New records of intracellular eukaryotic pathogens challenging brown macroalgae in the East Mediterranean Sea, with emphasis on LSU rRNA data of the oomycete pathogen *Eurychasma dicksonii*

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Abstract

For the Mediterranean and indeed most of the world's oceans, the biodiversity and biogeography of eukaryotic pathogens infecting marine macroalgae remains poorly known, yet their ecological impact is probably significant. Based on two sampling campaigns on the Greek island of Lesvos in 2009 and one in Northern Greece in 2012, this study provides first records of three intracellular eukaryotic pathogens, infecting filamentous brown algae at these locations: *E. dicksonii*, *Anisolpidium sphacellarum*, and *A. ectocarpii*. Field and microscopic observations of the three pathogens are complemented by the first *E. dicksonii* large subunit ribosomal RNA (LSU rRNA) gene sequence analyses of isolates from Lesvos and other parts of the world. The latter highlights the monophyly of *E. dicksonii* worldwide and confirms the basal position of this pathogen within the oomycete lineage (Peronosporomycotina). The results of this study strongly support the notion that the geographic distribution of the relatively few eukaryotic seaweed pathogens is probably much larger than previously thought and that much of the World's marine bioregions remains seriously undersampled and understudied in this respect.

Keywords

Brown algae, pathogens, oomycetes, hyphochytrids, infection, East Mediterranean Sea, phylogeny, LSU rRNA, SSU rRNA, *cox2*

Introduction

Like any other group of living organisms, seaweeds are subject to a permanent onslaught of a range of pathogens including viruses, bacteria, fungi, oomycetes, plasmodiophoraleans and endophytic algae (reviewed by Gachon et al. 2010). Much of the current knowledge of macroalgal-associated pathogens is based on decade- or century-old reports derived from field studies and is therefore limited to light microscopic observations and morphological classification. Although dating back half a century, Sparrow's monograph (1960) remains unsurpassed for the identification of marine fungi and fungus-like organisms. His terminology of aquatic phycomycetes, no longer in use, includes a polyphyletic group of zoosporic fungi and fungus-like organisms, mostly chytridiomycetes, oomycetes, and hyphochytridiomycetes, with the latter two actually being heterokonts (Stramenopiles). In most cases, such pathogens are holocarpic, biotrophic and intracellular. In many cases, a pathogen infection observed by light microscopy may have symptoms resembling non-pathological, subcellular structures of host cells, which are hard to distinguish by untrained observers. These features make their observation in field-collected material, but also their study in culture particularly difficult.

The ecological role of zoosporic fungi and fungus-like organisms is increasingly recognized in aquatic ecosystems and those organisms represent an important and non-negligible part of the microbial loop (Gleason et al. 2011, Sime-Ngando et al. 2011). Furthermore, previously unknown taxa are described at high pace in freshwater and marine systems, including parasites of algae.

Nevertheless, the knowledge of fungal-like organisms parasitizing macroalgae is by far less substantial compared to phytoplankton. This situation has certainly been driven by the strong research interest in the dynamics of diatom, coccolithophore and dinoflagellate blooms (Park et al. 2004, Chambouvet et al. 2008, Ibelings et al. 2011).

Overall, the biodiversity and biogeography of algal pathogens remains very poorly known in most of the world's seas. Traditionally, study areas were often in proximity to the investigators' home countries resulting in parts of the world that are comparatively well covered such as the European North Atlantic and both coasts of North America (Marano et al. 2012) whereas vast regions remain under- or unstudied, in particular warmer seas. While substantial, but probably incomplete records on the macroalgal flora are available from the East Mediterranean Sea (Parlakay et al. 2005, Tsiamis et al. 2010), macroalgal diseases in this region historically have not been investigated.

The Mediterranean Sea is a prominent example of an ecosystem with a multitude of human impacts. Despite a trend towards re-oligotrophication of coastal areas due to more widespread

sewage treatment (Tsiamis et al. 2013), in particular it remains a hotspot for alien species introductions, a number of those being invasive. Many of them are thought to have entered the Mediterranean via the Suez Canal (so-called Lessepsian migration; Boudouresque & Verlaque 2002, Streftaris & Zenetos 2006). Marine transport in particular enhances the spread of microorganisms including pathogens, while anthropogenic pollution may favour disease outbreaks (Ruiz et al. 2000). As in the terrestrial environment, the successful establishment and propagation of introduced species might at least to some extent be due to missing pathogens controlling alien seaweed populations in contrast to established pathogens controlling native populations (Torchin et al. 2003). Conversely, the introduction of a generalist pathogen is known to have devastating effects on native species (Andreou et al. 2012). Recently, the importance of pathogen spread and synergistic effects with abiotic stressors has come to light on the sea grass wasting disease caused by the protist *Labyrinthula* sp. affecting sea grass populations in the Mediterranean Sea and worldwide (McKone & Tanner 2009, Garcias-Bonet et al. 2011). Importantly, it provides one of the scarce examples on the impact of infectious diseases on aquatic plants and although not well studied similar scenarios can be assumed for seaweeds. Therefore, macroalgal pathologies cannot be neglected neither from an ecological point of view nor from an applied perspective considering macroalgae as source of nutrients and biofuels. However, correct assessment of changes in pathogen populations can only be made if base line data are available allowing determination of the frequency of infection and pathogen species compositions linked to abiotic factors and pollution.

The objective of the present study was to investigate the occurrence of eukaryotic pathogens affecting brown seaweeds in the East Mediterranean Sea. Observation of filamentous brown algal hosts by microscopy resulted in the detection of *Eurychasma dicksonii* (E. P. Wright) Magnus and two intracellular pathogens identified as members of the genus *Anisolpidium*. Furthermore, we report here the first large subunit ribosomal RNA (LSU rRNA) gene sequences for *E. dicksonii*, which has recently been used to delineate early-branching oomycete clades (Muraosa et al. 2009, Macey et al. 2011). The LSU rRNA gene sequence information from *E. dicksonii* isolates originating from the Pacific, South and North East Atlantic Ocean and the Aegean Sea not only confirms the identification of the Greek pathogen with molecular tools, but also underscores the monophyly of *Eurychasma* on a global scale. Finally, a phylogenetic analysis of combined LSU rRNA, small subunit (SSU) rRNA and mitochondrial cytochrome c oxidase subunit 2 (*cox2*) data confirms the basal position of *Eurychasma* among the oomycetes. The marker *cox2* was included since it is an established locus for phylogenies of marine oomycetes (Cook et al. 2001).

Material and Methods

Sampling and microscopic observation

Algal material was collected in the Aegean Sea (Greece), around the coastline of Lesvos (latitude: 39°10′N, longitude: 26°20′E) in February and March 2009 and around the small island of Panagia / Astris (latitude: 40°33′N, longitude: 24°37′E) off the south coast of Thasos Island in early May 2012. Particular focus was on filamentous brown algae of the order Ectocarpales since these are known hosts of the oomycete pathogen *E. dicksonii* (Müller et al. 1999) as well as due to our long track record studying these groups and due to the ease of subsequent microscopic investigation. Depending on the locality, sampling involved free diving, scuba diving and shore-based collection). At each site, algal samples were collected from different habitats (especially including rock pools, harbour structures including boat hulls, *Posidonia* sea grass meadows, *Cystoseira* forests, rocky seabed) with 5-10 specimens representing the host population depending on the overall host density. Specimens were immediately transferred into seawater and stored at ambient temperature until further analysis. Macroscopically, disease was never discernable and samples were thus collected from the field in an unbiased manner.

In the laboratory, specimens were examined for the presence of intracellular pathogens using conventional bright field microscopy (Olympus CH 20). Particular focus was laid on the detection of characteristic pathogen structures, namely the occurrence of parasitic spores on the algal host surface, intracellular pathogen thalli causing host hypertrophy and pathogenic sporangia. Depending on the thallus size of the individual algae between 5 and 10 tissue samples of each specimen were hereby analysed in order to ensure that even a low infection density was detected.

The Lesvos sampling campaign set in early February 2009 coincided with the start of the growth period of the targeted Ectocarpales species. One location on the east coast of Lesvos (Skala Neon Kydonion, Tab. 1) proved to be particularly abundant in terms of filamentous brown algae. The rocky shore at this location had numerous easily accessible rock pools which allowed land-based sampling. Therefore, this particular spot was re-visited and sampled on a weekly basis over a four week period during which the rapid growth of Ectocarpales biomass was readily recognizable

Preparation of permanent microscope slides and long-term storage of material for DNA extraction

Whenever intracellular pathogens were detected by microscopy, permanent mounts of these samples were subsequently prepared for documentation. Specimens were mounted on a microscope slide under a cover slip, fixed and stained with carmine red (0.1 -0.2 % w/v) in acetic acid (45%

v/v) and mounted with 50 % (v/v) Karo® light corn syrup. Microphotographs of fresh material and permanent slides were taken with an AxioCam HRc camera (Zeiss) using the AxioVision software (Zeiss, version 4.7.1). The remaining infected material was blotted dry on filter paper, wrapped in lens tissue and stored dehydrated in silica gel for subsequent DNA extraction. In this manner, one subsample of the infected individual specimen was used for genetic analysis.

Additional *Eurychasma*-infected algal material used in this study for molecular analysis originated from various localities including sites in Scotland, France, Argentina and the Falkland Islands and had been preserved under similar conditions by different isolators (Suppl. Tab. 1; Gachon et al. 2009).

DNA extraction and LSU rRNA amplification

A few milligrams of algal biomass dried in silicagel were transferred into a 2 mL Eppendorf tube containing a 5 mm stainless steel bead and DNA was extracted as previously described (Gachon et al. 2009). PCRs were run in a final volume of 20 µL or 50 µL containing 1x PCR mastermix (Qiagen Taq PCR mastermix or Eurogentec Goldstar Red'y mastermix), 0.4 µM primers and 2 or 4 µL of template DNA. The primers used for amplification of the LSU rRNA of the *Eurychasma dicksonii* gene were CG68 (5'-GATATCAGGTAAGAGTACCCACTGG-3') and the general oomycete primer LSU1170R (Van der Auwera et al. 1994). The primer CG68 has been designed based on a multiple sequence alignment of various oomycete and other stramenopile LSU sequences to anneal in a region conserved between the two *E. dicksonii* strains 05 and 96 which is notably divergent from other stramenopiles. The following PCR program was used for amplification: Initial denaturation of the DNA was carried out at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 2 min.

An aliquot of the PCR product was analyzed by agarose gel electrophoresis (1.5 % (w/v) agarose in 0.5x Tris-Borate-EDTA buffer) and checked for its correct size and quality. The remaining material was purified using the Qiaquick™ PCR purification kit according to the instruction manual (Qiagen). The bound purified PCR product was eluted from the spin columns with 40 µL elution buffer (Qiagen). Sequencing reactions on purified PCR products (20-100 ng) were performed using the sequencing primers LSU344F, LSU344R, LSU826F, LSU826R (Petersen & Rosendahl 2000) in addition to the PCR primers on an ABI3730 sequencer (Applied Biosystems) at the Edinburgh Sequencing Facility of the UK Natural Environment Research Council Molecular Genetics Facility.

Sequence assembly and phylogenetic analysis

The alignment of partial oomycete LSU rRNA gene sequence data comprised 44 taxa including 12 *E. dicksonii* strains and the two outgroup species, *Hyphochytrium catenoides* and *Developayella elegans*. The alignment had an initial length of 1198 characters. 577 ambiguous characters were removed (1-26, 103-120, 225, 425-427, 452-522, 585, 597-607, 613-615, 637-640, 655, 666-677, 686-716, 767-787, 825-1198) leaving 621 positions in the LSU alignment for the phylogenetic analysis. Similarly, the combined data set of LSU rRNA, SSU rRNA and *cox2* was reduced from initially 3648 to 2912 positions. The combined data set contained 19 taxa, including two representative *E. dicksonii* strains, for which sequences for all three markers were available, and the outgroup species *Hyphochytrium catenoides*.

Eurychasma dicksonii LSU rRNA gene sequences were assembled using the Geneious™ software (Drummond et al. 2009) and, if necessary, manually corrected. Additional LSU rRNA, SSU rRNA and *cox2* sequences were retrieved from GenBank (Supp. Tab. S1). The hyphochytrid *Hyphochytridium catenoides* and *Developayella elegans*, among the closest relatives to the oomycetes (Riisberg et al 2009) with respective sequences available in GenBank, were chosen as outgroup species for the LSU analysis, while sequences of the nearest known relatives beyond the Hyphochytrids, the Labyrinthulida *Thraustochytrium aureum* and the Bicosoecida *Caecitellus parvulus*, could not be unambiguously aligned with the ingroup over large portions of the LSU gene. No *cox2* sequence was available for *D. elegans*, leaving *H. catenoides* as the sole outgroup species for the combined analysis.

All sequences were initially aligned using MUSCLE on Geneious™ and then manually corrected in Se-Al v2.0a11 (Rambaut 2007). Ambiguously alignable positions were removed using the online tool GBlocks vs 0.91b (Castresana 2000, Talavera & Castresana 2007) under the following conditions: Smaller final blocks and gap positions within the final blocks were allowed, while areas of contiguous nonconserved positions were removed.

Phylogenetic analyses under the Maximum Likelihood (ML) criterion were performed on both data sets using the default GTRgamma model of rate heterogeneity in RAxML v.7.2.2 (Stamatakis 2006), with thorough bootstrap resampling set to 1000 replicates. All alignments were deposited in TreeBase accessible via http://purl.org/phylo/treebase/phylows/study/TB2:S13577?x-access-code=1627c81b884a86fb69436ea376dcf021&format=html.

Results

Detection of brown algal pathogens in the East Mediterranean Sea by microscopy

During the first visit to the Lesvos study sites the algal thalli were still very small and hardly pigmented. They showed strong diatom epiphytism and were not fertile. Within the next two weeks (around 23 Feb 2009), biomass in these rock pools increased visibly and plants became fertile (mainly plurilocular sporangia could be observed). With the onset of host growth, we could detect three different parasite species (Tab. 1).

The oomycete *E. dicksonii* was identified microscopically with a few developing thalli and numerous empty *E. dicksonii* sporangia (Fig. 1 a) that exhibited the net pattern characteristic for this species (Petersen 1905, Sekimoto et al. 2008). The pathogen was found parasitic on a host identified as *Acinetospora* sp.. Despite having analysed over 2000 *Ectocarpalean* samples, *E. dicksonii* could only be detected in one specimen over the six week sampling period (Tab. 1).

A second pathogen affecting Ectocarpales was detected in six independent samples from three different localities (Tab. 1). Parasitic spherical thalli developing endobiotically were detected in vegetative, apical cells (Fig. 1 b). In most cases a single parasitic thallus was observed developing holocarpically in the host cell; in rare cases two thalli were observed. The mature sporangium filled the host cell completely (Fig. 1 b). The evacuation tubes broke the algal cell wall in all instances and ended outside the host. Spores were not observed. Based on the morphology of the different infection stages and the host algae parasitized, this pathogen was identified as the hyphochytrid *Anisolpidium ectocarpii* Karling (1943).

A third pathogen infecting *Sphacelaria* sp. was detected in abundance from several localities around Lesvos and Panagia / Astris Island off Thasos (Tab. 1; Fig. 2). Infection structures were exclusively observed in the apical cells of the host (Fig. 2 a). The number of parasitic thalli developing within a single host cell varied between one and three (Figs. 2 a, b). Each parasitic sporangium showed one short evacuation tube (Fig. 2 c). In one instance, a parasitic spore could be detected at the surface of the host cell (Fig. 2 d). Based on the morphology of the different infection stages and the host algae, the pathogen was identified as the hyphochytrid *Anisolpidium sphacellarum* (Kny*)* Karling (1943).

While we could detect three different pathogens infecting brown algae around the coast of Lesvos, on Panagia / Astris Island we only found *A. sphacellarum* (Tab. 1).

Phylogenetic analysis of partial E. dicksonii LSU rRNA gene sequences from various localities and combined Maximum Likelihood Analysis

Due to limited quantities of infected host material and additional restrictions we did not succeed to establish live host-pathogen cultures. Nevertheless, by using silica-dried material of our Lesvos *E. dicksonii* collection (Eur Lesvos 34-4), we were able to acquire LSU rRNA gene sequence information of this Mediterranean sample and 11 additional *Eurychasma* samples from other parts of the world (Suppl. Tab. S1). In the present study, a new *Eurychasma*-specific primer CG68 was developed which targets the beginning (within the first twenty nucleotides) of the LSU rRNA gene. In the case of the *E. dicksonii* isolate from Lesvos), the amplification of the LSU rRNA gene with the primer combination CG68 / ITS1170R resulted in a specific PCR product with a length of 1087 bp. The alignment of the 12 individual *Eurychasma* LSU rRNA gene sequences resulted in a pairwise identity of 99.7%, with most mismatches to ambiguously called bases in either sequence. The nucleotide sequences were deposited at the European Nucleotide Archive (EMBL) under the accession numbers FR696310 - FR696320 (Suppl. Tab. S1).

In the LSU rRNA tree (Fig. 3), the positions of the major clades (i.e. the main "Saprolegnian" and "Peronosporalean" lines) within the oomycete ingroup could not be resolved, but they had a bootstrap support of 100% in the tree based on the combined data set of LSU rRNA, SSU rRNA and *cox2* gene sequences (Fig 4). In accordance with earlier studies (Küpper et al. 2006, Sekimoto et al. 2008) , the *E. dicksonii* strains formed a 100% supported monophyletic clade in both trees, which was positioned at the basis of the oomycete lineage, as the very first clade to branch off. The next clade to branch off in both trees contained species infecting crustaceans and shellfish, such as *Haliphthoros*, *Halodaphnea,* and *Halioticida*. However, this clade did not have sufficient bootstrap support in the LSU rRNA gene analysis (Fig. 3). Likewise, the relationship between the two entities included in the combined analysis, *Haliphthoros milfordensis* and *Haliphthoros*-like strain NJM 0034 did not have sufficient support (Fig. 4), suggesting that more species and more genes may need to be included in future studies, in order to fully resolve the relationships within this group of pathogens. The remaining oomycetes formed two well supported monophyletic clades, the Saprolegnian and the Peronosporalean clades (Figs.3 and 4). While the position of *Sapromyces elongatus* at the base of the Peronosporales did not have support in the LSU tree, it was included in that order in the combined analysis, with 91% support.

Discussion

The six-week long field work in Greece around the coastline of Lesvos and in Kavala/Thasos aimed to identify intracellular pathogens challenging brown seaweeds, with particular emphasis on hosts in the order Ectocarpales and Sphacelariales. The early spring season was chosen based on previous reports and observations according to which Ectocarpales are predominant in early spring due to lower water temperatures (Taskin & Ozturk 2007). Indeed the timing proved to be suitable since increasing abundance of host algae could be observed in early spring 2009. The target algae (mainly Ectocarpales and Sphacelariales) were not found at the same localities during a second campaign conducted in late October 2009. However, it cannot be ruled out that these species were still present in deeper waters which were not accessible to the sampling regime. *Eurychasma dicksonii* has previously been reported from the Western Mediterranean Sea, Italy and former Yugoslavia (Hauck 1878, Ercegovic 1955, Giaccone & Bryce Derni 1971, Strittmatter et al. 2009). In the present study, its occurrence in the Eastern Mediterranean Sea was demonstrated for the first time. The scarcity of findings during the six week long field work may have been caused by the well-known seasonality of the Mediterranean Ectocarpalean flora (Taskin & Ozturk 2007) . From our routine laboratory observations on the pathosystem, it appears plausible that *Eurychasma* infections might have been more abundant a few weeks later (early April) since the infection success of this obligate-biotrophic pathogen greatly depends on a good physiological status of the host. However, no comprehensive field studies are currently available addressing the question of *Eurychasma* seasonality with regard to host abundance and host status.

In addition to the already available marker genes of SSU rRNA and *cox2* (Küpper et al. 2006, Sekimoto et al. 2008), the first LSU rRNA gene sequence information of multiple *E. dicksonii* strains is provided in this study. The LSU rRNA gene has been used as a suitable marker for the phylogenetic reconstruction of the oomycete lineage over a decade ago which however did not include any of the so-called basal oomycetes (Petersen & Rosendahl 2000). Based on our experience, the amplification of the LSU rRNA gene of *E. dicksonii* proved to be less difficult compared to the SSU gene for which in many instances only fragmentary information could be obtained (Gachon et al. 2009). The LSU rRNA marker was suitable to identify the *E. dicksonii* isolate from Lesvos confirming the identification based on light microscopic analysis of the sample. However strain-specific differences with regard to their geographic origin - if any – were minimal as detected by this marker. The results obtained from the phylogenetic analysis of the LSU rRNA gene sequence confirmed the most basal position of this biotrophic seaweed pathogen within the oomycetes which is in agreement with SSU rRNA and *cox*2 gene sequence data on *E. dicksonii*

(Sekimoto et al. 2008). It is evident that more subclasses have to be created to accommodate especially the basal oomycetes (reviewed by Beakes et al. 2012). A multigenic approach is likely to solve the phylogenetic relationships among the oomycetes as previously demonstrated for the genera *Pythium* and *Phytophthora*, but also for the phylogenetic relationship between different heterokont lineages (Riisberg et al. 2009, Robideau et al. 2011). However, currently sequence information on different marker genes is in many instances restricted and fragmentary which especially holds true for the basal oomycetes (e.g. Hakariya et al. 2009, Muraosa et al. 2009, Sekimoto et al. 2009). The combined data set used in this study presents an updated view, but phylogenetic relationships could not be fully resolved, due to this lack of multi-gene sequence information for many basal oomycetes.

In contrast to *E. dicksonii*, the pathogens classified as *A. ectocarpii* and *A. sphacellarum* were found in numerous samples during this field work. *A. ectocarpii* has been reported to infect *Ectocarpus siliculosus* and *Ectocarpus mitchellae* (now *Hincksia mitchellae*; summarized by Sparrow 1960), while *A. sphacellarum* (formerly described as *Chytridium sphacellarum* (Kny), *Pleotrachelus sphacellarum* (Kny) Petersen and *Olpidium sphacellarum* (Kny) Fischer) has been described from hosts of the order of Sphacelariales including the genera *Cladostephus* sp., *Chaetopteris* sp. and *Sphacelaria* sp. (summarized by Sparrow 1960). Members of the Sphacelariales show apical growth pattern (Katsaros 1995). These cells are strongly polarized with a continuous flow of membrane material and polysaccharides to the tip of the cell (apical dome) resulting in cell wall expansion. In this region the cell wall is very thin and consists of only two layers whereas the cylindrical part of the apical cell consists of four layers (reviewed by Katsaros et al. 2006). In light of this, it is noteworthy that the infection by *A. sphacellarum* seems to be restricted to apical cells whereas other cells appear to be unaffected. In this instance a thinner cell wall or different composition of the cell wall could facilitate pathogen penetration and favour the infestation of apical host cells (Klochkova et al. 2012).

Apart from *Anisolpidium ectocarpii* and *A. sphacellarum* more species have been described for this genus (Tab. 2) including *A. rosenvingei, A. joklianum*, and *A. olpidium* (formerly *Pleotrachelus olpidium*) which infect morphologically very similar Ectocarpales species. However, Dick (2001) lists the latter two species of *Anisolpidium* as doubtful species. Although *A. sphacellarum* infects a different brown algal order compared to *A. ectocarpii, A. joklianum, A. olpidium* and *A. rosenvingei*, it cannot be said with certainty whether *A. sphacellarum* is indeed a different species bearing in mind that algal pathogens may have a broad host range, as for instance *E. dicksonii* which is able to infect around 13 different orders of brown algae (Müller et al. 1999).

Based on the scarcity of reports on infections by members of the genus *Anisolpidium* and considering the limited associated knowledge (microscopic observation of field material and permanent microscope slides), it cannot be determined at this point whether the pathogens observed in this study can actually be classified in *A. ectocarpii* and *A. sphacellarum* or whether more species of *Anisolpidium* exist. Ultimately, sequence data will be necessary to resolve not only the question about different species of *Anisolpidium* but also the phylogenetic position of this brown algal pathogen. So far, no molecular data are available for any members of the Anisolpidiaceae, Hyphochytriaceae and Rhizidiomycetaceae (Hyphochytriomycetes), which hampers the designs of PCR primers. First attempts with a hyphochytrid-specific primer (primer MS19: 5'-TCMAWCACCCAAGGGC-3') designed based on SSU rRNA sequence information of the only two hyphochytrids *Hyphochytridium catenoides* (X80344, Hausner et al. 2000) and *Rhizidiomyces apophysatus* (AF163295, Van der Auwera et al. 1995) were unsuccessful and we did not succeed to generate molecular information for our *Anisolpidium* samples. Therefore we have to rely on a classification based on morphological characteristics and host species.

It should be noted that our specimens of *A. ectocarpii* from Lesvos represent the first record of this species in Europe (Tab. 2). *A. sphacellarum* has previously been reported from various sites in Europe including France, Italy, Germany, Denmark, Sweden and Great Britain as well as in the USA and Japan (Tab. 2), but our findings are nevertheless the first in the Eastern Mediterranean and Greece. Despite the increasing occurrence of alien species in the Mediterranean (e.g. Boudouresque & Verlaque 2002, Streftaris & Zenetos 2006), there is no reason to assume that these pathogens are alien; it seems reasonable to assume that they have been overlooked by previous investigators.

All pathogens detected here display an intracellular life mode inside their host species except for their infective, motile stages. Their ecological implications on the algal hosts remain largely unknown. Generally, the infected host cells in a filament die off, but neighbouring cells are usually not affected. In the case of *Eurychasma*, infection results in fragmentation due to reduced mechanical resistance of affected cells after completion of the infection cycle and, consequently, vegetative propagation of host filaments (Wilce et al. 1982). The ecology of the two *Anisolpidium* species covered here is much less clear. Another representative of this genus, *A. rosenvingei*, targets exclusively sporangia of *Pylaiella* sp., a close relative of the Ectocarpalean brown algae covered here – epidemic outbreaks of this pathogen are thus inevitably linked to periods of high host fertility (Küpper & Müller 1999).

In summary, focussing on few brown algal families, this work demonstrated the presence of three eukaryotic pathogens in the East Mediterranean Sea with no previous records. Two of the

pathogens covered here have so far barely been described in the literature. Other authors are also contributing to filling this knowledge gap about seaweed pathogens worldwide (West et al. 2006, Sekimoto et al. 2009, Klochkova et al. 2012). Their work and ours provide circumstantial evidence that hints to an equal prevalence, and possibly equal ecological importance of seaweed parasites in warm seas compared to comparatively better known temperate ecosystems. Increased sampling effort (e.g. periodic, more diverse collection) will undoubtedly unravel more and so far undescribed organisms challenging macroalgae, and will aid to understand the impact on their host from ecological and genetic points of view.

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Table 1: Localities and microscopic diagnosis of samples collected during the field work on Lesvos Island (Greece).

Sample ID	Sampling locality	Habitat	Sampling date	Algal host
Eurychasma dicksonii				
34.4	Skala Neon Kydonion; 39° 14' 03 N 26° 27' 18 E	rock pools	02 Mar 09	*Acinetospora sp.*
*Anisolpidium ectocarpii**				
5	Skala Neon Kydonion; 39° 14' 03 N 26° 27' 18 E	rock pools	13 Feb 09	n/a
12.9	Skala Neon Kydonion; 39° 14' 03 N 26° 27' 18 E	rock pools	21 Feb 09	n/a
16	Aspropotamos; 39° 16' 47 N 26° 22' 40 E	rock pools	21 Feb 09	n/a
34.4	Skala Neon Kydonion; 39° 14' 03 N 26° 27' 18 E	rock pools	02 Mar 09	n/a
50.3	Skala Neon Kydonion; 39° 14' 00 N 26° 27' 12 E	epiphytes on harbour rope (1-2 m depth)	06 Mar 09	*Feldmannia* sp.
68	Skala Eresou; 39° 07' 44 N 25° 56' 08 E	on rocky surface at 3 m depth	09 Mar 09	*Feldmannia* sp.
*Anisolpidium sphacellarum**				
52.1	Skala Neon Kydonion; 39° 14' 27 N 26° 27' 11 E	epiphytes on fishing line	06 Mar 09	*Sphacelaria* sp.
74.3	Charamida ; 39° 00'58 N 26° 33'24 E	1 m depth on rocks: epiphytes	10 Mar 09	*Sphacelaria* sp.
010512-107	Panagia Island 40°33′33 N 24°37′13 E	> 15 m depth, epiphytes	01 May 12	*Sphacelaria* sp.
010512-109	Panagia Island 40°33′33 N 24°37′13 E	> 15 m depth, epiphytes	01 May 12	*Sphacelaria cirrhosa*
010512-111	Panagia Island 40°33′33 N 24°37′13 E	> 15 m depth, epiphytes	01 May 12	*Sphacelaria cirrhosa*

* identification based on morphology and host species

Table 2: Overview of *Anisolpidium* species described in the literature. *A. sphacellarum*, *A. ectocarpii* and *A. rosenvingii* are the currently recognized species whereas *A. minutum*, *A. joklianum* and *A. olpidium* are listed as doubtful species (Dick, 2001)
[*] Sparrow, 1960
[**] Küpper and Müller, 1999
[***] Dick, 2001
[****] Küpper et al., unpublished

Species	**Geographic record**	**Algal host species**
Anisolpidium sphacellarum[*]	Great Britain	*Cladostephus spongiosus*
	Germany (Helgoland)	*Cladostephus spongiosus*
	Denmark	*Chaetopteris plumosa*, *Sphacelaria cirrhosa*
	France	*Cladostephus verticillatus* *Sphacelaria cirrhosa*
	Ireland	*Sphacelaria sp.* *Cladostephus sp.*
	Italy	*Sphacelaria tribuloides*
	Japan	*Sphacelaria apicalis*, *Sphacelaria subfusca*
	Sweden	*Chaetopteris plumosa* *Sphacelaria sp.*
	United States	*Sphacelaria cirrhosa*
Anisolpidium ectocarpii	United States (East Coast)[*]	*Hincksia mitchellae*, *Ectocarpus siliculosus*
	Japan[****]	
	Chile[****]	
Anisolpidium rosenvingii	France[**]	*Pylaiella littoralis*
	Ireland[**]	*Pylaiella littoralis*
	Sweden[*]	*Pylaiella littoralis*
	Denmark[*]	*Pylaiella littoralis*
Anisolpidium minutum[***]	Denmark	*Chorda filum*
Anisolpidium joklianum[***]	Italy	*Hincksia granulosa*
Anisolpidium olpidium[***]	Denmark (Faroes Islands)	*Ectocarpus siliculosus*

Figure 1:

Micrographs of two intracellular pathogens in an Ectocarpalean host: The algal host showed numerous pathogenic sporangia of the oomycete pathogen *Eurychasma dicksonii* with the characteristic net sporangium (a, arrow). *Anisolpidium ectocarpii* (b) in this instance was found co-infecting the same sample. Infection structures were always found in apical cells and showed narrow evacuation tubes (arrow).

Scale bars: 25 µm.

Figure 2:

Microphotographs of the intracellular pathogen infecting *Sphacelaria* sp. identified as *Anisolpidium sphacellarum*: Intracellular infection structures stained with 0.2% (w/v) acetocarmine (a, b) were observed in the host's apical cells (a). The number of pathogenic thalli in a single apical cell varied (a, b and d). Each sporangium showed a single narrow evacuation tube (c, arrows). In the apical cell in d two pathogenic thalli developed and an empty, pathogen-derived spore could be detected at the surface of the alga (arrow and inlet). The pathogens illustrated in a, b and c were found on Lesvos and the pathogen shown in d was found on Thasos. Scale bars in a, b, c: 50 µm and in d 10 µm.

Figure 3:

Maximum likelihood tree based on LSU rRNA gene sequence data of 42 oomycetes containing 12 *Eurychasma dicksonii* strains from different geographic origin including one isolate from Lesvos (Aegean Sea, Greece). Two taxa, the hyphochytrid *Hyphochytridium catenoides* and *Developayella elegans* are used as outgroup. Bootstrap values in % represent 1000 replicates, bootstrap values below 50% are omitted.

Figure 4:

Maximum likelihood tree of the combined LSU rRNA, SSU rRNA and *cox2* sequence alignments of 18 oomycetes including the two *Eurychasma dicksonii* strains from Shetland Islands (96) and Brittany (05). *Hyphochytridium catenoides* is used as outgroup. Bootstrap values in % represent 1000 replicates, bootstrap values below 50% are omitted.

The following supplement accompanies the article

New records of intracellular eukaryotic pathogens challenging brown macroalgae in the East Mediterranean Sea, with emphasis on LSU rRNA data of the oomycete pathogen *Eurychasma dicksonii*

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Supplementary Table S1: LSU rRNA gene sequence information of *Eurychasma dicksonii* strains of various geographic origin. The isolate from Lesvos (Greece) is highlighted in bold.

Sample ID	**Strain details**	**Geographic origin of strain and year of isolation**	**Isolator**	**LSU gene sequence length (bp)**
Eur 05	monoeukaryotic live culture CCAP 4018/1	Le Caro, Brittany (France), 2005	D.G. Müller	933
Eur 96	monoeukaryotic live culture CCAP 4018/2	Bressay, Shetland (United Kingdom), 1996	D.G. Müller	1083
Eur 06-29-2	monoeukaryotic live culture	Dunstaffnage Bay, Oban (United Kingdom), 2006	C.M.M. Gachon	1085
Eur 06-29-4	monoeukaryotic live culture	Dunstaffnage Bay, Oban (United Kingdom), 2006	C.M.M. Gachon	1085
Eur 06-29-5	monoeukaryotic live culture	Dunstaffnage Bay, Oban (United Kingdom), 2006	C.M.M. Gachon	1084
Eur 06-29-7	monoeukaryotic live culture CCAP 4018/3	Dunstaffnage Bay, Oban (United Kingdom), 2006	C.M.M. Gachon	1087
Eur 07-1	monoeukaryotic live culture	Dunstaffnage Bay, Oban (United Kingdom), 2007	M. Strittmatter	649
Eur 07-2	monoeukaryotic live culture	Dunstaffnage Bay, Oban (United Kingdom), 2007	M. Strittmatter	1083
Eur 34-4	field-collected sample	Skala Neon Kydonion, Lesvos (Greece), 2009	M. Strittmatter	1087
Eur FI-S63	monoeukaryotic live culture	Falkland Islands, 2007	D.G. Müller	1087
Eur US-385B	monoeukaryotic live culture	Ushuaia (Argentina), 2007	D.G. Müller	1085

Supplementary Table S2: Species and sequence information of various oomycetes, the hyphochytrid *Hyphochytrium catenoides* and *Developayella elegans* used for phylogenetic analysis of LSU rRNA gene sequence data and the combined analysis of LSU rRNA, SSU rRNA and *cox2* gene sequences. Taxa defined as outgroup are underlined.

Species	**GenBank accession no. (reference)**
Achlya bisexualis	**LSU**: AF218187 (Leclerc et al. 2000)
Albugo candida	**LSU**: AF235938 (Petersen & Rosendahl 2000); **SSU**: JF504671 (Lara & Belbahri 2011); **Cox2**: AY927047 (Choi et al. 2006)
Aphanomyces astaci	**LSU:** AF235940 (Petersen & Rosendahl 2000)
Aphanomyces laevis	**LSU**: HQ665242 (Robideau et al. 2011); **SSU**: HQ343196 (unpublished); **Cox2**: HQ680584 (unpublished)
Aphanomyces piscicida	**LSU:** AF235941 (Petersen & Rosendahl 2000)
Atkinsiella dubia	**LSU**: AB285221 (Muraosa et al. 2009); **SSU** AB284575 (unpublished); **Cox2**: AF290312 (Cook et al. 2001)
Basidiophora entospora	**LSU**: EU287694 (unpublished)
Bremia lactucae	**LSU**: EF553478 (Voglmayr & Constantinescu 2008)
<u>*Developayella elegans*</u> CCAP1917/1	**LSU** FJ030882 (Riisberg et al. 2009)
Eurychasma dicksonii strain 05	**LSU**: FR696317 (this study); **SSU**: AB368176 (Sekimoto et al. 2008); **Cox2**: AB368177 (Sekimoto et al. 2008)
Eurychasma dicksonii strain 96	**LSU**: FR696318 (this study); **SSU**: AY032607 (Küpper et al. 2006); **Cox2**: AB368178 (Sekimoto et al. 2008)
Eurychasma dicksonii strain 06-29-2	**LSU**: FR696312 (this study)
Eurychasma dicksonii strain 06-29-4	**LSU**: FR696313 (this study)
Eurychasma dicksonii strain 06-29-5	**LSU**: FR696314 (this study)
Eurychasma dicksonii strain 06-29-7	**LSU**: FR696311 (this study)
Eurychasma dicksonii strain 07-1	**LSU**: FR696315 (this study)
Eurychasma dicksonii strain 07-2	**LSU**: FR696316 (this study)
Eurychasma dicksonii strain 34-4	**LSU**: FR696310 (this study)
Eurychasma dicksonii strain FI-S63	**LSU**: FR696320 (this study)
Eurychasma dicksonii strain US-385B	**LSU**: FR696319 (this study)
Halioticida noduliformans NJM 0631	**LSU**: AB285230 (Muraosa et al. 2009)
Haliphthoros milfordensis	**LSU**: AB285218 (Muraosa et al. 2009); **SSU**: AB178868 (Sekimoto et al. 2007); **Cox2**: AB178870 (Sekimoto et al. 2007)
Haliphthoros sp. NJM 0440	**LSU:** AB285225 (Muraosa et al. 2009)
Haliphthoros sp. NJM 0443	**LSU:** AB285226 (Muraosa et al. 2009)
Haliphthoros-like NJM 0034	**LSU**: AB178866 (Sekimoto et al. 2007); **SSU**: AB178865 (Sekimoto et al. 2007); **Cox2**: AB178867 (Sekimoto et al. 2007)
Halodaphnea baliensis	**LSU**: AB285222 (Muraosa et al. 2009)
Halodaphnea panulirata	**LSU**: AB285224 (Muraosa et al. 2009)
Halodaphnea parasitica	**LSU**: AB285223 (Muraosa et al. 2009)
Hyaloperonospora parasitica	**LSU**: AF235957 (Petersen & Rosendahl 2000); **SSU**: AY742752 (unpublished); **Cox2**: AY286223(Hudspeth et al. 2003)
Halophytophthora batemanensis	**LSU**: DQ361227 (Göker et al. 2007); **SSU**: GU994182 (unpublished); **Cox2**: DQ365703 (Göker et al. 2007)
<u>*Hyphochytrium catenoides*</u>	**LSU**: EF594059 (Gleason et al. 2008); **SSU**: AF163294 (Hausner et al. 2000); **Cox2**: AF086701 (Hudspeth et al. 2000)
Lagenidium chthamalophilum	**LSU**: AF235946 (Petersen & Rosendahl 2000)

Lagenidium giganteum	**LSU**: JQ745265 (unpublished); **SSU**: M54939 (Förster et al. 1990); **Cox2**: AF086697 (Hudspeth et al. 2000)
Leptolegnia caudata	**LSU**: AF218176 (Leclerc et al. 2000) **SSU**: AJ238659 (Dick et al. 1999), **Cox2**: AF086693 (Hudspeth et al. 2000)
Peronophythora litchii	**LSU**: AF235949 (Petersen & Rosendahl 2000); **SSU**: AY742750 (unpublished); **Cox2**: AF086698 (Hudspeth et al. 2000)
Peronospora ficariae	**LSU:** AF119600 (Riethmüller et al. 2000)
Phytophthora infestans	**LSU** HQ665217 (Robideau et al. 2011); **SSU**: AY742761 (unpublished); **Cox2**: JF771456 (unpublished)
Phytophthora sojae	**LSU**: EU079794 (Blair et al. 2008); **SSU**: AY742749 (unpublished); **Cox2**: DQ365753 (Göker et al. 2007)
Pythiopsis cymosa	**LSU**: DQ393490 (Hulvey et al. 2007); **SSU**: AJ238657 (Dick et al. 1999); **Cox2**:AF086689 (Hudspeth et al. 2000)
Pythium monospermum	**LSU**: AY035535 (Riethmüller et al. 2002); **SSU**: AJ238653 (Dick et al. 1999); **Cox2**: DQ365765(Göker et al. 2007)
Saprolegnia parasitica	**LSU**: HQ665197 (Robideau et al. 2011); **SSU**: HQ384412 (unpublished); **Cox2**: HQ660429 (unpublished)
Sapromyces elongatus	**LSU**: AF235950 (Petersen & Rosendahl 2000); **SSU**: AB548399 (unpublished); **Cox2**: AF086700 (Hudspeth et al. 2000)
Thraustotheca clavata	**LSU**: HQ665268 (Robideau et al. 2011)