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Widespread occurrence of endophytic *Labyrinthula* spp. in northern European eelgrass *Zostera marina* beds

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ABSTRACT: Seagrasses worldwide are commonly infected by endophytic protists of the genus *Labyrinthula*. To date, the nature of the interaction of endophyte and host is not well understood. In eelgrass *Zostera marina*, the endophyte *Labyrinthula zosterae* may become virulent (pathogenic) and lead to the loss of entire seagrass beds. One of the best known examples of any marine epidemic was a series of outbreaks of the 'wasting disease' on both sides of the Atlantic in the 1930s, but smaller infestations have been reported more recently. Until now, detection of infection by *Labyrinthula* spp. was based on the wasting index (i.e. the relative area of leaf lesions) or microscopy, while genetic data were virtually absent. We characterized a ~1400 bp portion of the 18S small subunit rDNA in *L. zosterae* isolates (N = 41) from 6 northern European sites and 1 southern location (Adriatic Sea) in order to assess the identity and potential diversity of endophytic protists. Because there are indications that low salinity impedes *Labyrinthula* growth, sampling sites included a wide range of salinities from 5 to 34 psu. A search against the non-redundant GenBank database revealed that most isolates are 99% similar to the only *L. zosterae* 18S sequence available from the database at all but the Finnish site (salinity values 5 to 7 psu). At this site, a different *Labyrinthula* species occurred, which was also found in fully marine Wadden Sea cultures. A third species was detected in Skagerrak, south-western Baltic and North Sea samples (20 to 25 psu). We conclude that *L. zosterae* is widespread among northern European eelgrass sites across wide ranges of salinity.

KEY WORDS: *Labyrinthula* infection · Eelgrass · 18S sequence analysis · Salinity · Wasting disease

INTRODUCTION

The importance of pathogens in marine ecosystems has recently become a major focus in marine biology (Harvell et al. 1999), particularly because there is evidence of an increase in marine diseases that may be linked to global climate change (Harvell et al. 2002, Ward & Lafferty 2004). Seagrasses are commonly infected by protists of the genus *Labyrinthula* (Vergeer & den Hartog 1994). Infection of *Zostera marina* by *Labyrinthula zosterae* in the 1930s led to a dramatic epidemic on the Atlantic coasts of both Europe (including France, The Netherlands, Germany, Denmark and England) and North America (New Brunswick to North Carolina; Cotton 1933, Cottam 1934). For northern Europe, published reports describe epidemics resulting in a severe decline and local elimination of entire eelgrass beds (e.g. Roskoff, France [Fischer-Piette et al. 1932]; Devon, England [Butcher 1934]; Island of Wieringen, The Netherlands [van der Werff 1938]; Königshafen on Sylt, Germany [Wohlenberg 1935]; also see Renn 1936). In the Wadden Sea, almost the entire subtidal eelgrass ecosystem disappeared (Nienburg 1927, Wohlenberg 1935) and has

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not recovered since, leaving only intertidal eelgrass in the Wadden Sea. In contrast, subtidal eelgrass populations in the Baltic remained relatively unaffected (Short et al. 1988). One hypothesis is that brackish water protects against *L. zosterae* because it slows down growth and replication of the endophyte (McKone & Tanner 2009). However, the fact that the very saline Mediterranean as well as the Pacific coast of North America remained unaffected (Cotton 1933) contradicts this hypothesis.

After the 1930s epidemic, no further large-scale epidemics were recorded, but several local- to medium-scale die-offs of *Zostera marina* beds infected by *Labyrinthula zosterae* have continued into the present (Hily et al. 2002). In Europe, a *Z. marina* decline in the Dutch Wadden Sea (Grevelingen lagoon) has been related to *L. zosterae* infection (Nienhuis 1994). With global warming, summer sea surface temperatures in the Wadden Sea and the Baltic Sea will probably increase (Wadden Sea: 16.5–19.0°C, van Aken 2008; predicted increase: 1.7–5.5°C, Philippart & Epping 2009; Baltic Sea: 15.5–19°C, HELCOM 2007; predicted increase: 2.5–3.7°C, Meier 2006) and together with other stressors lead to conditions more favourable for *L. zosterae* growth. The optimal temperature and salinity for growth and reproduction of this species are 25°C and 25 psu, respectively (McKone & Tanner 2009).

Although protists can only be conclusively identified to species by using appropriate DNA marker sequences (Adl et al. 2007), *Labyrinthula* species have mostly been identified by microscopy (Renn 1936), culture morphology (Vergeer & den Hartog 1994), host damage (the relative area of leaf lesions, i.e. the wasting index; Burdick et al. 1993), and infectivity trials (e.g. Muehlstein et al. 1988, Ralph & Short 2002). In terms of genetic data for *Labyrinthula zosterae*, the obtained information has been restricted to 2 partial 18S rDNA sequences: from the US east and west coasts (Leander & Porter 2001). As a baseline for further work on the potential role of *L. zosterae* in European eelgrass beds, genetically based species determination is urgently required. Accordingly, we characterize a ~1400 bp portion encoding the small ribosomal subunit (18S rDNA) in *Labyrinthula* spp. isolates from 6 sites in the Wadden, Baltic and Adriatic Seas of Europe in order to assess the identity and potential diversity of endophytic protists. Our sampling sites include a wide range of salinities (5 to 34 psu) from the Wadden Sea to the inner Baltic Sea (Finland, Fig. 1). Additional sequences from the Mediterranean *L. zosterae* (Adriatic Sea) were used for comparison.

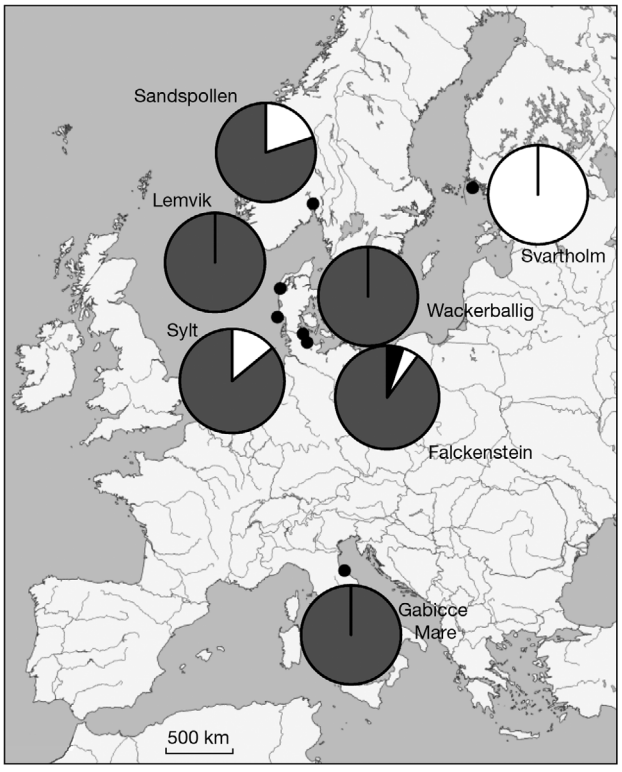

Fig. 1. *Labyrinthula* spp. isolated from *Zostera marina*. Sampling sites (●) throughout Europe and fraction of different *Labyrinthula* species corresponding to isolates (pie charts; *L. zosterae* = dark grey, *Labyrinthula* sp. A = black, *Labyrinthula* sp. B = white). Map by Stepmap (www.stepmap.de)

MATERIALS AND METHODS

Labyrinthula spp. cultures

Cultures of *Labyrinthula* spp. were prepared from 20 *Zostera marina* individuals at each of 5 different sites with sublittoral eelgrass beds (Fig. 1) between August and October 2010: Sylt (Wadden Sea, Germany), Lemvik (Limfjord, Denmark), Sandspollen (Oslo Fjord, Norway), Wackerballig (Flensburg Fjord Germany), and Svartholm (Archipelago Sea, Finland). Further cultures came from a collection in April 2008 for a mesocosm experiment in Falckenstein (Kiel Fjord, Germany) and the Gabicce Mare (Adriatic Sea, Italy).

For culturing, entire leaves (3rd or 4th leaf) showing visual symptoms of wasting disease (lesions) were dipped in 0.5% hypochlorite solution dissolved in seawater for 20 s for surface sterilization, and then rinsed with MilliQ-water for 10 s and in artificial sea water for 1 min. We made sure that only endophytic isolates were obtained by sterilizing the leaf surfaces. Washed

leaf samples were cut longitudinally. One half was dried for later genetic analysis. The other half was cut into 2 to 3 cm long pieces. Each leaf piece was placed separately on culture plates (10 cm diameter) with seawater agar according to a protocol modified from Muehlstein et al. (1988) that did not use germanium dioxide, *Rhodutorula rubra*, or Instant Ocean sea salt. Plates were incubated at 25°C in a climate cabinet, and cultures were checked after 3, 5 and 8 d. After 2 to 4 wk, cultures were transferred to new agar plates.

Isolation and DNA extraction

To extract DNA, growing culture was carefully scraped off the agar plates in 6 haphazardly chosen circles each with an area of 1.96 cm^2 that resulted in a total area of 11.76 cm^2. Removed culture was transferred into the extraction buffer of an Invitek tissue kit. DNA extraction followed the manufacturer's instructions. Growth forms of isolates from 4 sites (Wackerballig, Lemvik, Falckenstein, Sylt) differed very much (see Fig. 2). Therefore, up to 4 DNA extrations per culture from these 4 sites were performed here to check whether or not the cultures consisted of one or several labyrinthulid species.

Sequencing

Between 3 and 10 isolates per site were successfully sequenced for a significant portion of the small subunit (18S) ribosomal RNA gene (~1400 bp) using direct sequencing of the PCR product without cloning. The initial amplicon of the 18S rDNA gene was produced using the universal 18S F and R primers designed by Medlin et al. (1988) and had approximately 1680 bp. Based on initial sequencing and alignment with other published *Labyrinthula* sequences, 3 novel sequencing primers (18S_f2: 5′-CGA ATG TAG CGT TTA CTG TG-3′; 18S_r2: 5′-CCG TCA ATT CCT TTA AGT TTC AGC-3′, 18S_r3: 5′-GTG CCC TTC CGT CAA TTC C-3′) were designed within conserved portions of the 18S rDNA gene to enable the contiguous determination of the entire amplicon.

Amplicons of some representative isolates were also cloned into *Escherichia coli* competent cells in order to obtain full-length 18S sequences. For cloning, the PCR product was excised from a gel and purified, ligated into the pDrive vector (Qiagen) and transferred into *E. coli* competent cells (Qiagen EZ cells). After plasmid isolation and purification, sequencing was done using standard M13 forward and reverse primers, in addition to primers within the amplicon (see this section, above).

After standard Sanger sequencing on an ABI 3130xl genetic analyzer (Applied Biosystems) using the BigDye chemistry v3.1 (Applied Biosystems), raw DNA sequence reads for each isolate were inspected and manually edited in CodonCode aligner (CodonCode). The resulting consensus sequences for each isolate were exported to BioEdit (Hall 1999) and aligned using a combination of ClustalW and manual alignment (Larkin et al. 2007). A preliminary cluster analysis using CLUSTAL-X implemented in BioEdit was used to distinguish nearly identical sequences (1 to 3 bp difference) from different unique phylotypes.

All sequences were submitted to GenBank under accession numbers FR875306 to FR875362. We then subjected all unique sequences to BLAST search against the non-redundant nucleotide GenBank database and retrieved best matches for species assignment.

All unique 18S phylotypes obtained in this study were also subjected to a phylogenetic analysis. To this end, the data set was augmented by 24 representative Labyrinthulomycete sequences from the GenBank database with the following criteria. A core set of sequences were those that overlapped for at least 950 bp with the queries obtained here, had a BLAST E-value $<10^{-10}$ and showed a sequence similarity of ≥90%. Additional sequences from phylogenetically-related genera were chosen from published phylogenies (Kumon et al. 2003, Tsui et al. 2009, Collado-Mercado et al. 2010). As an outgroup we selected the chrysophyceae *Ochromonas danica* based on the phylogenetic analysis given in Kumon et al. (2003). We also included several recently identified terrestrial pathogenic *Labyrinthula* taxa (Craven et al. 2005, Douhan et al. 2009). The alignment was carefully inspected by eye, and only unambiguously aligned positions were included. The phylogenetic analysis was performed using maximum likelihood option in MEGA (Tamura et al. 2007) using complete deletion. We determined the best-fit substitution model using a model test (Posada & Crandall 1998). The robustness of tree topology was estimated via bootstrapping (500 times).

RESULTS

Cultures

Three to 5 d after culture start, *Labyrinthula* spp. isolates could be obtained for each sampling site. For

all isolates, spindle-shaped cells with ectoplasmatic networks were identified by light microscopy under 400× magnification (Fig. 2). Whereas most cultures grew on top of the seawater agar, isolates from Svartholm (Finland) grew within the agar. Moreover, cultures developed different morphological structures (Fig. 2b–d).

The number of isolates differed greatly between sites and was highest in samples from sites where we found only *Labyrinthula zosterae* (Table 1). Furthermore, cultures assigned to the 3 different putative species differed clearly in morphology under medium magnification (20×; Fig. 2b,c). *Zostera marina* leaves with lesions did not always lead to successful *Labyrinthula* spp. cultures, but isolates were always obtained from leaves with lesions, except for those from the Sylt sampling site.

Genetic identification and phylogenetic analysis

We extracted 18S sequences from cultures obtained from 41 infected *Zostera marina* host plants. In total, 3 unique 18S phylotypes were identified among 57 sequences based on sequence identity, including repeatedly analyzed cultures isolated from within single plants (Table 1). The majority of 18S sequences (49 out of 57) were very similar (99% identity) to 2 partial 18S rDNA sequences from North America, designated as *Labyrinthula zosterae* by Leander & Porter (2001). Two additional isolates found here were very similar to isolates that belong to 2 previously undetermined species of the genus *Labyrinthula*, hereafter designated *Labyrinthula* sp. A and sp. B. Each of these 2 isolates grouped within a well separated clade of other *Labyrinthula* species that was also differentiated to the clade containing putative *L. zosterae* (Fig. 3). Most repeated DNA extractions of cultures from the same host individual revealed identical 18S sequences, except for 1 isolate from Falckenstein, where 2 species were detected emerging from the same leaf (*Labyrinthula* sp. A and B). The closest match to *Labyrinthula* sp. A was a database sequence designated *Labyrinthula* sp. isolated from Japan (Kumon et al. 2003) with 99% identity (BLAST E-value < $10e^{-2000}$). Interestingly, the *Labyrinthula* sp. B found here is only 2 bp different to the 18S rDNA of an isolate (clone number AN-1565) found in Japan by Honda et al. (1999). In another isolate from Sylt, 2 species, *L. zosterae* and *Labyrinthula* sp. A, occurred within the same plant. Interestingly,

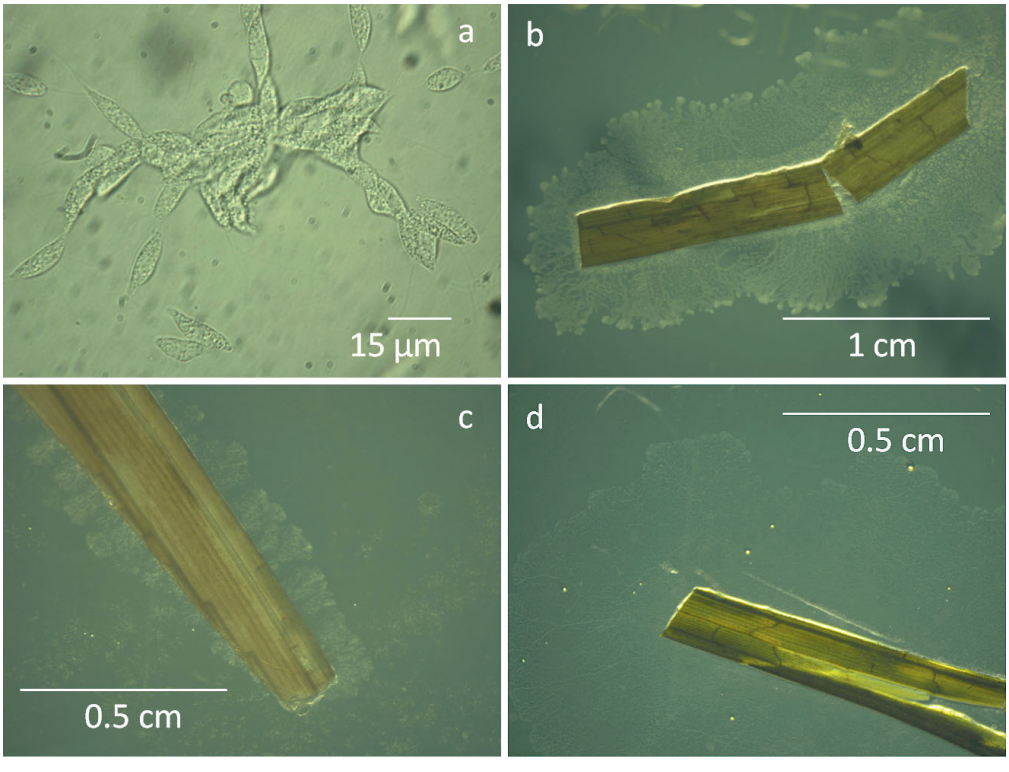

Fig. 2. *Labyrinthula* spp. growing on or in seawater agar: (a) spindle-shaped cells of *Labyrinthula zostera*, and culture of (b) *Labyrinthula zostera*, (c) *Labyrinthula* sp. A and (d) *Labyrinthula* sp. B surrounding a *Zostera marina* leaf

Table 1. Sample locations, number of cultures, 18S sequences obtained, and *Labyrinthula* isolates identified based on 18S rDNA sequencing in conjunction with BLAST-N searches against the non-redundant GenBank database. The species identification counts each culture only once. The percentage of positive cultures refers to 20 leaves tested per location. In one case, we identified 3 *Labyrinthula* spp. within the same plant (*L. zosterae*, *Labyrinthula* sp. A and B in a sample from Falckenstein), and in another case 2 species (*L. zosterae* and *Labyrinthula* sp. A in a sample from Sylt)

Location	Area	Geographic coordinates	Salinity (psu)	% positive cultures	No. of cultures analyzed	No. of 18S sequences	*L. zosterae*	*Labyrinthula* sp. A	*Labyrinthula* sp. B
Wackerballig	Baltic Sea, Germany	54° 75.57′ N, 09° 87.66′ E	15–20	70.0	6	10	6	0	0
Falckenstein	Baltic Sea, Germany	54° 39.25′ N, 10° 17.98′ E	15–20	na	6	8	5	1	1
Svartholm	Baltic Sea, Finland	60° 21.69′ N, 21° 69.17′ E	5	14.3	3	4	0	0	4
Lemvik	Limfjord, Denmark	56° 6.30′ N, 08° 9.61′ E	>30	47.4	10	14	14	0	0
Sylt	North Sea, Germany	55° 0.41′ N, 08° 04.13′ E	>30	21.4	8	12	7	1	0
Sandspollen	Skagerrak, Norway	59° 06.68′ N, 10° 57.10′ E	20–25	11.4	5	6	4	1	0
Gabicce Mare	Adriatic Sea	43° 96.79′ N, 12° 75.61′ E	35	na	3	3	3	0	0

the *Labyrinthula* isolates found inside a marine plant clearly separate from isolates found in terrestrial grasses with 100% bootstrap support (Craven et al. 2005, Douhan et al. 2009). Based on the initial screen, we selected 1 isolate putatively belonging to *Labyrinthula zosterae* from each of the 6 sites as well as replicate isolates of the putative *Labyrinthula* sp. A and B and obtained full-length 18S rDNA sequences for a total of 11 isolates. There were only 3 polymorphic sites among the 6 *L. zosterae* isolates, with a maximal pairwise difference of 2 bp among sites. Among the isolates of the other 2 putative *Labyrinthula* species, any pairwise divergence was no more than 3 bp, indicating the putative affiliation to the same species.

We then subjected the 3 isolates found here along with 24 representative 18S Labyrinthulomycete sequences deposited in GenBank to a phylogenetic analysis using maximum likelihood. As those sequences were often shorter than our 18S data, the entire data set had to be trimmed down to a length of 1075 bp for the entire alignment (total aligned sites = 791; number of parsimony informative sites = 423). No gap-coding was used as the phylogenetic signal was unambiguous. Our phylogeny revealed that our isolates likely represent 3 different endophytic labyrinthulid protist species living within eelgrass leaves in European coastal seas (Fig. 3). Note that we found a statistically significant bootstrap support for a split among the *Labyrinthula zosterae* identified here and the 2 isolates deposited in GenBank (accession nos. AF265334 and AF265335, Leander & Porter 2001) also designated as *L. zosterae*.

DISCUSSION

Using molecular genetic data, we confirm the widespread presence of a *Labyrinthula* species inside eelgrass *Zostera marina* leaves that is most similar to isolates designated as *Labyrinthula zosterae* in GenBank. This isolate is distributed across 6 sites in northern European seas and in the Adriatic, and is also 100% identical to other partial 18S sequences from isolates from 2 North American locations in Virginia and Washington state (total aligned length: 1400 bp; Bergmann et al. 2011). Only at the Finnish location (Baltic Sea) were we unable to isolate *L. zosterae*; instead, we found 1 other related isolate of the genus *Labyrinthula,* which was similar to phylotypes deposited as *Labyrinthula* sp. in GenBank but clearly distinct from *L. zosterae*. Because 2 additional distinct *Labyrinthula* species were identified in

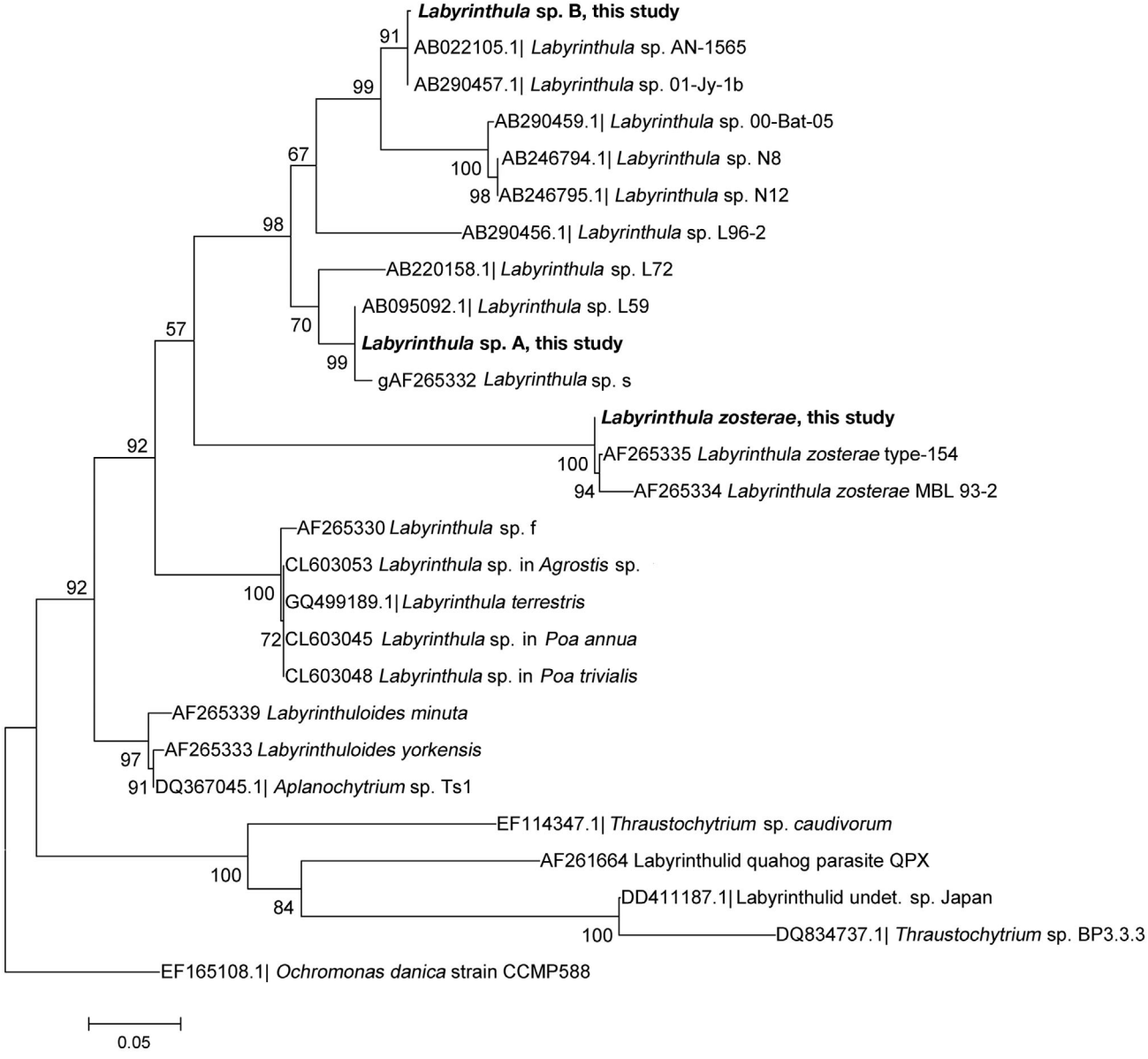

Fig. 3. *Labyrinthula zosterae* from *Zostera marina.* Phylogenetic analysis using maximum likelihood (ML) of 3 *Labyrinthula* spp. isolates identified in this study (in **bold**) along with 23 additional Labyrinthulomycete isolates obtained from GenBank. The tree is based on 791 unambiguously alignable positions including 423 parsimony informative sites. As best-fit substitution model, the Tamura-Nei model (Tamura & Nei 1993) including a discrete gamma distribution with 5 categories (TN93+G) was implemented. Branches were constructed using the nearest neighbour interchange option. Branches showing <50% bootstrap support are collapsed. As outgroup, *Ochromonas danica* was chosen

addition to *L. zosterae*, we stress that here molecular identification is as indispensible as in other protists (Adl et al. 2007). Among the full-length 18S sequences obtained from 1 isolate at each of the 6 sites with putative *L. zosterae*, pairwise differences were no more than 2 bp, although isolates came from locations as distant from each other as Italy and Norway.

Interestingly, the overall culturing success for samples from Falckenstein, Sandspollen, Sylt and especially Svartholm (sites with *Labyrinthula* sp. A or B in addition to *L. zosterae*) was considerably lower than for samples from the other 3 sites. Moreover, half of the cultures from these 4 sites survived only for a short period of time and disappeared after the 1st plate transfers (e.g. 2 to 3 wk; A. C. Bockelmann unpubl. data), suggesting that in culture, *L. zosterae* may be present in higher proportions than in *Zostera marina* in the field.

Our data from southwestern Baltic sites do not support the findings of McKone & Tanner (2009) that *Labyrinthula zosterae* is rare or absent in brackish water of medium salinity (~15 to 20 psu). Instead, *L. zosterae* may be absent at very low salinities (5 to 7 psu, Finnish site). Thus, the reason why *Zostera marina* in the southwestern Baltic Sea were unaffected by the wasting disease epidemic in the 1930s is probably not the absence of *L. zosterae*-like endophytic protists, but some other unknown process that renders this pathogen less virulent among Baltic seagrass populations. We also cannot rule out the possibility that the highly pathogenic strain is no longer present in Europe, given that 2 isolates obtained in the 1990s from North America (Leander & Porter 2001) differ from our isolates, a genetic separation that has high bootstrap support (Fig. 3). The widespread 18S sequence type identified here may thus be a different form of *L. zosterae* growing in the North Sea and Baltic area. However, our present northern European putative *L. zosterae* isolates have also caused severe lesions and mortality in plants in a recent mesocosm experiment (A. C. Bockelmann unpubl. data). Moreover, an 18S phylotype identical to the *L. zosterae* isolated in the present study also occurs in recent North American isolates from Washington State and Virginia obtained from the host plant *Z. marina* and is highly infectious upon leaf contact (Bergmann et al. 2011).

Muehlstein et al. (1988) also observed several different *Labyrinthula* isolates in North American eelgrass plants, only one of which was pathogenic. Unfortunately, the species determination at that time was entirely morphological, and no DNA exists from these initial tests. The most convincing evidence for the identification of the present *Labyrinthula zosterae*-like isolates being a potentially pathogenic form would be a direct isolation of material from a past or current wasting disease outbreak.

Despite its considerable ecological significance (e.g. Short et al. 1988, Nienhuis 1994, Short & Wyllie-Echeverria 1996), few molecular genetic data are available to support taxon identification in endophytic *Labyrinthula* species associated with seagrasses in general. Using culturing, Vergeer & den Hartog (1994) have convincingly demonstrated that many seagrasses worldwide carry *Labyrinthula*-like protists inside their tissue, but they only present light microscope data for identification. The present study provides the first data on the molecular identity of culturable *Labyrinthula* isolates in northern Europe, which will be the basis for any further ecological or epidemiological work.

Since our method is culture-based, many more different phylotypes could await discovery within seagrass plant tissue, similar to recent discoveries of the striking phylogenetic diversity of free-living Labyrinthulomycete species in the marine environment (Collado-Mercado et al. 2010).

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