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The First Isolation and Characterisation of the Protist *Labyrinthula* sp. in Southeastern Australia

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ABSTRACT

As a result of anthropogenic influences and global climate change, emerging infectious marine diseases are thought to be increasingly more common and more severe than in the past. The aim of our investigation was to confirm the presence of *Labyrinthula*, the etiological agent of the seagrass wasting disease, in Southeastern Australia and provide the first isolation and characterisation of this protist, in Australia. Colonies and individual cells were positively identified as *Labyrinthula* using published descriptions, diagrams and photographs. Their identity was then confirmed using DNA barcoding of a region of the 18S rRNA gene. Species level identification of isolates was not possible as the taxonomy of the *Labyrinthula* is still poorly resolved. Still, a diversity of *Labyrinthula* was isolated from small sections of the southeast coast of Australia. The isolates were grouped into three haplotypes that are biogeographically restricted. These haplotypes are closely related to previously identified saprotrophic clades. The study highlights the need for further investigation into the global distribution of *Labyrinthula*, including phylogenetic pathogenicity and analysis of host-parasite interactions in response to stressors. Given the results of our analyses, it is prudent to continue research into disease and epidemic agents to better prepare researchers for potential future outbreaks.

Keywords

Stramenopiles; *Labyrinthula*; seagrass; Australia; *Zostera*; *Halophila*; *Posidonia*; phylogeny; microscopy; culture

SEAGRASSES are an important group of marine flowering plants that provide numerous ecological and socioeconomic services globally (Cullen-Unsworth & Unsworth 2013) and that further contribute to substantial economic values (Dewsbury et al. 2016). As such, seagrasses are a critical component of nearshore ecosystems worldwide.

In recent decades habitat degradation has led to a consistent and often localised decline in seagrass health as well as the significant global habitat losses of seagrass ecosystems (Waycott et al. 2009; Short & Wyllie-Echeverria 1996), which is threatening extinction in some cases (Short et al. 2016). As a result of anthropogenic influences and global climate change, emerging infectious marine diseases are thought to be increasingly more common and more severe than in the past (Ward & Lafferty 2004; Lafferty 2009; Hoegh-Guldberg & Bruno 2010).

Labyrinthula is the only described genus in the family Labyrinthulaceae (class Labyrinthulomycetes, phylum Labyrinthulomycota) (Beakes et al. 2014), and is a well-known opportunistic, protistan pathogen found in association with marine vegetation, including seagrasses, around the world (Vergeer & Den Hartog 1994). In addition to seagrasses, *Labyrinthula* is also associated with infection of mangrove leaves (Bremer 1995) and marine algae (Pokorny 1967; Raghukumar 1987). Some *Labyrinthula* species are putative causative agents of wasting disease in seagrass, which has resulted in devastating losses of both seagrass habitat and their socioeconomic functions (Muehlstein et al. 1991; Robblee et al. 1991; Sullivan et al. 2013). Not all *Labyrinthula* isolates are pathogenic and some live as saprobic decomposers (Martin et al. 2016). Still, the cause of opportunistic pathogenicity is uncertain. Some hypotheses have emerged that opportunistic pathogenicity may be related to a) seagrass host stress and degraded health (Vergeer et al. 1995; Sullivan et al. 2013; Trevathan-Tackett et al. 2015) and/or b) linked to species lineages whereby some species are more virulent than others (Martin et al. 2016).

Australia is home to half the diversity of the world's seagrass species (Green & Short 2003), yet knowledge is lacking on the occurrence and role of *Labyrinthula* in the resilience of these meadows. In the past 50 years, just five isolates have been cultured from Australian (Vergeer & Den Hartog 1994; Martin et al. 2016) and New Zealand (Armiger 1964) seagrasses. In these efforts, the genus was primarily described through morphological observations of cultured isolates, namely cell size and the growth pattern of the colony. More recently, genetic sequencing using 18S and internal transcribed spacer (ITS) markers have improved our knowledge of *Labyrinthula* globally (Bockelmann 2012; Martin et al. 2016), and has included isolations of a potentially pathogenic strain from Queensland in Australia (Bockelmann 2012; Martin et al. 2016). While historically morphological investigations resulted in 10-12 marine and one terrestrial species being recognized (Pokorny 1967; Dick 2001; Bigelow et al. 2005), more recent phylogenetic investigations suggest a total of 16-17 species may be more accurate (Martin et al. 2016). Though there are comparable numbers of species recognized by both identification systems, within the global depository of nucleotide data, GenBank, interestingly 426 isolates have been entered into the database (<http://www.ncbi.nlm.nih.gov/genbank/>), but only two of these isolates are correlated with taxonomic epithets. Although a few studies have positively confirmed the presence of *Labyrinthula* in Western Australia, Queensland and New Zealand (Armiger 1964; Kirkman 1978; Vergeer & Den Hartog 1994; Martin et al. 2016), detailed observations and isolations

of *Labyrinthula* in association with possible wasting disease symptoms, genetic relatedness to global isolates and documentary photographs of isolates are lacking for Australia. Improving our understanding of the role of disease in seagrass meadows has been identified as a priority seagrass research area in Australia (York et al. 2016). This necessarily includes research of potential threats to seagrass from *Labyrinthula* and from other potential emerging marine infectious diseases, which should be carefully studied to prevent unexpected acute losses to valuable seagrass communities. In summary, although research is progressing with regards to the taxonomy and the phylogeny of this group, it remains understudied and poorly resolved in Australia and globally.

Our lack of knowledge of Australian *Labyrinthula* limits effective management of coastal ecosystems and biosecurity. Unfortunately, few resources are available to address emerging disease events when problems are discovered (Groner et al. 2016), thus ongoing efforts to understand recognised diseases is warranted. Further, as global climate change progresses, the prevalence of virulent strains and occurrences of seagrass wasting disease epidemics may be expected to increase (Harvell et al. 2002). Given the severity of change expected for Australia's climate in the coming decades, a collaborative and interdisciplinary effort to researching impacts in marine systems is urgently needed (Frusher et al. 2014). Monitoring *Labyrinthula* is vital because of the predicted general increase in diseases caused by microorganisms in marine biota due to climate change (Harvell et al. 2002) and the appearance of new species from many sources, such as ballast water (Hülsmann & Galil 2002). Distinguishing endemic species from potentially pathogenic invasive species is also essential. More knowledge of the occurrence and distribution of *Labyrinthula* in Australia and studies on host-pathogen is needed to increase our understanding of the role of *Labyrinthula* in the life cycle of seagrasses.

This study is the first systematic survey of seagrass-associated *Labyrinthula* in Australia using a multi-parameter approach to describe isolates through qualitative and quantitative microscopic and morphological analyses and phylogenetic relationships. The study provides more detailed baseline research on species occurrence, isolation and characterisation of *Labyrinthula* from Australia. It is the first study of its kind in Australia, and is thus essential for informing the growing knowledge on global *Labyrinthula* biogeography.

MATERIALS AND METHODS

Sample collection

Eight to twelve species of seagrass are known to populate the southeast coastal region of Australia (Kirkman 1997; Kuo 2005; Coyer et al. 2013). Of these, four species are commonly observed in the temperate seagrass meadows, including *Zostera muelleri*, *Halophila ovalis*, *Heterozostera nigricaulis*, and *Posidonia australis*. In order to investigate the occurrence of *Labyrinthula* infections in these species, the species were sampled from shallow coastal water bodies through field collection (Fig. S1).

Four seagrass species were sampled over 2 years from pre-selected study sites (Fig. S1) in Victoria (VIC) and New South Wales (NSW), representing a sample of typical seagrass meadow species. The VIC seagrass species were sampled in September 2015 from meadows in Port Phillip Bay along the Bellarine peninsula: Portarlington (PA; 38.112623 S, 144.650695 E), Indented Head (IH; 38.140231 S, 144.713504 E), and St. Leonards (SL; 38.170136 S, 144.719941 E). The NSW seagrass species were sampled over between March 2014 and October 2015 around the Sydney Region: Wyee Bay (WB; 33.158162 S, 151.531905 E), Lake Munmorah (LM; 33.233787 S, 151.564290 E), Budgewoi Lake (B; 33.240668 S, 151.563806 E), Tuggerah Lake (TL; 33.326755 S, 151.502806 E), Palm Beach (PB; 33.587723 S, 151.323617 E), Narrabeen Lagoon (NL; 33.719731 S, 151.294562 E), Watson's Bay North (WBN; 33.842424 S, 151.28122s2 E), Watson's Bay South (WBS; 33.846754 S, 151.280592 E), Rose Bay (RB; 33.872242 S, 151.262011 E), Narrabeen Beach (NB; 33.703349 S, 151.305114 E), and Botany Bay (BB; 34.007619 S, 151.194450 E).

The younger seagrass leaves (first to third oldest to avoid excessive epiphytic algae overgrowth) were collected from 10-25 seagrasses at each site and placed in separate resealable plastic storage bags with seawater to avoid cross contamination of samples. All seagrass specimen samples were transported immediately to the lab after collection.

Labyrinthula isolation

Following collection, all samples were kept cool and stored at 4 °C until the samples could be individually processed in the lab. Seagrass specimens were then cut into 1-2 cm segments and transferred to the solid agar medium (Muehlstein et al. 1988) [0.1g yeast extract, 0.1g peptone, 1g glucose, 0.003g germanium oxide, 12g agar, 300ml filtered sea water, in 690ml deionized H₂O]. Plates were observed daily until *Labyrinthula* isolates could be identified emerging from the seagrass samples. Once *Labyrinthula* morphologies were observed, the presence of *Labyrinthula* spp. was verified using a Radical inverted tissue culture

microscope. Once the presence of *Labyrinthula* sp. was identified and confirmed microscopically, the *Labyrinthula* specimen could be cut from the agar and re-plated on fresh agar. The cultures were determined to be axenic after daily observations of isolate growth for up to 4 weeks. During the incubation period, single cell extraction and re-plating from the cultures was performed on a regular basis to ensure pure cultures were obtained. The use of axenic cultures in the study was important for making accurate morphological observations. In order for DNA to be extracted, samples of successfully cultured and isolated *Labyrinthula* were transferred into liquid medium (media as described without agar) using the culture methods described by Martin and Boone (2009).

Morphological observations

Following successful acquisition of axenic cultures, morphology of *Labyrinthula* colonies on the agar was recorded through quantitative microscopy. Where axenic cultures were characterised, the plates were photographed using the Olympus CH2 inverted microscope fitted with a Nikon D700X camera. Images were used to verify the morphological characteristics and positively identify the isolated *Labyrinthula* sp. on agar. Further observations of colony formation and dynamic interactions were recorded using differential index contrast imaging (DIC) on the Olympus CH2 inverted microscope through an agar media. High-resolution micrographs of colony structures were made using the scanning electron microscope (SEM).

To prepare for SEM, rectangle agar segments (5 x 10 mm) were aseptically cut from axenic cultures. The segments were washed with sterile seawater and fixed with 3% glutaraldehyde overnight at room temperature. The fixed cultures were then washed for three 10-minute washes with 0.1 M Phosphate buffer, pH 7.0. The samples were then dehydrated in 30, 50, 70, 80, 90 and twice in 100% ethanol for 10 minutes each. The samples were then critical point dried and mounted using carbon paint before coating with 20 nm gold. The specimens were then observed using the JEOL JCM-6000 Benchtop SEM.

DNA sequence analysis

Agar segments from axenic cultures were obtained and photographed, positively colonised agar samples from the cultures were cut out and transferred to a standard liquid seawater culture (Martin et al. 2009). Transferred organisms were observed daily until positive samples growing away from the agar could be extracted using a sterile pipette and transferred to smaller vials containing liquid culture media [0.1 g yeast extract, 0.1 g peptone, 1 g

glucose, 0.003 g germanium oxide, 300 ml filtered seawater, 690 ml dH₂O]. The smaller vials of pure liquid culture were either processed immediately or stored at 4 °C until it was ready for DNA extraction.

DNA was extracted from agar or axenic liquid cultures, using either Econospin Micro Spin columns (Epoch Life Sciences) in combination with laboratory-made buffers or the Power Water DNA Isolation Kit (MO BIO Laboratories). A region of the 18S ribosomal RNA (rRNA) gene was amplified with PCR, using the universal primers, primerA and primerB (Medlin et al. 1988). Cycle parameters were: 94°C for 3 min, 35 cycles of 94°C for 30 s; 50°C for 30 s; 72°C for 2 min, followed by 72°C for 10 min. Sequencing of PCR products was performed by Macrogen (South Korea), using the above primers and the internal primers 18S_f2 and 18S_r3 (Bockelmann 2012). Preliminary DNA sequence analysis was performed using Geneious Pro 4.8.5 (Kearse et al. 2012).

Phylogenetic analysis

Phylogenetic analysis was performed with the *Labyrinthula* 18S rRNA sequences and a dataset of representative *Labyrinthula* 18S rRNA sequences downloaded from GenBank. The 18S rRNA sequences of a number of different stramenopile species, with varying relatedness to the genus *Labyrinthula*, were also included as outgroups. In order to create the representative *Labyrinthula* dataset, all of the *Labyrinthula* 18S rRNA sequences present in GenBank were assembled into groups within which the sequences had >99.0% identical sites. One representative sequence per group was then selected. Multiple sequence alignment was performed in MEGA7 (Kumar et al. 2016) using ClustalW with default parameters. To maximise the number of sequences retained for subsequent analysis, the alignment was trimmed to a length of 811bp. Sequences with incomplete coverage of this region were discarded. The Gblocks server (Castresana 2000) was used to select segments of the trimmed multiple alignment suitable for phylogenetic analysis. Bayesian analyses were performed with MrBayes 3.2.6 on the CIPRES Science Gateway (Miller et al. 2010; Ronquist et al. 2012). A Generalised Time Reversible (GTR) evolutionary substitution model was selected, with gamma distributed rate variation across sites (rate categories = 6). Two independent analyses were run for 50000 generations, with sampling every 1000th generation. Default values were used for all other parameters.

RESULTS

Morphological observations

Clear morphological characteristics of *Labyrinthula* growth patterns on agar were observed and recorded for *Labyrinthula* species isolated in Australia (Fig. 1-3). The observed network of growth and extension of ectoplasmodic nets characterised all the axenic cultures of *Labyrinthula* (Fig. 1). These growth patterns were observed for all three phylogenetic groupings (Hap1, Hap2 and Hap3), and are consistent with previous observations (Pokorny 1967; Porter 1969; Preston & King 2005; Dyková et al. 2008). In some cases the pattern of growth was complex and indeterminate, varying according to the age of the culture. As the axenic cultures of Hap1 matured, two *Labyrinthula* isolates exhibited growth that resembled whorls (Fig. 1a) or whirling aggregates (Nakatsuji et al. 1981). Morphological features for each isolate appear to change over time, depending on the density of growth and the level of aggregation (Fig. 1a,b). As axenic cultures matured, *Labyrinthula* growth into the agar was commonly observed (Fig. 1c,f). These branches or 'root-like' structures that extended into the agar were observed for all phylogenetic groups. Dense clusters or aggregates made up of multiple *Labyrinthula* formed in established cultures (Fig. 1g,h). These observations are similar to those made for *Labyrinthula terrestris* (Bigelow et al. 2005). Most of the cultures had a translucent to white pigment but there were individual strains of *Labyrinthula* in each phylogenetic group that had orange pigmentation (Fig. 1a,h). All *Labyrinthula* cultures had hyaline ectoplasmic secretions or extracellular secretions, which coated individual and colonies of *Labyrinthula*. Tracks formed by the hyaline secretions allowed cells to migrate as has been previously observed (Preston & King 2005; Dyková et al. 2008). The shape and size of individual *Labyrinthula* varied between strains, even within the same phylogenetic cluster (Fig. 1). The *Labyrinthula* in culture were observed to aggregate in air pockets in the agar to form colony structures. These structures may be artefacts of growing in agar but it may provide some insight as to the possible organisation of *Labyrinthula* in the lumen of seagrass blades. Structural variations in these bubble colonies varied in phylogenetic group Hap1 where they were observed (Fig. 2). Further investigation of Hap 2 and 3 are required to determine if similar patterns of growth are exhibited. Many of the strains had the characteristic spindle-shaped cells described previously (Pokorny 1967; Porter 1969; Dyková et al. 2008), however others when observed with the scanning electron microscope had long thread-like morphology (Fig. 3). Prior to the adoption of a preparation protocol for electron microscopy, several alternative protocols were tested so that a protocol could be selected that

eliminated artefacts and preserved tissue so that the results were consistent with the light microscope observations. The selected protocol was then used to prepare all isolates.

The light microscope observations (Fig. 1 and 2) indicate the range of morphological features exhibited by *Labyrinthula* on the medium used to isolate *Labyrinthula*. This approach was taken to minimise any variations in growth and morphology that may occur as a consequence of transfer to another medium or surface. The DIC images were taken using an inverted microscope through the agar media, which limited the resolution and the intensity of light transmitted through the medium.

Genetic variation among Australian *Labyrinthula* isolates

Sequence data was obtained for 34 different *Labyrinthula* isolates collected from 14 locations in Victoria and New South Wales. Sequencing of a ~1,600bp region of 18S rDNA identified three different haplotypes among the geographical regions surveyed, with 88.5-91.5% pairwise identity between haplotypes. All three haplotypes had high identity ($\geq 99\%$) to previously characterised *Labyrinthula* 18S sequences present in Genbank. A single haplotype (Hap1) was observed for all of the Victorian isolates. Two haplotypes (Hap2 and Hap3) were recorded among the NSW isolates, with the majority of isolates found to have haplotype Hap2. Four Hap2 sequence variants were identified, showing minor sequence variation (1-4bp) across samples. Haplotypes Hap1 and Hap3 were identical across samples.

Phylogenetic analysis

The phylogenetic patterns observed between representatives of the *Labyrinthula* and other related taxa were broadly consistent with those previously reported in the literature (Fig. 4) (Honda et al. 1999; Leander & Porter 2001; Bockelmann 2012; Martin et al. 2016). The *Labyrinthula* formed a monophyletic group within the stramenopiles, comprised of three main clades. One clade contained only terrestrial isolates. A second clade contained all isolates identified as *L. zosterae*. The third and largest clade included a combination of marine, terrestrial and endosymbiotic isolates, including the haplotypes identified in this study.

DISCUSSION

Both morphological and phylogenetic analyses support the presence of more than one species of *Labyrinthula* in Australia. Further, morphological investigations reveal similarities to other

disease studies (Muehlstein et al. 1988) where morphology of *Labyrinthula* was linked to pathogenicity and saprotrophic behaviours.

In contrast to these observations, phylogenetic investigations revealed all the species in our study were more closely related to saprotrophic clades identified by Martin *et al.* (2016). These findings are in contrast to the clear phylogeographic boundaries recorded for the Australian isolates, with Hap1 restricted to Victoria and Hap2/Hap3 found only in NSW. It is possible that the distribution of Hap1 extends to the NSW sites and Hap2/Hap3 to the VIC sites; however, this pattern was not detected by the sampling effort for this study. Alternatively, the distribution of the different *Labyrinthula* isolates may have been influenced by one or more as yet undetermined factors, such as physical or hydrologic barriers, or shift in species abundance due to annual climatic variations. For instance, it is known that marine flora and fauna in southeast Australia display strong regional genetic variations that suggest a lasting physical barrier to dispersal was present historically, and through several sea level change periods (Sinclair et al. 2016). These barriers resulted in what are known to be three biogeographical provinces (Peronian to the east, Flindersian to the south and west, and Maugean to the southeast) with clearly observed genetic variations that persist in both marine flora (Sinclair et al. 2016) and fauna (DiBattista et al. 2014; Charlton-Robb et al. 2015; Miller et al. 2016) of southeast Australia. Whether temporal or climatic variations influence biogeographical distribution of *Labyrinthula* needs to be investigated. Each of the three Australian haplotypes clustered with different previously described *Labyrinthula* isolates. The three resulting subclades identified in our study contained *Labyrinthula* representatives from a variety of international locations within the Northern Hemisphere (Fig. 5). However, it is interesting to note that the seagrass host species are themselves constrained to either the Northern or Southern Hemisphere. In fact, the members of each subclade collectively span two to three different climatic zones, from warm temperate to tropical.

Based upon previous pathogenicity studies (Martin et al. 2016), the Australian *Labyrinthula* characterised in this study appear to be host generalists. The Hap1 *Labyrinthula* were isolated from both *Heterozostera nigricaulis* and *Halophila ovalis* and are closely related to marine isolates that have been collected from *Zostera marina* and fallen leaves. Both Hap2 and Hap3 individuals were isolated from a number of seagrass species belonging to different families (Table S1). Unlike Hap1 and Hap2, which clustered with marine isolates only, Hap3 clustered with a mix of marine and terrestrial isolates. A recent experimental cross-infectivity study indicated that *Labyrinthula* are comprised of both host specialist

clades, the members of which tend to be restricted to either the tropics or the temperate zones and have a greater likelihood of being pathogenic, and host generalist clades that span a relatively broad climatic distribution and are more likely to be saprotrophic (Martin et al. 2016). Still, specific pathogenicity experiments are needed to test whether these isolates are in fact saprobes or if some of our isolated strains may be pathogenic.

The results of our phylogenetic analyses suggest that sufficient genetic divergence occurs within the genus *Labyrinthula* to justify the naming of one or more new species, in addition to the previously described *L. zosterae* and *L. terrestris*. Terrestrial *Labyrinthula* isolates occurred within two separate clades, forming a well-supported polyphyletic group (posterior probability = 1.00). This provides evidence that the terrestrial lifestyle is likely to have arisen independently more than once during the evolutionary history of the *Labyrinthula*. Similar conclusions were reached in another recent *Labyrinthula* study (Martin et al. 2016).

Directions for future research

More research into the timeline of global distributions and potential spread of *Labyrinthula* species are needed to determine if some species/strains may be considered true emerging infectious diseases, or if certain species are potentially more virulent than others, as may be suggested by phylogenetic and pathogenicity experiments (Martin et al. 2016), and the known history of wasting disease epidemics (Sullivan et al. 2013).

Host stress is thought to increase susceptibility of seagrass to disease. Yet results of studies into the role of environmental and physiological factors on disease susceptibility, such as increased temperature (Olsen et al. 2015) and salinity (Trevathan et al. 2011) offered surprisingly contrary results. This suggests further studies are needed to decouple host-pathogen-environment conditions that may lead to epidemic disease events. For instance, temperature is known to drive infectious disease prevalence in a variety of organisms, especially those without a known immune response to infection (Harvell et al. 2009). Yet, a recent study found that warming was not correlated with increased disease response in seagrasses infected with *Labyrinthula* (Olsen et al. 2015). Understanding host-parasite interactions in relation to temperature will require not only acknowledging the effects of temperature increase on tolerant species and their pathogenicity but also the impact on less tolerant species and their role in the ecosystem. Of further interest, Steele et al. (2005) found

that while turtlegrass (*Thalassia testudinum*) produces phenolic compounds near infection sites, the compounds are ineffective at controlling infections and are likely therefore to be a response to tissue damage, and not to the presence of *Labyrinthula* itself. Still, Trevathan-Tackett et al. (2015) were able to demonstrate the phenolic compounds were effective at controlling *Labyrinthula* infection in isolates. Further observations of how these symbiotic interactions may influence and moderate disease is required (Gleason et al. 2014).

Generally, reports of increased occurrence and severity of marine infectious diseases are being reported around the world (Burge et al. 2014). As the threat of global climate change becomes a reality, countries nearer to the poles are experiencing the brunt of altered climates first and with higher intensity (Barros et al. 2014). Therefore, threats of emerging infectious disease in critical and sensitive subpolar and temperate ecosystems must be studied more thoroughly. Increased temperatures, ocean acidification, UV-B radiation, changes to salinity and sea level rise are thought to be the most likely climate change threats that will affect seagrass ecosystems (Short et al. 2016). Thus, the effect of these conditional impacts on *Labyrinthula*-Seagrass infection and virulence must be studied. Further, due to the lack of seagrass host-specificity the pathogen should be given a higher priority in host-pathogen research for marine diseases (Sullivan et al. 2013).

Our results affirm previous Australian seagrass research gap analyses (York et al. 2016), which found research was lacking on the role of microbes in seagrass resilience, function and life history, and how changing environmental pressures influence microbial function in seagrass meadows. We believe studies are urgently needed to observe the immune response of seagrasses infected with various *Labyrinthula* strains and under variable environmental conditions. At a minimum, studies and inventories of *Labyrinthula* diversity, ecotypes, morphological variation, culture, parameter responses, and phylogenetics should be completed for other Australian states and seagrass species in the future, especially for *Labyrinthula* species inhabiting endemic and rare seagrass species. Studies aimed at developing baseline information on existing seagrass, *Labyrinthula* and parameter conditions, as well as testing for host pathogenicity and virulence of independent strains under a variety of altered scenarios is critical for preparing for emerging infectious disease in the future.

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FIGURE LEGEND

Fig 1: Light microscope images of axenic *Labyrinthula* strains. Networks surrounded by ectoplasm were present in all phylogenetic groups (Hap1, Hap2 and Hap3). As cultures matured and thickened, the growth patterns became irregular and either formed mats, extensive branches into the agar or aggregates of *Labyrinthula*. A) 14 day *Labyrinthula*

strains with tracks of growth (open arrow) forming swirls with individual cells moving along the tracks (open circles) (HAP1, Hn_SL, Ln14). B) 21 day culture with layers of *Labyrinthula* forming mats on the surface of the agar (HAP1, Hn_SL, Ln14). C) mature culture of *Labyrinthula* strain spreading into the agar, forming branching/root-like structures with tracks of *Labyrinthula* (arrow), whereas tracks characteristic of *Labyrinthula* (open arrow) are visible from the surface (HAP1, Hn_IH_B, Ln25). D) classic *Labyrinthula* networks present on the surface of agar (HAP1, Hn_IH_C, Ln17). E-F) classic *Labyrinthula* networks (open arrow) on the surface of agar (E), whereas the branching/root-like structures (arrow) were under the agar, (F) (HAP2a, Zm_WB_A, Ln4). G-H) some *Labyrinthula* strains formed aggregates of cells, these aggregates had tracks of ectoplasm (open arrow) that allowed individual cells (circles) to migrate (HAP3, Pa_PB_D, Ln5) (Bar=200µm).

Fig 2: Light microscope images of *Labyrinthula* strains using bubbles or imperfections in the agar to form colony-like structures. A-C) this structure was one of a number found in the agar of this *Labyrinthula* strain. Tracks (open arrow) are visible leading from one bubble to another. The tracks cover the surface of the bubble and eventually extend into the agar. Some tracks project out from the bubble (arrow)(HAP1, Ha_IH_A, Ln15). D) root-like extensions of the *Labyrinthula* strain radiate from the bubble. The ends of some branches are bulbous (open circles) and have been noted to release masses of cells (HAP1, Hn_PA_A, Ln13)(Bar=200µm).

Fig 3: SEM micrographs of axenic *Labyrinthula* strains. A-C) increasing magnification of a *Labyrinthula* isolate, with networks made up of multiple spindle-shaped cells. Junctions in the cluster are made up of multiple layers of cells (approx. 8-10µm long) with the tracks covered by ectoplasm. A break in the ectoplasm of one of the clusters (arrow) exposed cells underneath (HAP1, Hn_IH_C, Ln17). D-E) increasing magnification of a network, with small thin thread-like *Labyrinthula* cells (approx. 8-10µm long) (HAP1, Hn_SL, Ln14). f-h) increasing magnification of spindle-shaped *Labyrinthula* cells (approx. 8-10µm long) that are coated by ectoplasm with filopodia (*) present between some individual cells (HAP1, Hn_PA_A, Ln11).

Fig. 4: Bayesian phylogenetic analysis of *Labyrinthula* 18SrRNA sequences. All *Labyrinthula* sequences present in Genbank were assembled into groups with >99.0% identical sites. For clarity, one representative sequence per group was selected for phylogenetic analysis. A total of 602 nucleotide sites were used in constructing the tree.

Posterior probabilities and the expected number of substitutions per site (scale bar) are shown. The habitat from which isolates were collected is indicated as follows; M = marine (seagrass/fallen leaves), T = terrestrial (grass), E = as endosymbiont of amoeba (from fish gill). The distal clades shaded in grey each contain one of the three Australian *Labyrinthula* haplotypes identified in this study. The different geographic locations represented within each clade are listed. Please note that the countries correspond both to (1) representative sequences that were included in the phylogentic analysis and (2) omitted sequences with >99.0% identity to the representative sequences, as described above.

SUPPORTING INFORMATION

Fig. S1. Map of sampling locations from New South Wales (NSW) and Victoria (VIC). A total of 14 sites were sampled, including three in Victoria and 11 in New South Wales.

Table S1. Summary of collection sites along the southeast coast of Australia, the seagrass hosts and isolates.

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