

***Moestrupia oblonga* gen. & comb. nov. (syn.: *Gyrodinium oblongum*), a new marine dinoflagellate genus characterized by light and electron microscopy, photosynthetic pigments and LSU rDNA sequence**

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A small-sized, peridinin-containing, athecate dinoflagellate (13–17 µm long) was isolated into clonal culture from a water sample collected at a nearshore location in Tenerife, Spain (October 2004). Based on phenotypic characters (size, shape, pyrenoid and nucleus position), the culture was identified as *Gyrodinium oblongum*. However, a detailed ultrastructural examination revealed a number of features that did not fit into the current delineation of the genus *Gyrodinium* or any other dinoflagellate. Likewise, the molecular phylogeny and sequence divergence estimates based on partial LSU rDNA sequences indicated that this isolate has a taxonomically isolated position, and we therefore propose the new genus *Moestrupia* to accommodate this species within the Dinophyceae. The ultrastructural study uncovered a number of unique features for *Moestrupia*, for example, a microtubule supported ventral flange situated along the right cingular border and onto the episome. Also, the exit point of the peduncle, through a lip-like protrusion situated in a cavity on the episome, is new in dinoflagellates. The transverse flagellum appeared less coiled compared to other dinoflagellates, and its distal end terminated some distance within the wide sulcus. The upper cingulum border had a distinct rim supported by microtubules; whereas, the lower cingulum border was indistinct. The apical groove was short and curved and bordered on each side by a delicate rim, which may represent amphiesmal vesicles. It ran from the anterior part of the ventral flange to the middorsal side of the episome. Thus, it differs markedly from that in species of *Gyrodinium* and emphasizes it being distantly related to this genus.

INTRODUCTION

Traditionally dinoflagellates have been divided into armoured and unarmoured (i.e. naked) forms. The former possesses distinctive plates, composed of cellulose-like material, within flattened vesicles subtending the plasma membrane. The vesicles are the so-called amphiesmal vesicles or alveoli. The latter group is without light microscopically discernable plates, though often dark plate-like material may be present within the vesicles when observed in transmission electron microscopy (TEM). The taxonomy of armoured dinoflagellates is based primarily on the number and arrangement of the thecal plates (e.g. Balech 1980; Fensome *et al.* 1993). Contrary, the taxonomy of the unarmoured dinoflagellates has up until the molecular era been based solely on features such as location and displacement of the cingulum or girdle (i.e. a transverse furrow encircling the cell). For example, in the monumental monograph on unarmoured dinoflagellates by Kofoid and Swezy (1921), the genus *Amphidinium* is characterized by having a ‘girdle far anteriorly displaced’, *Torodinium* has an ‘episome several times the length of the hyposome’, *Gymnodinium* has a ‘girdle ... displaced less than one-fifth of the total length of the body’, *Gyrodinium* has ‘girdle ... displaced more than 0.2 of the total length of the body’, and so on. However, these generic boundaries were problematic,

as a continuum of species exist with cingular displacement around these borderlines. Moreover, early molecular studies indicated that the classification based on these generic characters were polyphyletic (Saunders *et al.* 1997). Subsequently, major emendations and revisions have been made for *Amphidinium* (Flø Jørgensen *et al.* 2004a, b; Calado & Moestrup 2005), *Gymnodinium* and *Gyrodinium* (Daugbjerg *et al.* 2000) based primarily on ultrastructural characters with support from molecular data. Particular emphasis has been given on the shape of a delicate anterior furrow termed the apical groove but also on details of the nuclear envelope and arrangement of the flagellar apparatus. For example, *Gymnodinium sensu* Daugbjerg *et al.* (2000) is characterized by a horseshoe-shaped apical groove running anticlockwise around the cell apex, a nuclear envelope with peculiar dilations, so-called nuclear chambers and attachment of the flagellar apparatus to the nuclear envelope by a fibrous fibre termed the nuclear fibrous connective. On the other hand, *Gyrodinium* is defined as having an amphiesma with longitudinal striations and a small elliptical apical groove (Daugbjerg *et al.* 2000). Later ultrastructural analyses of the type species *G. spirale* Bergh revealed that the nucleus is surrounded by a complex wall-like structure, a so-called nuclear capsule (Hansen & Daugbjerg 2004).

Several new genera have been erected to accommodate species not fulfilling the new fine structural criteria (e.g. *Akashiwo*, *Karenia*, *Karlodinium*, *Paragymnodinium* and

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Takayama). Additionally, species have been removed from *Gyrodinium* to *Gymnodinium* and vice versa, for example, *Gymnodinium aureolum* (Hulburt) Gert Hansen and *Gyrodinium helveticum* (Penard) Takano & Horiguchi formerly classified within *Gyrodinium* and *Gymnodinium*, respectively (Daugbjerg *et al.* 2000; Hansen *et al.* 2000a; de Salas *et al.* 2003; Takano & Horiguchi 2004; Kang *et al.* 2010).

The number of described marine species of *Gymnodinium* and *Gyrodinium* is little over 170 and nearly 90, respectively (Gómez 2005); whereas, the number of freshwater *Gymnodinium* and *Gyrodinium* species is considerably fewer. For example, Popovský & Pfiester (1990) listed 23 *Gymnodinium* and 2 *Gyrodinium* species. The majority of these species still awaits further investigations to clarify their generic relationship using the recently proposed criteria.

Gyrodinium oblongum J. Larsen & Patterson represent such a species. It was originally described from a marine sediment sample collected in Bowling Green Bay, Queensland. With a cingular displacement of 0.3 of the cell length, it was placed in the genus *Gyrodinium sensu lato*. It was characterized mainly by its small size and prominent pyrenoid (Larsen & Patterson 1990). To the best of our knowledge, it has been reported only from marine sediments in Australia. However, this species flourished in an enriched net sample collected at Tenerife, Spain. A clonal culture was established for detailed ultrastructural and molecular analyses intended for clarification of its generic relationship. Based on these analyses, it became obvious that *G. oblongum* did not belong to the genus *Gyrodinium* or any other dinoflagellate genus. Thus, to accommodate this species within the Dinophyceae, we propose a new genus, *Moestrupia*, and make the new combination *M. oblonga* (J. Larsen & Patterson) Gert Hansen & Daugbjerg *comb. nov.*

MATERIAL AND METHODS

The clonal culture used in this study was established from a net sample (20- μ m mesh size) collected from a pier at Callao Salvaje, Tenerife (October 2004). The sample was enriched with TL growth medium (Larsen *et al.* 1994) and incubated at 20°C for about a week. Single cells of potentially interesting small dinoflagellates were isolated using a hand-drawn micropipette and a Labovert inverted microscope (Leitz, Wetzlar, Germany). The clonal culture used in the present study was grown in TL medium, at 20°C and a 12:12-hour light:dark cycle. The light intensity was *c.* 40 μ mol m⁻² s⁻¹. The culture was maintained for nearly 3 years until a culture cabinet failure unfortunately terminated its growth.

Light microscopy

Live cells were observed using a Provis AX 70 with a 60 \times oil immersion lens, N.A. 1.40 (Olympus, Tokyo, Japan). Microphotography was made using an Axiocam HR digital camera (Zeiss, Jena, Germany).

Scanning electron microscopy

Cells were fixed in 4% OsO₄ made up in 0.2- μ m filtered seawater (final concentration *c.* 2%) and placed on a poly-L-lysine coated circular coverslip for 20 minutes. After washing in dH₂O for 1 hour, samples were dehydrated in an ethanol series: 30%, 50%, 70%, 96% and 99.9% for 10 minutes in each change and finally in two changes of 100% ethanol, 30 minutes in each change. Critical point drying was in a BAL-TEC CPD-030 (Balzers, Liechtenstein). The coverslip was then mounted on stubs and coated with palladium-platinum and examined in a JEOL JSM-6335F field emission scanning electron microscope (JEOL, Tokyo, Japan).

The SEM stub used in this study has been deposited at the Natural History Museum of Denmark under accession no. CAT 2460.

TEM

Two different fixations schedules were used. In schedule 1, one volume of culture was added to one volume of fixation cocktail consisting of 2.5% glutaraldehyde made up in 0.1 M Na-cacodylate buffer with 0.5 M sucrose. The culture was pelleted by centrifugation after 1 hour of fixation and washed in five changes of buffer with decreasing sucrose concentration: 0.5 M, 0.4 M, 0.2 M, 0.1 M and straight 0.1 M buffer, 20 minutes in each change.

Postfixation was 1 hour in 2% OsO₄ made up in 0.1 M Na-cacodylate buffer, and the material was dehydrated in an ethanol series and embedded in Spurr's resin via propylene oxide.

This fixation schedule proved the best for internal structures and is used for all TEM figures except Fig. 12.

A block of this fixation is considered as epitype material and has been deposited at the Natural History Museum of Denmark, under accession number CAT 2461.

In schedule 2, living cells were pelleted by centrifugation at 1500 rpm for 5 minutes. Supernatant, except for about 0.5 ml, was discarded and cells were resuspended in the remaining culture medium. Examination of resuspended cells by light microscopy (LM) revealed that these were surprisingly vigorous and unlike most other dinoflagellates seemed to be unaffected by centrifugation. A volume of 200 μ l of dense resuspended cells was fixed for 20 minutes in 6.4 ml fixation cocktail consisting of *c.* 3% glutaraldehyde and *c.* 0.35% OsO₄ made up in *c.* 0.1 M phosphate buffer. Cells were then pelleted and briefly washed in two changes of 0.1 M phosphate buffer. Postfixation was 1 hour in 2% OsO₄ made up in 0.05 M phosphate buffer, and the material was dehydrated in an ethanol series and embedded in Spurr's resin via propylene oxide.

This fixation schedule preserved the amphiesmal vesicles well but internal structures rather poorly. It is only used to illustrate Fig. 12.

A block of this fixation has been deposited at the Natural History Museum of Denmark, under accession number CAT 2462.

The material from both fixation schedules was sectioned on a Reichert Ultracut E ultramicrotome (Leica, Wetzlar, Germany) using a diamond knife. The sections were collected on slot grids and placed on formvar film. After staining in

uranyl acetate and lead citrate, sections were examined in a JEOL JEM-1010 electron microscope operated at 80 kV. Micrographs were taken using GATAN 792 or GATAN Orius SC1000 digital cameras (Pleasanton, CA, USA).

Pigment analysis

About 10 ml culture was filtered onto a 25-mm Whatman GF/F filter (Whatman International Ltd, Kent, UK). The filter was placed in a 5-ml syringe fitted with a 0.2- μ m filter, and 2.5 ml methanol was added. Cells were sonicated with a 6-mm probe for 30 seconds and filtered into a glass vial. One milliliter of this filtrate was mixed with 250 μ l distilled water immediately before analysis. The sample was analysed using a Waters high-performance liquid chromatography (HPLC) system with a Waters Symmetry C18 column (3.9 \times 150 mm, 5 μ m) following the methods of Wright *et al.* (1991). The system included a Waters 600 controller, a 717 plus autosampler and a Waters 996 photodiode array detector (Waters Corp., Milford, MA, USA). Pigments were identified by retention times and absorption spectra identical to those of standards. Pigments were quantified against standards purchased from DHI Lab Products (Hørsholm, Denmark).

DNA EXTRACTION

Ten milliliters of exponentially growing culture was harvested using a temperature-controlled centrifuge (Sigma-302K; Sigma-Laboratory Centrifuges GmbH, Osterode, Germany) set at 20°C and run at 2500 rpm (950 \times g) for 10 minutes. The pellet was transferred to an Eppendorf tube and following freezing at -18°C for 2 days total genomic DNA was extracted using CTAB (2 \times hexadecyltrimethylammonium bromide) as outlined in Daugbjerg *et al.* (1994).

POLYMERASE CHAIN REACTION

Approximately the first half of the nuclear gene encoding LSU rDNA was amplified using PCR. The 50- μ l reaction contained 5 μ l 10 \times Taq buffer [67 mM Tris-HCl, pH 8.5, 2 mM MgCl₂, 16.6 mM (NH₄)₂SO₄ and 10 mM β -mercaptoethanol], 20 μ l 0.5 μ M dNTPs, 5 μ l 10 μ M of each primer, 5 μ l 100 mM tetramethylammonium chloride and 1 U Taq polymerase (Ampliqon, Herlev, Denmark). Amplification primers were DIR-F (forward primer) combined with 28-1483 (reverse primer); for primer sequences, see Scholin *et al.* (1994) and Daugbjerg *et al.* (2000). PCR temperature profile was one cycle of denaturation at 94°C followed by 35 cycles, and each cycle consisted of three temperature steps: denaturation at 94°C for 1 minute, annealing at 52°C for 1 min and an extension at 72°C for 3 minutes. The PCR profile was ended by an extension step at 72°C for 10 minutes. To reveal DNA fragments of expected length (i.e. c. 1500 base pairs), PCR products were run for 20 minutes at 150 V in a 1.5% agarose gel containing ethidium bromide, and afterwards the gel was placed on a UV light table. The molecular marker used was Phi X175 HAE III (ABgene, Rockford, IL, USA). PCR fragments of correct length were purified using QIAquick PCR purification kit (Qiagen Inc., Valencia, CA,

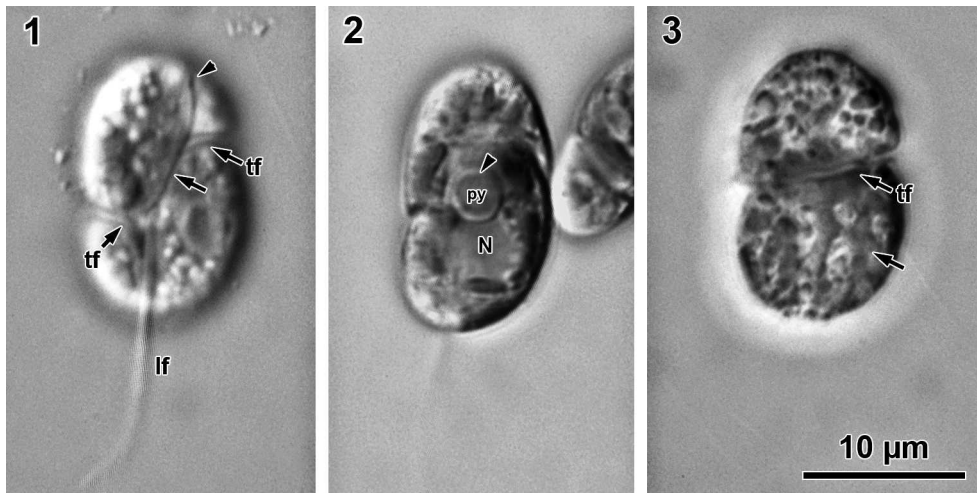
USA), and 30 ng DNA was used for sequence determination. Partial LSU rDNA sequence was determined using the dye terminator cycle sequencing ready reaction kit (Perkin Elmer, Foster City, CA, USA). Sequencing reactions were run on an automated sequencer (ABI PRISM model 3130 XL; Applied Biosystems, Foster City, CA, USA). For sequence determination in both directions, the two sequencing primers were used in addition to three internal primers (D3A and D3B; Nunn *et al.* 1996; and D2C; Scholin *et al.* 1994).

ALIGNMENT AND PHYLOGENY

The partial LSU rDNA sequence of *Moestrupia oblonga* was added to a data matrix comprising a highly diverse assemblage of dinoflagellates available in GenBank. This dinoflagellate in-group with 39 genera and 65 species was aligned with an out-group of ciliates (four species), apicomplexans (six species) and the perkinsid *Perkinsus*. GenBank accession numbers for all taxa included are provided in Fig. 39. For alignment purposes we used information from the secondary structure of the mature RNA molecule as suggested by de Rijk *et al.* (2000), and the data matrix was edited using MacClade ver. 4.08 (Maddison & Maddison 2003). We excluded the highly variable domain D2 *sensu* Lenars *et al.* (1989), as the DNA fragment could not be unambiguously aligned even among the dinoflagellates. This left 1141 base pairs, including introduced gaps for phylogenetic inference, and this was performed using Bayesian analysis as implemented in MrBayes ver 3.1.2 (Ronquist & Huelsenbeck 2003) and maximum likelihood as implemented in PhyML 3.0 (Guindon & Gascuel 2003). Bayesian analysis used a general time reversible (GTR) substitution model with base frequencies and substitution rate matrix estimated from the data. It was run for a total of 2 million Markov chain Monte Carlo generations with four parallel chains. A tree was sampled every 50th generation. The burn-in was evaluated by plotting LnL values as a function of generations. This resulted in a burn-in after 50,050 generations, leaving 39,000 trees for estimating posterior probabilities (i.e. 1001 trees were discarded). The online program AWTY by Wilgenbusch *et al.* (2004) was used to graphically evaluate that running 2 million generations in MCMC analysis was sufficient for the tree topologies to be sampled in proportion to their true posterior probability distribution. Posterior probabilities were obtained from a 50% majority-rule consensus of the saved trees using PAUP* ver. 4b10 (Swofford 2003). In maximum likelihood we used the parameter settings suggested by Modeltest ver 3.7 (Posada & Crandall 1998). To obtain an understanding of the robustness of the tree topology in maximum likelihood, we used bootstrapping with 1000 replications. All bioinformatic analyses were carried out on the freely available Biportal (www.biportal.uio.no).

SEQUENCE DIVERGENCE

We used MEGA ver. 4.1 (Tamura *et al.* 2007) to estimate the number of base substitutions per site from averaging over all sequence pairs between groups or single species. A total of



Figs 1–3. *Moestrupia oblonga*, LM of living cells.

Fig. 1. High focus reveals the ventral flange (arrow) and the barely visible apical groove (arrowhead). The longitudinal flagellum (lf) and transverse flagellum (tf) are also visible.

Fig. 2. Deeper focus reveals the pyrenoid (py) surrounded by a polysaccharide cap (arrowhead). Note also the posterior nucleus (N).

Fig. 3. Cell in dorsal view revealing the reticulated chloroplast (arrow). Notice the slightly coiled tf.

60 species divided into 20 groups (see Fig. 39) were included. The results are based on pairwise comparisons using the Kimura-2-parameter model as implemented in MEGA. Prior to sequence divergence estimates, all positions containing gaps and missing data were omitted from the data matrix, leaving 951 nucleotide positions to be analysed.

RESULTS

Taxonomy

Moestrupia gen nov.

Dinoflagellatae athecatae, ala ventrali microtubulis sustentata. Sulcus apicalis brevis, curvatus, ala ventrali ad partem medio-dorsalem cellulae transienti. Pedunculus e cavitate in episomate emergens. Amphiesma numerosis vesiculis penta-vel hexagonalibus constitutum, materiam laminae similem continentibus. Chloroplastus peridinin capiens tribus membranis circumcinctus. Pusula simplex sine receptaculo. Stigma absens.

Athecate dinoflagellates with ventral flange supported by microtubules. Short curved apical groove present, running from the ventral flange to the middorsal part of the cell. Peduncle emerging from a cavity in the episome. Amphiesma consisting of numerous penta- or hexagonal shaped vesicles containing plate-like material. Peridinin-containing chloroplast surrounded by three membranes. Simple pusule without collecting chamber. No eyespot present.

ETYMOLOGY: The genus name is chosen to celebrate Prof. Øjvind Moestrup on the occasion of his 70th birthday.

TYPE SPECIES: *Moestrupia oblonga* (J. Larsen & Patterson) Gert Hansen & Daugbjerg *comb. nov.*

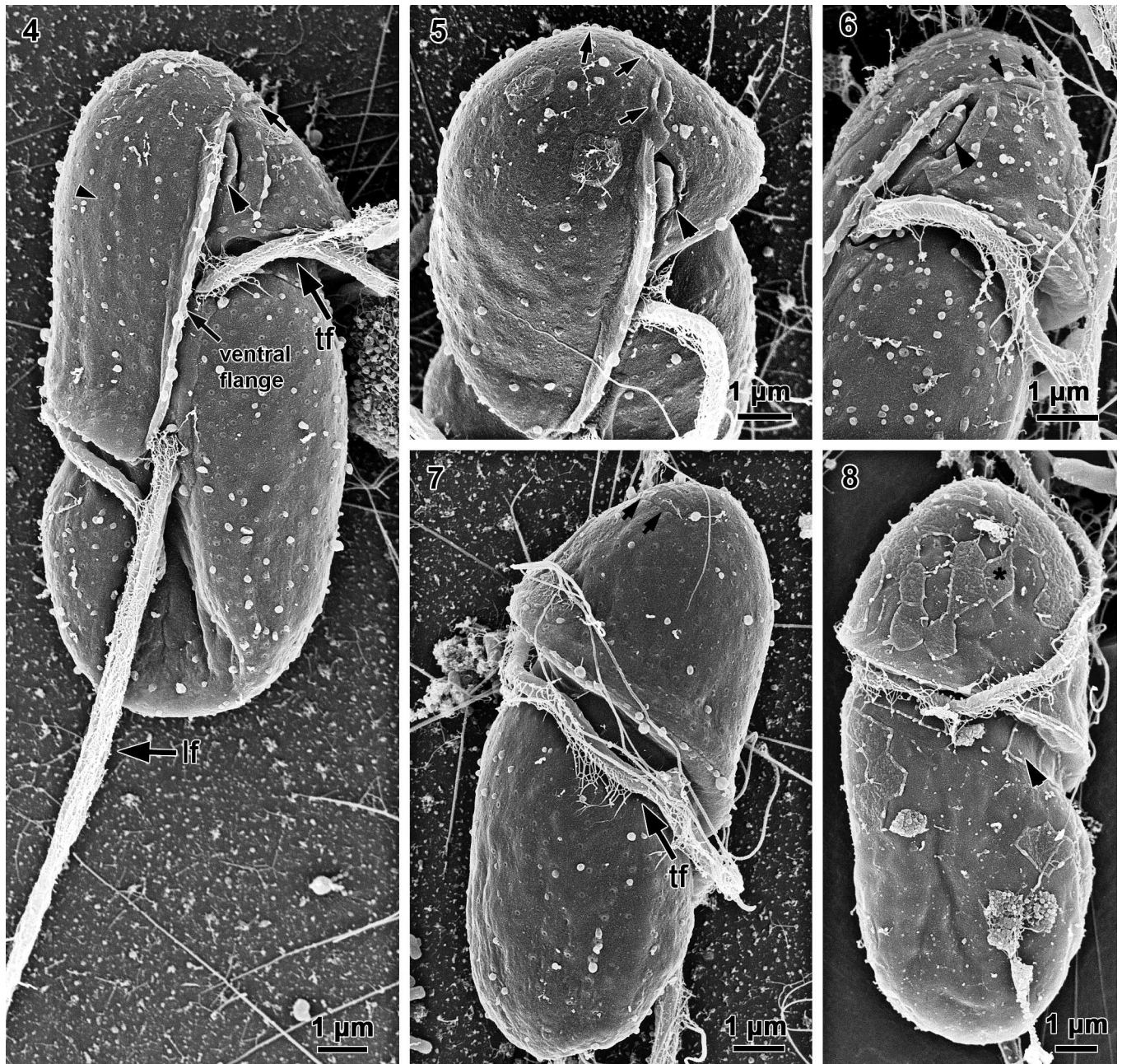
BASIONYM: *Gyrodinium oblongum* J. Larsen & Patterson (1990, p. 893, fig. 43g, h).

EMENDED DESCRIPTION: Cells are ovoid to oblong and 14–25 µm long (the upper range noted in Al-Qassab *et al.* 2002). The epi- and hyposome are almost equal in length.

The sulcus is relatively deep and becomes gradually wider towards the posterior end. The cingulum is displaced *c.* 0.3 times the cell length. The upper cingular border is distinct; whereas, the lower border is less clear. The cell is covered by many amphiesmal vesicles, and a prominent ventral flange is present. The apical groove is short and curved. The peduncle emerges from a small cavity situated in the episome. Transverse flagellum only slightly coiled and terminates within the sulcus. The chloroplast is reticulated with one associated pyrenoid of the single-stalked type. Peridinin is the major carotenoid pigment. The pusule is the simple type with spherical vesicles situated around each of the flagellar canals.

LM

Cells were elongated to ovoid and measured 14.6 µm in length (min. 12.6 µm, max 17.1 µm, SD 1.5, *n* = 10). The cingulum was displaced *c.* 0.3 times the body length (Fig. 1). A distinct dark line was apparent along the right border of the cingular displacement (Fig. 1). This structure, which we term the ventral flange, was more clearly visible in SEM (compare with Fig. 4). The same applied to the minute apical groove, which in LM was barely discernible and appeared as a slight bend of the ventral flange near the left apical part of the cell (Fig. 1, arrowhead). The sulcus was widening posteriorly and housed the longitudinal flagellum of about the same length as the cell body. The cingulum had a distinct upper border but no visible lower border (Figs 1, 2). The transverse flagellum was located in the cingulum (Fig. 3), but its distal end was situated within the sulcus (Fig. 1). The helical pitch of the transverse flagellum was much smaller compared to that of typical dinoflagellates (Figs 1, 3). This feature was also apparent in SEM (see below). The cell appeared to have one reticulated chloroplast (Fig. 3) and a centrally located distinct pyrenoid with a polysaccharide cap. The nucleus was situated posteriorly (Fig. 2).



Figs 4–8. *Moestrupia oblonga*, SEM.

Fig. 4. Cell seen in ventral view. The prominent ventral flange is evident as is the exit of the ‘lip-like’ peduncle (large arrowhead) within a cavity. Numerous longitudinal rows of pores are present on the cell surface (small arrowhead). The starting point of the apical groove is indicated by an arrow. tf and lf = transverse and longitudinal flagella, respectively.

Fig. 5. Apical view showing the apical groove (arrows) and the peduncle exit slit (arrowhead).

Fig. 6. Same features as in Fig. 5 but seen in left apical view.

Fig. 7. Cell seen in dorsal view. Imbrication of the apical groove is marked by arrows. Notice also the slightly coiled tf.

Fig. 8. Cell partly covered with amphiesmal vesicles. A single vesicle is indicated by an asterisk. Remnants of circular vesicles are marked by an arrowhead.

SEM

SEM clearly showed the ventral flange, a distinct wing-like structure along the right circular border continuing anteriorly onto the episome. This structure was differentiated in an outer somewhat thickened rib and a more narrow inner part (Figs 4, 5).

Another distinct structure was a small lip-like protrusion situated in a small cavity located on the episome. The right border of this cavity was formed by the anteriormost part of the ventral flange (Figs 4–6). TEM observations (see below) showed this structure to be the exit point of the peduncle.

A minute curved apical groove was running from the anterior part of the ventral flange to the middorsal side of

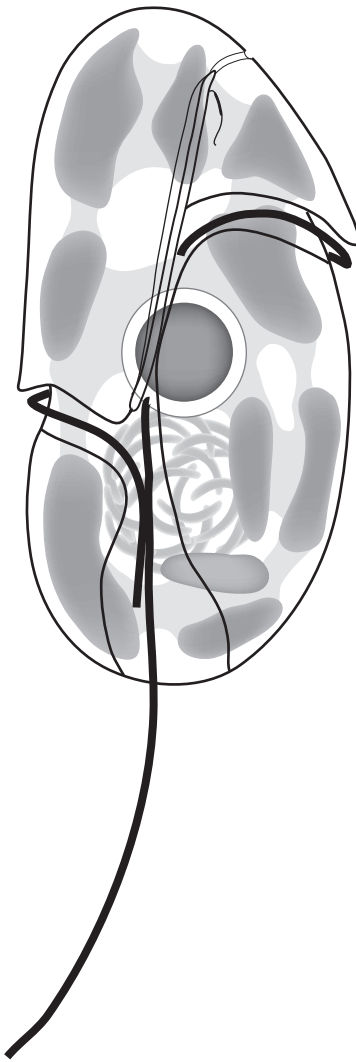


Fig. 9. Schematic drawing of *Moestrupia oblonga* based on LM and SEM observations.

the episome. The groove was bordered on each side by a delicate rim that might represent narrow amphiesmal vesicles (Figs 4–6). The amphiesmal vesicles were usually lost during the fixation, but imbrication of the groove was still apparent on the cell surface (Fig. 7).

In some cells penta- and hexagonally shaped amphiesmal vesicles were evident. The vesicles making up the cingulum were squarish rather than polygonal. Only one row was evident, though it seemed that a second narrow row had been present but was lost during the fixation (Fig. 8). The upper border of the cingulum was clearly marked by a thickened rim; whereas, no distinct rim was noticed along the lower border (Figs 4–8). Numerous small pores were present on the ‘naked’ cell surface (i.e. in cells where the

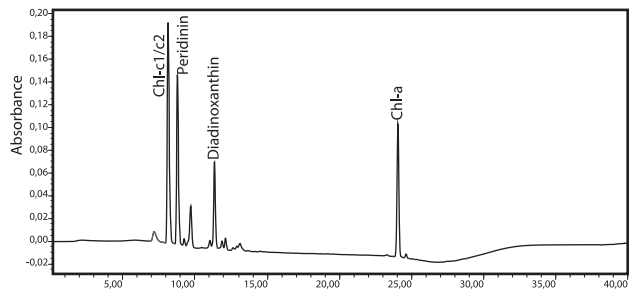


Fig. 10. Pigment profile of *Moestrupia oblonga*. Chlorophyll *c*1 and *c*2 could not be separated with the method used here.

amphiesmal vesicles were lost). The pores, which most likely represented trichocyst exit points, were arranged in regular rows on the episome but more randomly on the hyposome (Fig. 4).

The transverse flagellum was only slightly coiled (Fig. 7). This was originally thought to be a fixation artefact, but it was also noticed in living cells as stated above. Another unusual feature of the transverse flagellum was its termination within the sulcus. This is not so prominent in the specimen illustrated in Fig. 4, but in most other cases it continued the entire length of the sulcus. Figure 9 illustrates some of the features observed in LM and SEM.

Pigment profile

HPLC analysis of photosynthetic pigments revealed peridinin and diadinoxanthin as the major carotenoids (Fig. 10). Additional pigments identified were chlorophyll *a* and chlorophyll *c*1/*c*2. With the method used here, we could not differentiate between chlorophyll *c*1 and *c*2.

TEM

GENERAL ULTRASTRUCTURE: Thin sections revealed numerous chloroplast profiles that most likely comprised a single reticulated chloroplast (Fig. 11). This was also supported by observations using epifluorescence microscopy (data not shown). Three membranes enclosed the chloroplast, which is typical for peridinin-containing dinoflagellates (Fig. 16). The pyrenoid was the single stalked type and surrounded by an electron translucent polysaccharide cap (Fig. 11). Numerous mitochondrial profiles with tubular cristae were scattered throughout the cytoplasm but often closely associated with the chloroplast (Figs 11, 16). The nucleus was a typical dinokaryon with condensed chromosomes and normal nuclear pores (Figs 11, 17). Numerous starch grains were situated in the posterior part of the cytoplasm. Typical dinoflagellate trichocysts (i.e. extrusomes) were present (Fig. 15).

Numerous amphiesmal vesicles containing dark plate-like material subtended the outer plasma membrane

Figs 11–17. *Moestrupia oblonga*, TEM, general ultrastructure.

Fig. 11. Longitudinal section revealing the posterior nucleus with chromosomes (N), numerous chloroplast profiles (chl), pyrenoid with polysaccharide cap (py). Notice also the close association between the chloroplast and mitochondria (mi). Starch granules (asterisk) are situated in the posterior part of the cell. Arrow indicates the apical groove vesicles.



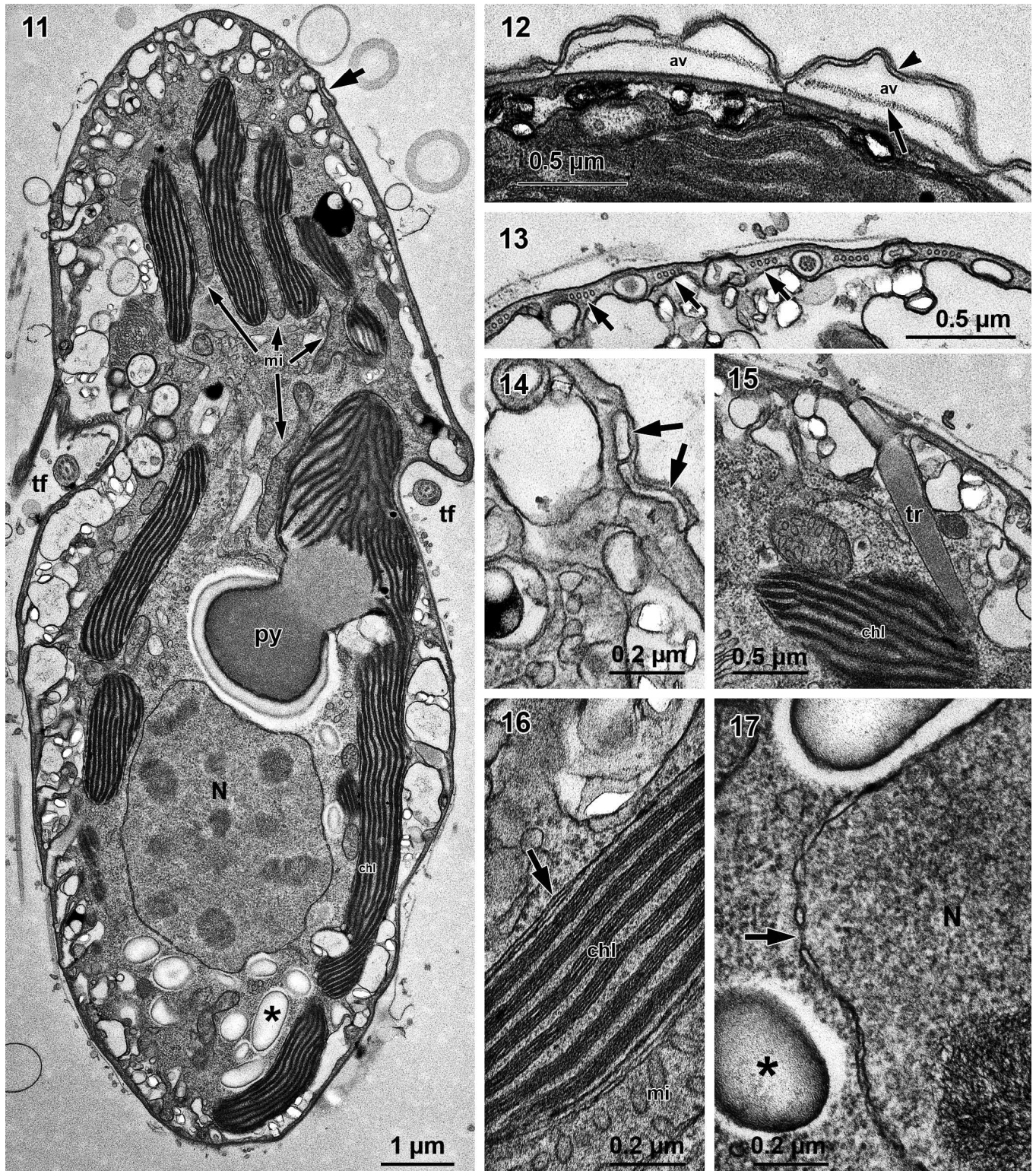


Fig. 12. Details of the amphiesma, fixation schedule 2. Plate-like material (arrow) is present within the amphiesmal vesicles (av). Plasmamembrane (arrowhead).
Fig. 13. Cell surface revealed by fixation schedule 1. The amphiesmal vesicles have been discarded, but subtheccal microtubules (arrows) are well preserved.
Fig. 14. The pair of putative apical groove amphiesmal vesicles (arrows).
Fig. 15. Trichocyst (tr) in longitudinal view and chloroplast (chl).
Fig. 16. The chloroplast (chl) is surrounded by three membranes (arrow).
Fig. 17. Details of the nuclear envelope, revealing the nuclear pores (arrow). Notice also part of a chromosome in lower right corner and starch granules (asterisk).

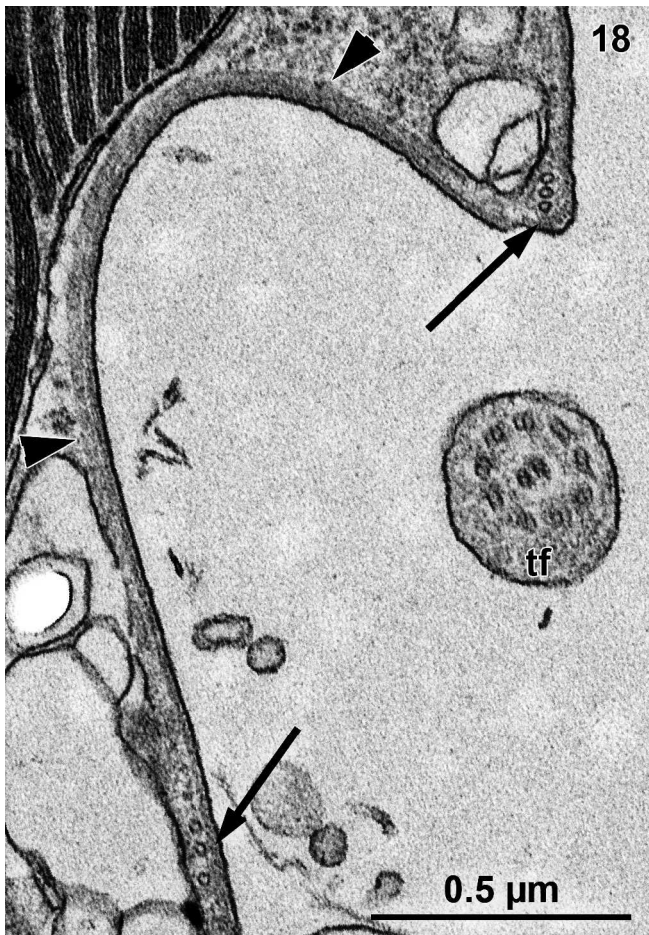


Fig. 18. Details of the cingulum in longitudinal section. Microtubules within the cingulum are marked with arrowheads. The microtubules making up the cingular border are marked with arrows.

(Fig. 12). Generally cells fixed according to schedule 1 had lost the amphiesmal vesicles. The seemingly fused inner membranes of the amphiesmal vesicles then made up the outer membrane of the cell. However, often two narrow vesicles remained in the apical part of the cell, exactly at the position of the apical groove, and we consider these to represent apical groove vesicles (Figs 11, 14).

Numerous cortical microtubules were located beneath the inner amphiesmal membrane, usually in groups of four or five (Fig. 13). The upper border of the cingulum was evident in longitudinal sections, and a group of three microtubules was running transversally around the cell supporting this structure. No lower cingular border was obvious, but another group of three microtubules more widely spaced marked this area (Fig. 18). Cingular microtubules were situated between these two groups of microtubules, and contrary to the other cortical microtubules, these occurred as a band of evenly spaced microtubules, *c.* 50 nm or more apart, around the cingulum (Figs 18, 26).

Spherical pusular vesicles were associated with both flagellar canals. These opened directly into the canals rather than into specialised collecting chambers (Figs 26, 36–38).

THE PEDUNCLE: Thin sectioning of the lip-like protrusion in the episomal cavity revealed that the tip of this structure

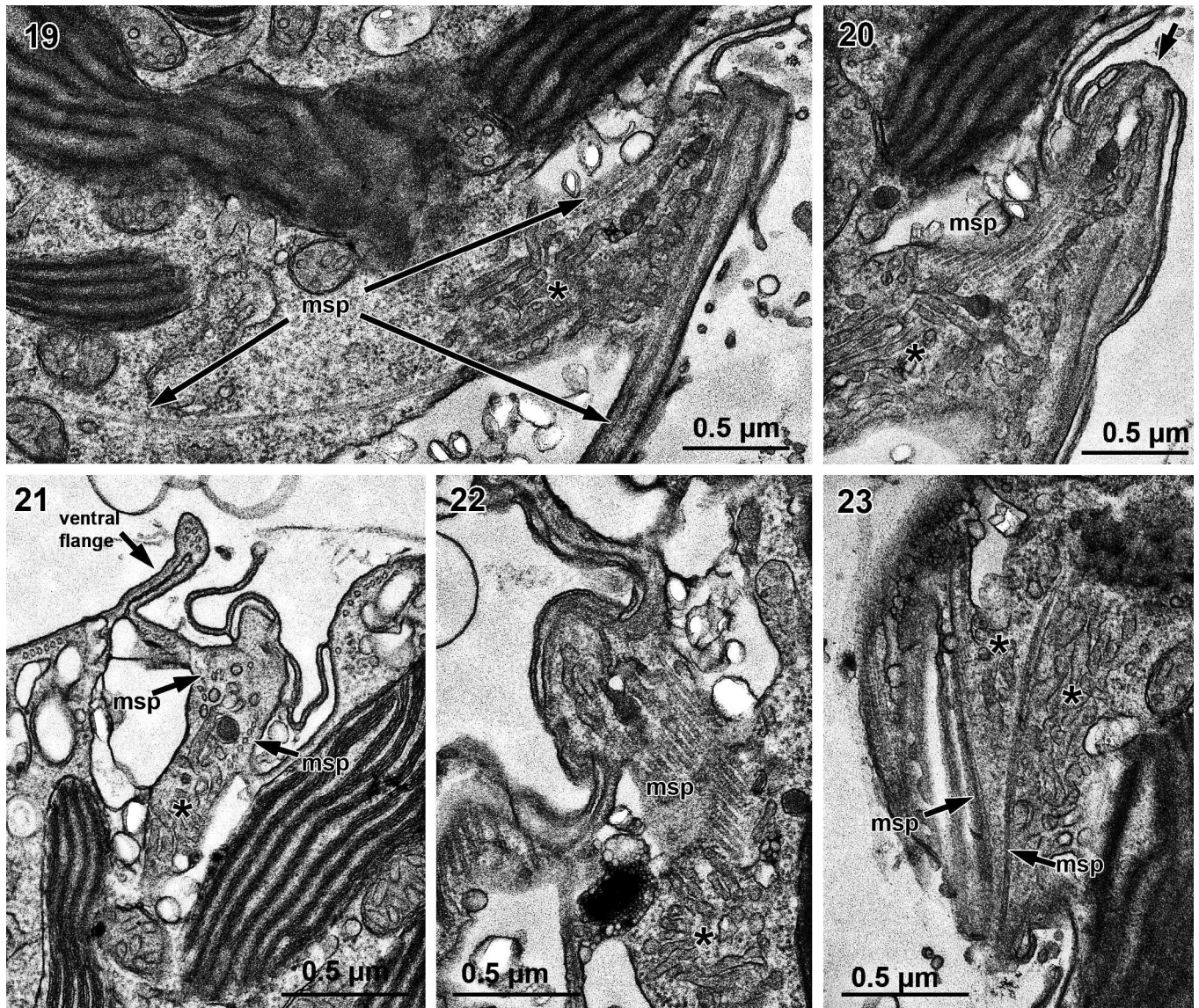
was devoid of amphiesmal vesicles (Figs 19–21). Numerous microtubules originating from deep within the cytoplasm terminated on this structure and made a funnel-shaped structure when observed in longitudinal sections (Fig. 23). The microtubules occurred as one major band consisting of seven to nine microtubules and a few scattered groups composed of one to two microtubules (Figs 21, 22). Tubules with dense content were associated with the microtubules (Figs 19–23). This arrangement is exactly the same as the feeding tube, the so-called peduncle, observed in the majority of dinoflagellates, and we therefore consider it to be a homologous structure.

VENTRAL FLANGE AND VENTRAL RIDGE: The ventral flange was formed by a long and narrow extension of the cell surface. The outer part of this structure was generally supported by two microtubules (Fig. 29), but up to five were observed at the level of the peduncle exit (Fig. 21). One of the peripheral vesicles or vacuoles formed a narrow extension into the inner part of the flange and continued along the entire length of the structure (Figs 21, 29).

Dark staining fibrous material was situated about 0.1 μm to the left of the ventral flange (Figs 29, 31, 33–38). This material seemed to span the distance between the two flagellar canals (not shown). At least it was no longer apparent at the level of the peduncular protrusion (Fig. 21). We consider this to represent the so-called ventral ridge fibre *sensu* Dodge & Crawford (1971). Cortical microtubules were situated next to the fibre, and one or two microtubules were situated beneath the fibre, though not easy to observe (Fig. 38).

THE FLAGELLAR APPARATUS: The three-dimensional configuration of the flagellar apparatus was not attempted, but we will provide some details for future reference. The basal bodies were inserted at an angle of about 140 degrees with respect to each other (Figs 24, 25). The root system associated with the flagellar basal bodies comprised four microtubular roots (R1–R4), two roots on each of the basal bodies. A multimembered root (R1) originated on the left side of the longitudinal basal body (LB) and continued posteriorly along the sulcus (Figs 24, 25, 31–38). It consisted of six microtubules at its starting point (Fig. 26), but this number gradually increased to 10–12 (Fig. 38). A single microtubular root (R2) initiated from the right side of the LB in a shroud of dense material and could be followed posteriorly along the longitudinal flagellar canal (Figs 26, 27, 33–38). A single stranded microtubular root (R3) was associated with the right dorsal side of the transverse basal body (TB). This root continued along the transverse flagellar canal, where it nucleated numerous microtubules, the so-called transverse microtubular root extension (TMRE; Figs 24, 25, 28–30). A compound root (R4) consisting of a single microtubular root and a striated fibre originated from the left proximal part of TB and continued along the transverse flagellar canal and the cingulum (Figs 24–27, 28, 29).

Numerous fibrous connectives interlinked the basal bodies and the flagellar roots. Thus, two small striated connectives, bbc_1 and bbc_2 , linked the basal bodies. The former was attached on the proximal ventral side of each basal body (Figs 30–32); the latter was attached to one of



Figs 19–23. The peduncle.

Fig. 19. Longitudinal section of the peduncle showing the microtubular strand(s) of the peduncle (msp) and the numerous electron-dense tubules (asterisk).

Fig. 20. Section from same cell as in Fig. 19; the exit point of the peduncle is devoid of amphiesmal vesicles (arrow).

Fig. 21. Transverse section of the peduncle. The ventral flange is also visible.

Fig. 22. Oblique section showing the msp.

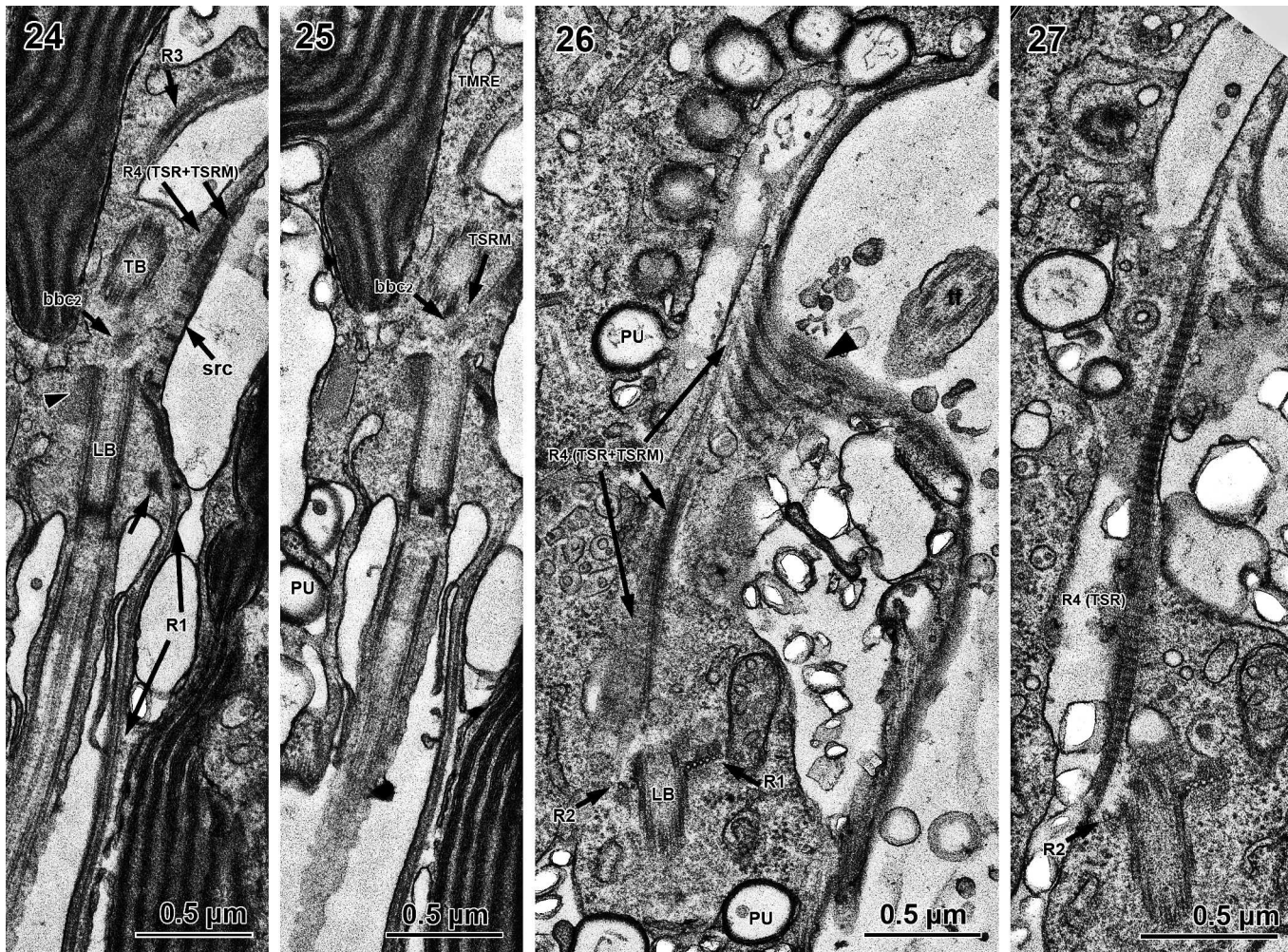
Fig. 23. Longitudinal section of the peduncle. The msp forms a funnel.

the triplets of the TB and the bottom part of the LB (Figs 30, 31). A striated connective, measuring about 0.5 µm in length, interlinked the R1 and R4 roots (Fig. 24). A dense fibre associated with the ventral side of the R1 root and diverged into three delicate threads each attaching to a triplet of the LB (Figs 34, 35).

Striated fibrous rings or sphincters around the flagellar canals were not present. These structures are often seen in other dinoflagellates.

MOLECULAR PHYLOGENY: The phylogeny of *Moestrupia oblonga* as suggested by Bayesian analysis of partial nuclear-encoded LSU rDNA is shown in Fig. 39. Here *Moestrupia* forms a sister taxon to all other species of

dinoflagellates included in the analysis. However, this branching pattern received only modest support from posterior probability (0.57) and less than 50% in maximum likelihood bootstraps (Fig. 39). Instead this single-gene analysis indicates that *M. oblonga* takes a phylogenetic position similar to well-established (monophyletic) evolutionary lineages of dinoflagellates representing families such as Kareniaceae, Tovelliaceae and Pfiesteriaceae or orders like Dinophysiales, Gonyaulacales, Gymnodinales (*sensu lato*), Peridinales (*sensu lato*) and Suessiales. The deepest lineages in the tree (including *Moestrupia*) form relatively short branches, and their interrelationship is unresolved. The unresolved topology of the deepest branches in Fig. 39 is similar to previously published phylogenetic analyses,



Figs 24–27. The flagellar apparatus (FA) in longitudinal view.

Figs 24, 25. Adjacent sections revealing major components of the FA. Arrow points to the connective between microtubular root R1 and the longitudinal basal body (LB); see also Figs 34, 35. Arrowhead indicates the fibrous material of the proximal part of the R2 microtubular root. R4 composite root consisting of the transverse striated root (TSR) and transverse striated root microtubule (TSRM); second basal body connective (bbc2), striated root connective (src), R1 (multistranded microtubular root), R3 (single stranded microtubular root) nucleating numerous microtubules (TMRE), TB (transverse basal body). Notice also pusule vesicles (PU) along the longitudinal flagellar canal.

Figs 26, 27. Adjacent sections of another cell showing the FA in a more oblique angle than previous sections. The striated pattern of TSR is evident. The TSRM is running along the cingulum. Several of the cortical microtubules in the cingulum are visible (arrowhead). The single microtubular root (R2) is situated on the right side (viewers left) of the LB. Notice that pusule vesicles (PU) are associated with both the transverse and longitudinal flagellar canals.

including a diverse assemblage of dinoflagellates using LSU rDNA sequence data (e.g. Moestrup & Daugbjerg 2007; Craveiro *et al.* 2010). The terminal taxa and their lineages are for the most part highly supported in terms of posterior probabilities and maximum likelihood bootstrap values.

SEQUENCE DIVERGENCE: To further explore the molecular data used here to infer the phylogeny and systematic position of *Moestrupia oblonga*, we estimated sequence divergences between 20 groups or single species of dinoflagellates as defined in Fig. 39. The maximum

→

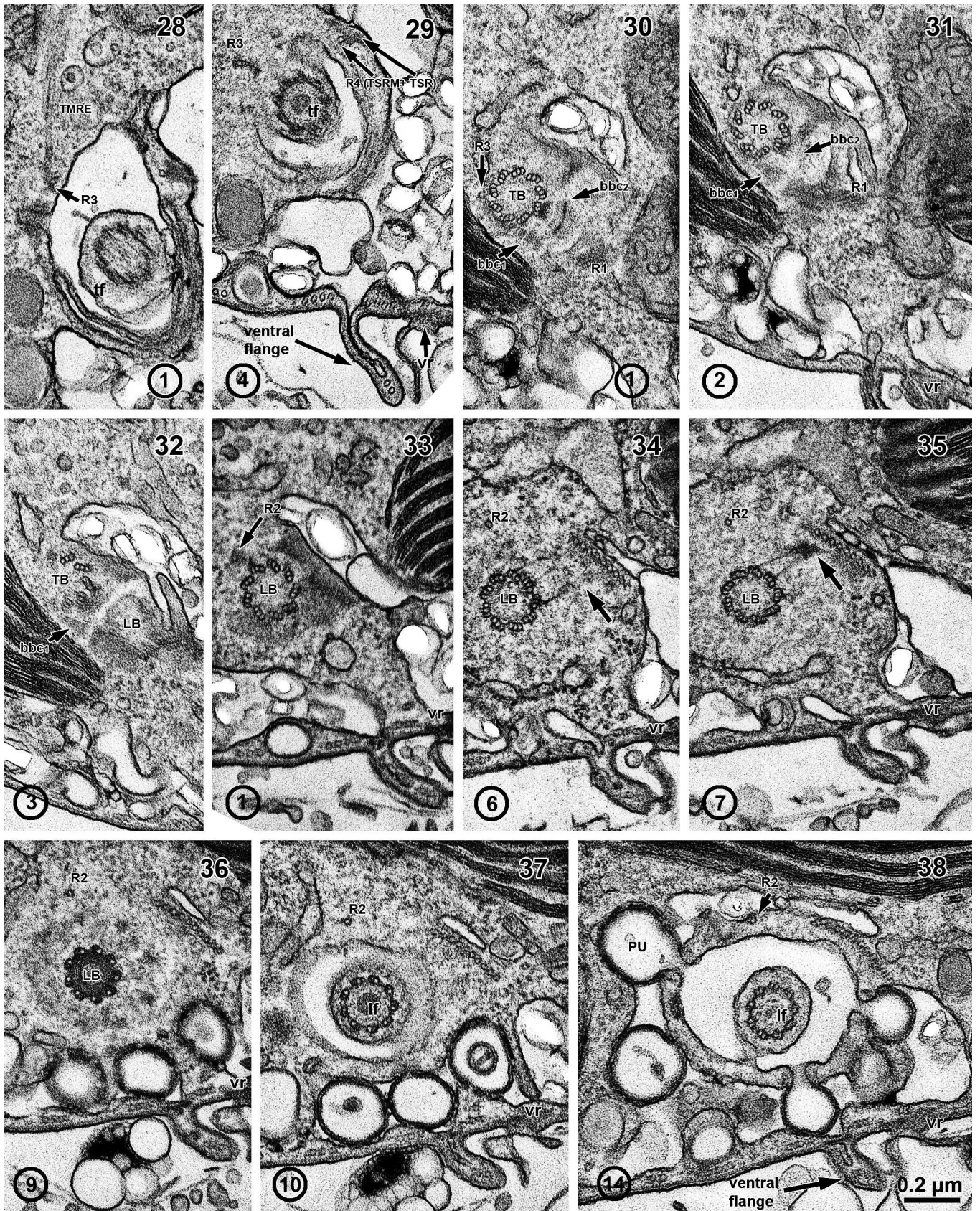
Figs 28–38. The flagellar apparatus (FA) in transverse view. Sections are from anterior to posterior and are represented by nonadjacent serial sections of three different cells: Figs 28–29, 30–32 and 33–38, respectively. Small encircled numbers refer to section number. Notice that sections have been tilted to reveal the basal bodies.

Figs 28, 29. The R3 nucleates numerous microtubules (TMRE) and the single microtubule (TSRM) associated with the TSR is particularly evident. The distal part of the ventral flange is supported by two microtubules. Notice also that the ventral ridge fibre (vr) is present at this level.

Figs 30–32. The two basal bodies are interconnected by two small connectives, bbc₁ and bbc₂, respectively.

Fig. 33. Origin of the R2 root.

Figs 34, 35. A connective interconnects the R1 microtubular root with three triplets of the LB (arrow).



Figs 36, 37. The transitional region of the LB, sections at the level of the diaphragm and central disc, respectively. Compare with Fig. 25. **Fig. 38.** The longitudinal flagellum (If) inside the flagellar canal. Numerous pusular vesicles (PU) open into the canal. Notice that R2 is still present at this level, as are the ventral flange and ventral ridge fibre (vr).

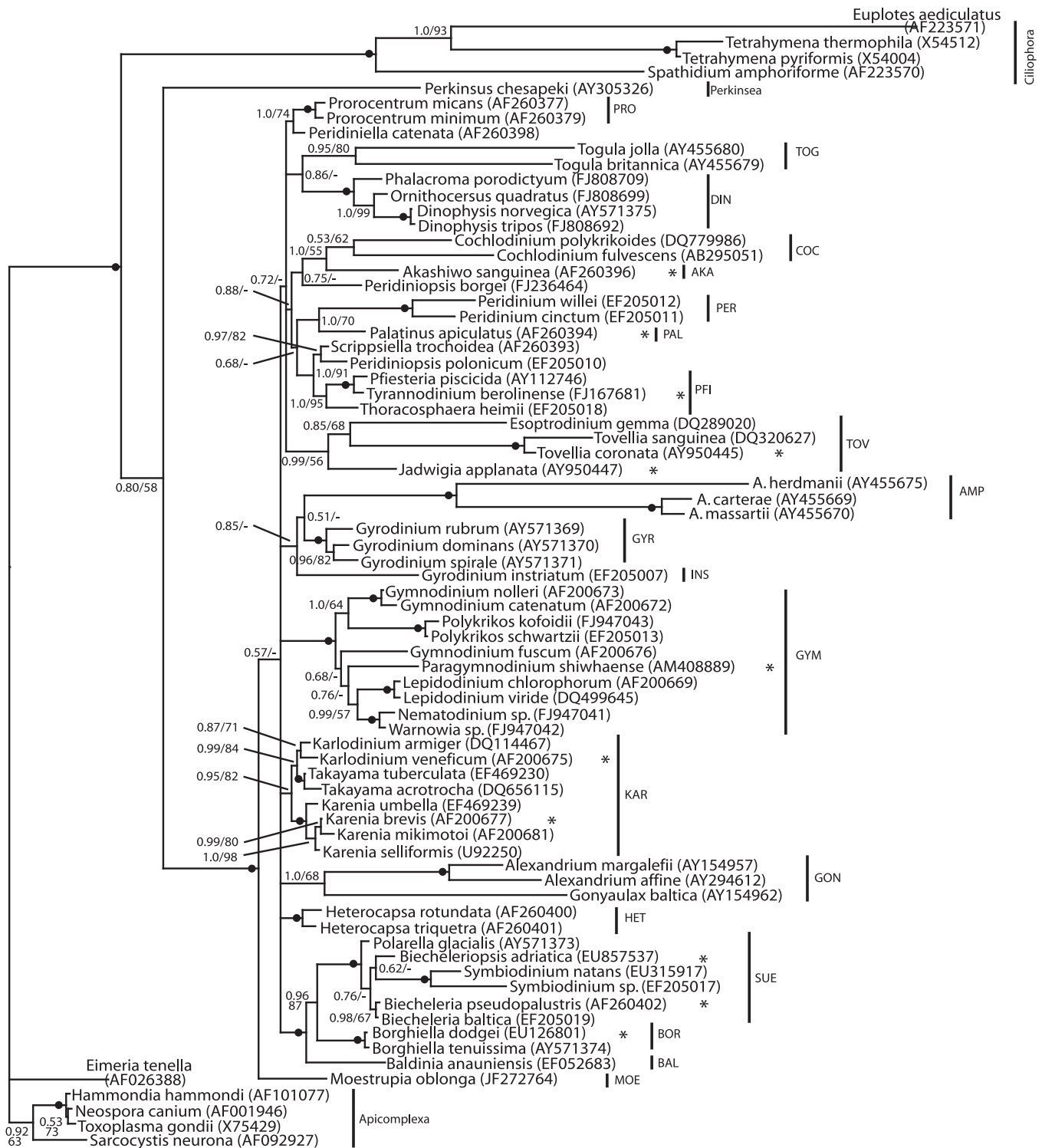


Fig. 39. Phylogeny of *Moestrupia oblonga* gen. & comb. nov. inferred from Bayesian analysis of nuclear-encoded LSU rDNA sequences. Four ciliates, five apicomplexans and *Perkinsus* comprised the out-group taxa. The first numbers at internal nodes are posterior probabilities (≥ 0.5) from Bayesian analysis; the last numbers are bootstrap values ($\geq 50\%$) from maximum likelihood with 1000 replications. A filled circle indicates the highest possible support in Bayesian analysis and maximum likelihood (1.0% and 100%, respectively). GenBank accession numbers are written in parentheses. Due to space limitations, the three species of *Amphidinium* are listed as *A. herdmanii*, *A. carterae* and *A. massartii*. The 11 asterisks (*) denote type species of dinoflagellates that Prof. Øjvind Moestrup and colleagues have erected since 2000. Capital three-letter codes to the right of vertical lines mark the 20 dinoflagellate clusters (groups or single species) used to estimate sequence divergences; see Table 1.

Table 1. Sequence divergence in percent of sequence pairs between groups or single species of dinoflagellates as they were defined in Fig. 39. A total of 60 species were included in the analysis.

	PRO	TOG	DIN	COC	AKA	PER	PAL	PFI	TOV	AMP	GYR	INS	GYM	KAR	GON	HET	SUE	BOR	BAL	MOE
PRO	—																			
TOG	16.0	—																		
DIN	8.2	17.8	—																	
COC	11.5	19.7	14.1	—																
AKA	8.7	18.8	12.0	11.7	—															
PER	9.1	19.1	12.3	15.6	13.1	—														
PAL	7.1	18.0	10.8	13.8	10.5	9.7	—													
PFI	5.5	17.2	10.0	12.4	9.5	10.5	7.1	—												
TOV	13.0	20.5	16.2	16.8	15.8	16.4	14.7	13.4	—											
AMP	22.0	28.6	23.7	24.5	23.9	24.3	23.2	22.2	25.1	—										
GYR	6.1	17.0	10.5	12.4	10.4	10.4	8.5	7.0	13.5	21.7	—									
INS	9.6	17.5	12.6	14.0	11.9	13.6	11.4	10.4	16.0	23.8	10.3	—								
GYM	9.2	19.1	13.2	13.7	12.0	13.3	11.7	10.5	15.7	24.2	9.7	12.7	—							
KAR	4.0	16.2	8.2	11.3	8.5	9.0	6.6	5.5	12.5	21.8	5.5	9.7	8.6	—						
GON	16.8	24.4	19.7	21.0	19.6	19.6	17.9	17.9	22.0	30.0	17.5	20.4	18.5	15.8	—					
HET	4.6	16.8	9.1	11.3	8.6	9.4	7.2	6.5	13.4	22.6	6.7	10.9	9.8	4.5	17.3	—				
SUE	8.4	17.6	13.0	14.4	12.1	13.1	11.4	10.4	16.1	24.1	9.9	12.5	12.6	8.7	19.3	9.1	—			
BOR	7.7	18.4	11.1	13.4	10.4	12.4	10.3	8.3	14.6	23.2	8.4	11.9	11.1	7.0	18.1	7.5	8.9	—		
BAL	8.2	17.2	10.9	14.6	12	12.4	11.1	9.0	15.3	23.2	9.0	10.9	12.1	8.3	18.3	9.6	10.1	8.0	—	
MOE	7.2	17.4	10.9	12.6	9.8	11.9	9.9	8.7	14.2	21.9	7.6	12.1	11.0	6.3	18.0	7.4	11.1	9.4	9.7	—

sequence divergences between any two groups (some as single species) were AMP-GON = 30% and AMP-TOG = 28.6%. The minimum sequence divergences were between KAR-PRO = 4%, KAR-HET = 4.5% and PRO-HET = 4.6% (Table 1). With respect to the dinoflagellate of interest here, *Moestrupia* had maximum sequence divergences to *Amphidinium* spp. (MOE-AMP = 21.9%), Gonyaulacales (MOE-GON = 18%) and *Togula* spp. (MOE-TOG = 17.4%). *Moestrupia* had the least sequence divergences to Kareniaceae (MOE-KAR = 6.3%), *Prorocentrum* spp. (MOE-PRO = 7.2%), *Heterocapsa* spp. (MOE-HET = 7.4%) and *Gyrodinium* spp. (MOE-GYR = 7.6%). From these comparisons it appears that the LSU rDNA of *Moestrupia oblonga* is equally divergent to any of the other 19 groups or species of dinoflagellates included here as each of these are to each other (Table 1).

DISCUSSION

Since its original description from sediment samples in Bowling Green Bay, Queensland (Larsen & Patterson 1990), *Moestrupia oblonga* has also been reported from sediments of the hypersaline Hamelin Pool, Shark Bay, Western Australia (Al-Qassab *et al.* 2002), in Broome, Western Australia and Botany Bay, Sydney (Murray *et al.*: <http://starcentral.mbl.edu/microscope/portal.php?pagetitle=assetfactsheet&imageid=13371>).

The Australian material has not been characterised by means of SEM, TEM or molecular data. Even so, its size, shape, distinct pyrenoid and position of the nucleus leaves no doubt that the isolate from Tenerife is identical to *Gyrodinium oblongum*. The fact that this species has now also been observed in Tenerife suggests a much wider distribution. The Australian specimens were observed in sediment samples; whereas, the material from Tenerife originated from a sample collected in the water column. Yet, the sample was collected near the shore during rather

rough weather conditions, and it was dominated by many species typically belonging to the benthic flora, such as pennate diatoms and dinoflagellates of the genera *Prorocentrum* and *Ostreopsis*. We therefore consider *Moestrupia oblonga* from Tenerife also to be a bottom dweller like the specimens from Australia. This was also supported by its behaviour in culture flasks. Here it either occurred in large immobile groups or swam at the bottom.

The present SEM, TEM and molecular data revealed details, to be discussed below, showing that this species does not fit the current concepts of the genus *Gyrodinium*, and we argue to transfer it to a new genus.

A number of recent studies on unarmoured and woloszynskioid dinoflagellates have stressed the taxonomic significance of apical structures such as the apical groove system (e.g. Daugbjerg *et al.* 2000; Moestrup *et al.* 2009a,b). The apical groove of *Moestrupia oblonga* does not really match any of the existing types and certainly not the elliptical bisected type found in *Gyrodinium*. It may have some resemblance to the *Gymnodinium* type, as it also continues in an anticlockwise direction onto the dorsal side of the episome. However, in most *Gymnodinium* species the groove forms a kind of a horseshoe-shaped loop around the cell apex. Though, in some species like *Gymnodinium aeruginosum* Stein, *G. palustriforme* Gert Hansen & Flaim, and *G. pseudomirabile* Gert Hansen & Flaim its shape is more like a fish-hook, and in *G. mirabile* Penard it forms an almost complete circular loop (Hansen & Flaim 2007). This indicates that finer details of the apical groove type may be used for further subdivision of *Gymnodinium*. This may therefore mirror the situation in the woloszynskioid dinoflagellates, where also the number of amphiesmal vesicles making up the groove is taken into account (Lindberg *et al.* 2005; Moestrup *et al.* 2006, 2008, 2009a, b). The number of amphiesmal vesicles making up the groove in *Moestrupia oblonga* was not determined with certainty but seemed to comprise two narrow vesicles. A pair of vesicles is also making up the groove system in the genus *Borghiella*. However, here one of the vesicles is

furnished with numerous knob-like protrusions, and the groove takes a straight line (Moestrup *et al.* 2008). This is different from the curved groove present in *M. oblonga*. The apical groove type does not provide any clue to the phylogeny of *M. oblonga*. It should be mentioned that the use of apical groove systems in gymnodinioid classification recently has been challenged, as *Paragymnodinium shiwhaense* Kang, Jeong, Moestrup, Shin, Nam, Park, de Salas, Kim & Noh does not possess the typical gymnodinioid apical groove (Kang *et al.* 2010). Rather, it possesses an apical groove that resembles the woloszynskioid type. Additionally no 'chambers' in the nuclear envelope and no connecting fibre between the nucleus and the flagellar apparatus are present in this species. These characters are normally present in 'true' gymnodinioids *sensu* Daugbjerg *et al.* (2000). Still, molecular analyses based on partial sequences of nuclear-encoded SSU and LSU genes propose a relationship between this species and the gymnodinioids (Kang *et al.* 2010).

The unique exit point of the peduncle on the episome was one of the most intriguing features of *M. oblonga*. Peduncles are common in dinoflagellates, but usually they emerge between the two flagella. However, in *Prosoaulax lacustris* (F. Stein) Calado & Moestrup, it also seems to emerge from the episome (Calado *et al.* 1998, as *Amphidinium lacustre* Stein) but not from a specialised structure as observed in *M. oblonga*. We did not observe any feeding by the peduncle, but on the other hand prey was not offered. In the culture flask it mostly swam in a wriggling fashion with the ventral side facing the bottom of the culture flask, as if it was grazing bacteria attached to the bottom, but remnants of bacteria were never observed inside sectioned cells. It cannot be excluded that this structure serves as holdfast organelle or any other unknown function rather than a feeding tube. However, its internal structure supports homology to a typical peduncle. In the majority of dinoflagellates, the peduncle consists of a single strand of microtubules, but in the pfiesterians and a species identified as *Gyrodinium lebouriae* Herdman by Lee (1977), it consists of several rows forming a so-called microtubular basket (Calado *et al.* 2009 and references therein). In *M. oblonga* the arrangement of the peduncle is somewhat different. Here one main strand consists of seven to nine microtubules in addition to groups of one to two microtubules.

The ventral flange represented another prominent feature in *M. oblonga*. Initially we considered it a well-developed ventral ridge. However, TEM revealed the presence of a typical ventral ridge next to ventral flange. A ventral ridge (i.e. an electron-dense, often slightly protruding fibre) is present in a number of dinoflagellates, but primarily in unarmoured or thinly thecate forms (e.g. Hansen *et al.* 2007a and references therein). It usually spans the distance between the flagellar pores or a short distance beyond (Dodge & Crawford 1968; Calado *et al.* 1998; Hansen & Daugbjerg 2004). Often the exit point of the peduncle is associated with the ventral ridge (e.g. Calado *et al.* 1998; Hansen *et al.* 2007a), but this seemed not to be the case in *M. oblonga*, as no trace of the ventral ridge fibre was observed at this position in the cell.

Likewise the general ultrastructure of *M. oblonga* left its closest relatives unresolved. The nuclear envelope was the

normal type found in other eukaryotes and most dinoflagellates and thus very different from the arrangements found in *Gyrodinium spirale* and 'typical' *Gymnodinium* species. The former has a nuclear capsule composed of two wall-like layers enclosed by membranes and nuclear pores restricted to globular invaginations of the inner layer (Hansen & Daugbjerg 2004), while the latter has nuclear pores restricted to special dilations of the nuclear envelope (e.g. Hansen *et al.* 2000b; Hansen 2001).

The pusule was identical to the 'simple type without collecting chamber' *sensu* Dodge (1972). In most dinoflagellates the pusule vesicles open into a large vesicle or tube, the so-called collecting chamber, rather than directly into the flagellar canals, as seen in *M. oblonga*. For example, *G. spirale* has a tubular collecting chamber extending deep into the cytoplasm (Hansen & Daugbjerg 2004); whereas, *Gymnodinium fuscum* (Ehrenberg) Stein has a rather complex type consisting of an internal collecting chamber *sensu* Dodge & Crawford (1969) with numerous merging pusular vesicles and an elaborate convoluted canal interconnecting the collecting chamber with the longitudinal flagellar canal (Hansen *et al.* 2000b). Other gymnodinioids have a more simple type with a collecting chamber that opens directly into the flagellar canal, such as *G. aureolum* and *G. catenatum* L.W. Graham. These species have spherical and tubular pusular vesicles associated with their collecting chamber, respectively (Hansen 2001; Rees & Hallegraeff 1991). This shows that large variations occur in pusular structure even within a single genus. The simple type present in *M. oblonga* is not so common but has been observed in such diverse and unrelated species as *Amphidinium carterae* Hulbert and *Akashiwo sanguinea* (Hirasaka) Gert Hansen & Moestrup (Dodge 1972; the latter species as *Gymnodinium nelsonii* Martin). Currently the taxonomic significance of the pusule type is unclear.

Details of the flagellar apparatus in dinoflagellates have gained increasing attention following the pioneering work of Roberts and coworkers (Roberts 1985, 1986, 1989; Roberts *et al.* 1988, 1995; Roberts & Timpano 1989). Its basic structure consisting of the three flagellar roots, R1, R3, and R4, following the terminology of Moestrup (2000), is very similar throughout the group. An additional root, the R2, was later discovered in certain gonyaulacoid dinoflagellates (Hansen *et al.* 1996, 1997; Hansen & Moestrup 1998) and has subsequently also been found in a number of peridinioid and woloszynskioid species (e.g. Calado *et al.* 1999; Hansen *et al.* 2007a, Hansen & Daugbjerg 2009). It is easily overlooked but appears to be absent in, for example, *Gymnodinium fuscum*, *G. aureolum* and *Gyrodinium spirale*, but it is present in the gymnodinioids *Lepidodinium chlorophorum* (Elbrächter & Schnepf) Gert Hansen, Botes & de Salas and *L. viride* M.M. Watanabe, S. Suda, I. Inouye, Sawaguchi & Chihara (Hansen & Moestrup 2005; Hansen *et al.* 2007b). The presence of R2 in *M. oblonga* with its isolated position (see below) suggests that this root is common but may have been secondarily lost during the evolutionary history of some dinoflagellate lineages.

A number of fibrous connectives are associated with flagellar roots and the basal bodies, but with a few exceptions their phylogenetic significance is unclear. Until recently a more or less conspicuous fibrous connective

between the R1 root and the nucleus was exclusive to the gymnodinioid taxa. However, its recent finding also in the thinly armoured *Biecheleriopsis adriatica* Moestrup, Lindberg & Daugbjerg has invalidated this character as unique to gymnodinioids (Moestrup *et al.* 2009b). Peridinioids have a characteristic layered connective between the R1 and R4 roots rather than a striated connective present in other groups (e.g. Calado *et al.* 2009), and most naked or thinly thecate groups have a connective linking the R1 root with the longitudinal basal body (Craveiro *et al.* 2010); the latter connective is also present in *M. oblonga*.

In conclusion, the fine structure of *M. oblonga* revealed a few unique features, such as the exit point of the peduncle and the ventral flange. These features clearly set it apart from other dinoflagellates. Similarly, the general ultrastructure did not render any obvious relatives. The molecular phylogeny based on partial LSU gene sequence did not provide any obvious relationships between *M. oblonga* and other dinoflagellates included in the analyses. Rather, it took a somewhat isolated position albeit weakly supported. The pairwise distances between *M. oblonga* and 19 major dinoflagellate groups (some as single species) were within the same range as between the other groups. This adds support for a distinct evolutionary history of *Moestrupia* and that it most likely does not belong in any of the already known families or orders of dinoflagellates. However, we refrain from suggesting a higher taxonomic rank for *Moestrupia oblonga* until more species of this lineage have been characterised.

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