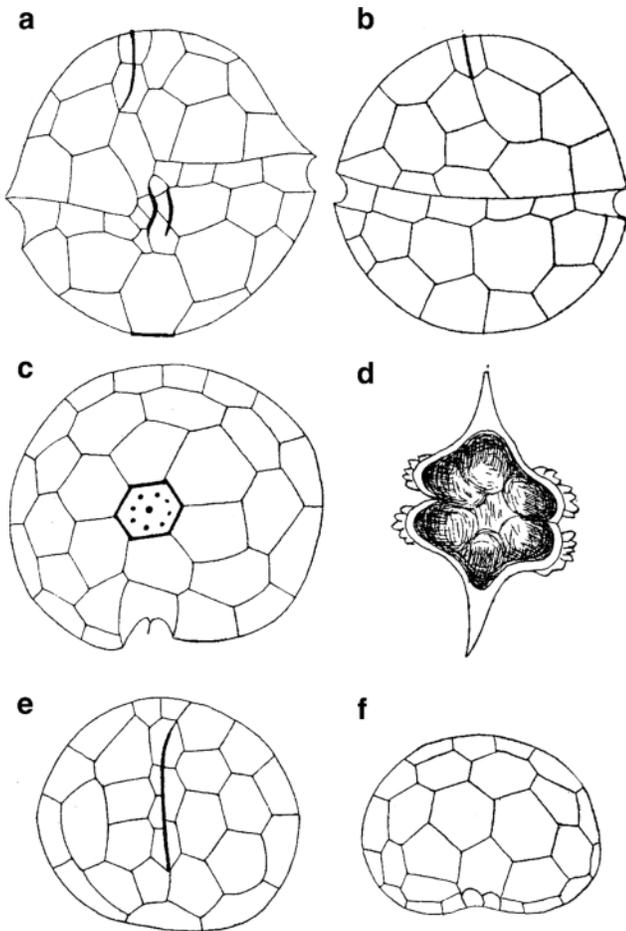
Fig. 38. The original drawings of Woloszynska (1917), illustrating *Gymnodinium coronatum* (Figs 38a–e), and the variety *glabra* (Fig. 38f). The latter differs in lacking the antapical hexagonal plate (compare with Fig. 38c). Fig. 38d illustrates the resting cyst.

Unknown species of *Tovellia: Woloszynskia tylota sensu* Bibby & Dodge

Bibby & Dodge (1972) reported on a dinoflagellate from a garden pond in Kent, England, identified by these authors as *Woloszynskia tylota*. Bibby & Dodge's material differs in cell shape and particularly in cyst morphology from the original description of *Gymnodinium tylotum* by Mapletoft *et al.* (1966), and it is very unlikely that it belongs to the same species. Mapletoft *et al.* provided no description of plates in their material, which they described as belonging to *Gymnodinium*. The material illustrated by Bibby & Dodge as figs 2, 3 (probably also fig. 4) certainly belongs to *Tovellia,* judging from the shape of the resting cyst. Its specific identity remains uncertain.

Jadwigia Moestrup, Lindberg & Daugbjerg *gen. nov.*

Dinoflagellata cellulas plerumque pentagonalibus aut hexagonalibus laminis obtectas habentia. Linea laminarum recta aut paulum curvata trans partem anteriorem cellulae currit, ab parte ventrali ad partem dorsalem cellulae. Linea laminarum serie laminarum pentagonalium utrinque circumcincta. Stigma extra chloroplastum, globuli stigmatis nudi. Hypnozygotae leves, rotundes.

Dinoflagellates in which the cells are covered with many usually pentagonal or hexagonal plates. The anterior end with a straight or slightly curved ALP that extends from the ventral to the dorsal side of the cell. The ALP is lined on each side by a row of pentagonal plates. The eyespot is extraplastidial, and individual eyespot globules are not surrounded by membranes. Hypnozygotes smooth, round.

ETYMOLOGY: The name is created in honour of Dr Jadwiga Woloszynska, whose exquisite work from the early part of the 20th century is a source of constant admiration.

TYPE SPECIES: *Jadwigia applanata* Moestrup, Lindberg & Daugbjerg *sp. nov.* (Figs 39–69).

Cellulae ovales – rotundae, fortiter dorsoventraliter complanatae. Epiconus et hypoconus paene eadem magnitudine, epiconus interdum paulo major. Cellulae plerumque 24–34 μm longae, 24–32 μm latae. Chloroplasti multi disciformes aurei-virides in peripheria cellulae siti. Nucleus allantoideus, praecipue in parte dextra cellulae. Linea anterior laminarum angustarum seriem laminarum pentagonalium utrinque habens. Linea anterior laminarum angustarum in parte ventrali ab parte sinistra cinguli una serie latitudinali laminarum separata, in parte dorsali 3–4 seriebus latitudinalibus laminarum. Cingulum latitudine unius cinguli remotum. Stigma ovalis, elongatum, in parte basali sulci sita. Laminae cristae ventralis et partium proximarum sulci crassae.

Cells oval-round, strongly compressed dorsoventrally. Epi- and hyposome of almost equal size, the episome sometimes marginally larger. Cells c. 24–34 μm long and 24–32 μm wide. Numerous golden-green discoid chloroplasts in the periphery of the cell. Nucleus sausage-shaped, mainly in the right side of the cell. Apical line of narrow plates (ALP) lined on each side by a row of pentagonal plates. ALP on the ventral side separated from the left part of the cingulum by one latitudinal row of plates, on the dorsal side by three to four latitudinal rows of plates. Cingulum displaced one cingulum width. Eyespot oval-elongate, located in the proximal part of the sulcus. Plates of the ventral ridge and nearby parts of the sulcus thicker than the other plates.

TYPE MATERIAL: A plastic-embedded fixation of culture CCAC 0021 has been deposited at the Botanical Museum, University of Copenhagen, C-AT-2377. Cells from the culture are illustrated in the present paper as Figs 44–69. Gene sequences are deposited at GenBank as number AY950447.

TYPE LOCALITY: small pond near Lochmühle, Germany.

REMARKS: The cell sizes given above are based on the following figures:

CCAC 0021: Cell length = 30.0 μm (s = 3.5 μm), cell width = 28.3 μm (sD = 3.6 μm) (n = 25);

FW 145: Cell length = 27.0 μm (s = 3.4), cell width = 23.6 μm (s = 3.1) (n = 22).

CYSTS: The spherical resting cysts (hypnozygotes) are illustrated in Fig. 49a, b. The cells are surrounded by a thick cell wall and remains of the gamete thecae are also present. Visible elements in the cell are chloroplasts, storage material and double eyespots (arrows), the latter a result of gamete fusion.

EXTERNAL STRUCTURE: Figs 50–53 illustrate cells seen in the SEM. The ALP is shown at low magnification in Figs 50–52 (arrowheads), and its detailed structure, including individual
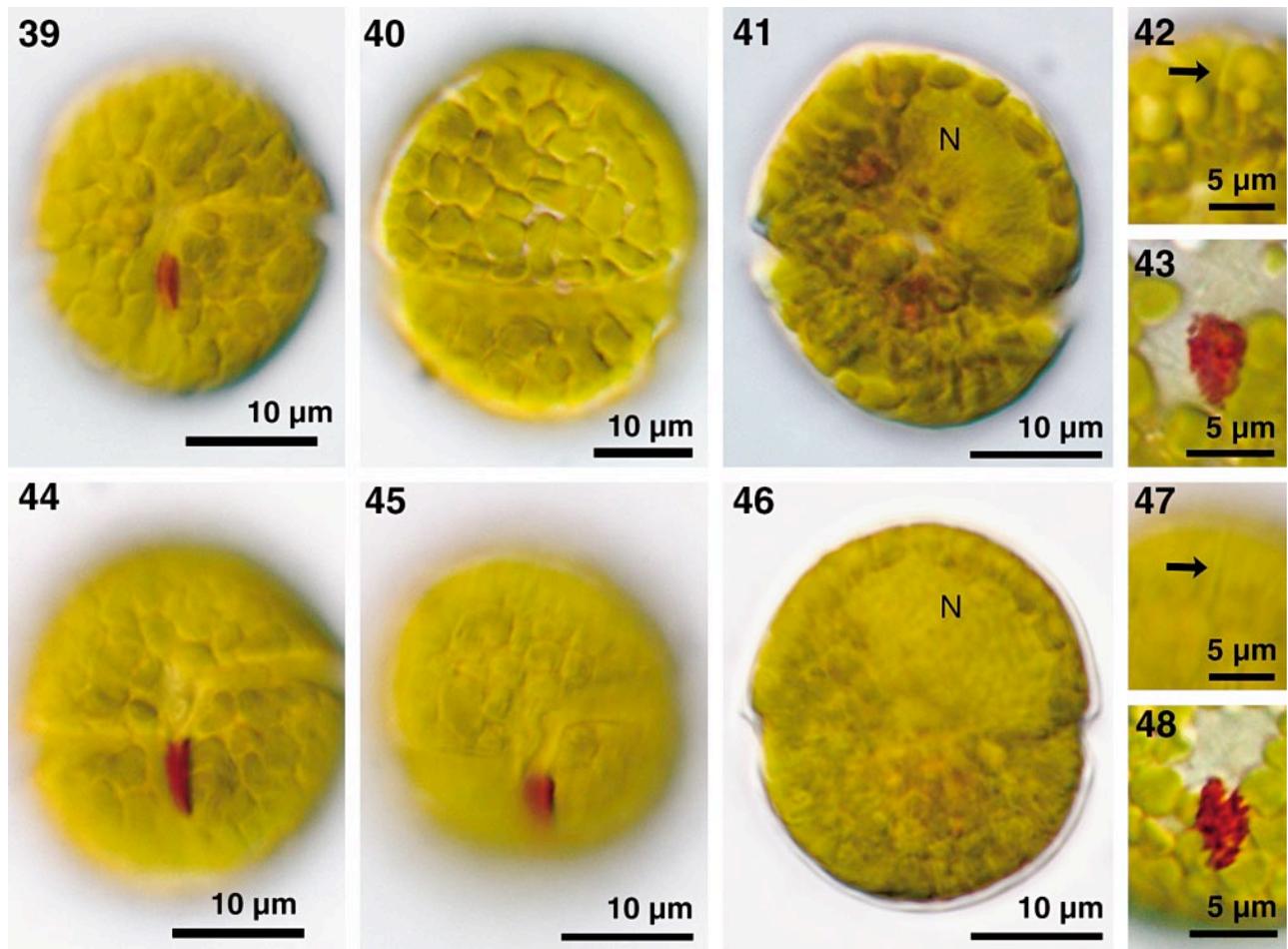
Figs 39–48. Differential Interference Contrast microscopy of *Jadwigia applanata* gen. et sp. nov. The ventral views of the almost spherical cells show the displaced cingulum, the elongate eyespot (Figs 39, 44, 45) and the ventral ridge (Fig. 45). The dorsal views show the discoid, peripheral chloroplasts (Fig. 40) and the sausage-shaped nucleus (N) (Figs 41, 46). The ALP is indicated by arrows in Figs 42, 47. The extraplastidial eyespot is shown in Figs 43, 48. Individual eyespot globules may be distinguished on the left in Fig. 43.
Figs 39–43: strain FW 145 from North America; **Figs 44–48:** strain CCAC 0021 from Germany.

plates, is visible at higher magnification in Fig. 53. The ventral ridge is seen as a bluntly triangular structure in the area between the two ends of the cingulum in Figs 50, 51.

INTERNAL STRUCTURE: Cells in longitudinal section are illustrated in Figs 54, 55 and in transverse section in Figs 56, 57. Although most of the nucleus is located in the right side of the cell (Figs 54, 57), part of it extends anteriorly along the dorsal side of the cell (Fig. 56) and another part extends into the hypocone (Fig. 55). The apical line of narrow plates (ALP) is shown in transverse section in Fig. 58, lined on each side

by amphiesmal plates. Further details of the ALP are illustrated in Figs 59–61, which show its very narrow plates, perforated by pores (Fig. 61) like the other amphiesmal plates. The ALP is also visible in the longitudinal section in Fig. 54 and in the two transverse sections (Figs 56, 57), almost opposite in Fig. 57 but closer to each other in Fig. 56. Adjacent plates always overlap, i.e. the sutures between adjacent plates are oblique (e.g. Fig. 58, left). This is seen in the tangential sections in Figs 59–61 as opaque areas along the sutures, especially in Fig. 60. The amphiesmal pores may at first sight be confused with the sutures between amphiesmal plates, but the pores differ by penetrating the amphiesmal plates at right angles (Fig. 58, right). The construction of the pores is unusual, and a literature search has shown that this structure has apparently not been described in dinoflagellates before. Figs 62, 63 illustrate the pores, whose lumen is taken up by a small cisterna (arrow in Fig. 63). Each cisterna is surrounded by the amphiesma vesicle (Fig. 63), but in contrast to trichocyst vesicles, the cisterna does not appear to extend into the cell. In other words, the pore cisternae appear to be located in depressions of the amphiesmal surface. In the cytoplasm below each pore is a complex vacuolar system that often contains

Table 4. Sequence divergence of *Tovellia glabra* and *T. coronata* based on LSU rDNA domain D1 (247 bp). Estimates of sequence divergence are based on 'uncorrected p' (above diagonal) and Kimura-2-parameter (below diagonal) using PAUP*. Strain numbers followed by GenBank accession numbers are given in brackets.

|	*Tovellia glabra*	*Tovellia coronata*
Tovellia glabra (1117/2, X61745)	—	25.7
Tovellia coronata (F1 and B1, AY950446, AY950445)	32.9	—

Figs 49–53. *Jadwigia applanata.*
 Fig. 49a, b. Light micrographs illustrating the spherical resting cyst. The arrows indicate the eyespot, which in Fig. 49b is clearly double.
 Figs 50–53. Scanning electron micrographs illustrating whole cell in ventral view (Fig. 50, and at higher magnification in Fig. 51) and in apical view (Fig. 52). The arrowheads indicate the ALP. The ALP is shown at higher magnification in Fig. 53, where individual plates of the ALP may be distinguished. d: dorsal side; v: ventral side.
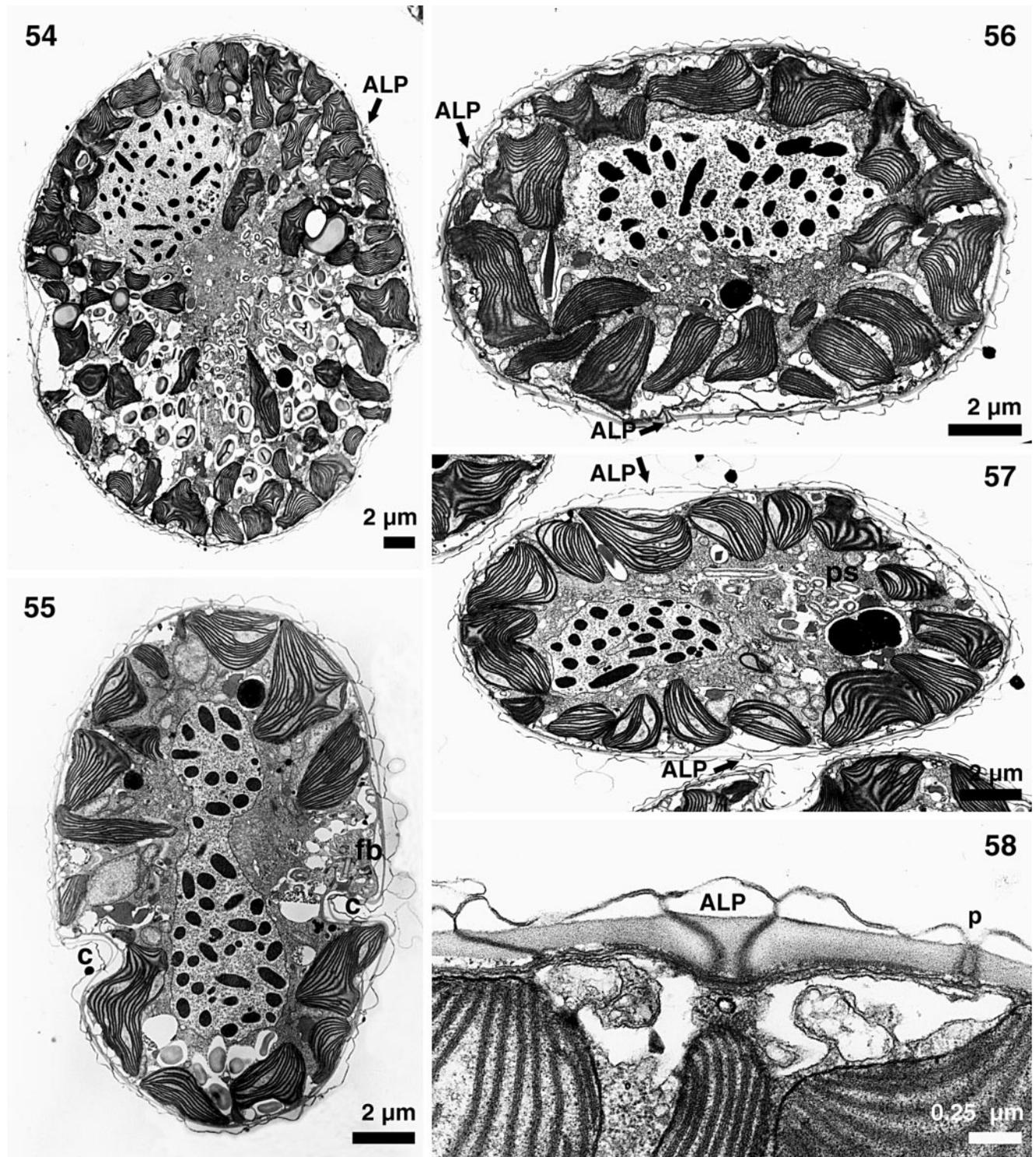
Figs 54–58. Ultrastructure of *Jadwigia applanata*.

Figs 54, 55. Longitudinal sections through the cell, Fig. 54 almost in the plane of the broad part of the cell (the ALP is visible on the right) and Fig. 55 through the narrow part of the cell. c: cingulum; fb: the two flagellar bases. Compare with the transverse sections (Figs 56, 57).

Figs 56, 57. Transverse sections through the flattened cell, Fig. 56 near the anterior end, Fig. 57 closer to the cingulum as indicated by the presence of a pusule system (ps). The ALP is visible on both cells, almost opposite in Fig. 57, closer to each other in Fig. 56.

Fig. 58. The ALP in transverse section, lined by more typical amphiesmal plates. The plate sutures are inclined to each other (left, see also Figs 64, 65). p: pore through amphiesmal plate.
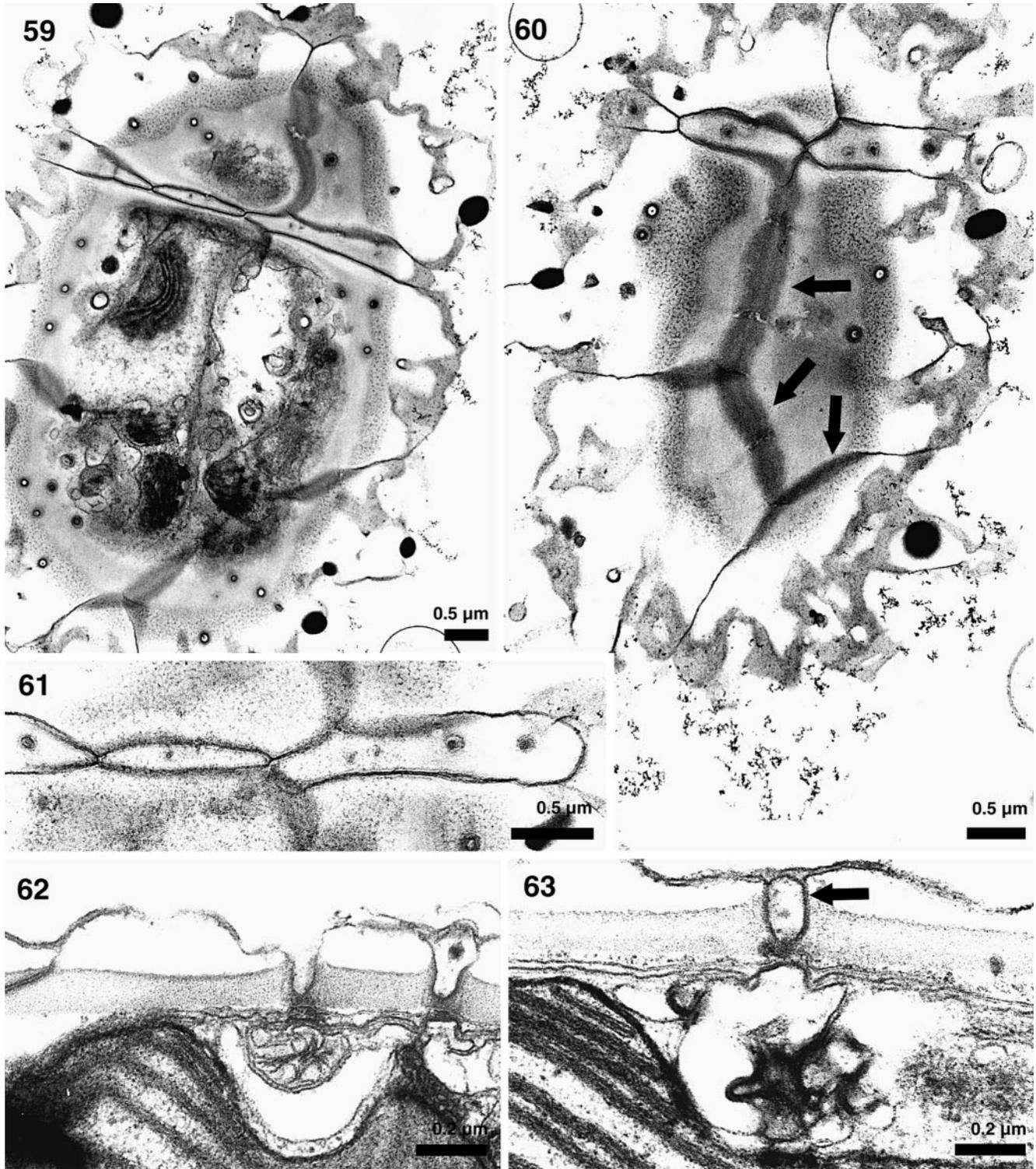
Figs 59–63. Ultrastructure of *Jadwigia applanata*.

Figs 59–61. Three sections selected from a series of tangential sections through the narrow plates of the ALP. Both the ALP and adjacent plates are perforated by pores. The oblique overlap between individual plates is particularly clear in Fig. 60 (arrows).

Figs 62, 63. Transverse sections through pores in the amphiesma. The pore lumen is filled with a cisterna (arrow), surrounded on the sides by the amphiesmal vesicles and on top by the cell membrane. It does not seem to penetrate the amphiesmal plate but the region of the cell beneath the pore is occupied by larger vesicles often containing membranous material.
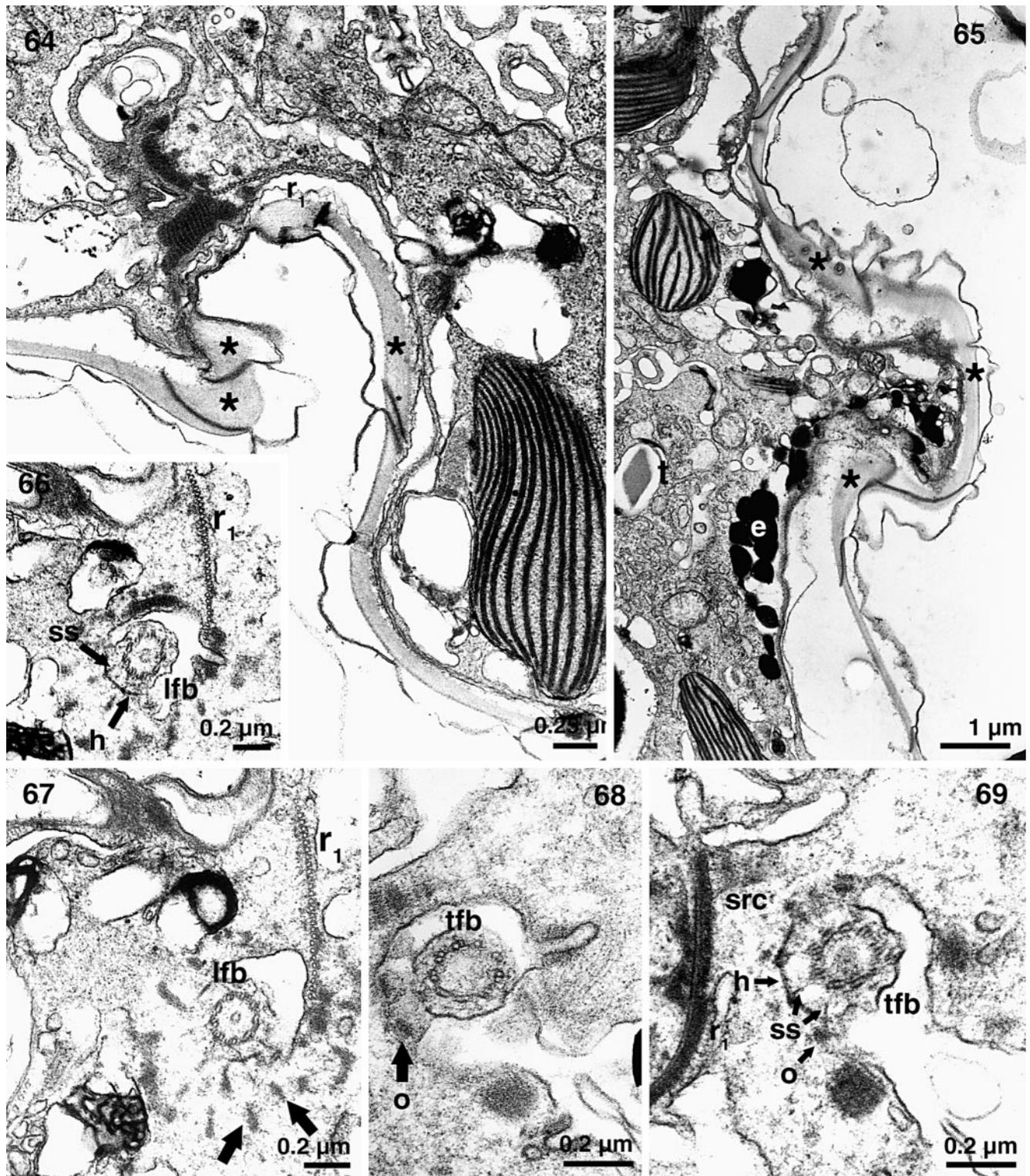
Figs 64–69. Ventral ridge region of *Jadwigia applanata*.

Figs 64, 65. Sections through the cingulum and the ventral ridge, illustrating the thick plates (asterisks) characteristic of this region. The extraplastidial eyespot (e) is visible in Fig. 65. r_1: flagellar root 1; t: trichocyst.

Figs 66, 67. Two adjacent sections from a series of transverse sections through the base of the longitudinal flagellum (lfb). Arrows: electron-opaque rods; h: 'hub'; ss: 'spokes'.

Figs 68, 69. Adjacent sections from a similar series through the base of the transverse flagellum (tfb). h: 'hub', o: opaque spheres; r_1: flagellar root 1; ss: 'spokes', src: the striated root fibre that extends from r_1.

membranous material. Externally each pore is covered by the plasmalemma (Fig. 63). The function of these complex pores obviously needs to be examined further.

One of the most characteristic features of *J. applanata* is the thickened plates of the ventral ridge and adjacent part of the cingulum, illustrated in Figs 64, 65. The thickness of the plates is somewhat variable but usually around 185–210 nm, whereas the normal plates measure about half that figure. Fig. 65 also illustrates the extraplastidial eyespot.

An ultrastructural description of clone FW 145 was provided by Roberts *et al.* (1995, as *Woloszynskia limnetica*). The sequence divergence between the German strain CCAC 0021 and the North American strain FW 145 is similar to that observed between different species within the genus *Ceratium* (Ellegaard *et al.* 2003), using the same DNA fragment. Because we have not been able to distinguish the two strains by light or electron microscopy we consider it premature to describe them as separate species, despite the fact that the DNA sequences indicate otherwise. The flagellar apparatus transition region of FW 145 contains a feature which Roberts *et al.* (1995) termed 'very unusual and to our knowledge not observed in other dinoflagellates' (Roberts *et al.* 1995, p. 956). Precisely the same structure was seen in our sections of CCAC 0021 from which the type material of *J. applanata* originates (Figs 66–69). It comprises a ring (the 'hub'), which interconnects the spokes that emanate from each of the flagellar triplets (Fig. 66, the longitudinal flagellum, and more clearly in the transverse flagellum in Fig. 69). On the outside of the hub is a row of opaque spheres (Figs 68, 69). Within the cell, the region is characterised by a series of electron-opaque rods, illustrated particularly well in the longitudinal flagellum in Figs 66, 67 (arrows in Fig. 67).

Family Tovelliaceae Moestrup, Lindberg & Daugbjerg fam. nov.

Dinoflagellata tenuiter thecata, autotrophica aut heterotrophica, habentia stigma globulis sine membraneis et non in chloroplasto sitis compositum.

Hodie due genera, *Tovellia* et *Jadwigia*.

Autotrophic or heterotrophic dinoflagellates with a thin theca and an eyespot composed of pigment globules not bound by membranes and not located in a chloroplast.

Presently with two genera, *Tovellia* and *Jadwigia*.

Family and ordinal affiliations of *Tovellia* and *Jadwigia*

We are creating a new family for species of dinoflagellates whose cells contain an eyespot, which is neither membrane bound nor part of a chloroplast. This is a very unusual type of eyespot in algae and its uniqueness is supported by DNA sequencing of the organisms available: *Tovellia coronata, Jadwigia applanata* (this study) and *Esoptrodinium* (A.J. Calado, unpublished observations). A micrograph of an organism identified as *K. campylops* by Wilcox (1989, fig. 3) illustrates the same type of eyespot and this organism almost certainly belongs in the same family. *Katodinium campylops* is not the type species of *Katodinium,* however, and its taxonomy is presently unclear.

It is likely that a separate order must be erected for the Tovelliaceae, but we prefer to await data on additional species of naked or thin-walled dinoflagellates before deciding on this issue.

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