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Frithjof C. Cohn, Ingo Maier, Dieter G. Müller, Susan Loiseaux-De Goer, Laure Guillou. Phylogenetic affinities of two eukaryotic pathogens of marine macroalgae, Eurychasma dicksonii (Wright) Magnus and Chytridium polysiphoniae. Cryptogamie Algologie, 2006, 27 (2), pp.165-184. hal-04800314

HAL Id: hal-04800314
https://hal.sorbonne-universite.fr/hal-04800314v1

Submitted on 24 Nov 2024

Cryptogamie, Algol., 2006, 27 (2): 165-184

Phylogenetic affinities of two eukaryotic pathogens of marine macroalgae, *Eurychasma dicksonii* (Wright) Magnus and *Chytridium polysiphoniae* Cohn

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(Received 3 August 2005, accepted 10 October 2005)

Abstract — The 18 S rRNA genes of *Eurychasma dicksonii* and *Chytridium polysiphoniae*, pathogens of brown algae, were sequenced and used to clarify their phylogenetic affiliations. *E. dicksonii* is consistently placed at the base of the Peronosporomycota (Oomycota) with high bootstrap support. Nevertheless, its sequence is clearly separated from other terrestrial and freshwater Oomycota. The closest related marine group is a clade entirely composed of environmental sequences retrieved from marine sediments and oceanic plankton samples. The genus *Chytridium* usually forms a clade that includes several other genera (alongside the clades of *Monoblepharis*-, *Rhizophydium*-, *Lacustromyces*-, *Nowakowskiella*-, *Neocallimastix*- and *Spizellomyces*-like organisms) within the Chytridiomycota, one of the principal lineages of the Eumycota. Interestingly, our sequence of *C. polysiphoniae* differs drastically from other sequences of the genus *Chytridium*, forming a novel clade of the Chytridiomycota, which also includes environmental sequences from water and soil samples. Consistent with these phylogenetic affiliations, *C. polysiphoniae* has a chitin cell wall, whilst *E. dicksonii* has cellulose. Together, these results suggest that *Eurychasma* and *Chytridium* may become interesting model organisms as the currently only culturable and morphologically known representatives of a poorly understood aquatic biodiversity, pointing out the necessity to include marine representatives for phylogenetic studies of the Oomycota and Chytridiomycota.

Chitin / *Chytridium* / Chytridiomycota / *Eurychasma* / Oomycota / *Pylaiella*

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Communicating editor: John Huisman

Résumé — Affinités phylogénétiques de deux parasites pathogènes eucaryotiques de macroalgues marines, *Eurychasma dicksonii* (Wright) Magnus et *Chytridium polysiphonia* Cohn. Les gènes codant pour l'ARN ribosomique 18S de deux agents pathogènes d'algues brunes, *Eurychasma dicksonii* et *Chytridium polysiphoniae*, ont été séquencés afin de clarifier leur position phylogénétique. La séquence d'*E. dicksonii* se situe toujours à la base des Peronosporomycètes (Oomycètes) avec des valeurs de bootstrap élevées. Cependant elle est clairement séparée de celles des autres Oomycètes terrestres ou d'eau douce. Le groupe le plus proche est un clade contenant uniquement des séquences environnementales provenant de sédiments marins et de plancton océanique. Les espèces du genre *Chytridium*, groupées avec plusieurs autres genres (entre autres, *Obelidium* et *Phlyctorhiza*), forment un clade qui est voisin des organismes rattachés aux genres *Monoblepharis*, *Rhizophydium*, *Lacustromyces*, *Nowakowskiella*, *Neocallimastix* et *Spizellomyces*, à l'intérieur des Chytridiomycètes, une des lignées principales des Eumycètes. La séquence de *Chytridium polysiphoniae* au contraire, forme avec des séquences environnementales aquatiques ou terrestres, un nouveau clade parmi les Chytridiomycètes, indiquant que la position systématique de cette espèce devra être revue. *C. polysiphoniae* contient de la chitine dans ses parois tandis que *E. dicksonii* contient de la cellulose, une composition pariétale en accord avec leurs positions phylogénétiques respectives. Ces résultats suggèrent qu'*Eurychasma* et *Chytridium* pourraient devenir des organismes modèles intéressants, étant les seuls représentants cultivables et morphologiquement connus d'une biodiversité aquatique très mal connue ; ceci met en évidence la nécessité d'inclure des représentants marins dans les études phylogénétiques des Oomycètes et des Chytridiomycètes.

Chitine / *Chytridium* / Chytridiomycota / *Eurychasma* / Oomycota / *Pylaiella*

INTRODUCTION

Eurychasma dicksonii (Wright) Magnus and *Chytridium polysiphoniae* Cohn are two widespread, but little studied, zoosporic "fungal" pathogens of marine macroalgae (Sparrow, 1960). On European coasts, they occur as massive epidemics in *Pylaiella littoralis* (L.) Kjellman (Küpper & Müller, 1999). Field observations indicated that they have a wide host range (Sparrow, 1960; Jenneborg, 1977), which was recently confirmed in laboratory experiments (Müller *et al.*, 1999). Both pathogens are holocarpic, infecting by a single spore.

The biflagellate *Eurychasma* has been generally placed in the Saprolegniales, within the biflagellate heterokont Oomycota (e.g. Aleem, 1950; Feldmann, 1954; Sparrow, 1960; Konno & Tanaka, 1988). In Dick's recent revision of Oomycota classification, *Eurychasma* is placed in the Eurychasmataceae within the group of holocarpic parasites, constituting a newly erected order, the Myzocytiopsidales (Hawksworth *et al.*, 1996; Dick, 2001), yet this is not supported by molecular results.

The uniflagellate fungus *Chytridium polysiphoniae* was first observed by Cohn (1865) on *Polysiphonia violacea* (Roth) Sprengel in Helgoland. Feldmann (1954) and Sparrow (1960) placed this pathogen in the Chytridiaceae within the uniflagellate Chytridiales, with no further phylogenetic discussion. In a more recent classification the genera *Chytridium* and *Rhizophydium* are placed in the Chytridiaceae, within the Chytridiomycota (Hawksworth *et al.*, 1996), but, like *Eurychasma*, without any support by molecular results. The first comprehensive molecular phylogenetic analysis of the chytrids has revealed that both the genera *Chytridum* and *Rhizophydium* are polyphyletic assemblages, within the Chytridiaceae (James *et al.*, 2000).

Until the recent availability of laboratory cultures (Küpper & Müller, 1999; Müller *et al.*, 1999), all observations on these marine parasites had been made by light microscopy on field-collected material (*Eurychasma*: e.g. Wright, 1879; Rattray, 1882; Magnus, 1905; Petersen, 1905; Aleem, 1950a, b, 1955; Sparrow, 1934, 1960, 1969; Jenneborg, 1977; Konno & Tanaka, 1988; *C. polysiphoniae*: Cohn, 1865; Petersen, 1905; Feldmann, 1954; Sparrow, 1960). No ultrastructural studies have been carried out on either organism. Molecular data are also not available for these pathogens and the morphological and biochemical knowledge of both organisms is not sufficient for a reliable phylogenetic classification. It is generally thought that holocarpic 'fungi' are likely to be more primitive than mycelial species (Barr, 1983, 1992). Recent molecular evidence seems to indicate that the biflagellate Oomycota are probably of marine origin (Gunderson *et al.*, 1987; Förster *et al.*, 1990; Beakes, 1998), a tempting hypothesis in light of this study. Rather surprisingly, the sister clade to the Oomycota in phylogenetic trees based on comparisons of the small subunit ribosomal RNA gene (18 S rDNA) has been shown to be the free-living marine bacterivorous flagellate *Developayella* (Leipe *et al.*, 1996). A recent molecular study based on the Cox II mitochondrial gene unexpectedly revealed that a number of closely related marine genera including *Haliphthoros* and *Halodaphnea* (both parasites of marine crustacea) formed a discrete clade at the base of the oomycete tree, branching before both the two main clades encompassing the Saprolegniales and Peronosporales (Cook *et al.*, 2001). Also, recent studies of environmental DNA samples by Massana *et al.* (2002) have identified novel marine stramenopile lineages representing sister clades to the Oomycota.

Although the uniflagellate chytrids have clearly been shown to be the most primitive representatives of the true fungi (Eumycota), no marine species have so far been analysed using molecular methods. This present study became feasible after the recent isolation of unialgal host cultures from an epidemic of these pathogens in a *Pylaiella* population in Shetland (Küpper & Müller, 1999; Müller *et al.*, 1999). This has enabled both an analysis of cell wall biochemistry and of the small subunit ribosomal RNA genes (18 S rDNA) in order to explore the phylogenetic affiliations of these two marine pathogens, especially in light of recent molecular phylogenetic studies of both oomycetes and chytrids, and the discovery of unexpected phylogenetic diversity based on environmental DNA extractions from water and soil samples.

MATERIALS AND METHODS

Biological material

Pylaiella littoralis infected by both *C. polysiphoniae* and *E. dicksonii* was collected at Aith Voe (Bressay, Shetland) in April 1996 (Küpper & Müller, 1999). Unialgal host cultures were established as described previously (Müller *et al.*, 1999). Briefly, small tufts of infected *Pylaiella* were co-incubated with aliquots of a unialgal clonal culture of *P. littoralis* (Pyl IR) from Isla Diego Ramirez (Chile, Drake Passage; Müller & Stache, 1989). Within several weeks, *E. dicksonii* became established on the new host. In a subsequent step, two Pyl IR subclones were initiated, each infected by one of the parasites only in the following way: 1) culture Eu Pyl IR 1 starting from spores of one *E. dicksonii* sporangium and 2) culture Chyt Pyl IR 14 starting from spores of 14 sporangia of *C. polysiphoniae*. These unialgal associations of the host *P. littoralis* and either *E. dicksonii* or *C. polysiphoniae* as parasites were used for all our experiments.

Cultures were maintained in plastic Petri dishes in Provasoli ES (Starr & Zeikus, 1987) prepared from filtered natural seawater (collected off Roscoff, Brittany). They were illuminated with daylight-type fluorescent lamps at an irradiance of 9 µE m^{-2} s^{-1} for 10 h day^{-1} and kept at 10 ± 1 °C. All cultures were transferred to fresh medium at one or two week intervals.

Herbarium specimens (microscope slides) have been deposited in the Jepson Herbarium (University of California, Berkeley / UC; *E. dicksonii*: UC 1726827, *C. polysiphoniae*: UC 1726828), the National Herbarium of Victoria (Melbourne / MEL; *E. dicksonii*: MEL 2068385, *C. polysiphoniae*: MEL 2068352), the Bolus Herbarium (BOL) of the University of Cape Town, in the Muséum National d'Histoire Naturelle – Cryptogamie (Paris, PC – this collection also received dried specimens in silicagel), and the collection of CAB International (Egham, Surrey, U.K.; *E. dicksonii*: IMI 385979, *C. polysiphoniae*: IMI 385980).

Light microscopy and chitin cytochemistry

The method for chitin detection was described by Maier *et al.* (2000). Briefly, parasitized algal filaments were fixed in ethanol/acetic acid (3 : 1). After two short washing steps in 70% ethanol, the material was equilibrated in phosphate-buffered saline (PBS, 13.7 mM NaCl, 3 mM KCl, 8.1 mM $Na_2HPO_4 \times 2\ H_2O$, 1.5 mM KH_2PO_4, pH 7.5) for 10 min. Unspecific protein binding sites were blocked by incubation on a shaker with 3% bovine serum albumin (BSA, Sigma) in PBS containing 0.05% sodium azide for 1 hour at room temperature. After removal of the blocking solution, a fluorescein isothiocyanate (FITC) - conjugated recombinant chitin binding protein (FUNGALASE™-F, Anomeric, Baton Rouge, USA) was applied according to the manufacturer's protocol (1:10 dilution), but omitting the periodic acid oxidation step. The staining reaction was carried out for 2 h at room temperature in the dark, followed by two washes with PBS for 5 min each. Afterwards, the specimens were mounted in SLOWFADE-LIGHT antifade solution (MoBiTec, Göttingen, Germany). Photographs were taken on Ilford 400 ASA HP5 Plus film (fluorescence) and Kodak 50 ASA Technical Pan film (bright field), using blue excitation light.

Cloning and sequencing of SSU rRNA genes

A visual inspection of the cultures used for this study showed that both *Eurychasma* and *Chytridium* had strongly infected the algal host, with up to approximately 10% of algal cells infected.

Infected algal material (either Eu Pyl IR 1 or Chyt Pyl IR 14) was dried with silicagel. Micro-extractions were carried out by grinding 1-2 mg dry weight with a micro-pestle in an Eppendorf tube, to which a tiny spatula (< 50 mg) of Fontainebleau sand, 600 µl of extraction buffer (25% sucrose, 50 mM TRIS, 1 mM EDTA, pH 7.5) and repeatedly small quantities of liquid nitrogen were added to keep the sample frozen. Grinding was carried out until the whole content of the tube had a homogenous, yellow-brownish appearance. Next 10 µl (6 U) of

Tab. 1 & 2. GenBank accession numbers of *Pylaiella littoralis*, *Eurychasma dicksonii, Chytridium polysiphoniae,* the other heterokonts and Chytridiomycota used for the alignments on which all ML, MP and NJ calculations are based (Figs 7, 8). The partial sequences (excluding primers) obtained in this study have been deposited in GenBank and assigned the accession numbers AY032606 (*P. littoralis*, 1780 bp), AY032607 (*E. dicksonii*, 1777 bp). Sequences for which only code numbers are available correspond to environmental samples studied by the following authors: OLI11026, OLI11008, OLI11006, OLI11066, OLI11150 (Moon-van der Staay *et al.*, 2001); BOLA515, BOLA320, BAQA232, BAQA21, BAQA72 (Dawson & Pace, 2002); CCW73 (Stoeck & Epstein, 2003); DH144-EKD10, ME1-21, ME1-22, DH1485-EKD, ME1-17, ME1-28, ME1-18, ME1-19, DH147-EKD10 (Massana *et al.*, 2002). The heterokont clades I-VII (Tab. 1, fig. 7) correspond to the terminology used by Massana *et al.* (2002), whilst the terminology of the clades of Chytridiomycota in tab. 2 and fig. 8 follow James *et al.* (2000).

Table 1.

Species	*Lineage*	*Label used in Fig. 2*	*Sequence accession no.*
Laminaria japonica	Phaeophyceae	*Laminaria japonica*	AF123575
Costaria costata	Phaeophyceae	*Costaria costata*	M97958
Desmarestia ligulata	Phaeophyceae	*Desmarestia ligulata*	L43060
Scytosiphon lomentaria	Phaeophyceae	*Scytosiphon lomentaria*	L43066
Ectocarpus siliculosus	Phaeophyceae	*Ectocarpus siliculosus*	L17015
Pylaiella littoralis	Phaeophyceae	*Pylaiella littoralis*	AY032606
Fucus distichus	Phaeophyceae	*Fucus distichus*	AB011423
Sargassum macrocarpum	Phaeophyceae	*Sargassum macrocarpum*	AB011432
Botrydium stoloniferum	Xanthophyceae	*Botrydium stoloniferum*	U41648
Tribonema aequale	Xanthophyceae	*Tribonema aequale*	M55286
Botrydiopsis intercedens	Xanthophyceae	*Botrydiopsis intercedens*	U41647
Giraudyopsis stellifera	Chrysomerophyceae	*Giraudyopsis stellifera*	U78034
Heterosigma akashiwo	Raphidophyceae	*Heterosigma akashiwo*	L42529
Pelagomonas calceolata	Pelagophyceae	*Pelagomonas calceolata*	U14389
Pelagococcus subviridis	Pelagophyceae	*Pelagococcus subviridis*	U14386
Mallomonas papillosa	Chrysophyceae	*Mallomonas papillosa*	M55285
Synura spinosa	Chrysophyceae	*Synura spinosa*	M87336
Chromulina chromophila	Chrysophyceae	*Chromulina chromophila*	M87332
Ochromonas danica	Chrysophyceae	*Ochromonas danica*	M32704
Nannochloropsis salina	Eustigmatophyceae	*Nannochloropsis salina*	AF045045
Nannochloropsis granulata	Eustigmatophyceae	*Nannochloropsis granulata*	U38903
Bacillaria paxillifer	Bacillariophyceae	*Bacillaria paxillifer*	M87325
Thalassionema nitzschioides	Bacillariophyceae	*Thalassionema nitzschioides*	X77702
Coscinodiscus radiatus	Bacillariophyceae	*Coscinodiscus radiatus*	X77705
Rhizosolenia setigera	Bacillariophyceae	*Rhizosolenia setigera*	M87329
Bolidomonas pacifica	Bolidophyceae	*Bolidomonas pacifica*	AF123595
Bolidomonas mediterranea	Bolidophyceae	*Bolidomonas mediterranea*	AF123596
Pythiopsis cymosa	Oomycota	*Pythiopsis cymosa*	AJ238657
Saprolegnia ferax	Oomycota	*Saprolegnia ferax*	AJ238655

Aplanopsis terrestris	Oomycota	*Aplanopsis terrestris*	AJ238658
Achlya apiculata	Oomycota	*Achlya apiculata*	AJ238656
Leptolegnia caudata	Oomycota	*Leptolegnia caudata*	AJ238659
Leptolegnia chapmanii	Oomycota	*Leptolegnia chapmanii*	AJ238661
Leptolegnia chapmanii	Oomycota	*Leptolegnia chapmanii*2	AJ238660
Achlya bisexualis	Oomycota	*Achlya bisexualis*	M32705
Apodachlya brachynema	Oomycota	*Apodachlya brachynema*	AJ238663
Phytophthora undulata	Oomycota	*Phytophthora undulata*	AJ238654
Pythium monospermum	Oomycota	*Pythium monospermum*	AJ238653
Lagenidium giganteum	Oomycota	*Lagenidium giganteum*	M54939
Uncultured stramenopile clone BOLA515	Oomycota	BOLA515	AF372763
Uncultured stramenopile clone BOLA320	Oomycota	BOLA320	AF372762
Uncultured stramenopile clone CCW73	Oomycota	CCW73	AY180031
Eurychasma dicksonii	Oomycota / this study	*Eurychasma dicksonii*	AY032607
Uncultured marine stramenopile DH144-EKD10	Clade I	DH144-EKD10	AF290063
Eukaryote clone OLI11026	Clade I	OLI11026	AJ402339
Eukaryote marine clone ME1-21	Clade I	ME1 21	AF363190
Eukaryote marine clone ME1-22	Clade I	ME1 22	AF363191
Uncultured stramenopile clone BAQA232	Clade I	BAQA232	AF372760
Eukaryote clone OLI11008	Clade I	OLI11008	AJ402350
Developayella elegans	(stramenopiles)	*Developayella elegans*	U37107
Hyphochytrium catenoides	Hyphochytriomycota	*Hyphochytrium catenoides*	X80344
Uncultured marine stramenopile DH148-5-EKD53	Clade II	DH148-5-EKD	AF290083
Eukaryote marine clone ME1-17	Clade II	ME1-17	AF363186
Uncultured eukaryote clone ME1-28	Clade III	ME1-28	AY116221
Eukaryote marine clone ME1-18	Clade III	ME1-18	AF363187
Oli11006	Clade III	OLI11006	AJ402357
Uncultured stramenopile clone BAQA21	Clade III	BAQA21	AF372755
Uncultured stramenopile clone BAQA72	Clade III	BAQA72	AF372754
Siluania monomastiga	Bicosoecida	*Siluania monomastiga*	AF072883
Cafeteria roenbergensis	Bicosoecida	*Cafeteria roenbergensis*	L27633
Eukaryote clone OLI11066	Clades IV, VI and VII	OLI11066	AJ402356

Eukaryote marine clone ME1-19	Clades IV, VI and VII	ME1-19	AF363188
Eukaryote marine clone ME1-20	Clades IV, VI and VII	ME1-20	AF363189
Eukaryote clone OLI11150	Clades IV, VI and VII		AJ402355
Eukaryote marine clone ME1-24	Clades IV, VI and VII	ME1-24	AF363207
Schizochytrium minutum	Labyrinthulida, Thraustochytriidae	*Schizochytrium minutum*	AB022108
Thraustochytrium multirudimentale	Labyrinthulida, Thraustochytriidae	*Thraustochytrium multirudimentale*	AB022111
Labyrinthuloides minuta	Labyrinthulida	*Labyrinthuloides minuta*	L27634
Uncultured marine labyrinthulid DH147-EKD10	Clade V	DH147-EKD10	AF290070
Amphidinium belauense	Dinophyceae	*Amphidinium belauense*	L13719
Prorocentrum minimum	Dinophyceae	*Prorocentrum minimum*	Y16238

Table 2.

Species	*Strain / clone identification*	*Order / Clade*	*Label used in Fig. 3*	*Sequence accession no.*
Rhizophydium sp.	UGA-F15	"Chytridium Clade"	F-15 *Rhizophydium* sp.	AF164319-20
Chytridium sp.	DU-DC2	"Chytridium Clade"	*Chytridium* sp.	AF164321-2
Chytridium confervae	BK M62706	"Chytridium Clade"	*Chytridium confervae*	M59758
Obelidium mucronatum	JEL 57	"Chytridium Clade"	*Obelidium mucronatum*	AF164309-10
Phlyctorhiza endogena	JEL 80	"Chytridium Clade"	*Phlyctorhiza endogena*	AF164313-4
Chytriomyces spinosus	JEL 59	"Chytridium Clade"	*Chytriomyces spinosus*	AF164323-4
Asterophlyctis sarcoptoides	JEL 186	«Chytridium Clade»	*Asterophlyctis sarcoptoides*	AF164317-8
Monoblepharis hypogyna		Monoblepharidales	*Monoblepharis hypogyna*	AF164334
Monoblepharis insignis	BK 59-7	Monoblepharidales	*Monoblepharis insignis*	AF164333
Monoblepharella elongata		Monoblepharidales	*Monoblepharella elongata*	AF164335
Harpochytrium sp.	JEL94	Monoblepharidales	*Harpochytrium* sp.	AF164331-2
Chytriomyces annulatus		Chytridiales	*Chytriomyces annulatus*	AF164303S1
Entophlyctis sp.	JEL122		*Entophlyctis* sp. JEL122	AF164257
Entophlyctis sp.			*Entophlyctis* sp.	AF164257
Rhizophydium sp.	JEL151		*Rhizophydium* sp. 151	AF164270-1
Allomyces macrogynus			*Allomyces macrogynus*	U23936
Rhizophydium sp.	UGA-F16	"Rhizophydium Clade"	*Rhizophydium* sp. F16	AF164264-5
Rhizophydium chaetiferum	JEL 39	"Rhizophydium Clade"	*Rhizophydium chaetiferum*	AF164263
Rhizophydium sphaerotheca	JEL 08	"Rhizophydium Clade"	*Rhizophydium sphaerotheca*	AF164259-60
Rhizophlyctis harderi	JEL 171	"Rhizophydium Clade"	*Rhizophlyctis harderi*	AF164272-3

Rhizophydium sp.	JEL138	"Rhizophydium Clade"	*Rhizophydium* sp. 138	AF164266-7
Lacustromyces hiemalis	JEL 31	"Lacustromyces Clade"	*Lacustromyces hiemalis*	AF164274-5
Polychytrium aggregatum	JEL 190	"Lacustromyces Clade"	*Polychytrium aggregatum*	AF164276-7
Chytridiales sp.	JEL 207	"Lacustromyces Clade"	Chytridiales sp. 207	AF164261-2
Karlingiomyces sp.	JEL93	"Lacustromyces Clade"	*Karlingiomyces* sp.	AF164278.1
Diplochytridium lagenarium	JEL 72	"Nowakowskiella Clade"	*Diplochytridium lagenarium*	AF164285-6
Nowakowskiella elegans	BK50-1	"Nowakowskiella Clade"	*Nowakowskiella elegans*	AF164281-1
Allochytridium expandens	BK 69-3	"Nowakowskiella Clade"	*Allochytridium expandens*	AF164291-2
Cladochytrium replicatum	JEL38	"Nowakowskiella Clade"	*Cladochytrium replicatum*	AF164297-8
Nephrochytrium sp.	JEL125	"Nowakowskiella Clade"	*Nephrochytrium* sp.	AF164295.1
Rhizophlyctis rosea	BK47-07		*Rhizophlyctis rosea* 47-07	AF164251-2
Rhizophlyctis rosea	BK57-5		*Rhizophlyctis rosea* 57-5	AF164249-50
Neocallimastix joyonii	NJ1	Neocallimastigales	*Neocallimastix joyonii*	M62705
Piromyces communis	FL	Neocallimastigales	*Piromyces communis*	M62706
Neocallimastix frontalis	MCH3	Neocallimastigales	*Neocallimastix frontalis*	M62704
Neocallimastix sp.	LM-2	Neocallimastigales	*Neocallimastix* sp. LM-2	M59761.1
Neocallimastix frontalis	L2	Neocallimastigales	*Neocallimastix frontalis* L2	X80341.1
Neocallimastix frontalis	MCH3	Neocallimastigales	*Neocallimastix frontalis* MCH3	M62704.1
Powellomyces variabilis	BK91-11	Spizellomycetales	*Powellomyces variabilis* 91-11	AF164241-2
Powellomyces hirtus	UGA-F18	Spizellomycetales	*Powellomyces hirtus*	AF164239-40
Powellomyces variabilis	BK85-1	Spizellomycetales	*Powellomyces variabilis* 85-1	AF164243.1
Powellomyces sp.	JEL95	Spizellomycetales	*Powellomyces* sp. 95	AF164245-6
Spizellomyces kniepii	UGA-F22	Spizellomycetales	*Spizellomyces kniepii*	AF164237-8
Spizellomyces acuminatus		Spizellomycetales	*Spizellomyces acuminatus*	M59759
Uncultured rhizosphere chytridiomycete	RSC-CHU-23		Uncultured rhizosphere chytridiomycete RSC-CHU-23	AJ506003.1
Uncultured rhizosphere chytridiomycete	RSC-CHU-18		Uncultured rhizosphere chytridiomycete RSC-CHU-18	AJ506000.1
Uncultured rhizosphere chytridiomycete	RSC-CHU-69		Uncultured rhizosphere chytridiomycete RSC-CHU-69	AJ506037.1
Uncultured rhizosphere chytridiomycete	RSC-CHU-20		Uncultured rhizosphere chytridiomycete RSC-CHU-20	AJ506002.1
Uncultured fungus clone	CCW64		Uncultured fungus clone CCW64	AY180029.1
Chytriomyces angularis			*Chytriomyces angularis*	AF164253
Chytridium polysiphoniae	Chyt Pyl IR-g14 – this study		*Chytridium polysiphoniae*	AY032608

Gaertneriomyces semiglobiferus	BK91-10	*Gaertneriomyces semiglobiferus*	AF164247-8
Pavlova gyrans		*Pavlova gyrans*	U40922
Chlamydomonas reinhardtii		*Chlamydomonas reinhardtii*	M32703
Chlorella lobophora	Andreyeva 750-I	*Chlorella lobophora*	X63504
Cyanophora paradoxa		*Cyanophora paradoxa*	X68483

proteinase K (Boehringer Mannheim, Germany) were added and the mixture was incubated at 37°C for two hours. After addition of 1 volume of phenol, the tube was gently shaken for 10 min. The aqueous phase containing the DNA was recovered after centrifugation at 12500 g for 15 min. Again, 1 volume of a 1:1 chloroform / phenol mixture was added and gently mixed to obtain a single phase. The aqueous phase was recovered after centrifugation (12500 g for 15 min) and the DNA was precipitated by addition of 1/10 volume of 3 M sodium acetate (pH 7.5) and 2 volumes of cold (- 20°C) ethanol (analytical grade). After 30 min at –80°C and centrifugation at 12500 g for 25 min, the supernatant was removed and the pellet was washed by addition of 1.5 ml 70% ethanol and centrifugation (12500 g for 15 min). After air-drying, the pellet was re-suspended in 500 ml H_2O and further purified using PhytoPure™ resin (Nucleon, Amersham Life Sciences, Little Chalfont, Buckinghamshire, England). The purified DNA was re-suspended in 50 µl sterile water.

PCR was carried out using 1 ml genomic DNA, 5 µl each (200 pmol/µl) of oligonucleotide primers # 328 (5‘-ACCTGGTTGATCCTGCCAG-3‘) and # 329 (5‘-TGATCCTTCYGCAGGTTCAC-3‘), 14 µl sterile water (i.e. 25 µl in total) and one Ready-to-go PCR bead™ (Amersham-Pharmacia) per reaction. The PCR program was as follows: denaturation at 94ºC for 1 min (initial denaturation 5 min), annealing at 55ºC for 2 min, and extension at 72ºC for 3 min (final extension 10 min). The reaction was cycled 30 to 35 times. The primers were complementary to conserved sequences close to the respective 5’ and 3’ termini of the 18 S rRNA gene, designed to amplify most eukaryotic 18 S rRNA genes (Moon–van der Staay *et al.*, 2000). Dilution (1/10 or 1/100) of the template proved to have a beneficial effect on PCR efficiency, presumably due to dilution of residual carbohydrate and polyphenol contaminants originating from the brown algal tissue. After verification of purity of the PCR product, cloning was carried out immediately using the TOPO TA Cloning® vector (Invitrogen®) in *Escherichia coli.* Plasmid DNA was prepared using the Flexiprep™ kit (Pharmacia). Different clones (of *Pylaiella littoralis* and the two pathogens) were distinguished by their restriction patterns obtained by a combined *Eco*RI and *Bam*HI digestion. Sequencing (double strand) was carried out by ESGS-Cybergene (Evry, France), using a primer-walking approach and yielding three different consensus sequences. Sequences were deposited in GenBank (Accession numbers: *Pylaiella* - AY032606; *Eurychasma* - AY032607; *Chytridium* - AY032608).

Phylogenetic trees

Three distinct sequences were obtained and manually aligned with other heterokont and fungal taxa, taking secondary structures into account. Poorly aligned positions and divergent regions were removed using Gblocks™

(Castresana, 2000) with a minimum length of a block of 5 and half allowed gap positions. We then processed 3 different phylogenetic analyses (maximum parsimony, MP, neighbor joining, NJ, and maximum likelihood, ML). For NJ and ML, gaps were treated as missing. For MP, gaps were treated as an additional state. Different nested models of DNA substitution and associated parameters were estimated using Modeltest 3.0 (Posada & Crandall, 1998). A heuristic search procedure using the tree bisection-reconnection branch-swapping algorithm (settings as in MP) was performed to find the optimal ML tree topology. NJ, MP, and ML were processed under the PAUP*4.0b10 software (Swofford, 2003). Bootstrap values were assessed from 1000 replicates for NJ and MP. For MP, the number of rearrangements was limited to 5,000 for each bootstrap replicate. The starting trees were obtained by randomized stepwise addition (number of replicates = 20).

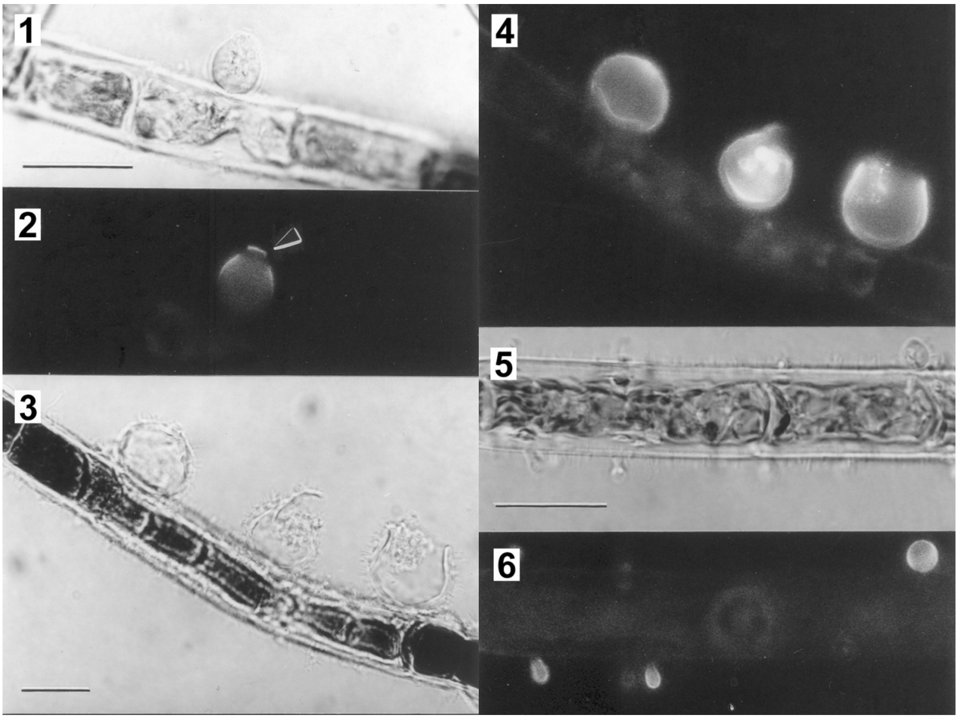
RESULTS

Histochemistry

Eurychasma does not produce chitin, as demonstrated by the absence of fluorescence upon Fungalase™ staining (not shown). This protocol clearly revealed the presence of chitin associated with the walls of *Chytridium* (Figs 1-6), of settled spores, and of developing and mature sporangia.

Nucleotide sequences

Unialgal host cultures of *Eurychasma* and *Chytridium* had been obtained by co-incubating field-collected, infected *Pylaiella littoralis* from Shetland with a unialgal *Pylaiella* strain from Isla Diego Ramirez (Chile), and establishing unialgal sub-isolates of the Chilean *Pylaiella* once its filaments had become infected by either *Eurychasma* or *Chytridium* (Müller *et al.*, 1999). These infected algal cultures were the material used for cloning the 18 S rRNA genes. Due to the mixed extraction of both *Pylaiella* and *Eurychasma* or *Pylaiella* and *Chytridium* DNA, respectively, the clones obtained were inevitably a mixture of brown algal and pathogen 18 S rRNA genes. The PCR products were cloned and a total of around 100 clones were screened by their restriction fragment length polymorphism (RFLP) patterns. In total, 3 different 18 S rDNA sequences were obtained from the two infected cultures, with each culture (Eu Pyl IR 1 and Chyt Pyl IR 14, respectively) yielding two different clones based on their RFLP patterns. One clone from each of the two cultures had the same *Eco*RI and *Bam*HI restriction pattern, which was attributed to *Pylaiella*, while the two other clones were distinctly different (not shown), attributable to the respective pathogen present in the culture from which the DNA had been extracted (all three confirmed by BLAST analyses). Manual alignments with heterokont and fungal representatives showed that one sequence possessed numerous signatures characteristic of Oomycota, confirmed by a BLAST search (Altschul *et al.*, 1990) in GenBank, and could be attributed to *Eurychasma.* Its restriction pattern was found only in one of the two types of clones from Eu Pyl IR 1, but not in those from Chyt Pyl IR 14. The second sequence in the pool of 18 S rDNA clones could



Figs 1-6. Light microscopy of *Chytridium*-infected *Pylaiella*, treated with Fungalase™ demonstrating the presence of chitin in the cell wall of this fungus: A mature *Chytridium* sporangium during detachment of the operculum (**1, 2**).- Older, empty sporangia – three remaining spores have germinated inside the parental sporangium (**3, 4**). Spores produce chitin soon after settling (the spores themselves are unwalled, e.g. James *et al.*, 2000), causing an intense fluorescence upon Fungalase™ staining and blue excitation (**5, 6**).- All scale bars correspond to 25 µm.

be attributed to *Pylaiella*, having clear brown algal signatures. According to its restriction pattern, it was present in the clones from both Eu Pyl IR 1 and Chyt Pyl IR 14. In contrast, the third sequence clearly aligned with the fungi (Eumycota), which was again confirmed by a BLAST search, and was attributed to *Chytridium*. Its restriction pattern was found only in one of the two types of clones from Chyt Pyl IR 14, but not in those from Eu Pyl IR 1. In the different cloning attempts, the number of brown algal clones was always far higher than that of supposed pathogen clones, with a ratio of around 1:30 for *Eurychasma* : *Pylaiella* 18 S rDNA and 1:50 for *Chytridium* : *Pylaiella* 18 S rDNA in mature, heavily infested cultures (> 5 weeks post-inoculation), respectively. The ratio was smaller in cultures inoculated more recently.

Using the alignments (of 1746 positions for the heterokonts and 1034 for the fungi; available at http://www.sb-roscoff.fr/Phyto/Databases/index.php3) to determine the 5' and 3' ends, the 18 S rDNA sequences of *Pylaiella littoralis*, *Eurychasma dicksonii* and *Chytridium polysiphoniae* were found to be 1823, 1820 and 1808 bp long, respectively, taking into account the length of the primers used here.

From an initial alignment of 1937 positions for stramenopiles, the program Gblocks™ left 1599 final positions (82% positions retained; available online at http://www.sb-roscoff.fr/Phyto/Databases/index.php3). Positions removed mainly correspond to the hypervariable regions located in the E21-1, 41, and 47 secondary structures in the 18 S rRNA of *Saccharomyces cerevisiae* (Lange *et al.*, 1996). 800 characters are constant, 215 are parsimony-uninformative, and 584 are parsimony-informative.

The tree topology obtained is congruent with previous 18 SSU rDNA sequence analyses, showing that most heterotrophic species are placed at the basal part of the tree, whereas all photosynthetic organisms emerged as a monophyletic group, in the upper part of the tree. Consistent with the tree of Massana *et al.*, (2002), Oomycota are part of a clade that also include the flagellate *Developayella elegans*, *Hyphochytrium* and the novel marine stramenopiles group 1. This was not supported by the bootstrap analyses, but it was nevertheless consistent between the three phylogenetic analyses made in this study. The Oomycota themselves were split into different clades, two of them corresponding to the Saprolegnian and Peronosporalean galaxies defined by Sparrow (reviewed in 1976), and another one composed of environmental sequences retrieved from anoxic marine sediments and the sequence of *Eurychasma dicksonii.*

The early divergence of *E. dicksonii* within the Peronosporomycota (Oomycota) branch, already suggested by its signatures (available online at http://www.sb-roscoff.fr/Phyto/), is confirmed by all phylogenetic analyses (Fig. 7). *Eurychasma* is always basal to the oomycete lineage with a bootstrap value of 100% in both MP and NJ, before the separation of the lineage into two main branches (the so called Saprolegniomycetidae and Peronosporomycetidae). This broad division of Oomycota into two major clades has recently been further supported by the molecular data of Dick *et al.* (1999), Petersen & Rosendahl (2000) and Hudspeth *et al.* (2000).

From an initial alignment of 1098 positions for Eumycota, the program Gblocks™ left 960 final positions (87% positions retained; available online at http://www.sb-roscoff.fr/Phyto/). 563 characters are constant, 100 are parsimony-uninformative, and 297 are parsimony-informative.

Our analyses, based on MP, NJ and ML, confirmed the repartition of the Chytridiales into a number of different clades (consistent with James *et al.*, 2000) and with several of traditional genera showing their polyphyletic origin, including *Chytridium*, *Rhizophydium* and *Chytriomyces* (Fig. 8). Our phylogenetic trees showed that *Chytridium polysiphoniae* is only distantly related to the two other members of the genus *Chytridium* for which sequences are available, *Chytridium confervae* and *Chytridium* sp. strain DU-DC2. Phylogenetic inference does not support in any way the inclusion of *C. polysiphoniae* in the clade which comprises these, which is also supported by characteristic signatures (available online at http://www.sb-roscoff.fr/Phyto/). Instead, it appears with 100 % bootstrap support by both MP and NJ in a novel clade, adjacent to *Chytriomyces angularis* as the only morphologically known species, and a number of uncultured soil organisms.

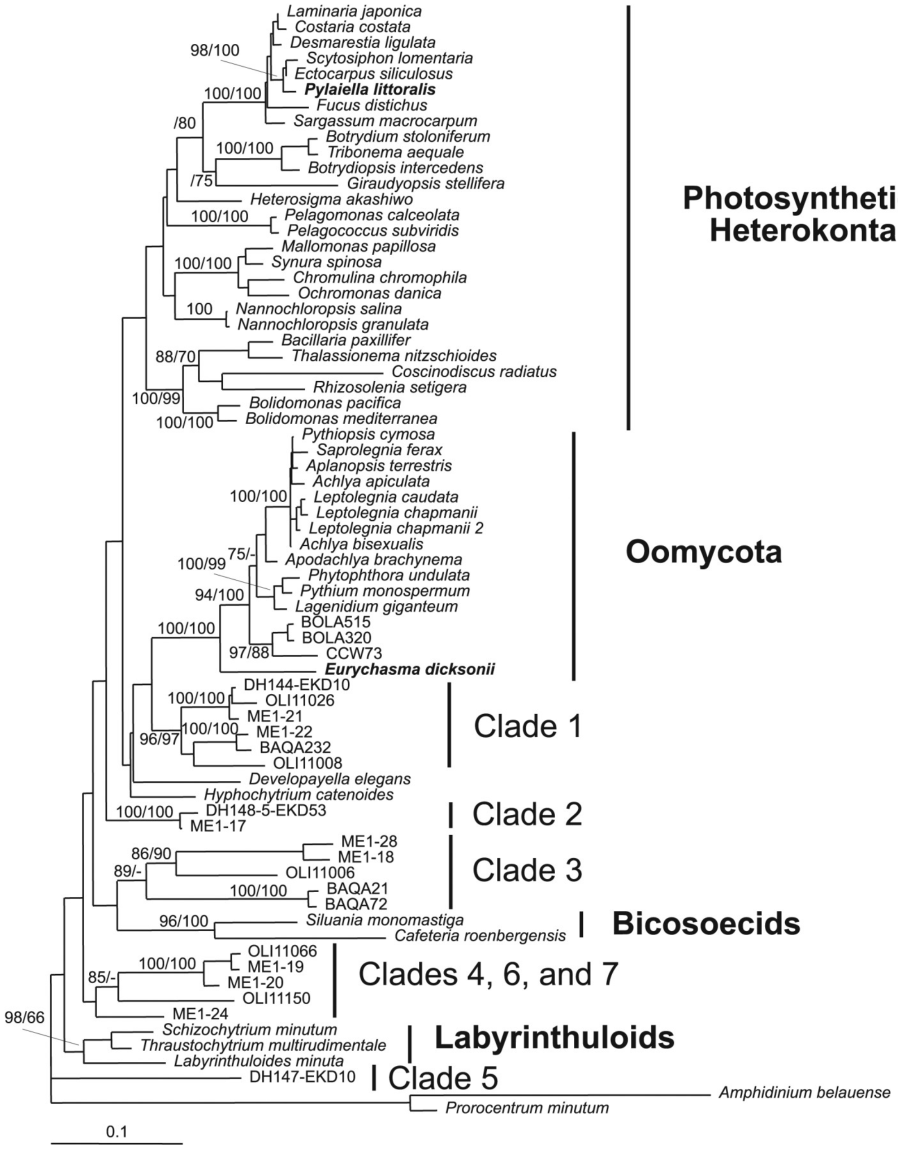


Fig. 7. Maximum likelihood tree of the sequences from *Pylaiella littoralis* and *Eurychasma dicksonii*, based on 71 SSU rDNA sequences and 1599 nucleotides in total. Best-fit DNA substitution model selected by Hierarchical Likelihood Ratio Tests using Modeltest (-lnL = 19702) was else described by Tamura & Nei (1993) with the following parameters: proportion of invariable sites (I) = 0.3279, gamma distribution shape parameter = 0.5419, and substitution models of R(b) [A-G] = 2.1193, R(e) [C-T] = 4.0823, and 1.0 for all other substitution rates. New sequences obtained from this study are in bold. Bootstrap values (1000 replicates) for major clades are indicated above internodes and correspond to NJ and MP respectively. Bootstrap values <75% are indicated by hyphens. Clade labeling of lineages including environmental sequences was defined by Massana *et al.* (2002). *Amphidinium belauense* and *Prorocentrum minutum* were chosen as outgroup.

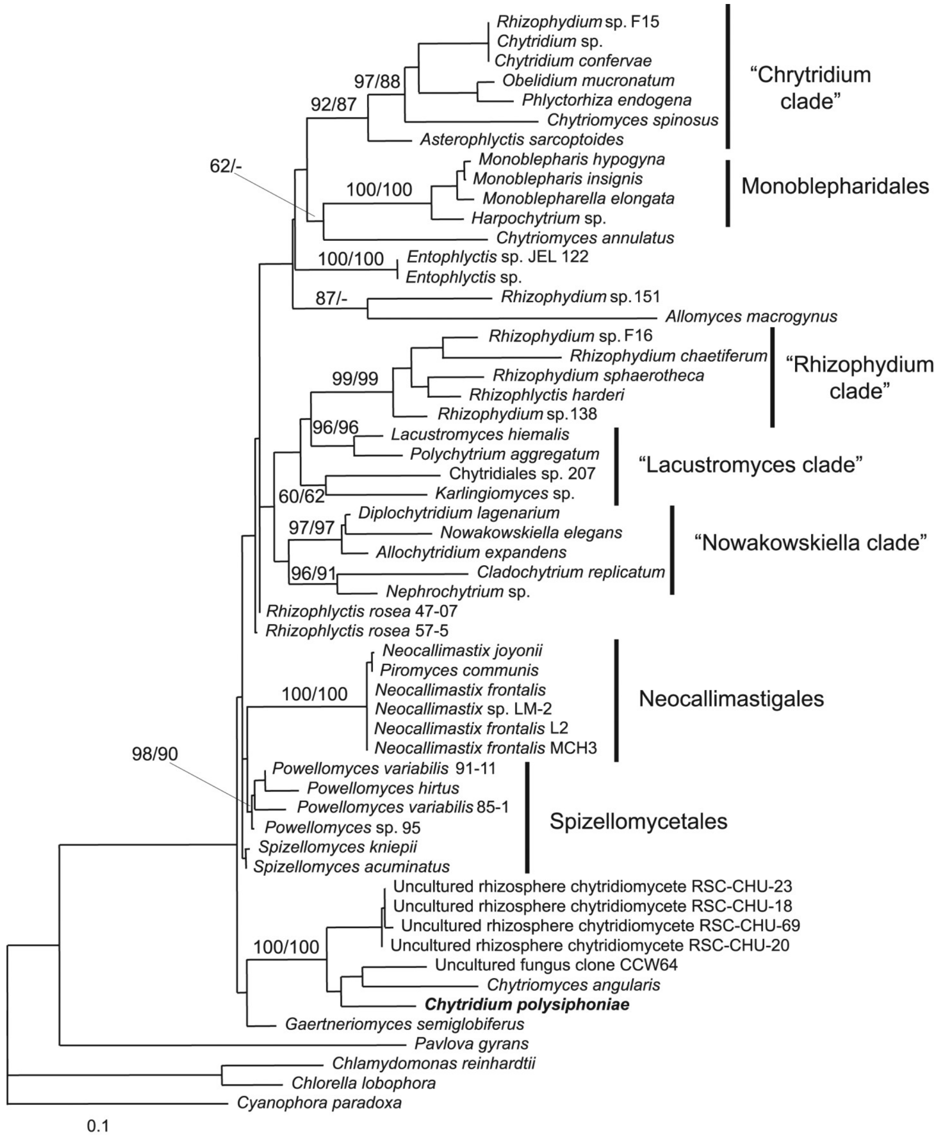


Fig. 8. Maximum likelihood tree of *Chytridium polysiphoniae* in the context of the Chytridiomycota. Parsimony analyses for *Chytridium* were done with 38 species and 1034 characters per species, respectively, whilst for neighbor joining (not shown) 35 species and 1027 positions were used. Branch support was determined by bootstrap analysis (Felsenstein 1985) using 100 replicates. *Pavlova gyrans*, *Chlamydomonas reinhardtii*, *Chlorella lobophora* and *Cyanophora paradoxa* were chosen as outgroup for the Chytridiomycetes. Best-fit DNA substitution model selected by Hierarchical Likelihood Ratio Tests using Modeltest (-lnL = 7681) had been described by Rodríguez *et al.* (1990; parameters: proportion of invariable sites (I) = 0.4408, gamma distribution shape parameter = 0.5679, and substitution models of R(b) [A-G] = 2.5562, R(e) [C-T] = 4.6751, and 1.0 for all other substitution rates). Bootstrap values (1000 replicates) for major clades are indicated above internodes and correspond to NJ and MP respectively. Bootstrap values <75% are indicated by hyphens.

DISCUSSION

The superficial morphological similarity of the holocarpic thalli of these two parasites has meant that historically there has been considerable confusion regarding the phylogenetic relationships of *Eurychasma* and *Chytridium*. Despite the research interest that these organisms have received for over a century, their phylogenetic affiliations have never been established unequivocally. In the nineteenth century both of these genera were placed together in the Chytridia (*sensu* Braun, 1844), which at that time was a term used for a polyphyletic assemblage, encompassing all of the aquatic plant pathogens known. The species we now refer to as *Eurychasma dicksonii* was first described by Wright in 1879 and Rattray (1885) referred to it as *Rhizophydium dicksonii*, a "chytridiaceous parasite". Wille (1899) subsequently placed it in the parasitic genus *Olpidium* as *O. dicksonii* (Wright) Wille, but this was not widely accepted. Finally, in 1905 Magnus elevated *Rhizophydium dicksonii* to the status of a genus of its own with the name *Eurychasma*. Based on the formation of a net sporangium in zoospore development, Petersen (1905) created the family Eurychasmaceae. Sparrow (1934) did not draw a clear separation between uniflagellate and biflagellate aquatic plant pathogens, which were placed within the all encompassing lower "phycomycete fungi". However he did point out that the zoospore structure (biflagellate) and behaviour of *Eurychasma dicksonii* and its possession of cellulose cell walls supported an affiliation with the Saprolegniales rather than with the Chytridiales. As the twentieth century progressed it became generally accepted that the biflagellate and uniflagellate zoosporic "phycomycete fungi" were phylogenetically unrelated groups (Sparrow, 1960). Doubts about the close affiliation of the biflagellate 'oomycete' fungi to the higher fungi have existed since the earliest studies of its members. As early as 1858, Pringsheim noted similarities in the sexual reproduction between the Saprolegniaceae and Vaucheriaceae. Indeed this led to both detailed comparative analysis of both zoospore ultrastructure (Manton *et al.*, 1951) and cell wall biochemistry (Parker *et al.*, 1963) of *Saprolegnia* and species of the phaeophyte and xanthophyte algae. These studies supported the close phylogenetic linkage between biflagellate oomycete fungi and the heterokont algae (reviewed by Beakes, 1989). Ribosomal RNA sequence homology studies finally proved beyond doubt the affiliation of the Peronosporomycota with the Chromista/Heterokonta rather than the fungi/Eumycota (Gunderson *et al.*, 1987; Förster *et al.*, 1990). Subsequently, more in-depth molecular studies of oomycete taxa seem to confirm Sparrow's (1976) conclusion that the 'oomycetes' could be apportioned to two main higher order 'subclasses', which Dick *et al.* (1999) has recently called the Saprolegniomycetidae and Peronosporomycetidae.

Few morphological and developmental characteristics of *Eurychasma* are known at this stage and need to be the subject of further study, and sexuality has never been observed. Yet, based on the molecular results discussed here, *Eurychasma* appears to have differentiated early, before the radiation of other known oomycetes into two lineages.

Whilst the vast majority of fungi have chitin in their cell walls, its occurrence in the heterokont Peronosporomycota is not uniform (Barr, 1983). The occurrence of chitin has been frequently considered as a phylogenetic marker in comparable studies of pathogens of aquatic organisms as a supplement to molecular or electron microscopic methods (e.g. Benny & O'Donnell, 2000; Uppalapati *et al.*, 2001). Unlike other Oomycota such as *Achlya radiosa* (Campos-

Takaki *et al.*, 1982), *Eurychasma* does not contain chitin. Even though Sparrow (1934) did not mention the absence of chitin in *Eurychasma*, he already based his argument to place the species within the Saprolegniales upon his findings of cellulose in its cell wall (determined by application of zinc chloriodide). In any case, Sparrow's (1934, 1960) classification of *Eurychasma* in the order Saprolegniales can clearly not be maintained. Instead, the phylogenetic vicinity of *Eurychasma* to morphologically uncharacterized marine and anaerobic members of the Oomycota (Dawson & Pace, 2002; Stoeck & Epstein, 2003), is an interesting result, at a considerable distance basal to the separation of the Oomycota into the two subclasses discussed above. This group obviously requires further taxonomic treatment, possibly including the creation of a new order at the basis of the Oomycota.

Our results also suggest that other members of the eukaryotic picoplankton, only known by their SSU rDNA sequence (Moon-van der Staay *et al.*, 2000; Massana *et al.*, 2002) branch as a sister clade (termed Clade I by Massana *et al.*, 2002) to the Oomycota. These sequences were retrieved from open-ocean water samples. This interesting result must be confirmed by more genetic information on the planktonic diversity.

These findings add an interesting aspect to the evolution of the Oomycota: Barr (1983) had suggested that terrestrial plant pathogens had evolved from aquatic saprobes belonging to the Saprolegniales. Our results show that the Oomycota comprise pathogens of marine algae, able to infect other phyla such as Chlorophyta, and suggest that obligate parasitism has evolved earlier than previously considered in the Oomycota lineage.

James *et al.* (2000) recently carried out a comprehensive molecular study on the systematics of the Chytridiomycota based on ribosomal RNA genes. In light of our findings, showing that *C. polysiphoniae* belongs to a clade only distantly related to that containing the other two *Chytridium* species for which SSU sequences are available, we conclude that a revision of the genus *Chytridium* as a whole is required, and that the availability of molecular data for the type species, *Chytridium olla* Braun (Braun, 1851) is essential for this. It also appears likely that the position of *C. polysiphoniae* in the genus *Chytridium* can no longer be maintained. *C. polysiphoniae* was initially placed in this genus by Sparrow (1934) based upon the operculate character of the sporangium, but unfortunately no more ultrastructural characteristics are available at this stage to provide reliable support for a taxonomic classification. According to our results, a re-classification and nomenclatural change of the taxon *Chytridium polysiphoniae* is inevitable. Furthermore, Sparrow (1960) has already pointed out that the species as described thus far is probably a composite one, requiring further study. As Barr (1990) and James *et al.* (2000) suggest in general for the Chytridiomycota, ultrastructural work on zoospores can be expected to contribute to a more accurate assignment of these aquatic fungi. Therefore, we propose to await such further ultrastructural evidence for a final judgement about the taxonomic status of *Chytridium polysiphoniae*. This result corroborates the statement of James *et al.* (2000) that the Chytridiales, in their current classification, are not monophyletic.

The basal position of both organisms, *E. dicksonii* and *C. polysiphoniae*, in relation to their respective phyla remains an interesting finding. One conceivable explanation could be that, as two of the few oceanic organisms studied among these phyla, they have to appear in an isolated position in a molecular phylogeny, since all other model organisms studied so far are terrestrial. And, possibly consistent with this hypothesis, these findings could suggest that they are indeed much more ancestral than the other members of the

Chytridiomycota and Oomycota studied to date. The close relationship of *E. dicksonii* and *C. polysiphoniae* with oceanic and soil-dwelling organisms of unknown morphology renders them particularly interesting as the only culturable organisms of these poorly understood groups available for further study. In this study, they appear as the only members with known morphology and accessible to laboratory studies of two obviously diverse groups from aquatic environments, highlighting their interest as model species for a better understanding of this poorly known biodiversity. Overall, this study underlines the need to increasingly consider marine representatives for a better understanding of the early evolution of these two groups of pathogens, and it appears highly desirable to include organisms such as the diatom pathogen *Ectrogella* and some of the numerous diatom pathogens among the Chytridiomycota in future studies.

Acknowledgements. We are grateful to Gordon W. Beakes (Newcastle) for useful suggestions when preparing this manuscript, and we would like to thank Catherine Leblanc (Roscoff) for support in molecular work. We gratefully acknowledge Daniel Vaulot (Roscoff) for sharing his sequence data and discussions. FCK was supported by fellowships from Studienstiftung des Deutschen Volkes (Bonn) and the European Commission (program MAST-III).

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