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Pathogenic *Labyrinthula* associated with Australian seagrasses: Considerations for seagrass wasting disease in the southern hemisphere

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Abstract

Marine disease ecology is a growing field of research, particularly for host organisms negatively impacted by a changing climate and anthropogenic activities. A decrease in health and increase in susceptibility to disease has been hypothesised as the mechanism behind wide-spread seagrass die-offs related to wasting disease in the past. However, seagrass wasting disease and the causative pathogen, *Labyrinthula*, has been vastly understudied in the southern hemisphere. Our aim was to build on the current knowledge of Australian *Labyrinthula* descriptions and phylogeny, while also providing a first look at wasting disease ecology in Australia. Five seagrass species along a 750 km stretch of coastline in southeastern Australia were sampled. The resulting 38 *Labyrinthula* isolates represented a diversity of morphotypes and five haplotypes of varying phylogenetic clade positions and virulence. The haplotypes clustered with previously-described phylogenetic clades containing isolates from Asia, USA and Europe. Pathogenicity tests confirmed, for the first time, the presence of at least two pathogenic haplotypes in Australia. While historically there have been no reports of wasting disease-related seagrass habitat loss, the presence of pathogenic *Labyrinthula* highlights the need for disease monitoring and research to understand seagrass wasting disease ecology in Australia.

Keywords: eukaryotic microbes; haplotype; morphotype; pathogenicity; seagrass wasting disease; stramenopile

Introduction

Disease ecology is a growing focal point for marine research, particularly for organisms that are commercially important to fisheries and aquaculture (Harvell et al., 1999; Harvell et al., 2002), as well as for marine keystone species such as corals that are being negatively impacted by a changing climate and anthropogenic activities (Bruno et al., 2007; Vega Thurber et al., 2014). Even moderate but chronic suboptimal environmental conditions together with reduced health of the host can exacerbate incidences of disease (Burge et al., 2014; Vega Thurber et al., 2014). Seagrasses, for example, are important components of coastal and estuarine ecosystems. However, the proximity of seagrasses to the coastline often increases their vulnerability to anthropogenic degradation, e.g. run-off, eutrophication and physical disturbances (Orth et al., 2006). Exposure of seagrasses to suboptimal conditions for long periods of time have been shown to cause a decline in seagrass health and even habitat loss (Koch et al., 2007; Carr et al., 2012). A decrease in health and increase in susceptibility to infection has been hypothesised as the mechanism behind wide-spread seagrass die-offs related to wasting disease in the past (Muehlstein, 1989; Blakesley et al., 2002). These large-scale die-offs in the past were damaging to local fisheries, water birds and water quality (Rasmussen, 1977; Muehlstein, 1989).

The causative agent of seagrass wasting disease, *Labyrinthula* spp., is a colonial Stramenopile protist that is ubiquitous to coastal and marine ecosystems (Raghukumar, 2002; Tsui et al., 2009). What is known about *Labyrinthula* ecology and seagrass wasting disease stems from research primarily performed in the Northern Hemisphere seagrass ecosystems. Using their ectoplasmic actin-myosin network for movement and attachment, these protists excrete enzymes to breakdown plant or algal detritus and thus are important to marine carbon cycling and food webs (Raghukumar, 2002). At other times, *Labyrinthula* may act as a seagrass pathogen by infecting living seagrass leaf cells, leading to the necrosis of chloroplasts and

distinct black lesions (Raghukumar, 2002). The dynamics among seagrass health, anti-microbial defences and *Labyrinthula* virulence is still a central topic for wasting disease research. It is thought that occurrences of wasting disease and seagrass die-off are linked both to genetic clades varying in virulence (Martin et al., 2016), as well as constitutive or induced defence metabolites produced by the host (Vergeer et al., 1995; Steele et al., 2005). *Labyrinthula* can easily be isolated into pure culture from living or senescent macrophytes in coastal ecosystems using standard isolation and culturing techniques. *Labyrinthula* in culture are identified through their characteristic spindle shape and ectoplasmic networks, though they can have a range of colony morphotypes ranging from lacy or branching to dense or bush-like formations (Muehlstein et al., 1988; Vergeer and den Hartog, 1994). However, the detection and identification of *Labyrinthula*-induced seagrass wasting disease in the field can be challenging and expensive to implement due to the need for regular, continued monitoring of seagrass populations as well as laboratory diagnostic testing strategies for symptomatic plants (Groner et al., 2016).

The most intensely studied wasting disease host-pathogen relationship is *Zostera marina* (eelgrass) and *Labyrinthula zosterae* in Europe, temperate North America and Japan (Sullivan et al., 2013). Investigations of the disease dynamics of (sub-) tropical *Thalassia testudinum* (turtlegrass) and Mediterranean *Posidonia oceanica* (neptune-grass) and *Cymodocea nodosa* (slender seagrass) have become increasingly common in recent years (Garcias-Bonet et al., 2011; Trevathan-Tackett et al., 2013; Martin et al., 2016). In comparison, research on seagrass wasting disease in the Southern Hemisphere is less common. The first observation of wasting disease occurred in New Zealand whereby *Labyrinthula* was associated with die-backs of *Zostera* spp. (Armiger, 1964). While the isolate was cultured, described and hypothesised to be the cause of the die-back, no pathogenicity test was done to confirm *Labyrinthula* as the causative agent of the disease outbreak (Armiger, 1964). Since then,

sampling efforts to identify and describe Southern Hemisphere *Labyrinthula* has been limited to a few studies in Western Australia (Vergeer and den Hartog, 1994), southeastern Australia (Sullivan et al., 2017) and Queensland (Kirkman, 1978). Only one pathogenicity study has been performed on an isolate from *Zostera muelleri* (subspecies *capricorni*) from Queensland (Martin et al., 2016). While this Australian *Labyrinthula* isolate clustered in a pathogenic phylogenetic clade, the pathogenicity test using the congener host *Z. marina* was negative (Martin et al., 2016), and thus virulence as shown by Koch's postulates remained inconclusive.

In this study, we isolated *Labyrinthula* from a broad range of temperate seagrass species along the southeastern Australian coast in order to identify occurrences of pathogenic genotypes of *Labyrinthula*. Our aim was to build on our current knowledge of Australian *Labyrinthula* phylogeny (Sullivan et al., 2017), while also providing a first look at wasting disease ecology in the Southern Hemisphere. The results will demonstrate the presence of new isolates of *Labyrinthula* in an understudied biogeographic region and add to the growing global database of *Labyrinthula* genetic diversity and virulence. This study will also identify potential areas for further seagrass wasting disease research and monitoring in Australian coastal ecosystems.

Methods

Sites, sampling and Labyrinthula *culturing techniques*

Seagrass leaves were collected in March 2016 (beginning of the Australian autumn) from five sites along the coast of Victoria from Lakes Entrance (LAK) in East Gippsland, Duck Point (DP) in Corner Inlet and Rhyll (RY), San Remo (SR) and Warneet (WAR) in Western Port Bay (approximately 750 km of coastline; Table S1, Fig. 1). Seagrass leaves were collected both from living plants ('attached') as well as fresh green 'floating' leaves from *Amphibolis antarctica* (Aa), *Halophila australis* (Ha), *Heterozostera nigricaulis* (Hn), *Posidonia australis*

(Pa) and *Zostera muelleri* (Zm; Table S1). Leaves were placed in individual, sealed plastic bags filled with local seawater until the leaves were transferred to agar plates within 48 hours.

Labyrinthula was isolated from the 1-3 cm sections of seagrass leaves using serum seawater agar media (Trevathan et al., 2011). Leaf tissue that was symptomatic of wasting disease (e.g. blackened areas surrounded by green tissue) was targeted for isolation, selecting for blackened tissue plus the immediate green border. *Labyrinthula* growth from the leaf sections was monitored daily using an inverted microscope (Olympus Model CK30 + CK40-RPSL; Olympus, Tokyo, Japan). Sections of the *Labyrinthula* cultures were immediately transferred to new agar plates in order to avoid overgrowth by fungi also originating from the seagrass leaf. To avoid biases in obtaining just one *Labyrinthula* isolate from a site or leaf segment, separate cultures denoted by letters were created for each separate 'colony' of *Labyrinthula* growing from the seagrass leaves.

Sequencing

Representative pieces of agar approximately 1 cm x 1 cm were cut from axenic *Labyrinthula* cultures for sequencing. DNA extraction was performed using standard lysis, wash and elution buffers, then eluted with Econospin Micro Spin columns (Epoch Life Sciences, Missouri City, TX) . A region of the 18S ribosomal RNA (rRNA) gene was amplified with PCR, using the universal primers: primer A (18S forward 5'-AACCTGGTTGATCCTGCCAGT-3'), and primer B (18S reverse 5'-TGATCCTTCTGCAGGTTCACCTAC-3') (Medlin et al., 1988). Sequencing of PCR products was performed by Macrogen (South Korea), using the above primers and the internal primers 18S_f2 (forward 5'-CGAATGTAGCGTTTACTGTG-3') and 18S_r3 reverse 5'-GTGCCCTTCCGTCAATTCC-3') (Bockelmann et al., 2012). Preliminary DNA sequence

analysis (i.e. trimming of poor quality ends and alignment using default settings) was performed using Geneious Pro 4.8.5 (Kearse et al., 2012).

Phylogenetic analysis was performed following the procedure described by Sullivan et al. (Sullivan et al., 2017). Briefly, 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 798 bp. Sequences with incomplete coverage of this region were discarded. The Gblocks server (Castresana 2000) was used to select segments of the trimmed multiple alignments 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 50,000 generations, with sampling every 1,000th generation. Default values were used for all other parameters.

Pathogenicity assays

Based on the colony morphology and growth rates of the agar cultures prior to sequencing, representative *Labyrinthula* isolates were chosen for pathogenicity tests. Pathogenicity tests were performed at the Victorian Marine Science Consortium (VSMC) research facilities during both winter (August/September 2016) and summer (February 2017) seasons. Local populations of *Zostera muelleri* (Barwon River, 38.273549 S, 144.506435 E) and *Heterozostera nigricaulis* (Swan Bay, 38.266161 S, 144.624941 E) were used as hosts for the pathogenicity studies. The host plants were cleaned of attached fauna, microalgae and

sediments by hand and acclimated for 24 hours before the start of the experiment. One experimental unit consisted of one seagrass ramet with attached rhizome/root tissues weighted down in a 15 L glass aquarium under a metal halide lighting system (12:12 h light:dark photoperiod). Seawater was provided by the research facilities pumped in from Victory Bight (40 μm filtered) and typically had a salinity of 35-40 psu during the experiments. Air stones were added to the mesocosms to provide oxygenation and gentle circulation. Temperature was controlled by the test room thermostat at 20-25°C. To ensure there was no cross-contamination of *Labyrinthula* between rounds of pathogenicity experiments or transference of *Labyrinthula* outside of the laboratory, the aquarium mesocosms, air stones, weights and seawater were cleaned with ≥10% v/v hypochlorite bleach before and after pathogenicity experiments.

The second rank blade (second youngest leaf) of the seagrass plants was used to test *Labyrinthula* pathogenicity (Muehlstein et al., 1988; Trevathan et al., 2011). Segments of *Z. muelleri* (~1.5 cm x 0.5 cm) were sterilised by autoclaving in seawater to create a vector substrate to transfer *Labyrinthula* from the agar culture to the seagrass leaf. Vectors were placed on 1-2 day old *Labyrinthula* cultures, and the colonies were allowed to completely cover the vectors (~2-7 days) before the vectors were used in the experiment. Sterile vectors were used as procedural controls. Vectors were attached to the middle of the second rank blade and attached with a clamp made from ~1 cm segments of PVC aquarium air tubing. The occurrence and spread of lesions were monitored daily during the length of the experiments (2-4 days), and deionised water was added to the mesocosms to account for evaporation.

At the end of the experiment, seagrass ramets were removed from the mesocosms and placed on sterile aluminium foil to be photographed for subsequent measuring. If lesion formation occurred, signaling possible infection, segments of the leaf were transferred to new agar plates. If pure cultures of *Labyrinthula* were isolated from the infected seagrass hosts, then Koch's postulates were confirmed, and the isolate was deemed pathogenic. Total leaf area and

lesion size at the end of the pathogenicity experiment for infected samples were measured using Image-J software (Rasband, 1997-2016).

Statistical analysis

A one-way Analyses of Variance (ANOVA) was used to test the variation in lesion size among the *Labyrinthula* isolates for each seagrass host (three ANOVA tests in total). Follow-up t-tests were performed to test for significant differences in lesion size between the seagrass host species for each *Labyrinthula* isolate for each season (six t-tests in total). Lastly, the effect of season was tested with a t-test using the data for the isolates tested in both seasons. Pairwise tests were performed with Tukey's post-hoc analyses. All statistical tests were performed at a 95% confidence level using SPSS v24 (IBM, Armonk, NY, USA).

Results and Discussion

The survey of southeastern, temperate Australian *Labyrinthula* from seagrass leaves in this study confirmed that this marine protist is ubiquitous to the region and seagrass host species. The isolated *Labyrinthula* cultures were both morphologically and genetically diverse and had a range of virulence attributes. Here, for the first time we confirmed the presence of pathogenic *Labyrinthula* present in coastal Australian waters.

Variation of Labyrinthula *isolates in southeastern Australia*

In total, 38 *Labyrinthula* isolates were cultured from five seagrass species along the Victoria coastline (Table S1). Five 18S rDNA haplotypes were identified across the *Labyrinthula* isolates. The haplotypes were named in continuation from previously described isolates in Australia (Aus1-3) by Sullivan et al. (2017): *Labyrinthula* sp. Aus2b (2 isolates),

Aus4 (17 isolates), Aus5 (8 isolates), Aus6 (7 isolates) and Aus7 (4 isolates) (Table S1). Except for one sample, the BLAST hits for Aus6 were low (<90%).

The majority of the isolates were similar to previous colony morphological descriptions of Australian *Labyrinthula* (Vergeer and den Hartog, 1994; Sullivan et al., 2017). Haplotype Aus2b was a thin morphotype found for both one Duck Point *P. australis* isolate and one San Remo *Z. muelleri* isolate from green tissue. Haplotype Aus5 from San Remo was from *A. antarctica* and *H. australis* seagrass hosts and consistently had very dense, globular formations or morphotypes with some instances of branching (Fig. 2a-d; Table S1). This morphotype also dominant in Haplotypes Aus6 (from living and drift *P. australis* leaves at Duck Point) and Aus7 (*Z. muelleri* isolates from Warneet and one *P. australis* drift isolate from Duck Point). Haplotype Aus4 was solely from *Z. muelleri*, and was found at most locations (Lakes Entrance, Duck Point, Rhyll and San Remo). Haplotype Aus4 also contained a wide range of morphotypes, including a lacy morphotype from RY_Zm_A and a swirling morphotype only found from the Lakes Entrance *Z. muelleri* hosts (LAK_Zm; Fig. 2e,f). The swirling, or spiraling, morphotype was also previously found for haplotype Aus1 isolated from *H. nigricaulis* in Port Phillip Bay in Victoria (Sullivan et al., 2017). The swirls have been thought to indicate a chemotactic interaction among cells (Nakatsuji et al 1991), though it is not clear if this is a function of the environment or a characteristic of the LAK isolates but not other Aus4 isolates.

The current database of sequenced *Labyrinthula* isolates indicates that the genus forms a monophyletic group within the stramenopiles, comprised of three main clades (Fig. 3). One clade contains only terrestrial isolates. A second clade contains all isolates identified as *Labyrinthula zosterae* and contains primarily pathogenic isolates (Martin et al., 2016). The third and largest clade includes a combination of marine, terrestrial, and endosymbiotic isolates, and recently has been shown include primarily non-pathogenic isolates (Martin et al.,

2016). In this study, two haplotypes Aus2b and Aus7 grouped within this third larger non-pathogenic clade, and were most closely related to *Labyrinthula* isolates from Japan, the Philippines and Vietnam (Fig. 3) (Sakata et al., 2000; Wahid et al., 2007; Tsui et al., 2009). Haplotype Aus4 grouped closely with *Labyrinthula zosterae* from the USA and Europe (Leander and Porter, 2001), while Aus5 haplotype grouped closely with a pathogenic isolate from Florida and a recently described isolate from Queensland, Australia (Martin et al., 2016). Haplotype Aus6 was most closely related to the pathogenic group, though it is a relatively long distance from the most recent common ancestor shared with the *L. zosterae* isolates (Fig. 3).

When comparing the *Labyrinthula* culture morphologies and the 18S rDNA haplotypes, it was apparent that colony morphotype was not bound within a phylogenetic group. This was particularly evident for haplotype Aus4, which included lacy, swirling, branching and dense morphotypes (Table S1). This result highlights two things. First, it shows that colony morphology may not be a particularly reliable indicator of evolutionary relationships between the *Labyrinthula* spp., as there is variation among isolates within the same haplotype. Clearly, future studies addressing *Labyrinthula* taxonomy should include phylogenetic data and would ideally examine more than one marker region to strengthen the inferred phylogenies (Martin et al., 2016). Second, it suggests that *Labyrinthula* colony growth morphology could be dependent on environment factors, such as substrate and resources. Do the differing morphologies represent an 'actively' produced adaptive response to chemotactic or environmental stimuli (phenotypic plasticity), or are they an artifact produced by some aspect of the culturing process or depletion of nutrients? Under the scenario of a plastic phenotypic response to stimuli, alternate morphotypes could be induced by (a) transient changes in gene regulation or (b) by epigenetic chromatin modifications that have the ability to persist in the absence of the stimuli that induced them. There has been little research on the relationship between morphology and environmental factors for *Labyrinthula* or the broader

Labyrinthulomycetes class (Leander et al., 2004). A distant stramenopile relative, the diatom *Phaeodactylum tricornutum* (Bacilliarophyceae), has been shown to have three distinct morphologies that vary based on environmental and resource conditions (Maumus et al., 2011). These morphotypes are associated with different transcriptome profiles (Maumus et al., 2011), but certain epigenetic machinery has been shown to be conserved in this species (reviewed in Tirichine et al., 2017). Future work on *Labyrinthula* understanding the connections between isolate phenotype, genetics and virulence would provide significant advances to understanding seagrass wasting disease ecology, particularly in the context of future environmental changes.

Pathogenicity of Labyrinthula *isolates*

Five of the 38 isolates were tested for pathogenicity on *Z. muelleri* and *H. nigricaulis* hosts. These isolates were originally chosen based on their different colony morphotypes and growth rates prior to genetic sequencing: LAK_Zm (isolate A, haplotype Aus4), DP1_Zm (isolate F, haplotype Aus4), SR_Zm (isolate C2, haplotype Aus4), SR_Aa (isolate D, haplotype Aus5) and SR_Ha (isolate C, haplotype Aus5). The remaining isolates were lost due to a laboratory-wide fungal contamination incident; thus, haplotypes Aus2b, Aus6 and Aus7 could not be tested for pathogenicity. However, isolates SR_Aa, SR_Ha, SR_Zm and LAK_Zm are still viable for future testing.

Three isolates (SR_Aa, SR_Ha and SR_Zm) tested positive for pathogenicity and were confirmed by Koch's Postulates (Figs. 4 & 5). The LAK_Zm and DP1_Zm isolates did not cause lesions to form on either seagrass species. Among the pathogenic isolates, SR_Ha caused the most rapid lesion formation compared to the other isolates, typically within 12-24 hours. For SR_Zm, lesion formation was noted after ~48 hours after infection, and induced a lower degree of infection seen by smaller lesion size compared to the other two isolates (Figs. 4 & 5). There was no significant difference in total lesion size among the isolates infecting *Z.*

muelleri in the winter (log transformed, $F_{1,5} = 7.110$, $p = 0.056$). However, when SR_Ha and SR_Zm were compared in a separate t-test (excluding negative tests of SR_Aa), there was a significant difference in lesion formation between the two isolates (df = 4, $p = 0.012$). In the winter, lesion size of infected *Z. muelleri* was also significantly higher than *H. nigricaulis* for SR_Ha isolates (d.f. = 6, $p = 0.018$).

The variability of infection of the individual isolates between the two hosts was also tested (Figs. 4 & 5). Isolate SR_Aa caused no infection for *Z. muelleri* but significant lesion formation for *H. nigricaulis* (Fig. 4a). For SR_Ha, the degree of infection was higher for *Z. muelleri* than *H. nigricaulis* in the winter with lesions covering the entire infected leaf in most replicates, and in some cases infecting the other leaves on the ramet (d.f. = 4, $p = 0.007$). However, in the summer, infection by SR_Ha was similar for both host species (lesion sizes < 50 mm^2 over three days of infection), with no significant difference in lesion size between the two seagrass host species (Fig. 4). Both SR_Ha and SR_Zm infected the seagrass hosts in both winter and summer, though it is unclear if the reduced lesion size during the summer was due to the season or a reduced length of infection time.

It also was noted that the pathogenic isolates had an in-agar or 'endo' growth characteristic that was not present in the non-pathogenic isolates (e.g. SR_Ha in Fig. 2d vs LAK_Zm in Fig. 2e). This 'endo' growth is a characteristic of other pathogenic *L. zosterae* isolates (Muehlstein et al., 1991; Martin et al., 2016). It has also been suggested that holes in epidermis of diseased seagrass leaves could have been a result of invasion of *Labyrinthula* (Muehlstein, 1992). We hypothesise that this endo-growth characteristic may be analgous to the infiltration of the leaf epidermis and evidence of a strategy for penetrating the cell wall and infecting living tissues (e.g. extracellular cellulolytic enzymes) (Bremer, 1995; Bremer and Talbot, 1995). However, the link between axenic culture morphology, extracellular enzyme production and virulence still needs to be explicitly tested for *Labyrinthula*-host interactions.

Australian and global Labyrinthula *biogeography*

The locations from which the phylogenetic groups were isolated in this study provide preliminary insight into *Labyrinthula* biogeography, both within Australia as well as across the globe. It is the general consensus that *Labyrinthula* is naturally transported through dispersal of the living host or detritus in the water column (Moore and Short, 2007). The furthest eastern sites, Lakes Entrance and Duck Point, are separated from the Western Port Bay sites by the Wilson's Promontory biogeographic barrier. Yet, phylogenetic haplotypes Aus4 and Aus7 contained isolates from both regions of the coast, suggesting a current-independent mode of dispersal, such as shipping or another form of human- or animal-mediated transport. On a broader scale, haplotype Aus2 was found in NSW (Sullivan et al., 2017) and this study, i.e. on both sides of the Bass Strait and in both Peronia and Maugea biogeographic provinces. These data indicate that ocean currents have a limited effect of the biogeographic isolation of *Labyrinthula* along the eastern Australian coast. It should be noted that there is relatively high genetic connectivity among seagrass populations along the Victoria coastline (Stafford-Bell et al., 2016). The rafting dispersal strategies of seagrasses for reproduction could also influence the dispersal of *Labyrinthula* and thus has important implications for regional disease connectivity.

On a global-scale, most of the Australian *Labyrinthula* isolates from this study and Sullivan et al. (2017) were placed within clades inclusive of overseas isolates from a diverse range of locations (Fig. 3). This cosmopolitan distribution of the isolates within each of the clades has important implications for global wasting disease ecology. Two of the haplotypes found in this study (Aus2b, Aus7) were within non-pathogenic clades described in Martin et al. (2016). Although dominated by Australasian isolates, these clades are diverse in both location as well as source (includes seagrass, mangrove, macroalgal and fish hosts). The other

three haplotypes (Aus4, Aus5, Aus6), two of which tested positive for pathogenicity in this study, fit within pathogenic clades. These pathogenic isolates are grouped in clades with isolates from seagrasses in the USA and Europe suggesting 'related' pathogenic haplotypes have made cross-ocean and cross-hemisphere dispersal at some point in their evolutionary history. Of these pathogenic haplotypes, we would expect that haplotype Aus4 in clade A would be consistently pathogenic and closely resemble *L. zosterae* (Martin et al., 2016). However, the isolates in haplotype Aus4 had the widest range of morphotypes and included both pathogenic and non-pathogenic isolates. Host specificity is not likely the reason for this range in pathogenicity since all the isolates came from *Z. muelleri*. Rather, these results suggest within-clade and within-haplotype variability in pathogenicity. Surveys of *L. zosterae* in Europe and USA has shown variable pathogenicity or wasting disease over a broad geographic range and could be linked to seagrass defense genes and age (Bockelmann et al., 2012; Bockelmann et al., 2013; Brakel et al., 2014; Groner et al., 2014). In contrast to Aus4, haplotype Aus5 was isolated from one area (San Remo) and included consistently dense, fast-growing isolates able to grow within the agar media. The isolate showing the highest virulence came from this group, but its original host showed no signs of wasting disease, suggesting that seagrasses with a high leaf turn-over rate like *Halophila* could act as a vector for pathogenic *Labyrinthula*. Haplotype Aus6 had a different position in the phylogenetic tree than then other isolates in this study. While Aus6 looks to be separate, the length of the branch separating it from the common ancestor it shares with the pathogenic *L. zosterae* clade is within the range of branch lengths seen within the non-pathogenic clades. These results make hypothesising haplotype Aus6's position in clades A-E or predicting its pathogenicity challenging. The population of *P. australis* from which Aus6 was isolated is the only population in Victoria. Since Aus6 was not yet found in NSW populations of *P. australis* and could represent a new diversity in *Labyrinthula* phylogeny, future work including deeper sampling will be important

to tease apart these potentially new dynamics of host and location specificity of Aus6. In addition, given the evidence for variability in virulence-clade framework in this and previous studies, it is a priority to further investigate the host- and pathogen-based attributes that drive the variability in wasting disease cause-and-effect.

Seagrass wasting disease in Australia

This study is the first to culture, genotype and confirm pathogenicity of *Labyrinthula* isolates in Australia. Our results showed that three pathogenic isolates were collected from three species in the same area and highlights a potentially important interaction between *Labyrinthula* and seagrass phylogeny in Australia. There has been no research describing a wasting disease-related die-off in the southern hemisphere since the 1960s, which suggests these die-offs have been over-looked or that the seagrasses have maintained sufficient chemical or life-history defence mechanisms to avoid meadow-wide spread of wasting disease.

The presence of pathogenic *Labyrinthula* in Australia provides an opportunity to further investigate unique aspects of wasting disease ecology. First, Australia is home to highly diverse and several endemic seagrass species, so they may possess equally diverse chemical, genetic or life-history traits (e.g. leaf turn-over rates, spatial heterogeneity of meadow formation) for disease avoidance (Zapata and McMillan, 1979; Irvine et al., 2016; Zidorn, 2016). Additionally, Australian seagrasses inhabit a range of habitat types including protected bays and exposed coastal environments, which could influence the rate of *Labyrinthula* transference and infection. For example, many estuaries along the coasts are intermittently blocked from tidal flow, i.e. intermittently closed/open lakes and lagoons or ICOLLs. Seagrasses in these environments would be subjected to brackish or freshwater conditions, particularly during heavy rains and flooding from the catchment (Mondon et al., 2003). With most *Labyrinthula* spp. less tolerant to low salinities, these freshwater events could allow for seagrasses to clear

their load of *Labyrinthula* and help control occurrences of disease (Blakesley et al., 2002; McKone and Tanner, 2009).

Conclusions

In summary, this study has provided novel data on the pathogenicity and phylogeny of *Labyrinthula* spp. along the southeastern Australian coast and a first in the southern hemisphere. We emphasise that more research is needed to understand seagrass wasting disease ecology in the southern hemisphere and in the context of global *Labyrinthula* biogeography. The results also highlight the importance of monitoring disease in Australian seagrass populations, especially in areas with anthropogenic and climatic stresses that may promote declines in seagrass health and enhance the risk of wasting disease.

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Author Contributions

STT, FG, BKS, OL, PIM conceived and/or designed the experiment. STT and BKS performed the research. STT and KR analysed the data. STT led the writing of the manuscript. All authors contributed to the data interpretation, writing and editing of the manuscript.

The authors have no competing interests.

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Figure Legends

Figure 1: Map showing the seagrass species sampled for *Labyrinthula* along the Victoria coastaline in southeastern Australia. Site abbreviations are provided in the parentheses. See Table S1 for details on site GPS and the isolates cultured from each site.

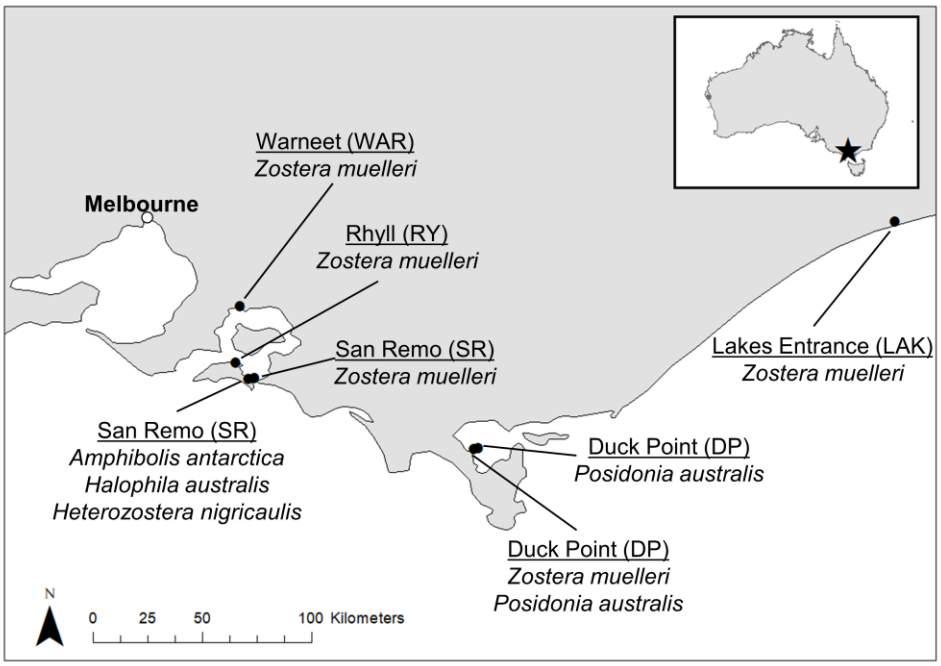

Figure 2: Representative morphotypes of *Labyrinthula* sp. cultured on serum-seawater agar from seagrasses along the Victoria coastline of Australia. (a-d) Dense and branching morphotypes dominated the cultures collected, while (e) swirling and (f) thin, lacy morphotypes were less common. The inset in panel (a) was magnified in panel (b) and shows a dense leading edge with thinner parallel formation just inside leading edge that was unique to SR_Zm isolate C2. The arrow in panel (d) shows cells growing within the agar in older parts of the colony. Double-sided arrows indicate the leading edge of the culture. Asterisks highlight visible ectoplasmic network between cells. Scale bars represent 1 mm (a, c, d), 0.1 mm (d, e) or 0.01 mm (f).

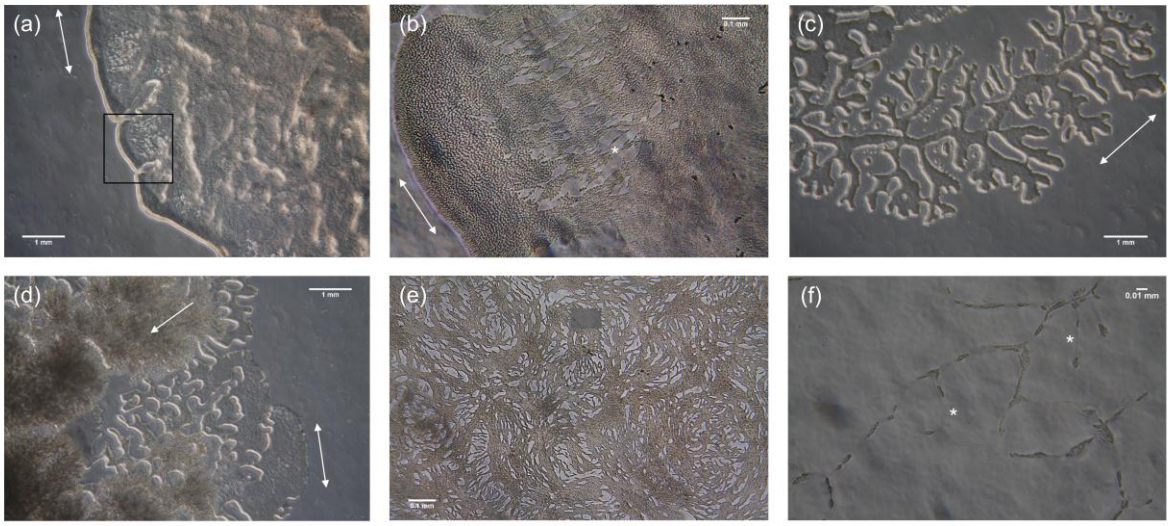

Figure 3: Bayesian phylogenetic analysis of *Labyrinthula* 18S rDNA sequences. All *Labyrinthula* sequences present in Genbank were assembled into groups with >99.0% identical sites. For clarity, one representative sequence per group was selected for phylogenetic analysis. A total of 612 nucleotide sites were used in constructing the tree. Posterior probabilities and the expected number of substitutions per site (scale bar) are shown. Bold (*) text indicates the isolates from the current study. Colours indicate pathogenic (red), non-pathogenic (blue), variable pathogenicity (purple) and unknown pathogenicity (black). Letters indicate clades from Martin et al. (2016). Original hosts include seagrasses (Aa = *Amphibolis antarctica*, Cn = *Cymodocea nodosa*, Ha = *Halophila australis*, Ho = *H. ovalis*, Hn = Heterozostera *nigricaulis*, Hw = *Halodule wrightii*, Pa = *Posidonia australis*, Po = *P. oceanica*, Tt = *Thalassia testudinum*, Zma = *Zostera marina*, Zmu = *Z. muelleri*), macroalgae (Ma), mangrove (Rx = *Rhizophora* sp.), Endosymbionts (E) and terrestrial plants (*Agrostis* sp., *Poa trivialis* and T = turfgrass).

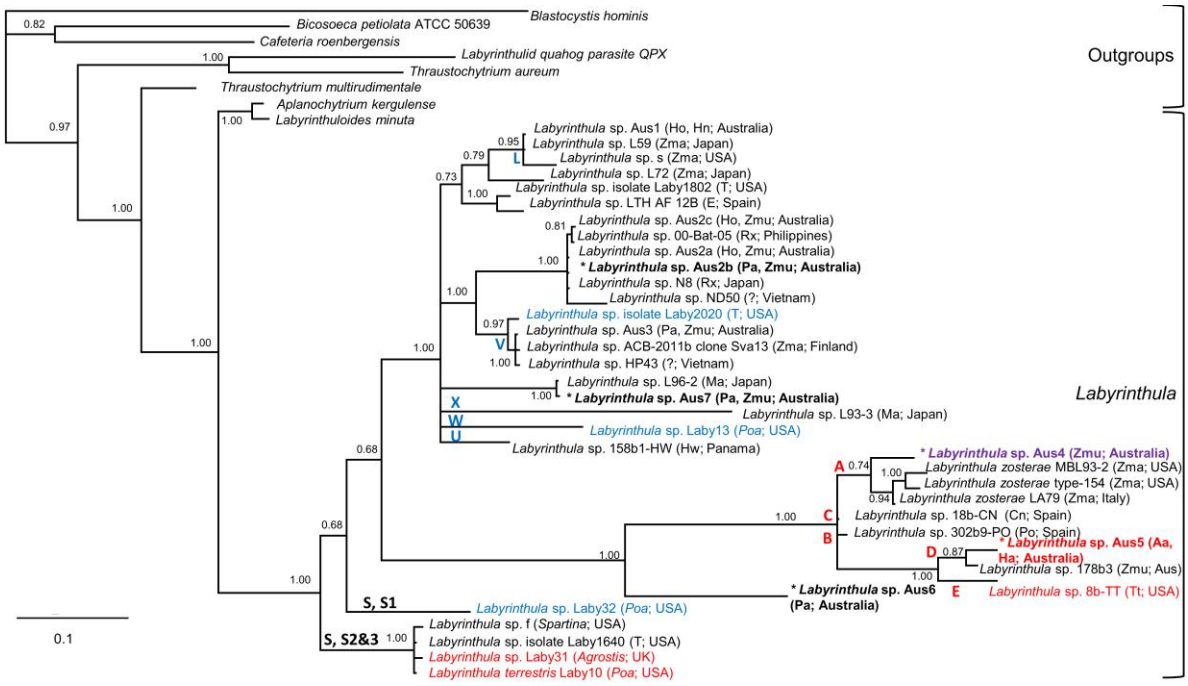

Figure 4: Lesion size on seagrass leaves (*Heterozostera nigricaulis*, *Zostera muelleri*) from three *Labyrinthula* isolates (SR_Ha isolate C, SR_Zm isolate C2, SR_Aa isolate D). The length of incubation (days) is indicated for each host-isolate combination to show differences in lesion growth rate. Two rounds of tests were performed in the Australian winter (August/September 2016) and summer (February 2017). Values represent means ± S.E.M (n = 3-4).

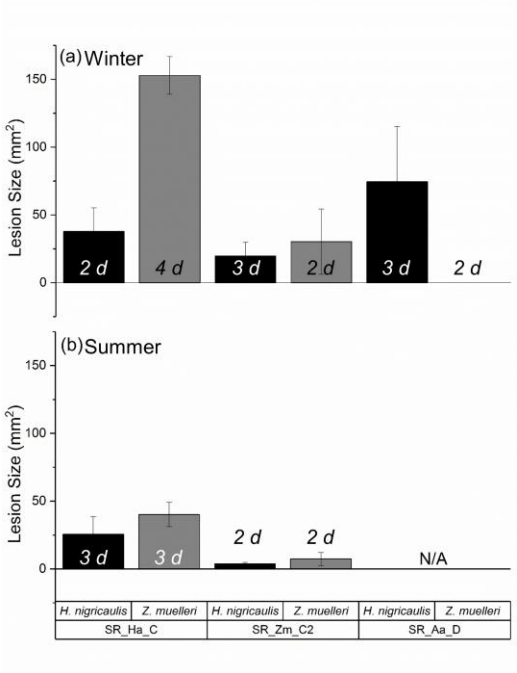

Figure 5: Examples of lesion formation after seagrasses were infected with *Labyrinthula* for three days. (a) Isolate SR_Ha_C infecting *Zostera muelleri* shows black lesions above and below vector placement. (b) Isolate SR_Zm_C2 infecting *Heterozostera nigricaulis* shows slower lesion formation near the vector only. (c) Isolate SR_Aa_D infecting *H. nigricaulis* shows extensive lesion formation over the entire leaf except around the vector as well as transference to an adjacent leaf. Arrows point to black lesions caused by *Labyrinthula* infection that can bisect the leaf width or spread along the leaf edge. Insets highlight the lesion details. Second-oldest leaf blades (second rank, 2°) were infected with vectors of *Labyrinthula* and held on by small PVC clamps (shown near infection site). Youngest leaves (first rank, 1°) and oldest leaves (third rank, 3°) are also shown in the photos. Scale bars = 1 cm.