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# 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

16
17 **Abstract**

18 Using laboratory cultures, we have documented the life cycle of *Anisolpidium ectocarpii,* a
19 pathogen of *Ectocarpus* and other filamentous brown algae, and presented preliminary
20 observations on *Anisolpidium rosenvingei*, a pathogen of *Pylaiella littoralis.* Consistent with
21 earlier reports, the zoospores of both species have a single anterior flagellum, which justified
22 the placement of *Anisolpidium* amongst the Hyphochytriales (Hyphochytridiomycota). We
23 have also shown that *A. ectocarpii* can complete its infection cycle in a broad selection of
24 species from various brown algal orders, whereas *A. rosenvingei* seemingly exhibits a strict
25 specificity for unilocular sporangia of *P. littoralis.* Unexpectedly, nuclear (18S rRNA) and
26 mitochondrial (*cox* 1, *cox*2) markers regroup *A. ectocarpii* and *A. rosenvingei*, into a hitherto
27 unrecognised monophyletic clade within the oomycetes (Oomycota), most closely related to
28 the Olpidiopsidales. The *Anisolpidium* genus is therefore entirely distinct from the
29 Hyphochytridiomycota and represents the first confirmed instance of an anteriorly uniciliate
30 oomycete. Finally, we suggest that a valid morphological criterion to separate true
31 hyphochytrids from oomycetes is the timing of zoospore cleavage. Given the evidence, we
32 propose to transfer the Anisolpidiales from the Hyphochytriales to the Oomycetes.

33

34 **Keywords:**

35 cox2, cox1, 18S rRNA, *Ectocarpus*, *Pylaiella*, host range, Hyphochytriales, Anisolpidiales

36 **Introduction**

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

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

54 In total, six marine species, all pathogens of filamentous brown algae, are currently
55 recognised in the genus *Anisolpidium* (reviewed in Marano *et al.*, 2012): *A. ectocarpii* Karling
56 on *Ectocarpus* and *Hincksia* spp.; *A. minutum* (H. E. Petersen) M. W. Dick on *Chorda filum*
57 (Linnaeus) C. Agardh; *A. sphacellarum* (Kny) Karling on *Sphacelaria* spp., *Chaetopteris*
58 *plumosa* (Lyngbye) Kützing, and *Cladostephus spongiosus* (Hudson) C. Agardh; *A. joklianum*
59 (M.W. Dick) on *Hincksia granulosa*; *A. olpidium* (H. E. Petersen) M. W. Dick on *E.*
60 *siliculosus*; and *A. rosenvingei* (H. E. Petersen) Karling on *Pylaiella littoralis*. *Anisolpidium* is

61 a widespread, perhaps even cosmopolitan genus reported in Japan, the North American East
62 coast and throughout European waters (reviewed in Marano *et al.*, 2012). In an
63 epidemiological study conducted over several years, Küpper & Müller (1999) found a strong
64 seasonal correlation between the prevalence of the pathogen *A. rosenvingei* in sporangia of
65 the filamentous brown alga *P. littoralis* and the breakdown of the algal population in late
66 autumn.

67 Most *Anisolpidium* species have been described using field-collected material and the limited
68 available information suggests that they share a common infection cycle. Species delimitation
69 is mostly based on the respective algal host, and ill-defined morphological characters such as
70 the size of the plasmodia inside their host. The Anisolpidiaceae as defined by Karling (1943)
71 have tentatively been placed by Dick (2001) within the order Hyphochytriales
72 (Hyphochytridiomycota or hyphochytrids), an order defined on the basis of a single anteriorly
73 directed flagellum, as opposed to the chytrids that have a posterior flagellum. Besides the
74 Anisolpidiaceae, the two other families currently recognised in the Hyphochytriales (Sparrow,
75 1960) are the freshwater, soil-dwelling or facultative parasites Hyphochytridiomycetaceae and
76 Rhizidiomycetaceae. Sequencing of the 18S rRNA gene showed that these two families were
77 monophyletic and they were proposed to be the closest relatives to the oomycetes (Hausner *et*
78 *al.*, 2000; Beakes *et al.*, 2014). To date, no molecular information is available for any
79 representative of the Anisolpidiaceae. In the most recent classification of eukaryotes (Adl *et*
80 *al.*, 2012), the Hyphochytriales are tentatively included among the typically biflagellated
81 stramenopiles, although this speculative placement has been a matter of debate for many years
82 (Beakes *et al.*, 2012).

83 Here, we report the establishment of stable laboratory cultures of *A. ectocarpii* and *A.*
84 *rosenvingei*. Since these organisms are only known from historic drawings, we documented
85 the life history of *A. ectocarpii* using a combination of bright field and epifluorescence

86 observations. In addition, we evaluated the host specificity of *A. ectocarpii.* We also assessed
87 the phylogenetic position of the species using three molecular markers, all of which reveal
88 that *Anisolpidium* falls within the oomycetes. Thus we note that oomycetes and hyphochytrids
89 cannot be distinguished by the presence of an anteriorly uniflagellate zoospore; instead we
90 suggest that the timing of zoospore cleavage might be a better criterion to separate the two
91 classes.

92

## 93 Material and methods

### 94 *Biological material*

95 Specimens of *Ectocarpus* sp. infected with *Anisolpidium ectocarpii* were collected in South
96 Chile (Table 1). In the laboratory, *A. ectocarpii* was transfected into a male *Ectocarpus* .
97 gametophyte (strain CCAP 1310/56). Successive cleaning steps removed all eukaryotic
98 contaminants. A culture of the original *Ectocarpus* host was established in parallel (strain *Ec*
99 QU 67-23). Finally, the parasite was re-transfected into this original host, leading to the host-
100 parasite system strain *Anisolpidium* / *Ectocarpus* QU 67-5. The cultures were maintained at
101 10 to 12°C under daylight-type fluorescent light at 10 µmol photons.$m^{-2}.s^{-1}$, with a 12h:12h
102 photoperiod. Culture medium was sterilized sea water containing 20 mL $L^{-1}$ Provasoli
103 enrichment. Parallel attempts with *A. ectocarpii* found in *Hincksia sandriana* (Zanardini) P.C.
104 Silva failed, and only preserved samples for DNA sequencing remained (strain *A. ectocarpii*
105 PM 76-6).

106 *A. rosenvingei* was encountered at two occasions in the tidal flats near Roscoff, France, in *P.*
107 *littoralis* growing on *Fucus serratus* Linnaeus receptacles. Culture conditions for this
108 pathogen were as described above but because of the high host-specificity of *A. rosenvingei*
109 towards unilocular sporangia of *P. littoralis,* the following modifications were necessary.

110 A unialgal clonal culture of healthy *P. littoralis* from Roscoff was established as a routine
111 source for unilocular sporangia. Repeated co-incubations were then undertaken of this new
112 host with *A. rosenvingei* plasmodia in detached unilocular sporangia in order to remove all
113 eukaryotic contaminants, whilst maintaining the pathogen. From this point onward, two
114 different techniques allowed the survival of clean host-pathogen cultures in the laboratory: 1)
115 a pulse system, by successive co-incubation with a new host culture in one to two week

116 intervals, and 2) a continuous system by selecting apical parts of fertile *P. littoralis* filaments
117 with infected unilocular sporangia in one to two week intervals for continuous growth.

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

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

131 ***Histological staining***

132 Beta 1-3 and 1-4 glucans were stained with a commercially available Calcofluor white
133 solution containing Blue Evans as a counterstain. Samples were incubated for 5 to 10 min in a
134 0.01 mg $mL^{-1}$ calcofluor solution, rinsed in sterile seawater and observed with a DAPI filter
135 (excitation: 365 nm, beam splitter: 395 nm, emission: long pass 420 nm). Alternatively, the
136 non-specific cellulose stain Congo Red was used, at a final concentration of 0.1 mg $mL^{-1}$,
137 followed by observation under differential interference contrast microscopy. Aniline blue
138 staining was performed as in Tsirigoti *et al.* (2014). In order to stain *Anisolpidium* nuclei,
139 samples were first fixed 1 h on ice with Microtubule Stabilizing Buffer (Katsaros & Galatis,

140 1992), followed by several washes in methanol until the algal filaments were discoloured. The
141 samples were then transferred into sterile seawater containing a $10^{-4}$ dilution of a commercial
142 Sybr Green solution (Sigma Aldrich), sometimes also containing aniline blue. Samples were
143 mounted in SlowFade (Life Techonologies) and observed on a Zeiss LSM 510
144 epifluorescence microscope coupled to an Axiocam HR digital camera, using a FITC filter
145 (excitation BP 450-490 nm; beam splitter 510 nm; emission LP 515 nm).

146 ***Molecular taxonomy***

147 DNA was extracted using a CTAB and phenol-chloroform method or Qiagen DNeasy minikit
148 as detailed in Gachon *et al.* (2009). The *cox*2 marker was amplified using the cox2HF -
149 cox2HR primer pair as described in (Hudspeth *et al.*, 2000). The *cox*1 marker was amplified
150 using Fm85mod and CoxleUp (Robideau *et al.*, 2011). The 18S rRNA marker was amplified
151 using the new primers F139 (5' AGTCTATTTGATAGTACCTTACTAC 3') and R1233 (5'
152 CAATCCTTACTATGTCTGG 3') with an annealing temperature of 45°C. After Sanger
153 sequencing, the resulting chromatograms were proofread manually in Geneious V6.1.8
154 (Kearse *et al.*, 2012) or BioEdit (Hall, 1999) and virtually translated (*cox*1/*cox*2).
155 Stramenopile-wide alignments were used to produce unrooted trees to ascertain the initial
156 branching position of *A. ectocarpii* and *A. rosenvingei* before final selection of suitable in and
157 outgroup species (Supplementary Info 1). Phylogenetic analyses were performed in MEGA v.
158 7 (Kumar *et al.*, 2016). Model tests were performed on the alignments in order to find the best
159 substitution models for subsequent maximum likelihood (ML) analysis. For the 18S rRNA,
160 Tamura-3-parameter was used (Tamura, 1992) with a discrete Gamma distribution to model
161 evolutionary rate differences among sites. The rate variation model allowed for some sites to
162 be evolutionarily invariable. For *cox*1 and *cox*2 the La Gascuel model (2008) with discrete
163 gamma distribution was used the general reversible mitochondrial model (Adachi &
164 Hasegawa, 1996) with discrete gamma distribution and for *cox*2 the La Gascuel model (2008)
165 with discrete gamma distribution was used. Additionally maximum parsimony analysis was

166 performed on all three datasets. Bootstrap re-sampling was set to 100 replicates. Sequence
167 data of all *Anisolpidium* strains were deposited in Genbank (see Table 1 for accession
168 numbers) and the alignments are given in Suppl. Info. 2-4.

169

170
171 **Results**

172 Life history and infection cycle

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

179 The release of infectious zoospores from *A. ectocarpii* sporangia could be triggered by
180 transferring the culture from 10°C into fresh medium at room temperature. Within a few
181 minutes, zoospores started to move inside the sporangium and escape in files through the exit
182 tubes. The swimming spores were globular bodies of 2.5 µm diameter, and became slightly
183 pyriform after a few minutes (Figs 3, 4). Nile Red staining indicated that they contain lipid
184 globules (not shown). They had one anterior flagellum, which pulled the spore forward by
185 rapid meandering oscillations (Suppl. Information 5). The liberated zoospores remained active
186 for about 30 minutes at room temperature before encysting at the surface of a new host cell
187 (Fig. 5). *A. ectocarpii* then penetrated into the algal cell and developed as an intramatrical,
188 unwalled plasmodium with a conspicuously granulose cytosol and a subspherical shape (Fig.
189 6). The algal cell components disaggregated rapidly and cell remnants were already visible at
190 this stage, with a typical chestnut brown colour. The growing plasmodium then differentiated
191 a thin cell wall, thus becoming spherical, whilst its cytoplasm became more uniformly
192 granular (Fig. 7). The pathogen progressively filled the host cell, closely moulding its shape
193 (Fig. 8) and differentiating one or several protruding exit tube(s) (Fig. 9); the final
194 plasmodium size depended on the cell type and the number of infections per host cell
195 (detailed below). For example, spore initials in *Ectocarpus* plurilocular sporangia were

196 commonly infected, and led to distinctively smaller pathogen sporangia compared to when
197 somatic cells were infected (Fig. 10). Throughout its growth, the pathogenic thallus was
198 monocentric and did not propagate to neighbouring algal cells. Likewise, *A. rosenvingei*
199 developed intracellularly, with a strict tissue specificity for unilocular sporangia of *P.*
200 *littoralis* (Figs 11, 12). Spore release typically occurred within 6-10 days following infection
201 (Fig. 13) and monoflagellation of the spores was observed. Virus-infected *P. littoralis* could
202 also undergo lysogeny, leading to the production of viral particles in the algal unilocular
203 sporangia. Though the association between both pathogens was not obligate, *A. rosenvingei*
204 infection of virus-producing unilocular sporangia was observed both in field material (Fig.
205 14) and in culture (not shown). Finally, and similar to *A. ectocarpii*, co-infection of the same
206 host cell by *A. rosenvingei* was also frequent (Fig. 15).

207 Young unwalled *A. ectocarpii* plasmodia did not react to any of the cell wall stains examined,
208 (Fig. 16). Typical of holocarpic pathogens, the developing thallus fully converted into a
209 sporangium with exit tube(s) that ruptured the wall of the dead host cell at full maturity. The
210 differentiating exit tubes were labelled by Congo red (Fig. 17); the entire cell wall of mature
211 sporangia reacted to this stain (Fig. 18), whilst spores gave a weak and inconsistent labelling.
212 However, these structures remained unstained when methylene blue was used (not shown).
213 The thin cell wall progressively built up by the early spherical unwalled thallus was most
214 easily visualised using the $\beta1\rightarrow3$ and $\beta1\rightarrow4$ glucan stain calcofluor white (Fig. 19). Mature
215 exit tubes were most intensely labelled (Fig. 20), strongly suggesting that the pathogen
216 sporangium forces its way out of the host cell. Calcofluor also stained the encysted *A.*
217 *ectocarpii* zoospores, revealing thin infectious germ tubes (ca. 1 μm in length) that penetrate
218 into the algal cell perpendicularly to its cell wall (Fig. 21). Therefore, we recommend
219 calcofluor as a rapid and convenient method to locate pathogen structures, in particular the
220 otherwise inconspicuous spores encysted at the surface of the algal cells. In addition, localised
221 modifications of the algal cell wall were visible in the immediate vicinity of encysted *A.*

222 *ectocarpii* spores both with calcofluor (Fig. 22, arrowhead) and aniline blue (Fig. 23). *A.*
223 *ectocarpii* structures were only weakly stained with aniline blue ($\beta 1 \rightarrow 3$ glucans), with again
224 the most intense labelling at the basis of exit tubes (Fig. 24).

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

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

242

243 **Host range of *A. ectocarpii* and *A. rosenvingei.***

244 Co-incubation experiments with representative brown algal cultures spanning 28 species
245 across ten brown algal orders revealed an unexpectedly broad host spectrum for *A. ectocarpii*
246 (Table 2). Algal strains were deemed susceptible if they allowed completion of the full life

247 cycle of the pathogen, i.e. penetration of spores, development of plasmodia, and spore release
248 through exit tubes (Figs 41-59). Susceptibility was the most frequent outcome of the
249 experiments, across all orders tested. However, we encountered several algal strains that
250 departed from this pattern, especially among uniseriate filamentous species. There was a
251 complete absence of any visible interaction for *Botrytella uvaeformis*, *Leptonematella*
252 *fasciculata*, *Feldmannia irregularis*, *Pylaiella littoralis*, *Cutleria multifida*, *Microzonia*
253 *velutina*, *Sphacelaria* sp. and *Sphacelaria rigidula*, although another *Sphacelaria* isolate was
254 successfully infected.

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

271

272 *Anisolpidium ectocarpii* and *A. rosenvingei* belong to the oomycetes

273 Partial sequence information for the 18S rRNA and *cox*1 genes was successfully obtained for
274 *A. ectocarpii* CCAP 4001/1, *A. rosenvingei* Ros and *A. rosenvingei* Per; partial *cox*2
275 sequences were obtained for *A. ectocarpii* CCAP 4001/1, *A. ectocarpii* PM 76-6 and *A.*
276 *rosenvingei* Ros. Our phylogenetic analyses of all three markers (Figs 60-62) placed *A.*
277 *ectocarpii* and *A. rosenvingei* within the oomycetes. The assumed placement of *Anisolpidium*
278 within the hyphochytrids was rejected, as seen from the phylogenetic analysis of 18S rRNA
279 and *cox*2. All *Anisolpidium* strains formed a monophyletic group with the three markers
280 investigated. Despite limited bootstrap support, both the 18S rRNA and *cox*1 marker placed
281 the three strains of *Anisolpidium* on a long branch, sister to the Olpidiopsidales. The *cox*2
282 marker lacked sufficient resolution to decipher the relationship between the *Anisolpidium* and
283 *Olpidiopsis* genera, but the tree topology retrieved was also consistent with the above
284 interpretation. Additionally, four environmental 18S sequences most closely related to
285 *Anisolpidium* were retrieved from Genbank (Table 3). All originate from coastal marine
286 habitats (pelagic or sediment), with a geographic coverage encompassing California,
287 Greenland and the Mediterranean Sea. Two of those sequences fell within the same clade as
288 the three *Anisolpidium* strains whereas the other two formed a sister clade (Suppl. Info. 6).

289

290 **Discussion**

291 Our observations of *A. ectocarpii* and *A. rosenvingei* in culture fully matched the meticulous
292 description of the type material given by Karling (1943). Novel aspects were the localised
293 thickenings of the algal cell wall at the pathogen penetration site, which are consistent with a
294 localised defence reaction similar to the one recently described in brown algal cells infected
295 by the oomycete *Eurychasma dicksonii* (Tsirigoti et al., 2015). As originally reported by
296 Karling, mitotic divisions were synchronous. However, we found that nuclear counts often

297 deviated from the exponential progression that would be expected if nuclei numbers doubled
298 at each division. Extensive observations, especially in multiple-infected host cells, suggested
299 that such non-canonical nuclei numbers were accounted for by the degeneration of some
300 parasite nuclei, with no evidence of any thallus fusion as recently reported in *Olpidiopsis*
301 *pyropiae* (Klochkova et al., 2015). Depending on resource availability from the host cell and
302 the resulting plasmodium size, this phenomenon would provide a possible mechanism to
303 regulate the number of spores produced per *A. ectocarpii* sporangium.

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

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

320 Despite being unexpected, the closeness of the relationship between *Anisolpidium* and
321 *Olpidiopsis* agrees with the observation of Johnson (1957), who already highlighted the

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

342 To the best of our knowledge this is the first report of an anteriorly uniflagellate oomycete*,*
343 yet flagellae were lost several times amongst Oomycota in obligate pathogenic clades such as
344 *Hyaloperonospora, Bremia*, and most *Peronospora* spp, as well as *Geolegnia* and some
345 *Haptoglossa* and *Myzocytiopsis*. Further variation is reported, for example the presence of two
346 smooth flagellae in *Haptoglossa dickii* (Beakes & Glockling, 1998), of two distinct
347 biflagellate patterns in *Olpidiopsis* (Whittick, 1972; West *et al.*, 2006; Sekimoto *et al.*, 2008)

348 and of differently flagellated zoospores at different life stages (e.g. *Saprolegnia* primary
349 spores vs secondary spores), suggesting that there is ample scope for variation in flagellation
350 amongst oomycetes. Among the heterokonts (Heterokonta), loss or strong reduction of the
351 posterior smooth flagellum is well documented in *Pelagomonas, Dictyota* (Kawai, 1992) and
352 in diatoms. In the latter group, the male gamete of all species investigated thus far has a single
353 tinselled flagellum. Hence, the loss of one flagellum in *Anisolpidium* represents the third
354 independent such event reported in the heterokonts. However, electron microscopy
355 investigation on *Anisolpidium* spores would be necessary in order to investigate a loss or
356 potential vestiges of the second flagellum.

357 As can be seen in Table 2, *A. ectocarpii* has a broad host spectrum, including several brown
358 algal orders with heteromorphic life histories, where both the micro- and macroscopic
359 generations are successfully attacked. Similarly, Müller *et al*. (1999) found that *Chytridium*
360 *polysiphoniae*, only described as infecting sporangia of *Pylaiella littoralis* in the field,
361 actually could infect 29 brown algal species across different brown algal orders. *Eurychasma*
362 *dicksonii* (Oomycota) has a similarly broad host range in marine Phaeophyceae (Müller *et al.*,
363 1999), as well as the Plasmodiophorid *Maullinia ectocarpii* I. Maier, E. R. Parodi, R.
364 Westermeier et D. G. Müller (Maier *et al.*, 2000). Over the years, our studies have revealed
365 that *M. ectocarpii*, *E. dicksonii* and *A. ectocarpii* all infect *Macrocystis* gametophytes in
366 laboratory cultures, begging to question as to what their impact might be on natural kelp beds.
367 The contrasting host spectra exhibited by our *A. rosenvingei* and *A. ectocarpii* isolates provide
368 further evidence against the widely-held view that all intracellular biotrophic pathogens are
369 necessarily highly host specific. Furthermore, the broad host range observed for *A. ectocarpii*
370 as well as other marine oomycetes and plasmodiophorids correlates with a unique ability to
371 perform cross-kingdom host shifts (extensively discussed in Beakes *et al.*, 2012; Neuhauser *et*
372 *al.*, 2014).

373 Altogether, our host range data also highlight the limitations of current species descriptions
374 within the genus *Anisolpidium*, which are almost exclusively based on morphological
375 characters that overlap between species, and observed host specificity in the field. It appears
376 necessary to reassess diversity and species delimitation with modern tools. For example, the
377 definition of *A. sphacellarum* as a pathogen of *Sphacelaria* is questioned by the fact that our
378 *A. ectocarpii* culture can infect at least one *Sphacelaria* isolate. However, the infection
379 structures that we observed were notably smaller than those reported in *Sphacelaria* spp. in
380 the field (Strittmatter *et al.*, 2013). It is also noteworthy that the observed preferential
381 infection of filament tips and apical cells of certain brown algal species by *A. ectocarpii*, for
382 example apical cells of several *Halopteris,* resonates with some other pathogens, such as
383 *Olpidiopsis porphyrae*, that recognise different lectins (Klochkova *et al.*, 2012).

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

396

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404

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536

537
538 **Table 1: Collection details of pathogen and algal strains reported in this study.**

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

541

| Sample name | Description | Genbank Accession number |
|---|---|---|
| ***An ectocarpii QU 67*** | *Ectocarpus* sp. infected with *A. ectocarpii.* Sample scraped from the bottom of a fishing boat in Quetalmahue (Chiloé, Chile) on Jan 31st, 2007 | |
| *Ec* QU 67-23 | Clonal, healthy *Ectocarpus* sp. isolate established from the infected material above. Available from the authors upon request. | |
| *An* QU 67-5 (CCAP4001/1) | Monoeukaryotic, non-axenic *A. ectocarpii* isolate transfected into the clonal strain *Ec* QU 67-23 | KU764786 (18S)<br>KX086261 (*cox*1)<br>KP420743.1 (*co*x2) |
| *An* QU 467-2 | Same monoeukaryotic, non-axenic *A. ectocarpii* isolate as above, artificially introduced into a clonal female *M. pyrifera* gametophyte (CCAP 1323/1) for easier maintenance | |
| ***An PM*** | *Hincksia sandriana* infected by *A. ectocarpii.* Sample obtained by scuba diving in a mariculture installation near Puerto Montt, Chile, at c. 13 m depth on Feb 2nd, 2007 | |
| *An PM 76-6* | Non-axenic *Anisolpidium ectocarpii* isolate transfected into the clonal strain *Ec* QU 67-23. This culture was lost during the course of the study. | KP420744.1 (*cox*2) |
| ***An rosenvingei Ros 2014*** | Sept 8, 2014, tidal flat outside Marine Station Roscoff. Infected *Pylaiella littoralis* epiphytic on receptacles of *Fucus serratus* found in the intertidal. | KU764783 (18S)<br>KU764784 (*cox*1)<br>KU764785 (*cox*2) |
| ***An rosenvingei Per 2015-4*** | Nov. 2015. Infected *Pylaiella littoralis* found on Perharidy beach, Roscoff, France. | KU752534 (18S)<br>KU764782 (*cox*1) |

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544

545 **Table 2: Broad host specificity of *Anisolpidium ectocarpii* as determined by co-incubation experiments with candidate brown algal hosts.**

546 dpi: days post infection

| Species | Taxonomic position | Origin of host culture, location, date | Dpi | Response and cell type affected |
|---|---|---|---|---|
| *Ectocarpus* sp. | Ectocarpales Ectocarpaceae | New Zealand, 1988 | 5 | susceptible; gametophyte filament |
| *Ectocarpus fasciculatus* Kützing | Ectocarpales Ectocarpaceae | Roscoff 1993 | 5 | gametophyte filament |
| *Myriotrichia clavaeformis* (Harvey) | Ectocarpales Chordariaceae | Argentina 1995 | 8 | somatic and hair meristem, hair cells, plurilocular sporangia |
| *Botrytella uvaeformis* (Lyngbye) Kornmann & Sahling | Ectocarpales Chordariaceae | Helgoland 1960 | 21 | no infection |
| *Leptonematella fasciculate* (Reinke) P.C.Silva | Ectocarpales Chordariaceae | Roscoff 1970 | 8 | no infection |
| *Elachista stellaris* Areschoug | Ectocarpales Chordariaceae | Canary Islands 1991 | 6 | filament cells |
| *Myriogloea chilensis* (Montagne) A.H.Llaña | Ectocarpales Chordariaceae | Chile 1985 | 5 | female gametophyte |
| *Feldmannia irregularis* | Ectocarpales | Canary Islands 1994 | 7 | no infection |

| | | | | |
|---|---|---|---|---|
| (Kützing) Hamel | Acinetosporaceae | | | |
| *Pylaiella littoralis* (Linnaeus) Kjellman | Ectocarpales Acinetosporaceae | Drake Passage 1986 | 13 | no infection |
| *Macrocystis pyrifera* (Linnaeus) C.Agardh | Laminariales | Chile 1999, CCAP 1323/1 | 9 | male and female gametophytes and juvenile sporophyte |
| *Asterocladon lobatum* D.G.Müller, E.R.Parodi & A.F.Peters | Asterocladales | Brazil 1996 | 7 | filament and apical cells |
| *Asterocladon rhodochortonoides* (Børgesen) S.Uwai, C.Nagasato, T.Motomura & K.Kogame | Asterocladales | South Africa 1993 | 8 | filament cells |
| *Scytothamnus fasciculatus* (J.D.Hooker & Harvey) A.D.Cotton | Scytothamnales | New Zealand 1981 | 8 | microthallus and juvenile macrothallus cells |
| *Tilopteris mertensii* (Turner) Kützing | Tilopteridales | Helgoland 1984 | 5 | filament and hair cells |
| *Cutleria multifida* (Turner) Greville | Tilopteridales | Canary Islands 1994 | 13 | no infection |
| *Microzonia velutina* (Harvey) J.Agardh | Syringodermatales | New Zealand 1981 | 9 | no infection |

| | | | | |
|---|---|---|---|---|
| *Desmarestia viridis* (O.F.Müller) J.V.Lamouroux | Desmarestiales | Helgoland 1978 | 6 | juvenile sporophyte |
| *Arthrocladia villosa* (Hudson) Duby | Desmarestiales | Villefranche 1980 | 10 | gametophyte |
| *Carpomitra costata* (Stackhouse) Batters | Sporochnales | Villefranche1981 | 23 | juvenile sporophyte |
| *Perithalia caudata*(Labillardière) Womersley | Sporochnales | Tasmania 1984 | 9 | female gametophyte |
| *Sphacelaria* sp. | Sphacelariales | Greece, 2009 | 15 | apical and hair cells |
| *Sphacelaria* sp. | Sphacelariales | Morocco 1964 | 19 | No infection |
| *Sphacelaria rigidula* Kützing | Sphacelariales | Netherlands 1968 | 20 | No infection |
| *Halopteris gracilescens* (J.Agardh) Womersley | Sphacelariales | Australia 1981 | 21 | young apical cells |
| *Halopteris* sp. | Sphacelariales | Australia 1988 | 11 | apical cells, hair cells |
| *Halopteris congesta* (Reinke) Sauvageau | Sphacelariales | New Zealand 1981 | 27 | apical cells |
| *Halopteris paniculata* (Suhr) Prud'homme van Reine | Sphacelariales | New Zealand 1981 | 27 | apical cells |

| | | | | |
|---|---|---|---|---|
| *Discosporangium mesarthrocarpum*(Meneghini) Hauck | Discosporangiales | Greece 2004 | 17 | death of filament cells, no repair |

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549

550 **Table 3**

551

552 18S rRNA environmental sequences most closely related to *Anisolpidium*.

| **Accession** | **Author** | **Geographic origin** | **Identity (%)** | | |
|---|---|---|---|---|---|
| | | | ***A. ectocarpii QU*** | ***A. ectocarpii Ros*** | ***A. ectocarpii Per*** |
| EF100276.1 | Stoeck *et al.*, unpub. | oxygen-depleted intertidal marine sediment, upper 2 cm sediment surface, Greenland | 97.6 | 99.0 | 96.4 |
| EF100297.1 | Stoeck *et al.*,unpub. | oxygen-depleted intertidal marine sediment, upper 2 cm sediment surface, Greenland | 97.6 | 99.1 | 96.5 |
| AY381206.1 | (Massana *et al.*, 2004) | coastal surface water, Northwestern Mediterranean, Blanes Bay | 91.7 | 91.6 | 89.5 |
| JQ781890.1 | (Lin *et al.*, 2012) | Pelagic sample, Monterey Bay, 5 m depth | 90.6 | 90.1 | 90.5 |

553

554

# Figure Legends

**Figs 1-15. Infection cycle of *Anisolpidium 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

(34). 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, B: bootstrap lower than 50 in MP.

**Fig. 61.** Maximum-likelihood (ML) tree of *cox*1 protein sequences of oomycetes including *Anisolpidium ectocarpii* and *Anisolpidium rosenvingei*, 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 *cox*2 protein sequences of oomycetes including *Anisolpidium ectocarpii* and *Anisolpidium rosenvingei*, 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 Information1:** 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