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European Journal of Phycology

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/tejp20>

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Published online: 03 Jun 2010.

To cite this article: Klaus Wenderoth, Jürgen Marquardt, Martin Fraunholz, Yves Van De Peer, Jürgen Wastl & Uwe-G. Maier (1999) The taxonomic position of *Chlamydomyxa labyrinthuloides*, *European Journal of Phycology*, 34:2, 97-108, DOI: [10.1080/09670269910001736152](https://doi.org/10.1080/09670269910001736152)

To link to this article: <http://dx.doi.org/10.1080/09670269910001736152>

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The taxonomic position of *Chlamydomyxa labyrinthuloides*

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(Received 9 July 1998; accepted 2 December 1998)

Chlamydomyxa labyrinthuloides is a heterokont alga known since the last century. It lives on *Sphagnum* and other water plants as aplanospores or plasmodia. We have investigated the taxonomic position of *Chlamydomyxa labyrinthuloides* by combining results from morphological studies, pigment analyses and a molecular phylogenetic analysis of the small subunit (SSU) rRNA gene. *Chlamydomyxa labyrinthuloides* shares morphological features with xanthophytes and chrysophytes, whereas pigment composition indicates a grouping with the phaeophytes, raphidophytes and chrysophytes. The sequence of the SSU rRNA gene and its phylogenetic reconstruction unambiguously demonstrate that *Chlamydomyxa labyrinthuloides* is related to the chrysophytes.

Key words: aplanospores, *Chlamydomyxa labyrinthuloides*, Chrysophyceae, plasmodium.

Introduction

Chlamydomyxa labyrinthuloides Archer lives as aplanospores (cysts) or plasmodia on *Sphagnum* and other water plants. The taxonomic position of this organism is confusing since it possesses only a few diagnostically useful characters. The organism was first reported by Janisch (1859), who described obvious aplanospores. However, he misinterpreted them as a developmental state of a diatom, due to the colour of the chromatophores and the presence within the aplanospores of diatom frustules, which probably came from an engulfed diatom. In 1875 Archer described *Chlamydomyxa labyrinthuloides* as a new species and genus. He observed the germination of the aplanospore resulting in an amoeboid organism with finely branched filopodia and yellowish-green plastids in the central cytoplasm, but did not detect nuclei. Archer assumed that *Chlamydomyxa* is related to the marine protist genus *Labyrinthula* Cienkowski, but Geddes (1882) finally recognized *Chlamydomyxa* as an alga. Hieronymus (1898) thoroughly investigated aplanospores and amoebae in samples from Riesengebirge (Poland) and first observed multinuclear amoebae, although he also found uninucleate forms. Pénard (1904) reported that the amoebae also produced flagellate states. It is possible that Pénard described a mixture of *Chlamydomyxa* and *Myxochloris sphagnicola* Pascher (see Discussion). Pascher (1930) distinguished *Chlamydomyxa* from *Myxochloris*, whose habitat is also in the hyaline cells of *Sphagnum*, where it has a complex life history (flagellate states, amoebae and aplanospores with one or more nuclei). In the case of

Myxochloris, membership of the Heterokontophyta is unequivocal. Pascher (1931) placed both *Myxochloris* and *Chlamydomyxa* in the Myxochloridaceae within the Heterokontae Luther (= Xanthophyceae), but noted that their taxonomic position was not clear.

Here we investigate the taxonomic position of *Chlamydomyxa labyrinthuloides* by both classical and modern methods, combining light and electron microscopy, pigment analysis and molecular biology.

Materials and methods

Isolation and cultivation

Aplanospores of *Chlamydomyxa labyrinthuloides* were isolated from the Seetaler See (Lungau), Centralalps (Austria) by H.A. von Stosch on 20 April 1974. They were also collected from this habitat and in other bogs (Dürreneck, Fuchsschwanzmöser) in the years 1987 and 1991 by K.W. The alga has been cultivated in small Petri dishes in a modified *Gleodinium* solution based on G 73 with a trace element solution and biotin (von Stosch, 1979). To 1000 ml of this solution 50.5 mg FeSO₄ · 7 H₂O, 0.2923 mg NaCl and 9.535 µg Na₂B₄O₇ · 7 H₂O were added. To induce germination of the aplanospores, the medium was changed daily. Light was provided under a light/dark regime of 12 h:12 h by fluorescent lamps at 3 W m⁻² or 9 W m⁻².

Microscopy

Living cells were examined with a Zeiss-Standard RA or a Leitz Orthoplan microscope equipped with water immersion objectives and differential interference contrast optics.

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For electron microscopy the material was fixed for 4 h at room temperature in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 6.5) containing 0.25 M sucrose. Cells were postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer overnight, dehydrated in an ethanol series and then embedded in Epon 812 (Serva). Sections were stained with methanolic uranyl acetate and lead citrate and examined with a Philips 301 G electron microscope.

For scanning electron microscopy (SEM) the fixed and dehydrated cells were transferred to acetone, critical point dried, sputter coated and viewed in a Hitachi S 530 microscope.

Pigment analysis

Chlamydomyxa cells were concentrated by centrifugation, resuspended in methanol and broken in a glass homogenizer with a Teflon piston. Residual pigments were extracted from the cell pellets with 80% acetone. Methanol and acetone extracts were combined and fractionated by high-pressure liquid chromatography (HPLC) as described previously (Marquardt & Rehm, 1995). Reference pigments were extracted with 80% acetone (final concentration) from thylakoids of the red alga *Galdieria sulphuraria* Merola and the diatom *Cyclotella cryptica* Reimann, Lewin *et al.* Thylakoid membranes were isolated as previously described (Marquardt & Rhiel, 1997; Rhiel *et al.*, 1997). Pigments of the green plant *Brassica oleracea* were extracted by grinding the soft parts of a leaf in 80% acetone with quartz sand in a mortar. The formation of antheraxanthin was induced by illuminating a leaf, which was heat-protected by a glass cuvette filled with water, with white light of 4000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ for 15 min prior to pigment extraction.

The HPLC run was monitored at 450 nm. The eluent was collected in 100 μl fractions. For absorption spectroscopy, the most concentrated pigmented fractions were diluted 1:20 with ethanol. Spectra were recorded with a Hitachi U-3200 spectrophotometer. The spectral characteristics of these samples obviously correspond to those of pigments in pure ethanol, since spectra of reference pigments treated in this manner were identical to spectra of pigments isolated by thin layer chromatography and eluted with ethanol. Occasionally, pigments were transferred from ethanol to diethyl ether by phase separation after adding 1 volume of diethyl ether and 1 volume of 5 M NaCl solution.

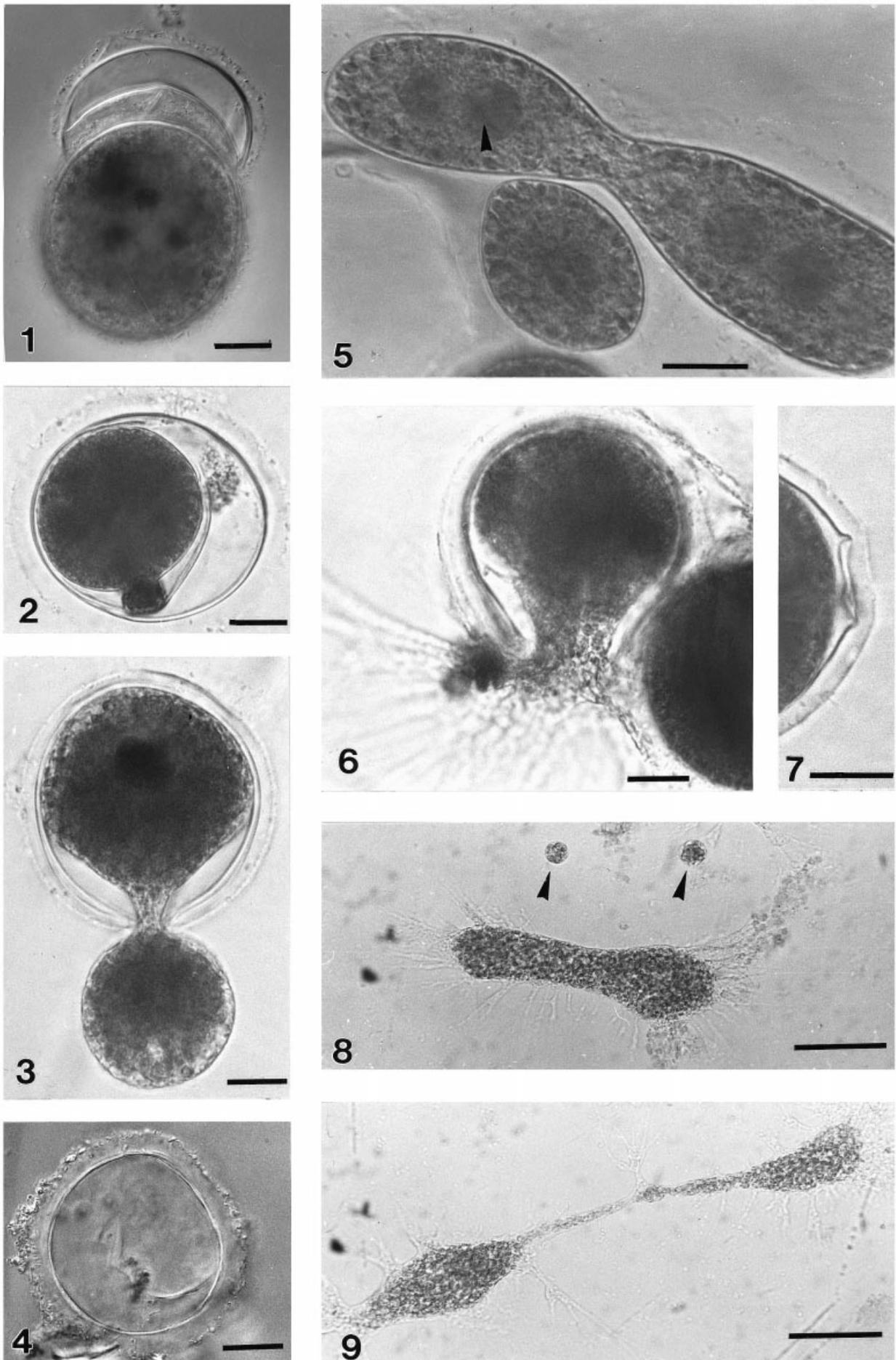
Cloning and analysis of SSU rRNA gene

Cells were harvested by centrifugation and resuspended in 250 μl DNA extraction buffer (0.1 M TrisCl pH 8;

1.4 M NaCl; 20 mM Na_2EDTA). After addition of glass beads (diameter 0.5 mm) the cells were broken up by vortexing vigorously for several minutes. Fifty microlitres of cetyltrimethylammonium bromide (CTAB); (10% in extraction buffer) was added and the preparation was incubated at 60 °C for 1 h. Proteins were removed by subsequent extraction with phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous layer was transferred to a new reaction tube, DNA precipitated by adding sodium acetate (final concentration: 0.3 M, pH 4.8) and 2 volumes ethanol, and pelleted by centrifugation (14000 rpm, 4 °C, 30 min). The pellet was washed twice with ethanol (75% v/v), pelleted, dried and resuspended in 25 μl sterile, distilled water. Two hundred nanograms of this nucleic acid preparation were used for polymerase chain reaction (PCR) amplification of small subunit (SSU) rDNA molecules using standard primers F (5'-GAAACTGCGAATGGCTC-3') and R (5'-CAATGATCCTTCCGCAGGT-3'). In a 0.5 ml Eppendorf reaction tube a PCR reaction with a final volume of 25 μl was mixed, containing water, primers (final concentration: 0.25 μM), 10 \times *Taq* buffer (1 \times), MgCl_2 (3 mM), dNTP (2 mM each), DNA (200 ng) and 1 U *Taq* polymerase (Pharmacia). The PCR was run in an Eppendorf MasterAmp thermal cycler. After an initial denaturing step of 95 °C for 3 min, there were 30 cycles of 1 min at 95 °C, 1 min at 52 °C and 2.5 min at 72 °C. At the end of the program an additional 5 min elongation step at 72 °C was appended.

The PCR product was analysed on a 0.8% agarose gel and the product band was excised with a clean scalpel. DNA was recovered using the JETSORB gel extraction kit (Genomed) following the instructions of the manufacturer. The PCR product was then cloned into Promega's pGEM-T vector and transformed into *E. coli* XL1-Blue MRF' (Sambrook *et al.*, 1989). Recombinant plasmids were identified by blue/white screening in LB plates containing XGal, IPTG and ampicillin. Overnight cultures of recombinant cells were grown in LB medium (Sambrook *et al.*, 1989) with ampicillin. Plasmid DNA was isolated from recombinants as described by Birnboim & Doly (1979) and digested with *SacI/SacII* restriction endonucleases to check for DNA inserts. Sequencing reactions of appropriate plasmids were performed with the Thermo-sequenase cycle sequencing kit from Amersham according to the manufacturer's instructions. The following CY5-fluorescent dye-labelled primers were used: standard sequencing primer M13(-20), M13(reverse), 301F (5'-CGGAGAGGGAGCCTG-3'), 690F (5'-AGAGGTGAAATTCT-3'), 690R (5'-AGAATTTACCTCTG-3'), 1055R (5'-CGGCCATGCACCACC-3'), 1200R (5'-GGGCATCACAGACCTG-3'), and the products resolved on an ALFexpress sequencer (Pharmacia).

Figs 1–9. *Chlamydomyxa labyrinthuloides*, light microscopy. Fig. 1. Aplanospore. The empty envelopes above result from repeated germination. Figs 2, 3. Germination of the aplanospore. Fig. 4. Empty aplanospore. Fig. 5. Division of the hatched plasmodium. Arrowheads, reddish globuli. Fig. 6. Germination of an aplanospore resulting in an amoeba with thin filopodia. Fig. 7. Germination pore in a cell wall of an aplanospore. Figs 8, 9. Division of the amoeba form. Arrows, very small amoebae, possibly containing only one nucleus. Scale bars represent 10 μm in Figs 1–7, 50 μm in Figs 8 and 9.



Figs. 1-9. For legend see facing page.

The *Chlamydomyxa labyrinthuloides* SSU rRNA gene sequence is deposited under the GenBank accession number AJ130893.

Phylogenetic analysis

The SSU rRNA sequence of *Chlamydomyxa labyrinthuloides* was added to the SSU rRNA sequence alignment of Van de Peer *et al.* (1998) and aligned by considering both primary and secondary structure. Phylogenetic trees were constructed using both pairwise distance methods and maximum parsimony. Estimation of evolutionary distances and construction of neighbor-joining trees were done with the software package TREECON for Windows (Van de Peer & De Wachter, 1997). Evolutionary distances were computed as described earlier (Van de Peer *et al.*, 1996) taking into account among-site rate variation. Bootstrap analysis, involving the computation of 200 trees from resampled data (Felsenstein, 1985), was also performed. Maximum parsimony trees were constructed with PHYLIP (Felsenstein, 1994).

Results

Light microscopy

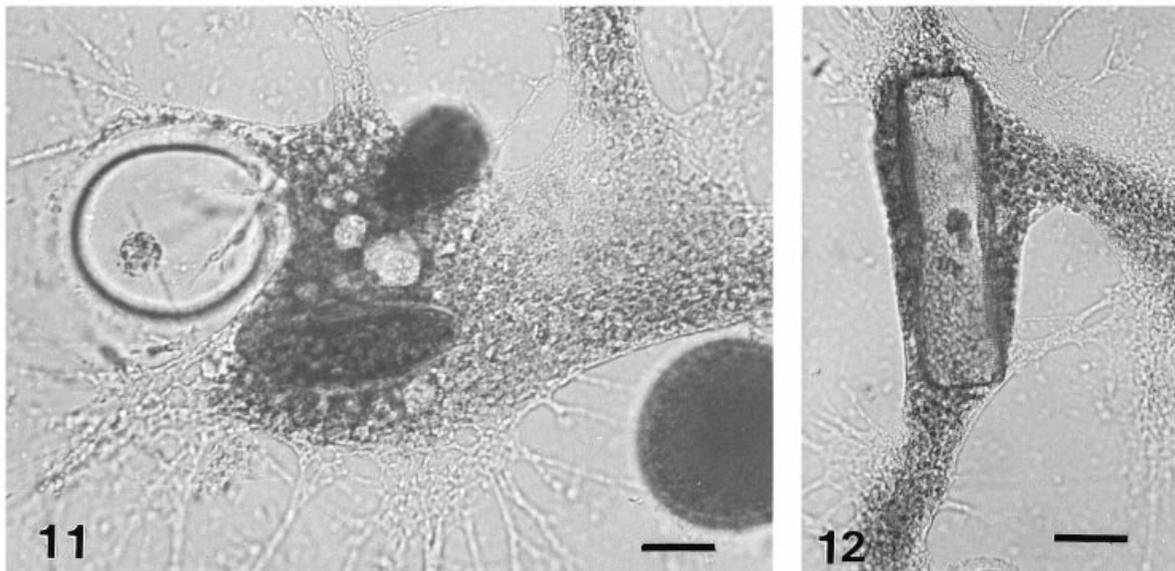
Germination of aplanospores. In contrast to earlier observations (Hieronymus, 1898; Pascher, 1930), aplanospores (Fig. 1) of *Chlamydomyxa* were rarely found within the

hyaline cells of *Sphagnum*, but were mostly between the moss 'leaves' and other water plants. In culture germination of the amoebae could be induced by changing the medium or by 'washing' the aplanospores with a Pasteur pipette. The germination rate was rather low – only 5–10% after 3 days – and started with partial disintegration of the cell wall (Figs 2, 3). Finally, the cell hatched out through a more or less circular opening (Figs 3, 7), leaving behind the remains of enclosed algae and reddish granules (Fig. 4), which usually accumulated in older aplanospores (Figs 1, 5). In many cases, cells left the aplanospores without filopodia. The plasmodium either divided and the new one formed an aplanospore (Fig. 5) or it generated an aplanospore without division (Fig. 1). In some cases cell division finished before the whole plasmodium hatched out of the cell wall and the new aplanospore developed within the old cell wall (Fig. 2). In other cases, the amoeba had thin multiple branches formed by the peripheral hyaline part of the cytoplasm even before the entire cell left the aplanospore (Fig. 6).

Plasmodia (= multinucleate amoebae). No dominant contractile vacuole was found. Instead many small vacuoles were present causing the cytoplasm to look frothy (Fig. 10). Furthermore, this part of the cytoplasm contained many granules that could not be stained with iodine–potassium iodide. The disc-shaped chloroplasts were located in the central portion of the cytoplasm, and were yellowish-brown under high-light and greenish-brown



Fig. 10. Plasmodium of *Chlamydomyxa labyrinthuloides*, viewed with DIC. Scale bar represents 10 μm .



Figs 11, 12. Phagocytosis by the plasmodium of *Chlamydomyxa labyrinthuloides*. Fig. 11. Part of a plasmodium with incorporated *Synechococcus* cells. At the left is an empty aplanospore. Fig. 12. Digested *Pinnularia* in a thin part of a plasmodium. Scale bars represent 10 μm .

under low-light conditions. Size of the plasmodia varied from 15 μm to 180 μm .

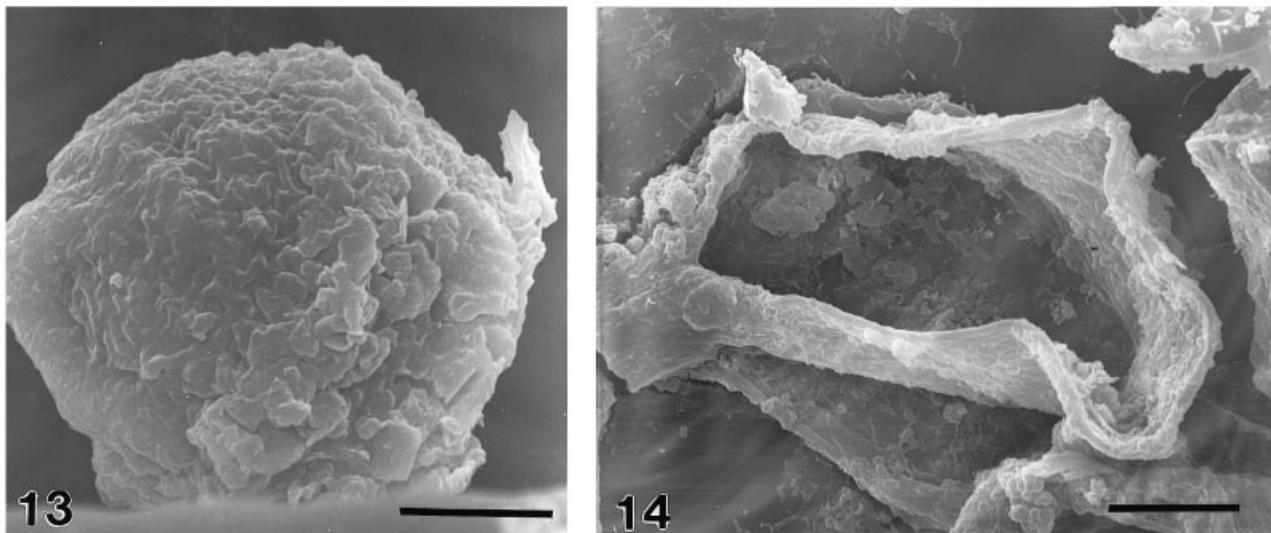
Division of plasmodia was initiated by a constriction of the cytoplasm, giving a dumbbell-like impression (Fig. 8). Finally the cytoplasmic connection between the two parts broke. Cell division was finished within 30 min (Fig. 9). Repeated division or separation of small parts of the plasmodium could result in fragments that usually still contained several nuclei stainable with iron–carmine–acetic acid. In a few cases we saw amoebae with only one nucleus and 7–10 chloroplasts (Fig. 8, arrows). These amoebae developed into multinuclear plasmodia again, which could fuse, thus forming larger plasmodia.

The plasmodia moved rather slowly, mostly by a

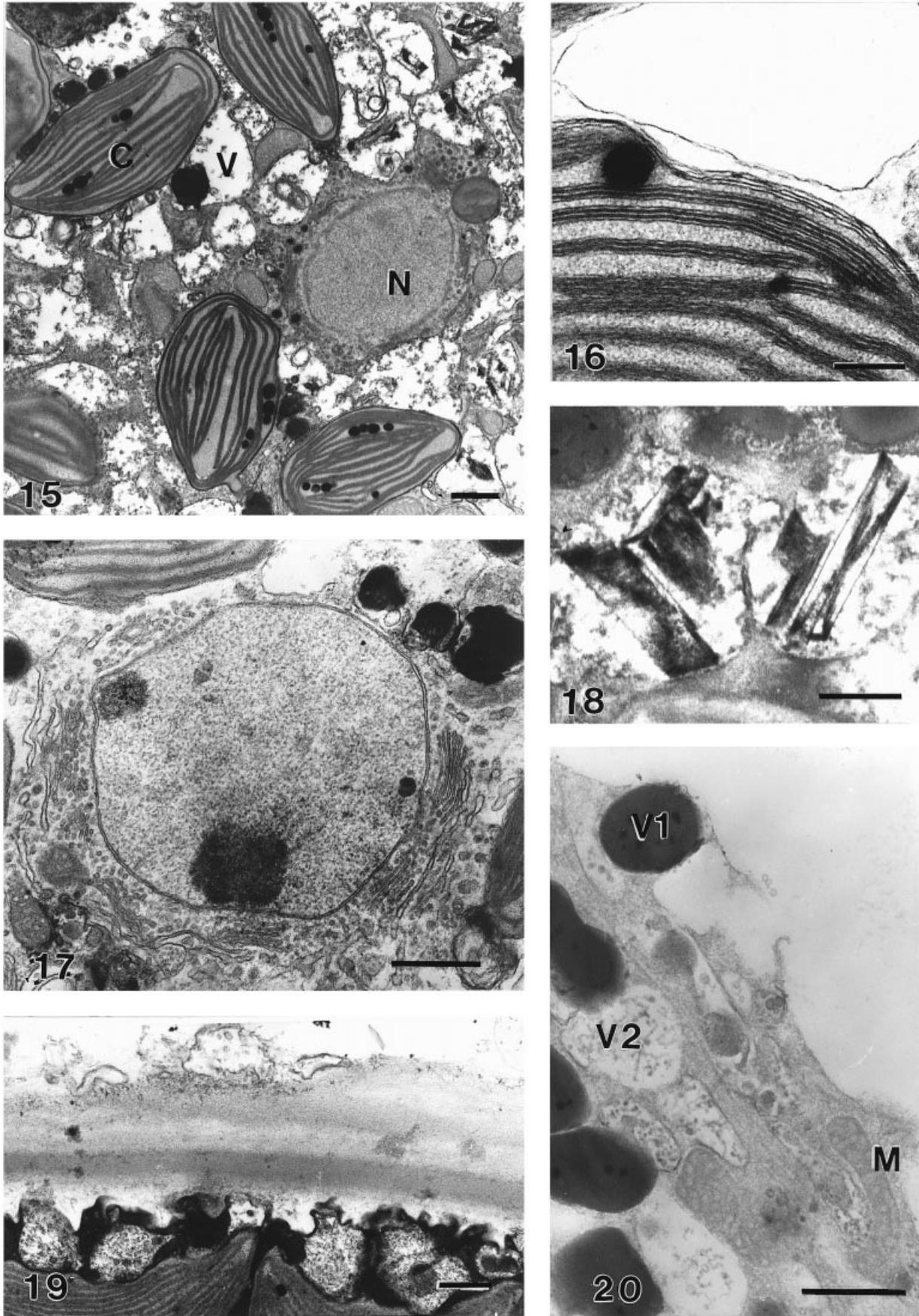
distance of their own diameter or less during 1 h. Germination rate and motility varied during daytime with a maximum in the morning.

In culture, *Chlamydomyxa* phagocytosed diatoms (*Eunotia*, *Frustulia*, *Pinnularia*; Fig. 12) and *Synechococcus* (Fig. 11), but not bigger desmids. To induce phagocytosis, plasmodia must be cultivated for several days in darkness. Prey organisms (encountered by chance) were surrounded by filopodia and transported to the central part of the plasmodium in a large vesicle, where they were digested within 2–4 h (Fig. 12).

Encystment. Approximately 1 week after germination the



Figs 13, 14. Ultrastructure (SEM) of *Chlamydomyxa labyrinthuloides* aplanospores. Fig. 13. Intact aplanospores. Fig. 14. Broken aplanospores showing attachment of the aplanospore on the substratum (left) and the germination pore (right). Scale bars represent 10 μm .



Figs 15–20. Ultrastructure (TEM) of *Chlamydomyxa labyrinthuloides*. Fig. 15. Part of the inner region of an amoeba. N, nucleus; C, chloroplasts in the cytoplasm with numerous vacuoles (V). Fig. 16. Chloroplast (detail): the chloroplast is surrounded by four membranes. Fig. 17. Nucleus with two nucleoli. The nucleus is surrounded by many dictyosomes. Fig. 18. Crystals in the vacuoles. Fig. 19. Cell wall of a cyst: cell wall with several layers. Fig. 20. Cross-section through a filopodium. M, mitochondrion; V1, vacuoles with stored food; V2, vacuoles with granular contents. Scale bars represent 1 μm in Figs 15, 17, 19, and 0.5 μm in Fig. 16.

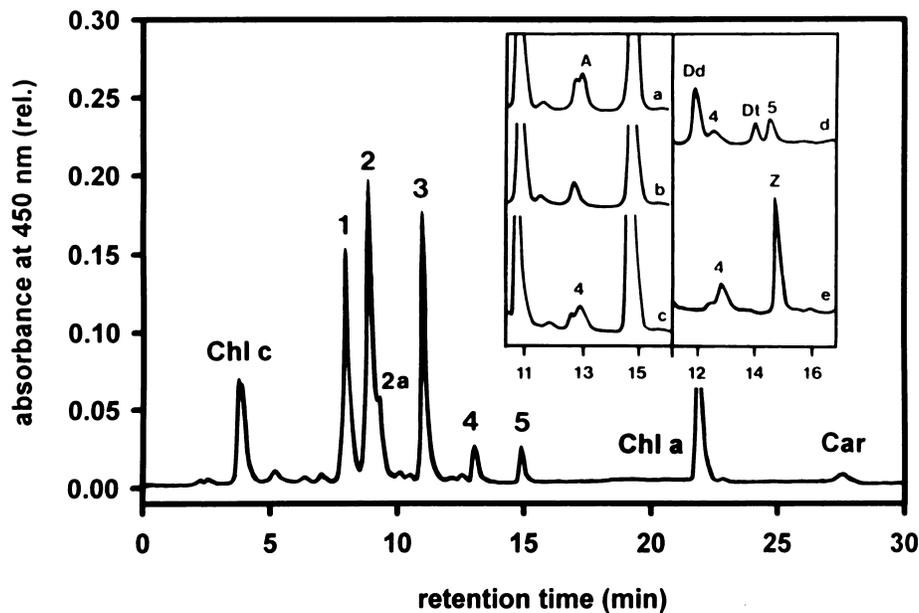


Fig. 21. HPLC trace of *Chlamydomyxa* pigments. Chl, chlorophyll; Car, carotene; xanthophyll peaks are numbered 1 to 5. The inset shows HPLC traces of (a) dark-adapted *Brassica oleracea*, (b) high-light treated *Brassica*, (c) a mixture of pigment extracts from *Chlamydomyxa* and dark-adapted *Brassica*, (d) a mixture of pigments from *Chlamydomyxa* and high-light adapted *Cyclotella cryptica*, and (e) a mixture of pigments from *Chlamydomyxa* and *Galdieria sulphuraria*. A, antheraxanthin; Dd, diadinoxanthin; Dt, diatoxanthin; Z, zeaxanthin.

plasmodium encysted. Aplanospore formation was initiated by retraction of the filopodia and parts of the central cytoplasm. After 1 day a cell wall was visible and after 1 week the wall often appeared multilayered. In some cases it could be seen that the cytoplasm had retracted slightly and a new layer of the cell wall had developed. In older cultures deficient in nitrogen and phosphorus, a red pigment accumulated in the aplanospores. To eliminate these pigmented granules, a part of the organism hatched out of the cell wall, the granules were ejected, and the cytoplasm retracted back and developed a new cell wall within the original cyst. *Chlamydomyxa* sometimes encysted inside the original aplanospore without developing new plasmodia, or division of the cytoplasm could take place prior to this encystment. We were not able to induce flagellate states even by varying culture conditions: strong light, dim light, nutrient deficiency, short day conditions or senescent cultures kept in the dark for several days all failed to induce flagellar formation.

Ultrastructure

By SEM both inner and outer surfaces of the cyst wall looked rugose (Figs 13, 14). In empty aplanospores a round aperture could be seen (Fig. 14).

Ultrastructurally the amoeba stage differed from the aplanospore only in the absence of the cell wall and the presence of filopodia. The cytoplasm was characterized by numerous small vacuoles. The disc-shaped chloroplasts were surrounded by two pairs of membranes: two membranes of the chloroplast envelope and two membranes of the periplastidal endoplasmic reticulum cisternae. The thylakoids were arranged in groups of three (Fig. 16).

A girdle lamella was more or less marked. Electron-dense globules were distributed between the lamellae and in the stroma (Figs 15, 16). No pyrenoids were found. In the amoeba stage, the nuclei were located only in the central cytoplasm. They were surrounded by large dictyosomes (Fig. 17). We often observed two nucleoli, but in our cross-sections we did not see division of the nuclei. The mitochondrial cristae were tubular. In some aplanospores or plasmodia vacuoles with crystals occurred (Fig. 18).

The cell wall of the aplanospore could be composed of several layers (Fig. 19). The ultrastructure of the filopodia (Fig. 20) differed from that of the cell body by the lack of nuclei, plastids and dictyosomes. They contained only electron-dense globules, mitochondria and vesicles with fibrillar-granular contents.

Pigment composition

The pigment composition of *Chlamydomyxa* was analysed by HPLC. Besides Chl *a*, Chl *c* and carotene a number of xanthophyll peaks were found. These peaks were numbered 1 to 5 with increasing retention time in Fig. 21. The pigments of peaks 1, 2 and 3 were the dominant xanthophylls while the pigments of peaks 4 and 5 were only minor components. The eluate was fractionated and the samples were analysed by absorption spectroscopy. Spectra of the major pigments are shown in Fig. 22.

The pigments of peak 2 had an absorption spectrum typical of fucoxanthin (Fig. 22*b*). Shape and position of the absorption maximum were identical to those of fucoxanthin isolated from the diatom *Cyclotella cryptica*. Additionally, the xanthophyll had a retention time identical to authentic fucoxanthin when co-migrating with

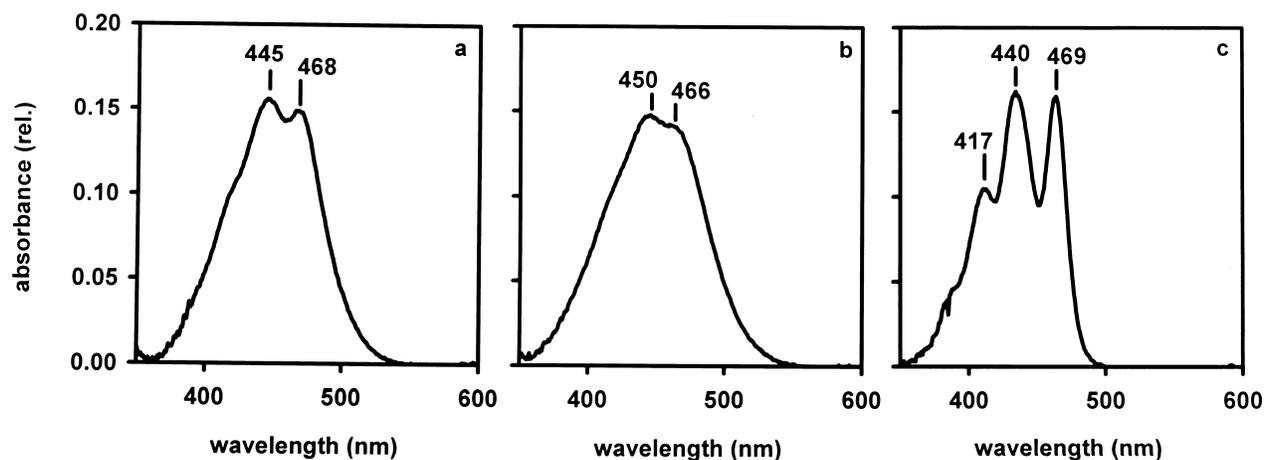


Fig. 22. Absorption spectra of the three major xanthophylls of *Chlamydomyxa*: pigment 1 (a), pigment 2 (b) and pigment 3 (c). The pigments are in ethanol.

Table 1. Comparison of absorption maxima of *Chlamydomyxa* xanthophylls with literature data and maxima of authentic pigments

Pigment	Source or reference	Maxima ^a	
No. 1	<i>Chlamydomyxa</i>	445	468
		445	467 ^b
19'-Butanoyloxyfucoxanthin-like	Wright & Jeffrey (1987)	446	470
		444	470 ^b
No. 2	<i>Chlamydomyxa</i>	450	466
Fucoxanthin	<i>Cyclotella cryptica</i>	450	467
	Davies (1976)	(426)	449 (465)
No. 2a	<i>Chlamydomyxa</i>	445	468
19'-Hexanoyloxyfucoxanthin-like	Wright & Jeffrey (1987)	446	470
No. 3	<i>Chlamydomyxa</i>	417	440
Violaxanthin	<i>Brassica oleracea</i>	416	439
	Davies (1976)	420	441
		(421)	446
No. 4	<i>Chlamydomyxa</i>	(421)	446
Antheraxanthin	Davies (1976)	422	444
Diadinoxanthin	<i>Cyclotella cryptica</i>	(423)	446
	Davies (1976)	(424)	445
		(427)	450
No. 5	<i>Chlamydomyxa</i>	(427)	450
Zeaxanthin	<i>Galdieria sulphuraria</i>	(426)	450
	Davies (1976)	(428)	450
Diatoxanthin	<i>Cyclotella cryptica</i>	(425)	450
	Davies (1976)	(425)	449
			475

If not specified, the solvent is ethanol.

^a Shoulders in parentheses.

^b In diethyl ether.

a pigment extract from *Cyclotella*. The absorption spectrum of pigment 3 (Fig. 22c) fitted rather well the available data for violaxanthin and was identical to that of authentic violaxanthin isolated from *Brassica oleracea*. When the

pigment was injected together with *Brassica* pigments, it also co-migrated perfectly with violaxanthin. The identical spectra and retention times indicated that pigments 2 and 3 were indeed fucoxanthin and violaxanthin.

Pigment 1 was more difficult to identify. Its spectral properties (Fig. 22a) did not fit those of the major pigments common in heterokont algae. In the shape of the spectrum and the position of the maxima it is similar to some fucoxanthin derivatives described by Wright & Jeffrey (1987). According to its polarity it is closest to the 19'-butanoyloxyfucoxanthin-like pigment found in some species from different heterokont algal groups (Wright & Jeffrey, 1987; Stauber & Jeffrey, 1988).

Pigments 4 and 5 had absorption characteristics similar to those of antheraxanthin and zeaxanthin, as compared with available data and the spectrum of authentic zeaxanthin (Table 1). In higher plants and some algal groups such as phaeophytes and chrysophytes, antheraxanthin and zeaxanthin are formed in the course of a light-induced xanthophyll cycle by de-epoxidation of violaxanthin (Hager & Stransky, 1970). Thus their occurrence in an organism containing violaxanthin is rather likely. However, the spectra of antheraxanthin and zeaxanthin are quite similar to those of diadinoxanthin and diatoxanthin, xanthophylls common in, for example, diatoms, eustigmatophytes and xanthophytes (Daugbjerg & Andersen, 1997) and even reported to be present in some chrysophytes (Withers *et al.*, 1981). The relative polarities of antheraxanthin and diadinoxanthin and of zeaxanthin and diatoxanthin are also similar.

In order to distinguish between these pigments, *Chlamydomyxa* extracts were run together with extracts from organisms containing these xanthophylls. We used

Fig. 23. Phylogenetic tree constructed by the neighbor-joining method (Saitou & Nei, 1987) from a distance matrix of 64 SSU rRNA sequences of heterokont algae and taking into account among-site rate variation (Van de Peer *et al.*, 1996). The bicosoecid *Cafeteria roenbergensis* was used to root the tree. Bootstrap values above 50% (out of 200 replicates) are shown at the internodes. The classification of chrysophytes denoted by an asterisk is under discussion (see Saunders *et al.*, 1995).

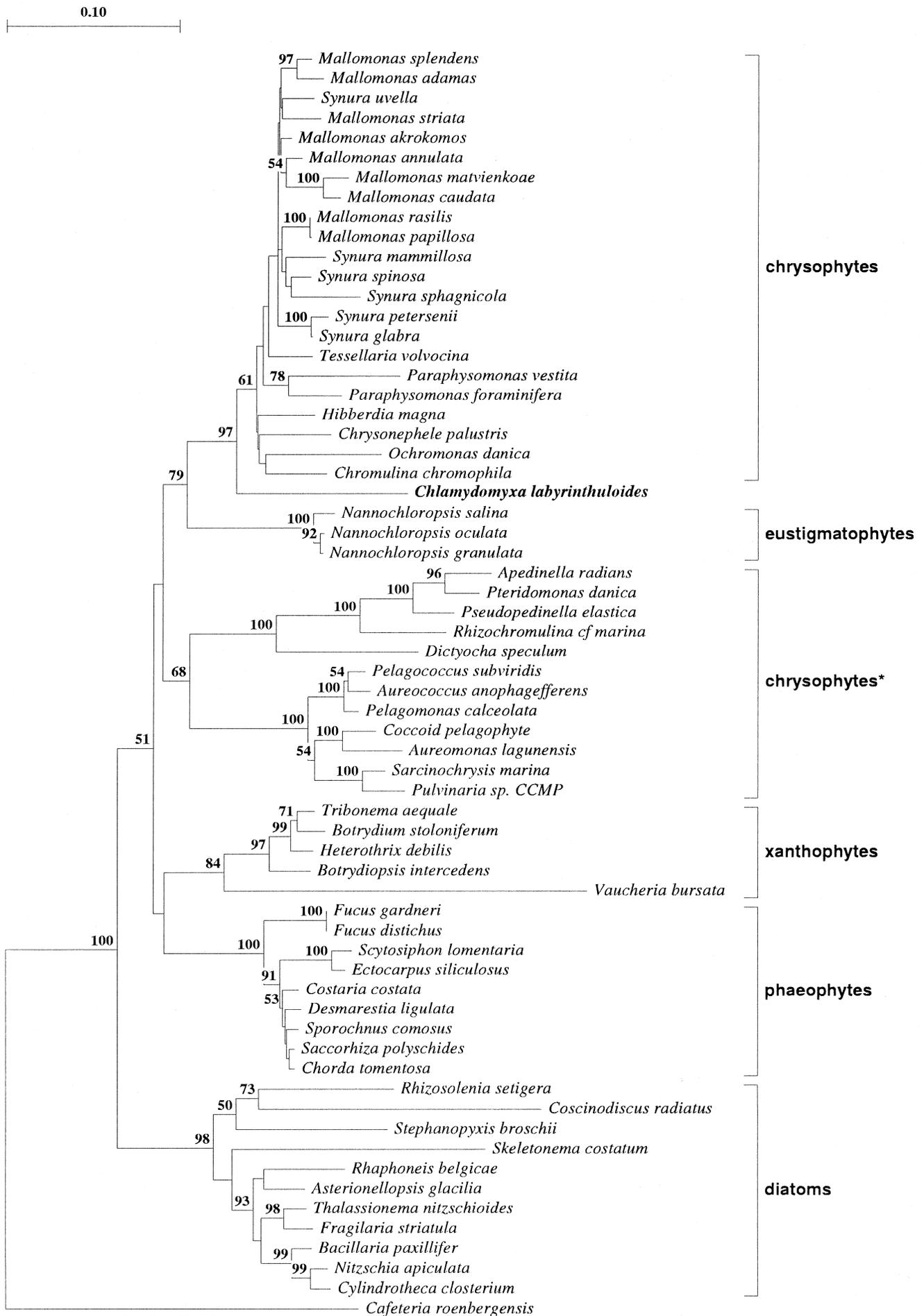


Fig. 23. For legend see facing page.

high-light adapted *Cyclotella cryptica* for diadinoxanthin and diatoxanthin (cf. Rhiel *et al.*, 1997) and *Galdieria sulphuraria* for zeaxanthin. Our reference organism for antheraxanthin was *Brassica oleracea*. Antheraxanthin was only present in high-light treated plants (Fig. 21, inset *a*), while dark-adapted leaves were devoid of this pigment (Fig. 21, inset *b*). Thus, the antheraxanthin peak was identified by comparing chromatograms of pigment extracts from dark-adapted and high-light treated leaves. A zeaxanthin peak was not found. Possibly, this pigment co-migrated with lutein. When *Chlamydomyxa* pigments and extracts from dark-adapted *Brassica* were mixed, we found the same pattern in the antheraxanthin region of the chromatogram as with high-light treated leaves (Fig. 21, inset *c*). When *Chlamydomyxa* pigments, however, were injected together with pigments from *Cyclotella* (Fig. 21, inset *d*), pigment 4 yielded a peak with a slightly higher retention time than diadinoxanthin. The same held true for pigment 5 and diatoxanthin, but pigment 5 co-migrated perfectly with zeaxanthin when *Chlamydomyxa* and *Galdieria* extracts were mixed (Fig. 21, inset *e*). Thus the occurrence of diadinoxanthin and diatoxanthin in *Chlamydomyxa* could be excluded and pigments 4 and 5 were most probably identical with antheraxanthin and zeaxanthin.

An additional xanthophyll (pigment 2a) was found as a small shoulder after the fucoxanthin peak. Its spectral properties were similar to those of pigment 1 (Table 1), and thus it was probably another fucoxanthin derivative. According to its retention time it was similar to the 19'-hexanoyloxyfucoxanthin-like pigment found by Wright & Jeffrey (1987) in *Phaeocystis pouchetii*. Using aged cultures, the peak eluting after fucoxanthin was much larger, but obviously caused by a different, as yet unidentified, pigment. Its spectrum showed a single maximum at 468 nm. This additional pigment might be connected with the carotenoid globuli accumulating in older aplanospores.

Phylogenetic analysis

Fig. 23 shows a neighbor-joining tree based on 64 sequences from heterokont algae.

As can be observed, *Chlamydomyxa labyrinthoides* is grouped with one clade of the chrysophytes at a very high bootstrap level (97%), strongly suggesting a common ancestry for *Chlamydomyxa* and these chrysophytes. And although *Chlamydomyxa* is basal within this group, it is clearly separated from the other heterokont algal groups such as the eustimatophytes, xanthophytes, phaeophytes, diatoms and another group of chrysophytes which is currently under discussion (Saunders *et al.*, 1995). A bootstrapped maximum parsimony consensus tree gave a very similar structure, although *Chlamydomyxa* now diverged from within the chrysophycean cluster (not shown). However, a close evolutionary relationship with a particular species of the Chrysophyceae could not be observed.

Discussion

Several descriptions of *Chlamydomyxa* have been available since the last century, but provide only a preliminary taxonomic characterization using morphological characteristics.

Morphology

We can confirm earlier reports by Hieronymus (1898) and Pascher (1930, 1939) that zoospores are not detectable. The irregular outer part of the cell wall as seen by SEM could be a result of different water contents in some regions of the cell wall. Although the ultrastructure of the chloroplasts is typical for heterokonts, differentiation between xanthophytes and chrysophytes on this basis was not possible.

However, morphological features do not unambiguously place *Chlamydomyxa* within the chrysophytes. Amoeboid or plasmodioid structures as the main stage in the life history are seen in both the Chrysamoebae or Myxochryidae. The Chrysamoebae (Poche, 1913) are defined as naked cells with a tendency to form lobed and branched extensions. Vegetative cells are amoeboid in most parts of the life history; flagellate states occur in some genera and in others were never observed. This difference was used to place those organisms with a flagellate state in the Rhizochrysidae Pascher *ex* Reichenau and those in which it is absent in the Chrysachniaceae Pascher (1931). Today the absence of flagellate states is not considered to be a good taxonomic feature (Preisig, 1995).

Amoeboid states occur in different groups of chrysophytes. O'Kelly & Wujek (1995), for example, have shown that *Chrysamoeba pyrendidifera* closely resembles chromulinal chrysophytes, whereas *Lagynion delicatulum* Skuja resembles hiberdialean chrysophytes. Formerly *Lagynion delicatulum* was classified as a member of the Stylocococaceae. The marine *Rhizochromulina marina* Hidderd & Chrétiennot-Dinet (1979) is closely related to the pelloids and silicoflagellates *sensu stricto*. So, on morphological characters, it was placed in a new order, Rhizochromulinales O'Kelly & Wujek (1995) (Class: Dictyochophyceae Silva) (Preisig, 1995).

Pigments

The pigment composition of *Chlamydomyxa* permits another evaluation of its taxonomic position. The lack of Chl *b* and the presence of Chl *c* are typical of heterokont algae, haptophytes, cryptophytes and dinophytes. Membership of *Chlamydomyxa* in the Cryptophyta can be excluded by its chloroplast ultrastructure and by the lack of biliproteins (not shown); membership in the Dinophyta is ruled out by the structure of the nuclei and the chloroplast. Therefore the xanthophyll pattern was only compared with that of haptophytes and heterokont algae.

Chlamydomyxa contains fucoxanthin, a pigment widespread in such groups of heterokont algae as phaeophytes,

chrysophytes and marine raphidophytes, but not in xanthophytes (Daugbjerg & Andersen, 1997). Typical xanthophyte xanthophylls have different spectral properties (cf. Davies, 1976). Thus the preliminary taxonomic position in the Xanthophyceae proposed by Pascher (1931) cannot be retained. The occurrence of violaxanthin gives a further hint about the systematic position. Violaxanthin is absent in haptophytes (Van den Hoek, 1993). In heterokont algae it occurs in chrysophytes and sinurophytes, phaeophytes, marine raphidophytes and eustigmatophytes (Daugbjerg & Andersen, 1997). The latter group, however, contains no fucoxanthin and can be excluded. Consequently, according to its pigment composition, *Chlamydomyxa* must be related to chrysophytes, phaeophytes or marine raphidophytes.

Besides these widespread pigments we also found some atypical xanthophylls, assumed from their absorption spectra to represent fucoxanthin derivatives. Similar carotenoids were described for diatoms (Stauber & Jeffrey, 1988), and for haptophytes and chrysophytes (Wright & Jeffrey, 1987). To our knowledge they have not yet been identified in phaeophytes and marine raphidophytes. However, since they were isolated from only a few species of rather unrelated algal groups, their occurrence may be of little taxonomic value. Thus membership of *Chlamydomyxa* in the phaeophytes and raphidophytes cannot be excluded on the basis of its pigment composition. Life cycle, ecology and morphology of the alga, however, indicate a closer relationship to the Chrysophyta than to the phaeophytes or marine raphidophytes.

SSU rRNA gene analysis

Finally, our phylogenetic analyses demonstrate with very good bootstrap support that *Chlamydomyxa labyrinthuloides* groups monophyletically with some Chrysophyta and is clearly separated from the other heterokont algae.

Conclusion

In our analysis we have re-examined *Chlamydomyxa labyrinthuloides* using morphological, biochemical and molecular techniques. We conclude from our results that *Chlamydomyxa labyrinthuloides* is a Chrysophyceae-like alga, with amoeba and aplanospore life stages.

Acknowledgements

The authors would like to thank M. Johannsen, A. Willanzheimer and C. Zimmermann for technical support, and Drs A.M. Schmid and S.E. Douglas for helpful discussions. This work was supported by the Deutsche Forschungsgemeinschaft. Y.V.deP. is Research Fellow of the Fund for Scientific Research (Flanders).

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