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# *Microspongium alariae* in *Alaria esculenta*: a widely-distributed non-parasitic brown algal endophyte that shows cell modifications within its host

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**Abstract:** *Alaria esculenta* is an important kelp species in northern Europe, Atlantic Canada and USA and the Arctic, with high economic potential. *Microspongium alariae*, a brown algal endophyte using *A. esculenta* as host, is reported for the first time from Scotland (Great Britain) and Brittany (France), suggesting a wide distribution in NW Europe. The alga was found growing epi-endophytically in *A. esculenta* stipes and was occasionally associated with warts. Isolated *Microspongium* thalli grew in host-free cultures and formed plurilocular sporangia in a broad range of temperature and irradiance conditions. DNA barcoding using the nuclear ribosomal ITS1, the mitochondrial COI and the plastidial *rbc*L confirmed the identity of the endophyte as *M. alariae*. Electron microscopy was used to compare the alga when endophytic in *Alaria* with a host-free culture. As an endophyte, cell diameter, pyrenoid diameter and cell wall thickness were reduced. In contrast, there were more plasmodesma connections between endophyte cells, possibly to enhance nutrient transport along the endophytic thallus. In the light of this evidence, a parasitic life style is considered unlikely for the species and the adaptive value of endophytism in *M. alariae* remains to be elucidated.

**Keywords:** Chordariaceae; COI; ITS1; *rbc*L; ultrastructure.

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## Introduction

*Alaria esculenta* (L.) Greville (a.k.a. winged kelp or Atlantic Wakame) is a large brown alga with populations encompassing the lowermost intertidal and the sublittoral in the northern hemisphere, where water temperatures do not exceed 16°C (Munda and Lüning 1977). It is the only species of *Alaria* in the North Atlantic. In Europe, the species is distributed in the British Isles, Faroe Islands, Iceland, Norway and the Swedish West coast, the Netherlands and France (see Guiry and Guiry 2017 and references therein). On the North American Atlantic coast, it occurs north of Cape Cod (Villalard-Bohnsack 1995), and it is a common kelp in the Arctic (Küpper et al. 2016). In addition to the significant importance of kelps to marine ecosystems (Dayton 2006), this species was used as food in the past, but currently it does not have noticeable landings in European fishery statistics (Mouritsen 2013). However, knowledge of mastering its cultivation has been substantially increased due to its use in gourmet cuisine (Chapman et al. 2015) and its potential for biofuel production (López Barreiro et al. 2015).

Like any organism, *Alaria* may be a partner in host-pathogen interactions, with so far only ascomycetes and algal endophytes being reported. Fungal symbionts in *A. esculenta* are limited to *Phycomelaina laminariae* (Rostrup) Kohlm., discovered once (Kohlmeyer 1968, Kohlmeyer and Kohlmeyer 1979). In contrast, there are several records of a brown algal endophyte infecting wild populations of *A. esculenta*. One of them corresponded to *Laminariocolax tomentosoides* (Russell 1964). The most common endophyte though is classified in the genus *Microspongium* (Peters 2003). As facultative endophytes (concept revisited by Correa 1994) of red or brown algae, *Microspongium* species may be found either growing as epibionts or sometimes deeply penetrating their hosts, and their presence may be associated with dark spots or areas or with deformations, such as warts, galls or twisted

thalli, the latter possibly due to the infection of the meristematic zone (Pedersen 1981, Peters 2003). *Microspongium alariae* has been found infecting *Alaria esculenta* but also other laminarean and fucalean species (Hardy and Guiry 2003, Peters 2003). *Microspongium alariae* (P.M. Pedersen) A.F. Peters was first described from Greenland by Pedersen (1981) as *Gononema alariae* P.M. Pedersen, after a reclassification from the genus *Entonema* Reinsch initially made by Jaasund (1965). It has also been recorded from Maine (USA), where it infected both *Alaria* and *Saccharina latissima* C.E. Lane, C. Mayes, Druehl and G.W. Saunders, and Tvärminne (Finland, inner Baltic Sea) where it was isolated from *Fucus* Linnaeus (Peters 2003). Using ITS1 and *rbc*L sequences, Peters (2003) classified *G. alariae* in *Microspongium* within Chordariaceae. *Microspongium alariae* has so far only been found as macroalgal endophyte in brown algae, in contrast to the closely related *M. tenuissimum* (Hauck) A.F. Peters, which was isolated from the interior of red algae (Burkhardt and Peters 1998). They have also been isolated from other substrata, viz. hydroids and pebbles and old shells, respectively (Pedersen 1981, Peters et al. 2015).

The effects of *M. alariae* on its host populations are not known. It can infect stipes and sporophylls of *A. esculenta* (Peters 2003) often causing warts (may not always be present, authors' personal observations), and may represent a potential disturbance in both natural stocks but also in the emergent aquaculture of this kelp. Details of the cell biology and the host-pathogen interface of *M. alariae* have not yet been studied. We have recently isolated *M. alariae* from Scottish and French populations of *Alaria*. After confirmation of the pathogen's identity by DNA barcoding, thalli of this species have been used in the present work to investigate the development and the ultrastructure of the endophyte inside its natural host or as a free-living culture.

## Materials and methods

### Field sampling

Individuals of *Alaria esculenta* were collected in October 2014 at two localities on the Scottish coast, at (1) Bullers O'Buchan, Aberdeenshire (57° 25′ 33″ N, 1° 49′ 6″ W, North Sea), 3 m depth below the low tide, by free diving (four specimens), and (2) Seil Island, Argyll (56° 17′ 26″ N, 5° 38′ 15″ W, West coast), beach cast (three specimens) (Figure 1).

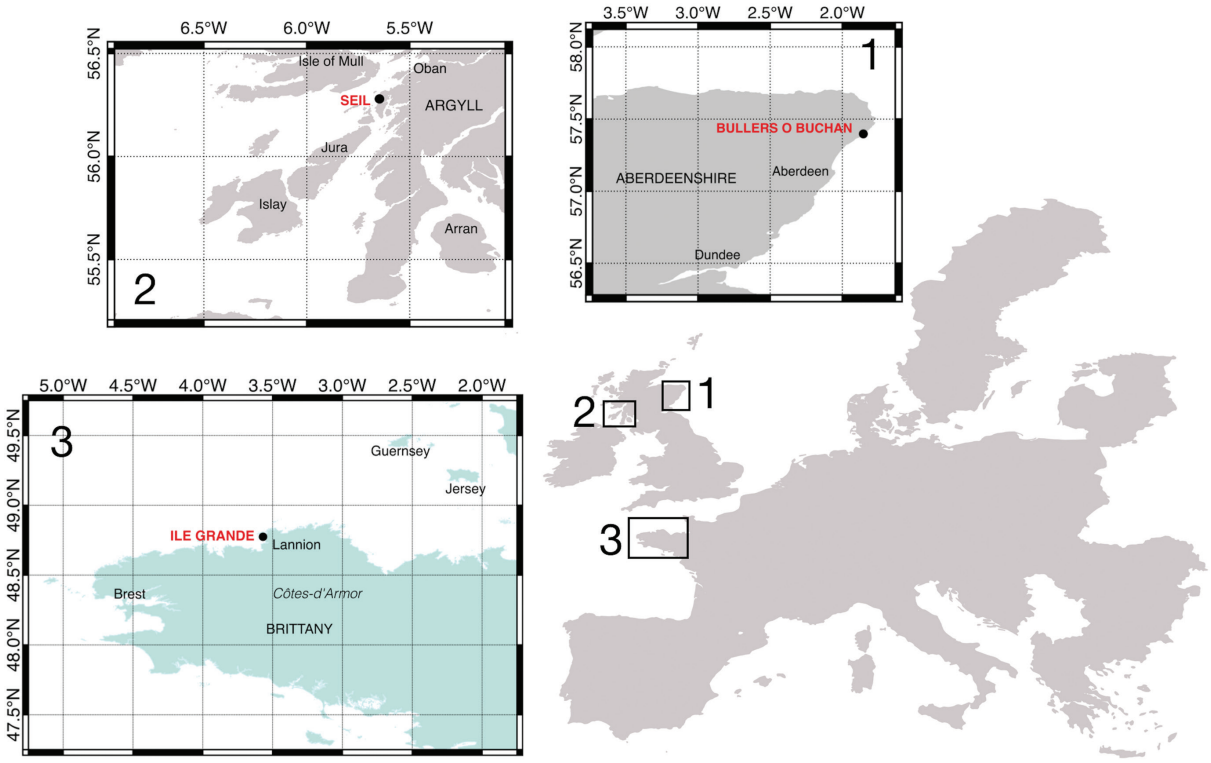


**Figure 1:** *Alaria esculenta* collection sites in Scotland, United Kingdom (1–2) and Brittany, France (3). Strain localities: (1) Mala CB, (2) Mala OB (CCAP 1317/1) and (3) Endo Aesc BR16-22 (CCAP 1317/2).

Additionally, an *A. esculenta* specimen from (3) the North coast of Brittany (48° 48′ 13″ N, 3° 35′ 15″ W, Ile Grande, Côtes-d'Armor) was collected in November 2016 (Figure 1). Samples were characterized by presence of dark surfaces or spots, warts or galls in stipes or fronds.

## Culture studies

After confirmation of endophyte presence, raw cultures (12–18 per locality) were initiated from transverse sections of the affected areas. The cortex was removed to avoid contamination from undesirable spores or epiphytes on the host surface. The resulting medullar fragments were cultured in Provasoli-enriched autoclaved seawater (PES, Starr and Zeikus 1993) in dishes containing 8 ml medium in white fluorescent light (General Electric, 35 W) at 40 µmol photons $m^{-2}$ $s^{-1}$, 12 h $day^{-1}$ photoperiod and 12°C. After 4–6 weeks, filaments emerged from the host tissues; apical fragments of them were cut with sterile surgical blades or Pasteur pipettes with sharp edges and transferred to new dishes with fresh medium to establish clonal isolates; subcultures were grown under the same conditions. The medium in the early raw cultures was supplied with 4–6 mg $l^{-1}$ germanium dioxide during the first month to suppress diatom growth. Following this approach, one isolate from Bullers of Buchan (Mala CB), one from Seil Island (Mala OB) and one from Ile Grande (Endo AescBR16-22) were produced. The Mala CB isolate is no longer available but the other strains were deposited at the Culture Collection of Algae and Protozoa (CCAP, The Scottish Association for Marine Science) under accessions CCAP 1317/1-2.

Subcultures were incubated at four temperatures (5, 10, 12 and 15°C) and two irradiances (60 and 5 µmol photons $m^{-2}$ $s^{-1}$) measured with an irradiance meter (QSL – 2100, Biospherical Instruments, San Diego, CA, USA), in order to detect developmental or morphological differences under different light and temperature regimes.

## Molecular analyses

From the strains Mala CB, Mala OB, Endo AescBR16-22 and *Microspongium radians* (Hauck) A.F. Peters (strain StraLM isolated 1988 from the red alga *Grateloupia* at Valdivia, Chile, see Burkhardt and Peters 1998), DNA was extracted using either the GeneJet™ DNA extraction kit (ThermoScientific) or the Nucleospin plant II kit (Macherey-Nagel, Germany), following the manufacturer's instructions, but improved by an initial CTAB buffer treatment according to Gachon et al. (2009). Polymerase chain reactions (PCR) were performed to amplify fragments of nuclear ribosomal (ITS1, primers ITSP1 and ITSKG4, Tai et al. 2001, Lane et al. 2006), mitochondrial (5′COI, primers GazF2 and GazR2, Lane et al. 2007) and plastidial DNA regions (*rbc*L, primers rbcL2P and rbcS139R; Peters and Ramírez 2001, Kawai et al. 2007). PCRs were conducted with an initial denaturation at 94°C for 5 min, followed by 35 cycles of amplification consisting of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and elongation at 72°C for 1 min. The 35 cycles were followed by a final extension at 72°C for 5 min. Amplicons were produced in a total volume of 25 µl, containing 2 mM $MgCl_2$, 1.5 µl template DNA, 1 µl of each primer at 10 mM and 21.5 µl Taq ready-mix (VWR® RedTAQ, PA, USA). PCR products were commercially sequenced by the Sanger method, chromatograms checked for quality, and sequences aligned and trimmed with Geneious v11.0.03 (Kearse et al. 2012). Consensus sequences were compared to published data by means of NCBI BLAST searches (Altschul et al. 1997) and imported into Geneious, containing several members of *Microspongium* and Chordariaceae and other representative brown algal taxa, with *Ascoseirophila violodora* Peters serving as outgroup for ITS1 analyses and *Fibrocapsa japonica* S. Toriumi and H. Takano for COI and *rbc*L. MAFFT was used as automated alignment method (Katoh and Standley 2013). Since ITS1 is challenging to align, in this case we first aligned the sequence manually using the areas in the ITS1 close to the SSU and the 5.8s rDNA regions (which have conservative motifs) and then applied MAFFT (Alignment view in Supplemental Figure S1). The final alignments were manually checked to ensure homology and tested by using the Randomized Accelerated Maximum likelihood method (RaxML; Stamatakis 2014) based on the General Time Reversible Model (1000 rapid bootstraps) and Bayesian inference using MrBayes V3.1.6 (Ronquist et al. 2012) (settings: chain length 2000, subsample frequency 1000, burn in of 10%). Sequences were deposited in GenBank with accession numbers MF040292–MF040299.

## Light and electron microscopy

Both *Alaria esculenta* infected tissue (Argyll population) and free-floating endophyte filaments from unialgal cultures (Mala OB) were fixed in 4% paraformaldehyde dissolved in sterile seawater for light microscopy. The samples were dehydrated in an ascending ethanol series (70% and 95% for 2 h and three series of 100%, 3 h each) and defatted/cleared in 1:1 xylene:chloroform solution (three times 1 h). Then, samples were wax-infiltrated

(Cellpath®) by two immersions of 3 h. Final blocks were sectioned at 5 µm on a Leica RM2125RT microtome and stained with 0.05% toluidine blue for 15 s. Bright field and DIC micrographs were obtained from a Zeiss Axio imager D2™ microscope.

For transmission electron microscopy (TEM), similar samples (from diseased and healthy tissues) were immersed for 3 days in fixation buffer (2.5% glutaraldehyde, 0.1 M cacodylate buffer at pH 7.4, 0.5% caffeine, 0.1% $CaCl_2$ and 3% NaCl in Provasoli-enriched seawater, after Murúa et al. 2017). Fixed material (in EM buffer) was washed three times in 0.1 M cacodylate buffer (pH 7.4) with 0.1% $CaCl_2$ and 3% NaCl, stained with 1% Osmium tetroxide in distilled water, washed again twice in distilled water and dehydrated in an increasing ethanol series (30%, 50%, 70%, 95%), followed by three washes in 100% acetone. Infiltration with Spurr's resin was performed by ascending Spurr's solutions dissolved in acetone (7:1, 3:1, 1:1, 1:3, 1:6) until 100% Spurr's. Polymerization was carried out at 70°C and resulting blocks were sectioned with an ultramicrotome (Leica UC6). Final sections cut at 90 nm were stained with lead citrate (3%) and uranyl acetate (2%) and imaged with a JEM-1400 Plus (JEOL) TE microscope with an AMT UltraVue™ camera at the University of Aberdeen microscopy facility.

For the examination of living endophytes, small amounts of biomass were harvested from the cultures, incubated for 15 min in a commercial Calcofluor white (CFW, Sigma-Aldrich™, USA, Missouri) solution (0.01 mg $ml^{-1}$) for β-1-3 and 1–4 glucan detection in the cell wall and mounted on slides in sterile seawater or PES. Imaging was carried out either with a Zeiss Primovert inverted microscope or a Zeiss Axio imager D2™ microscope. For epifluorescence (CFW), live samples were observed with a DAPI filter (excitation: 365 nm, beam splitter: 395 nm, emission: long pass 420 nm). CFW allows the staining of the cell wall, making it easier for measuring cells.

## Cell metrics

To evaluate cell ultrastructure modifications, cell morphometric measurements were calculated using FIJI (Schindelin et al. 2012) in TEM and epifluorescence images, measuring in every (up to 50) endophytic and free-living *Microspongium* cell: cell diameter, nucleus and pyrenoid diameter, chloroplast, cell wall and mitochondrion thicknesses. If more than one organelle was present (e.g. plastids, pyrenoids and mitochondria), only the biggest was measured. In order to compare cell size differences between endophytic and free-living *M. alariae* isolates, Mann-Whitney tests were performed. Plots were drawn using ggplot2 (Wickham 2009).

# Results

## Phylogenetic analyses

Our three *M. alariae* isolates had similar sequences and formed a well-supported clade using either nuclear, mitochondrial or plastidal markers, regardless of the phylogenetic method used. With ITS1 sequences all our *M. alariae* strains formed a clade together with previous *M. alariae* isolates from *Saccharina latissima* (AJ439844) and *A. esculenta* from the Northwest Atlantic (AJ439843) and from Baltic *Fucus* (AJ439845) (Figure 2). The ITS of Mala CB (MF040299) and Mala OB (MF040298) was 300–301 bp in length. Sequence identity among the five *M. alariae* sequences ranged from 94.9 to 99.7%; identities with the sequences in the sister clade (*M. tenuissimum*) were between 81.1 and 87.5%. The 5′-partial COI sequences from Mala CB (MF040293), Mala OB (MF040292) and Endo AescBr16 22 (MF040294) spanned 658 nucleotides.

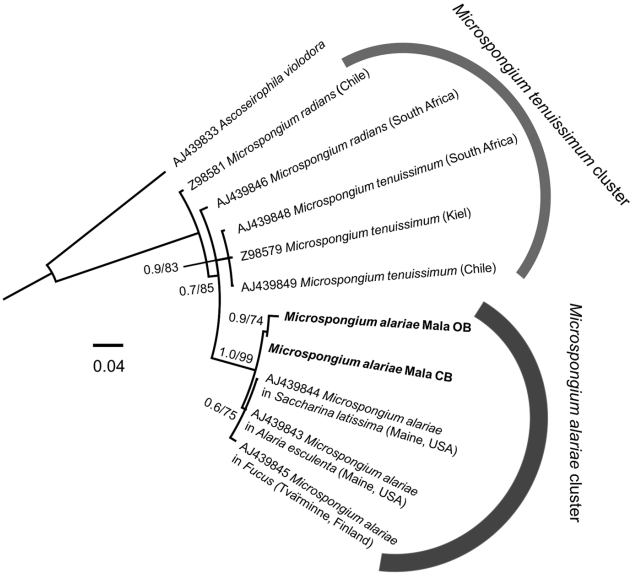


**Figure 2:** *Microspongium alariae* molecular phylogeny. MrBayes cladogram of *M. alariae* and representative taxa sequences of the *Microspongium* genus, based on the ITS1 nrDNA region. This tree contains a total of 11 sequences and 463 positions. Support values correspond to MrBayes posterior probabilities/RAxML boostrap values. Values lower than 0.5 (or 50%) were displayed as a hyphen. The scale bar indicates the number of substitutions per site. Alignment was deposited at figshare.org (https://doi.org/10.6084/m9.figshare.5962567.v1).

In phylogenetic trees inferred from COI our isolates formed a clade with *M. alariae* from the NW Atlantic (LM994993), being consistently separated (genetic distance 3.0–3.4%) from a sister clade comprising *M. radians* and *M. tenuissimum*, (Supplemental Figure S2). In phylogenetic inferences using *rbc*L (Supplemental Figure S3), sequences from our isolates were 1391 bp in length. Mala CB (MF040297) and Mala OB (MF040296) formed a clade together with *Microspongium tenuissimum* and *M. globosum*. As in the COI tree, the *Microspongium* clade was nested in the Chordariaceae.

## Microscopy

The surface of *A. esculenta* stipes and blades was smooth in uninfected areas but it had a "velvety" texture in zones with warts and dark spots (Figure 3a and b). Cross sections of such zones demonstrated the presence of heavily infected tissue with a thickening and disorganization of the outer layers (Figure 3d) in comparison to homogeneous healthy tissue (Figure 3c), in which different tissue layers were well-delimited. The irregular arrangement of the diseased host tissues made it difficult to discern the meristoderm-cortex boundary (which was evident in healthy kelps) and the host-pathogen interface. External filaments of *M. alariae* protruding from the host were uniseriate (average length of 45 µm but could reach up to 80 µm) and unbranched (Figure 3e), and consisted of regular, cylindrical cells (10–15 µm length). Some filaments were shorter (about 25 µm), consisted of cells of *ca.* 5 µm length and represented emerging plurilocular sporangia (Figure 3e).

Internal endophytic filaments were also uniseriate and penetrated deeply into the cortex, often reaching the medullary tissue (Figure 3f). In TEM, endophytic cells were discernible from those of the host by their smaller diameter, position in the interstitium of the host tissue and the organelle composition, in particular well-developed plastids (Figure 3g–j). They were devoid of physodes, in contrast to meristoderm and cortical cells of *A. esculenta* (Figure 3g). Endophytic *M. alariae* cells were 7–12 µm long and 4–7 µm in width, and followed a uniseriate growth from the surface to inner areas of the host tissue (usually perpendicular to the surface). Sometimes, however, this orientation suddenly changed, and the filaments were able to separate adjacent host cells, causing deformations of the latter (Figure 3h). The progress of the filaments was sometimes visualized with a disruption in the intercellular space separated by up to 3 µm from the tip of the endophyte (Figure 3h). Cross and longitudinal sections of the filaments also showed small pyrenoids (median = 800 nm; Figure 3h, inset), and small nucleus surrounded by parietal plastids (Figure 3i). Cell walls were up to *ca.* 200 nm in width. Endophytic adjacent cells almost always shared plasmodesma connections (Figure 3g and i). Plasmodesmata were occasionally seen between neighboring host cells as well, but not in host-endophyte interfaces.

The endophyte was able to grow without host in our laboratory conditions; in contrast, the co-inoculated host cells did not develop. Between 1 and 2 months were needed to observe the first filaments overgrowing the host medulla fragment (Figure 4a). After excision, the few-celled endophyte filaments remained uniseriate, branched and formed disorganized thalli of 1–2 mm in diameter (Figure 4b). Ultrastructure of free-floating filaments revealed that the cells were larger than in endophytic filaments (Figure 4c–e). Vegetative filaments usually consisted of squared to rectangular cells of 9 µm × 5 µm on average (Figure 4f), and were positive for CFW (Figure 4g). Plastids were disk-like, often showing a convex appearance (Figure 4g). We observed nuclei of 2 µm diameter, often with a nucleolus, 1–4 large convex plastids of up to 4 µm in length, several mitochondria (*ca.* 200–300 nm thick) and pyrenoids (>1 µm in diameter) associated with plastids. Cell walls were 500 nm or thicker, frequently with an additional outer cell wall layer of 0.5–1 µm (Figure 4e). Plasmodesmata were not seen.

Plurilocular sporangia were uniseriate and up to 150 µm long (90 µm on average, Figure 4h). All filaments reached fertility at 5°C and 5 µmol photons $m^{-2}$ $s^{-1}$, with formation of many plurilocular sporangia in the outer area of the mass of filaments (up to 45 per thallus of 1 mm). The thalli also became reproductive under higher temperatures (12°C and 15°C) and irradiance (60 µmol photons $m^{-2}$ $s^{-1}$), however the formation of plurilocular sporangia was reduced to a few small sporangia (no more than five per 1 mm filament of 25–40 µm, not shown) per filament. Zoospores were released two weeks after transfer of filaments to new dishes, they were approximately 5 µm in diameter and contained a long anterior and a short posterior flagellum (Figure 4i). Spore release lasted a few minutes and required the compression of the spore to less than 50% of its width in order to pass through the 2 µm sporangium exit orifice (Figure 4i).

Cell metrics in *Microspongium* revealed some quantifiable cellular re-arrangements after endophytic habitus (Figure 5). The cell diameter was significantly reduced, being shrunk by 30% inside *Alaria* tissue. Pyrenoid diameters did not surpass 1.5 µm in endophytic *Microspongium*, which was significantly smaller than

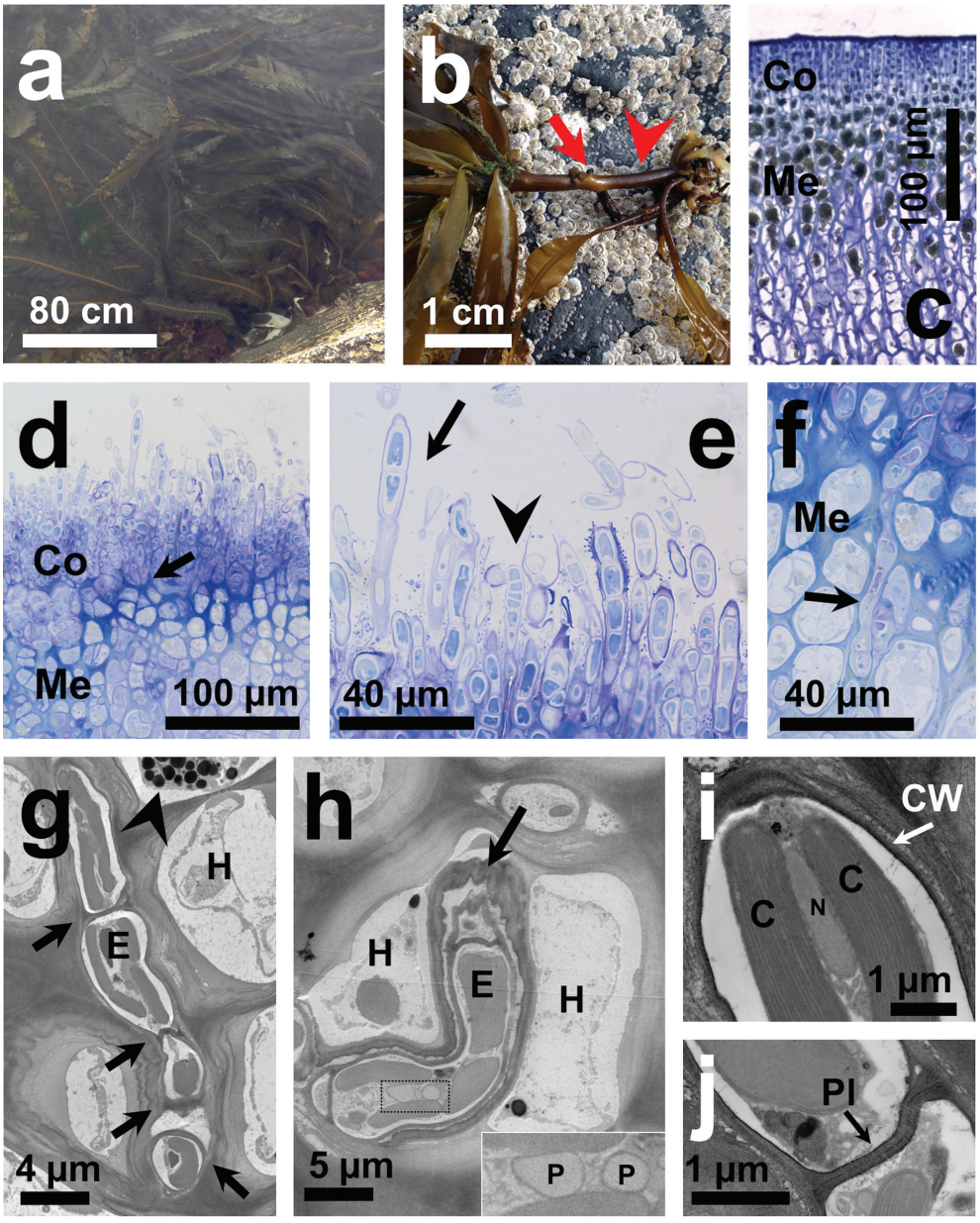


**Figure 3:** Morphology of the endophytic *Microspongium alariae* (Mala OB) in *Alaria esculenta* from Seil Island, Argyll (Scottish West coast). (a) Population of the host, showing healthy fronds without signs of infection. (b) Host stipe (*A. esculenta*) pointing out warts containing *M. alariae* epi-endobionts (arrow) and a healthy stipe area (arrowhead). (c) Cross section of an uninfected *A. esculenta* stipe. (d) Cross section showing external filaments of *M. alariae* and internal organization of infested *A. esculenta* tissue. Arrow: cortex-medulla transition. (e) *M. alariae* external filaments above the host surface (arrow) and a putative plurilocular sporangium (arrowhead). (f) Endophytic filament of *M. alariae* reaching the medulla (arrow) of *A. esculenta*. (g–j) Transmission electron microscopy of the infected host: (g) Longitudinal section through the host cortex showing a *M. alariae* endophytic filament and a typical host cell (arrows: plasmodesmata, arrowhead: host physodes). (h) *M. alariae* changing orientation, separating two neighbor host cells. Arrow indicates a disruption zone. Inset: pyrenoids of an endophytic *Microspongium* cell. (i) Cross section of an endophytic *M. alariae* cell indicating the cell nucleus, plastids and a thin cell wall (arrow). (j) Magnification of two neighboring endophyte cells showing interconnection by plasmodesmata (arrow). Co, cortex; Me, medulla; E, endophyte (*M. alariae*); H, host (*A. esculenta*); C, endophyte chloroplast; N, endophyte nucleus; CW, endophyte cell wall; P, pyrenoids; Pl, endophyte plasmodesmata.

in free-living cells. Cell wall thickness was probably the most altered cell feature, being reduced to less than 400 nm (median = 275 nm), equivalent to a *ca.* 4–5-times shrinkage. Other organelles such as plastids, mitochondria and nuclei did not present significant size modifications.

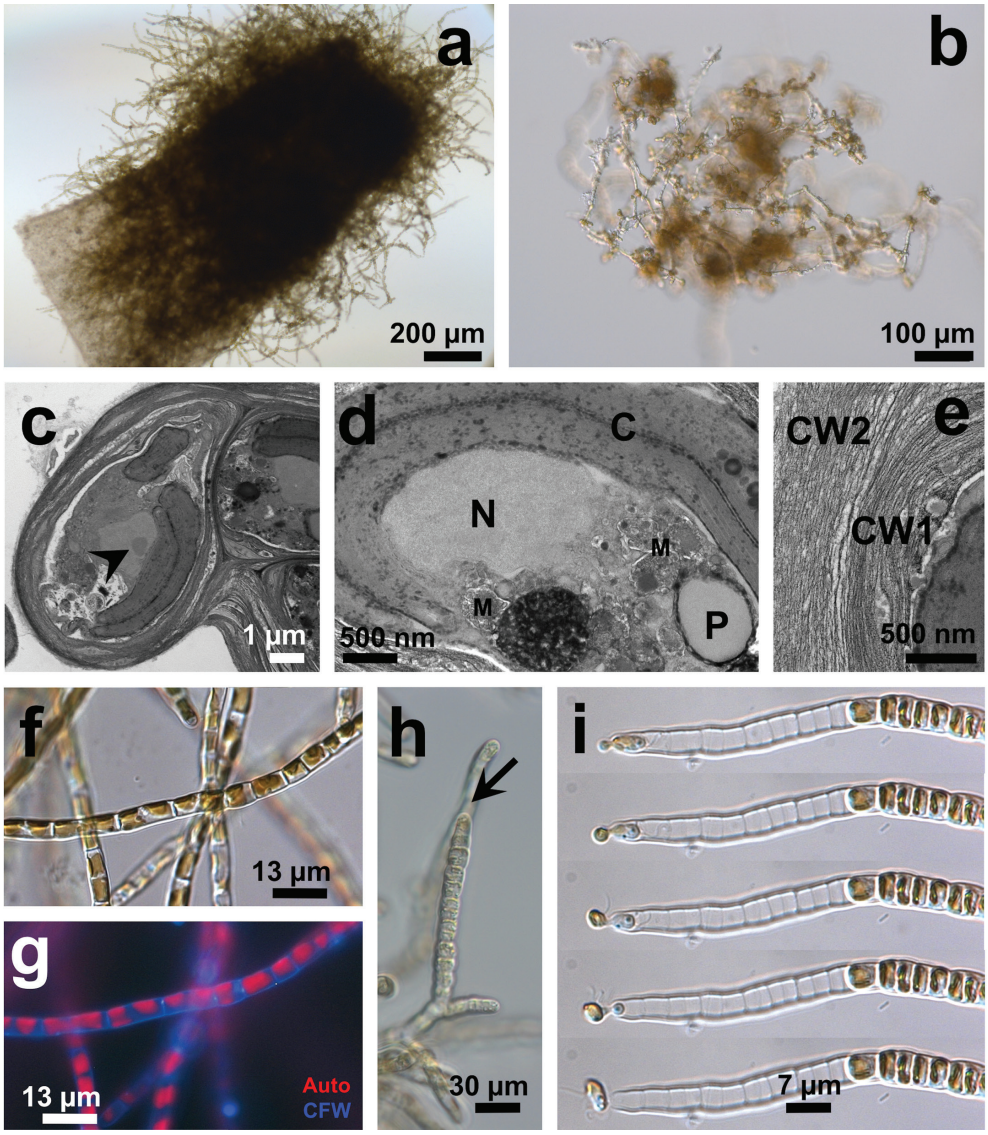


**Figure 4:** *Microspongium alariae* (Mala OB) in culture.
(a) Outgrowth of *M. alariae* filaments from an *A. esculenta* medullar cross section. (b) Mass of disorganized branched filaments of a *M. alariae* clonal isolate in unialgal culture. (c–e) Ultrastructure of free-floating *M. alariae* filaments: (c) Typical *M. alariae* cell from a free-floating filament (arrowhead: nucleolus). (d) Magnification of a *M. alariae* cell indicating cell nucleus (N), plate-shape chloroplasts (C), mitochondria (M), and one pyrenoid (P). (e) Magnification of the *M. alariae* outer part of the cell indicating the initial cell wall (CW1) and an a new polymerized cell wall component (CW2). (f and g) Magnification of a single filament of *M. alariae* under bright field and epifluorescence microscopy. Auto, chlorophyll autofluorescence; CFW, calcofluor white. (h) Uniseriate plurilocular sporangium (arrow). (i) Sequence of the release of a zoospore of *M. alariae* from a plurilocular sporangium.

## Discussion

Both morphology and DNA sequences confirmed that *Microspongium alariae* is one of the endobionts associated with warts in the brown alga in *Alaria esculenta*. This species was first described by Pedersen (1981) (as *Gononema alariae*) using unialgal isolates from Greenland (obtained from non-endophytic material). Our endophytes also had uniseriate branched filaments with diffuse growth, disc-shaped chloroplasts, developed uniseriate plurilocular sporangia at 4–15°C, unipolar germination and lacked unilocular sporangia, as described by Pedersen (1981). Peters (2003) also described phaeophycean hairs, which were not observed in our study. It is unclear whether genetics or different culture media or protocols used in this study were responsible for their lack in our isolates. In phylogenetic analyses based on ITS1, COI and *rbc*L, the new isolates from western Europe clustered

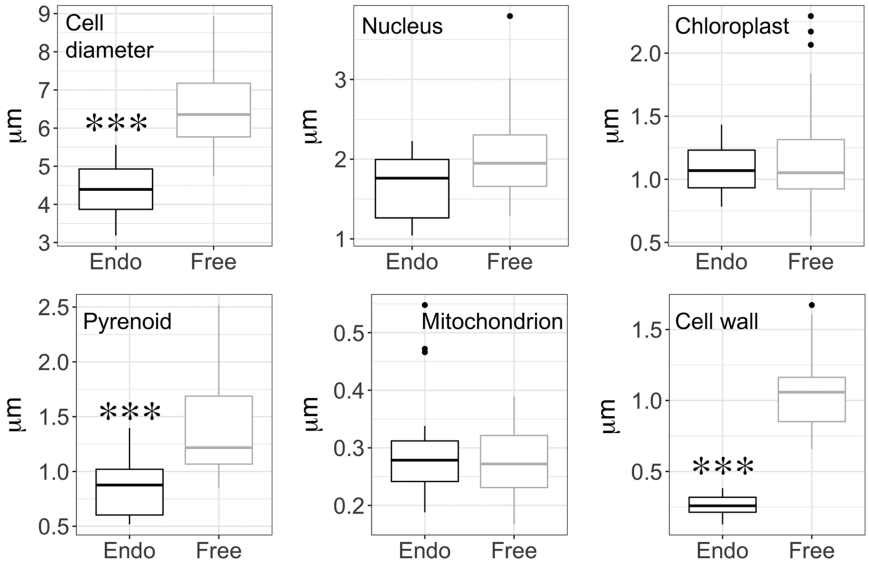


**Figure 5:** Cellular reductions in endophytic *Microspongium*.
Endo: endophytic. Free: free-floating *M. alariae*. Arrowheads indicate the organelle/cell structure quantified in every sampling (n = 7–50). Boxes show median (horizontal line) ±1.5 times the interquartile range (whiskers). Black dots represent deemed outliers. Asterisks on bars were used to designate statistical differences (Mann-Whitney-Wilcoxon rank-sum test), where ***: $p < 0.001$.

with *M. alariae* from the NW Atlantic and the inner Baltic. The information presented here, therefore, corresponds to the first records of this species from Britain and France.

According to our molecular data, the isolates of *M. tenuissimum* and *M. radians*, which together formed the sister clade of *M. alariae*, are conspecific. Their COI sequences showed 100% identity (BLAST). The species-level cut-off in COI in Ectocarpales lies at approximately 1.8% sequence divergence (Peters et al. 2015). In the more variable ITS1, the two taxa differed only by a 29-bp long indel in the highly variable first part, which in our analyses did not place them in different clades. Both species occur in red algal hosts (Table 1). In conclusion, the two taxa should be merged, with *M. tenuissimum* (Hauck 1884) having priority over *M. radians* (Howe 1914).

Morphological traits do hardly allow to distinguish *M. alariae* and *M. tenuissimum*/*radians* but they differ in their global distribution and in particular in their hosts (Table 1). Characters typically used to separate species like presence of phaeophycean hairs seem to be plastic in *Microspongium*, since material barcoded as *M. alariae* in different studies (Peters 2003; this study) may or may not develop hairs. In these species of *Microspongium* only one generation is known. If they are involved in a more complex life cycle, a possibly existing second generation might reveal differences to separate them morphologically at specific level.

Our results suggest that the endophytic behavior of *M. alariae* is associated with gall development in *A. esculenta*, since many galls contained well-developed endophytic filaments that led to *M. alariae* emergence when excised and cultivated. Typically, galls in *A. esculenta* were characterized by hyperplasia and hypertrophy, with a disorganized cortex and medulla development and no clear host/pathogen differentiation in the cortical zone. A complex *M. alariae* endophytic network was also conspicuous in the host medullary tissue. Gall formation has been related to biotic (virus, bacteria, fungal and algal endophytes, animals) or abiotic factors (e.g. carcinogenic compounds) (Apt 1988). In our study, no EM-based evidence of additional gall-triggering factors has been found. However, to confirm the pathogenic nature of *M. alariae* through Koch's postulates (Koch 1882), reinfection of healthy *A. esculenta* in controlled conditions and observation of symptoms would be required.

Cellular modifications appear to represent common adaptations to an endophytic habit within different algal lineages. Several endophytic algal parasites evolved to presumed simpler morphologies, smaller sizes and lower pigmentation levels in comparison to their free-living sister species (Goff 1979, Salomaki and Lane 2014). Algal parasites often also lack plastids and mitochondria or have reduced versions of them. Thus, most of them are biotrophic, i.e. they cannot be propagated without a proper host. In our case, endophytic cells of *M. alariae* were 25–50% smaller than in free-living culture, accompanied by a decrease in the nuclear volume (15–20%) and cell wall thickness (40–60%), as well as reduction

**Table 1:** Comparison of morphological characters of *Microspongium alariae* with *M. tenuissimum*.

| | ***Microspongium alariae*** | ***Microspongium tenuissimum*** |
|---|---|---|
| Habitus | Epi-endophytic (a) (b)/epilithic (c) | Epi-endophytic (a) (d)/epilithic (e) |
| Macroscopic appearance in the host | Warts, dark spots, felt-like cover, galls or twisted host thallus (b). May be asymptomatic (a) | Dark spots and lesions (d). May be asymptomatic (a) |
| Thallus organization | Microscopic uniseriate branched filaments, diffuse growth (b) (c) | Microscopic uniseriate branched filaments (d) |
| Phaeophycean hairs | May be present (b) (c) | May be present (d) |
| Plastids | Disc-shaped chloroplasts (c), may be slightly convex (b) | Subcuneate, or somewhat discoid, may be confluent or single (d) |
| Plurilocular sporangia | Uniseriate (b) (c) | Uniseriate (d) |
| Unilocular sporangia | Not reported | Not reported |
| Life history | Direct (only one generation known) (b) (c) | Direct (only one generation known) (d) |
| Geographic distribution | Temperate and polar ecosystems in the northern hemisphere (a) (b) (c) | Cosmopolitan in temperate waters (a) (d) (f) |
| Hosts | *Alaria esculenta* (a)<br>*Saccharina latissima* (a)<br>*Fucus vesiculosus* (a) | *Mazzaella laminarioides* (Bory de Saint-Vincent) Fredericq (a)<br>*Pachymenia* (*Aeodes*) *orbitosa* (Suhr) L.K. Russell (a)<br>*Grateloupia cutleriae* Kützing (d)<br>*Grateloupia doryphora* (Montagne) M. Howe (a) (f)<br>*Glaphyrosiphon chilensis* M.E. Ramírez, Leister and P.W. Gabrielson (a)<br>*Polysiphonia elongata* (Hudson) Sprengel (f) |

The latter includes *M. radians* (see Discussion section). (a) Peters (2003), (b) This study, (c) Pedersen (1981), (d) Howe (1914), (e) Peters et al. (2015), (f) Burkhardt and Peters (1998).

of plastids in both number and size. Disappearance of the mucilaginous extracellular matrix was also evident. This size and complexity reduction could enhance the penetration of host tissue or reduce the drag forces (friction), minimizing the damage to/by the host or, alternatively, could be just a consequence of being relieved from external pressures (e.g. desiccation, further biotic interactions). Similar observations were made in the brown alga *Herpodiscus durvilleae* (Lindauer) G.R. South, where internal cells were narrower (sometimes squeezed) than external filament cells and host penetration was likely led by apical cells (Heesch et al. 2008). Once endophytic, plastids of this parasitic species are reduced dramatically, however a functional *rbc*L sequence was detected as well as plastid autofluorescence in the epiphytic and presumably autotrophic gametophyte generation (Heesch 2005).

According to Blouin and Lane (2012), there is no record of algal parasites that do not develop secondary pit connections (or their analogs) with the host for the acquisition of nutrients. This supposition has been confirmed not only for red algal but also for green and brown algal parasites (Heesch 2005). In our case, plasmodesmata were not developed in the *A. esculenta*–*M. alariae* interface, suggesting that the relationship of *M. alariae* with its host is not parasitic. In addition, endophytic cells of *M. alariae* always contained plastids, ensuring autotrophy. Plasmodesma formation between *M. alariae* endophytic cells may represent a means to improve nutrient translocation along the endophyte filament. Endophytism of *M. alariae* may have evolved as protection against herbivory, competition or detachment, or sudden environmental changes such as desiccation.

A spatial separation of the endophytic filament from the starting point of disruption in the host tissue (Figure 3h) is suggestive of an enzymatic action that allows to penetrate into the intercellular space, possibly through hydrolysis of the polysaccharide matrices of the host. An enzymatic dissolution of host cell wall components by germinating zoospores has been suggested for *Laminariocolax aecidioides* (Rosenvinge) A.F. Peters and *Laminarionema elsbetiae* Kawai *et* Tokuyama because of the sharp edges around the entrance holes and the absence of inward deformation of the host and for some red algal parasites and epiphytes with some extent of host penetration (Heesch and Peters 1999, Leonardi et al. 2006 and references therein). It would be interesting to investigate the carbohydrate-modifying enzymes of the endophytes, which appear to facilitate a selective degradation of the host cell walls.

World-wide seaweed aquaculture has been increasing at a rate of 8% annually (Loureiro et al. 2015). However, its world-wide, large-scale commercial development has been delayed in many countries by emerging pests and pathogen outbreaks that can affect the desirable yield and quality of the biomass (Araújo et al. 2014) and more indirectly prevent competitive profit by increasing costs and inhibiting investment (Gachon et al. 2010, Westermeier et al. 2011). This situation may be worsened by global warming effects, which could have direct consequences on the prevalence of endophytes and other pathogens, possibly modifying host fitness and endophyte virulence (Eggert et al. 2010). *Alaria esculenta* is currently cultivated in Europe and in North America (Barrington et al. 2009). We found that *M. alariae* is a common endophyte of *A. esculenta*, which was present even in the population in Brittany, near the southern distribution limit of *Alaria*. Additional epidemiological data will be required to estimate whether the infection could represent an important bottleneck for *Alaria* aquaculture.

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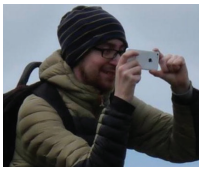
# Bionotes



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# Graphical abstract

Pedro Murúa, Frithjof C. Küpper, Liliana A. Muñoz, Miriam Bernard and Akira F. Peters

***Microspongium alariae* in *Alaria esculenta*: a widely-distributed non-parasitic brown algal endophyte that shows cell modifications within its host**

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**Research article:** New records of the phaeophycean endophyte *Microspongium alariae* are described, analyses which also determine that sister *M. tenuissimum* and *M. radians* are conspecific. Evidence from culturing/TEM revealed cell modifications for *M. alariae* endophytic lifestyle, where parasitism hallmarks were not found.

**Keywords:** Chordariaceae; COI; ITS1; *rbc*L; ultrastructure.