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Diversity, biogeography and host specificity of kelp endophytes with a focus on the genera *Laminarionema* and *Laminariocolax* (Ectocarpales, Phaeophyceae)

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ABSTRACT

Endophytic filamentous brown algae are known to invade stipes and fronds of kelps with potentially negative effects for the hosts. They have simple filamentous thalli and are difficult to identify based on morphology. We investigated the molecular diversity of 56 endophytes isolated from seven different kelp species from Europe, Chile, Korea and New Zealand by sequencing two unlinked molecular markers (5'COI and ITS1). A majority of 49 of the isolated endophytes (88%) belonged to the genera *Laminarionema* and *Laminariocolax*. The endophyte *Laminarionema elsbetiae* was isolated from *Saccharina latissima* and *S. japonica* tissues in Europe and Korea, respectively, and showed highly similar sequences in both regions. Three different species of the genus *Laminariocolax* were identified, the most common of which was *L. aecidioides*, an endophyte with a worldwide distribution and a broad host range. The other two species, *L. tomentosoides* and a species described here as *Laminariocolax atlanticus* sp. nov., were associated with different kelp species in the northern hemisphere and the North Atlantic, respectively. Our results suggest that specific host-endophyte patterns could exist locally, as found in kelps in Brittany where all endophytes isolated from *S. latissima* were *L. elsbetiae*, all endophytes isolated from *Laminaria digitata* were *Laminariocolax tomentosoides*, and those isolated from *Laminaria hyperborea* were *Laminariocolax atlanticus* and *L. aecidioides*. However, this pattern was not consistent with the results from other places, such as Western Scotland and Helgoland where the same kelp species are present.

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KEYWORDS 5'COI; barcoding; biogeography; endophytes; host specificity; ITS1; kelps

Introduction

Kelps are essential elements of lower eulittoral and sublittoral zones of rocky shore coastal ecosystems in temperate and northern polar seas (Bartsch *et al.*, 2008). While they serve as a food source or habitats for animals, they also provide a substratum for smaller algae growing on (epiphytes) and inside (endophytes) their thalli (Dayton, 1985; Bartsch *et al.*, 2008). Epiphytes penetrate into the outermost cell layers of the host tissue mainly for mechanical support (Setchell, 1918). Endophytes, on the other hand, may grow entirely within a host and only reproductive structures are formed at the host surface (Peters, 1991). A clear distinction between epi- and endophytes is not always possible because certain species may represent a continuum between an epiphytic and endophytic lifestyle (Peters, 2003; Gauna & Parodi, 2008). Furthermore, most of these associations are facultative, and life stages of such endophytic species may also be found outside their hosts (Peters *et al.*, 2015; Küpper *et al.*, 2016). In this study, we use the term endophyte to describe organisms that possess

the ability to penetrate deeper than the cortex and grow inside an algal host. Infections by filamentous endophytic brown algae have been reported from kelp species worldwide (e.g. Peters, 1991; Kawai & Tokuyama, 1995; Ellertsdóttir & Peters, 1997; Amsler *et al.*, 2009; Gauna *et al.*, 2009a, b), with a prevalence of up to 100% infected individuals within a population (Lein *et al.*, 1991). The presence of endophytes in kelps often coincides with disease symptoms, such as dark spots on fronds, warts or twisting of fronds and stipes (Yoshida & Akiyama, 1979; Apt, 1988; Peters & Schaffelke, 1996; Ellertsdóttir & Peters, 1997; Thomas *et al.*, 2009). However, not all infected hosts show morphological changes (Gauna *et al.*, 2009a; Bernard *et al.*, 2017), and the basic underlying molecular mechanisms of this interaction and the profits or disadvantages for either partner are still unclear.

Endophytes of kelps are in most cases microscopic brown algae with filamentous thalli, diffuse growth and plastids with pyrenoids (Burkhardt & Peters, 1998). Due to their morphologically reduced nature they are included in the Ectocarpales *sensu*

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lato, but their phylogenetic relationships are not fully explored, and classifications undergo continuous changes. The species *Laminariocolax aecidioides* (Rosenvinge) A.F.Peters, for instance, was originally classified in the genus *Ectocarpus*, as *E. aecidioides* Rosenvinge (1893). Later *L. aecidioides* was assigned to the genera *Phycocelis*, *Myrionema*, *Entonema*, *Gononema* and *Streblonema*, based on different aspects of its morphology (Burkhardt & Peters, 1998). A molecular systematic study finally classified it in the genus *Laminariocolax* within the Chordariaceae (Burkhardt & Peters, 1998). As the description of filamentous endophytic brown algae based exclusively on morphological characters has turned out to be insufficient, a combination of descriptive data and DNA barcoding emerged as a suitable method to catalogue the diversity and unravel the phylogeny of this group of organisms (Thomas *et al.*, 2009; Peters *et al.*, 2015). However, limited sampling due to the difficulty in isolating these algae from infected hosts has so far prevented a comprehensive revision of the endophyte taxonomy. Furthermore, little is known about their biogeographic distributions and host ranges (see Eggert *et al.* 2010 for a discussion of these aspects).

In this study, we isolated 56 endophyte strains from seven different kelp species in Europe, Korea, Chile and New Zealand and investigated their molecular diversity using two independent molecular markers, 5'COI and ITS1. The mitochondrial cytochrome oxidase I locus (5'COI) was proposed as a universal marker for DNA barcoding of animals by Herbert *et al.* (2003). It is suitable for species delimitation of various organisms, such as insects (Herbert *et al.*, 2004) and zooplankton (Bucklin *et al.*, 2010), but also red algae (Saunders, 2005; Le Gall & Saunders, 2010) and several brown algal groups, such as *Fucus* (Kucera & Saunders, 2008), Laminariaceae (McDevit & Saunders, 2010), *Sargassum* (Mattio & Payri, 2010), *Desmarestia* (Yang *et al.*, 2014) and Ectocarpales (Peters *et al.*, 2015; Montecinos *et al.*, 2017). The internal transcribed spacer 1 (ITS1) is a nuclear marker, separating the 18S and 5.8S subunits of the rDNA. While the 18S subunit is commonly used as a nuclear marker to roughly classify microbial eukaryotes (e.g. Tragin *et al.*, 2016), it is not sufficiently variable to distinguish between different species of brown algae (Saunders & Kraft, 1995). The ITS1 region, evolving much faster than the adjacent subunit regions of the rDNA (Baldwin, 1992; Goff *et al.*, 1994), has therefore been established as a common nuclear marker to distinguish closely related species in the Phaeophyceae (Burkhardt & Peters, 1998; Kucera & Saunders, 2008; Kogame *et al.*, 2015; Montecinos *et al.*, 2017).

The aims of this paper were to study the molecular phylogeny of the isolated endophytes and to compare their biogeographic distribution ranges based on published and new molecular data as well as on morphological records. Our data also allowed inferences on specific host-endophyte relationships.

Materials and methods

Sampling and isolation of endophytes

Endophytes were isolated from kelp tissue as described by Peters & Ellertsdóttir (1996), with most strains deriving from tissue showing obvious morphological alterations, such as dark spots, warts or twists in the kelp fronds and stipes (Eggert *et al.*, 2010). The kelps were usually collected *in situ* during low tide, and one endophyte strain was isolated per host individual. In total, 56 clonal endophyte strains were included in the study (Supplementary table S1). They were isolated from seven different kelp species: *Laminaria digitata* (Hudson) J.V.Lamouroux, *L. hyperborea* (Gunnerus) Foslie (Brittany, Helgoland), *Saccharina latissima* (L.) C.E.Lane *et al.* (Brittany, German Baltic and North Sea, Scotland and England), *Saccharina japonica* (Areschoug) C.E.Lane *et al.* (Korea), *Saccharina nigripes* (J.Agardh) Lontin & G.W.Saunders (Svalbard), *Lessonia berteroaana* Montagne (Chile) and *Macrocystis pyrifera* (L.) C. Agardh (New Zealand). Furthermore, a filamentous brown alga (BI-041) isolated from incubated abiotic substratum from Baffin Island in the Canadian Arctic (Küpper *et al.*, 2016) has been added to the present study. The endophytes from temperate regions were cultivated at 14°C and Arctic isolates at 4°C, with monthly changes of the culture medium (half-strength Provasoli enrichment, Coelho *et al.*, 2012). Light was supplied at 5 $\mu\text{mol photons s}^{-1} \text{ m}^{-2}$ for 12 h per day.

DNA extraction, barcode markers, amplification and sequencing

Algal material from actively growing cultures was freeze-dried and ground in a mechanical bead grinder (Tissuelyser II, Qiagen, Germany) twice for 2 min at 30 Hz. DNA was extracted using the Nucleospin Plant II kit (Macherey-Nagel, Germany). The mitochondrial marker (5'COI, primers GazF2 and GazR2, Lane *et al.*, 2007) was PCR-amplified in all samples. Additionally, the nuclear ribosomal marker (ITS1, primers AFP4L and 5.8S1R, Peters & Burkhardt, 1998) was amplified in representative isolates (at least one isolate from each locality). The total PCR reaction volume consisted of 20 μl , containing 3 mM MgCl_2 , 5 \times Green GoTaq Flexi buffer (Promega,

USA), 1 µl template DNA, primers at 400 nM, 0.2mM dNTP each and 1 unit of GoTaq Flexi Polymerase (Promega, USA). An initial 4 min denaturation step at 95°C was followed by 35 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C and a final extension at 72°C for 10 min. PCR products were commercially Sanger sequenced using the primers mentioned above and each resulting chromatogram was checked for quality by eye.

Data analysis

The 5'COI sequences were edited in MEGA7 (Kumar *et al.*, 2016) and aligned by MUSCLE (Edgar, 2004). Consensus sequences were compared to published data by NCBI BLAST searches (Altschul *et al.*, 1990), and close matches (>97% identity) were included in the phylogenetic analyses (Supplementary table S2). The kelp species *Laminaria digitata*, *L. hyperborea* and *S. latissima* were used as the outgroup. ITS1 sequences were too divergent for common alignment and were therefore aligned separately for *Laminarionema* and *Laminariocolax*.

5'COI and ITS1 sequences were analysed using the same methods. Maximum likelihood analysis (1000 bootstraps, General Time Reversing Model GTR; henceforth ML) was performed with MEGA7. Bayesian analysis (BI) was performed with Beast 2 (Bouckaert *et al.*, 2014) using the HKY substitution model, default settings for temperature and branch-swapping, 8 million generations and samplings of every 1000 generations. The first 10% of obtained trees were discarded as burn-in. Trees were edited in TreeGraph 2 (Stöver & Müller, 2010). Kimura two-parameter distances (Kimura, 1980, henceforth K2P) between and within the resulting clades were calculated in MEGA7. The gap between intraspecific diversity and interspecific diversity for 5'COI sequences of the genus *Laminariocolax* was determined with the web version of Automatic Barcode Gap Discovery (ABGD, Puillandre *et al.*, 2012) using the Jukes–Cantor model with a relative gap width of 1.5 and 10 steps. Prior maximum divergence of intraspecific diversity was set between 0.001 and 0.012. All sequences were submitted to GenBank with the accession numbers MG770493–MG770548 for 5'COI sequences and MG781159–MG781176 for ITS1 sequences (Supplementary table S1).

Distribution maps were constructed in R using the packages `mapdata`, `maps` and `mapproj` (R Development Core Team, 2013) based on genetic sequences of endophytes isolated in this study, sequences available in public databases and morphological records obtained from Algaebase (Guiry & Guiry, 2017) and published articles.

Results

Molecular systematics

For molecular analyses of the 5'COI region, we constructed an alignment of 77 sequences (Fig. 1), which included 21 reference sequences obtained from public databases and 56 newly determined sequences. The length of the 5'COI alignment used for the phylogenetic analysis was 591 bp. All isolated strains were members of the Ectocarpales. The topology of the 5'COI tree was independent of the phylogenetic reconstruction method used (PhyML or BI). For molecular analyses of the ITS1 region of the genus *Laminarionema*, we used an alignment of six sequences, which included a reference sequence obtained from public databases and five newly determined sequences; the aligned sequences had a length of 278 bp (tree not shown because all sequences were highly similar). For molecular analyses of the ITS1 region of the genus *Laminariocolax*, we used an alignment of 23 sequences (Fig. 2), which included 10 reference sequences obtained from public databases and 13 newly determined sequences. Due to several indels in the alignment, the length of ITS1 and the flanking subunit sequences ranged from 323 to 839 bp. The topology of the *Laminariocolax* ITS1 tree was independent of the method used (PhyML or BI). The choice of setting had a minor impact on the bootstrap/posterior probabilities values, but not on the general topology of the tree. Overall, the phylogenetic analyses of the endophyte strains with the two different markers supported the same clades.

Forty-nine of the isolated endophytes (88%) belonged to the genera *Laminarionema* and *Laminariocolax*. Furthermore, seven epi-endophytic species were isolated (Fig. 1), comprising a so far unidentified member of Chordariaceae, a strain of *Hecatonema maculans* (Collins) Sauvageau, two isolates of *Hincksia hincksiae* (Harvey) P.C.Silva, an unidentified member of Acinetosporaceae, and two isolates of *Ectocarpus fasciculatus* Harvey. In the following, the focus will be on the endophytic genera *Laminarionema* and *Laminariocolax*.

The genus *Laminarionema* consisted of a single species, i.e. *L. elsbetiae* H.Kawai & Tokuyama. Analysis of 5'COI (Fig. 1) did not show any intra-specific variability, whereas ITS1 sequences showed a low intraspecific variability of 0.6% (Table 1).

The genus *Laminariocolax* consisted of three clades, which were supported statistically by high bootstrap and posterior probability values (Figs 1, 2). Three congruent primary partitions were obtained by ABGD analysis of the 5'COI sequences for prior distances ranging from 0.001 to 0.091 (Supplementary fig. S1). Higher prior distances resulted in one partition only (Supplementary fig.

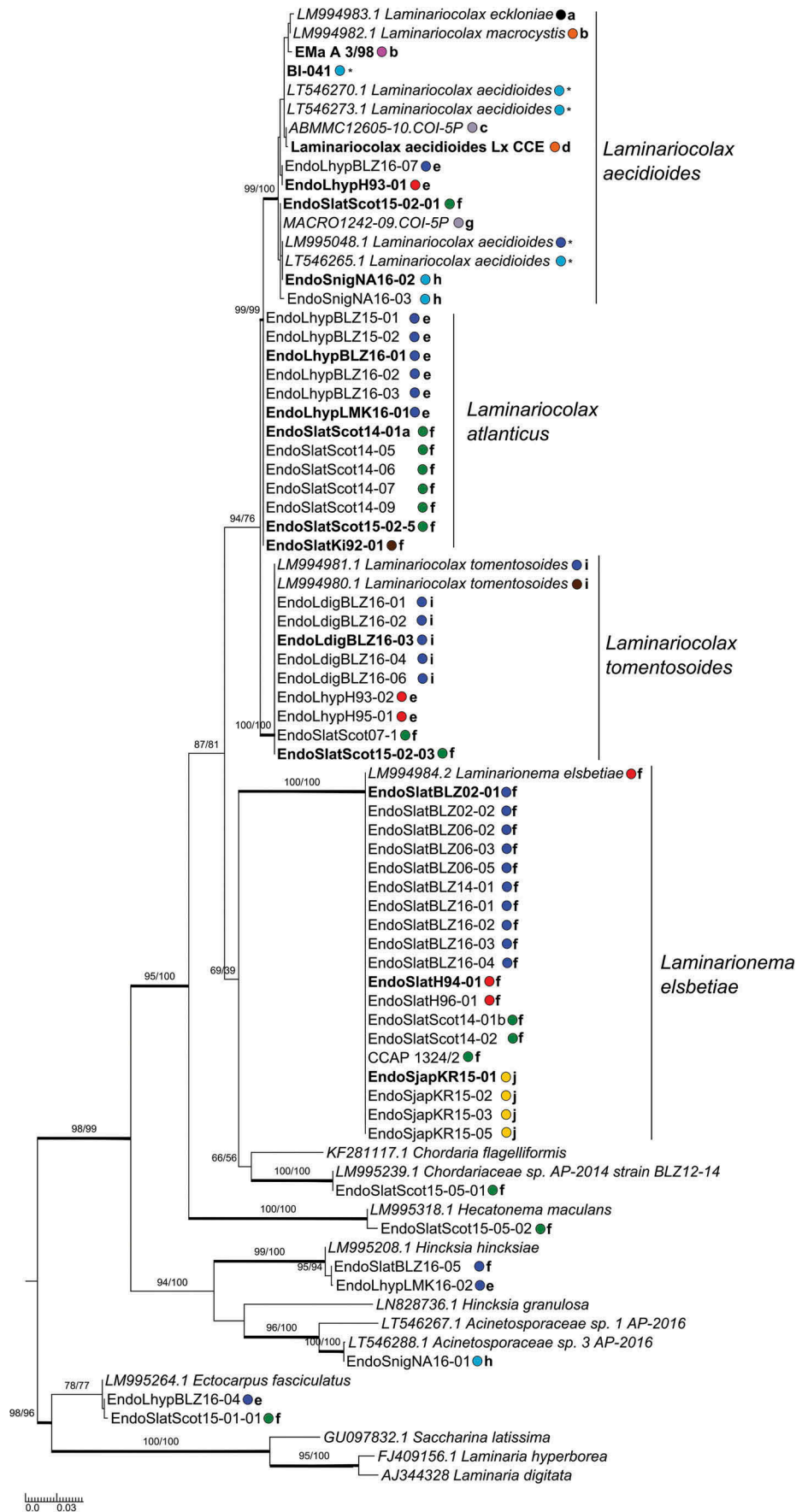


Fig. 1. Phylogenetic tree of 5'COI sequences. Values at nodes indicate bootstrap support obtained by ML/BI analysis. Bootstrap supports >95 in both analyses are indicated by a thicker line. Reference sequences from public databases are printed in italics and using the identities given in the original publications. ITS1 sequences are available for specimens shown in bold. The colours and letters behind the strain names indicate the geographic origin and host species, respectively. Origins: black = South Africa; orange = Chile; pink = New Zealand; light blue = Arctic; grey = Canadian Pacific coast; dark blue = Brittany; red = Helgoland; green = UK; brown = Kiel, western Baltic; yellow = Korea. Hosts: a = *Ecklonia maxima*; b = *Macrocystis pyrifera*; c = *Saccharina sessilis*; d = *Lessonia berteroa*; e = *Laminaria hyperborea*; f = *Saccharina latissima*; g = *Costaria costata*; h = *Saccharina nigripes*; i = *Laminaria digitata*; j = *Saccharina japonica*; * = grown from incubated substratum.

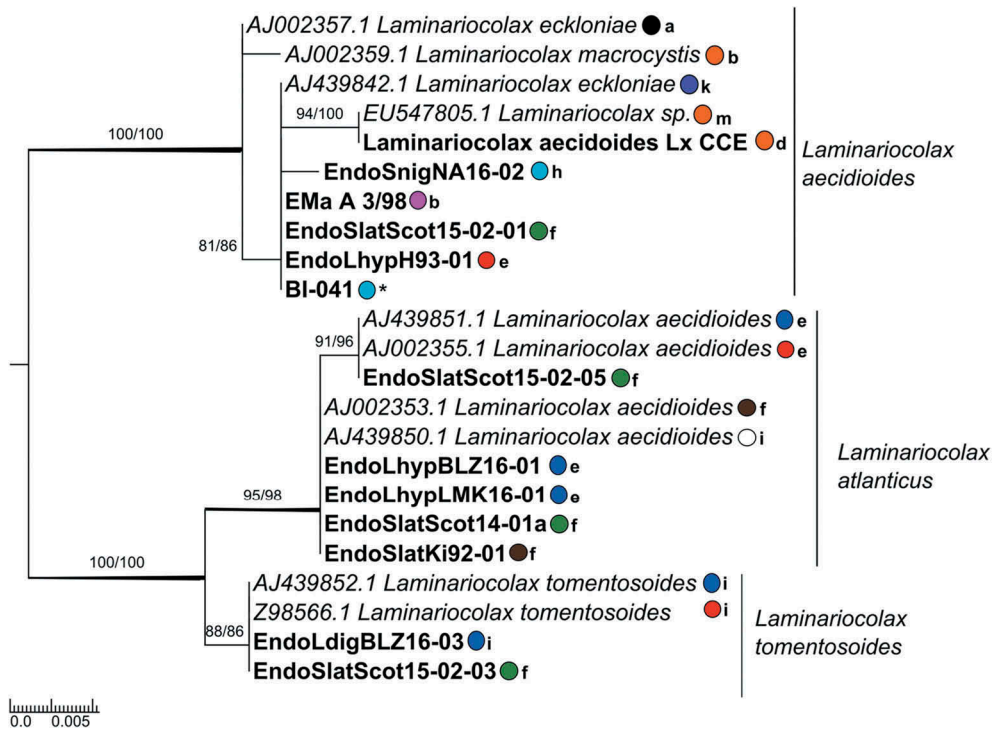


Fig. 2. Phylogenetic tree of ITS1 sequences from strains of the genus *Laminariocolax*. Values at nodes indicate bootstrap support obtained by ML/BI analysis. Bootstrap supports >95 in both analyses are indicated by a thicker line. Reference sequences from public databases are printed in italics and using the identities given in the original publications. Specimens printed in bold are also presented in the 5'COI tree (Fig. 1). The colours and letters behind the strain names indicate the geographic origin and host species, respectively. Origins: black = South Africa; orange = Chile; purple = Antarctica; light blue = Arctic; pink = New Zealand; green = UK; red = Helgoland; dark blue = Brittany; brown = Kiel, western Baltic; white = US Atlantic coast. Hosts: a = *Ecklonia maxima*; b = *Macrocystis pyrifera*; d = *Lessonia berteroana*; e = *Laminaria hyperborea*; f = *Saccharina latissima*; h = *Saccharina nigripes*; i = *L. digitata*; k = *Himantothallus grandifolius*; m = *Lessonia nigrescens*; * = grown from incubated substratum.

S1). The first clade – *L. aecidioides* – clustered together with published sequences of *L. aecidioides*, *L. eckloniae* A.F.Peters and *L. macrocystis* (A.F.Peters) A.F.Peters. The second group did not have any matches in public databases for 5'COI sequences (Fig. 1). However, it formed a clade with four published sequences labelled as *L. aecidioides* in the ITS1 analysis (Fig. 2). The third clade represented *L. tomentosoides* (Farlow) Kylin. Interspecific K2P pairwise genetic differences of *Laminariocolax* ranged from 1.4 to 3% for 5'COI and from 2.6 to 5.8% for ITS1 (Table 2). Intraspecific K2P pairwise significant differences were 0 to 0.8% in the 5'COI analysis and 0 to 1.1% in the ITS1 analysis. They were higher within the *L. aecidioides* clade than in the other clades of the genus *Laminariocolax* (Table 1).

Hosts and geographic origin of the isolated strains

Laminarionema elsbetiae was isolated from tissue of *S. latissima* in Scotland, France and Helgoland and from *S. japonica* in Korea (Fig. 4). A putative distribution along the northern hemisphere Atlantic and Pacific coasts is suggested based on molecular records (Fig. 3A).

Laminariocolax aecidioides showed the broadest host range of all endophytes included in this study. It was isolated from *Macrocystis pyrifera*, *Lessonia berteroana*, *Laminaria hyperborea*, *S. latissima* and *S. nigripes* (Figs 1, 2). Furthermore, it has been cultivated from incubated abiotic substratum (isolate BI-041). In this study, *L. aecidioides* was found in Brittany, Helgoland, Scotland, Svalbard, Baffin Island, New Zealand and Chile (Fig. 3B). Published sequences and records of the species based on morphological identification suggest a worldwide distribution in temperate to polar regions.

The second clade of *Laminariocolax* was isolated from *Laminaria hyperborea* and *S. latissima* (Figs 1, 2) in Brittany, Scotland and Kiel. Additionally, ITS1 sequences of strains isolated from *L. hyperborea* in Helgoland and from *L. digitata* in Maine (Fig. 2) are available in public databases, suggesting a distribution of this species in kelp populations along American and European North Atlantic coasts (Fig. 3D).

Laminariocolax tomentosoides was isolated from *Laminaria digitata*, *L. hyperborea* and *S. latissima* (Figs 1, 2) in Brittany, Helgoland and Scotland. Published sequences and records based on

Table 1. Kimura two-parameter genetic distances for pairwise comparisons within 5'COI and ITS1 sequences in % \pm SE for *Laminariocolax aecidioides*, *L. atlanticus*, *L. tomentosoides* and *L. elsbetiae*.

	d \pm SE (5'COI)	d \pm SE (ITS1)
<i>Laminariocolax aecidioides</i>	0.8 \pm 0.01	1.1 \pm 0.12
<i>Laminariocolax tomentosoides</i>	0	0
<i>Laminariocolax atlanticus</i>	0	0.3 \pm 0.05
<i>Laminarionema elsbetiae</i>	0	0.6 \pm 0.18

Table 2. Kimura two-parameter genetic distances for pairwise comparisons between 5'COI (below diagonal) and ITS1 (above diagonal) sequences in % \pm SE for *Laminariocolax* species.

	<i>L. aecidioides</i>	<i>L. tomentosoides</i>	<i>L. atlanticus</i>
<i>L. aecidioides</i>	-	5.3 \pm 0.15	5.8 \pm 0.09
<i>L. tomentosoides</i>	3 \pm 0.02	-	2.6 \pm 0.05
<i>L. atlanticus</i>	1.8 \pm 0.03	1.4 \pm 0	-

morphological identification suggest a distribution of this species along northern hemisphere Atlantic and Pacific shores (Fig. 3C).

Based on these results we describe the second clade of *Laminariocolax* as a new species. Its distinction from the sister species (Figs 5, 6) is shown in Table 3.

***Laminariocolax atlanticus* M.S. Bernard,
Strittmatter & A.F. Peters, sp. nov**

DIAGNOSIS AND DESCRIPTION: Microscopic filamentous thallus, branched, endophytic in the sporophytes of *Laminaria hyperborea*, *L. digitata* and *Saccharina latissima* on North Atlantic coasts, recognized macroscopically as dark spots on the host. Phaeophyceae hairs sticking out from host surface. Plurilocular sporangia in groups on the host surface (Fig. 5), 30–33 μ m long (7–8 loculi), 7–9 μ m in diameter (values from measurements in field material used for isolation of the authentic strain). Plurilocular sporangia similar in unialgal culture (Fig. 6). Unilocular sporangia not seen. Vegetative cells 10–20 μ m long with several discoid or shortband-shaped plastids (Fig. 7).

HOLOTYPE: Kiel (western Baltic, Germany); coll. A. F. Peters, 23/11/1992; fixed material of cultivated authentic strain; deposited in the Natural History Museum, Paris, France (MNHN_PC_PC0786150).

ISOTYPE: Deposited in the Natural History Museum, Paris, France (MNHN_PC_PC0786151) and the Natural History Museum, London, UK (BM000701859).

AUTHENTIC STRAIN: CCAP 1322/3.

TYPE LOCALITY: Isolated on 23/11/1992 from plurilocular endophyte infesting a sorus of *Saccharina latissima* collected in Kiel (western Baltic, Germany).

ETYMOLOGY: The name refers to the putative distribution of the species along (North) Atlantic coasts.

HABITAT: Marine, endophytic in kelps, so far isolated from *L. hyperborea*, *L. digitata*, *S. latissima*.

REPRESENTATIVE BARCODES: MG770512 (5'COI) and MG781174 (ITS1).

Discussion

Molecular phylogeny of kelp endophytes

In this study we performed a broad sampling of kelp endophytes, isolation into clonal cultures and identification of the strains by means of DNA barcoding. All isolated endophytes were brown algae belonging to the Ectocarpales and 88% to the endophyte genera *Laminarionema* and *Laminariocolax*. The phylogenetic trees obtained using 5'COI and ITS1 sequences were in concordance, and the resolution of the markers was sufficiently variable to distinguish different clades within the genus *Laminariocolax*.

Laminarionema was monospecific with no genetic variability in the 5'COI sequences and low variability in ITS1 sequences despite its geographic separation on Atlantic and Pacific coasts. This raises the question whether the endophyte has been exchanged between the two oceans only recently. While ITS sequences have been used to follow the dispersal of other algal species, such as the invasive green alga *Caulerpa taxifolia* (M.Vahl) C.Agardh (Jousson *et al.*, 1998; Schaffelke *et al.*, 2002), the ITS1 data of *Laminarionema* obtained in our study are not sufficiently variable to distinguish European and Asian populations. Additional sampling and more sensitive markers are necessary to further investigate this question. Our isolates represent the first records of this species in Great Britain, France and Korea. Previously, it was only known from the type locality in Northern Japan (Kawai & Tokuyama, 1995) and from Helgoland, North Sea, Germany (Peters & Ellertsdóttir, 1996). We became aware of *L. elsbetiae* in natural populations of European and Korean *Saccharina* because it was associated with twisting of stipes (Fig. 4). The symptoms were similar but usually less dramatic than those seen previously in *S. latissima* at Helgoland (Peters & Ellertsdóttir, 1996). In *S. japonica*, symptoms like the ones we saw in Korea had not been observed from infected hosts in northern Japan (Kawai & Tokuyama, 1995). They were similar to symptoms referred to as 'twisted-frond disease' in cultivated *S. japonica* in China (Wu *et al.*, 1983). However, Wu *et al.* (1983) detected a mycoplasma-like organism in sections of diseased tissue and regarded it as a probable causative agent. In Brittany, the presence of *L. elsbetiae* in *S. latissima* often does not cause any obvious morphological changes (Bernard *et al.*, 2017).

There is a surprising morphological record of *L. elsbetiae* infecting *Rhodymenia pseudopalmata* (J.V.

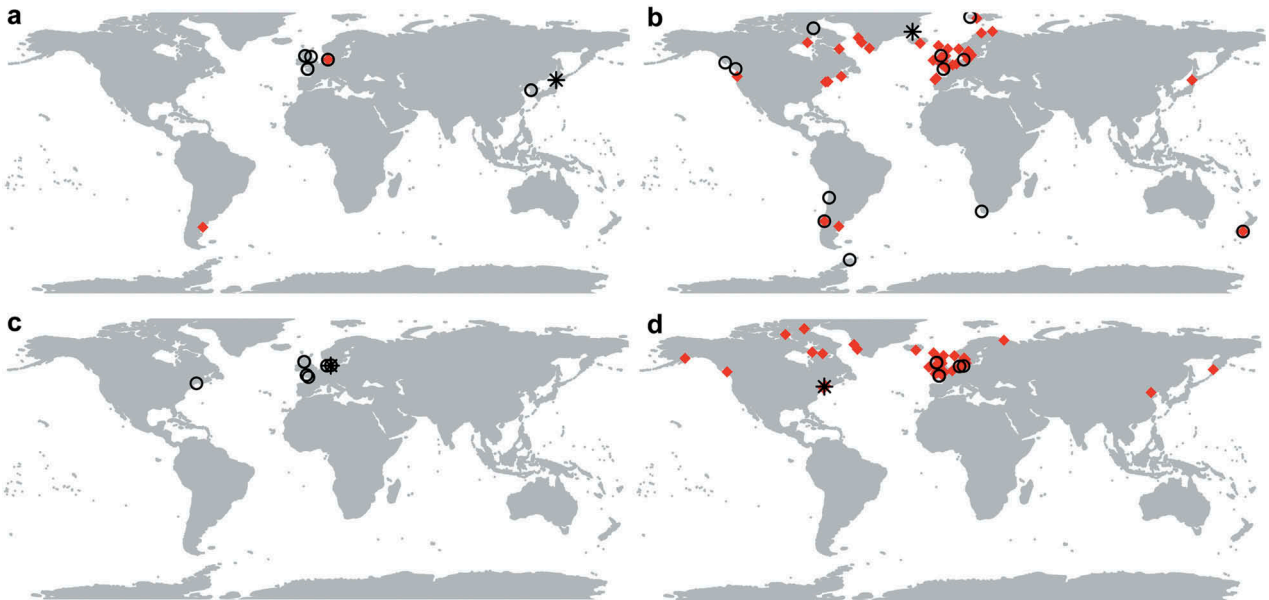
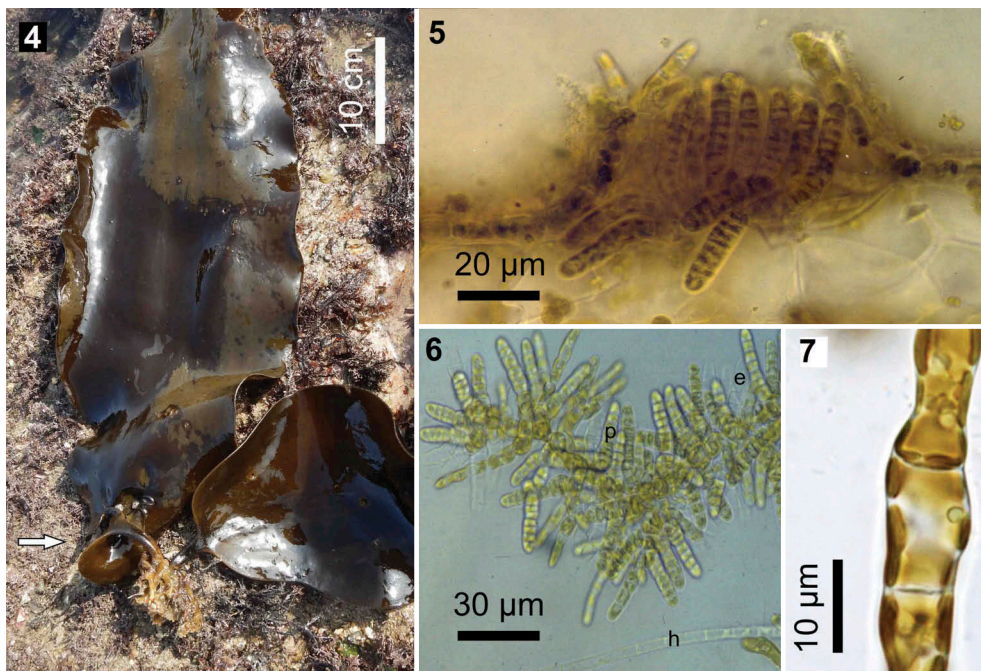


Fig. 3. Biogeographic distribution maps of A. *Laminarionema elsbetiae*, B. *Laminariocolax acidioides*, C. *Laminariocolax atlanticus*, D. *Laminariocolax tomentosoides*. Black circles indicate records based on sequence data (Supplementary table S2), red diamonds indicate records based on morphological records (Supplementary tables S3–S5), black asterisks indicate the type localities.



Figs 4–7. **Fig. 4.** *Saccharina japonica* sporophytes from Padori Beach, Taeon, Chungnam Province, Korea. The left individual presents symptoms of putative infection by *Laminarionema elsbetiae*, which we isolated from similar individuals: twisted lower part of frond (arrow). The right individual has a regular morphology. **Fig. 5.** Plurilocular sporangia of *Laminariocolax atlanticus* sp. nov. on the surface of a field sample of *Saccharina latissima* from Kiel, Germany (transverse section). Large cells in lower part of the micrograph belong to the host. **Fig. 6.** Authentic strain of *L. atlanticus* in culture. h = phaeophycean hair, p = plurilocular sporangium, e = empty plurilocular sporangium (also published in Eggert *et al.* 2010). **Fig. 7.** Authentic strain of *L. atlanticus* in culture. Vegetative cells.

Lamouroux) P.C.Silva from Argentina (Gauna *et al.*, 2009b). *L. elsbetiae* has characteristic large zoospores (Kawai & Tokuyama, 1995; Peters & Ellertsdóttir, 1996) and was therefore clearly recognized by Gauna *et al.* (2009b). It is possible that the species has been introduced to Argentina with macroalgae of

North-East Asian origin such as *Undaria pinnatifida*. Re-isolation and sequence data are nevertheless required to confirm the identity of this endophyte, especially since it represents the first record of *L. elsbetiae* from a red algal host and from the southern hemisphere.

Table 3. Comparison of *Laminariocolax tomentosoides* and *L. aecidioides* with the new species *L. atlanticus*.

	<i>Laminariocolax aecidioides</i>	<i>Laminariocolax atlanticus</i>	<i>Laminariocolax tomentosoides</i>
Macroscopic appearance	Dark spots ^a , galls ^b	Dark spots	Dark areas, distorted phylloids, twisted cauloids ^a , felt-like cover on the host ^k
Thallus organisation	Sporophyte: microscopic uniseriate branched endophytic filaments; gametophyte: epiphytic uniseriate filaments up to 200 µm in length ^c	Microscopic uniseriate branched filaments	Microscopic, uniseriate branched endophytic filaments and epiphytic uniseriate filaments up to 1 cm in length ^k
Hairs	Present ^c	Present	Absent ^l
Plastids	Several (2–10), discoid or band-shaped, with pyrenoids ^c	Several, discoid or band-shaped, with pyrenoids	Small number (usually 2), irregularly band-shaped, with pyrenoids ^l
Plurilocular sporangia	Uniseriate (both on sporophyte and gametophyte) ^c	Uniseriate	Uniseriate ^l
Unilocular sporangia	Solitary, ovoid ^c	Not observed	Not observed ^l
Life history	Diploid-haploid; also, direct replication of both generations by means of spores from plurilocular sporangia or parthenogenesis of gametes of both sexes ^c	Direct	Direct ^l
Hosts	Kelps: <i>Costaria costata</i> ^d , <i>Ecklonia maxima</i> ^e , <i>Laminaria hyperborea</i> , <i>L. digitata</i> , <i>Lessonia berteroa</i> , <i>L. nigrescens</i> , <i>M. pyrifer</i> ^c , <i>Saccharina latissima</i> , <i>S. nigripes</i> , <i>S. sessilis</i> ^c , <i>Undaria pinnatifida</i> ^g Other brown algae: <i>Fucus vesiculosus</i> ^h , <i>Himantothallus grandifolius</i> ⁱ , <i>Saccorhiza polyschides</i> ^j	Kelps: <i>L. hyperborea</i> , <i>L. digitata</i> ⁱ , <i>S. latissima</i>	Kelps: <i>Alaria esculenta</i> ^l , <i>L. digitata</i> , <i>L. hyperborea</i> , <i>S. latissima</i> Other brown algae: <i>S. polyschides</i> ^l , <i>Himantothalia elongata</i> ^m Red algae: <i>Palmaria palmata</i> ^l , <i>Grateloupia turuturu</i> ⁿ
Geographic distribution	Worldwide temperate to polar	Temperate North Atlantic	Northern hemisphere temperate to polar, Atlantic and Pacific

*: Information based exclusively on morphological records. (a) Ellertsdóttir & Peters (1997); (b) Apt (1988); (c) Peters (1991); (d) host information by G. W. Saunders, pers. communication; (e) Burkhardt & Peters (1998); (f) Setchell & Gardner (1922); (g) Yoshida & Akiyama (1979); (h) Nielsen & Gunnarsson (2001); (i) Peters (2003); (j) Dixon (1961); (k) Kornmann & Sahling (1977); (l) Russell (1964); (m) Cotton (1912); (n) Villalard-Bohnsack & Harlin (2001).

Our *Laminariocolax* isolates belonged to three different species, the distinction of which was supported by high bootstrap and posterior probability values and congruent with the primary partitions obtained by the ABGD analysis (Supplementary fig. S1). The interspecific K2P pairwise genetic difference between 5'COI sequences of *L. tomentosoides* and *L. atlanticus* sp. nov. (1.4%) is lower than the general species-level cut-off of 1.8% proposed by Peters *et al.* (2015) for Ectocarpales. However, the value of 1.8% must not be regarded as a strict criterion. We think it is required and justified to describe *L. atlanticus* sp. nov. as a separate species because intraspecific variability is absent in the clades of *L. tomentosoides* and *L. atlanticus* for 5'COI and negligible for ITS1. The small genetic distance between *L. tomentosoides* and *L. atlanticus* sp. nov. suggests that they may have diverged recently, possibly in North Atlantic waters where their assumed distribution ranges overlap. However, geographically extended sampling is necessary to further support this hypothesis.

Species delimitation in *Laminariocolax*

The data obtained in this study support the proposition of Peters *et al.* (2015) to include the previously described species *L. eckloniae* and *L. macrocystis* in

L. aecidioides. The original description of these taxa as distinct species was based on the occurrence of large indels in ITS1 sequences, geographic separation and occurrence in different hosts (Burkhardt & Peters, 1998). However, the importance of indels as phylogenetic markers can easily be overestimated, leading to incorrect conclusions (Babteste & Philippe, 2002). In fact, indels in *L. aecidioides* affect mainly the first part of the ITS1 region, which shows extremely high variability in Ectocarpales (e.g. Montecinos *et al.*, 2017). As *L. aecidioides* was originally described from Greenland (Rosenvinge, 1893), we decided that the name *L. aecidioides* should be applied to the clade that includes Arctic isolates. Logistic constraints inhibited us from re-collecting at the type locality, but the isolates from similar habitats at Svalbard and Baffin Island were used to molecularly define *L. aecidioides*. In our study, *L. aecidioides* was isolated from *Laminaria*, *S. latissima* and *M. pyrifer*, but it is known to infect a broader range of kelps, including *Costaria costata* (C.Agardh) De A.Saunders (host information by G. W. Saunders, pers. communication), *Ecklonia maxima* (Osbeck) Papenfuss (Burkhardt & Peters, 1998), *Saccharina sessilis* (C.Agardh) Kuntze (Setchell & Gardner, 1922) and *Undaria pinnatifida* (Harvey)

Suringar (Yoshida & Akiyama, 1979). Additionally, it was found on other brown algal hosts such as *Fucus vesiculosus* L. (Nielsen & Gunnarsson, 2001), *Himantothallus grandifolius* (A.Gepp & E.S.Gepp) Zinova (Desmarestiales, Peters, 2003) and *Saccorhiza polyschides* (Lightfoot) Batters (Tilopteridales, Dixon, 1961). *L. aecidioides* has previously also been isolated from abiotic substratum at sites where potential hosts were present (Supplementary table S2; Peters *et al.*, 2015; Küpper *et al.*, 2016). It is found in temperate to polar regions worldwide, and the adaptation to different hosts and geographic regions could be a possible explanation for the higher intraspecific divergence within this species (Ramel, 1998). Additionally, *L. aecidioides* is the only species in the genus in which unilocular sporangia (the possible site of meiosis in brown algae) have been described (Rosenvinge, 1893, in the type) and sexuality has actually been observed (Peters, 1991), which could be another reason for the larger intraspecific genetic variability (Bengtsson, 2003).

The newly described species *L. atlanticus* sp. nov. did not return any matches in public databases for the 5'COI sequences, but formed a clade with four ITS1 sequences previously identified as *L. aecidioides* (Peters, 2003). The new species has so far been isolated from *S. latissima*, *L. hyperborea* and *L. digitata* in the North Atlantic. While it is morphologically similar to the sporophyte of *Laminariocolax aecidioides* (Table 3), no unilocular sporangia, which are known to be present in *L. aecidioides* (Rosenvinge, 1893; Peters, 1991; Burkhardt & Peters, 1998), have been observed in field material or any of the *L. atlanticus* sp. nov. isolates. The new species is morphologically distinct from *L. tomentosoides* in having generally more plastids per cell and lacking epiphytic assimilatory filaments. It possesses phaeophycean hairs, which have not been reported in *L. tomentosoides* (Table 3; Russell, 1964; Kornmann & Sahling, 1977). However, the presence of phaeophycean hairs may depend on environmental conditions (Pedersen 1984), making them a less reliable classification criterion.

L. tomentosoides was first described as *Ectocarpus tomentosoides* by Farlow (1889) infecting *Laminaria* species in Massachusetts (USA, see asterisk in Fig. 3B). It is the only *Laminariocolax* species that has been found not only in brown algal hosts, but also in the red algae *Palmaria palmata* (L.) F.Weber & D.Mohr (Russell, 1964) and *Grateloupia turuturu* Yamada (Villalard-Bohnsack & Harlin, 2001), based on morphological records. Published sequences and our new molecular data confirm the presence of *L. tomentosoides* in the North Atlantic. However, there are several morphological records of *L. tomentosoides* infecting Pacific kelps (Lee, 1980; Lindstrom, 2006; Liu, 2008; Klochkova *et al.*, 2009), and a molecular

characterization of Pacific strains is necessary to clarify its actual distribution range.

The morphological species concept has dominated algal systematics for decades but numerous cases of cryptic (= morphologically indistinguishable) species have been revealed by the use of molecular data (De Clerck *et al.*, 2013; Peters *et al.*, 2015; Montecinos *et al.*, 2017). Consequently, species delimitation based on morphological data can lead to an underestimation of diversity, especially in organisms with a low morphological complexity, such as endophytic brown algae. *L. aecidioides*, *L. atlanticus* sp. nov. and *L. tomentosoides* were observed sympatrically, with their distribution ranges overlapping on the European Atlantic coast. Although slight morphological and ecological differences between the *Laminariocolax* species exist, our study stresses the importance of molecular barcoding or related methods (e.g. Bernard *et al.*, 2017) for reliable species identification in endophytic brown algae.

In addition to the three species of *Laminariocolax* included in this study, *L. draparnaldioides* (Noda, 1971) has been recorded from Japan (Noda, 1971; Yoshida *et al.*, 1990), the Russian Far East (Perestenko, 1980), and China (Liu, 2008). It was found as an epiphyte on *Stephanocystis hakodatensis* (Yendo) Draisma *et al.*, a member of the Fucales. Re-isolation and molecular data are required to confirm its belonging to this genus.

Variability of host specificity

Host ranges of the endophyte species differed across localities. The strains isolated from kelps in Brittany showed a clear host specificity: all endophytes isolated from *Saccharina latissima* in Brittany were identified as *Laminarionema elsbetiae*, all endophytes isolated from *Laminaria digitata* were identified as *Laminariocolax tomentosoides*, and the two endophytic species *L. atlanticus* sp. nov. and *L. aecidioides* were isolated from *Laminaria hyperborea*. However, this pattern was not consistent with the results from other localities, where the same kelp species are present. According to Ellertsdóttir & Peters (1997), both *Laminariocolax tomentosoides* and *L. aecidioides* were isolated from *Laminaria hyperborea* at Helgoland. In Scotland, all three species of *Laminariocolax* as well as *Laminarionema elsbetiae* were isolated from *S. latissima*; none of these endophyte species had been described from the Scottish sampling site before.

Electron microscopic observations by Heesch & Peters (1999) showed that *L. elsbetiae* and *Laminariocolax atlanticus* (described as *L. aecidioides*) infect their hosts by penetration of the host cell wall, suggesting an enzymatic dissolution. However, the underlying molecular mechanism of the infection and kelp responses are still unclear.

Differences in the cell wall composition of the host species, for instance in the content of celluloses, hemicelluloses and alginates (Siegel & Siegel, 1973), could play an important role in defining specific host-endophyte relationships.

The strains isolated in this study hardly represent the diversity of all endophytic taxa as there was a sampling bias towards species that coincide with morphological changes. However, not all hosts infected with endophytes show morphological changes (Gauna *et al.*, 2009a; Bernard *et al.*, 2017). More complete sampling campaigns, including a broader range of kelp hosts, disease symptom-free hosts, additional sampling sites and advanced identification techniques avoiding time-consuming isolation and cultivation of endophytes (Bernard *et al.*, 2017) are necessary to further investigate specificity in host-endophyte interactions. Moreover, Laminariales are known to induce specific defence reactions towards biotic attacks, such as oxidative bursts (Küpper *et al.*, 2002) or transcriptional reprogramming (Cosse *et al.*, 2009). Physiological and co-cultivation studies are essential to further investigate the ability of endophyte species to infect different hosts to finally obtain a comprehensive knowledge of this interaction.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Supplementary information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at <http://doi.org/10.1080/09670262.2018.1502816>

Supplementary table S1: Collecting information. ¹: representative strain of *L. atlanticus*, original name SaecKi92-5. Culture was also used in Burkhardt & Peters (1998, table 1, strain 1) and Heesch & Peters (1999) as *L. aecidioides*. ²:

sequence has been updated. *: strain isolated from incubated abiotic substratum.

Supplementary table S2: Sequences obtained from public databases.

Supplementary table S3: *L. elsbetiae* records used to build Fig. 3A.

Supplementary table S4: *L. aecidioides* records used to build Fig. 3B.

Supplementary table S5: *L. tomentosoides* records used to build Fig. 3D.

Supplementary fig. S1: Results of the automatic barcode gap discovery (ABGD) showing the initial primary partitions (i.e. number of groups) for a range of prior maximum divergence of intraspecific diversity. Partition 1 (N = 16): LM994983.1, LM994982.1, EndoMpyrNZ98-01, BI-041, LT546270.1, LT546273.1, ABMMC12605-10.COI-5P, Laminariocolax aecidioides Lx CCE, EndoLhypBLZ16-07, EndoLhypH93-01, EndoSlatScot15-02-01, MACRO1242-09.COI-5P, LM995048.1, LT546265.1, EndoSng16-02, EndoSng16-03

Partition 2 (N = 13): EndoLhypBLZ15-01, EndoLhypBLZ15-02, EndoLhypBLZ16-01, EndoLhypBLZ16-02, EndoLhypBLZ16-03, EndoLhypLMK16-01, EndoSlatScot14-01a, EndoSlatScot14-05, EndoSlatScot14-06, EndoSlatScot14-07, EndoSlatScot14-09, EndoSlatKi92-01, EndoSlatScot15-02-05 Partition 3 (N = 11): LM994980.1, LM994981.1, EndoLdigBLZ16-01, EndoLdigBLZ16-02, EndoLdigBLZ16-03, EndoLdigBLZ16-04, EndoLdigBLZ16-06, EndoLhypH93-02, EndoLhypH95-01, EndoSlatScot07-1, EndoSlatScot15-02-03.

Author contributions

Miriam S. Bernard: original concept, collection of field samples and isolation of strains, generation of morphological and molecular data, data analysis, writing of manuscript. M. Strittmatter: collection of field samples and isolation of strains, generation of molecular data, manuscript editing. P. Murúa: collection of field samples and isolation of strains, generation of molecular data, manuscript editing. S. Heesch: collection of field samples and isolation of strains, generation of molecular data, manuscript editing. G. Youn Cho: sample collection, manuscript editing. C. Leblanc: original concept, manuscript editing. A. F. Peters: original concept, collection of samples and isolation of strains, generation of morphological data, data analysis, manuscript editing.

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