Genetic diversity of *Labyrinthula* spp. in seagrass *Zostera marina* beds in the North Atlantic Ocean and the Baltic Sea

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Abstract

Die sogenannte Seegras – „Wasting Disease“ führte in den 1930er Jahren zu einem Massenrückgang des Seegrases *Zostera marina* im nördlichen Atlantik. *Labyrinthula zosterae* ist der potentielle Erreger der Krankheit, der zu dem Verlust von Seegrasbetten führen kann. Dieser Organismus infiziert die Blätter des Seegrases. Frühere Studien, die die Vielfalt der *Labyrinthula* spp untersuchten, basierten auf der Kultivierung von *Labyrinthula*. Diese Studie ist die erste Studie, die Blattproben zur molekularen Identifizierung von *Labyrinthula* verwendet und erforscht die Vielfalt der Gattung *Labyrinthula*. Proben von sechs Standorten wurden untersucht, dabei wurde ein 290 bp lange Teilsequenz der 18S rDNA kloniert und Sanger sequenziert. Das Abgleichen der erhaltenen Sequenzen mit NCBI GenBank mittels des blast Algorithmus ergab, dass die meisten Sequenzen von 77 bis 100% mit *L. zosterae* übereinstimmen, während inige Sequenzen der Standorte Wackerballig und Sandspollen eine 84-94 prozentige Übereinstimmung mit *Labyrinthula* spp zeigten. Keine der mutmaßlichen *Labyrinthula* spp. Sequenzen wurden mit bekannten Sequenzen aus der Datenbank zusammengefasst. Es wurden sieben OTUs generiert. Der Test von verschiedenen DNA-Extraktionmethoden ergab für ein Pflanzen- und ein Gewebe-Extraktionskit ähnliche Ergebnisse. Nichtsdestotrotz hat das Pflanzen-Kit mehr Vorteile wegen geringerer Kosten und dem geringeren Zeitaufwand. Zusammenfassend zeigt diese Studie, dass bisher unbekannte *Labyrinthula* spp. in den Seegraspopulationen vorkommen, und dass das in dieser Studie verwendete Verfahren für weitere Anwendungen geeignet ist.

Abstract

Seagrass wasting disease in the 1930s resulted in mass decline of seagrass *Zostera marina* in the Northern Atlantic Ocean. *Labyrinthula zosterae* is the potential pathogen of this disease that can lead to seagrass bed loss. This organism infects eelgrass' leaf. Previous studies to identify the diversity of *Labyrinthula* spp. were based on culture method. This present study is the first study to use leaf samples for *Labyrinthula* molecular identification and explores diversity of the genus *Labyrinthula*. I investigated samples from six locations by cloning and Sanger sequencing of a 290 bp partial sequence of the 18S rDNA. Blast search against GenBank revealed that most sequences are 77-100% similar to *L. zosterae*, while some sequences from Wackerballig and Sandspollen were 84-94% matched *Labyrinthula* spp. None of that putative *Labyrinthula* spp. was grouped with known sequences from the database. There were seven OTUs generated. DNA extraction gave similar results for both tested extraction kits, a plant and a tissue kits. Nonetheless, the plant kit has more advantage due to its lower cost and time effort. In conclusion, this study shows undiscovered *Labyrinthula* spp. in the investigated eelgrass populations, second it shows that the method used in this study is suitable for further applications.

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Chapter 1: Introduction

1.1 Seagrass

Seagrasses are monocotyledonous marine angiosperms (Procaccini et al., 2007). They play important roles in marine ecosystems such as nursery grounds for many animals, protecting shelters from sediments between mangrove and coral reefs and food sources for dugongs, manatees, turtles and herbivorous fish (Björk et al. 2008; Unsworth et al. 2008). The most important function of the seagrass bed is the large amount of carbon that is fixed by photosynthesis and becomes available via the detrital food chain and an important carbon sink (Duarte et al., 2010; Fourqurean et al., 2012)

There are 14 genera of seagrasses belonging to four families, Cymodoceaceae, Hydrocharitaceae, Posidoniaceae and Zosteraceae; all are in subclass Alismatidae and consist of less than 0.1% of all flowering plants found in the world (Waycott et al. 2002; Lucas et al. 2012).

Zostera (commonly known as eelgrass) is very widespread and so occurs in temperate and as well as in the tropical climates (Hemminga and Duarte, 2000). It belongs to family Zosteraceae. The genus is characterized by the species that are monoecious with creeping rhizome and are usually perennial. There are nine species within this genus (Larkum et al., 2007). *Zostera* is considered a colonizer as well as a climax species - that is, the single species represents the entire range of system development (Den Hartog, 1971). *Zostera marina* L. occurs predominantly in a monoculture throughout most of its distribution, although it sometimes co-occurs with a variety of species.

Z. marina is widely distributed in the coastal and estuarine areas of the northern Atlantic, Mediterranean, and Eastern Pacific (Jacobs, 1979; Short and Short, 2003). *Z. marina*'s blade is about 80 centimeters long, with 3 to 12 millimeters wide (Larkum et al., 2007). It forms dense beds and found in sheltered bays and lagoons from the lower shore to about 7.6 m depth in the West Baltic, generally on sand and sandy mud (Dale et al., 2007; Schubert et al., 2015).

Current studies reveal the declining size and abundance of seagrass meadows around the world (Waycott et al., 2009). Environmental changes including physical disturbance, aquacultures, nutrients runoff, climate change, human activities as well as disease accelerate the decline (Orth et al., 2006; Rasmussen, 1977; Short et al., 2011).

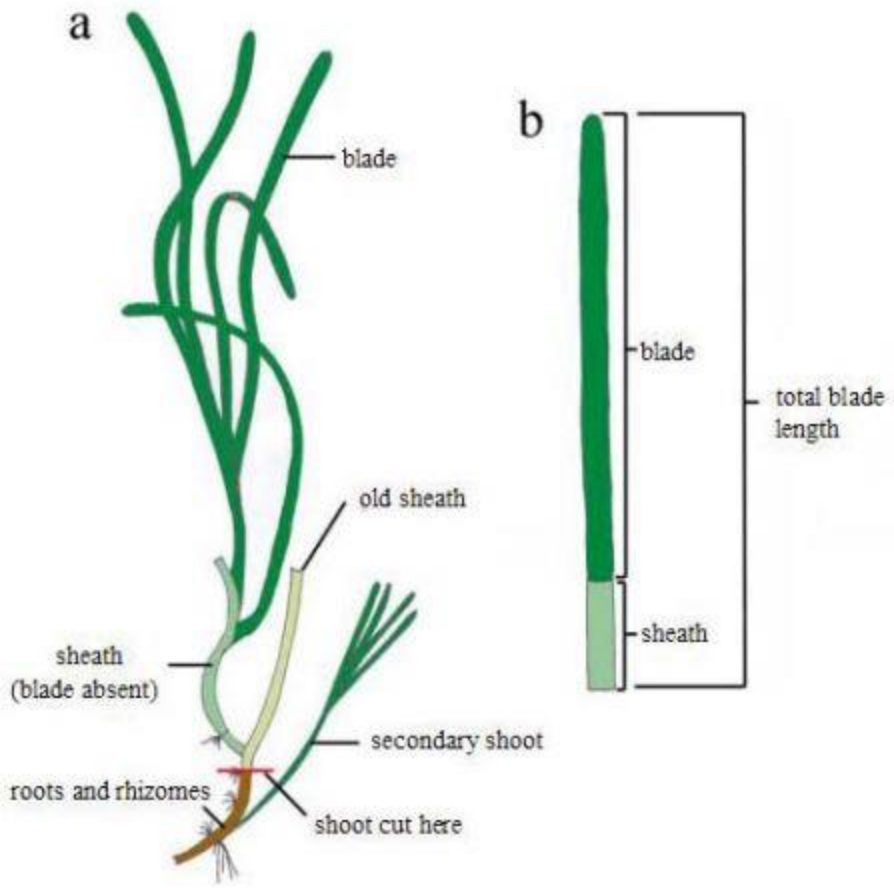

Figure 1.1 Diagram of *Z. marina* (Dale et al., 2007).

1.2 Wasting disease

The mass declination event of nearly all eelgrass *Z. marina* populations in the Northern Atlantic in the 1930s is wasting disease. The wasting disease was firstly reported in populations of eelgrass *Z. marina* in the North American Atlantic in 1931, and later in the Atlantic European populations in 1932. The disease in Northern America and Northern Europe between 1930 and 1940 resulted in significant eelgrass beds lost, coastal erosion and loss of fisheries habitat. However, since the eelgrass is a fast-growing species with rapid colonization capacity most of the eelgrass meadows were reestablished within 10 years, mainly after 1960 (Blois et al., 1961; Larkum et al., 2007). There are some locations that the eelgrass has never recovered after the disease, for example, at the Dutch Wadden Sea (van Katwijk et al., 2000) and the French coasts (Godet et al., 2008). Not only *Z. marina*, *Labyrinthula* spp. also associated in mass declination of *Z. capricorni* in New Zealand in 1960 (Armiger, 1964) and putative cause of mass mortality of seagrass *Thalassia testudinum* in the Florida Bay in 1987 (Blakesley et al., 2001; Durako and Kuss, 1994; Robblee et al., 1991).

It was at first thought to be caused by the slime mold *L. macrocystis* Cienkowski, pollution, climatic factors especially extreme precipitations, or even high summer temperature (Den Hartog, 1987). Eventually, Muehlstein, Porter, & Short (1991) isolated *Labyrinthula* spp. from infected leaves of *Z. marina.* Among various culture of *Labyrinthula*, only one strain causes the symptom of wasting disease. They identified that strain as *Labyrinthula zosterae* Porter & Muehlstein as a causing agent of this disease, based on host specificity, cytology, growth pattern, cell size, color in mass, and aggregation structure. Supported by infection test by Muehlstein (1992). He found that direct contact between infected leaf and healthy leaf was the necessary mechanism of the symptoms.

The symptoms of the disease are very characteristic. Small brown spots develop on the leaves; these spots spread and become darker, and finally cover large parts of the leaves, which eventually are detached. The whole process takes a few weeks. The rhizomes also show discoloration. The infected plants often survive for up to a year, but repeated defoliation exhausts the plants and eventually, lead to death (Den Hartog, 1987; Rasmussen, 1977).

L. zosterae is mostly found at the margin of necrosis from both surface of the leaf. At the beginning of infection, *Labyrinthula* cells were mainly located in mesophyll cells taken from marginal areas of necrosis of small necrotic spots (Armiger, 1964). The cells are able to invade neighboring cells by making hole in the plant cell wall and moving through it. The cells are found in the vascular tissue. In later phases of infection, characterized by leaf tissue that was completely brown, *Labyrinthula* cells are more common in epidermal cells than in earlier stages and are occasionally found in lacunae (Muehlstein, 1992).

Figure 1.2 Infected *Z. marina* leaves (Ralph and Short, 2002).

1.3 Marine microorganisms

There are about 100 million species predicted but fewer than 2 million species are described to now (May, 1988). The species catalog is quite complete for large body size organisms. Nonetheless, we are missing the vast majority of diversity as since the estimated 99% of small organisms such as bacteria and single cell eukaryotes cannot cultivated (Woese, 1996). Microorganisms in marine ecosystems play fundamental ecological roles such as primary producers, consumers and decomposers in aquatic food webs (Caron et al., 2012). However, diversity of marine organism is still unclear. The early method to access the diversity like cultivation-dependent method gave low diversity and abundance (Galand et al., 2009). Pace (1997) estimated the marine species is about hundred times higher than the estimates based on the cultivation. The emerging field of DNA sequence-based method in the last fifteen years has revealed the diversity of all types of microorganisms (Galand et al., 2009; Pedrós-Alió, 2006). Sogin et al. (2006) revealed bacterial communities in the deep sea in the North Atlantic are about one to two times more complex than previously reported. There are thousands of low abundance populations containing most of the diversity, which is called "rare biosphere".

1.4 *Labyrinthula*

Labyrinthulomycetes is a group of protists that mainly lives in marine environment. This class consists of two groups: Labyrinthulids and Thraustochytrids. Porter and Kochert, (1978) firstly used ribosomal DNA molecular weights to reclassify the phylogenetic relationship of this group. Cavalier-Smith, Allsopp, & Chao (1994) reclassified phylum Heterokonta putting Labyrinthulomycids and Thraustochytrids in subphylum Labyrinthista, families Labyrinthulidae and Thraustochytridae, respectively by using evidence of 18S small subunit ribosomal RNA gene. They also found that Labyrinthulids, along with Thraustochytrids are closely related and from a deeply divergent branch of the phylum Heterokonta.

Recently, Tsui et al. (2009) studied phylogeny of Labyrinthulomycetes using sequences of the actin, beta-tubulin, and elongation factor 1-alpha gene fragments and ribosomal small subunit (SSU) genes. This was the first time using multiloci nuclear sequences to reconstruct phylogenetic tree of this group. They found that Labyrinthulomycetes and the bicoecean are sister groups. Labyrinthulomycetes is monophyletic consists of two clades, one with *Labyrinthula* spp. and *Aplanochytrium* spp., and another clade has Thraustochytriidae and *Oblongichytrium minitum*. Thus, Labyrinthulomycetes is sister to the other nonpigmented stramenopiles (the Oomycetes, Developayella, and Hyphochytrium) (Leander and Porter, 2001).

Genus *Labyrinthula* was firstly described by Cienkowski (1867) as marine slime molds. *Labyrinthula* are characterized by spindle-shaped or spherical, non-amoeboid cells, live in colony, biflagellate zoospores, and multilamellate cell walls composed of Golgi body-derived scales. One feature thing is the members of this genus can produce ectoplasmic network from one or more points on the cell. These networks are produced by a special organ called sagenogenetosome or bothrosome (Perkins, 1972a; Porter, 1972). The network helps cell in locomotion, digestion, increasing surface area and protection (Perkins, 1972b).

The number of species in this genus is uncertain. There are eight or nine recognized species. The characteristics used in species delineation include cell size, color in mass and reproductive or resting stages (Muehlstein et al., 1991). *L. zosterae* has a spindle-like cell, 15.5-19.5 µm long and 3.5- 5 µm wide. It forms a dense, bushy colony with an evenly expanding margin, hyaline to pale yellow in soft agar, vacuole 0.4 µm in diameter and ectoplasmic network branching and anastomosing (Muehlstein et al., 1991; Young, 1943).

Almost all members of this genus live in marine environment except some species. *L. terrestris* is known to be a pathogen causing rapid blight disease of terrestrial plants, mostly turfgrasses such as *Agrotis capillaris* and *Poa annua* (Bigelow et al., 2005; Entwistle et al., 2006; Olsen, 2007). *Labyrinthula cienkowski* Zopf, isolated from *Vaucheria sessilis*, a freshwater alga (Zopf 1892). Aschner (1958) cultured *Labyrinthula macrocystis* Cienk. from soil around roots of a diseased *Carica papaya* in Israel.

The use of rDNA sequences to explore microbial diversity has become popular in the last decades (Nakamura et al., 2015; Yuasa et al., 2006) because sequences of these genes provide taxonomic characters for species that have few distinctive morphological features, or that require involved microscopy or laboratory culture and testing (Countway et al., 2005). The rDNA, particularly 18S rDNA for eukaryotes overcome other genes due to its ubiquity, size, and low evolutionary rate, the small subunit ribosomal RNA is an important tool in molecular evolution (Van de Peer and De Wachter, 1997).

Bockelmann et al. (2012) firstly used 18s ribosomal DNA for phylogenetic analysis of *Labyrinthula* spp. They cultured *Labyrinthula* from leaves showing symptoms of wasting disease. With direct sequencing of PCR products they found *L. zosterae* in most populations of eelgrass analyzed with two putatively new species in Northern European Waters with different salinity, however, higher diversity is expected for the culture-independent study (Pace, 1997). In addition, Bockelmann et al. (2013) studied prevalence and abundance of *L. zosterae* in 19 *Z. marina* populations in the Baltic Sea and North Sea by quantitative PCR using internal-transcribe-spacer 1 (ITS1). They found that *L. zosterae* occurs mainly in summer especially between June to September, none during winter and early spring; the abundance ranges between 0 and 271 cells per mg *Z. marina* dry weight.

1.5 DNA extraction kit comparisons

High quality of DNA is required for molecular systematics. Genomic DNA extraction is different depends on organisms and tissues (Aljanabi, 1997). Isolation of DNA in plant is more complicated than in animal tissue due to its rigid cell wall surrounding the cell (Manen et al., 2005) along with polysaccharides and secondary metabolites (Pandey et al., 1996).

Standard extraction methods is often a limiting factor in genetic study in plant fields (Xin and Chen, 2012) while silica-based commercially available methods give stable DNA extracts, highly sensitive and are generally faster than the standard methods (Demeke and Jenkins, 2010). The detection of microbial nucleic acid for the diagnosis of infection is dependent on the successful separation of nucleic acid from material (Maropola et al., 2015; Queipo-Ortuño et al., 2008). Many of the extraction kits use spin column technology because this approach is usually easily integrated into laboratories with standard equipment, which contains four major steps; lysis, binding, washing and elution. Nucleic acid is released using chaotropic buffer to denature proteins. The separation of nucleic acid from other components is achieved by temporary nonspecific adsorption to a silica membrane within a column. High salt concentration washes and removes proteins and other impurities. The subsequent applications of a low salt concentration buffer elutes the purified nucleic acid in a form suitable for enzymatic processes (Queipo-Ortuño et al., 2008). In recent years, many extraction methods have released to give higher yield of nucleic acids (Akkurt, 2012; Boudon-Padieu et al., 2015; Maropola et al., 2015; Xin and Chen, 2012). The polymerase chain reaction (PCR) has been used with increasing frequency for detecting and identifying plant pathogens. A prerequisite of PCR amplification is the preparation of good quality DNA with a consistent yield from infected hosts. Although PCR is sensitive, research has shown that amplification of target microbial DNA from within another organism can be inhibited by the presence of host DNA (Cating et al., 2012).

1.6 DNA isolation of *Labyrinthula*

Bergmann et al. (2011) used Invisorb spin tissue mini kit (Stratec Biomedical, Berlin, Germany) with addition of UltraPure Salmon Sperm DNA solution (Invitrogen, Life Technologies, USA) for DNA extraction of *L. zosterae* cultures isolated from infected *Z. marina*. This method was later used by Bockelmann et al.(2013) with extra purification step using One-Step PCR inhibitor removal kit (Zymo Research, USA) fresh *Z. marina* leaves DNA isolation.

Presently there are various kind of DNA extraction methods. Each method produces different yield of DNA even from the same material and may have significant effects in

the downstream processes (Akkurt, 2012; Fredricks et al., 2005; Queipo-Ortuño et al., 2008). Plant DNA extraction kit is widely used to isolate pathogens from plant specimens (Boudon-Padieu et al., 2015; Li et al., 2013).

1.7 Aims

Firstly, this study aimed to use 18S small subunit ribosomal DNA to detect eelgrass pathogens, especially *Labyrinthula* spp. in eelgrass populations in the North Atlantic and Baltic Sea using PCR amplification, cloning and Sanger sequencing to reveal their diversity using phylogenetic analysis, species richness estimators, and rarefaction curves. The second aim was to compare DNA extraction efficiency of commercially available plant and tissue extraction kits to detect *Labyrinthula* in *Z. marina* leaf samples.

Chapter 2: Methods

2.1 Diversity of *Labyrinthula* across the North Atlantic and the Baltic Sea

Sample collections

Extracted DNA samples obtained from (Bockelmann et al., 2013, 2012) were used in this study. These samples were collected within the affected areas of 1930 wasting disease in the North Atlantic and Baltic Sea except samples from Puck, Poland and Rannakula, Estonia (Table 1).

Table 2.1 Locations of samples used in this study. * data obtained from (Maar et al., 2011).

Location	GPS Coordinates	Collection date	Number of samples	Salinity (psu)
Lemvig, Denmark	N 56.6300 E 08.2961	February 2011	2	>30
Wackerballig, Germany	N 54.7557 E 09.8668	February 2011	2	15-11
Puck, Poland	N 54.7383 E 18.4471	April 2012	2	5-10*
Rannakula, Estonia	N 59.2547 E 23.6575	February 2011	2	5-10*
Sandspollen, Norway	N 59.6657 E 10.5869	February 2011	2	20-25
Kungälv, Sweden	N 57.5405 E 11.4083	August 2011	2	6-14

Primer design

Sequences of *Labyrinthula*, *Phytophthora* and other related species 18S ribosomal DNA obtained from GenBank were aligned using Mega version 6 (Tamura et al., 2013). Two sets of primers were designed; *Labyrinthula* and *Phytophthora* specific and *Phytophthora* specific primers. These primers contain some degenerate sites to bind conserved regions, which give the products about 250 base pairs long. Additional *Labyrinthula* specific primers (Reusch, unpublished) were tested. USEARCH (Edgar, 2010) was used to test these primers *in silico* against *Labyrinthula* and *Phytophthora* sequences about their binding sites and expected amplicon length. To select the primers with the best coverage, we used TestPrime version 1.0 (Klindworth et al., 2013) available on Silva database (Quast et al., 2013) with Parc database (release 119).

Primers test

All primers pairs (Eurofins, Ebersberg, Germany) were optimized by testing annealing temperatures between 52°C to 58°C with previously extracted *Zostera marina* leaf DNA samples available in the laboratory. PCR reaction mixture (20 µl) contains 10 µg of DNA, 1X of multiplex PCR master mix (QIAGEN, Hilden, Germany) and 5 pg of each primer.

Amplification was conducted in Veriti 96-well Thermal Cycler (Life Technologies, USA) with the following steps: denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C 35 seconds, 52-58°C for 40 seconds and 72°C for 1 minute, and final extension at 72°C for 15 minutes. Quantity and quality of PCR products were tested with 2% agarose gel electrophoresis. The PCR products were cut from the gel and were purify with Nucleospin Gel and PCR Clean-Up (Macherey-Nagel, Düren, Germany). Exonuclease I and alkaline phosphatase FastAP (Invitrogen, Life Technologies, USA) were used to remove excess primers, nucleotides and phosphate at 3' and 5' end of the positive PCR products prior to cloning.

Cloning of PCR products

Purified PCR products were cloned into pCRII-TOPO vector (Invitrogen, Life Technologies, USA) (Figure 1) using 3:1 PCR product to vector ratio and ligation at 4°C for 16-18 hours. Recombinant vector was transformed into TOP10 chemically competent *Escherichia coli* cells (Invitrogen, Life Technologies, USA), and plated onto LB plates containing 50 mg ampicillin and 50 mg Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Transformants containing recombinant plasmid were randomly selected by blue/white screening and suspend in 25µl water.

Colony PCR

Colony PCR reaction mixture (10 µl) contains 10 µg of DNA, 1.5 units of DreamTaq DNA polymerase (Life Technologies, USA), 1X of DreamTaq Buffer, dNTPs and 5 pg of each M13 primers. Cycling conditions were initial denaturation at 95°C for 1 minute followed by 35 cycles of 95°C 30 seconds, 50°C for 15 seconds and 72°C for 1 minute, and final extension at 72°C for 7 minutes. PCR amplification products were size fractionated by 2% agarose gel electrophoresis. Amplicons of the right size were cleaned by Exonuclease I and alkaline phosphatase FastAP (Invitrogen, Life Technologies, USA).

Sequencing

Cycle sequencing was performed in 10-µl reaction containing 0.25µl BigDye v3.1, 1X BigDye buffer and 2.5 pmol of primer. Cycling conditions were 96°C for 1 minute, 25 cycles of 96°C for 1 minute, 50°C for 5 seconds and 60°C for 4 minutes. The products were cleaned to remove excess fluorescent dye with BigDye XTerminator Purification Kit (Applied Biosystems, USA) and were sequenced in both directions using automated DNA sequencer (Applied Biosystems, USA).

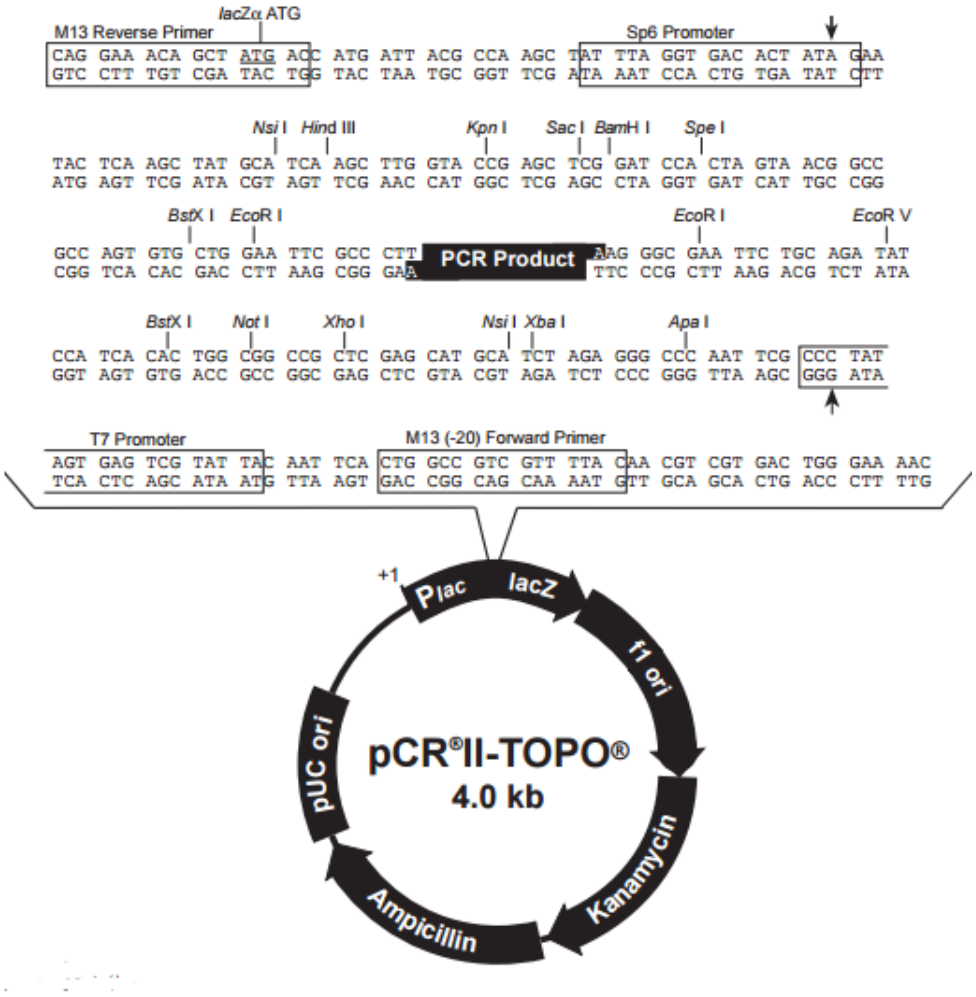

Figure 2.1 pCRII-Topo cloning vector map (Invitrogen, Life Technologies, https://tools.lifetechnologies.com/content/sfs/vectors/pcriitopo_map.pdf).

Sequence editing and alignment

Raw sequences were manually edited using Geneious (Biomatters). Sequences with clear chromatogram and longer than 200 bp were used for the analysis. Sequences of the cloning vector were removed prior to assemble. Forward and reverse sequences were *de nevo* assembled. The sequences were blasted against nucleotide database on GenBank – fungal and other non-*Labyrinthula* sequences were excluded. Multiple alignment was done by Clustal W implemented on Mega 6 (Tamura et al., 2013) with additional 18s rDNA sequences of *Labyrinthula* spp. and *Aplanochytrium* spp. from GenBank (Table 2).

Table 2.2 Additional *Labyrinthula* and *Aplanochytrium* sequences obtained from GenBank for phylogenetic analysis.

Species	Accession number
Aplanochytrium spp.	EU851174.1
	EU851172.1
Aplanochytrium stocchinoi	AJ519935.1
Labyrinthula spp.	FR875359.2
	FR875362.2
	FR875356.2
	FR875355.2
Labyrinthula zosterae	FR875352.2
	FR875335.2
	JN121408.1
	JN121407.1
	JN121406.1
	JN121405.1
	JN121404.1
	FR875309.1
	FR875307.1
	FR875306.1
	AF265335.1

Phylogenetic analysis

The alignment file containing additional sequences was used. All sites were included. The best fit evolutionary model of the data set was done by jModelTest 2.1.7 (Darriba et al., 2012). Likelihood setting were: 203 substitution schemes (which calculates 1,624 models), include models with unequal base frequencies (+F), models with rate variation among sites and number of categories (+G), models with a proportion invariable sites (+I). Maximum likelihood optimized was set as a base tree, and using the best of Nearest Neighbor Interchange (NNI) and Subtree Pruning and Regrafting (SPR) for tree search. Best models calculated with the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) were taken into consideration. RAxML version 8.1.2 (Stamatakis, 2014) was used to generate maximum likelihood phylogenetic tree reconstruction with 100 bootstraps. Phylogenetic tree was then edited with FigTree version 1.4.2 (Rambaut, 2014).

OTU calling

Sequences from the six clone libraries were pooled and grouped into operational taxonomic units (OTUs) using UPARSE (Edgar, 2013) at a sequence similarity cutoff of 97%, that means, sequences that are less than 3% difference were grouped together into one OTU. Consensus sequences of each OUT were aligned. Evolutionary model of this data set was calculated by jModeltest. Phylogenetic tree were generated by PhyML (Guindon et al., 2005) with HKY model and following parameters; transition/transversion ratio 1.3778, gamma shape 0.595.

Species diversity and species prediction

Operational taxonomic units generated from UPARSE were used as an input file for Species Prediction And Diversity Estimation (SPADE) program (Chao and Shen, 2010) to calculate richness (Chao-1, bias-corrected Chao-1, ACE and ACE-1) and species prediction with these parameters; multinomial model, 1,000 prediction size, and cut-off value = 5). Sample-size-based rarefraction and extrapolation sampling curves, sample completeness curve, and coverage-based rarefraction sampling curve were plotted using iNEXT software (Hsieh et al., 2013) with endpoint setting 224 and 40 knots.

2.2 Extraction kit comparison

Plant samples

Z. marina leaf samples stored in silica gel from two locations were used (Table 3). Samples from Waquoit Bay was affected by the wasting disease in the 1930s (Muehlstein et al., 1988). Two commercially available kits were compared: Invisorb Spin Plant Mini Kit and Invisorb Spin Tissue Mini Kit (Stratec Biomedical, Berlin, Germany). Three samples from each location were divided into two parts - one for each extraction kit and were homogenized by TissueLyser II (QIAGEN, Hilden, Germany) with frequency 1/30s for 8 minutes.

Table 2.3 Samples used for the DNA extraction kit comparison.

Location	GPS Coordinates	Collection date	Number of samples
Chioggia, Italy	N 45.2233 E 12.3130	May 2010	3
Waquoit Bay, USA	N 41.5578 E -70.5168	May 2010	3

Extraction methods

The extractions were performed following manufacturers' instructions. For Invisorb Spin Tissue Mini Kit, 10 µg of UltraPure Salmon sperm solution (Invitrogen, Life Technologies, USA) was added to each sample to improve the binding efficiency of the DNA to the silica membrane column, and additional purification step with OneStep™ PCR Inhibitor Removal Kit (Zymo Research, USA) to remove inhibitors in the downstream process. Total DNA was eluted with elution buffer in 50 µl for both extractions. DNA concentration was measured by NanoDrop ND-1000 spectrophotometer.

PCR amplification

Extracted DNA was amplified with 10 mM of each *Labyrinthula* specific primer (Table), DreamTaq PCR Master Mix (Life Technologies) that contains DreamTaq DNA Polymerase, 1X DreamTaq buffer, 2 mM $MgCl_2$, and dNTPs in total of 10-µl reaction. PCR conditions included 3 minutes' initial denaturation at 95°C, followed by 25-30 cycles of 30 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C, and final elongation for 15 minutes at 72°C. PCR products were checked by electrophoresis with 2% agarose gel. Bands of expected size were cut off, purify with Nucleospin Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany), and were measured for the concentration of the products with NanoDrop ND-1000 spectrophotometer.

Cloning

Cloning was performed as described above.

Restriction analysis

Plasmid preparation was performed relying on the principle of alkaline lyses using Zyppy Plasmid Miniprep Kit (Zymo Research, USA). Plasmid was digested by FastDigest *Eco*RI (Life Technologies, USA) for 15 minutes at 37°C and was fractionated by 1% agarose gel electrophoresis.

Colony PCR

Colony PCR reaction mixture (10 µl) contains 10 µg of DNA, 1X of DreamTaq PCR Master Mix and 5 pg of each M13 primers. Cycling conditions were initial denaturation at 95°C for 1 minute followed by 35 cycles of 95°C 30 seconds, 50°C for 15 seconds and 72°C for 1 minute, and final extension at 72°C for 7 minutes. PCR amplification products were size fractionated by 2% agarose gel electrophoresis. We selected eight corrected inserts to be cleaned by Exonuclease I and alkaline phosphatase FastAP (Invitrogen, Life Technologies, USA). Cycle sequencing was performed in 10-µl reaction containing 0.25µl BigDye v3.1, BigDye buffer and 2.5 pmol of primer. Cycling conditions were 96°C for 1 minute, 25 cycles of 96°C for 1 minute, 50°C for 5 seconds and 60°C for 4 minutes. The products were cleaned with EDTA/ethanol prior sequencing and were sequenced in forward directions with automated DNA sequencer (Applied Biosystems, USA).

Bioinformatic analysis

Vector sequences were trimmed and the sequences were edited with SeqMan Pro (DNAStar, Madison, USA). To remove the fungal and other non *Labyrinthula* sequences were excluded, those sequences were blasted against GenBank database. Clustral W implemented in Mega version 6 (Tamura et al., 2013) aligned the remaining sequences. The sequences were defined into two groups by the extraction methods. DNAsp (Librado and Rozas, 2009) was used to calculate number of haplotype. Haplotype network for each data set was created and edited in Network (Polzin and Daneshmand, 2003). We used UPARSE (Edgar, 2013) to group these sequences into operational taxonomic units (OTUs).

Calculation of the extraction costs

The cost per extraction was calculated by dividing the cost by the number of extractions that could be performed with the kit.

Chapter 3: Results

3.1 Diversity of *Labyrinthula* across the North Atlantic and the Baltic Sea

Primer testing

I designed two new primer pairs to detect *Labyrinthula* spp. and *Phytophthora* spp. 18S rDNA sequences; Tom/Jerry to amplify *Labyrinthula* spp. and *Phytophthora* spp. and Mickey/Minnie amplifying only *Phytophthora* spp. These degenerated primer pairs have an expected product size around 250 base pairs. *In silico* test of their binding sites with USEARCH showed that these primer pairs were perfectly bind to the target region. With Silva 18S rDNA database, Tom and Jerry bind to *Labyrinthula* spp. and *Phytophthora* spp. with 85.1 and 95.6% respectively. For those *Labyrinthula* and *Phytophthora* specific primers, their coverages were 95.7% and 96.4% respectively (Table 3.1).

Table 3.1 Primers used in this study. *obtained from (Reusch, unpublished)

Primers	Sequences (5'-3')	Tm (°C)	Coverage (%)		Amplicon size (bp)
			Labyrinthula	*Phytophthora*	
Tom	GCGTATATTAAMGTTGTTGCAGTT	57	85.1	95.6	250
Jerry	ATTATTCCATGCTARTGTATKCA				
Fold*	GCAGTTAAAAAGCTCGTAGTTGAA	58	95.7	-	290
Rold*	AGAATTTCACCTCTGACATRCTC				
Mickey	CTTAACGAGGATCAATTGGAG		-	96.4	250
Minnie	AACGCCTGCTTTAAACACTCT				

However, when the primer pair Tom/Jerry was tested, I found that these primers also amplified *Zostera marina* 18S rDNA instead of only *Labyrinthula* and *Phytophthora*, so we decided not to use them. For further studies, we used primer pair obtained from (Reusch, unpublished). These primers worked well, however, they are limited the genus of *Labyrinthula*. The average sequence length produced by these primers was about 290 base pairs. Unfortunately, there was no *Phytophthora* spp. DNA that could be used as positive control available, so we could not test primers Mickey/Minnie.

Phylogenetic analysis

Blasting the obtain sequences from Wackerballig revealed *L. zosterae* FR875335.2 with 77-100% identity, and *Labyrinthula* spp. AB220158.1, FR875356.2 and EU431330.1 with 85-94%, 84%, and 93% identity respectively. The sequences from Sandspollen matched to *L. zosterae* FR875335.2 with 94-100% identity, and *Labyrinthula* spp. EU431330.1 with

94%. Samples from other four locations have only sequences that match to *L. zosterae* FR875335.2.

Maximum likelihood phylogenetic tree of all sequences from six locations are shown in figure 3.1. Additional sequences of *Labyrinthula zosterae*, *Labyrinthula* spp. and *Aplynochytrium* spp. were included. The *Labyrinthula* were grouped into two clades; *L. zosterae* (red) and *Labyrinthula* spp. (blue), with Ap*lynochytrium* spp. appeared as the outgroup. However, *Labyrinthula* spp. clade is ambiguous. The *Labyrinthula* spp. found in this study divided into three clades.

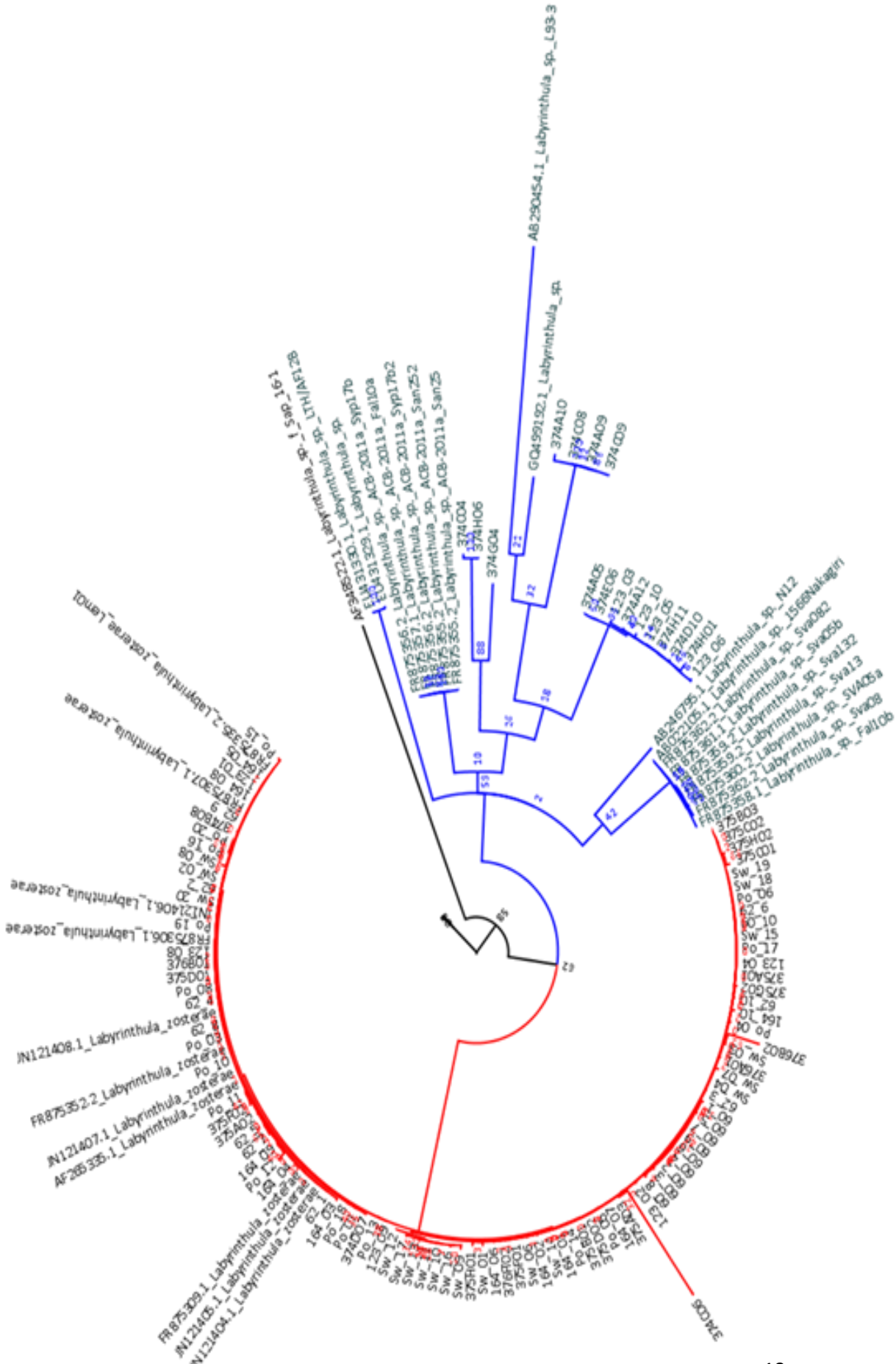
AB290454.1_Labyrinthula_sp._L93-3
GQ459192.1_Labyrinthula_sp.
374A10
374C08
374A09
374C09
AF348522.1_Labyrinthula_sp._LTH/AF12B
EU131330.1_Labyrinthula_sp.
EU131329.1_Labyrinthula_sp._Syp17b
FR875356.2_Labyrinthula_sp._ACB-2011a_Fall10a
FR875357.1_Labyrinthula_sp._ACB-2011a_Syp17b2
FR875356.2_Labyrinthula_sp._ACB-2011a_San25.2
FR875355.2_Labyrinthula_sp._ACB-2011a_San252
374G04
374H06
374G04
374A05
374E06
123_03
374A12
123_10
123_05
374H11
374D10
374H01
123_06
AB246795.1_Labyrinthula_sp._N12
AB022105.1_Labyrinthula_sp._1568Nakagiri
FR875362.2_Labyrinthula_sp._Sva082
FR875361.1_Labyrinthula_sp._Sva056
FR875359.2_Labyrinthula_sp._Sva132
FR875360.2_Labyrinthula_sp._Sva13
FR875362.2_Labyrinthula_sp._SVA05a
FR875358.1_Labyrinthula_sp._Sva08
FR875358.1_Labyrinthula_sp._Fal10b
375B03
375C02
375H02
375D01
Sw_19
Sw_18
Po_06
62_6
Po_10
Sw_15
Po_17
123_04
375A01
375G02
62_10
164_10
Po_04
376B02
Sw_04
374C06
JN121408.1_Labyrinthula_zosterae
FR875352.2_Labyrinthula_zosterae
JN121407.1_Labyrinthula_zosterae
AF285335.1_Labyrinthula_zosterae
FR875309.1_Labyrinthula_zosterae
JN121405.1_Labyrinthula_zosterae
JN121404.1_Labyrinthula_zosterae

Figure 3.1 Maximum likelihood phylogenetic tree with 100 bootstraps of all samples from six locations. *Aplynochytrium* spp. were used as the outgroup. Operational taxonomic units (OTUs)

Seven operational taxonomic units (OTUs) were generated from all the sequences. OTU1, OTU2 and OTU7are best match to *L. zosterae.* OTU1 contains the 93 sequences with 99 to 100% similarity within this group. OTU3 to OTU6 cluster close to *Labyrinthula* spp. and are closely related (Table 3.2). OTU1 appeared as a sister group of *Labyrinthula* spp. clade, while OTU2 and OTU7 are not fully resolved (Figure 3.2). The phylogenetic tree looks similar to the tree in figure 2 except for position of OTU3 and OTU4, and OTU2 and OTU7. Regarding of the locations, samples from Wackerballig contain highest number of OTUs, followed by samples from Sandspollen (Figure 3.3).

Table 3.2 OTU table.

Name	Best blast hit	Percent hit	Number of sequence
OTU 1	*L. zosterae* FR875355.2	99	93
OTU 2	*L. zosterae* FR875355.2	99	1
OTU 3	*Labyrinthula* sp. AB220158.1	92	2
OTU 4	*Labyrinthula* sp. FR875356.2	84	1
OTU 5	Labyrinthula sp. EU431330	94	10
OTU 6	*Labyrinthula* sp. AB220158.1	84	4
OTU 7	*L. zosterae* FR875335.2	94	1

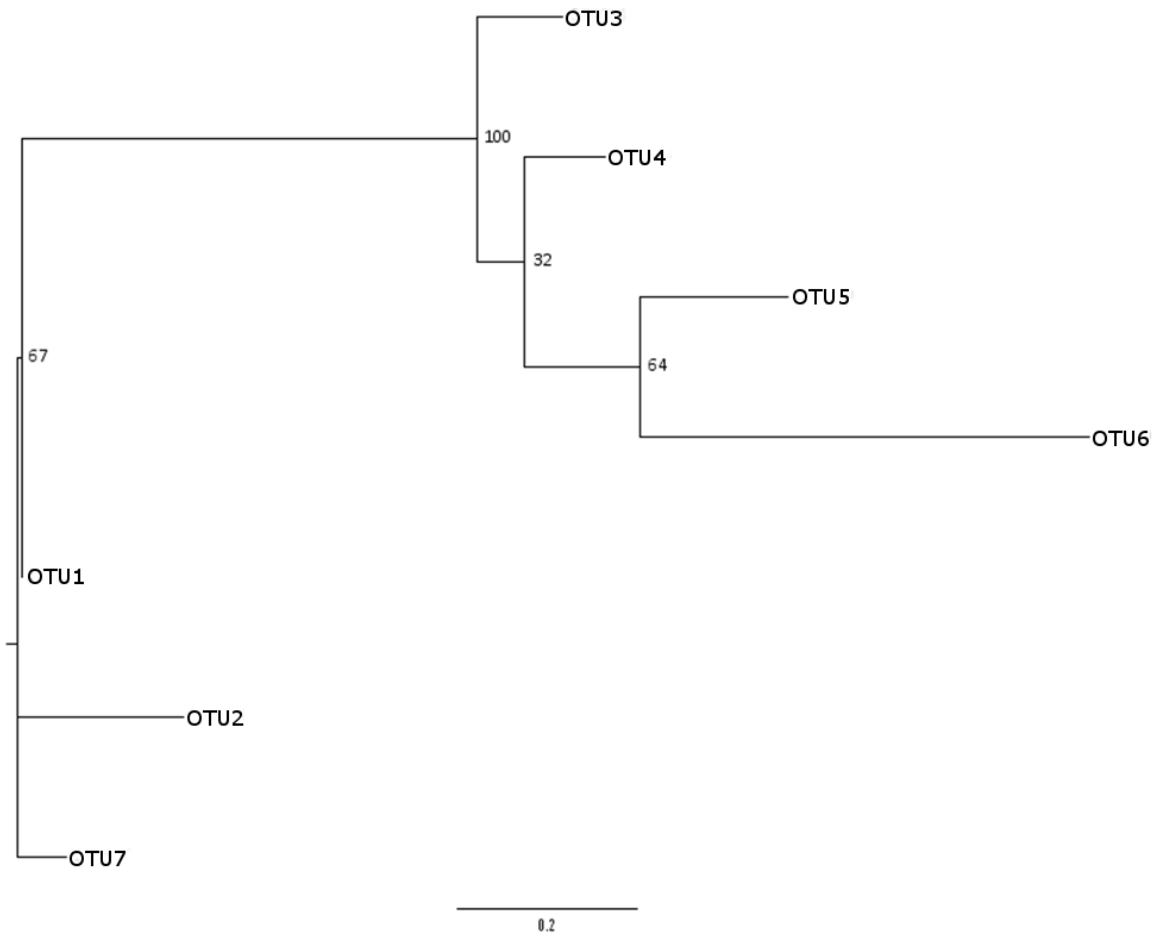

Figure 3.2 Maximum likelihood unrooted phylogenetic tree with 100 bootstraps of seven OTUs.

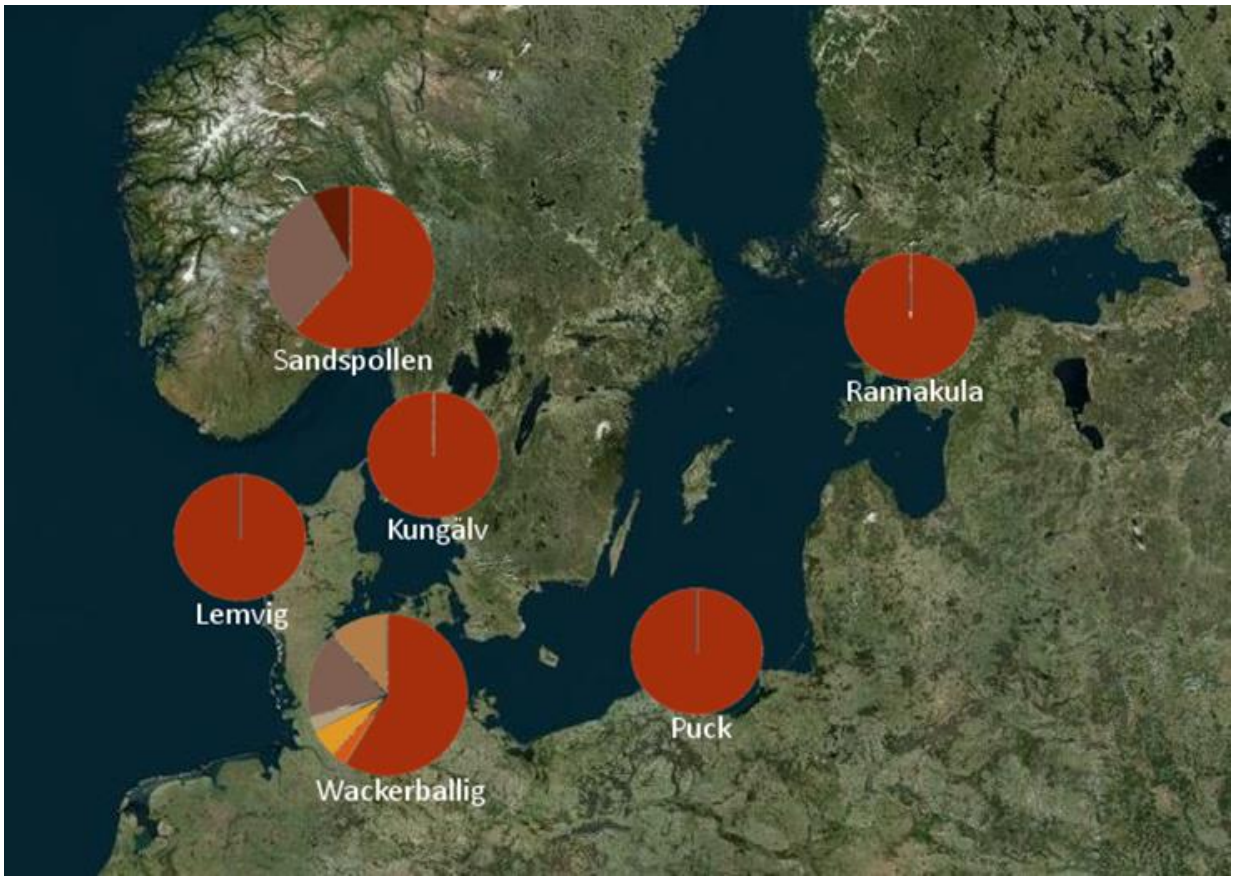

Figure 3.3 Portion of OTUs of sequences from all locations. Six OTUs are found in Wackerballig, 3 OTUs from Sandspollen and one OTU from Lemvig, Kungälv, Puck and Rannakula. ■ = OTU1, ■ = OTU2, ■ = OTU3, ■ = OTU4, ■ = OTU5, ■ = OTU6 and ■ = OTU7.

Species prediction and richness

New species prediction for 1,000 samples ranged from 1.4±0.5 to 4.5±5.5. Species richness ranged between 8.5±2.6 to 13.4±9.9 (Table 3.3). Estimated sample coverage for our current dataset is 97.3% (Figure 3.4A, B). Rarefraction curves (Figure 3.4C) revealed species richness estimates for a rarefied and extrapolated samples.

Table 3.3 Prediction of the number of new species in a further survey and estimation of species richness of samples from these six locations.

Estimator/Model	Estimate	S.E.	95% Confidence interval
Species prediction			
Boneh et al. (1998)	1.4	0.5	(0.4, 2.5)
Solow & Polasky (1999)	4.5	6.7	(0.0, 17.5)
Shen et al. (2003)	4.5	5.5	(0.0, 15.4)
Species richness			
Chao1 (Chao, 1984)	11.5	7.2	(7.5, 47.9)
Chao1-bc	8.5	2.6	(7.1, 22.1)
ACE (Chao & Lee, 1992)	11.6	6.1	(7.6, 40.2)
ACE-1 (Chao & Lee, 1992)	13.4	9.9	(7.7, 63.2)

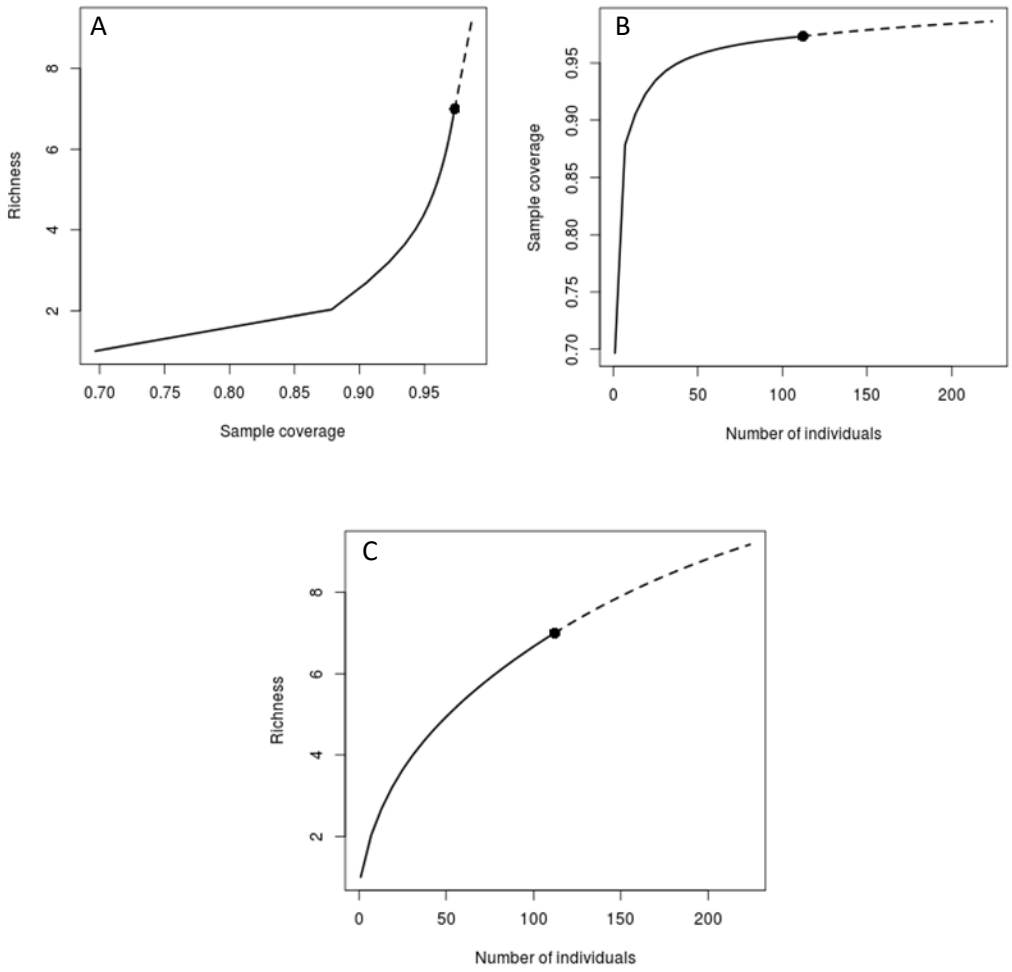

Figure 3.4 Coverage-based rarefaction and extrapolation sampling curve (A), sample completeness curve (B) and sample-size-based rarefaction and extrapolation sampling curve (C).

3.2 DNA extraction kit comparison

Two DNA extraction kit we used for comparison have same principle – incubation of homogenized sample with proteinase K, followed by silica membrane binds DNA, and contaminants pass through spin column. The lysis temperatures differ between kits: 65°C for 30 minutes for the Invisorb Spin Plant Mini Kit and 52°C up to overnight for the Invisorb Spin Tissue Mini Kit. These kits share same chemicals used in most steps, however, the Invisorb Spin Plant Mini Kit has an extra washing step prior elution of DNA. We used OneStep PCR Inhibitor Removal Kit to eliminate contaminants such as polyphenolic compounds from the Invisorb Spin Tissue Mini Kit extracts. Summary of these kits is shown in Table 3.5.

When performing PCR, we found that samples extracted with the Invisorb Spin Tissue Mini Kit showed nonspecific amplification and smears.

There are no differences in sequences between these kits – most of them differ in one or two nucleotides. This also confirmed by one OTU generated from pooled sequences.

Table 3.5 Summary of DNA extraction kits. * Without lysis time.

Kit	Vendor	Price per reaction (€)	Time (minutes)
Invisorb Spin Plant Mini Kit	Stratec	2.70	43
Invisorb Spin Tissue Mini Kit	Stratec	2.50	14*
OneStep PCR Inhibitor Removal Kit	Zymo Research	2.32	5

Chapter 4: Discussion

This is the first report of *Labyrinthula* diversity in eelgrass leaf sample from six locations in the North Atlantic and the Baltic Sea. With DNA extracted directly from the samples, we found higher diversity of *Labyrinthula* than previous studies that relied on culture-based method. *Labyrinthula* was found in every location. Samples collected from Wackerballig and Sandspollen exhibit higher diversity than others.

Difficulty culturing *Labyrinthula* spp. has been shown by numerous studies. Recently, Chitrampalam et al. (2015) showed that only 30% of isolates were successfully maintain in their study and those *Labyrinthula* spp. with small cell size are likely to disappear during culturing. This problem with culturing has also been reported by Bockelmann et al. (2012) and Garcias-Bonet et al. (2011) concluded that this difficulty is common in this group. This present study bypassed the culturing method, thus, all available cells presented in the samples were used.

Designate *Labyrinthula zosterae* partial 18S sequences in this study are mostly 100% identical to those 18S full-length deposited in the GenBank. Sequences which have highest blast hit to *L. zosterae* but their identities are less than 99% may be because of the major problems in the sequencing, for example, PCR amplification bias, sequencing errors, and chimeric sequences (Schloss et al., 2011).

In addition to those sequences which best match to *L. zosterae*, the remaining sequences from Wackerballig and Sandspollen are best matched to *Labyrinthula* spp. deposited on GenBank, however, their identities are quite low (84-94%). In addition, the phylogenetic tree revealed that none of these *Labyrinthula* sequences found in this study were placed within known sequences. This might suggest that sequences found in this study are novel sequences and might correspond to new *Labyrinthula* spp.

There are three OTUs found in Sandspollen: OTU1, OTU5 and OTU7. *Labyrinthula* spp. found in this location was different from previous study. Culturing method by Bockelmann et al. (2012) found putatively two species (*L. zosterae* and *Labyrinthula* sp. A) at this site, however, the OTU5 sequences found in this study are only 84% similar to sequences of *Labyrinthula* species A. It can imply that this study found another *Labyrithula* sp. that cannot be cultured or some samples had not used in other previous studies. In addition, sampling period might cause effect of our study. Our study used samples which came from various places and time. Abundance of *L. zosterae* is highest during the summer but low in the winter and early spring (Bockelmann et al., 2013). Hence, there may be a huge variation between samples. OTU5 also present in Wackerballig. The consensus sequence of this OTU is 84-85% identity to *Labyrinthula* species A and B (FR875355.2 and FR875362.2 respectively). This OTU, however, is 94% match to *Labyrinthula* sp. EU431330.1 which mutualistic live with amoeba *Thecamoeba hilla* isolated from fish *Psetta maxima* (Dyková et al., 2008).

OTU2 and OTU7 are 99% and 94% identity to known *L. zosterae* (FR875355.2) sequences respectively (Table 3.2). These OTUs have only one sequence each. We could merge these OTUs to OTU1 because they are similar to each other and also supported by phylogenetic tree (Figure 3.2). UPARE uses consensus sequences of all amplicons cluster to an individual OTU. These consensus sequences are the average of two or more sequences (Hildebrand et al., 2014). It generally classifies sequences, which are less than 3% difference together. OTU designation in this present study used *de novo* clustering method. In some cases, *de novo* and reference-based clustering methods may show difference results. For well-known taxa, He et al. (2015) showed that *de novo* clustering are unstable to some extent, but reference-based clustering generates more stable OTUs, and vice versa for novel taxa. Furthermore, *de novo* clustering method gives higher richness than reference-based (Hildebrand et al., 2014). Blaxter et al., (2005) discussed that the common way to identify the best match in a reference database such as BLAST is not good enough to identified barcoded samples and especially in not well-studied organisms because its algorithm takes the highest scoring match with some quality score cutoff.

In contrast to Bockelmann et al. (2012), this study found higher diversity of samples in Wackerballig. There are six OTUs in this location: OTU1-OTU6 while the cultured-dependent study found only *L. zosterae* (OTU1 in this study). Novel *Labyrinthula* sequences OTU2, 3, 4 and 6 are exclusively found in this location. This suggests that most of *Labyrinthula* spp. is uncultivable. Supported by the study of Bockelmann et al. (2012) reported that the success of culturing samples from Sandspollen was lower than other locations and the cultures disappeared after short period. They suggested that *Labyrinthula* might exhibit higher diversity in the field. This study showed the potential of molecular taxonomy compared to traditional taxonomies. Although, we revealed some novel OTUs, the connection of OTUs with species may fail if two or more species have genes that are more than 97% similar, causing the OTU contains many species (Edgar, 2010).

Labyrinthula zosterae was found in every location in this study. It was found also in Puck, Poland and Rannakula, Estonia where the salinity ranged between 5 to 10 psu. This finding is in contrast to Bockelmann et al. (2012) that found no *L. zosterae* in sites of very low salinity (Svartholm, Finland, 5 psu). Although the *L. zosterae* was found in these two locations, no wasting disease has been reported (Krause-Jensen et al., 2004; Möller and Martin, 2007). This phenomenon may explained that the pathogenic activities of *Labyrinthula* is reduced in the water with salinities lower than 20-25 ppt, especially if it's lower than 10 psu (Muehlstein et al., 1988; Ralph and Short, 2002). McKone and Tanner (2009) also showed that the lesion areas affected by *L. zosterae* are reduced in the seagrass at salinity lower than 15 psu. Effect of salinity on *Labyrinthula* diversity was reported by L. K. Muehlstein et al. (1988). With culturing method, they found two *Labyrinthula* spp. from eelgrass: the species P and S, which are pathogenic and non-pathogenic respectively. The *Labyrinthula* species P found in every location, which has

the salinity higher than 10 psu, while the species S, can be found from 5 psu on. Anyhow, this present study could not detect any species besides *L. zosterae* from the sites where the salinity is about 5 psu. In addition, the pathogenicity of *L. zosterae* is not only controlled by salinity, high temperature or low light availability which make seagrass under stress condition can also cause the disease (Brakel et al., 2014; Vergeer et al., 1995).

Compare to previously study of Bockelmann et al. (2012), this present report reveal higher diversity of *Labyrinthula*. Even though, two up to five new species of *Labyrinthula* could be found for the next thousand samples as suggested by species estimators. With this, one popular species estimator was made by Solow and Polasky (1999). This estimation of species richness works well if the further sampling size is similar to the previous sampling because it assumes that all unobserved species in the initial sample have equal relative abundances but it gives negative bias when apply to a larger sampling size (Shen et al., 2003).

Species richness calculated from our current dataset revealed similar values. In combination between species prediction and species richness, Chao1 and ACE indices give the best estimations. Chao1 is mainly based on species that has only one individual (singleton). The greater the singleton, the higher the estimate (Colwell and Coddington, 1994). The corrected estimate of Chao1 is close to the number of OTUs found in this current study. Confidence interval of 95% of new species is predicted to fall within range 7.1-63.2 by these estimates. Both Chao1 and ACE may underestimate the true diversity if the sample size is low (Colwell and Coddington, 1994; Hughes et al., 2001).

With novel sequences and OTUs, supported by phylogenetic trees, our study revealed higher diversity of *Labyrinthula* spp. than other previously studies. However, according to the accumulation curve (Figure 3.4), about 97% of total diversity was already found in our sampling size. Furthermore, it implies that the diversity is still dependent of the sample size. Thus, more samples are needed to reveal higher diversity.

Due to specific characteristics such as its ubiquity, size, and low evolutionary rate, the small subunit ribosomal RNA (SSU rRNA) has widely used in molecular evolution (Van de Peer and De Wachter, 1997). Besides rRNA genes, numerous lines of evidence now suggest that variation in the faster-evolving ITS regions provide a better marker for speciation in many groups (Stoeck et al., 2010), while Bachy et al. (2013) found both 18S rDNA and ITS were efficient in detecting some groups of protists species and gave similar phylogenetic structures of the protist community at the species level.

The primers newly designed for this study based on the conserved regions of both *Labyrinthula* spp. and *Phytophthora* spp. and contain one and two degenerate sites for forward and reverse primer, however, non-specific and low sensitivity of amplification happened. Losing of primers' specificity can happen when the primers contain high

degeneracy, predominantly if amplify sequence of closely related species (Linhart and Shamir, 2002).

DNA extraction comparisons

The second aim of this study was to compare extraction of DNA extracted directly from dried *Z. marina* leaf. Using Invisorb Spin Tissue Mini Kit alone without purification step resulted in brownish solution and inhibits PCR. Remaining polyphenolic compounds and polysaccharides mainly causes this brown color. Their oxidized form covalently binds to DNA giving brown color making PCR not amplifiable (Dhanya et al., 2007). Generally, molecular enzymes, such as, DNA ligase and DNA polymerases are inhibited by polysaccharides and other secondary metabolites (Maropola et al., 2015).

PCR amplification of DNA extracted from Invisorb Spin Tissue Mini Kit resulted in smeared products. Thus, reduction of cycle by ten and shorter annealing time are required for the PCR amplification. This smear might due to excess DNA used which caused non-specific amplification or due to incorrect competitive binding (mispriming) (Cousins et al., 1992; Mamedov et al., 2008). However, we did not try the extraction method without addition of salmon sperm DNA to check whether this smear happened due to additional foreign DNA. Besides this, the results of the two DNA extraction kits were similar, however, the Invisorb Spin Plant Mini Kit outcompete another kit in these following ways: shorter lysis time, no extra purification steps and cost per extraction is cheaper than another kit.

According to manufacturer's protocol, the Invisorb Spin Tissue Mini kit calls for the use of tissue sample, rodent tail, biopsy material, insects, paraffin embedded tissues or eukaryotic cell swab. It is widely used for animal tissue DNA extraction (Broll et al., 2007; Goebbels et al., 2010), moreover, it also used to isolate microbial DNA from culture plates (Lau et al., 2005) but never found in other plant studies.

Furthermore, DNA isolation from dried specimens usually requires some modifications to frequently used protocols (Rogers, 1994). This is due to small amount of sample available. (Maropola et al., 2015) compared classical DNA extraction methods with commercial kits on sorghum. They found that the classical protocols (CTAB and SDS) revealed high DNA yields from sorghum tissues but were less reproducible than the commercial kits. The commercial kits had higher quality of DNA, but with lower endophytic bacterial diversities compared to classical protocols. Although, classical protocols such as CTAB method may give a better result, it would be difficult to extract DNA from a number of samples. Besides conventional PCR, the high-fidelity PCR was reported up to six times more efficient than conventional PCR in detecting pathogens from freshly inoculated plants, demonstrating its increased sensitivity in early detection of fungal and bacterial pathogens that are difficult to identify in disease development (Cating et al., 2012). I recommend comparing between conventional and high fidelity PCR to detect *Labyrinthula* in eelgrass samples for future study.

Conclusion

This study reported successful in using partial sequence of 18S rDNA to explore diversity of *Labyrinthula* spp. in eelgrass leaves samples collected across the Baltic and Northern Atlantic Ocean. This current study provides the first insight of *Labyrinthula* molecular diversity from the leaf samples. For better understanding the diversity, more samples should be used. Medinger et al. (2010) showed that Next generation amplicon sequencing based on rDNA was superior in detecting rare protist species. This advantage is due to the large amount of sequence reads provide a unique opportunity to resolve the gap between morphological and molecular studies. However, it is necessary to take into account three potential sources of error which may artificially inflate the apparent levels of diversity detected: the combined effects of nucleotide mis-incorporation and read errors during PCR and sequencing, PCR chimaera formation, and intragenomic polymorphism among multiple copies of the rRNA within a single nucleus (Stoeck et al., 2010).

Chapter 5: References

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