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The life cycle and taxonomic affinity of the coccolithophore *Jomonlithus littoralis* (Prymnesiophyceae)

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Abstract – *Jomonlithus littoralis* is a coccolithophore exhibiting unusual coccolith morphology that has previously only been reported from Japanese coastal waters. *Jomonlithus* is a mono-specific genus that has remained *incertae sedis* within the Prymnesiophyceae since its original description. We isolated a culture of the calcifying (diploid) stage of this species from Mediterranean coastal waters that subsequently produced a non-calcifying life cycle stage in culture. The non-calcifying (haploid) stage of *J. littoralis* is described for the first time through light and electron microscope observations of cytology, scale ornamentation and ultrastructure. A 28s rDNA molecular phylogeny is presented and the systematic affinities of *Jomonlithus* are discussed. This genus is placed in the Hymenomonadaceae and the diagnosis of this family is emended.

Coccolithophore / *Jomonlithus littoralis* / life cycle / ultrastructure / Hymenomonadaceae / Pleurochrysidaceae

INTRODUCTION

Jomonlithus littoralis Inouye & Chihara is a coccolithophore exhibiting unusual coccolith morphology first described from a culture isolated from samples taken at the mouth of the Nakagawa river in Japan (Inouye & Chihara, 1983). The monospecific genus *Jomonlithus* Inouye & Chihara is characterized by the coccolith which is composed of an organic base plate scale with a lightly calcified rim made up of small rectangular inner and outer sub-elements. In what was interpreted by Inouye & Chihara (1983) as incompletely formed coccoliths, rosary-like strands of calcite beads are sometimes observed around the rim of the base plate scale. These authors suggested that the rectangular elements of the fully developed coccoliths may be formed by fusion of these rosary-like strands. The rosary-like structures (from which the genus derives its name reflecting the

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similarity to the ornamentation of the ancient Japanese earthenware called "Jomon") are unique among coccolithophores studied to date. Inouye & Chihara (1983) conducted a detailed TEM study of *J. littoralis* and concluded that the ultrastructure of this species was similar to that of members of the genera *Pleurochrysis* Pringsheim and *Hymenomonas* Stein, all of these having bulging pyrenoids and well developed flagellar root systems with two compound and two simple microtubular roots. Due to uncertainties as to which of the families Pleurochrysidaceae or Hymenomonadaceae *Jomonlithus* most closely resembled and due to the unusual coccolith structure of this genus, *Jomonlithus* has remained *incertae sedis* within the Prymnesiophyceae since its original description. In the most recent revision of haptophyte taxonomy, Jordan *et al.* (2004) classified *Jomonlithus* as a genus "with possible affinities to the Pleurochrysidaceae or the Hymenomonadaceae".

The alternation of independent haploid and diploid generations within a haplo-diplontic life cycle is now believed to be widespread, if not ubiquitous, among prymnesiophytes (e.g. Houdan *et al.*, 2004a). The ornamentation of the organic body scales (when present) has been shown to consistently and predictably differ between diploid and haploid life cycle stages in prymnesiophytes. In addition, a common feature of this life cycle in coccolithophores appears to be that the diploid stage bears heterococcoliths (complex structures consisting of interlocking crystal units), while the haploid stage may bear holococcoliths (coccoliths made of numerous minute euhedral crystals), nanoliths, or be devoid of calcification. Despite increasing documentation of the nature and importance of coccolithophore life cycles, very few comparative ultrastructural studies within life cycles have been reported and it is generally assumed that diploid and haploid cells exhibit identical fine structure.

In this paper a non-calcifying stage in the life cycle of *Jomonlithus littoralis* is described for the first time through light and electron microscope observations of cytology, scale ornamentation and ultrastructure. A 28s LSU rDNA molecular phylogeny is presented and the systematic affinities of *Jomonlithus* are discussed.

MATERIAL AND METHODS

Cultures and culture conditions

The original clonal culture strain of *Jomonlithus littoralis* used in this study (RCC1354, Roscoff Culture Collection, France: www.roscoff-culture-collection.org) was isolated by micro-pipetting a single cell from an enriched water sample collected from El Perelló (39°16'45"N 0°16'45"W), approximately 25 km south of Valencia on the Spanish Mediterranean coast in December 1999. The sample was collected from the mouth of a small river draining the Albufera lagoon to the sea. Cultures of the haptophytes *Pavlova* sp. and *Platychrysis* sp. were also initiated from this sample. Initially isolated in filter-sterilized ES-Tris medium (Cosson, 1987) with a seawater base (salinity ~ 35), the *J. littoralis* batch culture was found to grow better and was therefore routinely maintained in 1:1 L-C (Lefebvre-Czarda, 1948):ES-Tris medium (salinity ~ 20). The culture was maintained at 17°C with daylight fluorescent tubes providing an irradiance of ~ 50 $\mu Em^{-2}s^{-1}$ with a photoperiod of 14L:10D. The original culture contained only the lightly calcified cells of *J. littoralis* described by Inouye & Chihara (1983).

Table 1. Summary of strains and nucleotide sequences used in the present study

Species	*Strain code*	*Isolator, year*	*Authentic culture*	*Genbank accession number*
Pavlova pinguis	RCC1538	S. Maestrini, 1969	no	EU502883
Phaeocystis sp.	AC618	I. Probert, 2003	no	EU502882
Prymnesium parvum	RCC1435	J. Fresnel, 1992	no	EU502881
Emiliania huxleyi	RCC1215	I. Probert, 2001	no	EU502880
Syracosphaera pulchra	RCC1460	I. Probert, 1999	no	EU502879
Calcidiscus leptoporus	RCC1147	I. Probert, 1999	no	EU502878
Pleurochrysis pseudoroscoffensis	RCC1403	J. Fresnel, 1979	yes	EU502877
Pleurochrysis placolithoides	RCC1401	J. Fresnel, 1983	yes	EU502876
Jomonlithus littoralis (calcifying phase)	RCC1354	J. Fresnel, 2000	no	EU502875
Jomonlithus littoralis (non-calcifying phase)	RCC1355	J. Fresnel, 2001	no	EU502874
Hymenomonas lacuna	RCC1340	I. Probert, 2003	no	EU502873
Hymenomonas globosa	RCC1338	C. Billard, 1971	no	EU502872
Ochrosphaera neapolitana	RCC1361	J.A. West, 1969	yes	EU502871

After a partial phase change in this culture (see results section), the mixed-phase culture was streaked onto agar plates (1.8% plant cell culture grade agar in 1:1 L-C:Es-Tris) and after a few weeks growth, an individual colony of the alternate phase (RCC1355) was transferred into liquid medium and maintained in conditions identical to the original culture.

Details of all of the clonal culture strains used to construct the molecular phylogeny are provided in Table 1. Batch cultures of these strains were grown in either ES-Tris medium or K/2 (-Tris, -Si) medium (Keller *et al.*, 1987) at 17°C with a light intensity of ~ 50 $\mu Em^{-2}s^{-1}$ and a photoperiod of 14L:10D.

Microscopy

Light microscope observations were conducted with a Leitz Orthoplan microscope equipped with differential interference contrast (DIC) optics. For fluorescence microscopy, pellets of cells were fixed for 1 hour with paraformaldehyde (2%) in phosphate buffered saline (PBS) with 5% NaCl, rinsed in PBS and stained with DAPI (5 µg/ml). Preparations were viewed with a Zeiss standard LAB16 epi-flourescence microscope. For scanning electron microscopy, cell suspensions were gently filtered onto 0.6µm pore-size isopore filters (Millipore) which were dried, mounted onto stubs, sputter coated with gold/palladium and viewed with a Phillips XL-30 field emission microscope. For whole mounts, a cell suspension was fixed with a few drops of osmium tetroxide (buffered at pH 7.4). The material was gently centrifuged (to observe scales of the coccolith bearing stage, coccoliths were dissolved by the addition of a few drops of dilute HCl to the centrifuge tube) and rinsed in double distilled water. Drops of the rinsed cell suspensions were mounted onto formvar coated grids, dried and shadow-casted with gold-palladium at an angle of about 20°. For the ultrastructural study, cells were fixed overnight at 4°C in a 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer (pH 7.4) containing 0.25M sucrose. The material was then rinsed in the same buffer containing decreasing

concentrations of sucrose (0.25 M, 0.125 M, the last wash without sucrose) for 15 minutes each time and post-fixed for 2 hours in 1% osmium tetroxide in 0.1 M sodium cacodylate. After rinsing in double distilled water, the cell pellet was embedded in 1.5% purified agar, dehydrated in a graded ethanol series and embedded in Spurr's resin. Thin sections were cut using a diamond knife and double stained for 30 minutes in 2.5% uranyl acetate in 50% ethanol followed by 10 minutes in lead citrate. All TEM preparations were viewed with a JEOL JEM-1011 electron microscope.

DNA extraction, amplification and sequencing

Exponential phase cultures were harvested by centrifugation (1000 r.p.m. for 5 minutes) and 100 µl of GITC* DNA extraction buffer (4M guanidine thiocyanate, 50 mM Tris-HCl (pH 7.6), 2% N-Lauroyl-sarcosine, 0.1 M β-mercaptoethanol) was added to the cell pellet. Cells in buffer were stored at – 20°C until analysis. Total DNA was extracted using a Qiagen DNeasy Plant MiniKit (Qiagen, Düsseldorf, Germany) following the manufacturers instructions. The 28S rDNA gene was amplified using a set of eukaryotic primers: Leuk2 (5'-acccgctgaacttaagcatatcact-3') and Leuk34r (5'-gcatcgccagttctgcttacc-3') with Taq DNA polymerase (Sigma) and a PCR enhancer system (Invitrogen) in order to amplify high GC-content DNA. PCR amplification was carried out with an initial denaturing step at 96°C for 2 min, 39 cycles of denaturing at 94°C for 30s, annealing at 55°C for 30s, extension at 68°C for 2 min and a final extension step at 68°C for 5 min. Amplified DNA was then sequenced in both directions on an ABI 3100-Avant Genetic Analyser (Applied Biosystems).

Phylogenetic analyses

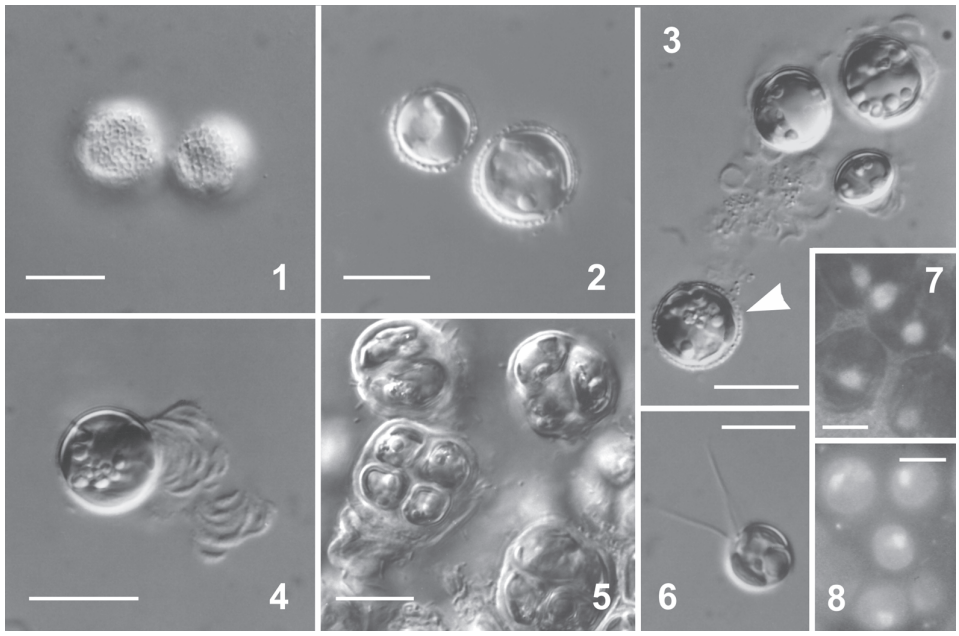
Sequences were manually aligned using the Genetic Data Environment (GDE) 2.2 software (Larsen *et al.,* 1993). Neighbour-joining (NJ, Saitou, 1987), maximum likelihood (ML, Felsenstein, 1981), PhyML (Guindon *et al.*, 2005) and Bayesian statistics (Ronquist & Huelsenbeck, 2003) methods were used to estimate the tree topology. For ML and PhyML analyses, the MODELTEST program (Posada & Crandall, 1998) was used to compare the likelihood scores while applying different nucleotide substitution models of the nested general time reversible (GTR) family to find the best fit model. The Bayesian approach was implemented in MrBayes ver. 3.1.2 (Ronquist & Huelsenbeck, 2003). The Markov chain Monte Carlo (MCMC) sampler was run for two million steps; two independent chains were run in parallel; split frequencies were checked to be < .015. The first 148800 steps were discarded as burn-in.

RESULTS

Light microscopy

The coccolith-bearing phase

For two years following isolation of our *Jomonlithus littoralis* culture strain, only the lightly calcified coccolith-bearing cells typical of the species were present. The non-motile cells of this phase were often flattened disc-shaped



Figs 1-8. DIC light micrographs of *Jomonlithus littoralis*. **1.** Coccolith-bearing cell observed in surface view with lightly calcified oval coccoliths. **2.** Coccolith-bearing cells seen in median optical section. **3.** Mixed culture with coccolith-bearing cell (arrowhead) and non-calcified cells. **4.** Non-calcified cell showing the refringent layer peeling away from the cell. **5.** Old culture with packets of non-calcified cells. **6.** Flagellated cell of the non-calcified generation. **7-8.** DAPI-stained nuclei of cells of the two generations: **7.** coccolith-bearing cells; **8.** non-calcified cells. (Scale bars = 10 μm.)

(10-12 μm) and were covered with small oval coccoliths (Fig. 1). The cells contained two parietal chloroplasts each with a prominent pyrenoid (Fig. 2). Inoculation into fresh medium provoked the formation of flagellated cells which exhibited a strongly positive phototactic response. These cells bore the same small oval coccoliths. Under our culture conditions the flagellated cells were observed over approximately a one month period following sub-culture, after which they lost/retracted their flagella and became non-motile. The coccolith-bearing phase was described in detail by Inouye & Chihara (1983).

Appearance and isolation of the non-calcifying phase

In late 2001 these coccolith-bearing cells were observed to be mixed with another type of cells, darker in colour, which did not possess coccoliths but which were surrounded by a refringent layer (Fig. 3). In light of the reported observations for *Hymenomonas* (Fresnel, 1994), the possibility that the two types of cells observed in our *Jomonlithus* culture represented different stages in a life cycle was immediately envisaged. The two types of cells were easily separated into pure culture by streaking onto agar medium and subsequent inoculation into liquid media of individual colonies (colonies of the cells without coccoliths being much darker in colour).

The non-calcifying phase

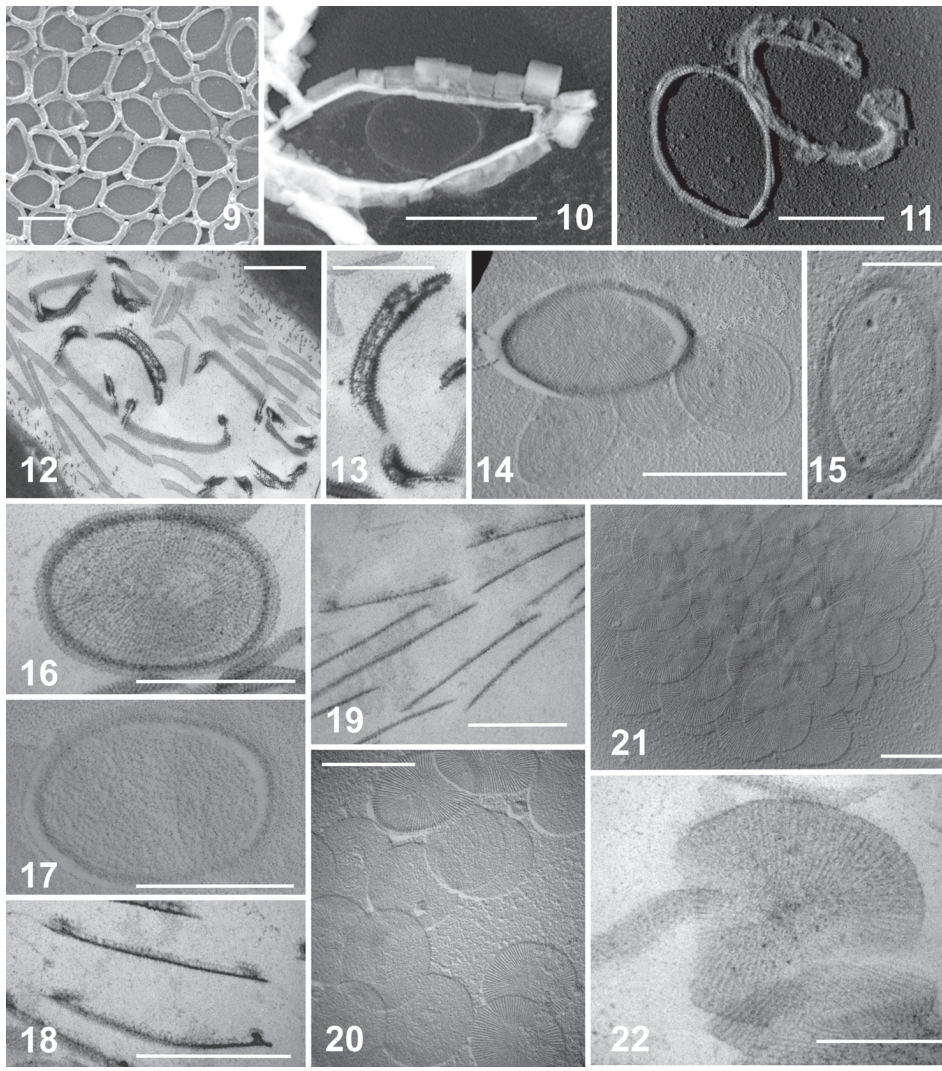
The non-motile cells of the non-calcifying phase were more or less spherical (never flattened like the coccolith-bearing cells) (Figs 3, 4). Slightly smaller (7-10 μm) than coccolith-bearing cells, cells of this phase contained two dark brown parietal chloroplasts. In LM, the pyrenoids were typically masked by numerous refringent inclusions (Fig. 4). There was no significant production of mucous around the cells. Cells tended not to separate after successive divisions and hence formed aggregations of four or more cells (Fig. 5). The refringent layer surrounding the cells was often detached around individual cells (Fig. 4) and was particularly thick around aggregations of cells (Fig. 5). Inoculation of these non-motile cells into fresh medium provoked the formation of numerous flagellated cells (Fig. 6) which, like flagellated cells of the coccolith-bearing phase, exhibited a strongly positive phototactic response. Following formation, these motile cells were ovoid and possessed two sub-equal flagella (12 and 14 μm in length), but did not possess a visible emergent haptonema (Fig. 6). These flagellated cells remained present for a few weeks following sub-culture, subsequently becoming rounded, losing/retracting their flagella and adhering to the walls of the culture vessel nearest to the light source.

Comparison of the size of the nuclei of the two phases by DAPI-staining revealed that the nuclei of the coccolith-bearing cells were roughly twice the size of those of the non-calcifying phase (Figs 7, 8).

Electron microscopy

The coccolith-bearing phase

Cells were covered by coccoliths and two types of unmineralized organic scales (Figs 9-15). The morphology of the calcified rim of the coccoliths varied, as observed by Inouye & Chihara (1983). In our culture strain the type 3 coccoliths of Inouye & Chihara (1983) were most frequently observed. This coccolith type consisted of 25-30 smooth and solid elements arranged around the rim of the base-plate scale (Fig. 9). Inouye & Chihara (1983) suggested that individual crystal units of these coccoliths consisted of two kinds of sub-elements that differed in size and shape: a cycle of relatively large square to rectangular sub-elements arranged subjacent to (and coincident with) a cycle of smaller rectangular sub-elements (respectively the inner and outer sub-elements). We interpret the structure slightly differently as crystal units consisting of a single element which appears to be cubic-shaped with a relatively well developed distal flange (corresponding to the outer sub-element) and a slight proximal flange (Fig. 10). Type 1 coccoliths consisting of characteristic rosary-like strands were observed mainly in shadowcast preparations of acid-treated (partly decalcified) preparations. The characteristic rosary-like strands were superposed around the rim of the base-plate scale, giving a coiled rope appearance (Fig. 11). In Type 2 coccoliths, the stacked strands formed rectangular elements with the "beads" still clearly visible (Figs 11-13). The elliptical coccolith base-plate scale, which measured 0.9-1.4 × 0.5-0.8 μm, had a raised rim and a pattern of radiating microfibrils arranged in quadrants on the distal face (Fig. 14) and an amorphous covering on the proximal face (Fig. 15). Unmineralized body scales, present in several layers underneath the coccoliths (Fig. 12), were circular to oval, 0.7-0.85 × 0.65-0.8 μm in size, and exhibited a pattern of concentrically arranged microfibres



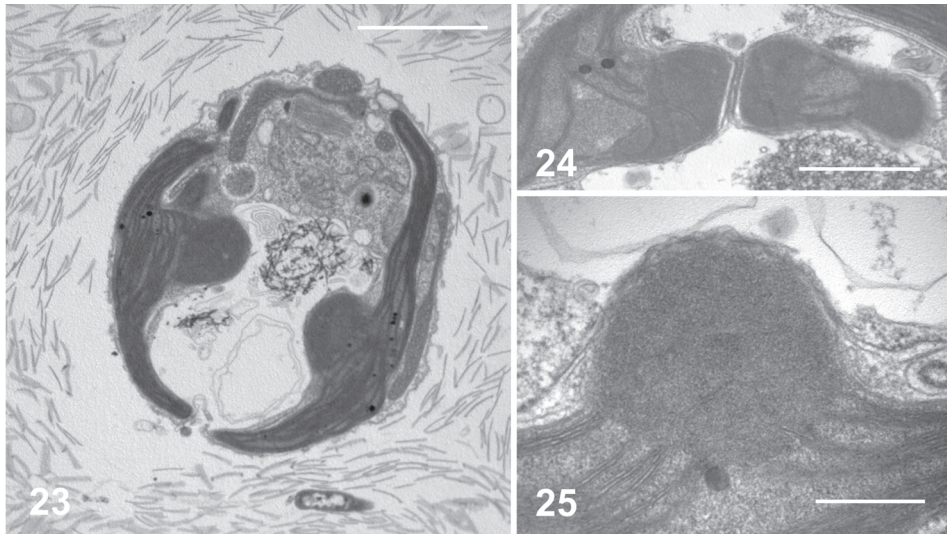
Figs 9-22. Electron micrographs of coccoliths and scales. **9-15.** Diploid calcifying generation: **9.** Part of the coccosphere; **10.** Detail of coccolith in proximal view showing proximal flange (arrowhead) and distal flange (arrow); **11.** Partially decalcified coccoliths corresponding to type 2 (arrowhead) and type 1 (arrow); **12.** Section of periplast showing coccoliths and underlying non-mineralized body scales; **13.** Detail of coccolith in longitudinal section; **14.** Coccolith base-plate scale in proximal view (arrowhead) and body scales; **15.** Decalcified base-plate scale in distal view. – **16-22.** Non-calcifying haploid generation: **16.** Tangential section of distal scale; **17.** Distal scale in distal view; **18.** Distal scale in transverse section showing upright rim (arrowhead); **19.** Transverse section showing single layer of distal scales (arrowhead) and several layers of flat body scales; **20.** Body scales in in distal view (arrowheads); **21.** Group of body scales in proximal view; **22.** Tangential section of body scale. (Fig. 9. SEM; Figs 10, 11, 14, 15, 17, 20, 21: shadowcast TEM. Figs 12, 13, 16, 18, 19, 22. TEM sections. Scale bars: Figs 9, 14 = 1 µm; Figs 10, 11, 12, 13, 15, 16, 17, 18, 19, 20, 21 = 0.5 µm: Fig. 22 = 0.25 µm.)

on both faces and a narrow rim (Fig. 14). A second type of smaller, elliptical scale (0.45 × 0.2 μm) with the same ornamentation was also occasionally observed (not illustrated).

The non-calcifying phase

TEM observation revealed the nature of the cellular covering of the non-calcifying cells. It consisted of layers of scales of two types: multiple layers of body scales and a single external layer of distal scales. Distal scales were more or less oval (0.75-0.8 × 0.5-0.6 μm) and exhibited different ornamentation on either face: radial microfibres arranged in quadrants on the proximal side (Figs 16, 20) and a concentric spiral pattern of microfibres, more or less masked by an amorphous covering, on the distal face (Figs 17, 20). Distal scales possessed a raised rim near the edge of the scales (Figs 18, 19). Body scales were most often circular (0.5-0.65 μm), but sometimes oval (0.55-0.65 × 0.45-0.5 μm), and exhibited the same ornamentation as distal scales (i.e. radial microfibres on the proximal side, concentric microfibres on the distal side), but were rimless (Figs 20-22).

The internal organisation of cells of the non-calcifying phase was similar to that of the calcifying phase (Inouye & Chihara, 1983), showing many of the features characteristic of the Haptophyta. Adjacent to the nucleus was a single Golgi body, the cisternae in the middle portion of the stack showing intercalary dilations characteristic of the haptophytes (not illustrated). The entire cytoplasm except the flagellar region was enclosed by peripheral endoplasmic reticulum (PER) situated immediately beneath the plasmalemma (Fig. 23). The two parietal chloroplasts possessed lamellae of three thylakoids (Figs 23-25). From the inner face of each chloroplast, a pyrenoid bulged towards the centre of the cell (Fig. 23), the two pyrenoids sometimes coming into contact with each other (Fig. 24). The pyrenoids were traversed by a few paired lamellae of thylakoids (Figs 24, 25).

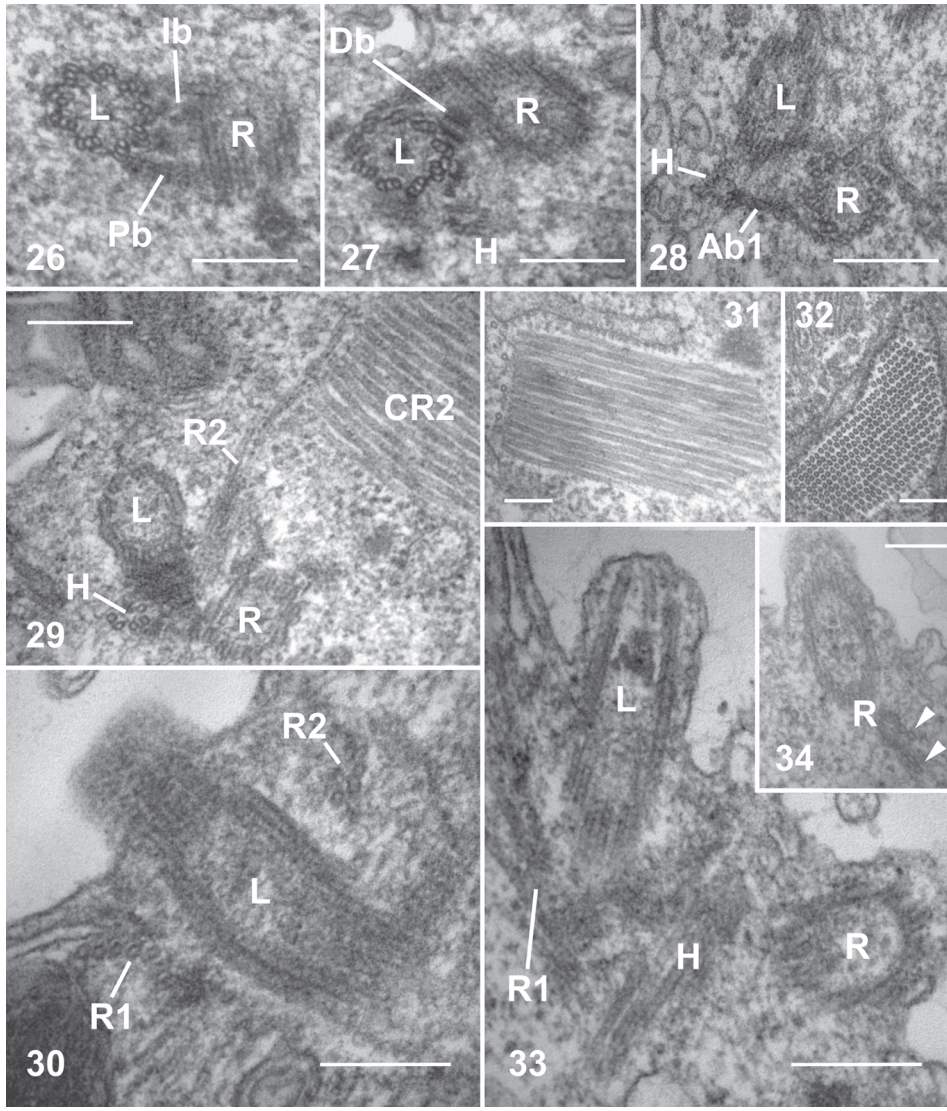


Figs 23-25. Ultrastructure of the non-calcifying phase. **23.** Section of a whole cell surrounded by multiple layers of scales. **24.** Bulging pyrenoids in contact with each other. **25.** Detail of a bulging pyrenoid, with a few paired lamellae of thylakoids. (Scale bars: Fig. 23 = 5 μm; Fig. 24 = 1 μm; Fig. 25 = 0.5 μm.)

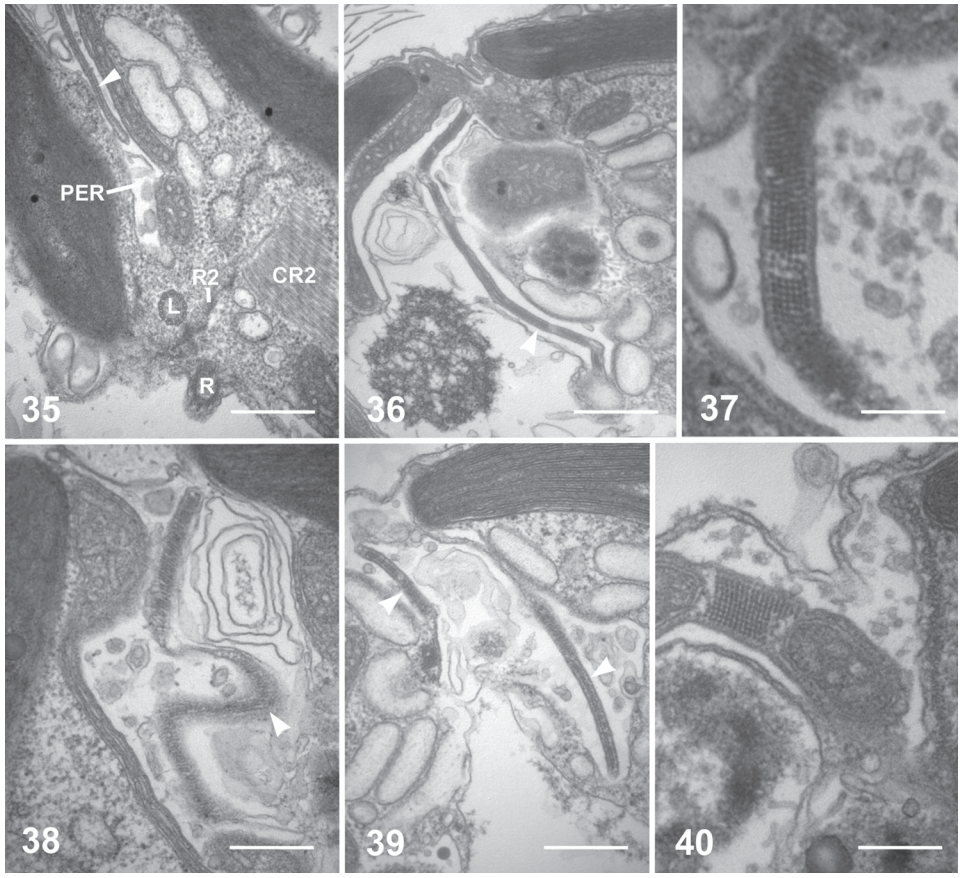
Girdle lamellae were not present in the chloroplast, the outer ER membrane of which was continuous with the nuclear membrane. Elongated sections of mitochondrion with tubular cristae were often situated adjacent to the inner surface of the chloroplast and in the antapical region of the cell (Fig. 23). Unmineralized scales were produced in Golgi vesicles, several scales often observed within one vesicle towards the mature face of the stack.

The structure of the flagellar apparatus was identical to that of the calcifying phase (Inouye & Chihara, 1983), except in one important respect. The two basal bodies and the haptonematal base were joined by connecting bands (Figs 26, 27). The haptonematal base, connected to the right basal body by an accessory band (Fig. 28), consisted of 5 microtubules (Fig. 29). Four types of microtubular roots (R1, R2, R3 and R4) were observed. As reported by Inouye & Chihara (1983) for the calcifying phase, the R2 consisted of a set of 5 microtubules extending from between the flagellar bases towards the cell surface, with a secondary bundle ("CR2") of more than 200 microtubules extending at a right angle towards the inner surface of one of the chloroplasts (Figs 29-32). R1 consisted of a sheet-like set of approximately 10 microtubules extending from the left basal body to a level just below the cell surface (Figs 30, 33), but in contrast to the calcifying phase (Inouye & Chihara, 1983), we found no evidence of a CR1 associated with this root. The fibrous root, which in the calcifying phase originates at the left basal body on the opposite side to the haptonematal base (Inouye & Chihara 1983), was not observed in the non-calcifying phase. R3 and R4 (together the R3 of Inouye & Chihara, 1983), both originating at the right basal body, each consisted of 2 microtubules at origin (Fig. 34) and these two roots joined some distance away from the basal body by which time the bundle had increased in number to 7 microtubules.

A structure which in some respects resembled the R1-fibrous root / cytoplasmic tongue complex in *Pleurochrysis carterae* (Braarud & Fagerland) Christensen described by Beech & Wetherbee (1988) was frequently observed in cells of the non-calcifying phase of *Jomonlithus littoralis* (Figs 35-41). As in *P. carterae*, the PER at the antapical end of cells of the non-calcifying phase of *J. littoralis* invaginated into the cell via the gap between the chloroplasts. This invagination penetrated towards the opposite, apical end of the cell in the vicinity of the flagellar bases (Fig. 35). A tongue of cytoplasm traversed this invagination of PER. In *P. carterae* elements of the microtubular sheet of R1 merged with a fibrous root (both originating at the left basal body) are incorporated into this cytoplasmic tongue and this root complex traverses the cell and attaches to the plasma membrane at the opposite, antapical end of the cell (Beech & Wetherbee, 1988). In *J. littoralis* the root-like structure within the cytoplasmic tongue was pseudo-crystalline in nature rather than microtubular (Figs 37, 40) and did not appear to originate directly from a flagellar root. The lattice of the pseudo-crystalline matrix was typically oriented in the direction of the axis of the cell as it traversed the cytoplasmic tongue, bending to form a plate more or less parallel to the cell surface near the antapical pole (Fig. 36). Sometimes, however, the cytoplasmic tongue and associated pseudo-crystalline structure zig-zagged through the PER (Figs 38-39). As in *P. carterae*, branches of the mitochondrion were always closely adpressed to some parts of the inner membrane of the PER adjacent to the cytoplasmic tongue and distinctive decorations were observed on the cytoplasmic face of the inner envelope of the PER. Loose membranous profiles and sometimes darkly stained stringy aggregations were observed within the PER and numerous vacuoles containing light amorphous material were present in the cytoplasm around (and sometimes fused with) the PER. Near the



Figs 26-34. Flagellar/haptonematal roots of the non-calcifying phase. **26.** Left (L) and right (R) basal bodies connected by a proximal band (Pb) and an intermediate band (Ib). **27.** Basal bodies connected by distal band (Db). **28.** Haptonematal base (H) connected to the right basal body by an accessory band (Ab1). **29.** R2 extending from between the flagellar bases towards the cell surface, with a secondary bundle (CR2) extending at a right angle towards the inner surface of one of the chloroplasts. **30.** R2 consisting of 5 microtubules and R1 extending from the left basal body to a level just below the cell surface. **31.** Longitudinal section of CR2. **32.** Cross section of CR2. **33.** Glancing section of flagellar and haptonemetal bases. **34.** R3 and R4, both originating at the right basal body, each consisted of 2 microtubules at origin. (Scale bars = 100 nm.)



Figs 35-40. Pseudo-crystalline root-like structure in the non-calcifying phase. **35.** Invagination of the peripheral endoplasmic reticulum (PER) containing a cytoplasmic tongue (arrowhead) penetrating towards the flagellar bases at the apical end of the cell. **36, 38, 39.** Various orientations of the cytoplasmic tongue and associated pseudo-crystalline structure (arrowheads) as they pass through the cell. **37.** Detail of the pseudo-crystalline structure. **40.** Cytoplasmic tongue merged with a cytoplasmic bridge containing mitochondrial profiles that surround the plate of pseudo-crystalline elements. (Scale bars = 0.5 μm.)

antapical surface of the cell the cytoplasmic tongue was observed to merge with a cytoplasmic bridge containing mitochondrial profiles which surrounded the plate of pseudo-crystalline elements which was angular in cross section (Fig. 40). In *P. carterae* the composite root was observed to attach to the plasma membrane at the antapex of the cell via a fibrous holdfast-like structure (Beech & Wetherbee, 1988). Such a structure was not observed in *J. littoralis*.

Molecular phylogeny

A phylogenetic reconstruction based on nucleotide sequences of the 28S rRNA gene is presented in Figure 41. Sequences of pure cultures of the two phases of *J. littoralis* were identical, confirming that the non-calcifying phase was

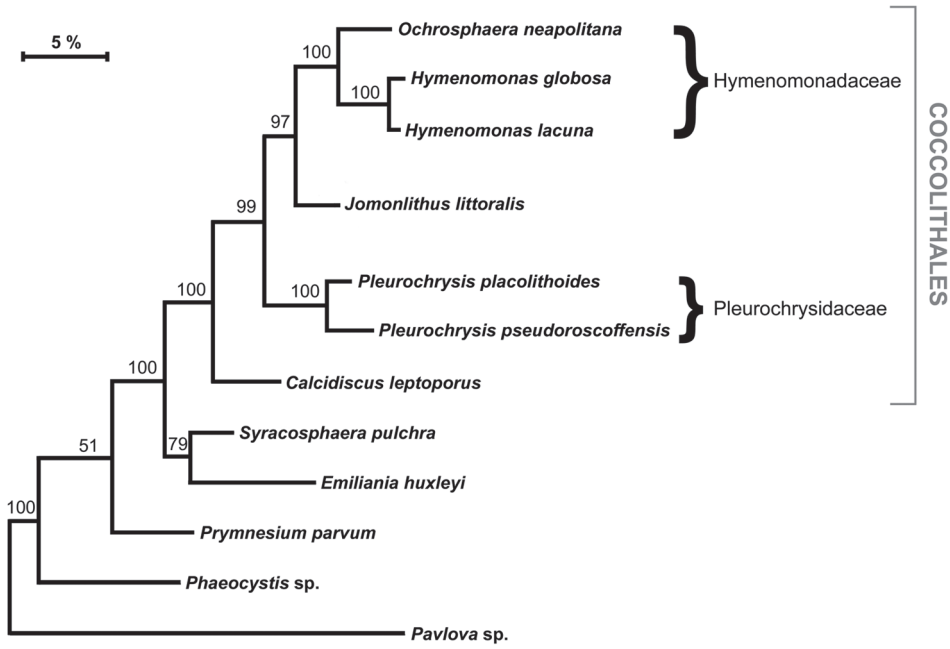


Fig. 41. Phylogenetic tree based upon a Bayesian analysis (50% majority rule) showing the relationships of a selection of haptophyte taxa. The tree is rooted on the branch leading to the outgroup, *Pavlova* sp.. Posterior probabilities are represented at internal nodes.

indeed a life cycle stage of this species and not a contaminant. *J. littoralis*, together with members of the Hymenomonadaceae and Pleurochrysidaceae, formed a monophyletic clade within the Coccolithales. Within this clade, *J. littoralis* grouped more closely with members of the Hymenomonadaceae (*Hymenomonas* and *Ochrosphaera* Schussnig), but did form a sub-clade distinct from the monophyletic grouping of *Hymenomonas* and *Ochrosphaera*.

DISCUSSION

Jomonlithus littoralis was reported by Inouye & Chihara (1983) in samples from several coastal locations around Japan, but since this publication this species has not, to our knowledge, been reported from any other location. *J. littoralis* has a very unusual coccolith structure, particularly in terms of the rosary-like strands of incompletely calcified coccoliths. Despite the lack of living reference material or sequence data from the authentic strain, the similarity of the morphology of the coccoliths of our culture strain to those reported by Inouye & Chihara (1983) leave no doubt that the culture strain from the Mediterranean Sea observed here is indeed a member of the morphospecies *J. littoralis*. This species evidently has a broad global distribution, but current evidence suggests that it is restricted to coastal (littoral or brackish water) locations.

The rectangular elements of the fully developed coccoliths of *J. littoralis* were noted by Inouye & Chihara (1983) to be reminiscent of the coccoliths of

Wigwamma Manton *et al.* and to be morphologically similar to those of *Pappos*phaera Tangen and *Pappomonas* Manton et Oates, three genera which were at the time (and still are) classified *incertae sedis* within the Prymnesiophyceae. However, several aspects of the ultrastructure of the cells left Inouye & Chihara (1983) under little doubt that the organism they described was closely related to members of the families Hymenomonadaceae and Pleurochrysidaceae. At the time it was not possible to state with confidence to which of these families *Jomonlithus* bore the closest affinities. A review of the current literature, which includes several detailed ultrastructural studies on members of the Hymenomonadaceae and Pleurochrysidaceae conducted since the study of Inouye & Chihara (1983), provides certain indications that *Jomonlithus* is more closely related to members of the Hymenomonadaceae. The flagellar root systems of members of the Hymenomonadaceae and Pleurochrysidaceae are broadly similar, being well developed with both R1 and R2 being compound (with the notable exception of *Hymenomonas coronata* Mills which does not possess a CR1 – see discussion below). However, the haptonema and haptonematal root differs between the two families: the Pleurochrysidaceae typically possess a short bulbous haptonema with a root consisting of 8 microtubules, whereas the Hymenomonadaceae do not possess an emergent haptonema (except the short "Stummel" in the freshwater species *H. roseola* Stein, see Manton & Peterfi, 1969), but have a haptonematal root consisting of 5 microtubules. In this respect, *J. littoralis* is identical to members of the Hymenomonadaceae. In addition, the coccolith base plate scale in both *Jomonlithus* and *Hymenomonas* possesses a rim, unlike those of *Ochrosphaera* or the Pleurochrysidaceae. The pyrenoids of the calcifying phase of *J. littoralis* as illustrated by Inouye & Chihara (1983) more closely resemble those of *Pleurochrysis*, i.e. bulging and traversed by many intact paired thylakoids, whereas those of the non-calcifying phase illustrated here are of the *Hymenomonas* type, bulging more and containing less paired thylakoids.

Despite these ultrastructural similarities between *J. littoralis* and the Hymenomonadaceae, contradictory evidence exists from comparative biochemical studies. In the study of Van Lenning *et al.* (2004), the photosynthetic pigment composition of *J. littoralis* more closely resembled that of most members of the Pleurochrysidaceae than members of the Hymenomonadaceae, but the differences were minor (absence of 4-keto-fucoxanthin/19'butanoyloxyfucoxanthin in *J. littoralis* and 6 out of 8 members of the Pleurochrysidaceae, and presence of divinyl chlorophyll c3 in *Ochrosphaera*). In addition, Houdan *et al.* (2004b) reported that *J. littoralis* and several members of the Pleurochrysidaceae were toxic to *Artemia* larvae, whereas *Hymenomonas coronata*, *H. globosa* (Magne) Gayral et Fresnel and *Ochrosphaera neapolitana* Schussnig were non-toxic.

Our discovery of a heteromorphic (and digenetic) life cycle in *J. littoralis* provides clear evidence that *Jomonlithus* bears greater affinities to members of the Hymenomonadaceae than to members of the Pleurochrysidaceae. Due to the relative ease with which they can be collected and cultured, the Hymenomonadaceae and Pleurochrysidaceae are the most extensively studied coccolithophore families in terms of life cycles. Von Stosch (1955, 1967) demonstrated an alternation of heterococcolith (cricolith)-bearing cells with scale-bearing, non-calcifying pseudofilaments in *Pleurochrysis carterae*. The majority of cricolith-bearing (*Pleurochrysis*) species have also been shown to exhibit an alternate non-calcifying pseudofilamentous stage (Leadbeater, 1970, 1971; Inouye & Chihara, 1979; Gayral & Fresnel, 1983; Fresnel & Billard, 1991). Another life cycle type, involving the alternation of heterococcolith (tremalith)-bearing cells with scale-bearing, solitary non-calcifying cells, both stages being non-motile or

motile, was observed in two species of *Hymenomonas* (Fresnel, 1994). The life cycle of *Ochrosphaera neapolitana* (which also bears tremaliths) is very similar to that of *Hymenomonas*, one difference being that non-motile cells of the non-calcifying phase are typically surrounded by a thick layer of mucous (Fresnel & Probert, 2004). The absence of pseudofilaments in the non-calcifying (haploid) phase of the life cycle of *J. littoralis* clearly situates *Jomonlithus* closer to members of the Hymenomonadaceae, and in particular to *Hymenomonas* due to the lack of mucous production by the non-calcifying stage.

The phylogenetic analysis based on the 28s LSU rDNA gene presented here corroborates the conclusions based on comparison of cytological characters and life cycle characteristics. The consensus Bayesian tree indicates (with high posterior probabilities) that *Jomonlithus* and the monophyletic grouping of *Hymenomonas* and *Ochrosphaera* share a more recent common ancestor than either do with members of the Pleurochrysidaceae.

Given the multiple lines of evidence that indicate the close evolutionary relationship between *Jomonlithus* and members of the Hymenomonadaceae, the question arises as to whether this genus can now be placed in this family. The original description of the family Hymenomonadaceae, which has actually never been emended, was made by Senn (1900) who divided the Chrysomonads (unicellular algae with yellow-brown chloroplasts and oil and leucosin as storage products) into three families (the Chromulinaceae, the Hymenomonadaceae and the Ochromonadaceae) based on number and relative length of flagella (1 flagellum, 2 equal flagella, 2 unequal flagella, respectively). The Hymenomonadaceae were described as: "*Mit zwei gleich langen Geisseln versehene, ovale, langliche oder dreieckige Formen mit 1-2 Chrysochromplatten. Bewegliche Zellen nackt oder von Gallerte oder schalenartigen Hullen umgeben, einzeln oder in Colonien lebend*" (*with two equal length flagella, oval, elongated or triangular form with 1-2 chloroplasts. Flexible cell naked or surrounded with mucous or a flaky shell, living solitary or in colonies*). The genera included in this family were *Hymenomonas*, *Phaeocystis*, *Wystozkia*, *Naegeliella*, *Stylochrysalis*, *Derepyxis*, *Synura*, *Chlorodesmus* and *Syncrypta*. The first two of these genera are haptophytes (the non-calcifying *Phaeocystis* now being classified within the family *Phaeocystaceae L*agerheim, order *Phaocystales* Medlin in Edvardsen *et al.*, 2000), whilst the rest have been transferred to other classes. This diagnosis and circumscription are totally misleading for the current usage so this family clearly requires a formal emendation. In a recent guide to the identification of extant coccolithophores, Young *et al.* (2003) described the coccoliths of the Hymenomonadaceae as being goblet-shaped muroliths (also termed tremaliths) with an open central-area, well-developed proximal flange, and a narrow distal flange or flaring end, entirely formed of a single cycle of < 15 crystal units. Muroliths (from the latin *murus* meaning wall) are defined by Young *et al.* (1997) as being coccoliths with an elevated rim, but without the well-developed shields of placoliths. The coccoliths of *J. littoralis* are muroliths apparently with proximal and distal flanges, but are comprised of more than 15 crystal units. An emended description of the Hymenomonadaceae inclusive for features of the coccoliths of the genera *Hymenomonas*, *Ochrosphaera* and *Jomonlithus* is given below. Coccoliths of the Hymenomonadaceae differ from those of the Pleurochrysidaceae by the absence of a second cycle of elements *i.e.* R-units; the cricoliths of the Pleurochrysidaceae are a type of placolith with the R-units forming the distal shield (note that in *J. littoralis* the elements are too small to determine crystal orientation).

The rosary-like strands observed in the type 1 and type 2 coccoliths clearly distinguish *Jomonlithus* from *Hymenomonas* (and indeed all other

coccolithophores studied to date). We observed these relatively infrequently compared to Inouye & Chihara (1983) and we are not able to provide clear evidence as to whether these indeed represent incompletely formed coccoliths, or whether this is an artefact resulting from decalcification during sample preparation. In either case, to our knowledge these structures have never been observed in *Hymenomonas* or other coccolithophores and they remain a distinctive feature of the genus *Jomonlithus* and may represent a deviation from (or possibly a deeper insight into) the V/R model of coccolith structure and ontogeny with individual crystals nucleating to form a protococcolith ring (Young *et al.*, 1992).

Distinctive cytological features of *Jomonlithus* relative to other Hymenomonadaceae include the R2 consisting of 5 (as opposed to 4) microtubules (note that *H. coronata* was described (but not illustrated) as having a R2 consisting of 5-6 MTs by Roberts & Mills, 1992, but despite extensive searching we have always observed 4 MTs in the R2 of this species) and the pseudo-crystalline nature of the structure associated with the cytoplasmic tongue (at least in the non-calcifying phase). Both Gayral & Fresnel (1983) and Beech & Wetherbee (1988) hypothesized that the R1/fibrous root complex within the cytoplasmic tongue served a contractile function involved in the export of scales. It seems reasonable to assume that the pseudo-crystalline structure in *J. littoralis* is homologous and therefore potentially involved in the export of the copious amounts of scales observed in simultaneous production within the cell and adorning the cell surface. The ultrastructure of the haploid non-calcifying life cycle phase was very similar to that of the diploid calcifying phase as reported by Inouye & Chihara (1983), one notable difference being the lack of a CR1 in the former. In this respect, the haploid phase of *J. littoralis* groups with the diploid phase of *H. coronata* (Fresnel, 1989; Roberts & Mills, 1992) rather than with it is alternate diploid phase and all other (diploid) Hymenomonadaceae (Manton & Peterfi, 1969; Pienaar, 1976; Gayral & Fresnel, 1976; Fresnel & Probert, 2004). This raises interesting questions about the functional significance of flagellar roots and differential gene expression within the life cycle, but may compromise the use of flagellar root structure as a phylogenetic character.

Diagnosis

***Hymenomonadaceae* Senn *emend.* Probert, Fresnel & Young**

Calcifying diploid generation: Cells with coccoliths (muroliths) and non-calcified body scales. Coccoliths elliptical, consisting of base plate scale with or without upright rim on the distal face, 5-30 solid calcareous rim elements and open central area. Calcareous rim consisting of either solid rectangular elements with distal flange/pointed end and proximal flange or stacked strands. Body scales with identical ornamentation on both faces. Cells non-motile or motile; if motile, two equal flagella. Usually without emergent haptonema and haptonematal root consisting of 5 microtubules.

Non-calcifying haploid generation: Cells without coccoliths. Periplast covered by two types of scale: several layers of rimless body scales and a single layer of distal scales with upright rim on distal face. Body scales with differing ornamentation on distal and proximal faces. Cells non-motile or motile; if motile, two equal flagella. Usually without emergent haptonema. Cells solitary or in aggregations, with or without mucous covering, never pseudofilamentous.

Type genus: *Hymenomonas* Stein emend. Gayral & Fresnel.

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