Protist, Vol. 165, 230–244, March 2014
http://www.elsevier.de/protis
Published online date 1 March 2014

Protist

ORIGINAL PAPER

Dinomyces arenysensis gen. et sp. nov. (Rhizophydiales, Dinomycetaceae fam. nov.), a Chytrid Infecting Marine Dinoflagellates

Frédéric Lepelletier[a,b], Sergey A. Karpov[c,d], Elisabet Alacid[e], Sophie Le Panse[f,b], Estelle Bigeard[a,b], Esther Garcés[e], Christian Jeanthon[a,b], and Laure Guillou[a,b,1]

[a]CNRS, UMR 7144, Place Georges Teissier, CS90074, 29688 Roscoff Cedex, France
[b]Université Pierre et Marie Curie (Paris VI), Station Biologique de Roscoff, Place Georges Teissier, CS90074, 29688 Roscoff Cedex, France
[c]Zoological Institute RAS, St. Petersburg, Russia
[d]St. Petersburg State University, St. Petersburg, Russia
[e]Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar, CSIC, Pg. Marítim de la Barceloneta, 37-49, E08003 Barcelona, Spain
[f]CNRS, FR 2424, Plate-forme Merimage, Station Biologique de Roscoff, Place Georges Teissier, CS90074, 29688 Roscoff Cedex, France

Submitted January 14, 2014; Accepted February 21, 2014
Monitoring Editor: Michael Melkonian

Environmental 18S rRNA gene surveys of microbial eukaryotes have recently revealed the diversity of major parasitic agents in pelagic freshwater systems, consisting primarily of chytrid fungi. To date, only a few studies have reported the presence of chydrids in the marine environment and a limited number of marine chytrids have been properly identified and characterized. Here, we report the isolation and cultivation of a marine chytrid from samples taken during a bloom of the toxic dinoflagellate *Alexandrium minutum* in the Arenys de Mar harbour (Mediterranean Sea, Spain). Cross-infections using cultures and natural phytoplankton communities revealed that this chytrid is only able to infect certain species of dinoflagellates, with a rather wide host range but with a relative preference for *Alexandrium* species. Phylogenetic analyses showed that it belongs to the order Rhizophydiales, but cannot be included in any of the existing families within this order. Several ultrastructural characters confirmed the placement of this taxon within the Rhizophydiales as well its novelty notably in terms of zoospore structure. This marine chytridial parasitoid is described as a new genus and species, *Dinomyces arenysensis*, within the Dinomycetaceae fam. nov.

Key words: Fungi; chytrid; microbial parasitoids; dinoflagellates; *Dinomyces arenysensis*; Rhizophydiales.

[1]Corresponding author; fax +33 2 98 29 23 24
e-mail lguillou@sb-roscoff.fr (L. Guillou).

http://dx.doi.org/10.1016/j.protis.2014.02.004

Introduction

Chytrids are true fungi that produce motile zoospores with a single posteriorly directed flagellum. Barr (2001) originally placed them in the phylum Chytridiomycota. Asexual reproduction results in the formation of a sac-like structure, the zoosporangium, in which zoospores are produced. Chytrids may also produce sexual and asexual resting spores that eventually germinate into a sporangium after a period of dormancy. Most chytrids produce a rhizoidal system for absorption of nutrients. Many chytrids have been reported as saprobes and/or parasites in a wide range of habitats, notably in freshwater and soil ecosystems (Powell 1993; Shearer et al. 2007; Sime-Ngando 2012; Sparrow 1960), but only a few species have been found in marine environments (Gleason et al. 2011; Sparrow 1960). In freshwater, chytrid parasitoids of planktonic microalgae and chytridiomycoses play a crucial role in the regulation of sizes of populations of phytoplanktonic species (Ibelings et al. 2004; Sime-Ngando 2012; Sparrow 1960). In particular, they infect large and inedible diatoms and green algae (Kagami et al. 2007; Rasconi et al. 2012). Because chytrid zoospores are also grazed by predators, such parasitoids are considered as key players in the transfer of organic matter from such inedible phytoplanktonic cells to higher trophic levels in the microbial food web (Kagami et al. 2007; Miki et al. 2011; Sime-Ngando 2012). Environmental SSU rDNA surveys have revealed widespread occurrence and high diversity of chytrids in lakes (Lefèvre et al. 2007, 2008). Diverse and abundant environmental fungal sequences have been retrieved from particular marine habitats, such as deep and anoxic marine ecosystems (Jebaraj et al. 2010; Le Calvez et al. 2009; Orsi et al. 2013). At the sea surface, where microalgae are abundant, their contribution is considerably lower (Massana and Pedrós-Alió 2008). Although literature on freshwater chytrids infecting microalgae is extensive, very few chytrid species infecting microalgae have been properly identified in marine ecosystems (Gleason et al. 2011). To date, infections by chytrids have only been reported in marine diatoms (Elbrächter and Schnepf 1998; Hanic et al. 2009). The putative capacity of chytrids to adapt to and/or compete with other parasites of microalgae in marine environments has been proposed (Richard et al. 2012).

In this study, we report the isolation into culture and characterization of the first chytrid known to infect marine dinoflagellates. This species was isolated during a bloom of *Alexandrium minutum*, a widespread toxic marine bloom-forming species (Anderson et al. 2012). Dinoflagellates of the genus *Alexandrium* are known to produce toxins that can be accumulated along the food chain and cause paralytic shellfish poisoning (PSP) events in the case of human ingestion of contaminated shellfish. It was recently demonstrated that *A. minutum* populations could be efficiently controlled by highly virulent protozoan parasitoids (Chambouvet et al. 2008; Montagnes et al. 2008). Several pathogens of *A. minutum* have recently been identified and described within the kingdom Alveolata (Chambouvet et al. 2008; Figueroa et al. 2008; Lepelletier et al. 2014). This study demonstrates that the diversity of their parasitoids also extends to another eukaryotic lineage.

Results

Isolation and Culturing Methods

Natural samples were collected weekly during a bloom of *A. minutum* in the Arenys de Mar harbour (Mediterranean Sea, Spain) in 2012. Maximal density of the toxic dinoflagellate (10^6 cells L^{-1}) was observed on February 15th. Cell density slowly decreased over the following weeks (8.9×10^5 cells L^{-1} and 6.2×10^5 cells L^{-1} on February 22nd and March 14th, respectively). Chytrids (6 strains) were isolated during the first two weeks of the survey period (February 15th and 22nd, Supplementary Material Table S1). Chytrids could easily be maintained in culture with their hosts at salinities ranging from 27 to 35. Strains RCC3404 and RCC3408 were not able to form colonies on either of the agar media tested without their hosts (Koch's K-1 and F/2 supplemented with soil extract).

Host Specificity

Diatoms (5 strains), cryptophytes (3 strains), haptophytes (3 strains) and prasinophytes (2 strains) were resistant when cross infected with fungal strains RCC3404 and RCC3408 (Table 1). Among dinoflagellates, *Akashiwo sanguinea*, *Amphidinium carterae*, *Coolia monotis*, *Gymnodinium* spp., *Karlodinium veneficum*, *Kryptoperidinium foliaceum*, and *Prorocentrum* spp. were also resistant. Both fungal strains could infect most *Alexandrium* species. However, some host cells survived infections after 20 days in most of cases, except for few strains of *A. minutum*, *A. ostenfeldii*, *A. tamarense* and *A. cf. catenella* where host cells disappeared totally. Intra-specific variability in host response was observed: of fifteen *A. minutum*

Table 1. Cross-infections between the chytrid parasitoid *Dinomyces arenysensis* and selected hosts belonging to different microalgal lineages (for details see supplementary Table 1). Resistant= —, moderately resistant=-, sensitive=+++, moderately sensitive=+. ND= Not Done.

Host Species	Strain	Ocean	*Dinomyces arenysensis* RCC3404	*Dinomyces* sp. RCC3408
Dinophyceae				
Akashiwo sanguinea	RCC3040	The Channel	—	ND
Alexandrium andersonii	ICMB222	Med Sea	+	+
Alexandrium cf. catenella (Group IV)	ACT2	Med Sea	+++	ND
Alexandrium cf. catenella (Group IV)	ACT3	Med Sea	+	ND
Alexandrium cf. catenella (Group IV)	AC2C	Med Sea	—	—
Alexandrium cf. catenella (Group IV)	VGO599	Med Sea	+	-
Alexandrium cf. tamarense (Group II)	VGO1042	Med Sea	—	+
Alexandrium tamarense (Group III)	AT5	North Sea	+++	ND
Alexandrium minutum	AL2V	Atlantic	+	ND
Alexandrium minutum	AL9C	Med Sea	+	+
Alexandrium minutum	AMP4	Med Sea	-	-
Alexandrium minutum	RCC3020	The Channel	+	ND
Alexandrium minutum	SZN030 CC1	Med Sea	+	ND
Alexandrium minutum	CBA38	Med Sea	+++	ND
Alexandrium minutum	RCC3021	Atlantic	+	ND
Alexandrium minutum	RCC2646	The Channel	+	ND
Alexandrium minutum	RCC3022	The Channel	+	ND
Alexandrium minutum	RCC3416	Med Sea	+	ND
Alexandrium minutum	RCC3409	Med Sea	+	ND
Alexandrium minutum	RCC3410	Med Sea	+	ND
Alexandrium minutum	RCC3411	Med Sea	+++	ND
Alexandrium minutum	RCC3412	Med Sea	+++	ND
Alexandrium minutum	RCC3413	Med Sea	+++	ND
Alexandrium ostenfeldii	CCAP1119/45	North Sea	+++	ND
Alexandrium ostenfeldii	VGO956	Med Sea	+	+
Alexandrium tamutum	SZN029	Med Sea	+	+
Alexandrium tamutum	A8	Med Sea	+	+
Alexandrium taylori	VGO703	Med Sea	—	—
Amphidinium carterae	RCC1522	The Channel	—	—
Coolia monotis	VGO941	Med Sea	—	—
Gymnodinium impudicum	GY3VA	Med Sea	—	—
Gymnodinium instriatum	ICMB234	Med Sea		—
Gymnodinium litoralis	ICMB226	Med Sea	—	—
Heterocapsa triquetra	RCC3044	The Channel		ND
Heterocapsa triquetra	RCC3043	The Channel	+	ND
Karlodinium veneficum	ICMB256	Med Sea	—	—
Kryptoperidinium foliaceum	AR	Med Sea	—	—
Kryptoperidinium foliaceum	RCC3045	The Channel	—	ND
Ostreopsis cf. ovata	VGO1052	Med Sea	+	+
Ostreopsis cf. siamensis	VGO978	Med Sea	-	+
Prorocentrum cf. cassubicum	VGO835	Med Sea	—	—
Prorocentrum micans	RCC3046	The Channel	—	ND

Table 1. (*Continued*).

Host Species	Strain	Ocean	*Dinomyces arenysensis* RCC3404	*Dinomyces* sp. RCC3408
Prorocentrum rathymum	VGO761	Med Sea	—	—
Protoceratium reticulatum	VGO758	Med Sea	-	-
Scrippsiella donghaienis	RCC3414	The Channel	—	ND
Scrippsiella donghaienis	RCC3415	The Channel	—	ND
Scrippsiella ramonii	VGO1053	Med Sea	—	—
Scrippsiella sp.	071005E5	Med Sea	-	+
Scrippsiella sp.	RCC2610	North Sea	+	ND
Scrippsiella trochoidea (clade STR2)	RCC1720	The Channel	+++	ND
Others				
Chaetoceros affinis	Marine Zooplankton Group ICM	Med Sea	—	—
Chaetoceros curvisetus	Marine Zooplankton Group ICM	Med Sea	—	—
Chaetoceros sp.	RCC2968	Med Sea	—	—
Guinardia striata	RCC2966	Med Sea	—	—
Thalassiosira sp.	RCC436	Med Sea	—	—
Cryptophyceae	RCC2341	Med Sea	—	—
Cryptophyceae	RCC2334	Med Sea	—	—
Cryptophyceae	RCC439	Med Sea	—	—
Emiliania huxleyi	RCC1821	Med Sea	—	—
Pavlova pinguis	RCC1538	Med Sea	—	—
Prymnesium faveolatum	VGO557	Med Sea	—	—
Tetraselmis sp.	RCC233	Med Sea	—	—
Tetraselmis sp.	RCC235	Med Sea	—	—

strains cross-infected with fungal strain RCC3404, most underwent low infection level (presence of only few infected cells in the culture), five were sensitive and one was resistant (no infection at all). Infections also occurred in a few other dinoflagellate species (Fig. 1). In *Ostreopsis cf. ovata*, host cells became granulated, and then turned black. One strain of *Scrippsiella trochoidea* (RCC1720, genetic clade STR2) was sensitive to infection, including its resting cysts (Fig. 1).

Mixed phytoplankton samples, containing *A. minutum*, collected in the Arenys de Mar harbour on the 6th of June 2013 were inoculated with strain RCC3404. No fungal infection was observed in controls (natural samples, without the addition of the fungal strain). In inoculated tests, fungal infections were observed after 4 days on *Scrippsiella* sp., *Heterocapsa* sp. and *A. minutum*. Other dinoflagellates species, including *Dinophysis sacculus*, *Gymnodinium instriatum* and *Prorocentrum micans* as well as diatoms (the most abundant taxa being *Chaetoceros* spp. and *Pseudo-nitzschia* spp.) were not infected.

Ultrastructure

General Morphology

Zoospores of strain RCC3404 are oblong (2-4 μm) to circular with a long posterior flagellum (12-13 μm) and a lipid globule (Fig. 2A). Production of a cyst wall is a necessary prerequisite to production of a germ-tube and vegetative growth. Thus, zoospores attach to the host (Fig. 2B), encyst and germinate into the host cell (Fig. 2B, C). Multiple infections are frequent. Zoospores attach to the host near the gaps between thecal plates with mucilage material ensuring adhesion to the theca (Fig. 3A). After attachment, the parasitoid resorbs its flagellum and produces a thin outer cyst wall (Fig. 3B, C). Cysts contain usual zoospore organelles and microtubules of the resorbed axoneme (Fig. 3C). Encysted cells produce a germ tube that penetrates into the host through the gap between thecal plates (Fig. 3D). Immediately beneath the host periplast, the germ tube has an apophysis-rhizoid swelling, which contains endoplasmic reticulum and mitochondria

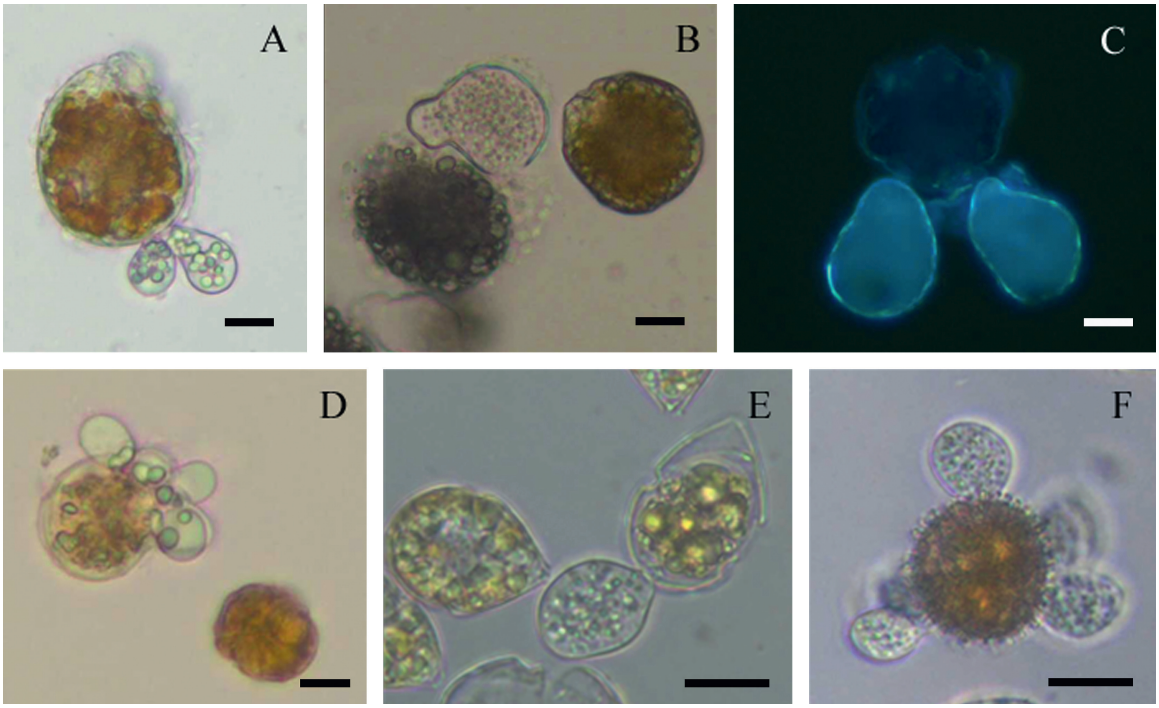

Figure 1. Infection by *Dinomyces arenysensis*. **A.** Early infection in *Ostreopsis cf. ovata*. Host cell starts to become granulated inside. **B.** Late infection in *O. cf. ovata*. Cytoplasm of the host cell became black, compare with a healthy cell in the same picture. **C.** Fungus sporangia infecting *O. cf. ovata* stained with calcofluor under epifluorescence microscopy. **D.** *Alexandrium andersonii*, polyinfection. **E.** Infection on one strain of *Scrippsiella trochoidea* vegetative cell. **F.** Infection of *S. trochoidea* resting cyst (same strain as precendently). Scale bars = 10 μm.

(Figs 2D, E; 3D, E). Branching rhizoids penetrate the host cytoplasm while the body of the fungus remains outside of the host (Fig. 2E). Rhizoids feed the parasitoid (the trophont stage) which grows for about 48 hours to become a young sporangium, which has variable shape from pyriform to spherical (Fig. 2C-E). Mature sporangia can be as large as their host, but this depends on the number of sporangia infecting the host (Fig. 2D-F). The outer sporangial wall is smooth without an operculum (Fig. 2F).

In the sporangium, the body of the parasitoid undergoes multiple divisions, producing immature zoospores (Fig. 3F). At this stage, cytokinesis occurs and flagella are observed within the cell interior (channels). Multinucleate sporangial contents are cleaved into tightly packed, nearly orthogonal immature zoospores, which become rounded when mature (Figs 3F, G; 4A). Zoospores are released into the water through a break in the sporangium wall (not shown).

Zoospore Ultrastructure

The general ultrastructural organization of zoospores is shown in Figure 5. Mature zoospores within sporangia already possess a free flagellum before release (Fig. 3G). They have a set of organelles and cellular organization typical for chytrid zoospores (Fig. 5). The nucleus lies anteriorly and slightly shifts from the centre to one (centriole) side of the cell (Fig. 4A, B, D). A vesiculated zone is present between the nucleus and the kinetid (Fig. 4C). The vesiculated zone includes many types of small vesicles, the contents of which are either translucent or electron dense (Fig. 4C). All of these vesicles are of the same size and have transitions from light to dense contents. The origin of these of so-called dense vesicles seems to be connected with vesicles produced by perinuclear cisternae. The core of ribosomes occupies the centre of the zoospores separating the nucleus from the MLC (microbody–lipid globule complex). This latter complex contains a simple flat microbody partially surrounding a large lipid globule, separating the latter from the ribosomal core (Fig. 4A-B). A prominent fenestrated cisterna (rumposome) faces to outside of the cell. A large mitochondrion with flat cristae is associated with the ribosomal core (Fig. 4D). Dense bodies are scattered throughout the cytoplasm, but often are

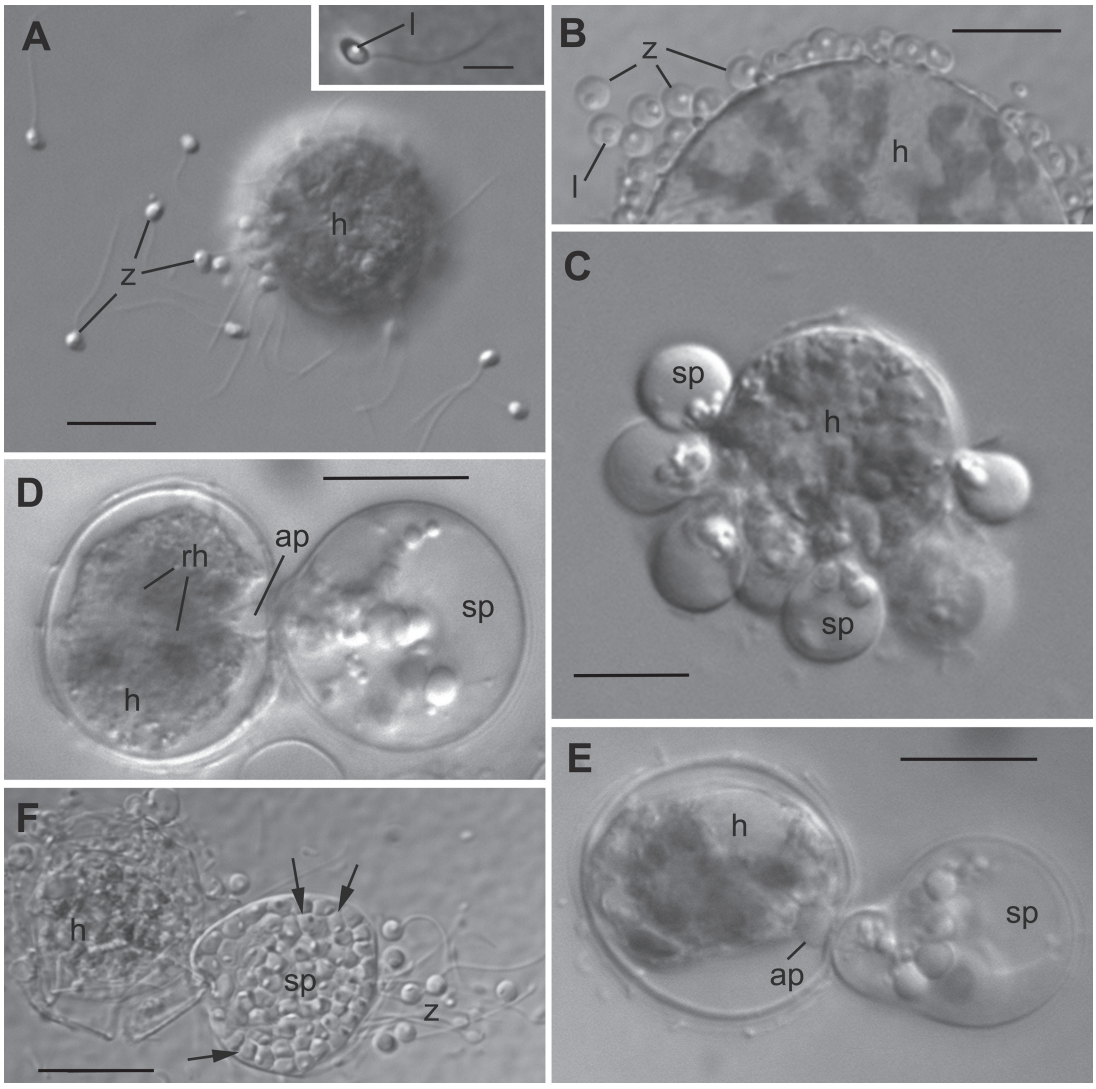

Figure 2. Light microscopy of the main stages of *Alexandrium* infection by *Dinomyces arenysensis*. **A.** Free swimming zoospores around *Alexandrium* cell. Insert. Single zoospore at higher magnification. **B.** Numerous of encysted cells at the surface of the host. Note a lipid globule in cyst. **C.** Seven young sporangia on *Alexandrium*. **D,E.** Large sporangium with apophysum. Note variations of the sporangia shape and less cytoplasm in the host in E. **F.** Nearly mature zoopores in sporangium with still intact wall, totally degraded host cell on the left. Arrows show squared immature zoospores. Scale bars: Insert in A = 5 μm, A-F = 10 μm. DIC of living material. Insert in A in phase contrast. Abbreviations: ap-apophysum, h-host, l-lipid globule, rh-rhizoids, sp-sporangium, z-zoospore.

concentrated at the anterior end or in the vicinity of the kinetid (Fig. 4A-C).

Kinetid Structure

The kinetid of the parasitoid is also typical for chytrids. It has a spiral filament in the transition zone, an inconspicuous transverse plate, developed transitional fibers (props), and characteristic hooks at the distal end of the kinetosome (Fig. 4A, E-H). Transverse consecutive sections of the root, which contains five stacked microtubules, are typical for Rhizophydiales (Fig. 3E, F). A kinetosome formed by triplets, about 400 nm long, produces a short curved spur and a root of 5 stacked microtubules (Fig. 4E-G). A spur, or kinetosome associated structure (KAS), has a peculiar structure: its proximal part is a thin dense plate that passes along the kinetosome, while its distal part curves and associates with a thick amorphous plate

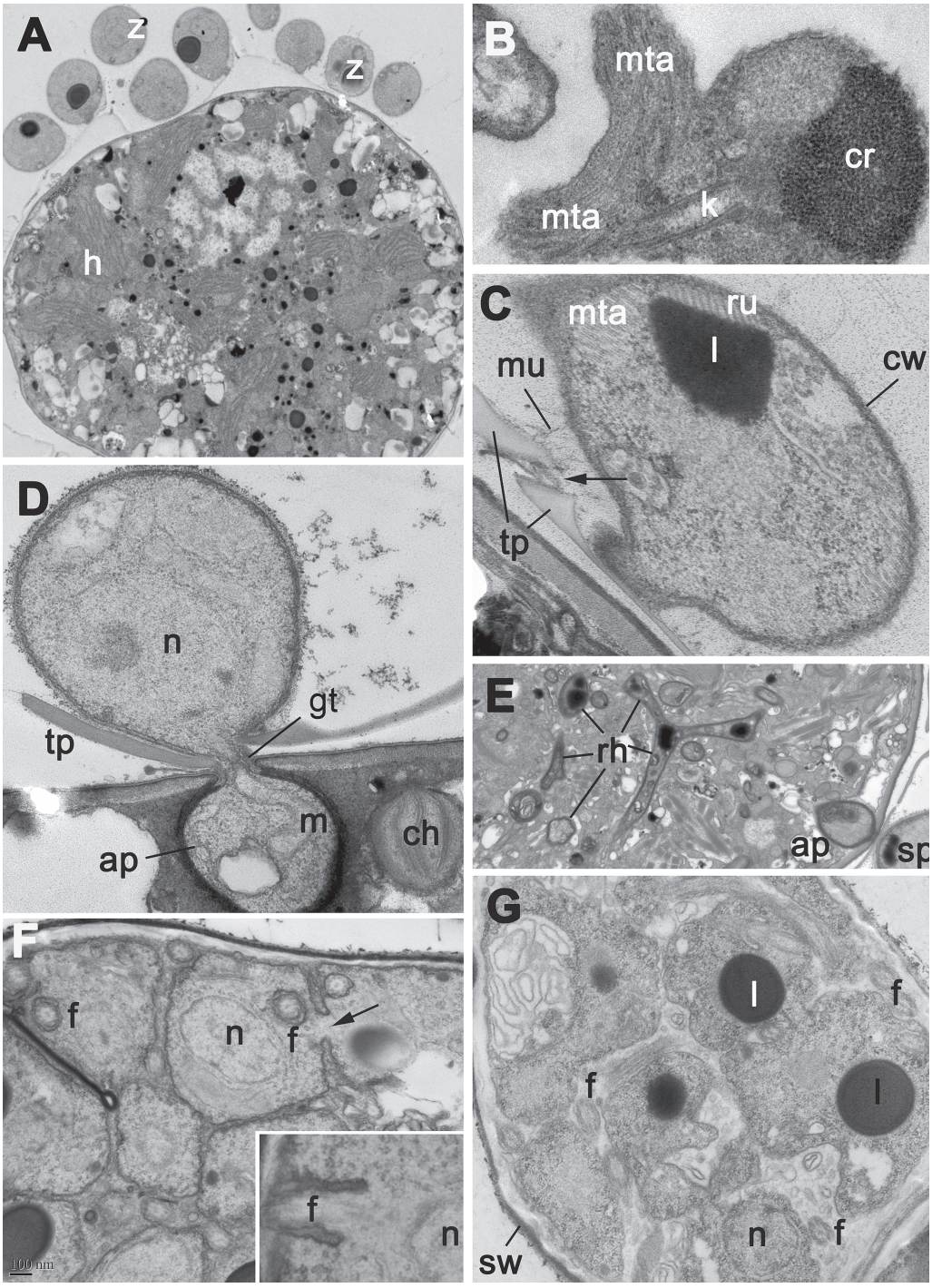

Figure 3. Ultrastructure of the main stages of *Dinomyces arenysensis* infecting *Alexandrium minutum* (Dinophyceae). **A.** Zoospores (z) attack the *Alexandrium* cell (h). **B.** Flagellum involvement in zoospore. **C.** Encysted zoospore attaches to the theca of the host. Arrow shows a gap between the thecal plates. **D.** Cyst germination. **E.** Apophysum and branched rhizoids in the host. **F.** Premature sporangium. Arrow shows a cytoplasmic bridge between divided cells. Insert: LS through newly forming flagellum in maturating zoospore. **G.** Mature sporangium, containing zoospores with formed flagella. Scale bars: F = 200 nm (insert = 100 nm). Abbreviations: ap-apophysum, ch-chloroplast, cr-core of ribosomes, cw-cyst wall, f-flagellum, gt-germ tube, h-host, k-kinetosome, l-lipid globule, m-mitochondrion, mta-microtubules of involved axoneme, mu-mucilage, n-nucleus, rh-rhizoids, ru-rumposome, sp-sporangium, sw-sporangium wall, tp-thecal plate, z-zoospore.

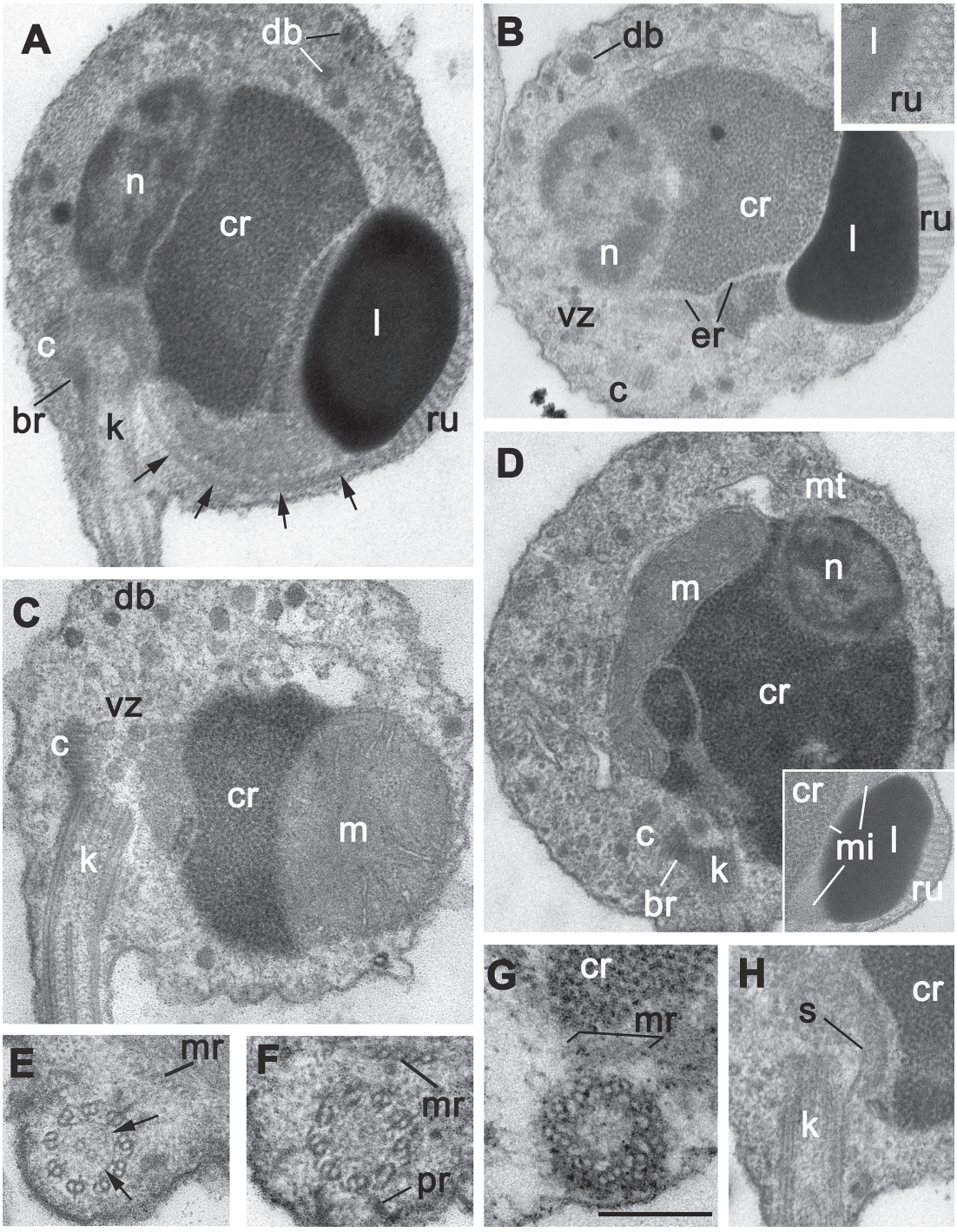

Figure 4. Zoospore ultrastructure of the chytrid *Dinomyces arenysensis*. **A-D.** Anterior-posterior LSs to show organelle disposition. Arrows on A show microtubular root. Insert in B: tangential section of rumposome. Insert on D: ribosomal aggregate (cr) and MLC: microbody (mi)–lipid globule complex (l). **E-G**. TSs of flagellar transition zone (E: arrows show coiled fiber), distal end (F) and proximal end (G) of kinetosome. **H.** LS of kinetosome. Scale bar on G: A, B, D = 500 nm, C = 400 nm, E-G = 200 nm, H = 300 nm. Abbreviations: br-bridge, c-centriole, cr-core of ribosomes, db-dense bodies, er- endoplasmic reticlum, l-lipid globule, k-kinetosome, m-mitochondrion, mi-microbody, mr-microtubular root, mt-bandle of microtubules at the cell anterior, n-nucleus, pr-prop, ru-rumposome, s-spur.

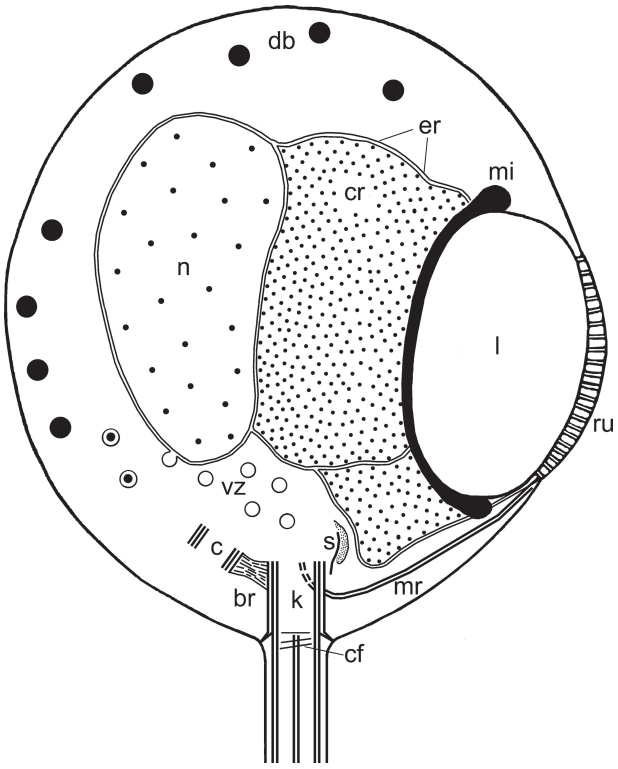

Figure 5. General scheme of *Dinomyces arenysensis* zoospore. Mitochondrion is not shown, as it masks the MLC lying at the same level. Abbreviations: br-bridge, c-centriole, cf – coiled fiber in flagellar transition zone, db-dense bodies, er-endoplasmic reticlum, k-kinetosome, l-lipid globule, mi-microbody, mr-microtubular root, n-nucleus, cr-core of ribosomes, ru-rumposome, s-spur, vz-vesiculated zone.

from the ribosomal side (Fig. 4H). This bilaminated part of the spur is rather short, ending in the vesiculated zone. Two microtubules adjacent to the thick part of the spur may be part of the microtubular root system. The microtubular root runs posterior then turns lateral and back underlying the plasma membrane, and ends at the rumposome (Fig. 4A, E-G). A short centriole (up to 100 nm) lies at an acute angle to the kinetosome, and is connected to the latter by a broad fibrillar bridge as large as the centriole (Fig. 4A, C-D). Zone of convergence was not found.

Phylogeny

28S rDNA and ITS of the 6 chytrid strains were all identical, except for RCC3408 which differed by 164 nucleotides over 1547 positions (10.6% divergence) from other strains. Concatenated analyses of 28S rDNA and ITS1-5.8S-ITS2 rDNA molecular sequences place these new chytrid strains within the order Rhizophydiales. MP and Bayesian tree topologies were congruent and adequately separated the 10 families recognized within the Rhizophydiales (Letcher et al. 2008b), expect the genus *Betamyces* that was described within the Alphamycetaceae (Letcher et al. 2012) but was closest to the Kappamycetaceae in our phylogeny (Fig. 6). Our fungal isolates do not belong to any of these well-resolved family-level clades. The closest related species are *Coralloidiomyces digitatus* (strain PL163L, Letcher et al. 2008a) and *Operculomyces laminatus* (JEL 223, Powell et al. 2011), both described as incertae sedis Rhizophydiales.

Discussion

Chytrids (zoosporic true fungi) include a diverse group of fungi that produce motile spores and require water to complete their life cycles. They grow as saprobes and/or parasites in many freshwater and soil ecosystems (Barr 2001; Powell 1993; Shearer et al. 2007; Sparrow 1960). Chytrids infecting dinoflagellates in freshwater lakes have been well known for a long time. For example, the dinoflagellate *Peridinium gatunense* was reported to be chronically infected by *Phlyctochytrium* sp. (Spizellomycetales, Alster and Zohary 2007) and *Ceratium hirundinella* infected by *Amphicypellus elegans* (Chytridiales, Ingold 1944; Canter 1961).

Only a few species of putative chytrids have been reported in brackish and marine ecosystems (Gleason et al. 2011). Among them only *Rhizophydium* spp. (Rhizophydiales) and *Olpidium* spp. (incertae sedis) have been reported to infect marine diatoms (Elbrächter and Schnepf 1998; Hanic et al. 2009). However, in both cases the identity of these parasitoids is questionable and likely includes several species/genera. Our study is the first report of a chytrid infecting marine dinoflagellates. The fact that two genotypes, that may correspond to more than one species, were isolated during the same dinoflagellate bloom highlights the fact that chytridial parasitoids have likely been largely overlooked in marine waters. These strains are well adapted to grow in marine waters with salinities up to 35 and can easily be maintained at lower salinities (for example at salinity 27). These parasitoids are thus probably euryhaline rather than strictly marine species. The marine isolates did not grow on agar under the tested experimental conditions without their hosts and are therefore probably biotrophs. However, since close relatives have been reported to be able to grow saprotrophically on agar (see Powell et al. 2011), more tests are necessary

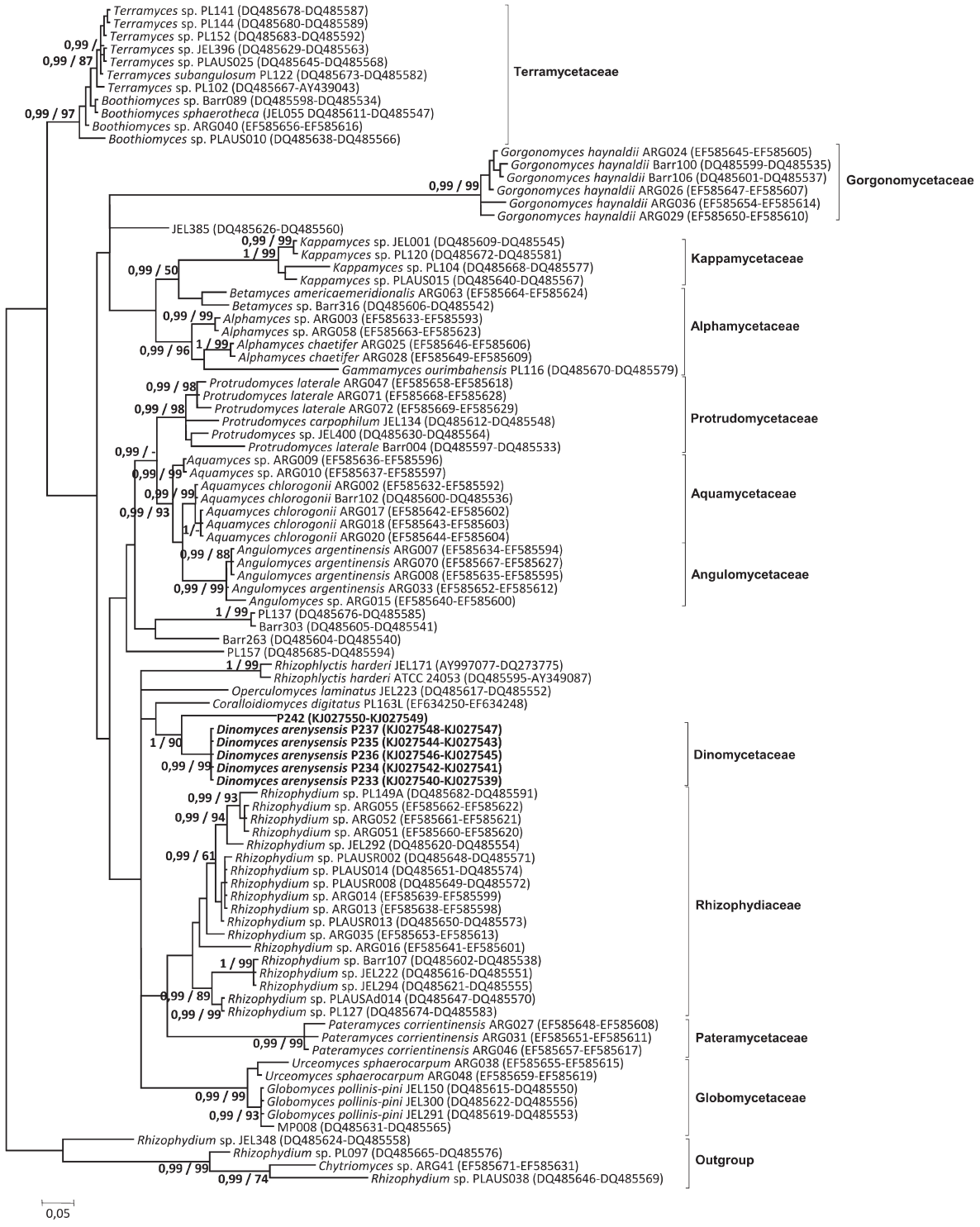

Figure 6. Bayesian inferences using a concatenated alignment (801 bp) of intergenic ribosomal spacer (ITS) and partial large subunit rRNA gene sequences (LSU) of Rhizophydiales families (Fungi, chytrid). On the nodes are indicated the bayesian posterior probabilities followed by maximum-likelihood bootstrap values.

before any conclusion can be drawn about their trophic status.

Like many other marine parasitoids, such as *Amoebophrya* spp. (Syndiniales) and *Parvilucifera* spp (Perkinsozoa), the marine chytrids we isolated only infect dinoflagellates (demonstrated both with cultures and from field samples). Within dinoflagellates, the host range of these marine chytrids are rather wide, but a particular sensitivity of *Alexandrium* species to infection seems to exist. Their rather wide host range contrasts with many freshwater species which are often considered to be host specific (Ibelings et al. 2004).

These marine isolates belong to Rhizophydiales which is the largest and most diverse order in the Chytridiomycetes (Letcher et al. 2006, 2008b). Many of Rhizophydiales are parasites of microalgae, invertebrates and other chytrids in freshwater environments (Powell 1993). Ribosomal gene sequences and zoospore ultrastructural analyses are the most important taxonomic characteristics used in the discrimination of ten currently accepted families. Zoospores of strain RCC3404 have morphological characteristics typical of the Rhizophydiales: the MLC (microbody–lipid globule complex) lies laterally with the fenestrated cisterna facing outwards, the root of five stacked microtubules connects a kinetosome to the fenestrated cisterna, the nucleus is at the opposite side of the MLC, and the ribosomal core is delimited with the endoplasmic reticulum (ER) and occupies the central part of the cell (Letcher et al. 2006, 2008b). The spur is short and curved as in many representatives of the Rhizophydiales. However, some ultrastructure aspects are relatively rare in the order: the centriole is not parallel to the kinetosome and an ER cisterna crosses the ribosomal core. The former character is extremely rare in the Rhizophydiales, having only been observed in *Kappamyces laurelensis* (Letcher and Powell 2005). The latter taxon cannot be confused with our marine strains, since it has the MLC in the centre of the cell and the kinetosome attached to the mitochondrion, and since it lacks microtubular roots (Letcher and Powell 2005; Letcher et al. 2008b). A septate ribosomal core also occurs in *Rhizophydium aestuarii* and *R. littoreum* and both are classified incertae sedis (Amon 1984). The estuarine ecotype of *R. littoreum* grows on the surface of the siphonous green algae *Bryopsis* and *Codium* (Chlorophyta), whilst the marine ecotype is a saprobe/parasite of eggs of the yellow rock crab *Cancer anthonyi* (Shields 1990). *R. aestuarii* grows on pollen, in submersed estuary sediments. The presence of a single rather large mitochondrion is also rather rare in families of this order, as zoospores normally have several small mitochondrial profiles around the ribosomal core (Letcher et al. 2006). Such a mitochondrion has nevertheless been described in *Kappamyces* and *Alphamyces*. *Pateramyces*, *Rhizophydium aestuarii*, and *R. littoreum* also have two mitochondrial profiles according to the drawings provided by Amon (1984) and Letcher et al. (2008b). All of these taxa that share rare ultrastructural features with our chytrid strains belong to different families or are incertae sedis, and are not related to our strains genetically.

By the organelle disposition, zoospores of *Pateramyces* are probably the most similar, but unlike RCC3404, the *Pateramyces* zoospore has the centriole parallel to the kinetosome, no ER septation of the ribosomal core and a spur, and has a shifted posterior MLC, which is rather small. By molecular phylogeny, the Pateramycetaceae is sister to the Rhizophydiaceae, and these together with the Globomycetaceae form a branch that is sister to the cluster RCC3403-RCC3408. Marine strains weakly branched with several incertae sedis chytrids, namely *Operculomyces laminatus* (JEL 223), *Coralloidiomyces digitatus* (PL163L) and *Rhizophlyctis harderi* (ATCC 24053) all of which live as saprobes in soil. The zoospore organization of *R. harderi* (Powell and Roychaudhary 1992) differs from that of RCC3404 by having two lipid globules, a centriole parallel to the kinetosome, several small mitochondria profiles, absence of dense bodies and of ER septation of the ribosomal core. Zoospores of *O. laminatus*, *R. harderi*, and *C. digitatus* are characterized by a laminated spur. Interestingly, isolate RCC3404 also has a solid bilaminated short spur.

In conclusion, according to the general organization of its zoospore, the taxon represented by strain RCC3404 clearly belongs to the order Rhizophydiales, but cannot be included in any of the 10 families described to date. Its phylogenetic position confirms the ultrastructural peculiarities of the strain, which we consider warrant family level separation. RCC3408 is closely related to the new species described here, but further evidence is required to assess whether this strain should be included within the same species.

Diagnosis

Dinomycetaceae fam. nov. Karpov and Guillou (Chytridiomycotina, Rhizophydiales)

MycoBank MB 807620**.** Parasitic marine chytrid. Zoospore has centriole not parallel to kinetosome with bilaminated spur, ribosomal core crossed by endoplasmic reticulum.

Dinomyces gen. nov. Karpov and Guillou
MycoBank MB 807621**.** Parasitoid of marine dinoflagellates with simple thallus with inoperculate, monocentric, epibiotic sporangium having endogenous development, with apophysum and branching rhizoidal axis. Zoospore with central ribosomal aggregation separating nucleus from Microbody-Lipid-Complex, which contains a single microbody enveloping a large lateral lipid globule with fenestrated cisterna. One mitochondrion is associated with MLC. Small dense bodies present in peripheral cytoplasm. Kinetid is adjacent the ribosomal core. Flagellar transition zone contains a spiral fibre. Centriole at angles from 80 to 45° to kinetosome; both connected to each other by a broad fibrillar bridge. Lateral root composed of 5 microtubules passes from kinetosome to fenestrated cisterna. Etymology. *Dino* – root of Dinophyta, *myces* – fungus. Type species *Dinomyces arenysensis*

Dinomyces arenysensis sp. nov. Karpov and Guillou
MycoBank MB 807622**.** GenBank numbers: KJ027541 and KJ027542. Figures 1–5. Parasitoid of dinophytes, with some preference for *Alexandrium* species. Mature inoperculate epibiotic sporangium spherical to pear-shaped 18-20 μm in diameter without papillae. Zoospores slightly elongated 2-4 μm long with single lipid globule. Zoospores released by sporangium break. Etymology. By the name of type location Arenys de Mar harbour.Type strain: RCC3404 of the Roscoff Culture Collection. Isolated on the host *Alexandrium minutum* from samples from the Arenys de Mar harbour (Mediterranean Sea, Spain) in 2012. Holotype, a fixed specimen derived from the strain RCC3404 embedded in a resin block for electron microscopy deposited in the RCC.

Methods

Strain isolation and cultivation: In Roscoff (France), infected and uninfected host cultures are maintained in F/2 medium (Marine Water Enrichment Solution, Sigma) using autoclaved natural seawater from the Penzé estuary (salinity 27) collected at least 3 months prior to use and stored in the dark. This medium was supplemented with 5% (v/v) soil extract (Starr and Zeikus 1993). A final filter sterilization step using a 0.22 μm pore size filter was performed under sterile conditions. All stock cultures and experiments were conducted at 19 °C and on a L:D cycle of 12:12 h at 80 μmol m^2 s^{-1}. In Barcelona (Spain), host cultures were maintained in 50-mL polystyrene tissue culture flasks filled with 20 mL of L1 medium (Guillard 1995) without silica. The medium was prepared with filtered (0.2-μm pore size), autoclaved natural seawater from Blanes bay, adjusting the salinity to 31 psu by the addition of sterile MilliQ water. Cultures were grown at 19±1 °C with a photoperiod of 12:12 h (light:dark) with a photon irradiance of 90 μmol photons m^{-2} s^{-1}.

The origin and details of strains used in this study are provided in Supplementary Material Table S1. Cultures of parasitoids were initiated in Roscoff. Natural samples (500 μL) were incubated in 24 well plates together with 500 μL of exponentially growing host cultures (*A. minutum*, strain RCC3018). Presence of parasitoids was regularly checked by microscopy until 15 days of incubation. Single cell-derived cultures were then established using a glass micropipette to transfer a single infected host cell to 1 mL of exponentially growing host culture. Strains were re-isolated twice using this procedure. During this period, strains were maintained by weekly transfer of 100 μL of infected host culture into 1 mL exponentially growing host culture. Single cell-derived cultures of chytrid strains were subsequently maintained in the same host strain (*A. minutum* strain RCC3018).

New strains isolated in this study have been deposited in the Roscoff Culture Collection (RCC, http://www.sb-roscoff.fr/Phyto/RCC/).

Sequencing: Zoospores of parasitoids were separated from host cells by filtration through a 5 μm cellulose acetate filter (Minisart, SARTORIUS, Germany). Cells were then harvested by centrifugation and stored at -20 °C. DNA was extracted using a modified GITC (guanidinium isothiocyanate) protocol (Chomczynski and Sacchi 2006). Cells were submerged in 50 μL of the GITC extraction buffer and incubated at 72 °C for 20 min. One volume of cold isopropanol was then added and samples stored at -20 °C overnight for DNA precipitation. Tubes were then centrifuged (20,000 g, 15 min at 4 °C) and supernatants removed. The DNA pellet was cleaned with 70% ethanol (100 μL), followed by a last centrifugation (20,000 g, 10 min). The supernatant was removed and the DNA pellet was hydrated in 20 μL of sterile distilled water and stored at -20 °C until used.

The PCR mix (15 μL final volume) contained 1 μL of the DNA extract, 330 μM of deoxynucleoside triphosphate (dNTP), 2.5 mM of $MgCl_2$, 1.25 U of GoTaq® DNA polymerase (Promega Corporation), 0.17 μM of both primers (see list Supplementary Material Table S2), 1X PCR buffer (Promega Corporation). PCR cycles, run in an automated thermocycler (GeneAmp®PCR System 9700, Applied Biosystem), were programmed to give an initial denaturating step at 95 °C for 5 min, 35 cycles of denaturating at 95 °C for 1 min, annealing at 58 °C for 45 s and extension at 72 °C for 1 min 15 s, and a final extension step at 72 °C for 7 min.

PCR products were purified (ExoSAP-IT® for PCR Product Clean-Up, USB®) and sequenced using the Big Dye Terminator Cycle Sequencing Kit version 3.0 (PE Biosystems®) on an ABI PRISM model 377 (version 3.3) automated sequencer. Sequences were edited using the BioEdit 7.0.5.3 program and complete sequences deduced from runs using both external and internal primers (Supplementary Material Table S2).

New sequences are available from the GenBank database under the following accession numbers: KJ027539-KJ027550.

Phylogenetic analyses: A concatenated alignment (801 bp) was obtained using the online version of MAFFT (http://mafft.cbrc.jp/alignment/server/, Katoh and Toh 2010) using the secondary structure of RNA (Q-INS-I option). Non-informative sites were removed using Gblocks (http://molevol.cmima.csic.es/castresana/Gblocks_server.html, Castresana 2000) using the least stringent conditions. A Bayesian phylogenetic tree was constructed with MrBayesv3.1.2 (Ronquist and Huelsenbeck 2003) using a GTR substitution model with gamma-distributed rate variation across sites (GTRCI) as suggested as the best-fit

model in JModeltest v2.1.1 (Darriba et al. 2012). Four simultaneous Monte Carlo Markov chains were run from random trees for a total of 1,000,000 generations in two parallel runs. A tree was sampled every 100 generations, and a total of 2,500 trees were discarded as 'burn-in' upon checking for stationarity by examination of log-likelihood curves over generations, and posterior probabilities were calculated in MrBayes. A consensus tree (50% majority rule) was constructed from the post-burn-in trees and posterior probabilities were calculated in MrBayes. Maximum Likelihood analyses were performed with MEGA5.1 (Tamura et al. 2011) using the GTR substitution model with gamma-distributed rate variation across sites. Bootstrap values were estimated from 1,000 replicates.

Cross infection: Exponentially growing hosts were obtained in 50 mL vented culture flasks by diluting once a week and during two consecutive weeks 5 to 10 mL into 30 to 40 mL of fresh medium. Freshly produced zoospores were obtained after 3-5 days of incubation in 6 mL well plates, by inoculating 500 μL of infected culture into 5 mL of exponentially growing *A. minutum* culture (strain RCC3018). In order to remove remains of the initial host, zoospores were filtered through a 5-μm cellulose acetate filter (Minisart, SARTORIUS, Germany). Aliquots (100 μL) were then inoculated into 1 mL of dinoflagellate host culture. For a given parasitoid strain, tests of the different hosts were undertaken on the same date and using the same initial parasitoid batch culture. Triplicates were processed at different dates. Results of cross-infections were recorded by visual inspection under light microscopy after 5, 10 and 20 days. Results were classified into four categories. Hosts were classified as either resistant (no trace of infection), moderately resistant (trace of infection but the culture resisted the infection and more than 10 cells were observed after 20 days), sensitive (100% cells killed by the parasite), or moderately sensitive (culture well infected but some cells, less than 10, persisted after 20 days).

Chytrid infection of natural samples: Live natural samples obtained from Arenys de Mar harbour (Catalan Coast), NW Mediterranean Sea, during an *Alexandrium minutum* bloom (from the end of January until April 2013), were incubated during 4 days for parasitoid detection. After this incubation period, those samples where the chytrid appeared, infection prevalence was calculated as a percentage of dinoflagellate species infected with respect to the total dinoflagellate population. Prevalence was counted using a Leica–Leitz DMIRB inverted light microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Five mL of the concentrated natural sample from the 6th of June 2013, collected in Arenys de Mar harbor, were transferred to sterile polystyrene Petri dishes (Iwaki, Japan, 32-mm diameter) and then inoculated with 1 ml of freshly produced chytrid zoospores (strains RCC3404 and RCC3408) obtained as explained above. We used 5 mL of of the concentrated natural sample without adding the chytrid as a control. Live samples were observed after 4 days under inverted light microscopy, detection of the various stages of infection confirmed cell infection.

Ultrastructure: For transmission electron microscopy (TEM), the intracellular stages of the fungal strain RCC3404 in *A. minutum* (strain RCC3018) were followed every 6 h during the first 60 h of infection. Samples were fixed for 5 hours at 4 °C in a fixative containing 4% glutaraldehyde, 0.2 M sodium cacodylate buffer (pH 7.4) and 0.25 M sucrose (final concentrations). The samples were then rinsed in a series of buffer solutions containing graded concentrations of sucrose and NaCl (from 0.25 M sucrose, 13 g/L NaCl in 0.2 M sodium cacodylate to 0.35 M NaCl in 0.2 M sodium cacodylate) and post-fixed for 1 hour at 4 °C in 1% osmium tetroxide buffered in 0.2 M sodium cacodylate and 0.33 M NaCl. Samples were then rinsed three times for 15 min using a 0.35 M NaCl and 0.2 M sodium cacodylate solution. Dehydratation was carried out in a graded alcohol series (from 30 to 100%) and samples were finally embedding in Spurr resin (Delta microscopies, France). Sections were cut using a diamond knife on a Leica ultracut UCT ultramicrotome, stained with uranyl acetate and lead citrate and viewed with a JEOL JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan). Micrographs were taken using a Gatan Orius camera.

Acknowledgements

We warmly thank Nicolas Gayet from the "Laboratoire Environnement Profond", Ifremer Brest (France), for his technical assistance during SEM analyses. We acknowledge Morgan Perennou and Gwenn Tanguy (FR2424) at the Roscoff Biological Station (Biogenouest platform) for sequencing. Funding for SAK was granted by the CNRS-INEE (France), RFBR grant No. 12-04-01486 and by the RAS Presidium program "Problems of life origin and biosphere development". Funding for CSIC was granted by the Spanish project PARAL (CTM2009-08399). Microalgal cultures were provide by the culture collection of CCVIEO Vigo, Spain and by the following researchers A. Reñé, A. Calbet, N. Sampedro and S. Anglès. FL was supported by a grant of the French Direction Générale de l'Armement (Gilles Vergnaux) and the Région Bretagne. This work was financially supported by the French ANR project PARALEX ("The sixth extinction") and the European Project MaCuMBa (FP7-KBBE-2012-6-311975).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2014.02.004.

References

Alster A, Zohary T (2007) Interactions between the bloom-forming dinoflagellate *Peridinium gatunense* and the chytrid fungus *Phlyctochytrium* sp. Hydrobiologia **578**:131–139

Amon JP (1984) *Rhizophydium littoreum*: a chytrid from siphonaceous marine algae- an ultrastructural examination. Mycologia **76**:132–139

Anderson DM, Alpermann TJ, Cembella AD, Collos Y, Masseret E, Montresor M (2012) The globally distributed genus *Alexandrium*: multifaceted roles in marine ecosystems and impacts on human health. Harmful Algae **14**:10–35

Barr DJS (2001) Chytridiomycota. In McLaughlin DJ, McLaughlin EG, Lemke PA (eds) The Mycota VII, Part A: Systematics and Evolution. Springer-Verlag, Berlin, pp 93–112

Canter HM (1961) Studies on Bristish chytrids XVIII. Further observations of species invading planktonic algae. Nova Hedwiga **3**:73–82

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol **17**:540–552

Chambouvet A, Morin P, Marie D, Guillou L (2008) Control of toxic marine dinoflagellate blooms by serial parasitic killers. Science **322**:1254–1257

Chomczynski P, Sacchi N (2006) The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on. Nature Protoc **1**:581–585

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. Nature Meth **9**, 772–772

Elbrächter M, Schnepf E (1998) Parasites of Harmful Algae. In Anderson DM, Cembella AD, Hallegraeff GM (eds) Physiological Ecology of Harmful Algal Blooms. Berlin, Heidelberg, Springer-Verlag, pp 351–369

Figueroa RI, Garcés E, Massana R, Camp J (2008) Description, host-specificity, and strain selectivity of the dinoflagellate parasite *Parvilucifera sinerae* sp. nov. (Perkinsozoa). Protist **159**:563–578

Gleason FH, Küpper FC, Amon JP, Picard K, Gachon CMM, Marano AV, Sime-Ngando T, Lilje O (2011) Zoosporic true fungi in marine ecosystems: a review. Mar Freshwater Res **62**:383–393

Guillard RRL (1995) Culture Methods. In Hallegraeff GM, Anderson DM, Cembella AD (eds) IOC Manuals and Guides: Manual on Harmful Marine Microalgae. Intergovermental Oceanographic Commission of UNESCO, Paris, France, pp 45–62

Hanic LA, Sekimoto S, Bates SS (2009) Oomycete and chytrid infections of the marine diatom *Pseudo-nitzschia pungens* (Bacillariophyceae) from Prince Edwards Island, Canada. Botany **87**:1096–1105

Ibelings BW, de Bruin A, Kagami M, Rijkeboer M, Brehm M, Van Donk E (2004) Host parasite interactions between freshwater phytoplankton and chytrid fungi (Chytridiomycota). J Phycol **40**:437–453

Ingold CT (1944) Studies of British chytrids II. New chytrids on *Ceratium* and *Peridinium*. Trans Brit Mycol Soc **27**:93–96

Jebaraj CS, Raghukumar C, Behnke A, Stoeck T (2010) Fungal diversity in oxygen-depleted regions of the Arabian Sea revealed by targeted environmental sequencing combined with cultivation. FEMS Microbiol Ecol **71**:399–412

Kagami M, de Bruin A, Ibelings BW, Van Donk E (2007) Parasitic chytrids: their effects on phytoplankton communities and food-web dynamics. Hydrobiologia **578**:113–129

Katoh K, Toh H (2010) Parallelization of the MAFFT multiple sequence alignment program. Bioinformatics **26**:1899–1900

Le Calvez T, Burgaud G, Mahé S, Barbier G, Vandenkoornhuyse P (2009) Fungal diversity in deep-sea hydrothermal ecosystems. Appl Environ Microbiol **75**:6415–6421

Lefèvre E, Bardot C, Noel C, Carrias JF, Viscogliosi E, Amblard C, Sime-Ngando T (2007) Unveiling fungal zooflagellates as members of freshwater picoeukaryotes: evidence from a molecular diversity study in a deep meromictic lake. Environ Microbiol **9**:61–71

Lefèvre E, Roussel B, Amblard C, Sime-Ngando T (2008) The molecular diversity of freshwater picoeukaryotes reveals high occurrence of putative parasitoids in the plankton. PloS ONE **3**:e2324

Lepelletier F, Karpov SA, Le Panse S, Bigeard E, Skovgaard A, Jeanthon C, Guillou L (2014) *Parvilucifera rostrata* sp. nov. (Perkinsozoa), a novel parasitoid that infects planktonic dinoflagellates. Protist **165**:31–49

Letcher PM, Powell MJ (2005) *Kappamyces*, a new genus in the Chytridiales (Chytridiomycota). Nova Hedwigia **80**:115–133

Letcher PM, Powell MJ, Viusent MC (2008a) Rediscovery of an unusual chytridiaceous fungus new to the order Rhizophydiales. Mycologia **100**:325–334

Letcher PM, Powell MJ, Churchill PF, Chambers JG (2006) Ultrastructural and molecular phylogenetic delineation of a new order, the Rhizophydiales (Chytridiomycota). Mycol Res **110**:898–915

Letcher PM, Velez CG, Schultz S, Powell MJ (2012) New taxa are delineated in Alphamycetaceae (Rhizophydiales, Chytridiomycota). Nova Hedwigia **94**:9–29

Letcher PM, Velez CG, Barrantes ME, Powell MJ, Churchill PF, Wakefield WS (2008b) Ultrastructural and molecular analyses of Rhizophydiales (Chytridiomycota) isolates from North America and Argentina. Mycol Res **112**:759–782

Massana R, Pedrós-Alió (2008) Unveiling new microbial eukaryotes in the surface ocean. Curr Opin Microbiol **11**:213–218

Miki T, Takimoto G, Kagami M (2011) Roles of parasitic fungi in aquatic food webs: a theoretical approach. Freshwater Biol **56**:1173–1183

Montagnes DJS, Chambouvet A, Guillou L, Fenton A (2008) Can microzooplankton and parasite pressure be responsible for the demise of toxic dinoflagellate blooms? Aquat Microb Ecol **53**:201–210

Orsi W, Biddle JF, Edgcomb V (2013) Deep sequencing of subseafloor eukaryotic rRNA reveals active Fungi across marine subsurface provinces. PLoS ONE **8**:e56335

Powell MJ (1993) Looking at mycology with a janus face: a glimpse at Chytridiomycetes active in the environment. Mycologia **85**:1–20

Powell MJ, Roychaudhary S (1992) Ultrastructural organization of *Rhizophlyctis harderi* zoospores and redefinition of the type I microbody-lipid globule complex. Can J Bot **70**:750–761

Powell MJ, Letcher PM, Longcore JE (2011) *Operculomyces* is a new genus in the order Rhizophydiales. Mycologia **103**:854–862

Richard TA, Jones MDM, Leonard G, Bass D (2012) Marine fungi: their ecology and molecular diversity. Annu Rev Mar Sci **4**:495–522

Rasconi S, Niquil N, Sime-Ngando T (2012) Phytoplankton chytridiomycosis: community structure and infectivity of fungal parasites in aquatic ecosystems. Environ Microbiol **14**:2151–2170

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19**:1572–1574

Shearer CA, Descals E, Kohlmeyer B, Kohlmeyer J, Marvanová L, Padgett D, Porter D, Raja HA, Schmit JP, Thorton HA, Voglymayr H (2007) Fungal biodiversity in aquatic habitats. Biodivers Conserv **16**:49–67

Shields JD (1990) *Rhizophydium littoreum* on the eggs of *Cancer anthonyi*: parasite of saprobe? Biol Bull **179**:201–206

Sime-Ngando T (2012) Phytoplankton chytridiomycosis: fungal parasites of phytoplankton and their imprints on the food web dynamics. Frontiers Microbiol **3**:1–13

Sparrow FK Jr (1960) Aquatic Phycomycetes. 2nd ed. University of Michigan Press, Ann Arbor MI, 1187 p

Starr RC, Zeikus J (1993) The Culture Collection of Algae at the University of Texas at Austin. J Phycol **29**:1–106

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol **28**:2731–2739