1 **Diversity and microhabitat associations of *Labyrinthula* spp. in the Indian River Lagoon**
2 **System**

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8 Running Head: Diversity of *Labyrinthula* spp.

9   ABSTRACT

10   Seagrasses create foundational habitats in coastal ecosystems. One contributing factor to their

11   global decline is disease, primarily caused by parasites in the genus *Labyrinthula*. To explore the

12   relationship between seagrass and *Labyrinthula* spp. diversity in coastal waters, we examined the

13   diversity and microhabitat association of *Labyrinthula* spp. in two inlets on Florida's Atlantic

14   Coast, the Indian River Lagoon (IRL) and Banana River. We used amplicon-based high

15   throughput sequencing (HTS) with two newly designed primers to amplify *Labyrinthula* spp.

16   from five seagrass species, water, and sediments to determine their spatial distribution and

17   microhabitat associations. The SSU primer set identified 12 *Labyrinthula* zero-radius operational

18   taxonomic units (ZOTUs), corresponding to at least eight putative species. The ITS1 primer set

19   identified two ZOTUs, corresponding to at least two putative species. Based on our phylogenetic

20   analyses, which include sequences from previous studies that assigned seagrass-related

21   pathogenicity to *Labyrinthula* clades, all but one of the ZOTUs that we recovered with the SSU

22   primers were from non-pathogenic species, while the two ZOTUs recovered with the ITS1

23   primers were from pathogenic species. Some of the ZOTUs were widespread across the sampling

24   sites and microhabitats (e.g., SSU ZOTU_10), and most were present in more than one site. Our

25   results demonstrate that targeted metabarcoding is a useful tool for examining the relationships

26   between seagrass and *Labyrinthula* diversity in coastal waters.

27   Keywords: *Labyrinthula*, biodiversity-disease relationship, host specificity, seagrass,

28   metabarcode, biodiversity

1. INTRODUCTION

Seagrasses are aquatic angiosperms that occur globally in shallow coastal waters (Short et al. 2007). They perform many vital functions such as providing essential nursery habitat for many aquatic species, creating a carbon sink, stabilizing sediments, increasing water clarity, and impacting nutrient cycling (Duffy 2006). Unfortunately, seagrasses are undergoing a dramatic reduction worldwide, with a 30% decline in coverage within the last 130 years (Waycott et al. 2009). One contributing factor to these declines has been seagrass wasting disease (SWD), which impacts seagrass species worldwide (Martin et al. 2016, Sullivan et al. 2017). This disease blocks photosynthetic capabilities and prevents the plant from building up carbohydrate reserves, eventually leading to carbon deficiency, secondary infection from bacteria or fungi, and death of the plant (Renn 1936).

Parasites in the genus *Labyrinthula* are the etiological agents of SWD (Muehlstein et al. 1991). This genus comprises the monotypic family Labyrinthulaceae (Stramenopiles, Labyrinthulomycetes; Adl et al. 2012) and consists of ~15-20 presumed terrestrial and marine species (Chitrampalam et al. 2015, Martin et al. 2016), with many more likely undetected. Previous research demonstrated that species in one major clade, consisting of at least 5-7 putative species of *Labyrinthula,* are likely pathogenic and specific to seagrasses (Martin et al. 2016, Trevathan-Tackett et al. 2018), with variations in their host-specificity, pathogenicity, and virulence (Martin et al. 2016). However, we know relatively little about how host diversity impacts the ecology of SWD.

Historically, research examining the impacts of host diversity on disease has utilized terrestrial plant pathosystems (a subset of an ecosystem characterized by parasitism), in particular examining how host-species richness and composition can change disease dynamics. There are three primary factors that influence how host diversity effects disease dynamics including 1) variation in host competence (i.e., the ability of the parasite to reproduce within a host across potential host species, 2) high density communities containing poor-quality hosts, and 3) the ability for genetic and community diversity to decrease the encounter rate of the parasite with high-quality hosts (Ostfeld & Keesing 2012). Variations across these factors ultimately determine whether host composition has a "dilution" (decreasing overall parasite population and subsequent prevalence) or "amplification" (increasing overall parasite population and subsequent prevalence) effect. For seagrasses, previous work demonstrated potential variation in host competence, with seagrass species differing in their ability to resist fungal and labyrinthulid infection through the production of secondary metabolites (Ross et al. 2008, Trevathan-Tackett et al. 2015) and reactive oxygen species (ROS) (Loucks et al. 2013). Thus, it is likely that these differing plant immune responses would influence parasite diversity and distribution.

While evidence exists both for and against the role of host biodiversity in decreasing disease risk, the majority of these studies have been conducted in terrestrial, rather than marine, systems (Civitello et al. 2015). Thus, we chose the *Labyrinthula*-seagrass pathosystem as a potential model system to explore how host diversity may impact *Labyrinthula* spp. diversity and

69 SWD. To do this, we examined the range and diversity of *Labyrinthula* species across five
70 potential seagrass hosts as well as local water and sediment microhabitat associations within the
71 Indian River Lagoon (IRL) and Banana Rivers, which are two components of the greater Indian
72 River Lagoon system located on the Atlantic coast of Florida, USA. The IRL system contains
73 seven species of seagrasses and is geographically situated on a transition zone between temperate
74 and warmer sub-tropical biological provinces, making it one of the most diverse seagrass
75 locations in the USA (Dawes et al. 1995).
76   In past surveys of *Labyrinthula* diversity, researchers primarily used traditional culturing
77 techniques to isolate and coarsely identify the parasite (Muehlstein et al. 1988, Vergeer & den
78 Hartog 1994). More recent studies used genetic methods to increase the resolution of the culture-
79 based parasite identifications (Martin et al. 2016, Trevathan-Tackett et al. 2018). However, both
80 are arduous approaches to parasite identification, given the length of time required to culture
81 certain species, the ever-present threat of fungal contamination, and the uncertainty regarding
82 what diversity may be unculturable or missed. Thus, we chose instead to use a targeted
83 metabarcode approach, allowing us to directly amplify and sequence *Labyrinthula* spp.
84 associated with seagrass blades or environmental samples. This approach has been recommended
85 as a more cost and time-effective method to assess the diversity of protistan parasites from
86 environmental samples (Bass et al. 2015).
87   In this study, we used amplicon-based high throughput sequencing (HTS) to estimate the
88 diversity of *Labyrinthula* species present on five seagrass species as well as surrounding water
89 and sediments collected from multiple locations within the IRL and Banana River. Our
90 objectives were to 1) design primers suitable for high throughput sequencing that targeted
91 *Labyrinthula* spp., 2) obtain information on the diversity, spatial distribution, and disease
92 impacts of these *Labyrinthula* spp. in relation to the diversity and spatial distribution of potential
93 seagrass hosts and 3) determine the microhabitat (i.e., seagrass, water, sediment) associations of
94 these parasites.
95
96 **2. MATERIALS AND METHODS**
97 **2.1 Sample Collection**
98   In 2015, we collected environmental samples (i.e., water, sediment) and seagrass blades
99 from five species of seagrasses (*Thalassia testudinum*, *Syringodium filiforme*, *Halodule wrightii*,
100 *Halophila engelmannii*, and *Halophila johnsonii*) across four sites in the IRL and two sites in the
101 Banana River (Table S1). We attempted to choose sites where three seagrass species co-
102 occurred, but collected from sites where at least two species co-occurred and collected each
103 species from at least two of six sampling sites. Water (n=3 per site; total = 12) and sediment (n=
104 3 per site; total = 12) samples were collected first to minimize disturbance (Table S1). Due to
105 time and supply limitations, we were unable to take water and sediment samples at one location
106 in the IRL and one location in the Banana River (Table S1). Using sterile Nalgene bottles, 1 L
107 water samples were collected above the seagrass bed ≥ 5m apart from each other, put on ice, and
108 immediately filtered upon returning to the laboratory (<4 hr). We filtered 500 mL of water

109   through 3 µm nucleopore Whatman filters (VWR International, Radnor, PA, USA) on sterile
110   single-use disposable filter apparatuses (Fisher Scientific, Pittsburgh, PA, USA). Filters were
111   placed into individual 1.5 mL microcentrifuge tubes and stored at -80ºC. Sediment was collected
112   using a bleach-washed syringe core directly below where the water sample was collected. A 1.5
113   mL microcentrifuge tube was filled with sediment taken from the top 1 inch of the sediment core
114   (i.e., surface sediment) using sterile forceps. Samples were then placed on ice and immediately
115   frozen at -80°C upon returning to the laboratory.

116   For each seagrass species, blades were collected along a single transect per site within the
117   seagrass bed, ≥ 5m apart from each other. For each sample, a single blade was cut at the base of
118   the shoot, placed leaf-tip-first into a single Ziploc bag, then stored in a cooler on ice. At a single
119   site (i.e., Harbor Branch), multiple blades from the same shoot were sampled; however, due to
120   time constraints for sample processing, all other collections included only a single blade per
121   shoot. Upon returning to the laboratory, the full length of the blade was rinsed with sterile
122   seawater and any epibionts were removed. The full length of the blade was measured to the
123   closest mm and the disease index score (%) was recorded (Table S2), based on the wasting key
124   index from Burdick et al. (1993). The top half of the blade, which is the oldest part and most
125   likely to be associated with *Labyrinthula* spp., was removed using flame sterilized instruments,
126   placed into a 15 mL conical tube and preserved in 95% ethanol. Due to the small blade size of
127   *Halophila* spp., the blade was preserved whole. Between each sample, the workspace was
128   sterilized with a 10% bleach solution and instruments were flame-sterilized. The environmental
129   and seagrass blades were shipped to the Smithsonian Environmental Research Center in
130   Edgewater, MD, for further genetic processing. Maps showing the sampling locations were
131   generated using ArcGIS 10.5 for Desktop (Esri, Redlands, CA).

132   **2.2 DNA extraction, Amplification, and Sequencing**

133   We extracted genomic DNA from water and sediment samples using a MoBio
134   PowerWater DNA Isolation Kit and a MoBio PowerSoil DNA Isolation Kit (Qiagen Sciences,
135   Germantown, MD, USA), respectively, following the manufacturer's protocols. For water
136   samples, an entire filter was extracted. For the sediment samples, 0.2 g of sediment per sample
137   was extracted and eluted in a final volume of 100 µL. A negative control containing no sample
138   was included with every set of extractions. These negative controls were treated exactly the same
139   as the samples through the entire process, including amplification and sequencing, in order to
140   account for the sequencing of aerosolized contaminants in the laboratory.

141   For each blade, a 12 mm section from the tip, targeting sections with lesions when
142   present, was combined with one stainless steel bead and beat for 2 min at 30Hz using a
143   TissueLyser. This process was repeated 1-2x until the plant tissue was pulverized. We extracted
144   genomic DNA from seagrass leaves using a Qiagen DNeasy Plant Mini DNA Isolation Kit
145   following the manufacturer's protocols. The final elution volume for each DNA sample totaled
146   100 µL. All extractions completed within the same day included a blank extraction, which served
147   as a negative extraction control for PCR. The original DNA elutions were stored at -20ºC while
148   separate aliquots (20 µL), which were made to avoid contamination of original DNA elutions,

149   were stored at 4ºC. Each DNA sample was quantified using a NanoDrop 2000 (Thermo Fisher
150   Scientific, Waltham, MA, USA).
151   A general plant PCR primer set, ITS1-P (5'-TTATCATTTAGAGGAAGGAG-3'; T. D.
152   Bruns, https://nature.berkeley.edu/brunslab/tour/primers.html) and ITS1-4 (5'-
153   TCCTCCGCTTATTGATATGC-3'; White et al. 1990) was used to determine if amplifiable
154   DNA was present in the extracted DNAs. PCR reagents consisted of 1X PCR Buffer, 1.5 mM
155   $MgCl_2$, 0.2 mM dNTP, 0.5 µM each primer, 0.2 mg/mL BSA, 0.03 units Taq polymerase, and
156   water to a final volume of 19 µl. Thermocycling was carried out using a Bio-Rad C1000 or
157   S1000 ThermoCycler (Bio-Rad, Hercules, CA) with an initial denaturation of 94ºC for 10
158   minutes followed by 35 cycles of 94ºC for 30 sec, 50ºC for 1 min, 72ºC for 1 min, and a final
159   extension of 72ºC for 5 min. An aliquot of the PCR product (5 µL) was electrophoresed on
160   agarose gel (2% w/v) and visualized under ultraviolet (UV) light after GelRed staining. Only
161   seagrass samples positive for this PCR reaction were subsequently screened.
162   We designed new primers to target the small subunit (SSU or 18S) gene or the first
163   internal transcribed spacer (ITS1) region of the ribosomal gene complex of *Labyrinthula* species.
164   Primers were designed by aligning previously generated SSU and ITS1 sequences in Geneious
165   using alignment parameters described in Martin et al. (2016). Identified primer sequences were
166   then run through Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) to check for potential problems.
167   Based on these results, we produced the following primers to amplify the SSU gene: Ill-
168   LabPathSSU-F1 (5' –IlluminaAdaptor - CACCTCTGACATACTCATACG-3') and Ill-
169   LabPathSSU-R1 (5' - IlluminaAdaptor - CAATCCTGATACGGGGAGG-3'), and the ITS1
170   region: Ill-LabPathITS1-2F (5' IlluminaAdaptor - AACTCAATGAATATCTTGGTTTCC-3')
171   and Ill-LabPathITS1-2R (5' IlluminaAdaptor - GATATGCTTAAATTCAGGGAGT-3').
172   Following the two-step PCR approach to amplicon metabarcoding, we added 20 bp of the
173   Illumina adaptor sequences to these primers to be used as a priming site in a second indexing
174   PCR (Illumina Support 2013).
175   The initial PCR reactions for each primer set consisted of the following final
176   concentrations: 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 (SSU) or 0.3 (ITS1) µM
177   each primer, 0.4 mg/mL BSA, 0.025 units Taq polymerase, 3 µL of DNA template, and water to
178   a final volume of 25 µL. Thermocycling was carried out using a Bio-Rad C1000 or S1000
179   ThermoCycler (Bio-Rad) with an initial denaturation of 94ºC for 10 minutes followed by 40
180   cycles of 94ºC for 30 sec, 53ºC for 45 sec, 72ºC for 45 sec, and a final extension of 72ºC for 5
181   min. An aliquot of each PCR product (5 µL) was electrophoresed on agarose gel (2% w/v) and
182   visualized under UV light after GelRed staining. The expected fragment sizes were 507 bp and
183   316 bp for the SSU and ITSI primer sets, respectively.
184   Due to budget constraints, we used both primer sets on the environmental samples, but
185   only the SSU primer set on the seagrass samples. All samples were amplified in triplicate for
186   each primer set applied. Triplicate PCRs were pooled based on relative gel band intensity.
187   Excess primers and nucleotides were removed from pooled amplicons using ExoSAP-IT PCR

188 Product Cleanup Reagent (diluted 1:10) with an initial incubation at 37ºC for 40 minutes and a
189 final incubation at 80ºC for 15 min.
190   We used a dual-indexing scheme with Nextera adaptors, ensuring a unique combination
191 was applied to each sample. PCR reagents consisted of 12.5 µL KAPA Ready Mix, 1µL each
192 index (i5 or i7), 1 µL amplicon (pooled and cleaned product), and 9.5 µL water for a final
193 volume of 25 µL. Thermocyling was carried out with an initial denaturation of 95ºC for 5 min,
194 followed by 12 cycles of 98ºC for 20 sec, 60ºC for 45 sec, 72ºC for 45 sec, and a final extension
195 of 72ºC for 5 min. An aliquot of the indexed PCR product (3 µL) and unindexed PCR product (3
196 µL) were both electrophoresed on agarose gel (2% w/v) and visualized under UV light after
197 GelRed staining to verify that indexing was successful.
198   The indexed PCR product was cleaned using Agencourt AMPure XP beads (Beckman
199 Coulter, Indianapolis, IN, USA), following the manufacturer instructions using a 10 µL sample
200 reaction volume. An aliquot of the PCR product (3 µL) was electrophoresed on an agarose gel
201 (2% w/v) and visualized under UV light after GelRed staining to verify that cleaning was
202 successful. The bead-cleaned products were quantified using a Qubit dsDNA HS Assay Kit
203 (Thermo Fisher Scientific, Grand Island, NY, USA) with a Qubit 2.0 Fluorometer following
204 manufacturer instructions. All bead-cleaned samples were subsequently pooled based on
205 equimolar concentrations. The final pooled volume was sequenced with a 2x250 MiSeq Reagent
206 Kit v2 (Illumina, San Diego, CA, USA) on an Illumina MiSeq at the Laboratories of Analytical
207 Biology (LAB) at the Smithsonian National Museum of Natural History (NMNH). Raw
208 sequences can be retrieved from NCBI SRA BioProject PRJNA575863.
209 **2.3 Bioinformatics**
210   Forward and reverse reads for both the SSU and ITS1 sequences were merged using
211 USEARCH v10.0 (Edgar 2013) with a maximum of 10 differences allowed in the overlapping
212 region (-fastq_mergepairs). Post-merging, the allowable sequence range was 420-470bp for the
213 SSU and 200-275bp for the ITS1. All sequences with maximum expected error rates >1 (-
214 fastq_filter) and primers (-fastx_truncate) were removed. For each dataset, unique sequences
215 were identified and de-replicated (-fastx_uniques), then sequences were sorted by abundance (-
216 sortbysize). To explore strain level differences across hosts and microhabitats, we used zero-
217 radius operational taxonomic clustering (ZOTU), allowing for a minimum of four copies, which
218 is recommended for smaller datasets (Edgar 2016). Reads were then mapped to the
219 representative ZOTUs at 99% similarity to generate the ZOTU table (-usearch_global). For the
220 SSU sequences, taxonomy was assigned using the RDP classifier (Wang et al. 2007) as
221 implemented in QIIME (Caparoso et al. 2010) using the PR2 database v4.3 (Guillou et al. 2013).
222 The ITS1 sequences were blasted against the NCBI database through Geneious v11.1.5
223 (Biomatters, Ltd.) using megablast.
224   Sequences in the negative control samples were considered for removal from both
225 datasets based on criteria from Pagenkopp Lohan et al. (2017). Briefly, this included any ZOTUs
226 present 1) only in negative controls, 2) 10X more abundant in negative controls than in any

227   single sample, or 3) not 10X more abundant in any one sample than in the negative control.
228   Based on these criteria, no SSU or ITS1 ZOTUs were removed from the dataset.
229   To verify sequence identifications, we made phylogenetic trees, which included all
230   sequences identified as *Labyrinthula* spp. from this dataset and the majority of *Labyrinthula* spp.
231   sequences from GenBank. For the SSU and ITS alignments, we also used parameters similar to
232   those in Martin et al. (2016), using the ClustalW plugin (Thompson et al. 1994) in Geneious,
233   with a gap open cost = 50 and a gap extend cost = 6.66 for the SSU analysis, and a gap open cost
234   = 20 and a gap extend cost = 6.66 for ITS analysis. Both alignments were trimmed to the length
235   of the shortest sequence, so that the final SSU alignment included 62 sequences and was 426 bp
236   long, while the ITS1 alignment included 91 sequences and was 239 bp long.
237   These alignments were then run through the online Gblocks server v0.91b
238   (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) and both conservative and
239   relaxed options were tested to determine the impacts on both alignments. The conservative
240   approach allowed for 299bp and 141bp in the SSU and ITS1 alignments, respectively. The
241   relaxed approach allowed for 396bp and 211bp in the SSU and ITS1 alignments, respectively.
242   The evolutionary models for all four alignments were determined using jModeltest v2.1.6
243   (Darriba et al. 2012) with three substitution schemes (for MrBayes) and starting with a BIONJ
244   base tree for likelihood calculations, then using AICc calculations. Phylogenetic trees were
245   generated in Geneious using the MrBayes v2.2.4 (Ronquist & Huelsenbeck 2003) plug-in. For
246   the two SSU alignments, the HKY85+G model was used with the default parameters for MCMC
247   settings and unconstrained branch lengths. For the two ITS1 alignments, the JC69 model with
248   equal rate variation was used with default parameters for MCMC settings and unconstrained
249   branch lengths.
250   We used the plyr package (Wickham & Wickham 2018) in the statistical program R (R
251   Core Team 2017) to combine ZOTUs by species, microhabitat, and location for both markers.
252   Due to variation and low sequence counts across samples, we decided to convert ZOTU tables to
253   binary for comparisons. Finally, correlations and regression analyses comparing *Labyrinthula*
254   and seagrass species across locations were conducted in Microsoft Excel.
255   **3. RESULTS**
256   **3.1 Primer Specificity**
257   Of the 220 seagrass shoots that were collected and screened, we obtained SSU sequences
258   from 57% of the seagrass shoots (n=126) and 100% of the water and sediment samples (n=24).
259   Of those, SSU sequences identified as Labyrinthulea were obtained from 48% of seagrass
260   samples (n=107) and 100% of water and sediment samples (n = 24), while sequences identified
261   as *Labyrinthula* sp. were obtained from 25% of seagrass samples (n=54), and 92% of water and
262   sediment samples (n=22). For the SSU dataset, 639,243 sequences remained after all quality
263   filtering. Of the resulting 369 ZOTUs generated, 56% could not be assigned to any taxonomic
264   group (Table 1), accounting for 64% of all the sequences generated. Of the 161 ZOTUs that were
265   assigned to a taxonomic group, 156 ZOTUs were assigned to Labyrinthulea, with 12 ZOTUs
266   identified as *Labyrinthula* spp. (27,723 sequences) and another 34 identified as *Aplanochytrium*

spp. (36,004 sequences). Thus, these primers appear to target members of the Labyrinthulea, but do not readily distinguish within the group (e.g. *Aplanochytrium* vs. *Labyrinthula* or putative pathogenic *Labyrinthula* spp.) and include many off-target amplifications.

Of the 24 water and sediment samples collected, ITS1 sequences were obtained from 75% (n=18), with 128,701 sequences remaining after quality filtering. Of the resulting 5 ZOTUs, two were identified as *Labyrinthula* spp., while the other three could not be assigned to a taxonomic group (Table 1). Of the two ZOTUs identified as *Labyrinthula* spp., these accounted for the majority of the sequence data generated, with ZOTU_1 accounting for 99.8% of all sequences. Thus, two ZOTUs identified as *Labyrinthula* spp. were amplified from the environmental samples with the ITS1 primers, while 12 ZOTUs identified as *Labyrinthula* spp. were amplified from the environmental samples with the SSU primers.

3.2 *Labyrinthula* spp. Diversity and Disease Measures

We conducted phylogenetic analyses to determine how many distinct *Labyrinthula* spp. were found in the IRL system and how they related to previously described *Labyrinthula* spp. from around the world. As the results from both the strict and relaxed GBlock settings were similar, only the phylogenetic trees generated from the strict alignments are shown. From the SSU dataset, the 12 ZOTUs identified as *Labyrinthula* spp. fell into eight distinct clades (Figure 1), likely representing eight different species of *Labyrinthula*. Only three of those clades were previously given species-level designations (Martin et al. 2016). Only ZOTU_109 grouped with *Labyrinthula* spp. previously identified as pathogenic (as defined in Martin et al. 2016), specifically grouping with sequences from *Labyrinthula zosterae*. All other ZOTUs grouped within clades containing *Labyrinthula* spp. previously described as non-pathogenic (Martin et al. 2016). From the ITS1 dataset, the two ZOTUs identified as *Labyrinthula* spp. fell into two distinct clades, likely representing two separate species of *Labyrinthula,* both of which were previously given species-level designations and described as pathogenic (Figure 2; Martin et al. 2016). Overall disease scores across species were very low, indicating little to no disease for most of the blades collected. For the 215 blades with disease scores (the remaining were too heavily fouled to assess), 87% (n = 188) had a score of zero (indicating no disease lesions present), 2% (n = 4) had a score of <1%, 9% (n = 20) had a score of 1% (indicating few, small lesions present), and only 1% (n = 3) had a score of >1% (Table S3). No lesions were observed on any *H. engelmanni* or *S. filiforme*. Lesions with scores >1% were only observed on *T. testudinum*.

3.3 Geographic Distribution and Microhabitat Associations

For the SSU dataset, ZOTU_10 and 22 were found most often across sites and on seagrass shoots (Figures 1 & 3). Five ZOTUs were found in all three microhabitats, while three were found a single time in a single microhabitat (Figure 1). Across samples sites, ZOTU_109 was only found in two locations, Big Island (in a water sample) and Sykes Creek (in a sediment sample) (Figure 3). The remaining SSU ZOTUs were recovered between 2-52 times with all but one (ZOTU_80) of them being recovered from multiple sites (Figure 3). There was higher total diversity across the IRL compared to the Banana River, with Harbor Branch and Big Island

307   having the most ZOTUs (Figure 3). For the ITS1 dataset, ZOTU_1 was present at four sites,
308   while ZOTU_3 was only present at two sites (Figure 4). ZOTU_3 was found in three samples,
309   including both sediment and water. ZOTU_1 was the most widespread, found in 18
310   environmental samples, including both water and sediment (Figure 2).
311   Parsing out occurrences only recovered from the seagrasses, turtlegrass (*T. testudinum*)
312   and shoalgrass (*H. wrightii*) had the most occurrences of *Labyrinthula* ZOTUs (Figure 5). There
313   was variation in ZOTU richness across seagrass species, with *H. wrightii* hosting the most
314   *Labyrinthula* ZOTUs (n = 6), followed by *T. testudinum* (n = 4). The greatest number of
315   occurrences was found on *T. testudinium* (46%, n= 37, Figure 5), which was expected as we
316   sampled the most shoots from that species. ZOTU_10 was the only ZOTU found on all five
317   species of seagrasses, and the only one found on *H. engelmanii* or *H. johnsonii* (Figure S1). After
318   grouping these ZOTUs into presumptive species, there appears to be little specificity across
319   seagrasses, with two putative species (ZOTU_50 and ZOTU_36) recovered from a single
320   seagrass species (Figure S1).
321   While the total number of seagrass species appears positively correlated with the total
322   number of *Labyrinthula* ZOTUs detected per sampling location (Figure 6), our regression
323   analysis showed that number of seagrass species sampled at a site explained 57% of the variation
324   in *Labyrinthula* ZOTUs across sites, but was not significant (F=5.38, p=0.08), which is likely
325   due to low statistical power from our six sampling sites. We also tested for a correlation between
326   the number of ZOTU occurrences (i.e., the number of times a ZOTU was detected on a single
327   blade) and the total number of seagrass species in a single sampling location (Figure S2). Upon
328   correcting for the number of blades sampled per species, the correlation between these factors is
329   positive, but weak, indicating there are likely additional factors influencing this relationship.
330   **4. DISCUSSION**
331   Our results demonstrate the utility of a targeted metabarcode approach for assessing
332   *Labyrinthula* spp. and their microhabitat associations in coastal ecosystems. We identified 12
333   *Labyrinthula* ZOTUs from three microhabitats, and a weak, positive correlation between
334   *Labyrinthula* ZOTUs and seagrass species diversity. With this targeted approach, we
335   successfully amplified a variety of *Labyrinthula* genotypes (i.e., ZOTUs) and species (identified
336   in phylogenetic analyses) without the need for tedious culturing or expensive cloning to identify
337   different isolates co-habitating in a single sample, allowing for the examination of intra- and
338   inter-specific variation of these parasites across microhabitats and seagrass species.
339   **4.1 Targeted Metabarcoding for *Labyrinthula* spp. Detection**
340   The use of DNA-based tools for assessing biological diversity in the environment has
341   been rapidly increasing, including for elucidating parasite diversity (Bass et al. 2015).
342   Researchers have used HTS approaches to study a range of aquatic parasites including X-cells
343   (Freeman et al. 2017), myxozoans (Hartikainen et al. 2016), and paramyxids (Ward et al. 2016).
344   These attempts often demonstrate that there is high parasite diversity within environmental
345   samples, implicating undiscovered host-parasite relationships (Bass et al. 2015). In this study, we
346   used an amplicon-based HTS approach to examine the diversity of *Labyrinthula* spp. within two

347   coastal lagoons. Previous culture-independent assessments of the diversity of
348   Labyrinthulomycetes utilized primers that amplified more than one genus (Collado-Mercado et
349   al. 2010) or amplified fragments that were not sufficient in length (202bp; Bergmann et al. 2011)
350   for taxonomic assignment (current HTS platforms from Illumina can sequence 400-500bp
351   amplicons using dual-indexing). Additionally, as Martin et al. (2016) was the first paper to
352   attempt species-level identification, primer sets developed prior to this had significantly less
353   information on the relatedness of *Labyrinthula* spp. The primer sets that we designed using the
354   sequence data from Martin et al. (2016) appear more specific than "universal primers", with clear
355   variation across the two markers. The SSU primer set amplified other genera in the
356   Labyrinthulea and a number of additional off-target organisms (Table 1). While these primers
357   were not as specific as intended, the large number of sequences generated through this method
358   means that that even after non-target sequences are removed, many sequences remain for
359   analyses.
360   The ITS1 primer set that we designed appeared more specific, particularly amplifying
361   primarily putative seagrass-pathogenic species (sensu Martin et al. 2016) of *Labyrinthula* spp.
362   (Figure 2). When comparing the specificity of these primer sets across the environmental
363   samples, the SSU primer set recovered more *Labyrinthula* ZOTUs (n=12), as well as more non-
364   target amplification and more putatively non-pathogenic species. The ITS1 primer set recovered
365   less total *Labyrinthula* ZOTUs (n=2), but only recovered putatively pathogenic species of
366   *Labyrinthula*. Thus, the ITS1 primers appear promising as a targeted primer set for studying
367   pathogenic *Labyrinthula* spp. on seagrasses, while the SSU primers appear promising for broader
368   surveys of total *Labyrinthula* spp. diversity (including non-pathogenic species). Overall,
369   targeted metabarcoding is a viable approach for studying the diversity and biogeography of
370   putatively pathogenic and non-pathogenic *Labyrinthula* spp. and the mechanisms that influence
371   them.
372   **4.2 Parasite Diversity and Specificity**
373   Our study joins a few others at examining the genetic diversity of *Labyrinthula* spp.
374   associated with different seagrass species (Bockelmann et al. 2012, Martin et al. 2016, Sullivan
375   et al. 2017, Trevathan-Tackett et al. 2018). These have occurred at large spatial scales, showing
376   that *Labyrinthula* spp. can have broad geographic distributions including across vast oceans
377   (Martin et al. 2016, Trevathan-Tackett et al. 2018). In our study across a much smaller
378   geographic region, we found some of the same presumptive species (three in the SSU dataset,
379   two in the ITS1 dataset) as well as a number of novel presumptive species of *Labyrinthula* (four
380   in the SSU dataset)*,* though none were presumptive seagrass-pathogenic types. Although only
381   environmental samples were analyzed with the ITS1 primers, the species with the greatest
382   occurrence was the wide-spread *Labyrinthula* sp. D, which had been documented previously on
383   various tropical Atlantic and Indo-Pacific seagrasses, including *S. filiforme* and *H. wrightii*
384   (Martin et al. 2016). *Labyrinthula* sp. E, previously reported only from *T. testudinum* and *H.*
385   *wrightii* (Martin et al. 2016), was also found in the environment, but less frequently. For the SSU
386   gene, also consistent with previous studies, we primarily found *Labyrinthula* species that were

associated with clades previously designated as non-pathogenic (Figure 1 & 2), though on living seagrass shoots. The two most dominant of these, by sample occurrence and geographic distribution, were ZOTU_10 (n = 52) and ZOTU_22 (n = 37). The former is closely associated with *Labyrinthula* sp. X (Martin et al. 2016), which was reported from a "seagrass surface", while ZOTU_22 is closely associated with a previous species from "seaweed surface" samples. In addition, there were two ZOTUs (ZOTU_249 and ZOTU_50) not associated with any previously identified species of *Labyrinthula*. ZOTU_249 was found in the water and associated with *H. wrightii* (n = 1 occurrences) and *S. filiforme* (n=1 occurrence). ZOTU_50 was found in water, sediment, and associated with only *H. wrightii* (n=7 occurrences).

Labyrinthulids are assumed to be primarily saprobic (Raghukumar 2002); however, whether those that cause disease are obligate or facultative parasites is a subject of on-going research (Burge et al. 2013, Martin et al. 2016). Our results show that the majority of *Labyrinthula* ZOTUs and species associated with seagrasses are presumably non-pathogenic. Our data (i.e., ITS1 and SSU ZOTUs from environmental samples) also could indicate that *Labyrinthula* spp. in the seagrass-pathogenic clade (e.g. ZOTU_109) are capable of living in the environment without a host. This finding leads to questions about the transmission of SWD, which may be through multiple mechanisms including contact with infected leaves or parasite-laden detritus (Moore & Short 2006) or through water-borne dispersal of the parasite. A waterborne transmission route is consistent with the widespread geographic ranges of some *Labyrinthula* spp. (Martin et al 2016, Trevathan-Tackett et al. 2018), as this would most likely occur through natural means (e.g. ocean currents, rafting on seagrass) and anthropogenic vectors, such as ballast water (Galil & Hülsman 1997, Pagenkopp Lohan et al. 2016, 2017). Additional research is required to assess whether the *Labyrinthula* spp. that cause pathology can also survive as saprobes, to determine if all *Labyrinthula* spp. have any impact on living seagrasses, even if overt signs of wasting disease do not appear, and the importance and contribution of different transmission pathways to the ecology of SWD.

4.3 Parasite Microhabitat and Geographic Distributions

Labyrinthula species occur globally, with infections reported from nine seagrass species (Sullivan et al. 2017) as well as occurrences on macroalgae and detritus (Martin et al. 2016). Our results match those of previous studies, showing that *Labyrinthula* species and ZOTUs can be found across microhabitats within and across sampling sites. In the ITS1 dataset, the two ZOTUs both occurred in water and sediment, with ZOTU_1 occurring at four sites and ZOTU_3 occurring at two adjacent sites (Figure 4) within the same geographical range of ZOTU_1. These findings correlate with previous results on a larger scale, where *Labyrinthula* sp. D (ZOTU_1) was previously found in both northern and southern hemispheres, indicating a wide geographic range (Martin et al. 2016), while *Labyrinthula* sp. E (ZOTU_3) apparently has a narrower geographic range (i.e., only occurring on *T. testudinum* and *H. wrightii* in a single bioregion, Martin et al. 2016). In the 18S dataset, five ZOTUs (i.e., ZOTU_10, ZOTU_22, ZOTU_36, ZOTU_329, ZOTU_50), corresponding to four presumptive species (i.e., *Labyrinthula* sp. X and three unnamed species), were recovered from all three microhabitats examined (Figure 1). Not

427 surprisingly, those ZOTUs that occurred over the most microhabitats also occurred in the most
428 sampling sites and had the greatest number of occurrences across the dataset (Figure 3). Thus,
429 while there appears to be variation in geographic and microhabitat range across ZOTUs within
430 this system, additional research is required to tease apart the myriad of biotic or abiotic factors
431 that could differentially impact strains of *Labyrinthula* spp. to cause these patterns.

4.4 Seagrass Diversity and Wasting Disease

433 The notion that host diversity begets parasite diversity is generally acknowledged (Poulin
434 & Morand 2004). How the diversity of the community impacts disease systems is less well
435 known, particularly in marine systems (Civitello et al. 2015), though work with terrestrial plants
436 has shown that host diversity and composition are both important factors in disease systems
437 (Mitchell & Power 2006). Extrapolating this to seagrasses seems logical, particularly given
438 previous work demonstrating that seagrass species generate different compounds for thwarting
439 infection from *Labyrinthula* spp. (Ross et al. 2008, Trevathan-Tacket et al. 2015). In fact, the
440 most virulent strain of *Labyrinthula* in Trevathan-Tackett et al. (2018)*,* as indicated through
441 pathogenicity testing on *Zostera muelleri* and *Heterozostera nigricaulis,* came from an individual
442 (*Halophila australis*) with no signs of wasting disease.

443 Our results demonstrated a weak, positive correlation between the number of seagrass
444 species and *Labyrinthula* ZOTUs within sampling locations (Figure 6). Thus, it is possible that
445 diversity can beget diversity even at the ZOTU level regardless of *Labyrinthula* spp.
446 pathogenicity, but the inclusion of additional sampling sites is required to increase the statistical
447 power of the test and confirm this finding. Our results also indicate, as expected, that increasing
448 the number of shoots examined also increases the *Labyrinthula* ZOTU diversity recovered
449 (Figure S2). Given the extensive evidence that host diversity begets parasite diversity (e.g.,
450 Kamiya et al. 2014, Poulin and Morand 2014), we expected a higher diversity of pathogenic
451 species due to the higher number of host species sampled. However, it is possible that other
452 factors limit the diversity of pathogenic *Labyrinthula* species in this system (e.g., Duffin 2018),
453 such as the intra-specific diversity of the seagrasses. *Thalassia testudinum* was shown to have
454 extremely low intra-specific diversity within the IRL, with a single clone dominating most of the
455 area (Bricker et al. 2018). Unfortunately, the intra-specific diversity of the other seagrass species
456 is not known. It is possible that the diversity of pathogenic *Labyrinthula* spp. is linked to the
457 genetic diversity of the host species or that there is haplotype-level variation in seagrass response
458 to *Labyrinthula* spp. infection, both of which could cause a decrease in the diversity of
459 pathogenic *Labyrinthula* spp.

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608 Table 1. The taxonomic classifications of the ZOTUs identified in this study. For both the SSU
609 and ITS1 primer sets, the number of ZOTUs per taxonomic classification is listed along with the
610 total number of ZOTUs recovered after quality filtering.

Taxonomic Classification			Number of ZOTUs	
			SSU	ITS1
Unclassified			208	3
Alveolata				
	Dinophyta			
		Syndiniales	1	
Stramenopiles			4	
	Labyrinthulea		55	
	Labyrinthulales			
	Labyrinthulaceae		55	
		Aplanochytrium	34	
		Labyrinthula	12	2
		Total	**369**	**5**

611

612 Figure 1. The phylogenetic tree constructed using SSU ZOTUs identified as *Labyrinthula* spp.
613 from this study (in bold) and related sequences from GenBank. The shown topology was
614 generated using the conservative options from Gblocks and the HKY85+G model for the
615 Bayesian analysis. Posterior probabilities are shown. Species level designations are shown with
616 bars, for multiple haplotypes, or circles, for single haplotypes, and letters associated with
617 previously defined species-level designations (Martin et al. 2016) are shown where applicable.
618 For each ZOTU, the number of occurrences within a habitat (seagrass, water, or sediment) is
619 shown in pie charts.

AB022103 *Aplanochytrium kerguelense*
CL603053 UTI3 *Labyrinthula* KDC2004
GQ499189 *Labyrinthula terrestris*
AF265330 *Labyrinthula* sp.
GQ499191 *Labyrinthula* sp. (Laby31)
AF348522 *Labyrinthula* sp.
GQ499190 *Labyrinthula* sp. (Laby 32)
MF872140 *Labyrinthula* sp. Ln11
MF872144 *Labyrinthula* sp. Ln23
MF872147 *Labyrinthula* sp. Ln9
MF872141 *Labyrinthula* sp. Ln12
MF872142 *Labyrinthula* sp. Ln15
AB095092 *Labyrinthula* sp. L59
KU559379 *Labyrinthula* sp. 215b
AF265332 *Labyrinthula* sp.
FR875355 *Labyrinthula* sp. ACB2011aclone_San25
AB220158 *Labyrinthula* sp. L72
ZOTU_138
ZOTU_22
AB290456 *Labyrinthula* sp. L962
AB290455 *Labyrinthula* sp. L951
ZOTU_352
ZOTU_357
ZOTU_329
ZOTU_249 ●

AF265334 *Labyrinthula zosterae* MBL93_2
AF265335 *Labyrinthula zosterae* type 154
ZOTU_109
KU559372 *Labyrinthula zosterae* 316b
JN121404 *Labrinthula zosterae*
FR875335 *Labyrinthula zosterae*
KU559376 *Labyrinthula zosterae* 4b
KU559377 *Labyrinthula zosterae* 95w1
JN121405 *Labyrinthula zosterae*
KU559374 *Labyrinthula* sp. 18b
KU559375 *Labyrinthula* sp. 302b9
KU559371 *Labyrinthula* sp. 8b
KU559373 *Labyrinthula* sp. 178b3

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D/E

ZOTU_50 ●
AB290454 *Labyrinthula* sp. L93_3
ZOTU_10
EU431329 *Labyrinthula* sp. LTH
GQ499192 *Labyrinthula* sp. (Laby13)
AB022105 *Labyrinthula* sp. (Nakagiri)
ZOTU_72
AB290457 *Labyrinthula* sp. (Jy_1b)
FR875360 *Labyrinthula* sp. ACB2011clone SVA05a
MF872149 *Labyrinthula* sp. Ln41
MF872145 *Labyrinthula* sp. Ln5
ZOTU_36
ZOTU_80
MF872146 *Labyrinthula* sp. Ln8
MF872138 *Labyrinthula* sp. L7
AB246794 *Labyrinthula* sp. N8
MF872136 *Labyrinthula* sp. L11
MF872135 *Labyrinthula* sp. L1
MF872137 *Labyrinthula* sp. L4
MF872143 *Labyrinthula* sp. Ln20
MF872135 *Labyrinthula* sp. Ln1
MF872148 *Labyrinthula* sp. Ln2
AB290458 *Labyrinthula* sp. 00Bat03
KU559378 *Labyrinthula* sp. 158b1

X
V

100
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● Seagrasses
● Water
● Sediment

ZOTU_138 n=3
ZOTU_22 n=37
ZOTU_352 n=2
ZOTU_357 n=4
ZOTU_329 n=6
ZOTU_249 n=3
ZOTU_109 n=2
ZOTU_50 n=9
ZOTU_10 n=52
ZOTU_72 n=2
ZOTU_36 n=12
ZOTU_80 n=2

620
621

622   Figure 2. The phylogenetic tree constructed using ITS1 ZOTUs identified as *Labyrinthula* spp.
623   from this study (in bold) and related sequences from GenBank. The shown topology was
624   generated using the conservative options from Gblocks and the JC69 model with equal rate
625   variation for the Bayesian analysis. Posterior probabilities are shown. Species level designations
626   are shown with bars, for multiple haplotypes, or circles, for single haplotypes, and letters
627   associated with previously defined species-level designations (Martin et al. 2016) are shown
628   where applicable. For each ZOTU, the number of occurrences within a habitat (water, or
629   sediment) is shown in pie charts.

JN121411 *Labyrinthula zosterae*
JN121413 *L. zosterae*
JN121412 *L. zosterae*
KU559384 *L. zosterae* 113b
KU559405 *L. zosterae* 34b
KU559397 *L. zosterae* 144bb
KU559412 *L. zosterae* 50b
KU559396 *L. zosterae* 144ba
KU559391 *L. zosterae* 133b
KU559390 *L. zosterae* 131b2
KU559408 *L. zosterae* 3b
KU559386 *L. zosterae* 119b
KU559415 *L. zosterae* 58b
KU559392 *L. zosterae* 134b
KU559389 *L. zosterae* 131b1
KU599398 *L. zosterae* 205w
KU559401 *L. zosterae* 246b
KU559402 *L. zosterae* 256b
KU559406 *L. zosterae* 36b
KU559395 *L. zosterae* 142b
KU559394 *L. zosterae* 138bm
KU559413 *L. zosterae* 53b
KU559409 *L. zosterae* 46b
KU559393 *L. zosterae* 135b
KU559385 *L. zosterae* 114b
KU559383 L. zosterae 107b
JN121409 *L. zosterae* ZM-VA-FL-1a &12b
KU559416 *L. zosterae* 90b
JN121410 *L. zosterae* ZM-WA_SUI-SBb &1b
KU559407 *L. zosterae* 37b
KU559400 *L. zosterae* 209b
KU559404 *L. zosterae* 2b
KU559403 *L. zosterae* 261b
KU559399 *L. zosterae* 206w
KU559411 *L. zosterae* 4b
KU559418 *L. zosterae* 95w1
KU559414 *L. zosterae* 54b
KU559417 *L. zosterae* 91b
KU559387 *L. zosterae* 120b
KU559382 *L. zosterae* 106b
KU559381 *L. zosterae* 105b
KU559380 *L. zosterae* 104b
KU559410 *L. zosterae* 49b
KU559388 *L. zosterae* 130b
KU559419 *L. zosterae* 140b
KU559420 *L. zosterae* 155b
A
92

KU559422 *Labyrinthula* sp. 23b
KU559423 *Labyrinthula* sp. 24b
KU559425 *Labyrinthula* sp. 297b
KU559421 *Labyrinthula* sp. 10b
KU559424 *Labyrinthula* sp. 26b
KU559426 *Labyrinthula* sp. 302b9
100
KU559430 *Labyrinthula* sp. 18b
KU559431 *Labyrinthula* sp. 287b
KU559429 *Labyrinthula* sp. 17b
KU559428 *Labyrinthula* sp. 16b
KU559427 *Labyrinthula* sp. 110b
KU559432 *Labyrinthula* sp. 229b
100
99

KU559451 *Labyrinthula* sp. 160b1
KU559457 *Labyrinthula* sp. 8b
KU559455 *Labyrinthula* sp. 324w2
KU559456 *Labyrinthula* sp. 76b
KU559454 *Labyrinthula* sp. 324w1
ZOTU_3
KU559452 *Labyrinthula* sp. 268b
KU559453 *Labyrinthula* sp. 296b
KU559458 *Labyrinthula* sp. 316w2
E **100** **84** **99**

KU559433 *Labyrinthula* sp. 158b3
ZOTU_1
KU559439 *Labyrinthula* sp. 38b
KU559444 *Labyrinthula* sp. 83b
KU559441 *Labyrinthula* sp. 74b
KU559442 *Labyrinthula* sp. 78b
KU559435 *Labyrinthula* sp. 179b
KU559438 *Labyrinthula* sp. 291b
KU559437 *Labyrinthula* sp. 180b3
KU559443 *Labyrinthula* sp. 79b
KU559436 *Labyrinthula* sp. 179b2
KU559440 *Labyrinthula* sp. 73b
KU559434 *Labyrinthula* sp. 177b1
D **100** **100**

KU559447 *Labyrinthula* sp. 177b2
KU559449 *Labyrinthula* sp. 178b1
KU559446 *Labyrinthula* sp. 176bb1
KU559448 *Labyrinthula* sp. 177b3
KU559450 *Labyrinthula* sp. 178b3
KU559445-- *Labyrinthula* sp. 175b
94

KU559454 *Labyrinthula* sp. 324w3
KU559476 *Labyrinthula* sp. 68b
KU559474 *Labyrinthula* sp. 290b
KU559473 *Labyrinthula* sp. 229b
KU559478 *Labyrinthula* sp. 276b
100 **100** **87**

100

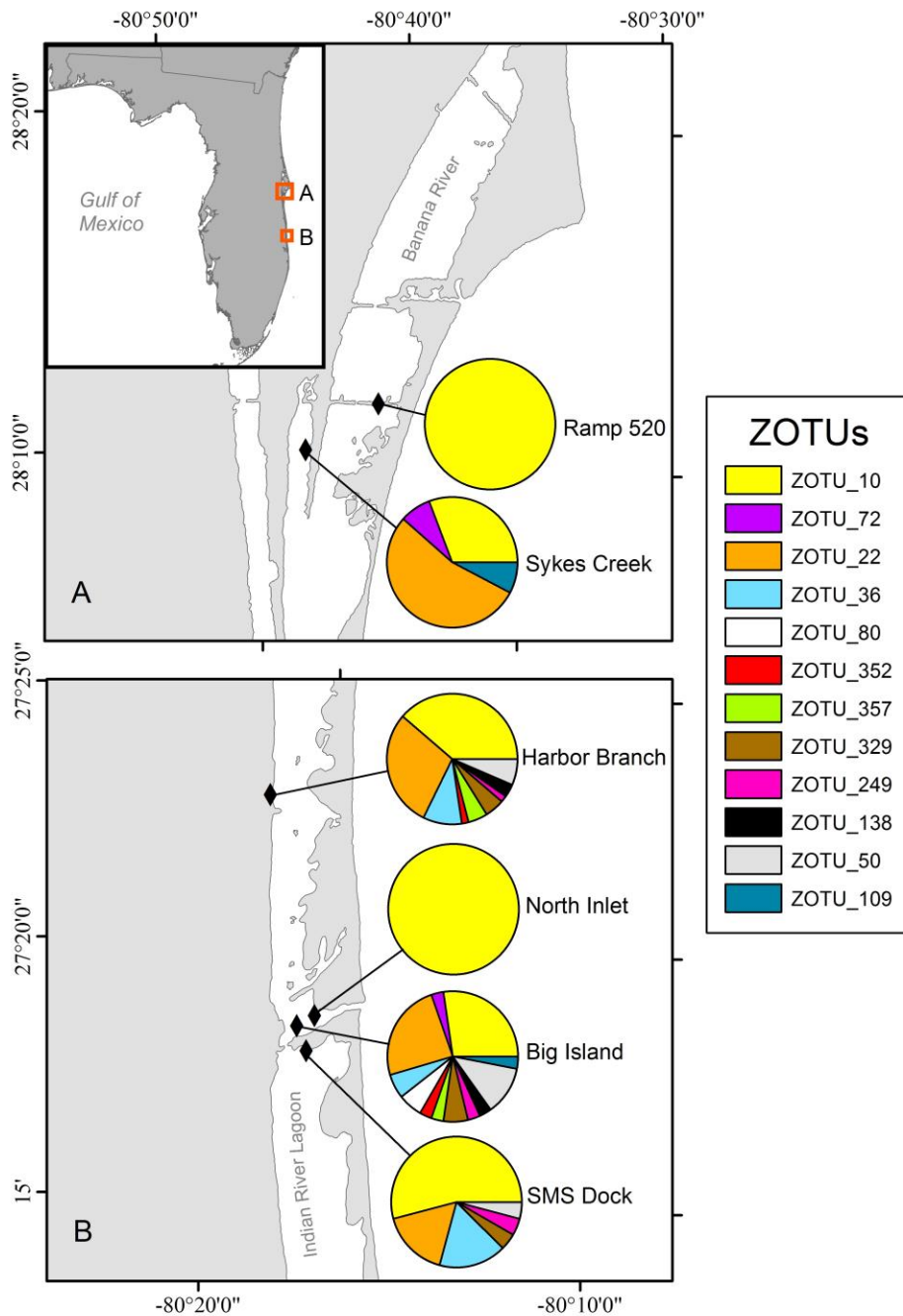● Sediment
● Water

ZOTU_3 n=3

ZOTU_1 n=18

0.06

630
631 Figure 3. Map showing the sampling locations in the Banana River (A) and Indian River Lagoon
632 (B). The number of occurrences for ZOTUs identified as *Labyrinthula* spp. using the SSU primer

633   set is also shown.

634
635   Figure 4. Map showing the sampling locations in the Banana River (A) and Indian River Lagoon
636   (B). The number of occurrences for ZOTUs identified as *Labyrinthula* spp. using the ITS1
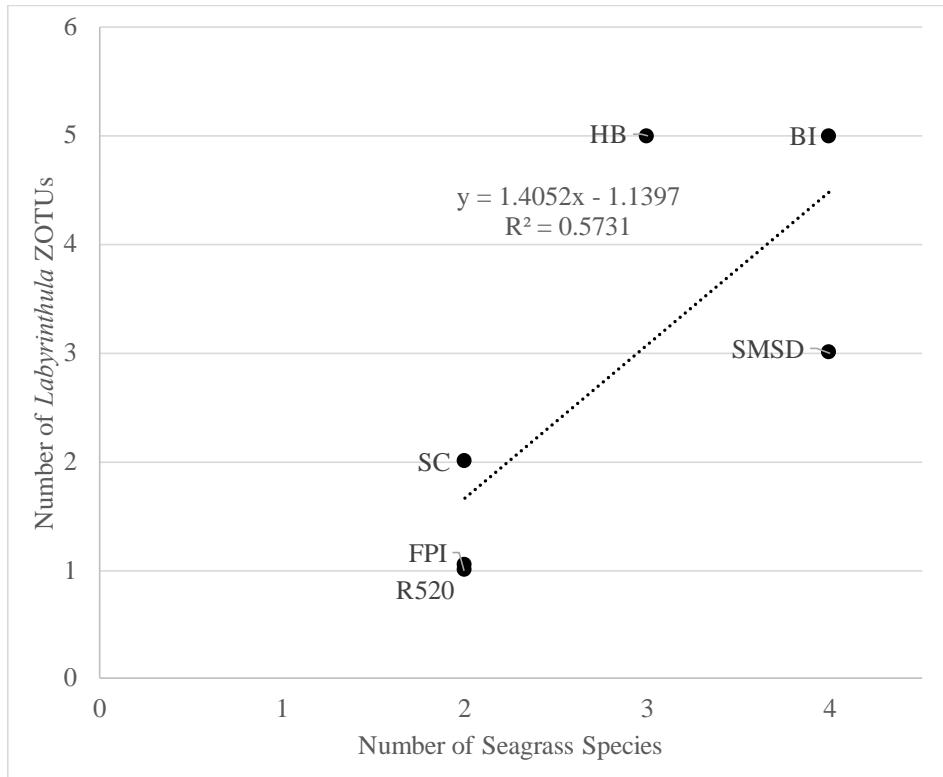637   primer set is also shown.

638
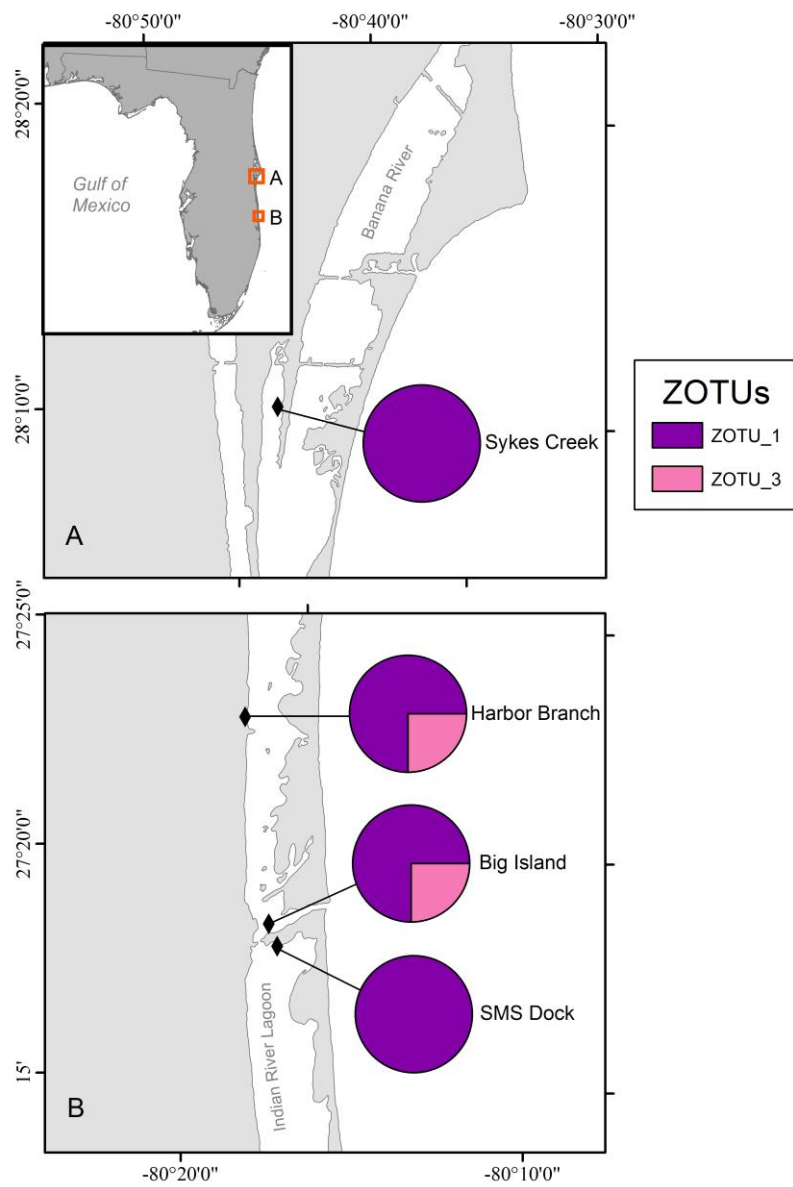
Figure 5. The number of occurrences of *Labyrinthula* ZOTUs identified using the SSU primer set that were recovered from shoots of the five different seagrasses sampled in this study. Numbers above the bars indicate the total number of blades collected for each species.
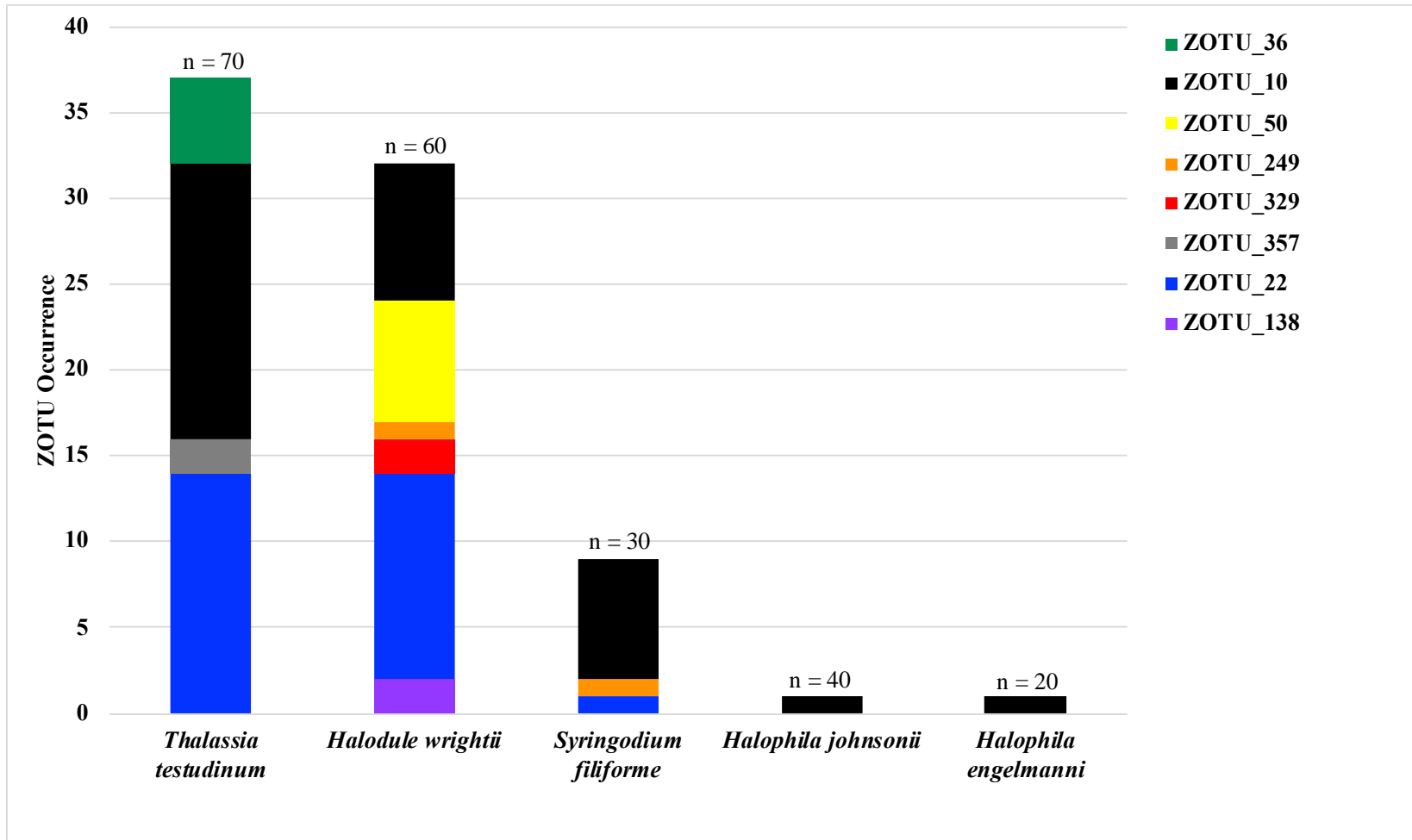
643 Figure 6. The number of *Labyrinthula* ZOTUs vs. the number of seagrass species occurring at the locations sampled. Note that there
644 were two sites with a single ZOTU and two seagrass species, so one of these values was manipulated so it would appear on the graph
645 Labels on points indicate sampling locations: Harbor Branch (HB), Big Island (BI), SMS Dock (SMSD), Sykes Creek (SC), Fort
646 Pierce Inlet-North (FPI), and Ramp @520 (R520)..

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