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Studies on woloszynskioid dinoflagellates II: On *Tovellia sanguinea* sp. nov., the dinoflagellate responsible for the reddening of Lake Tovel, N. Italy

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The organism responsible for the former annual reddening of Lake Tovel in the Italian Alps (up to 1964) has been identified and studied in detail. Considerable confusion exists regarding the identity of this organism, and the detailed description by Baldi in 1941 is now believed to be based on more than one organism. Baldi's red and green forms appear to be two different organisms, both of which have now been isolated into unialgal culture and studied using light microscopy, electron microscopy, and sequencing of the large subunit of ribosomal DNA (LSU rDNA). The organism has been found in three lakes in the area, but only in Lake Tovel have conditions allowed for reddening of the water during summer. The name of the organism believed to be the cause of the reddening, *Glenodinium sanguineum* Marchesoni, used in numerous publications, is an illegitimate homonym of *G. sanguineum* H.J. Carter, and the organism is described here as a new species, *Tovellia sanguinea* sp. nov., the seventh species of the newly described genus *Tovellia*. *T. sanguinea* is closely related to the other red-coloured species of *Tovellia*, *Tovellia coronata* (previously known as *Woloszynskia coronata*) but differs in several morphological features, notably the chloroplast arrangement, and in LSU rDNA sequence divergence (11–12%). Cells preserved from Lake Tovel during a reddening phenomenon in 1938 have been re-examined by scanning electron microscopy and agree morphologically with the new isolates. *Tovellia sanguinea* is a species of oligotrophic or mesotrophic–oligotrophic cold-water lakes, in which the average summer temperature does not exceed 15°C. It occurs on both calcareous (as in Lake Tovel) and non-calcareous substrata (as in the other two lakes).

Key words: Dinophyceae, freshwater phytoplankton, *Glenodinium sanguineum*, red tide, *Tovellia*, *LSU rDNA*, phylogeny

Introduction

Lake Tovel is a small lake in the Italian Alps renowned for the red colouring of its waters during summer (Fig. 1). The lake is situated in the Brenta Adamello Natural Park and the red colouring has been a major tourist attraction in the area. Disappearance of the phenomenon after 1964 was the subject of much controversy (Cavalca *et al.*, 2001) and finally led to a major interdisciplinary project (SALTO: Studio al mancato Arrossamento del Lago di Tovel 2001–2004), during which the possible causes of bloom disappearance were investigated.

Reddening of the water was caused by a dinoflagellate, studied in detail by Baldi (1941) and known as *Glenodinium sanguineum* Marchesoni. It was discovered during the SALTO project that the situation in the lake is complex and at least three very similar dinoflagellates are present in the lake (Flaim *et al.*, 2004). All three species have now been established in unialgal culture, and they are being examined in detail by light and electron microscopy, combined with DNA nucleotide sequencing.

In the original, very detailed, description of '*Glenodinium sanguineum*', Baldi (1941) described a red and a green form in its life cycle, but we now believe that these belong to different species. Two of the species established in culture resemble the green form, and the third resembles the red form (Flaim *et al.*, 2004). The two green taxa appear to have dominated since 1964, while the red is presently very rare. The underlying causes for this change, which may be related to a change of nutrient inflow to the lake, were discussed by Flaim *et al.* (2004).

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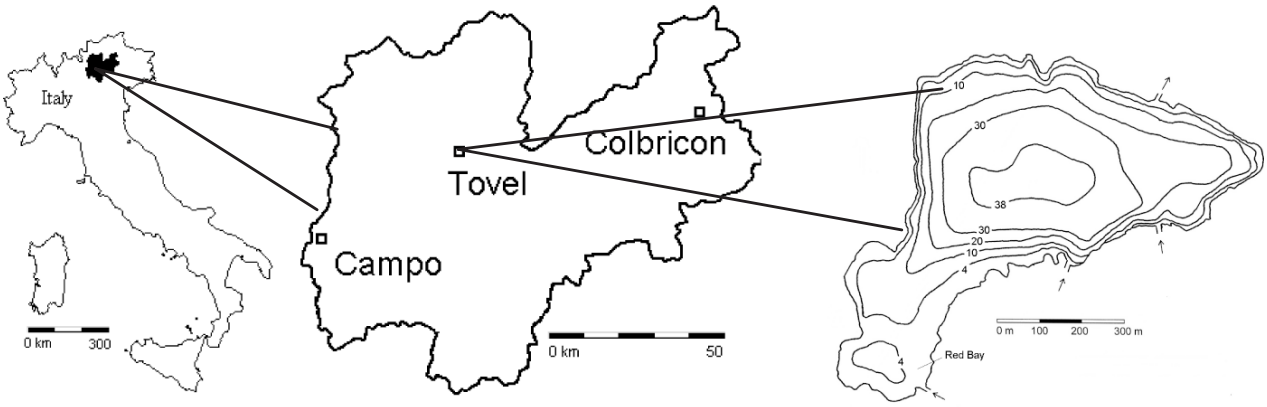


Fig 1. Location of the three lakes in the Trentino Province in Northern Italy: Lake Tovel, Lake Campo and Lake Colbricon, and bathymetric map with Red Bay in Lake Tovel. Arrows indicate inflows and outlets.

The three taxa belong to three different genera. *Tovellia* Moestrup, Lindberg & Daugbjerg was described recently (Lindberg *et al.*, 2005) and two additional genera will be described in detail in separate publications. In this paper, we provide information on the species we believe to be responsible for the former red colouring of Lake Tovel, *Tovellia sanguinea* sp. nov.

Materials and methods

Lake Tovel sediments were collected in autumn 2003 and winter 2004 by scraping emerged rocks when a drop in the water level exposed the Red Bay (Fig. 1). Lake Campo sediments were collected in September 2003 with an Ekman grab in various parts of the lake and combined. Lake Colbricon sediments were collected in October 2004 in the same way. All sediments were stored in the dark at 4°C until processed. They were sonicated in a MT Branson 1510 sonicator (Danbury, CT, USA), filtered through 100, 50 and 20 µm sieves, re-sonicated and concentrated by SPT centrifugation following the protocol of Bolch (1997). Cysts were then isolated under a stereomicroscope using a micropipette. Isolated cysts were washed with sterile DY IV medium (Andersen *et al.*, 1997), placed in 24-well plates (1 cyst/well), covered with aluminium foil and kept at 4°C for at least 1 month for maturation. They were allowed to germinate in a growth chamber at 13°C with a 16:8 h light–dark cycle and 75 µmol m^{-2} s^{-1} of cool white fluorescent light. Length and width of vegetative cells and cysts were measured using Leica Image Manager software (Leitz, Wetzlar, Germany).

Live vegetative cells and cysts were examined from a 20-µm net plankton sample collected from Lake Campo in August 2003, and cultures were established from cysts collected September 2003. Live vegetative cells from Lake Colbricon were studied in plankton samples collected during July 2004, and cysts were collected in October 2004.

Plankton samples collected and preserved by Baldi from the Red Bay in August and October 1938 were also analysed.

Light microscopy

Light microscopy was performed using a Diaplan microscope fitted with a DFC300FX digital camera (Leica, Wetzlar, Germany) or an Olympus AX70 Provis microscope (Olympus, Tokyo, Japan) fitted with a Zeiss Axiocam HR digital camera (Zeiss, Oberkochen, Germany).

Scanning electron microscopy (SEM)

100 µl culture was fixed by adding 100 µl fixative consisting of 1 part saturated $HgCl_2$, 1.5 parts 4% OsO_4 and 1.5 parts distilled water (Pàrducz, 1967). Cells were allowed to settle on poly-L-lysine covered circular coverslips for 20 min and then washed in two changes of distilled water, 30 min in each change. They were dehydrated in a graded ethanol series and critical point dried using a Baltec CPD-030 critical point drier (Bal-Tec, Liechtenstein). The coverslips were subsequently mounted on aluminium stubs using carbon-discs and coated with Pt before being examined in a JEOL J-6335 field emission scanning electron microscope (JEOL, Tokyo, Japan), at the Zoological Museum, University of Copenhagen. Baldi's samples from 1938 had been fixed in 4% formalin, and we processed them for SEM as described in Flaim *et al.* (2004).

Transmission electron microscopy (TEM)

The strain from Lake Campo, maintained in medium DYIV at 15°C, was mixed 1:1 (v:v) with

cold 2% glutaraldehyde in 0.2 M phosphate buffer, pH 7.25. The cells were fixed for 85 min at 4°C, centrifuged into a pellet which was retained throughout the following steps of the procedure, and then washed in three changes of cold buffer over 105 min. They were post-osmicated in cold 2% osmium tetroxide in 0.1 M phosphate buffer for 75 min, rinsed briefly in buffer and dehydrated in a cold ethanol series: 20 min in each change of 30, 50, 70 and 96% ethanol. Dehydration was completed at room temperature: 40 min in 99% ethanol (two changes) followed by 10 min in propylene oxide (two changes), and a 1:1 mixture of propylene oxide and Spurr's resin overnight. Embedding was in Spurr's resin: 2 h at room temperature followed by polymerization in a fresh change of resin in an oven at 70°C overnight.

Serial sections were prepared on a LKB 8800 ultramicrotome (LKB, Bromma, Sweden), mounted on slot grids, stained for 30 min in uranyl acetate at room temperature, followed by 30 min at room temperature in lead citrate. The sections were examined and photographed in a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) at the Biological Institute, University of Copenhagen.

LSU rDNA amplification of the flagellated stage of Tovellia sanguinea

Clonal cultures of the flagellated stage of *T. sanguinea* from Lake Tovel and Lake Campo were treated slightly differently prior to PCR amplifications. Since cultures from Lake Tovel never grew very dense, single cells from a clonal culture were isolated using a micropipette under the dissecting microscope. The cells were transferred to a 0.5-ml PCR tube and used as template in PCR reactions. The clonal culture from Lake Campo grew better and we used pelleted material as template directly in PCR amplifications. Hence, total genomic DNA was not extracted prior to PCR amplification. PCR set-up and thermal cycle conditions were as outlined in Hansen *et al.* (2003). Weak PCR bands of appropriate length were excised from a NuSieve agarose gel under UV-light. The excised bands were re-suspended in 300 µl double-distilled water and incubated at 80°C for approximately 30 min. 1 µl of the re-suspended PCR products was used as template for nested PCR reactions using the following terminal and internal primer combinations: D1R–D3B and D3A–28-1483R (for primer sequences, see Daugbjerg *et al.*, 2000). The reaction conditions were the same as before but the PCR program consisted of only 18 cycles and the annealing temperature was 55°C.

LSU rDNA amplification from single cysts of Tovellia sanguinea

Tovellia sanguinea produces cysts with relatively thick cell walls. These proved very resistant to the disruption methods commonly used to break the cyst wall, such as liquid nitrogen (Bolch, 2001) or boiling at >90°C. Instead we physically ruptured the isolated cysts using a sterile needle under the dissecting microscope. Punctured cysts and contents were transferred to 0.5-ml PCR tubes containing 5 µl double-distilled water. Single cysts were amplified by hot start PCR using primers D1R and 28-1483R. Following the initial denaturation step at 94°C for 10 min, 0.2 µl Taq polymerase was added to each tube and the LSU rDNA fragments were amplified using the conditions described for PCR of the clonal cultures.

Sequence determination of LSU rDNA

The PCR products from cysts and flagellated stages were purified using the QIAquick PCR purification kit (Qiagen) and quantified with a spectrophotometer. The Big Dye Terminator Cycle sequencing Ready Reaction kit (PE Biosystems, Foster City, California, USA) was used to determine the partial LSU with the following primers: D1FR, 28-1483R, D2R, D3B and D3A (Hansen *et al.*, 2000). Cycle sequence reactions were run on an ABI PRISM 377 DNA sequencer (Perkin Elmer, Foster City, California, USA).

The LSU rDNA sequence of the *Tovellia sanguinea* cyst isolated from Lake Colbricon was determined at Istituto Agrario (Italy) whereas the LSU rDNA sequences of the flagellated stage and cyst stage from material collected in Lake Tovel and Lake Campo, respectively, were determined at Biological Institute (Denmark). The LSU rDNA sequences determined in this study have been deposited in GenBank and accession numbers are given in Table 1.

Table 1. GenBank accession numbers for LSU rDNA of *Tovellia sanguinea* from three Italian lakes.

Location and sample	Accession number
Lake Tovel and Lake Campo:	
Single cysts and cultured flagellates[a]	DQ 320627
Lake Colbricon:	
LSU rDNA sequence from a single cyst	DQ 320628

[a] The LSU rDNA sequence of cysts and flagellated stages sampled in Lake Tovel and Lake Campo were identical, hence only a single GenBank accession number is provided.

Alignment and phylogenetic analyses of nuclear-encoded LSU rDNA

The five LSU rDNA sequences determined from cysts and flagellated stages of *T. sanguinea* originating from the three Italian lakes were compared with 40 dinoflagellate LSU rDNA sequences retrieved from Genbank. Two species of ciliates (viz. *Tetrahymena pyriformis* (Ehrenberg) A. Lwoff and *T. thermophila* Nanney & McCoy) were added to the data matrix and subsequently used for outgroup-rooting. The alignment incorporated information from the secondary structure of the LSU rDNA gene as suggested by de Rijk *et al.* (2000). The alignment was edited manually using MacClade v. 4.06 (Maddison & Maddison 2003) and consisted of 1,440 base pairs (bp), including introduced gaps. Compared with the LSU rDNA model suggested by Lenaers *et al.* (1989) our rDNA fragment started 36 bp upstream of domain D1, and stopped 10 bp within domain D6. Due to ambiguous alignment of domain D2 we excluded this fragment (361 bp) from the phylogenetic analyses. Hence, a total of 1,070 bp were analysed with Maximum parsimony (MP) and Neighbour-joining (NJ) methods using PAUP* v. 4b10 (Swofford, 2003), and Bayesian analysis (BA) using MrBayes v. 3.1 (Ronquist & Huelsenbeck, 2003). MP analyses were completed with 1,000 random additions in heuristic searches and a branch-swapping algorithm (tree-bisection-reconnection). All characters were unordered and equally weighted. Gaps were treated as missing data. MP bootstrap analyses included 1,000 replications. We used Modeltest v. 3.6 (Posada & Crandall, 1998) to search for the best model for the LSU rDNA sequences using hierarchical likelihood ratio tests. The best-fit model was TrN + I + G (Tamura & Nei, 1993) with among sites rate heterogeneity ($\alpha = 0.6090$), an estimated proportion of invariable sites (I = 0.2523), and two substitution-rate categories (A–G = 2.9199 and C–T = 6.7603). Base frequencies were set as follows A = 0.289, C = 0.1749, G = 0.2652 and T = 0.2709. The TrN + I + G model was applied to compute dissimilarity values and we used the resulting distance matrix to build a tree with the NJ method. NJ bootstrapping included 1,000 bootstrap replications. Bayesian analysis was conducted using a GTR substitution model with base frequencies and substitution rate matrix estimated from the data. We used 1 million Markov chain Monte Carlo (MCMC) generations with four parallel chains (one cold and three heated) and a tree sampled every 50 generations and a prior burn-in of 20,000 generations equalling 400 trees. The log likelihood value converged at a value of approximately –11,915. The 19,600 sampled trees were imported into PAUP* and a majority rule (50%) consensus tree was constructed.

Observations

Tovellia sanguinea sp. nov.

Cellulae 24 μm longae et 19 μm latae. Et epiconus et hypoconus plus minusve conici fere in amplitudine aequales. Cingulum latitudine una dispositum. Cellulae plerumque lateritiae causa guttarum numerosarum manifestarumque rubrarumque. Chloroplasti flavovirentes ex centro cellulae radiantes. Nucleus posticus. Stigma prominens iuxta sulcum locatum. Amphiesma ex catellis numerosis tenuibus multangulisque composita. Extrema antica catillorum postcingularium in cingulum extensa. Linea catillorum angusta anticaque super partem anticam cellulae extensa plus minusve latus mediventrale versus ad latus medidorsale versus. Catillus antapicalis plerumque sexangularis interdum leviter ex cellula protrudens. Cystae lateritiae et typicae cornibus bipolaribus et constructione centrali interdum secus paracingulum torulosae.

Terra typica: Lacus Campo, Trentino, Italy.

Cells on average 24 μm long and 19 μm wide. Epicone and hypocone usually more or less conical, almost equal in size. Cingulum displaced about one cingulum width. Cells usually bright red due to the presence of numerous distinctly red-pigmented bodies. Chloroplasts yellow-greenish, radiating from the central part of the cell. Nucleus posterior. A prominent eyespot located next to the sulcus. The amphiesma comprises numerous thin polygonal plates. The anterior ends of the postcingular plates extend into the cingulum. A narrow apical line of plates extends over the anterior part of the cell, approximately in a mid-ventral to mid-dorsal direction. Antapical plate usually hexagonal, sometimes protruding slightly from the cell. Cysts are bright red in colour and typically have bipolar horns and a central constriction (paracingulum), sometimes with knobs along the paracingulum.

TYPE LOCALITY: Lake Campo, Trentino, Italy.

TYPE MATERIAL: The plastic block containing cells of the strain from which the TEM illustrations have been made, has been deposited at the Botanical Museum, Copenhagen (C) as No. A-2383.

Description of motile cells as seen in the light and scanning electron microscope

Table 2 provides information on the size of vegetative cells, gametes and resting cysts. In old or ageing cultures, small (putative gametes, Fig. 5)

Table 2. Mean cell measurements (μm) for vegetative cells, putative gametes, and resting cysts of *Tovellia sanguinea*.

	Vegetative cells		Gametes		Resting cysts (sediment)			Resting cysts (culture)		
	l	w	l	w	l[a]	w	d	l[a]	w	d
Mean	24	19	13	10	37	27	21	32	22	20
SD	2.5	4.1	1.2	1.5	3.7	2.9	3.1	2.4	1.8	1.5
n	112	112	10	10	72	83	11	100	106	6

Abbreviations: l: maximum length; l[a]: length from horn tips; w: maximum width; d: depth; SD: standard deviation; *n*: number of cells measured.

and large cells (putative planozygotes, not shown) occurred, which measured 12–15 μm and 26–43 μm in length, respectively.

Although usually more or less conical, the epi- and hypotheca were sometimes hemispherical (Figs 2–9). The hypotheca occasionally showed a slight antapical protrusion although this feature was generally more prominent in SEM (Fig. 9). The cingulum was median or slightly sub-median. The bright-red bodies responsible for the red colour of the cell are visible in Figs 2–4 and 6. The chloroplast(s) radiate from the central part of the cell but this was often obscured by the red pigment bodies (Fig. 6). The eyespot was usually prominent (Fig. 2).

The thin, polygonal plates surrounding the cell were sometimes visible in the light microscope using x100 oil-immersion phase contrast microscopy (not shown), and empty thecae of ecdysed cells are visible at low magnification in Fig. 7. The epi- and hypotheca each comprised *c*. 30 plates, each plate measuring 2–4 μm across. The number of plates varied, however, and the epi- and hypotheca of cells from Lake Colbricon usually comprised 35–40 plates (not shown).

All cells had a distinct antapical plate (Fig. 11), positioned at the end of the antapical protrusion (Figs 9, 16). TEM observations revealed this plate to be considerably thicker than the other plates (see below). The cingulum comprised about 20 plates, but the exact numbers have not been determined. The anterior part of the 'postcingular' series of plates curved smoothly into the cingulum (Fig. 9). The sulcus comprised 5–7 small platelets (not shown). A significant feature was the presence of a so-called ALP (apical line of plates) *sensu* Lindberg *et al*. (2005). This delicate structure measured about 4.5–5.6 μm in length and comprised about five narrow plates, each plate measuring *c*. 0.2 μm across and perforated by pores of about 65 nm in diameter. The ALP was bordered on each side by 3–4 platelets, each measuring *c*. 0.5 μm across (Figs 10, 12, 13, 16b). Considerable length variation was observed in the ALP. Thus in Lake Colbricon specimens it was up to 10 μm long and bordered by up to 9 platelets (not shown).

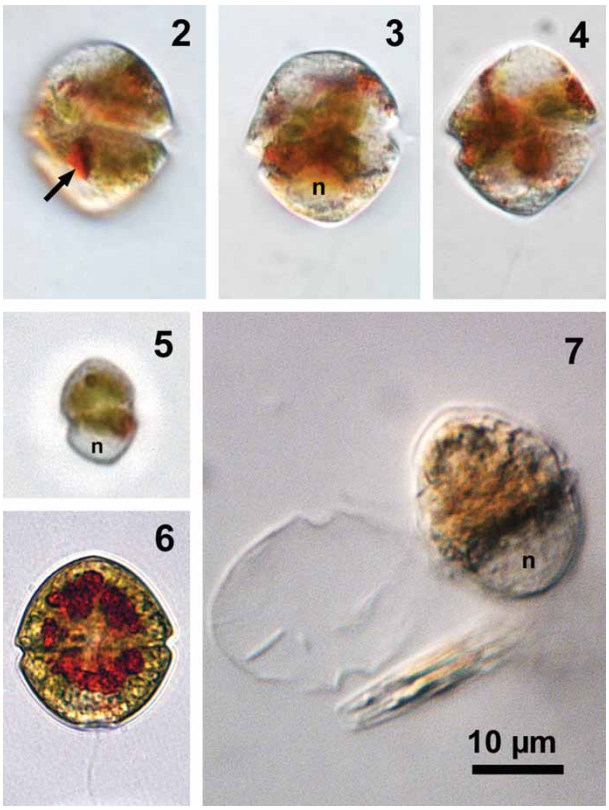
There was no major morphological difference between the preserved material collected by Baldi during the reddening phenomenon in Lake Tovel in August 1938 and recent material. Cells from 1938 had the same type of ALP, number of plates, arrangement of cingular plates and the same appearance of the antapical plate (Figs 12–15).

Cysts

Cysts from sediment samples displayed two bipolar horns and a central constriction, the paracingulum (Figs 17–23), but with considerable variation in morphology, especially horn length. Some specimens also had protuberances or knobs situated along the border of the paracingulum (Figs 17, 23). Cysts had a distinct red colour and usually contained one dark red accumulation body (Figs 18, 20). Cysts produced in culture showed considerably less variation in morphology; horn formation and paracingulum were less pronounced, and knobs along the paracingulum were never observed (Fig. 19). Cysts produced in culture were also consistently smaller (32 μm in length) than wild-type cysts (37 μm, Table 2) because of less pronounced horn formation. The cysts in the plankton samples collected by Baldi in October 1938 were very similar to those produced in culture (Figs 21, 22).

Transmission electron microscopy: general features of the cell

A longitudinal section through the cell is reproduced in Fig. 24. It illustrates the main features of the cell, including the posterior nucleus (n) with the nucleolus, numerous chloroplast profiles, the central pyrenoid complex (py), some large opaque aggregates (o) in the epicone, and the eyespot (e) just below the flagellar insertion area. The thick, antapical plate (ant) is also visible (compare with SEM, Fig. 9, also Fig. 16). The area between the plate and the nucleus is occupied by a small number of opaque vesicles, a very unusual feature, and this area is usually devoid of chloroplasts. A canal of the pusule complex is also present in the figure (pu). Its interior surface is coated by a



Figs 2–7. Light micrographs of *Tovellia sanguinea* sp. nov. Figs 2–5. Cultured material based on germinated cysts from Lake Tovel. The stigma is visible in Fig. 2 (arrow), and the posterior nucleus (n) in Fig. 3. Note the variations in epitheca shape, hemispherical in Fig. 3 and conical in Fig. 4. Fig. 5. Putative gamete. Fig. 6. Cell from Lake Campo filled with red pigment aggregates. Fig. 7. Cell from a sample collected by Baldi August, 1938 in Lake Tovel. Note the posterior nucleus and the empty theca to the left.

layer of opaque bodies, which are barely visible at this low magnification. Several trichocyst profiles are also present (tr). The cell is more or less rounded in transverse section (Fig. 25).

The red colour of the cell

The cells' visible red colour is caused by pigment globules, usually present as large, conspicuous aggregates (Figs 24–26), sometimes as more numerous, smaller bodies (Fig. 27). The large aggregates may appear almost brain-like (Fig. 26) and reach a size of 2–3 µm. Three such aggregates are visible in Fig. 24.

The chloroplasts

The characteristic appearance of the chloroplasts is illustrated in Figs 28–30. The chloroplasts join together centrally in the cell (Fig. 28) and from this area extend as long narrow bands toward the cell periphery, taking up much of the cytoplasmic space between the posterior nucleus and the anterior end of the cell. Centrally the chloroplasts contain a morphologically distinct area interpreted as a pyrenoid system (Fig. 30). The pyrenoid region has fewer thylakoid lamella but each lamella possesses two or three thylakoids (Fig. 29). The pyrenoid-bearing regions of the chloroplasts are located rather close together; giving the appearance of a somewhat loosely aggregated compound pyrenoid system. Connections are sometimes visible between the pyrenoids (Fig. 28) and it is possible that the chloroplasts form a single, very complex, system.

The trichocysts

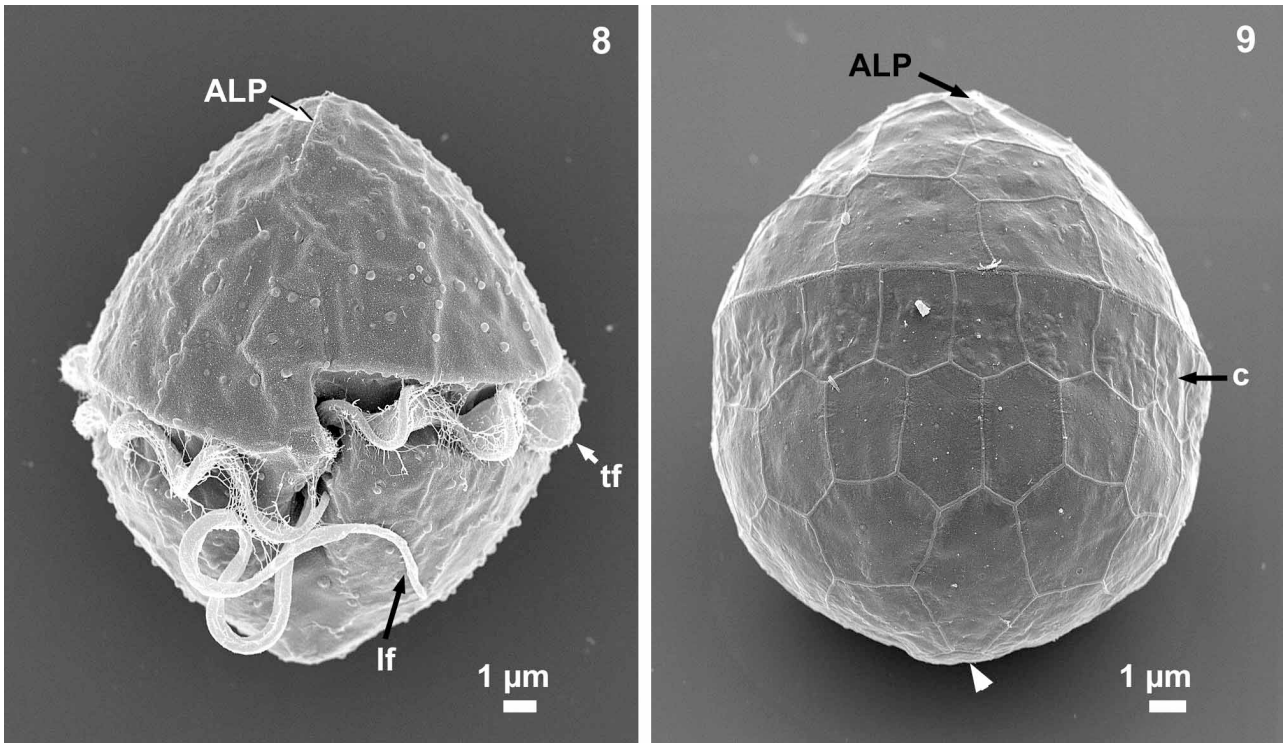
While the trichocysts did not appear to differ in structure from the usual dinoflagellate trichocyst, a somewhat unexpected feature was the common presence of three membranes around each trichocyst vesicle (Fig. 31). At higher magnification the two outermost membranes were seen to be continuous with endoplasmic reticulum (ER) in the area near the trichocyst (Fig. 31). The ER cisterna does not ensheath the trichocyst vesicle throughout its length; part of the vesicle is naked. The outermost of the three membranes often bears ribosomes.

The cell posterior

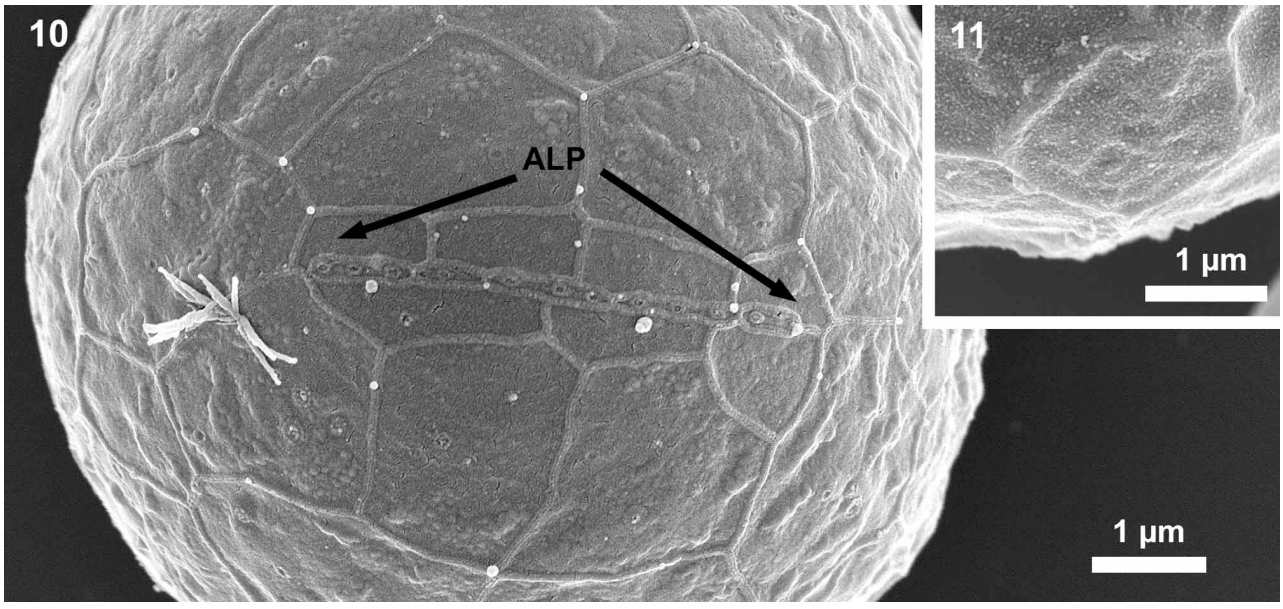
This is one of the most unusual parts of the cell in *T. sanguinea* (Figs 32–34). As mentioned above, the cell is bounded by a posterior antapical plate and Fig. 32 shows this characteristic, very thick plate, the only thick amphiesma plate in *T. sanguinea*. It also illustrates the opaque vesicles that abound in the area immediately above the plate. There may be high numbers of vesicles: 20 profiles are visible in Fig. 33.

Food uptake, the peduncle

A complex opaque body within a vacuole is shown in Fig. 34, but whether it represents a food vacuole is not known. However, this is likely, considering that a peduncle is a prominent part of the cell (Figs 35–37). The peduncle microtubules (msp) emerge from the cell interior and extend towards the so-called ventral ridge of the cell, accompanied by numerous vacuoles with opaque contents (pv, Figs 35, 36). The microtubules pass close to the flagellar bases and may be seen in transverse section in Fig. 37, which also illustrates the two basal bodies (LB and TB), the flagellar canals (LFC and TFC), and two flagellar roots, r_3 and r_4. The latter root is seen as a dense area next to the so-called striated root connective (src), which interconnects r_4 and the broad root associated



Figs 8, 9. Scanning electron micrographs of *Tovellia sanguinea*, seen in ventral (Fig. 8) and dorsal view (Fig. 9). ALP, anterior line of narrow plates; c, cingulum; tf, transverse flagellum; lf, longitudinal flagellum. Arrowhead in Fig. 9 indicates posterior plate (cf. Figs 16, 24, 32).

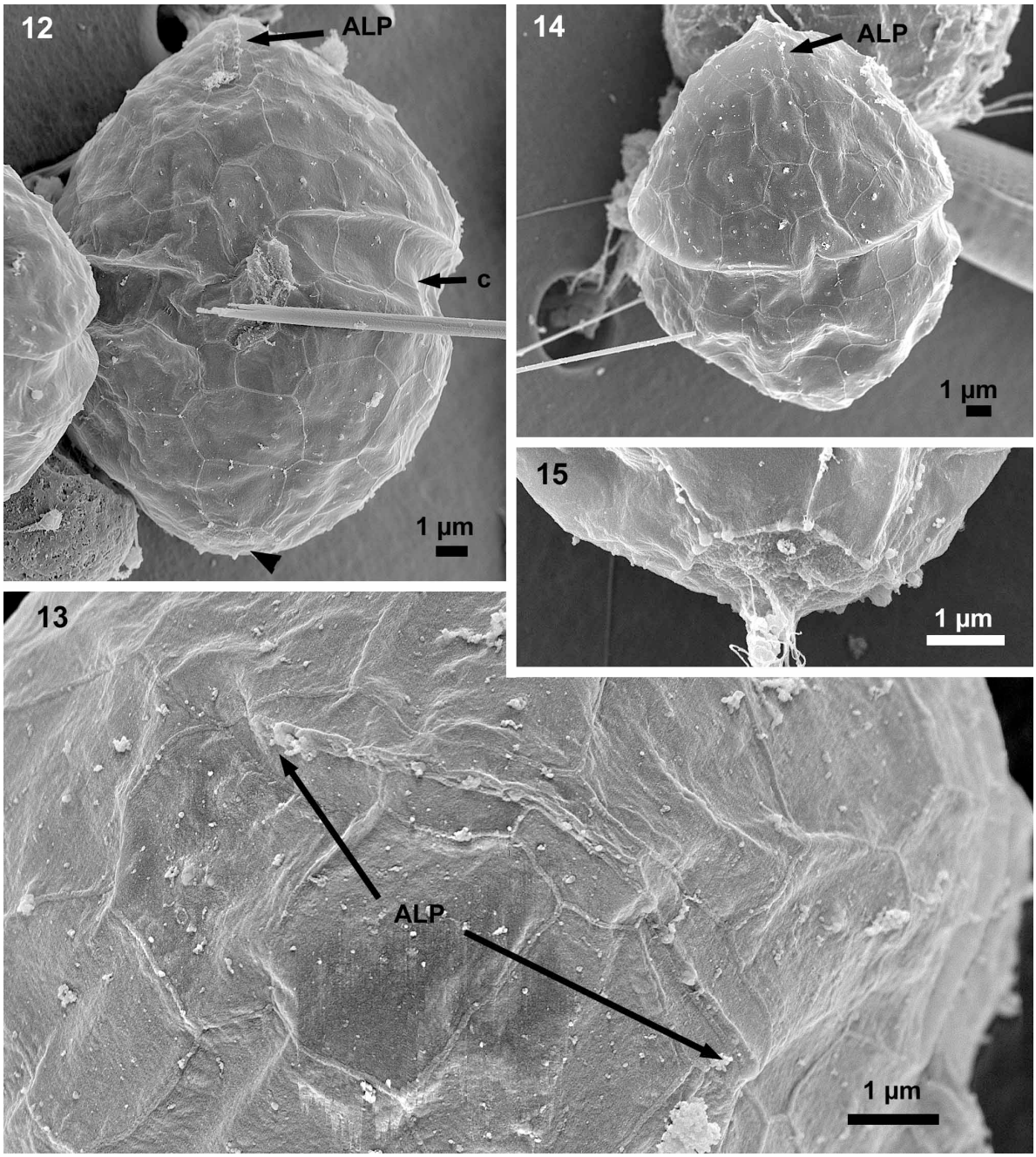


Figs 10, 11. Anterior and posterior views of *Tovellia sanguinea*, respectively. The ALP appears to comprise four very narrow plates, lined on each side by a row of 3–4 elongate plates. Fig. 11 shows the antapical plate.

with the basal body of the longitudinal flagellum, r_1 (this root is not visible in the section). One end of the row of msp has been sectioned transversely in Fig. 37 while the other end is more oblique. The msp terminates near opaque material at the cell surface, probably a collar-like structure (Fig. 35). Food uptake was not studied and the peduncle was not seen in the extended position.

Molecular data and phylogeny of Tovellia

The LSU rDNA sequence data of *T. sanguinea* from the three Italian lakes were identical or nearly identical. No differences among the 1,366 bp included in the comparison (four LSU rDNA sequences; Table 3) were detected between the cysts and flagellated stages from Lake Tovel and Lake Campo. These taxa form a polytomy in



Figs 12–15. Scanning electron microscopy of Baldi's preserved material from 1938. Figs 12 and 14 are ventral and dorsal views, respectively (ALP is the apical line of plates). Fig. 13 is an apical view at higher magnification, illustrating both the ALP and the row of plates on either side of the ALP. Fig. 15 illustrates the antapical plate.

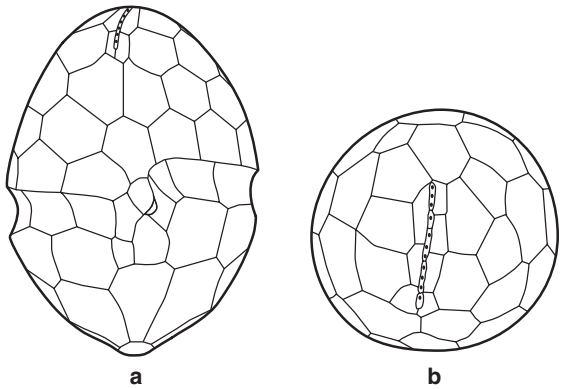
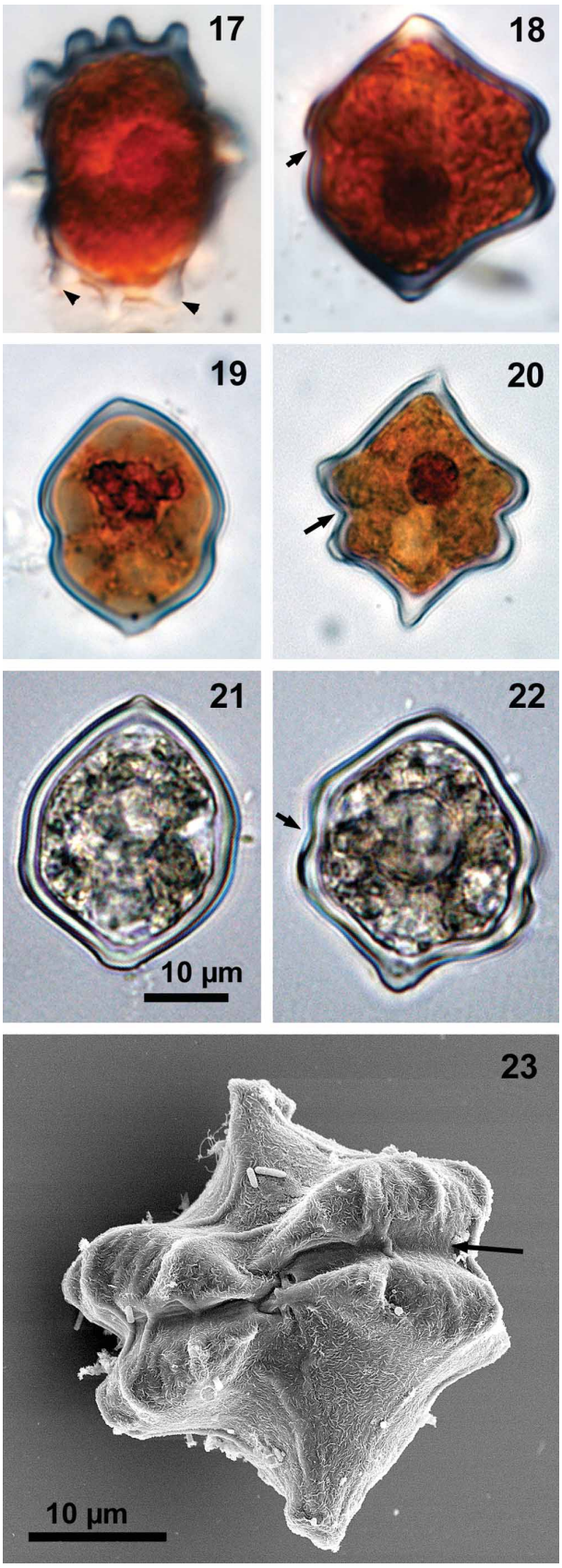


Fig 16. Diagrammatic reconstruction of *Tovellia sanguinea*, in ventral (a) and apical view (b).

the phylogeny shown in Fig. 38. The LSU rDNA sequence of the *T. sanguinea* cyst isolated from Lake Colbricon differed by two nucleotides compared with *T. sanguinea* from Lake Tovel and Lake Campo, resulting in a sequence divergence of 0.15% (Table 3). Only one of these substitutions resided in the highly variable domain D2 whereas the other substitution occurred 66 bp downstream of domain D1 compared to the secondary structure model of *Prorocentrum micans sensu* Lenaers *et al.* (1989). This explains the divergent branching of the *T. sanguinea* cyst from Lake Colbricon (see Fig. 38) compared with the other *T. sanguinea* sequences. Comparing our



Figs 17–23. Cysts of *Tovellia sanguinea*. Figs 17, 18, 23 from sediment in Lake Tovel; Figs 19–20 from a Lake Campo culture; Figs 21–22 from a plankton sample from Lake Tovel collected by Baldi in October, 1938. Notice the large variation in cyst shape and in paracingulum structure (arrows), features which are particularly clear in SEM (Fig. 23).

T. sanguinea LSU rDNA sequences to those of the closely related *T. coronata* (Woloszynska) Moestrup, Lindberg & Daugbjerg, recently described by Lindberg *et al.* (2005), revealed a sequence divergence of 11–12% (Table 3).

The phylogenetic inference based on parsimony analysis of LSU rDNA sequences is shown in Fig. 38. Only the terminal branches are well supported with high bootstrap values and posterior probabilities. Hence, this data matrix does not support the relationships between higher taxonomical groups (i.e. families and orders). The recently proposed family Tovelliaceae, comprising *Tovellia* and *Jadwigia* (Lindberg *et al.*, 2005), received bootstrap support only in the neighbour-joining analysis, and posterior probability in the BA analysis (Fig. 38). Nevertheless, the strict consensus tree of four equally parsimonious trees showed that *Jadwigia* and *Tovellia* are sister groups (constituting group I *sensu* Lindberg *et al.*, 2005). The two other groups of woloszynkioids (viz. group II and group III) formed sister groups supported by high bootstrap values in all analyses (≥75%).

Discussion

Taxonomy

All available evidence indicates that the species examined and illustrated here is identical to the organism that caused the red colour of the lake water of Lake Tovel until 1964. The phenomenon and its possible causes were discussed in detail by Baldi (1938, 1941), who attributed the colour to the dinoflagellate *G. sanguineum* Marchesoni, a species described from the lake by Marchesoni (1941) but previously identified by Largaiolli (1907) as *G. pulvisculus* (Ehrenberg) Stein var. *oculatum*. We have previously argued that Baldi's description of *G. sanguineum* is based on more than one species (Flaim *et al.*, 2004). Baldi described both a green and a red form of *G. sanguineum* but we now know that the two forms belong to different genera. The green form is being described separately using light and electron microscopy as well as LSU rDNA sequence data (Hansen & Daugbjerg, unpublished). However, the red form cannot retain the name *G. sanguineum* Marchesoni. This name is illegitimate, being a younger homonym of *G. sanguineum* (H.J. Carter) Diesing (Diesing, 1866). Marchesoni (1941) overlooked that the name had been given to another dinoflagellate, collected by Carter (1858) on the shores of the island of Bombay, India, where it was also a cause of red water. The Italian and the Indian taxa are different, however, and the oldest name takes priority. The Italian taxon is described here as

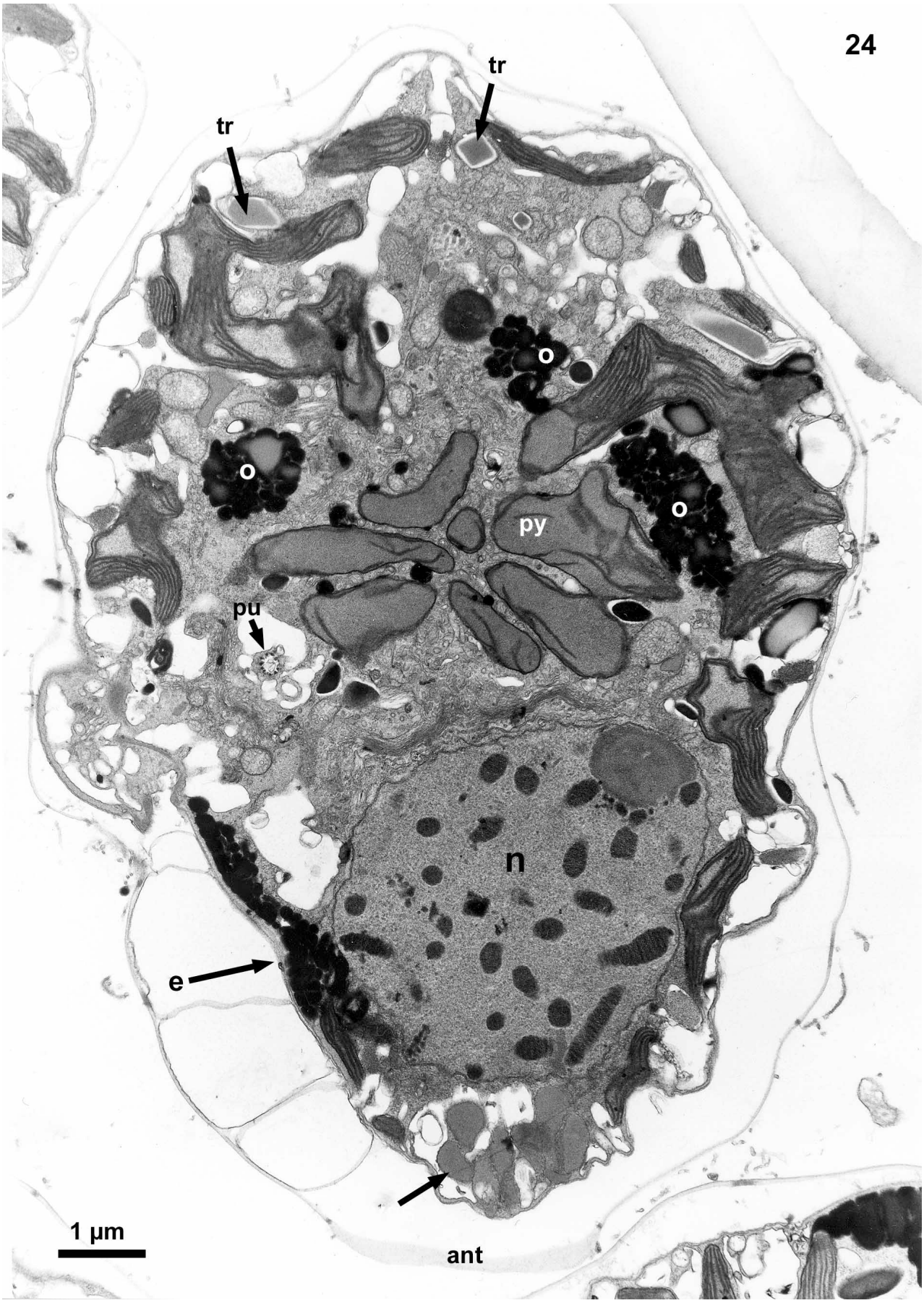
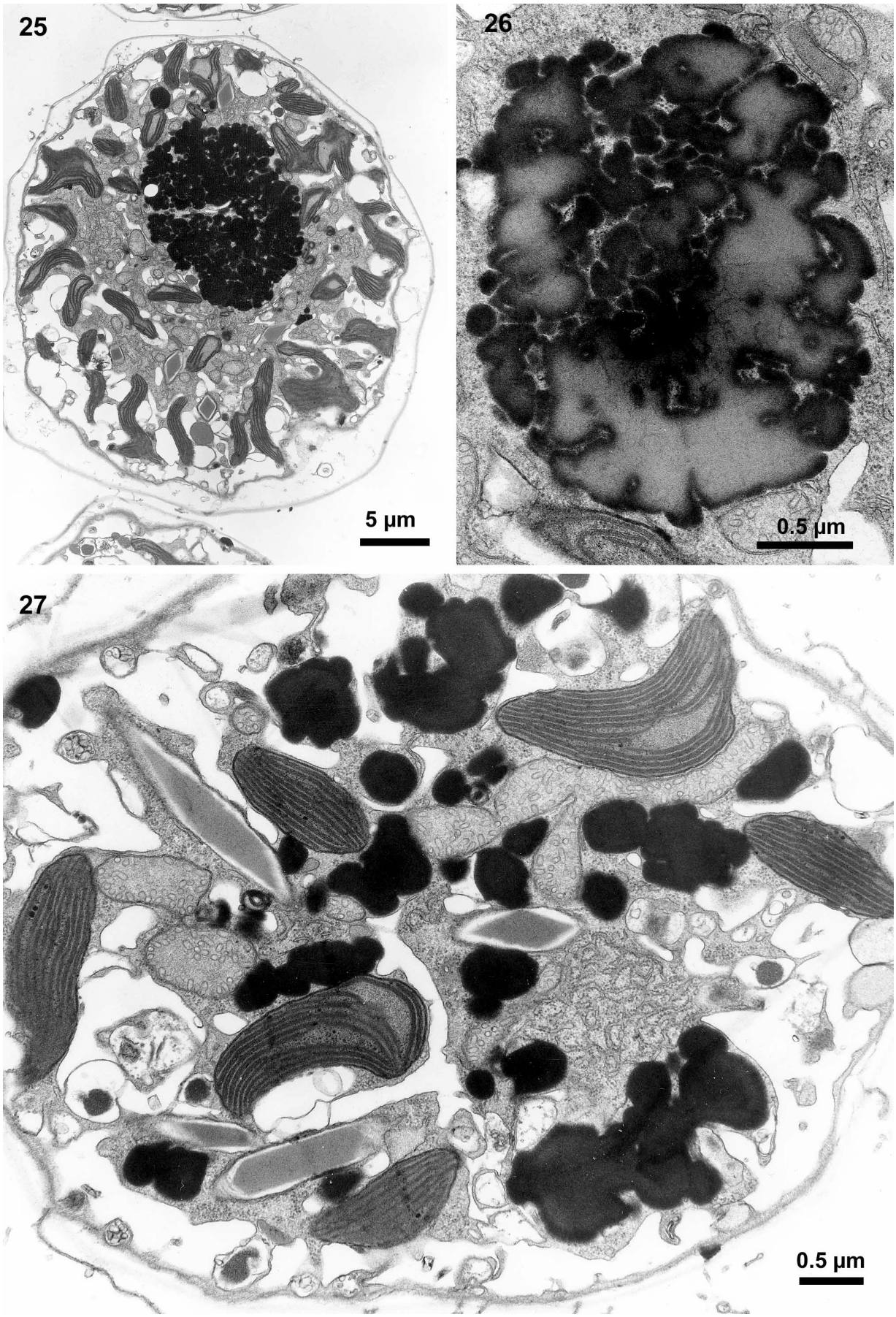
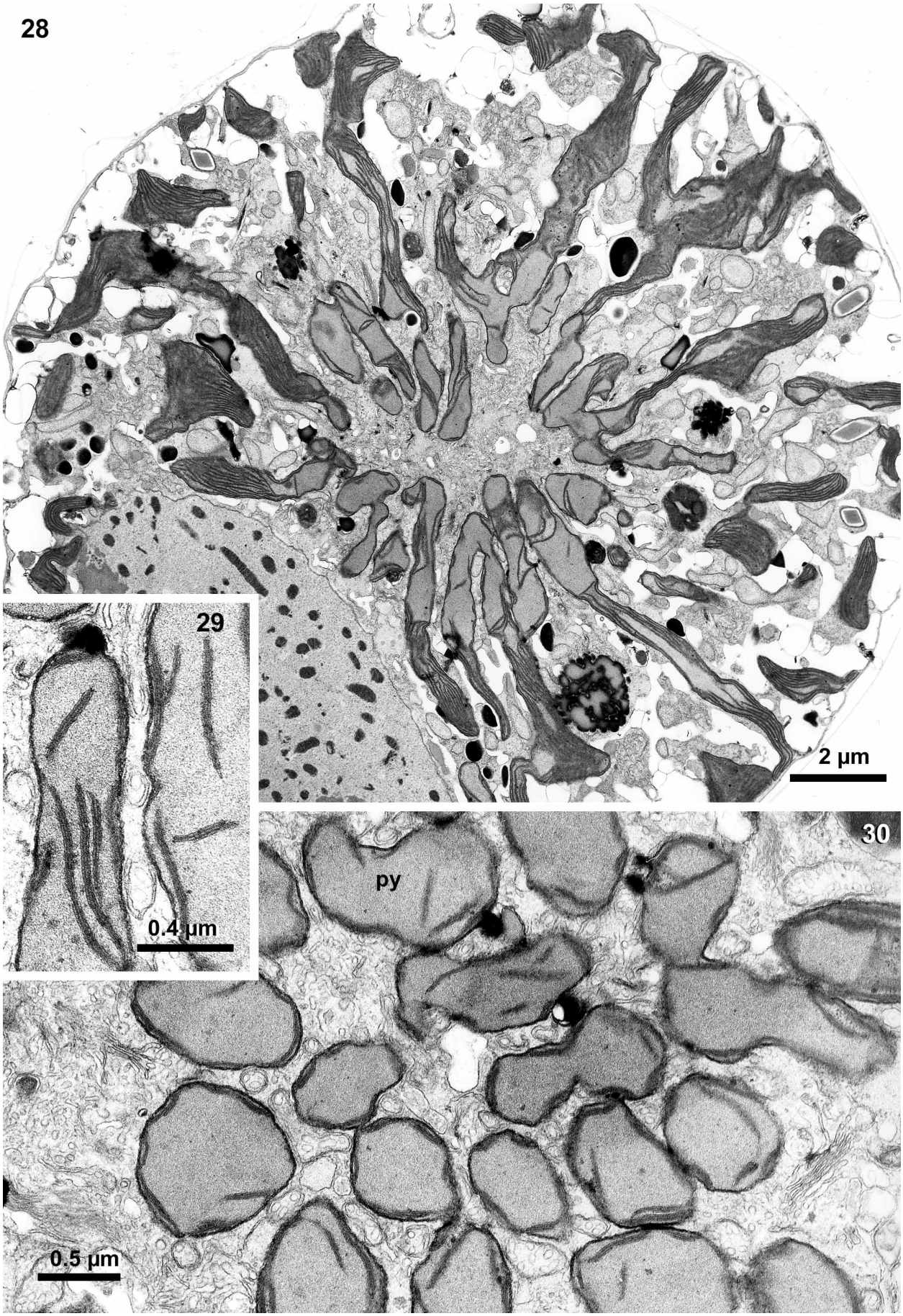


Fig 24. Longitudinal thin section through a cell of *Tovellia sanguinea*, illustrating most of the organelles. Abbreviations: ant: antapical plate; e: eyespot; n: nucleus; o: opaque pigment aggregates; pu: pusule canal; py: pyrenoid complex; tr: trichocysts. Notice the opaque vesicles in the antapical region (arrow).



Figs 25–27. *Tovellia sanguinea*. Fig. 25. Transverse section of the cell, which is barely compressed. Fig. 26. Close-up of pigment aggregate. Fig. 27. Section through another cell in which the pigment granules occur scattered or in smaller aggregates.



Figs 28–30. The plastidome. Long chloroplast bands radiate from the cell centre towards the periphery. The central part of each arm contains fewer chloroplast lamellae and this area is interpreted as a loosely organized compound pyrenoid (py). The pyrenoid regions are traversed by fewer lamellae with 2 or 3 thylakoids (Fig. 29).

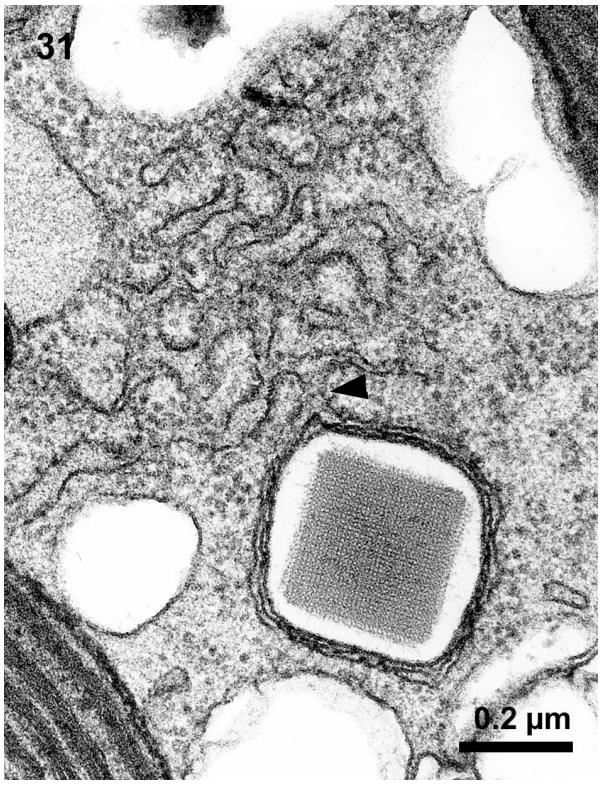


Fig 31. The trichocyst-containing vesicles are commonly surrounded by two additional membranes, which are continuous with endoplasmic reticulum located near the vesicle (arrowhead).

a new species belonging to the genus *Tovellia*, but we have refrained from using the name of the variety, *G. pulvisculus* var. *oculatum* in the interest of maintaining the widely used specific epithet *sanguineum*.

Comparison with other woloszynskioid dinoflagellates

Tovellia sanguinea differs from the other known red species of *Tovellia*, *T. coronata*, in the arrangement of the chloroplasts: parietal and without pyrenoids in *T. coronata*, but radiating from a common compound pyrenoid in *T. sanguinea*. The sequence divergence between the two *Tovellia* species is 11–12% for 1,366 bp of the LSU rDNA. They were incorrectly considered to be identical by Popovsky & Pfiester (1990, as *Woloszynskia coronata* (Woloszynska) R.H. Thompson). The majority of the brown (greenish) species of *Tovellia* also usually possess parietal chloroplasts, the exception being *Tovellia nygaardii* (Christen) Moestrup, Lindberg & Daugbjerg, described from Switzerland (Christen, 1958). Christen described the cells of *T. nygaardii* as having radially arranged chloroplasts, which were rarely more irregularly arranged. The latter condition may represent stressed or old cells, in which the chloroplasts have separated from the central, compound pyrenoid, a phenomenon also known in other flagellates with a central compound pyrenoid, such as the euglenophyte *Eutreptiella eupharyngea* Walne & Moestrup (Walne *et al.*, 1986). Cells of *T. nygaardii* are very similar to *T. sanguinea*, and the only difference appears to be the colour: yellow-green or brownish green in *T. nygaardii* (Christen, 1958), reddish in *T. sanguinea*.

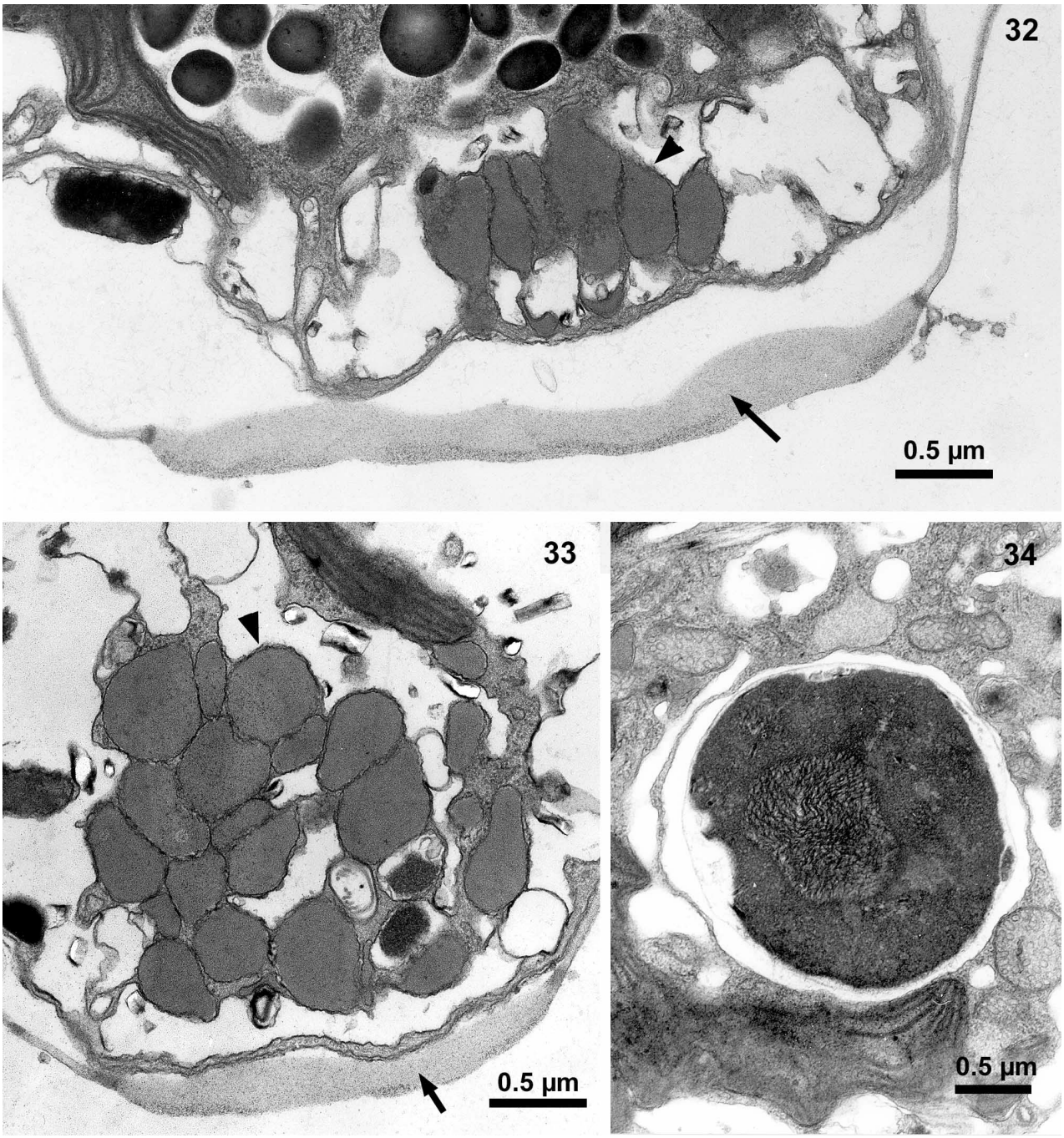
When examined in SEM and TEM, the presence of the flattened, thick antapical amphiesma plate is a striking feature of *T. sanguinea*. A somewhat similar, but thinner, plate occurs in *T. coronata*, and Christen (1958) mentions that the plates of the hypocone in *T. nygaardii* also radiate from a small antapical plate which he, however, did not illustrate. Both red species contain a posterior nucleus. In *T. sanguinea* this is separated from the antapical amphiesma plate by a group of opaque vesicles, while chloroplasts of *T. coronata* occur throughout the peripheral part of the cell, including the area between the cell and the antapical plate.

The peduncle

While food uptake has not yet been documented in *T. sanguinea*, the presence and path of the microtubular band associated with opaque vacuoles indicates that this species is potentially mixotrophic. Little is known about mixotrophy in freshwater dinoflagellates, but we have also observed the microtubular band and the associated opaque vesicles in *Tovellia coronata*. It may be more widespread than indicated by the few available accounts. A peduncular band was found in *Prosoaulax lacustre* (von Stein) Calado & Moestrup (formerly *Amphidinium lacustre* von Stein) (Calado *et al.*, 1998) and in *Peridiniopsis borgei* Lemmermann (Calado & Moestrup, 2002) and *P. berolinensis* (Lemmermann) Bourrelly (Calado & Moestrup, 1997). It is absent in *Peridinium cinctum* Ehrenberg (Calado *et al.*, 1999), however, since a previous report of a 'microtubular basket' was based on confusion with a flagellar root. The peduncular microtubules occur both in strictly heterotrophic species (e.g. *Prosoaulax lacustre* (F. Stein) Calado & Moestrup), and in autotrophic species, which therefore appear to have the potential for mixotrophy. Studies on feeding behaviour in these freshwater dinoflagellate species are therefore needed to explore whether the peduncle is functional or simply a relict feature of a common heterotrophic ancestor.

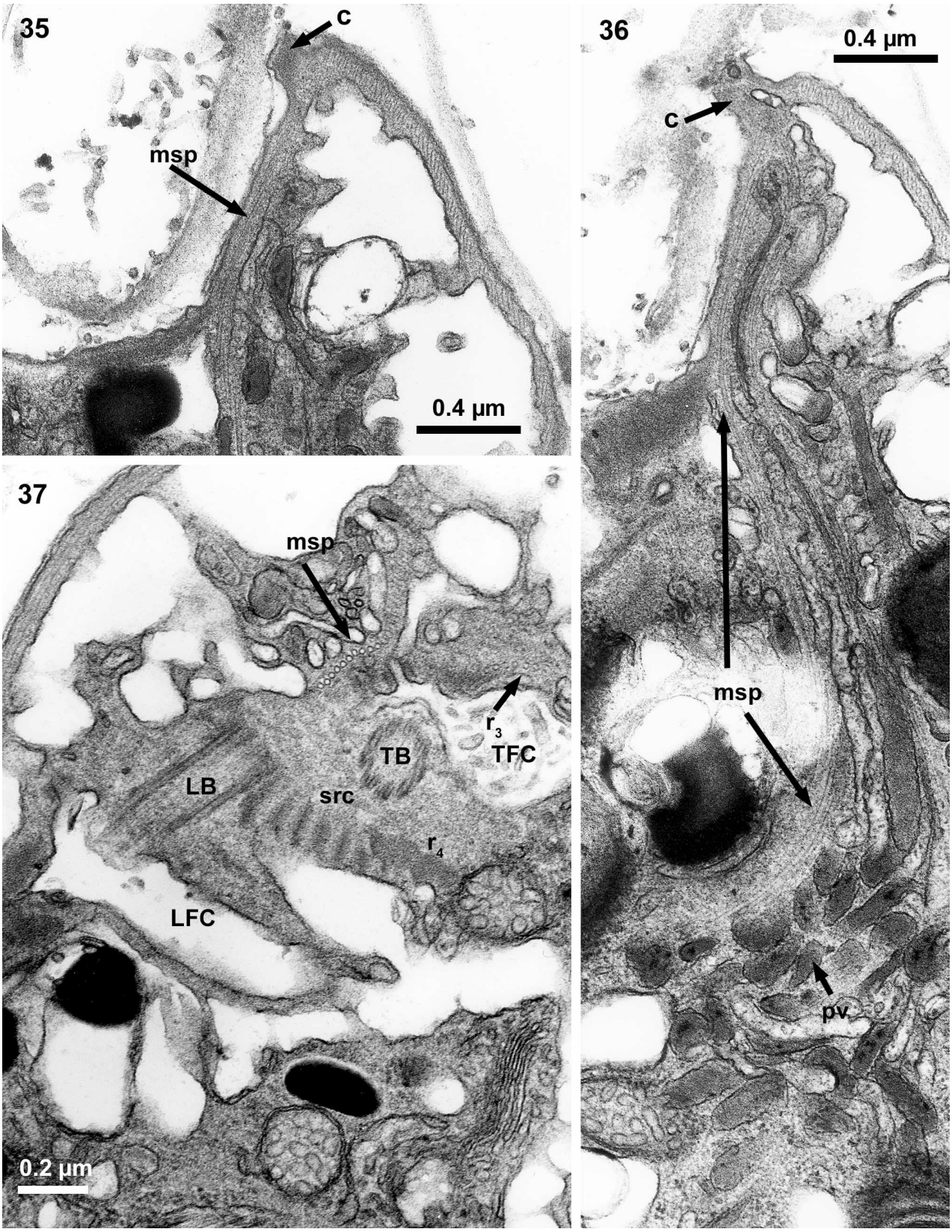
Trichocysts

Trichocysts have been illustrated in numerous dinoflagellates from Grell & Wohlfarth-



Figs 32–34. *Tovellia sanguinea*. Fig. 32. The antapical end of the cell is covered with a notably thickened plate (arrow), several times thicker than the adjacent plates (cf. Figs 9, 16). Opaque vesicles are present in the area adjacent to the antapical plate (arrowhead). Fig. 33. The many opaque vesicles (arrow head) in the region of the antapical plate (arrow). More well-defined contents were never seen in the vesicles. Fig. 34. Large inclusion body in the cell, probably a food vacuole. The fibrous part in the centre resembles chromosomal DNA.

Bottermann (1957) in *Amphidinium operculatum* Claparède & Lachmann (as *Amphidinium elegans* Grell & Wohlfarth-Bottermann, Flø Jørgensen *et al*., 2004), and Bouck & Sweeney's (1966) illustrations of *Prorocentrum micans* Ehrenberg and *Lingulodinium polyedrum* (F. Stein) Dodge (as *Gonyaulax polyedra* F. Stein). Bouck & Sweeney (1966) found trichocysts were formed in vesicles located near the Golgi apparatus, and they concluded, with some reservation, that the vesicles were probably Golgi derived. The vesicles initially contained amorphous material, but a crystal lattice subsequently appeared, which developed into a trichocyst. In *T. sanguinea* the common occurrence of the trichocyst-containing vesicle surrounded by a cisterna of rough endoplasmic reticulum is striking. This cisterna is continuous with ER present in the vicinity of the trichocyst vesicle. The ER cisterna does not surround the entire trichocyst vesicle, part of which is bounded by only a single membrane. The structural association indicates a functional



Figs 35–37. Some details of the peduncle microtubules in *Tovellia sanguinea*. Figs 35, 36. The microtubular strands of the peduncle (msp) extend from the cell interior towards the cell surface, terminating near electron-opaque material which may represent a collar (c). The microtubules are always joined by numerous vesicles containing opaque material (pv). Fig. 37. Peduncle microtubules sectioned more or less transversely and seen to pass very near the flagellar apparatus. LB, TB, LFC, TFC: the longitudinal and transverse basal bodies, and their respective flagellar canals. Parts of two flagellar roots are visible: the extension of root 3 (r_3) extending along the canal of the transverse flagellum, and the striated part of root 4 (r_4) (striation not visible in this angle of sectioning); src is the striated connecting fibre between root 4 and root 1.

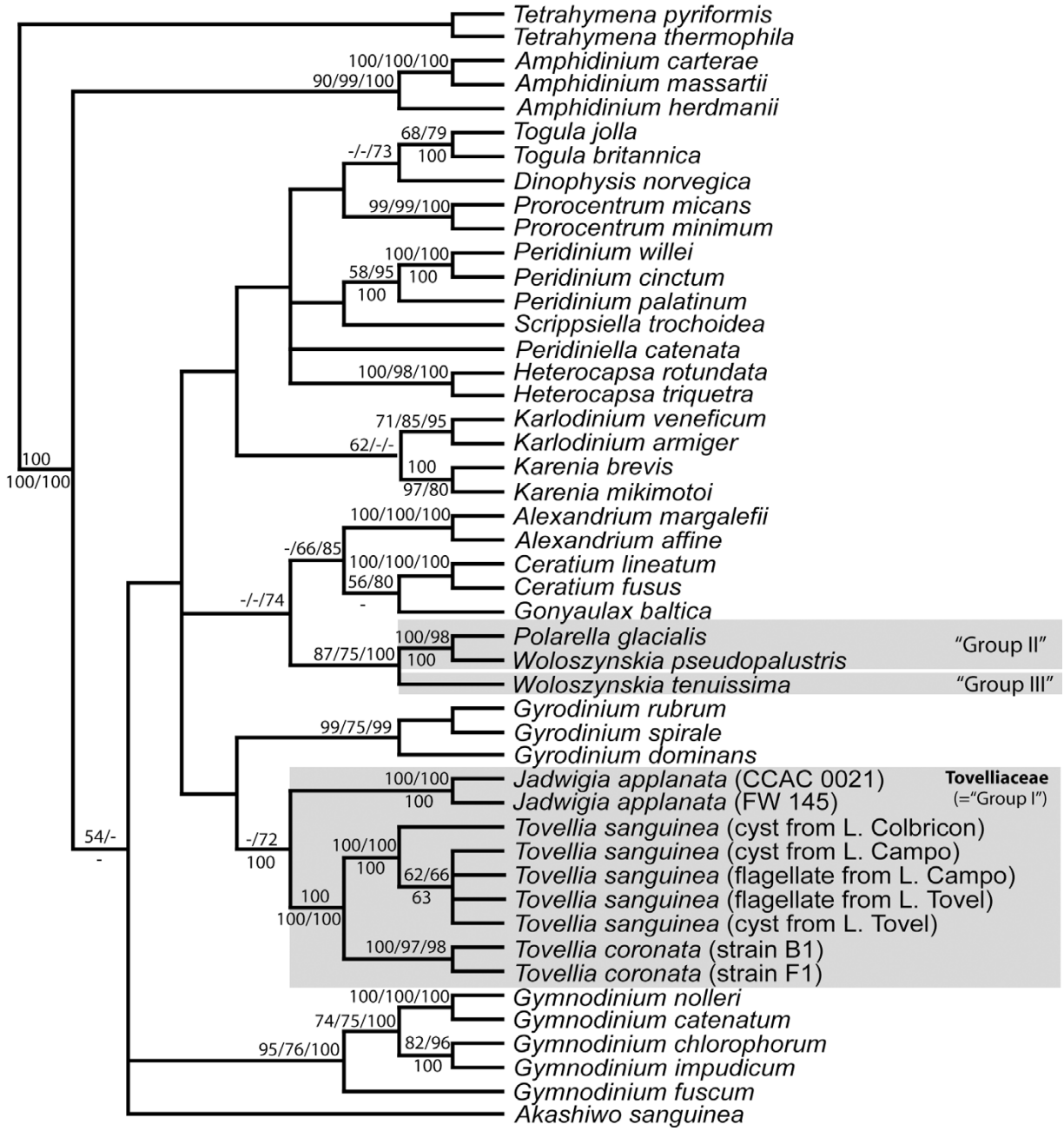


Fig 38. Phylogeny of *Tovellia* species inferred by unweighted parsimony analysis. Of the 1,079 characters included, 506 were parsimony informative and the analyses produced four equally parsimonious trees, each comprising 2,384 steps (CI = 0.445, RI = 0.626). The tree shown is a strict consensus of the four equally parsimonious trees. Bootstrap values or support from posterior probabilities of 50% or above are written to the left of internal nodes. The first number is from parsimony analyses (1,000 replications), the second number is from neighbour-joining analyses based on the maximum likelihood settings obtained using Modeltest (TrN + I + G model; see Materials and methods) and with 1,000 replications. The third number is the posterior probability from Bayesian analyses. Two ciliate species belonging to *Tetrahymena* were chosen as out-groups. Woloszynskioids assigned to groups I, II and III (*sensu* Lindberg *et al.*, 2005) are highlighted in grey.

Table 3. Sequence divergence (in percent) of isolates of *Tovellia sanguinea* from Lake Tovel, Lake Campo and Lake Colbricon, and *Tovellia coronata* from Sweden, based on 1366 unambiguous aligned LSU rDNA nucleotides. Uncorrected distances (p-values in PAUP*) are given above the diagonal, and distance values calculated using Kimura 2-parameter model are given below the diagonal.

	T. sanguinea (Lakes Tovel & Campo)	*T. sanguinea* (Lake Colbricon)	*T. coronata* (Strains B1 & F1)
T. sanguinea (Lakes Tovel & Campo)	–	0.15	11.3
T. sanguinea (Lake Colbricon)	0.15	–	11.2
T. coronata (Strains B1 & F1)	12.3	12.1	–

significance, which most likely involves formation of proteins that become transferred to the trichocysts. We are not aware of any studies addressing the chemical composition of the trichocysts in dinoflagellates but, in the phylogenetically related group, the ciliates, trichocysts are thought to be proteinaceous (Steers *et al*., 1969; Pollack & Steers, 1973), and neither carbohydrates nor nucleic acids have been found. This agrees with the observation that the trichocysts of ciliates originate in the endoplasmic reticulum (e.g. Hausman, 1978). Whatever its origin in dinoflagellates, the trichocyst-forming vesicle, surrounded in part by ER, appears to represent a stage in trichocyst formation, and it is surprising that this association has not previously been noticed. The association between ER and a trichocyst vesicle is also visible in a micrograph of *T. glabra* Moestrup, Lindberg & Daugbjerg published by Dodge (1973, fig. 9.1 as *W. coronata*, see Lindberg *et al*. 2005). More attention needs to be paid to the origin and the chemical composition of trichocysts in dinoflagellates.

Occurrence

Although only the water of Lake Tovel has been known to turn red in summer, we have observed the causative species, *T. sanguinea*, in two additional lakes in the area (viz. Lake Campo and Lake Colbricon). In August–September 2003 we collected *T. sanguinea* in both Lake Tovel and Lake Campo, although it was rather sparse in Lake Tovel. In July 2004, only a few cells were observed in Lake Tovel while it was relatively abundant in Lake Colbricon.

It is likely therefore that *T. sanguinea* is more widely distributed than presently thought, but only past conditions in Lake Tovel have allowed it to occur in bloom proportions. Table 4 provides some limnological information on the three lakes. All are small, but they vary in area, volume and depth; Lake Campo and Lake Colbricon stratify stably in summer while stratification in Tovel is more haphazard. Probably most significant, all are cold-water lakes, with mean summer temperatures of surface waters below 15°C, suggesting that *T. sanguinea* is a cool stenotherm (also indicated by laboratory observations). The lakes have restricted nutrient supplies, with Lake Tovel and Lake Campo being oligotrophic and Lake Colbricon meso-oligotrophic, as indicated by total phosphorus and chlorophyll *a* concentrations, and Secchi disk transparency. Land use for all three lakes occasionally causes sporadic and localized increases in nutrients. Specific conductivity as a measure of mineral content of the water varies greatly, reflecting three different geological substrata and indicating that *T. sanguinea* is not limited to a strict range of mineral content. It should be stressed that the species identified as *G. sanguineum* by Dodge *et al*. (1987), and claimed to be responsible for colouring of Lake Tovel, has been obtained in culture by us and will also be described in this series of articles. In dense cultures the colour of this taxon, which does not belong in the genus *Tovellia*, is brown rather than bright red.

Genetic divergence and phylogeny of Tovellia

Based on comparative morphology, *T. sanguinea* possesses a number of distinct features separating it

Table 4. Some physical and trophic parameters[a] for Lakes Tovel, Campo and Colbricon.

	Tovel	Campo	Colbricon
Physical parameters:			
Geographical coordinates	46°15′N, 10°57′E	46°02′N, 10°30′E	46°17′N, 11°46′E
Mountain range	Brenta Dolomites	Adamello	Lagorai
Altitude (m a.s.l)	1177	1943	1926
Geological substratum	Dolomitic limestone	Mica schists, moraines	Poryphyry
Land use	Forest, pasture	Mountain, pasture	Mountain, tourism
Area (km^2)	0.38	0.09	0.03
Volume (m^3)	7,367,600	1,190,000	162,900
Maximum depth (m)	39	27	12
Lake renewal (yrs)	$\ll 1$	< 1	< 1
Trophic parameters:			
Trophic status	Oligotrophic	Oligotrophic	Meso-oligotrophic
Water temperature (°C)[b]	14.0 ($n = 46$)	12.6 ($n = 2$)	13.6 ($n = 18$)
Conductivity ($\mu S\,cm^{-1}$)[c]	200	90	45
Total phosphorus ($\mu g\,l^{-1}$)[c]	6	8	12
Chlorophyll *a* ($mg\,l^{-1}$)[c]	< 2.5	5.8	9.4
Secchi disk transparency (m)[c]	11	13	5

[a]Data from annual reports (IASMA 1995–2000) and unpublished data.
[b]Mean values from surface waters (centre of lake) from July to September.
[c]Mean of four sampling dates (1997) for Lake Campo and multi-annual means (1995–2004) for Lake Tovel and Lake Colbricon.

from the similar species *T. coronata*. The morphological differences between the two species are supported by genetic differences in the LSU rDNA gene (sequence divergence of 11–12%). Similar sequence divergence values are found between other species of dinoflagellates (e.g. species of *Gyrodinium* Kofoid & Swezy, *Peridinium* Ehrenberg and *Alexandrium* Balech, data not shown). The genus *Amphidinium* Claparède & Lachmann is a notable exception with sequence divergence values above 25% for some species (e.g. *Amphidinium herdmanii* Kofoid & Swezy *versus A. carterae* Hulburt and *A. massartii* Biecheler *versus A. herdmanii*). The difference in sequence divergence between dinoflagellate genera reflects that there is no universal percentage value (or specific range) for the nuclear-encoded LSU rDNA gene to indicate species boundaries.

The partial LSU rDNA of *T. sanguinea* from Lake Tovel/Lake Campo and Lake Colbricon differed by only two substitutions. We consider that this reflects a difference at the population level. Hansen *et al*. (2000) observed a similar level of substitutions for isolates of the marine dinoflagellate *Karenia mikimotoi* (Oda) G. Hansen & Moestrup (as *Gymnodinium mikimotoi* Oda). However, their comparison included isolates that were geographically much more widely separated than the Italian lakes in which *T. sanguinea* was sampled, which are less than 100 km apart.

The methods used to infer the phylogeny of *Tovellia* and a diverse assemblage of other dinoflagellates by sequence analyses of partial LSU rDNA did not provide support for the most divergent branching. This is similar to other studies using partial LSU rDNA (e.g. Daugbjerg *et al*., 2000; de Salas *et al*., 2003; Flø Jørgensen *et al*., 2004), complete SSU rDNA sequences (e.g. Saldarriaga *et al*., 2004), and even concatenated analyses using LSU and SSU rDNA (Saldarriaga *et al*., 2004). It is unlikely therefore that addition of further sequences to the SSU and LSU rDNA data matrices will improve our understanding of the sister group relationship among dinoflagellate lineages using these genes. The nuclear-encoded ribosomal genes probably do not contain enough genetic information to resolve the topology of the deep branches as the branch lengths are very short.

However, the addition of one more species of *Tovellia* (*T. sanguinea*) to the recently suggested family Tovelliaceae (comprising only *Tovellia* and *Jadwigia*) still supported this as a distinct lineage. It was, however, only favoured in terms of bootstrap support in the NJ analysis and by a posterior probability in BA analysis.

While the present manuscript was being reviewed prior to publication, an article appeared in which a brackish water species, *Gymnodinium halophilum* Biecheler, was examined (Kremp *et al*., 2005). The authors concluded that *G. halophilum* belongs in the genus *Woloszynskia* R.H. Thompson, to which genus it was transferred as *Woloszynskia halophila* (Biecheler) Elbrächter & Kremp. It differs from species of *Tovellia* most notably in the structure of the eyespot and is a close relative of *W. pseudopalustris* (Schiller) Kiselev. These *Woloszynskia* species do not belong in the family Tovelliaceae, however, and molecular data suggest that they form another evolutionary line ('Group II' in Lindberg *et al*. 2005; Fig. 38), also including *Symbiodinium* Freudenthal, *Protodinium simplex* Lohmann (syn. *Gymnodinium simplex* (Lohmann) Kofoid & Swezy) and *Polarella glacialis* Montresor *et al*. (Kremp *et al*. 2005). This lineage forms the order Suessiales (Montresor *et al*., 1999), defined by Fensome *et al*. (1993), also including numerous extinct species of dinoflagellates extending back to the Triassic and Jurassic. Although superficially similar to species of *Tovellia*, *W. halophila* (and Group II in general) does not occupy a sister group position to the Tovelliaceae in the molecular phylogenies (Fig. 38) and additional studies are needed on members of Group II.

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