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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*, have 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.

1. 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, 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 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

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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* spp. 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* (neptunegrass) 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* have 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 demonstrated the presence of new isolates of *Labyrinthula* in an understudied biogeographic region and added 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.

2. Methods

2.1. 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 h.

Labyrinthula was isolated from 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.

2.2. Sequencing

Representative pieces of agar approximately 1 cm × 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. (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 1000th generation. Default values were used for all other parameters.

2.3. 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 h 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