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Published in:
European Journal of Phycology

Publication date:
2017

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Download date: 03. Aug. 2019
Pathogens of brown algae: culture studies of Anisolpidium ectocarpii and A. rosenvingei reveal that the Anisolpidiales are uniflagellated oomycetes.

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Running title: Anisolpidium is a uniciliate oomycete genus
Abstract

Using laboratory cultures, we have documented the life cycle of *Anisolpidium ectocarpii*, a pathogen of *Ectocarpus* and other filamentous brown algae, and presented preliminary observations on *Anisolpidium rosenvingei*, a pathogen of *Pylaiella littoralis*. Consistent with earlier reports, the zoospores of both species have a single anterior flagellum, which justified the placement of *Anisolpidium* amongst the Hyphochytriales (Hyphochytridiomycota). We have also shown that *A. ectocarpii* can complete its infection cycle in a broad selection of species from various brown algal orders, whereas *A. rosenvingei* seemingly exhibits a strict specificity for unilocular sporangia of *P. littoralis*. Unexpectedly, nuclear (18S rRNA) and mitochondrial (*cox1*, *cox2*) markers regroup *A. ectocarpii* and *A. rosenvingei*, into a hitherto unrecognised monophyletic clade within the oomycetes (Oomycota), most closely related to the Olpidiopsidales. The *Anisolpidium* genus is therefore entirely distinct from the Hyphochytridiomycota and represents the first confirmed instance of an anteriorly uniciliate oomycete. Finally, we suggest that a valid morphological criterion to separate true hyphochytrids from oomycetes is the timing of zoospore cleavage. Given the evidence, we propose to transfer the Anisolpidiales from the Hyphochytriales to the Oomycetes.

Keywords:

*cox2, cox1, 18S rRNA, Ectocarpus, Pylaiella, host range, Hyphochytriales, Anisolpidiales*
Algae are susceptible to disease outbreaks both in natural and cultivated populations, hence their pathogens are increasingly recognised as key ecosystem drivers (Gachon et al., 2010). Diseases also cause significant economic damage, the severity of which is worsening with the development of seaweed aquaculture worldwide (Kim et al., 2014; Loureiro et al., 2015). Despite renewed interest, the biodiversity and physiology of intracellular eukaryotic pathogens of seaweeds remain poorly known. These pathogens span several protistan groups that are themselves little studied (oomycetes, hyphochytrids, chytrids and plasmodiophorids; see Gachon et al., 2010; Neuhauser et al., 2011). In addition, they tend to be inconspicuous in the field, their diagnosis requires specialist training and pathogen-host systems are difficult to bring into stable laboratory culture. Furthermore, many reports of these pathogens in the literature date back many decades and generally consist of rather limited descriptions of field-collected material.

The genus *Anisolpidium* was originally described by Karling (1943) and tentatively regrouped with *Canteromyces stigeoclonii*, a pathogen of green freshwater algae within the class *Anisolpidiaceae* (Sparrow, 1960; Adl et al., 2012). Soil-dwelling *Anisolpidium saprobium* (Karling) isolated from pollen grains and freshwater *Anisolpidium elongatum* (Karling) have been reassigned to *Hyphochytrium saprobium* and *Hyphochytrium elongatum* (Dick, 2001).

In total, six marine species, all pathogens of filamentous brown algae, are currently recognised in the genus *Anisolpidium* (reviewed in Marano et al., 2012): *A. ectocarpii* Karling on *Ectocarpus* and *Hincksia* spp.; *A. minutum* (H. E. Petersen) M. W. Dick on *Chorda filum* (Linnaeus) C. Agardh; *A. sphacellarum* (Kny) Karling on *Sphacelaria* spp., *Chaetopteris plumosa* (Lyngbye) Kützing, and *Cladophyllum spongiosus* (Hudson) C. Agardh; *A. jokljanum* (M.W. Dick) on *Hincksia granulosa*; *A. olpidium* (H. E. Petersen) M. W. Dick on *E. siliculosus*; and *A. rosenvingei* (H. E. Petersen) Karling on *Pylaiella littoralis*. *Anisolpidium* is...
a widespread, perhaps even cosmopolitan genus reported in Japan, the North American East
coast and throughout European waters (reviewed in Marano et al., 2012). In an
epidemiological study conducted over several years, Küpper & Müller (1999) found a strong
seasonal correlation between the prevalence of the pathogen *A. rosenvingei* in sporangia of
the filamentous brown alga *P. littoralis* and the breakdown of the algal population in late
autumn.

Most *Anisolpidium* species have been described using field-collected material and the limited
available information suggests that they share a common infection cycle. Species delimitation
is mostly based on the respective algal host, and ill-defined morphological characters such as
the size of the plasmodia inside their host. The Anisolpidiaceae as defined by Karling (1943)
have tentatively been placed by Dick (2001) within the order Hyphochytriales
(Hyphochytridiomycota or hyphochytrids), an order defined on the basis of a single anteriorly
directed flagellum, as opposed to the chytrids that have a posterior flagellum. Besides the
Anisolpidiaceae, the two other families currently recognised in the Hyphochytriales (Sparrow,
1960) are the freshwater, soil-dwelling or facultative parasites Hyphochytridiomycetaceae and
Rhizidiomycetaceae. Sequencing of the 18S rRNA gene showed that these two families were
monophyletic and they were proposed to be the closest relatives to the oomycetes (Hausner et
al., 2000; Beakes et al., 2014). To date, no molecular information is available for any
representative of the Anisolpidiaceae. In the most recent classification of eukaryotes (Adl et
al., 2012), the Hyphochytriales are tentatively included among the typically biflagellated
stramenopiles, although this speculative placement has been a matter of debate for many years
(Beakes et al., 2012).

Here, we report the establishment of stable laboratory cultures of *A. ectocarpii* and *A.*
*rosenvingei*. Since these organisms are only known from historic drawings, we documented
the life history of *A. ectocarpii* using a combination of bright field and epifluorescence
observations. In addition, we evaluated the host specificity of *A. ectocarpii*. We also assessed the phylogenetic position of the species using three molecular markers, all of which reveal that *Anisolpidium* falls within the oomycetes. Thus we note that oomycetes and hyphochytrids cannot be distinguished by the presence of an anteriorly uniflagellate zoospore; instead we suggest that the timing of zoospore cleavage might be a better criterion to separate the two classes.
Material and methods

Biological material

Specimens of *Ectocarpus* sp. infected with *Anisolpidium ectocarpii* were collected in South Chile (Table 1). In the laboratory, *A. ectocarpii* was transfected into a male *Ectocarpus*. gametophyte (strain CCAP 1310/56). Successive cleaning steps removed all eukaryotic contaminants. A culture of the original *Ectocarpus* host was established in parallel (strain Ec QU 67-23). Finally, the parasite was re-transfected into this original host, leading to the host-parasite system strain *Anisolpidium / Ectocarpus* QU 67-5. The cultures were maintained at 10 to 12°C under daylight-type fluorescent light at 10 µmol photons.m⁻².s⁻¹, with a 12h:12h photoperiod. Culture medium was sterilized sea water containing 20 mL L⁻¹ Provasoli enrichment. Parallel attempts with *A. ectocarpii* found in *Hincksia sandriana* (Zanardini) P.C. Silva failed, and only preserved samples for DNA sequencing remained (strain *A. ectocarpii* PM 76-6).

*A. rosenvingei* was encountered at two occasions in the tidal flats near Roscoff, France, in *P. littoralis* growing on *Fucus serratus* Linnaeus receptacles. Culture conditions for this pathogen were as described above but because of the high host-specificity of *A. rosenvingei* towards unilocular sporangia of *P. littoralis*, the following modifications were necessary. A unialgal clonal culture of healthy *P. littoralis* from Roscoff was established as a routine source for unilocular sporangia. Repeated co-incubations were then undertaken of this new host with *A. rosenvingei* plasmodia in detached unilocular sporangia in order to remove all eukaryotic contaminants, whilst maintaining the pathogen. From this point onward, two different techniques allowed the survival of clean host-pathogen cultures in the laboratory: 1) a pulse system, by successive co-incubation with a new host culture in one to two week
intervals, and 2) a continuous system by selecting apical parts of fertile *P. littoralis* filaments with infected unilocular sporangia in one to two week intervals for continuous growth.

In contrast to *A. rosenvingei*, the broad host spectrum of *A. ectocarpii* tolerated a convenient long term culture system. Transfection to a female gametophyte clone of *Macrocystis pyrifera* (strain CCAP 1323/1) resulted in a stable host-pathogen co-culture, which only needed supply of fresh host biomass in one to two monthly intervals (strain *A. ectocarpii* QU 467-2).

Evaluation of host compatibility followed the techniques described by Müller *et al.* (1999). *M. pyrifera* gametophytes infected with *A. ectocarpii* were co-incubated with a culture of the potential host in 60 mm plastic Petri dishes. Interactions were deemed susceptible if mature and discharged *A. ectocarpii* plasmodia with exit tubes were detected in cells of the new host. The earliest infection symptoms appeared after five days, and were subsequently recorded with digital photography. For additional documentation, permanent mounts of samples with acetocarmine staining were prepared and mounted in Karo® syrup. A selection of microscope slides have been deposited in the Natural History Museum of London (BM), under the accession numbers BM000701847-BM000701849.

**Histological staining**

Beta 1-3 and 1-4 glucans were stained with a commercially available Calcofluor white solution containing Blue Evans as a counterstain. Samples were incubated for 5 to 10 min in a 0.01 mg mL$^{-1}$ calcofluor solution, rinsed in sterile seawater and observed with a DAPI filter (excitation: 365 nm, beam splitter: 395 nm, emission: long pass 420 nm). Alternatively, the non-specific cellulose stain Congo Red was used, at a final concentration of 0.1 mg mL$^{-1}$, followed by observation under differential interference contrast microscopy. Aniline blue staining was performed as in Tsirigoti *et al.* (2014). In order to stain *Anisolpidium* nuclei, samples were first fixed 1 h on ice with Microtubule Stabilizing Buffer (Katsaros & Galatis,
1992), followed by several washes in methanol until the algal filaments were discoloured. The samples were then transferred into sterile seawater containing a $10^{-4}$ dilution of a commercial Sybr Green solution (Sigma Aldrich), sometimes also containing aniline blue. Samples were mounted in SlowFade (Life Technologies) and observed on a Zeiss LSM 510 epifluorescence microscope coupled to an Axiocam HR digital camera, using a FITC filter (excitation BP 450-490 nm; beam splitter 510 nm; emission LP 515 nm).

**Molecular taxonomy**

DNA was extracted using a CTAB and phenol-chloroform method or Qiagen DNeasy minikit as detailed in Gachon *et al.* (2009). The cox2 marker was amplified using the cox2HF - cox2HR primer pair as described in (Hudspeth *et al.*, 2000). The cox1 marker was amplified using Fm85mod and CoxleUp (Robideau *et al.*, 2011). The 18S rRNA marker was amplified using the new primers F139 (5’ AGTCTATTTGATAGTACCTTACTAC 3’) and R1233 (5’ CAATCCTTACTATGTCTGG 3’) with an annealing temperature of 45°C. After Sanger sequencing, the resulting chromatograms were proofread manually in Geneious V6.1.8 (Kearse *et al.*, 2012) or BioEdit (Hall, 1999) and virtually translated (cox1/cox2).

Stramenopile-wide alignments were used to produce unrooted trees to ascertain the initial branching position of *A. ectocarpii* and *A. rosenvingei* before final selection of suitable in and outgroup species (Supplementary Info 1). Phylogenetic analyses were performed in MEGA v. 7 (Kumar *et al.*, 2016). Model tests were performed on the alignments in order to find the best substitution models for subsequent maximum likelihood (ML) analysis. For the 18S rRNA, Tamura-3-parameter was used (Tamura, 1992) with a discrete Gamma distribution to model evolutionary rate differences among sites. The rate variation model allowed for some sites to be evolutionarily invariable. For cox1 and cox2 the La Gascuel model (2008) with discrete gamma distribution was used the general reversible mitochondrial model (Adachi & Hasegawa, 1996) with discrete gamma distribution and for cox2 the La Gascuel model (2008) with discrete gamma distribution was used. Additionally maximum parsimony analysis was
performed on all three datasets. Bootstrap re-sampling was set to 100 replicates. Sequence data of all *Anisolpidium* strains were deposited in Genbank (see Table 1 for accession numbers) and the alignments are given in Suppl. Info. 2-4.
Results

Life history and infection cycle

Field-collected material was morphologically identified as *Anisolpidium ectocarpii* Karling (Figs 1, 2) and *A. rosenvingei* (not shown). Subsequent observations were based on laboratory cultures of *A. ectocarpii* in its original host *Ectocarpus* sp. (strain *A. ectocarpii* QU 67-5) or in female gametophytes of *Macrocystis pyrifera* (strain *A. ectocarpii* QU 467-2). Both cultures contained naturally-occurring commensal bacteria but were devoid of eukaryotic contaminants.

The release of infectious zoospores from *A. ectocarpii* sporangia could be triggered by transferring the culture from 10°C into fresh medium at room temperature. Within a few minutes, zoospores started to move inside the sporangium and escape in files through the exit tubes. The swimming spores were globular bodies of 2.5 µm diameter, and became slightly pyriform after a few minutes (Figs 3, 4). Nile Red staining indicated that they contain lipid globules (not shown). They had one anterior flagellum, which pulled the spore forward by rapid meandering oscillations (Suppl. Information 5). The liberated zoospores remained active for about 30 minutes at room temperature before encysting at the surface of a new host cell (Fig. 5). *A. ectocarpii* then penetrated into the algal cell and developed as an intramatrical, unwalled plasmodium with a conspicuously granulose cytosol and a subspherical shape (Fig. 6). The algal cell components disaggregated rapidly and cell remnants were already visible at this stage, with a typical chestnut brown colour. The growing plasmodium then differentiated a thin cell wall, thus becoming spherical, whilst its cytoplasm became more uniformly granular (Fig. 7). The pathogen progressively filled the host cell, closely moulding its shape (Fig. 8) and differentiating one or several protruding exit tube(s) (Fig. 9); the final plasmodium size depended on the cell type and the number of infections per host cell (detailed below). For example, spore initials in *Ectocarpus* plurilocular sporangia were
commonly infected, and led to distinctively smaller pathogen sporangia compared to when somatic cells were infected (Fig. 10). Throughout its growth, the pathogenic thallus was monocentric and did not propagate to neighbouring algal cells. Likewise, *A. rosenvingei* developed intracellularly, with a strict tissue specificity for unilocular sporangia of *P. littoralis* (Figs 11, 12). Spore release typically occurred within 6-10 days following infection (Fig. 13) and monoflagellation of the spores was observed. Virus-infected *P. littoralis* could also undergo lysogeny, leading to the production of viral particles in the algal unilocular sporangia. Though the association between both pathogens was not obligate, *A. rosenvingei* infection of virus-producing unilocular sporangia was observed both in field material (Fig. 14) and in culture (not shown). Finally, and similar to *A. ectocarpii*, co-infection of the same host cell by *A. rosenvingei* was also frequent (Fig. 15).

Young unwalled *A. ectocarpii* plasmodia did not react to any of the cell wall stains examined, (Fig. 16). Typical of holocarpic pathogens, the developing thallus fully converted into a sporangium with exit tube(s) that ruptured the wall of the dead host cell at full maturity. The differentiating exit tubes were labelled by Congo red (Fig. 17); the entire cell wall of mature sporangia reacted to this stain (Fig. 18), whilst spores gave a weak and inconsistent labelling. However, these structures remained unstained when methylene blue was used (not shown). The thin cell wall progressively built up by the early spherical unwalled thallus was most easily visualised using the β1→3 and β1→4 glucan stain calcofluor white (Fig. 19). Mature exit tubes were most intensely labelled (Fig. 20), strongly suggesting that the pathogen sporangium forces its way out of the host cell. Calcofluor also stained the encysted *A. ectocarpii* zoospores, revealing thin infectious germ tubes (ca. 1 µm in length) that penetrate into the algal cell perpendicularly to its cell wall (Fig. 21). Therefore, we recommend calcofluor as a rapid and convenient method to locate pathogen structures, in particular the otherwise inconspicuous spores encysted at the surface of the algal cells. In addition, localised modifications of the algal cell wall were visible in the immediate vicinity of encysted *A.
ectocarpii spores both with calcofluor (Fig. 22, arrowhead) and aniline blue (Fig. 23). A. ectocarpii structures were only weakly stained with aniline blue (β1→3 glucans), with again the most intense labelling at the basis of exit tubes (Fig. 24).

The dynamics of nuclear division during vegetative growth was followed using Sybr-Green (Figs 25-40). Consistent with the rapid degeneration of the host cell mentioned above (Fig. 6, also visible in Fig. 16), the nucleus of the algal host cell was rarely seen past the 2-nucleus plasmodium stage (Fig. 26). Throughout the plasmodium development, nuclei became smaller and more compact (see the progression between Figs 25-34). All nuclei within the syncytium were always at the same stage, and divisions were synchronous (Figs 35-38). The observed trails of fluorescence during the anaphase suggest closed mitosis, a frequent trait of syncytial organisms (Fig. 37).

Multiple infections of the same host cell were commonly observed, leading to the successful, yet not necessarily synchronous, differentiation of one to five sporangia per host cell (see for example one empty and one dehiscing sporangium in the same host cell on Fig. 33). In extreme cases however, over fifteen plasmodia were observed in a single host cell (Figs 31-32). The number of spores produced per A. ectocarpii sporangium varied from a handful to several hundred for the biggest observed sporangia. Finally, it was repeatedly noted that A. ectocarpii spores preferentially encyst on the filament cells close to the apical tips of Ectocarpus filaments and M. pyrifera gametophytes, although no quantification of this phenomenon was attempted (Figs 39-40).

Host range of A. ectocarpii and A. rosenvingei.

Co-incubation experiments with representative brown algal cultures spanning 28 species across ten brown algal orders revealed an unexpectedly broad host spectrum for A. ectocarpii (Table 2). Algal strains were deemed susceptible if they allowed completion of the full life
cycle of the pathogen, i.e. penetration of spores, development of plasmodia, and spore release through exit tubes (Figs 41-59). Susceptibility was the most frequent outcome of the experiments, across all orders tested. However, we encountered several algal strains that departed from this pattern, especially among uniseriate filamentous species. There was a complete absence of any visible interaction for *Botrytella uvaeformis*, *Leptonematella fasciculata*, *Feldmannia irregularis*, *Pylaiella littoralis*, *Cutleria multifida*, *Microzonia velutina*, *Sphacelaria* sp. and *Sphacelaria rigidula*, although another *Sphacelaria* isolate was successfully infected.

In sharp contrast to *A. ectocarpii* both *A. rosenvingei* isolates tested infected exclusively unilocular sporangia initials of *P. littoralis*. This strict host and cell specificity required intense observations of unilocular sporangia of *Pylaiella* at the initial stage of the study. Among field samples from Perharidy, in addition to *A. rosenvingei*, we found evidence for a DNA virus. Our material agreed in all light-microscopic details with the symptoms known for the PlitV-1 virus infecting *P. washingtoniensis* C.C. Jao in Alaska, as described in detail by Maier et al. (1998). The virus genome is integrated into the nuclear genome of the host and systemically present in each somatic host cell. It becomes virulent exclusively in unilocular sporangia, and causes their cytosol to appear translucent and unstructured. In field samples and by infection experiments we could verify that virion formation and plasmodium development of *A. rosenvingei* could occur simultaneously in the cytoplasm of a unilocular sporangium initial on a virus-infected *Pylaiella littoralis* specimen. Since this aspect extends beyond the scope of the present study, we refer to Fig. 14 as a representative field sample. A series of permanent light microscopic mounts documenting the Perharidy *Pylaiella* virus have been deposited in the Paris Natural History Museum (D.G. Müller 2015, accession numbers PC0723465-PC0723473 and PC0723476).
Anisolpidium ectocarpii and A. rosenvingei belong to the oomycetes

Partial sequence information for the 18S rRNA and cox1 genes was successfully obtained for
A. ectocarpii CCAP 4001/1, A. rosenvingei Ros and A. rosenvingei Per; partial cox2
sequences were obtained for A. ectocarpii CCAP 4001/1, A. ectocarpii PM 76-6 and A.
rosenvingei Ros. Our phylogenetic analyses of all three markers (Figs 60Q62) placed A.
ectocarpii and A. rosenvingei within the oomycetes. The assumed placement of Anisolpidium
within the hyphochytrids was rejected, as seen from the phylogenetic analysis of 18S rRNA
and cox2. All Anisolpidium strains formed a monophyletic group with the three markers
investigated. Despite limited bootstrap support, both the 18S rRNA and cox1 marker placed
the three strains of Anisolpidium on a long branch, sister to the Olpidiopsidales. The cox2
marker lacked sufficient resolution to decipher the relationship between the Anisolpidium and
Olpidiopsis genera, but the tree topology retrieved was also consistent with the above
interpretation. Additionally, four environmental 18S sequences most closely related to
Anisolpidium were retrieved from Genbank (Table 3). All originate from coastal marine
habitats (pelagic or sediment), with a geographic coverage encompassing California,
Greenland and the Mediterranean Sea. Two of those sequences fell within the same clade as
the three Anisolpidium strains whereas the other two formed a sister clade (Suppl. Info. 6).

Discussion

Our observations of A. ectocarpii and A. rosenvingei in culture fully matched the meticulous
description of the type material given by Karling (1943). Novel aspects were the localised
thickenings of the algal cell wall at the pathogen penetration site, which are consistent with a
localised defence reaction similar to the one recently described in brown algal cells infected
by the oomycete Eurychasma dicksonii (Tsirigoti et al., 2015). As originally reported by
Karling, mitotic divisions were synchronous. However, we found that nuclear counts often
deviated from the exponential progression that would be expected if nuclei numbers doubled at each division. Extensive observations, especially in multiple-infected host cells, suggested that such non-canonical nuclei numbers were accounted for by the degeneration of some parasite nuclei, with no evidence of any thallus fusion as recently reported in *Olpidiopsis pyropiae* (Klochkova et al., 2015). Depending on resource availability from the host cell and the resulting plasmodium size, this phenomenon would provide a possible mechanism to regulate the number of spores produced per *A. ectocarpii* sporangium.

Anteriorly monoflagellated zoospores, hitherto believed to be a hallmark of hyphochytrids, were clearly detected in both *A. ectocarpii* and *A. rosenvingei*. Unexpectedly, all three nuclear and mitochondrial markers used here point to *A. ectocarpii* and *A. rosenvingei* defining a novel monophyletic clade within the oomycetes. We therefore propose that the order Anisolpidiales, represented by the marine members of the genus *Anisolpidium*, should be transferred into the oomycetes (subphylum Oomycota) and given equal status to the Olpidiopsidales and Haliphthorales (as defined by Beakes et al., 2014). The former order, erected by Dick (2001), regroups obligate, initially plasmodial, holocarpic endoparasites of plants, algae and oomycetes, of which only marine species have been characterised molecularly. The latter order contains three marine genera of molluscan and crustacean parasites, that produce polycentric hyphal structures and can be cultivated saprophytically on agar. The status of the freshwater *Canteriomyces* and soil-dwelling *Anisolpidium saprobium* Karling remains unresolved,

It is noteworthy that all available molecular data derived from both our cultures and environmental samples fully agree with the concept of Anisolpidiales as erected by M.W. Dick (2001), which solely groups marine, obligate endoparasites of marine algae.

Despite being unexpected, the closeness of the relationship between *Anisolpidium* and *Olpidiopsis* agrees with the observation of Johnson (1957), who already highlighted the
morphological similarities between their sporangia. Another similarity between both genera is
that zoospores are cleaved within the sporangia before the exit tubes are opened, in contrast to
terrestrial hyphochytrids, many of which release a naked, partially differentiated, protoplasm
into a restraining vesicle or directly into the environment (e.g. *Hyphochytrium catenoides*
(Karling, 1939) and *Rhizidiomyces apophysatus* (Karling, 1943). Additionally, the central
vacuole witnessed in *Anisolpidium* species (Karling, 1943), might be similar to that found
recently in *Olpidiopsis bostrychiae* S. Sekimoto, T. A. Klochkova, J. A. West, G.W. Beakes
& D. Honda (Sekimoto *et al.*, 2009) and *Olpidiopsis feldmannii* (Fletcher *et al.*, 2015), though
it is absent in *Olpidiopsis porphyrae* (Sekimoto *et al.*, 2008) and *Olpidiopsis pyropiae*
(Klochkova *et al.*, 2015). Finally, the genus *Olpidiopsis* was originally defined by the
occurrence of sexual union between two contiguous thalli originating from different zoosporic
infections (see details in Dick, 2001), which echoes the intracellular sexual conjugation of
young uninucleate parasitic thalli described in *A. ectocarpii* (Johnson, 1957; Karling, 1977).
Unfortunately, we were unable to observe such conjugation events in our cultures, and our
observations of *A. ectocarpii* nuclear divisions (Fig. 25-40) did not suggest any non-mitotic
division pattern. Therefore, the sexual potential of our *Anisolpidium* cultures remains
unresolved; likewise, sexuality in marine *Olpidiopsis* species remains to be described.
However, a distinct possibility is that as data become available, intracellular sexual
conjugation in host cells might appear as a defining feature (synapomorphy) of the
Olpidiopsidales and Anisolpidiales.

To the best of our knowledge this is the first report of an anteriorly uniflagellate oomycete,
yet flagellae were lost several times amongst Oomycota in obligate pathogenic clades such as
*Hyaloperonospora*, *Bremia*, and most *Peronospora* spp, as well as *Geolegnia* and some
*Haptoglossa* and *Myzocytiospis*. Further variation is reported, for example the presence of two
smooth flagellae in *Haptoglossa dickii* (Beakes & Glockling, 1998), of two distinct
biflagellate patterns in *Olpidiopsis* (Whittick, 1972; West *et al.*, 2006; Sekimoto *et al.*, 2008)
and of differently flagellated zoospores at different life stages (e.g. *Saprolegnia* primary spores vs secondary spores), suggesting that there is ample scope for variation in flagellation amongst oomycetes. Among the heterokonts (Heterokonta), loss or strong reduction of the posterior smooth flagellum is well documented in *Pelagomonas, Dictyota* (Kawai, 1992) and in diatoms. In the latter group, the male gamete of all species investigated thus far has a single tinselled flagellum. Hence, the loss of one flagellum in *Anisolpidium* represents the third independent such event reported in the heterokonts. However, electron microscopy investigation on *Anisolpidium* spores would be necessary in order to investigate a loss or potential vestiges of the second flagellum.

As can be seen in Table 2, *A. ectocarpii* has a broad host spectrum, including several brown algal orders with heteromorphic life histories, where both the micro- and macroscopic generations are successfully attacked. Similarly, Müller *et al.* (1999) found that *Chytridium polysiphoniae*, only described as infecting sporangia of *Pylaiella littoralis* in the field, actually could infect 29 brown algal species across different brown algal orders. *Eurychasma dicksonii* (Oomycota) has a similarly broad host range in marine Phaeophyceae (Müller *et al.*, 1999), as well as the Plasmodiophorid *Maullinia ectocarpii* I. Maier, E. R. Parodi, R. Westermeier et D. G. Müller (Maier *et al.*, 2000). Over the years, our studies have revealed that *M. ectocarpii, E. dicksonii* and *A. ectocarpii* all infect *Macrocystis* gametophytes in laboratory cultures, begging to question as to what their impact might be on natural kelp beds. The contrasting host spectra exhibited by our *A. rosenvingei* and *A. ectocarpii* isolates provide further evidence against the widely-held view that all intracellular biotrophic pathogens are necessarily highly host specific. Furthermore, the broad host range observed for *A. ectocarpii* as well as other marine oomycetes and plasmodiophorids correlates with a unique ability to perform cross-kingdom host shifts (extensively discussed in Beakes *et al.*, 2012; Neuhauser *et al.*, 2014).
Altogether, our host range data also highlight the limitations of current species descriptions within the genus *Anisolpidium*, which are almost exclusively based on morphological characters that overlap between species, and observed host specificity in the field. It appears necessary to reassess diversity and species delimitation with modern tools. For example, the definition of *A. sphacellarum* as a pathogen of *Sphacelaria* is questioned by the fact that our *A. ectocarpii* culture can infect at least one *Sphacelaria* isolate. However, the infection structures that we observed were notably smaller than those reported in *Sphacelaria* spp. in the field (Strittmatter et al., 2013). It is also noteworthy that the observed preferential infection of filament tips and apical cells of certain brown algal species by *A. ectocarpii*, for example apical cells of several *Halopteris*, resonates with some other pathogens, such as *Olpidiopsis porphyrae*, that recognise different lectins (Klochkova et al., 2012).

The biogeography and epidemiology of *Anisolpidium* remain very imperfectly known. This is expected, as infections of filamentous seaweeds are inconspicuous to the naked eye. It is therefore unsurprising that this study is a first record of the genus for South America. We should stress however, that this finding is the outcome of a relatively short field campaign held in summer, similar to another one recently conducted in Greece that also led to the finding of *A. ectocarpii* and *A. sphacellarum* (Strittmatter et al., 2013). Taken together with the available environmental sequences from California, Greenland and the Spanish Mediterranean Coast, our repeated findings via space- and time-limited sampling efforts reinforce the notion that *Anisolpidium* pathogens are geographically widespread in marine environments, perhaps even cosmopolitan. We hope that the unprecedented availability of cultures and molecular data will foster further research on oomycetes pathogens of marine algae.

Acknowledgements
Thanks are due to A. F. Peters for field collections at Perharidy, to F. Küpper for collection of specimens in South Chile and to R. Westermeier for organising the sampling campaign in Chile and providing laboratory infrastructure. CMMG and YB are funded by the FP7 Marie Curie program (PERG03-GA-2008-230865) and the UK Natural Environment Research Council (NE/J00460X/1 and NE/L013223/1). MS acknowledges funding from the Genomia Fund (project HERDIR).
References


Table 1: Collection details of pathogen and algal strains reported in this study.

An = *Anisolpidium*, Ec = *Ectocarpus*, Pyl = *Pylaiella*. Field samples are highlighted in bold italics; derived laboratory cultures are in normal font.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Description</th>
<th>Genbank Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An</em> ectocarpii QU 67</td>
<td><em>Ectocarpus</em> sp. infected with <em>A. ectocarpii</em>. Sample scraped from the bottom of a fishing boat in Quetalmahue (Chiloé, Chile) on Jan 31st, 2007</td>
<td>KU764786 (18S) KX086261 (cox1) KP420743.1 (cox2)</td>
</tr>
<tr>
<td>Ec QU 67-23</td>
<td>Clonal, healthy <em>Ectocarpus</em> sp. isolate established from the infected material above. Available from the authors upon request.</td>
<td></td>
</tr>
<tr>
<td><em>An</em> QU 67-5 (CCAP4001/1)</td>
<td>Monoecuakaryotic, non-axenic <em>A. ectocarpii</em> isolate transfected into the clonal strain <em>Ec</em> QU 67-23</td>
<td></td>
</tr>
<tr>
<td><em>An</em> QU 467-2</td>
<td>Same monoecuakaryotic, non-axenic <em>A. ectocarpii</em> isolate as above, artificially introduced into a clonal female <em>M. pyrifera</em> gametophyte (CCAP 1323/1) for easier maintenance</td>
<td></td>
</tr>
<tr>
<td><em>An</em> PM</td>
<td><em>Hincksia sandriana</em> infected by <em>A. ectocarpii</em>. Sample obtained by scuba diving in a mariculture installation near Puerto Montt, Chile, at c. 13 m depth on Feb 2nd, 2007</td>
<td>KP420744.1 (cox2)</td>
</tr>
<tr>
<td><em>An</em> PM 76-6</td>
<td>Non-axenic <em>Anisolpidium ectocarpii</em> isolate transfected into the clonal strain <em>Ec</em> QU 67-23. This culture was lost during the course of the study.</td>
<td></td>
</tr>
<tr>
<td><em>An</em> rosenvingei Ros 2014</td>
<td>Sept 8, 2014, tidal flat outside Marine Station Roscoff. Infected <em>Pylaiella littoralis</em> epiphytic on receptacles of <em>Fucus serratus</em> found in the intertidal.</td>
<td>KU764783 (18S) KU764784 (cox1) KU764785 (cox2)</td>
</tr>
<tr>
<td><em>An</em> rosenvingei Per 2015-4</td>
<td>Nov. 2015. Infected <em>Pylaiella littoralis</em> found on Perharidy beach, Roscoff, France.</td>
<td>KU752534 (18S) KU764782 (cox1)</td>
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</table>
Table 2: Broad host specificity of *Anisolpidium ectocarpii* as determined by co-incubation experiments with candidate brown algal hosts.

dpi: days post infection

<table>
<thead>
<tr>
<th>Species</th>
<th>Taxonomic position</th>
<th>Origin of host culture, location, date</th>
<th>Dpi</th>
<th>Response and cell type affected</th>
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<tbody>
<tr>
<td><em>Ectocarpus sp.</em></td>
<td>Ectocarpales Ectocarpaceae</td>
<td>New Zealand, 1988</td>
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<td>susceptible; gametophyte filament</td>
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<tr>
<td><em>Ectocarpus fasciculatus</em> Kützing</td>
<td>Ectocarpales Ectocarpaceae</td>
<td>Roscoff 1993</td>
<td>5</td>
<td>gametophyte filament</td>
</tr>
<tr>
<td><em>Myriotrichia clavaeformis</em> (Harvey)</td>
<td>Ectocarpales Chordariaceae</td>
<td>Argentina 1995</td>
<td>8</td>
<td>somatic and hair meristem, hair cells, plurilocular sporangia</td>
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<tr>
<td><em>Botrytella uvaeformis</em> (Lyngbye) Kornmann &amp; Sahling</td>
<td>Ectocarpales Chordariaceae</td>
<td>Helgoland 1960</td>
<td>21</td>
<td>no infection</td>
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<tr>
<td><em>Leptonematella fasciculate</em> (Reinke) P.C.Silva</td>
<td>Ectocarpales Chordariaceae</td>
<td>Roscoff 1970</td>
<td>8</td>
<td>no infection</td>
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<td><em>Elachista stellaris</em> Areschoug</td>
<td>Ectocarpales Chordariaceae</td>
<td>Canary Islands 1991</td>
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<td>filament cells</td>
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<tr>
<td><em>Myriogloea chilensis</em> (Montagne) A.H.Llaña</td>
<td>Ectocarpales Chordariaceae</td>
<td>Chile 1985</td>
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<td>female gametophyte</td>
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<td><em>Feldmannia irregularis</em></td>
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<td>no infection</td>
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<tr>
<td>Species</td>
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<td>Location</td>
<td>Year</td>
<td>Details</td>
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<td>------------------------------------</td>
<td>-------------------------</td>
<td>-----------------------------------</td>
<td>-------</td>
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<tr>
<td><em>Pylaiella littoralis</em> (Linnaeus) Kjellman</td>
<td>Ectocarpales Acinetosporaceae</td>
<td>Drake Passage 1986</td>
<td>13</td>
<td>no infection</td>
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<td><em>Macrocystis pyrifera</em> (Linnaeus) C.Agardh</td>
<td>Laminariales</td>
<td>Chile 1999, CCAP 1323/1</td>
<td>9</td>
<td>male and female gametophytes and juvenile sporophyte</td>
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<td><em>Asterocladon lobatum</em> D.G.Müller, E.R.Parodi &amp; A.F.Peters</td>
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<td>Brazil 1996</td>
<td>7</td>
<td>filament and apical cells</td>
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<td><em>Asterocladon rhodochortonoides</em> (Børgesen) S.Uwai, C.Nagasato, T.Motomura &amp; K.Kogame</td>
<td>Asterocladales</td>
<td>South Africa 1993</td>
<td>8</td>
<td>filament cells</td>
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<td><em>Scytothamnus fasciculatus</em> (J.D.Hooker &amp; Harvey) A.D.Cotton</td>
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<td>microthallus and juvenile macrothallus cells</td>
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<td><em>Tilopteris mertensii</em> (Turner) Kützing</td>
<td>Tilopteridales</td>
<td>Helgoland 1984</td>
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<td>filament and hair cells</td>
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<td><em>Cutleria multifida</em> (Turner) Greville</td>
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<td><em>Microzonia velutina</em> (Harvey) J.Agardh</td>
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<tr>
<td>Species</td>
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<td>Collection Year</td>
<td>Number</td>
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<td><em>Desmarestia viridis</em> (O.F.Müller) J.V.Lamouroux</td>
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<td><em>Arthrocladia villosa</em> (Hudson) Duby</td>
<td>Desmarestiales</td>
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<td><em>Carpomitra costata</em> (Stackhouse) Batters</td>
<td>Sporochnales</td>
<td>Villefranche</td>
<td>1981</td>
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<td><em>Perithalia caudata</em> (Labillardière) Womersley</td>
<td>Sporochnales</td>
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<td><em>Sphacelaria rigidula</em> Kützing</td>
<td>Sphacelariales</td>
<td>Netherlands</td>
<td>1968</td>
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<td><em>Halopteris gracilescens</em> (J.Agardh) Womersley</td>
<td>Sphacelariales</td>
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<td><em>Halopteris</em> sp.</td>
<td>Sphacelariales</td>
<td>Australia</td>
<td>1988</td>
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<td><em>Halopteris congesta</em> (Reinke) Sauvageau</td>
<td>Sphacelariales</td>
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<td><em>Halopteris paniculata</em> (Suhr) Prud'homme van Reine</td>
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<td>New Zealand</td>
<td>1981</td>
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<td><em>Discosporangium mesarthrocarpum</em> (Meneghini) Hauck</td>
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<td>Greece 2004</td>
<td>17</td>
<td>death of filament cells, no repair</td>
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</table>
18S rRNA environmental sequences most closely related to *Anisolpidium*.

Table 3

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<th>Accession</th>
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<th>Geographic origin</th>
<th>Identity (%)</th>
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<td>EF100276.1</td>
<td>Stoeck <em>et al.</em>, unpub.</td>
<td>oxygen-depleted intertidal marine sediment, upper 2 cm sediment surface, Greenland</td>
<td>97.6 99.0 96.4</td>
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<td>EF100297.1</td>
<td>Stoeck <em>et al.</em>, unpub.</td>
<td>oxygen-depleted intertidal marine sediment, upper 2 cm sediment surface, Greenland</td>
<td>97.6 99.1 96.5</td>
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<tr>
<td>AY381206.1</td>
<td>(Massana <em>et al.</em>, 2004)</td>
<td>coastal surface water, Northwestern Mediterranean, Blanes Bay</td>
<td>91.7 91.6 89.5</td>
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<tr>
<td>JQ781890.1</td>
<td>(Lin <em>et al.</em>, 2012)</td>
<td>Pelagic sample, Monterey Bay, 5 m depth</td>
<td>90.6 90.1 90.5</td>
</tr>
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Figure Legends

Figs 1-15. Infection cycle of *Anisoldidium ectocarpii* CCAP 4001/1 (1-10) and *A. rosenvingei* (11-15).

Figs 1-2. Acetocarmine-stained field-collected *Ectocarpus* sp. with developing *A. ectocarpii* intramatrical plasmodia (1, arrowheads) and mature empty sporangia with typical exit tubes (2, arrowheads). Fig. 3. Anteriorly monoflagellated spores (arrowheads). See Supplementary Information 1 for a time-lapse movie illustrating their swimming behaviour. Fig. 4. Detail of a spore (phase contrast). Fig. 5. The arrows point to two *A. ectocarpii* spores encysted at the surface of the same *Ectocarpus* cell. Fig. 6. Two young unwalled plasmodia, with a conspicuously granulous cytoplasm. Fig. 7. Spherical walled thallus, with homogeneous cytoplasm. Fig. 8. Late intramatrical plasmodium filling entirely the host cell, preceding zoosporangium differentiation. Fig. 9. Differentiating exit tube (arrowhead). Fig. 10. Infection of *Ectocarpus* spore initials in plurilocular sporangia. Figs 11-12. Healthy (11) vs. *A. rosenvingei*-infected (12) unilocular sporangia of *Pylaiella littoralis*. Fig. 13. Spore release, 6 days after infection. Fig. 14. Acetocarmine-stained field specimen with virus symptoms (double arrowhead) and *A. rosenvingei* exit tube (arrowheads). Fig. 15. As for *A. ectocarpii*, multiple infections of the same host cell by *A. rosenvingei* are frequent (arrowheads point to exit tubes of sporangia formed in the same host cell).

Scale bars: 1-2, 11-15: 50 μm; 3, 5-10: 10 μm; 4: 5 μm.

Fig 16-24. Cell wall differentiation of *A. ectocarpii* CCAP 4001/1 and algal defence.

Figs 16-18. Staining pattern obtained with Congo red. 16. Unlabelled, unwalled young granulose plasmodium. Fig. 17. Differentiating exit tube forcing its way outside the host cell. Fig. 18. Mature empty sporangia in dead algal cells. Note also that encysted spores (arrowheads) are weakly
labelled. Figs 19-22. Calcofluor white. Fig. 19: walled plasmodium (inset: corresponding bright field image). Fig. 20. Empty mature sporangia with a distinctive annular thickening at the basis of the exit tubes. Fig. 21. Encysted spores at the surface of an Ectocarpus cell. Note the very thin penetration apparatus (arrowhead). Fig. 22. Cell wall thickening underneath an encysted spore, tentatively attributed to a defence reaction of the alga. Figs 23-24. Aniline blue. Fig. 23: Cell wall thickening (arrowheads) underneath encysted spores, similar to Figs 22. 24. Weak labelling of the sporangium cell wall, again with an annular thickening at the basis of the exit tube. Scale bars: 16-22, 24:10 μm. 23: 20 μm.

**Fig. 25-40. Nuclear dynamics of A. ectocarpii CCAP 4001/1.**

Arrowheads and arrows point to A. ectocarpii and host structures, respectively. All pictures illustrate Ectocarpus sp. except Figs 39-40, which represent M. pyrifera. Fig. 25. Young uninucleate thallus. Fig. 26. Interphasic nuclei of a binucleate Anisolpidium thallus; the arrow points to the nucleus of the infected host cell. Fig. 27. Eight-nucleus stage, corresponding to the granulose stage illustrated in Fig. 16. Note the perinuclear mitochondria visible as minute fluorescent dots. Fig. 28. At a later stage, the syncytium contains small compact nuclei, whilst the younger thallus at the bottom is at a similar stage as in Fig. 27. Fig. 29. Late walled Anisolpidium thallus, with multiple small compact nuclei; the arrow points to the nucleus of a healthy algal cell. Fig. 30. Spore encysted at the algal host cell surface (arrowhead). Note also a late-stage walled thallus with compact nuclei on the right. Figs 31-32. Multiple infections of a single host cell that contained sixteen A. ectocarpii plasmodia. In Fig. 31 (bright field), numerous empty infectious spores (arrowheads) are encysted at the surface of the host. In Fig. 14, each A. ectocarpii plasmodium contains a small number (max. 8) of small compact nuclei, illustrating the regulation of the pathogen development by available host resources. Figs 33-34. Mobile zoospores in a dehiscent sporangium just before release, viewed under bright field (33) and epifluorescence
Figs 35-38. Synchronous mitoses of *A. ectocarpii* nuclei. Fig. 35. Metaphase. Fig. 36. Anaphase. Fig. 37. Late anaphase, with trails of fluorescence suggestive of a closed mitosis. Fig. 38. Telophase. Figs 39-40. Preferential infection of distal end of filaments in *M. pyrifera* gametophytes. Both pictures depict the same field of view under bright field and epifluorescence. Developing (arrowheads on 39 & 40) plasmodia and mature dehiscent sporangia (additional arrowheads on 40) are disproportionately located at the tips of the host filaments. Scale bars: Figs 25-38: 10 μm; Figs 39-40: 20 μm.

**Fig. 41-59. Host range of *A. ectocarpii* CCAP 4001/1**

Arrowheads point to *A. ectocarpii* structures. Figs 41-43. *Macrocystis pyrifera*: Infected young sporophyte at 12 dpi (41), oogonia on a female gametophyte (42), and mature empty sporangium on a male gametophyte (43). Figs 44-45. *Asterocladon lobatum*: developing plasmodium (44) and mature empty sporangium (45). Figs 46-48. *Myriotrichia clavaeformis*: multiple (46) and single (47) infection in hair cells; infected plurilocular sporangium (48). Figs 49-50: *Myriogloea chilensis* female gametophyte: developing plasmodium (49) and mature sporangium (50). Figs 51-52. *Elachista stellaris*: developing plasmodia. Fig. 53. *Ectocarpus fasciculatus*: mature sporangium at 6dpi. Fig. 54. *Desmarestia viridis* juvenile sporophyte: developing plasmodia and mature sporangium (inset) at 8 dpi. Fig. 55-56. *Tilopteris mertensii*: developing plasmodia in somatic filament (55) and hair cell (56, stained with acetocarmine) at 8 dpi. Fig. 57-58. *Halopteris gracilescens*: infected apical cells containing motile zoospores just before release (57) and empty sporangium (58). Fig. 59. *Halopteris* sp.: apical cell with three mature sporangia. All scale bars: 20 μm.

**Fig. 60.** Maximum-likelihood (ML) tree of 18S rRNA gene sequences of oomycetes including *Anisolpidium ectocarpii* and *Anisolpidium rosenvingei*, the three hyphochytrids *Hyphochytrium*
catenoides BR217, ATCC18719 and Rhizidiomyces apophysatus and the two marine flagellates Cafeteria sp. and Cafeteria roenbergensis, which were used to root the tree. Bootstrap values are given for ML and Maximum parsimony (MP) phylogenetic analyses. Bootstrap values in % represent 100 replicates; bootstrap values below 50 are omitted. Branch lengths represent substitutions per site. A/X: bootstrap lower than 50 in ML and no bootstrap support for this branch in MP.

Fig. 61. Maximum-likelihood (ML) tree of cox1 protein sequences of oomycetes including Anisolpidium ectocarpii and Anisolpidium roenvingei, the brown alga Ectocarpus sp., the thraustochytrid Thraustochytrium aureum and the marine flagellate Cafeteria roenbergensis. Thraustochytrium aureum and Cafeteria roenbergensis were used to root the tree. Bootstrap values are given for ML and Maximum parsimony (MP) phylogenetic analyses. Bootstrap values in % represent 100 replicates; bootstrap values below 50 are omitted. Branch lengths represent substitutions per site. A: bootstrap lower than 50 in ML, B: bootstrap lower than 50 in MP X: no bootstrap support for this branch in MP.

Fig. 62. Maximum-likelihood (ML) tree of cox2 protein sequences of oomycetes including Anisolpidium ectocarpii and Anisolpidium roenvingei, and the hyphochytrid Hyphochytrium catenoides which was used to root the tree. Bootstrap values are given for ML and Maximum parsimony (MP) phylogenetic analyses. Bootstrap values in % represent 100 replicates; bootstrap values below 50 are omitted. Branch lengths represent substitutions per site. A: bootstrap lower than 50 in ML, X: no bootstrap support for this branch in MP.
Legends for Supplementary information

Supplementary Information 1: Genbank accession number of all organisms used in the phylogenetic analyses of the markers 18S rRNA, cox1 and cox2.

Supplementary Information 2: Multiple sequence alignment of the marker 18S rRNA.

Supplementary Information 3: Multiple sequence alignment of the marker cox1.

Supplementary Information 4: Multiple sequence alignment of the marker cox2.

Supplementary Information 5: Time lapse video of freshly released A. ectocarpii zoospores, illustrating their swimming behaviour, in particular their anterior flagellation. Pictures were taken over a 2 min period and the video is accelerated 10 times. The scale bar is indicated on the first image.

Supplementary Information 6: Maximum-likelihood (ML) tree of 18S rRNA sequences of environmental sequences (Table 3) and the basal oomycetes Olpidiopsis pyropiae, Olpidiopsis porphyrae, Olpidiopsis feldmannii, Olpidiopsis sp. Anisolpidium ectocarpii, Anisolpidium rosenvingei, Haliphthoros milfordensis and Haliphthoros sp., The latter two were used to root the tree. Bootstrap values are given for ML and Maximum parsimony (MP) phylogenetic analyses. Bootstrap values in % represent 100 replicates; bootstrap values below 50 are omitted. Branch lengths represent substitutions per site. B: bootstrap lower than 50 in ML, X: no bootstrap support for this branch in ML.