

1 **Title:** Characterization of the mycobiome of the seagrass, *Zostera marina*, reveals putative
2 associations with marine chytrids

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41 **Abstract**

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Seagrasses are globally distributed marine flowering plants that are foundation species in coastal ecosystems. Seagrass beds play essential roles as habitats and hatcheries, in nutrient cycling and in protecting the coastline from erosion. Although many studies have focused on seagrass ecology, only a limited number have investigated their associated fungi. In terrestrial systems, fungi can have beneficial and detrimental effects on plant fitness. However, not much is known about marine fungi and even less is known about seagrass associated fungi. Here we used culture-independent sequencing of the ribosomal internal transcribed spacer (ITS) region to characterize the taxonomic diversity of fungi associated with the seagrass, *Zostera marina*. We sampled from two *Z. marina* beds in Bodega Bay over three time points to investigate fungal diversity within and between plants. Our results indicate that there are many fungal taxa for which a taxonomic assignment cannot be made living on and inside *Z. marina* leaves, roots and rhizomes and that these plant tissues harbor distinct fungal communities. The most prevalent ITS amplicon sequence variant (ASV) associated with *Z. marina* leaves was classified as fungal, but could not initially be assigned to a fungal phylum. We then used PCR with a primer targeting unique regions of the ITS2 region of this ASV and an existing primer for the fungal 28S rRNA gene to amplify part of the 28S rRNA gene region and link it to this ASV. Sequencing and phylogenetic analysis of the resulting partial 28S rRNA gene revealed that the organism that this ASV comes from is a member of Novel Clade SW-I in the order Lobulomycetales in the phylum Chytridiomycota. This clade includes known parasites of freshwater diatoms and algae and it is possible this chytrid is directly infecting *Z. marina* leaf tissues. This work highlights a need for further studies focusing on marine fungi and the potential importance of these understudied communities to the larger seagrass ecosystem.

81 Introduction

82
83 Seagrasses are fully submerged marine flowering plants (angiosperms) that play essential roles in
84 marine ecosystems as foundation species. Although angiosperms are the most diverse terrestrial
85 plant group with over 250,000 species, there are only around 70 species of seagrasses. There are
86 three main lineages of seagrass in the order Alismatales that separately moved into and adapted
87 to the marine ecosystem through convergent evolution between 70 and 100 million years ago
88 (Les et al., 1997; Wissler et al., 2011). Seagrasses are important keystone species in most coastal
89 environments around the world with ecosystem services comparable to those of tropical
90 rainforests (Costanza et al., 1997). However, seagrass beds are increasingly impacted by climate
91 change, pollution and habitat fragmentation and restoration is expensive and has a low success
92 rate (Orth et al., 2006). Although many studies have focused on the ecological importance of
93 seagrasses, relatively little is known about the fungi associated with these species.

94
95 Fungi are known to affect land plant fitness both in detrimental ways (e.g., as pathogens) and
96 beneficial ways (e.g., mycorrhizae are involved in facilitating phosphorus and nitrogen uptake
97 for their plant hosts (Bonfante and Anca, 2009)). It is estimated that 82-85% of angiosperm
98 species have mycorrhizal fungal associations (Radhika and Rodrigues, 2007; Wang and Qiu,
99 2006). Mycorrhizae were previously thought to not colonize aquatic environments, but have
100 since been found in wetlands, estuaries, mangrove forests and freshwater ecosystems (Beck-
101 Nielsen and Madsen, 2001; Bohrer et al., 2004; Kohout et al., 2012; Radhika and Rodrigues,
102 2007; Šraj-Kržič et al., 2006). Mycorrhizal associations are believed to be at least 400 million
103 years old, critical for plant terrestrialization (Humphreys et al., 2010; Redecker et al., 2000;
104 Wang et al., 2010) and the ancestral state for angiosperms (Brundrett, 2002). Despite their
105 importance to land plants, seagrasses are believed to not form mycorrhizal associations (Nielsen
106 et al., 1999), even though freshwater relatives of seagrasses have been observed to sporadically
107 form mycorrhizal associations (Beck-Nielsen and Madsen, 2001; Brundrett, 2009; Clayton and
108 Bagyaraj, 1984; Khan and Belik, 1995; Radhika and Rodrigues, 2007; Šraj-Kržič et al., 2006;
109 Wang and Qiu, 2006; Wigand and Court Stevenson, 1994).

110
111 In contrast to their apparent lack of mycorrhizal associations, some studies of seagrasses have
112 observed associations with novel fungal endophytes similar to dark septate endophytes (DSE)
113 common in land plants (Borovec and Vohník, 2018; Torta et al., 2015; Vohník et al., 2015, 2016,
114 2017, 2019). DSE are a morphology based type and not a phylogenetic group, and are largely
115 uncharacterized. In some cases, DSEs have been shown to transfer nitrogen and receive carbon
116 from plants as well as increase overall plant nutrient content and growth (Porrás-Alfaro and
117 Bayman, 2011; Usuki and Narisawa, 2007). DSEs are not the only fungi that have been observed
118 to form associations with seagrasses. Additional culture based studies have found fungi
119 associated with the leaves, roots and rhizomes of different seagrasses; however, many of these
120 studies often conflict on the prevalence and taxonomic identities of the fungi observed and suffer

121 from the limitations of culture-based methods (Cuomo et al., 1985; Devarajan and
122 Suryanarayanan, 2002; Gnavi et al., 2014; Kirichuk and Pivkin, 2015; Kuo, 1984; Ling et al.,
123 2015; Mata and Cebrián, 2013; Newell, 1981; Panno et al., 2013; Phongpaichit and Supaphon,
124 2014; Sakayaroj et al., 2010; Shoemaker and Wyllie-Echeverria, 2013; Supaphon et al., 2013,
125 2017; Torta et al., 2015; Venkatachalam, 2015; Venkatachalam et al., 2015; Vohník et al., 2016).
126 This is compounded by the fact that marine fungi are understudied and that marine fungal culture
127 collections are limited with only around ~1100 cultured isolates, despite estimates that there are
128 10,000 or more marine fungal species (Amend et al., 2019; Jones et al., 2015; Jones and Gareth
129 Jones, 2011). Here, we use culture-independent methods to characterize the taxonomic diversity
130 of fungi associated with the seagrass, *Z. marina*, in order to build a framework from which we
131 can begin to determine the evolutionary, ecological, and functional importance of these
132 associations.

133

134 **Methods**

135 *Sample collection*

136

137 Roots, leaves and rhizome tissues from individual *Z. marina* plants and adjacent sediment were
138 collected by coring into the sediment using a 2.375 inch diameter modified PVC pipe
139 (McMaster-Carr, Elmhurst, IL, USA). PVC pipes were modified such that one end of the pipe
140 was cut at an angle to make insertion into the sediment easier. Samples were collected from two
141 sites in Bodega Bay, CA, Gaffney Point (GPS: 38°18'45.05"N, 123° 3'16.66"W) and Westside
142 Point (GPS: 38°19'10.67"N, 123° 3'13.71"W), at three timepoints two weeks apart spanning
143 July-August 2016. Samples from GP were collected only at the first timepoint. Samples were
144 dissected using sterile scissors into different bulk sample types (e.g. roots, leaves, rhizome
145 tissues) in the field, placed into 1.5 mL centrifuge tubes which were immediately placed on dry
146 ice for transport and then kept at -80 °C until analyzed. Sediment was collected from the 2.375
147 inch PVC pipe using an 11 mm diameter straw which was placed directly next to the individual
148 seagrass shoot tissue in order to assure sediment was collected as close to the plant as possible.
149 For the third timepoint (T3), multiple samples were collected from the same plant. Leaf tissue
150 was cut into ~5 inch long segments moving along the length of the leaf tissue, individual roots
151 were collected and an additional 7 mm diameter straw core was obtained and then dissected into
152 ~1.5 cm sections of sediment. Cores of unvegetated sediment from between sites were obtained
153 as a biological control at each time point. For sample sizes, refer to Tables S1-3.

154

155 *Molecular methods*

156

157 DNA was extracted from samples with the PowerSoil DNA Isolation kit (MO BIO Laboratories,
158 Inc., Carlsbad, CA, USA) with minor changes to the manufacturer's protocol as follows. To
159 improve fungal lysis, samples were heated at 70 °C for 10 minutes between steps 4 and 5 as

160 suggested by the manufacturer. For step 5, samples were bead beaten on the homogenize setting
161 for 1 minute using a BioSpec Products mini-bead beater. Samples were placed into randomized
162 blocks prior to extraction using a random number generator.

163
164 In order to obtain loosely associated, or epiphytic, fungi, tissue samples were washed in 1 mL of
165 a 1:50 dilution of Redford buffer solution (1 M Tris-HCL, 0.5 M NaEDTA, 1.2% Triton-X)
166 (Kadivar and Stapleton, 2003; Kembel et al., 2014). Samples were left in this solution for 5
167 minutes during which they were periodically vortexed. Tissues were then removed and placed
168 into a new microcentrifuge tube for use in endophyte analyses. The epiphyte wash solution was
169 centrifuged at 4000 g for 20 minutes and the pellets were resuspended in MoBio PowerSoil C1
170 solution, the first step in the DNA extraction process.

171
172 After being washed to obtain epiphytes, a subset of samples collected at the third sampling
173 timepoint were further surface cleaned with the goal of removing any leftover loosely associated
174 fungi. This was done in an effort to obtain samples containing only endophytes, which we define
175 here as being tightly associated to the outside or inside the tissue. Tissues were rinsed with
176 autoclaved nanopure water (30 seconds), immersed in 95% ethanol (5 seconds), immersed in
177 0.5% NaOCl (~10% bleach) (2 minutes), immersed in 70% ethanol (2 minutes), then rinsed with
178 autoclaved nanopure water (1 minute) three times. Tissues were then placed into MoBio
179 PowerSoil C1 solution and crushed using flame sterilized tweezers.

180 181 *Sequence generation*

182
183 Using a random number generator, samples were randomly assigned places in 96-well plates and
184 sent to Zymo Research, Inc for sequencing via their ZymoBIOMICS Service. We briefly
185 summarize their protocol here. Fungal ITS2 amplicon sequencing was performed using the
186 Quick NGS Library Preparation Kit (Zymo Research, Irvine, CA). The ITS2 region was
187 amplified via PCR using the ITS5.8S_Fun and ITS4_Fun (McHugh and Schwartz, 2015; Taylor
188 et al., 2016) primers which were chosen to try to minimize the amount of host plant
189 amplification. Final PCR products were quantified with qPCR fluorescence readings and pooled
190 together based on equal molarity. The final pooled library was cleaned up with Select-a-Size
191 DNA Clean & Concentrator™ (Zymo Research, Irvine, CA), then quantified with TapeStation
192 (Agilent, Santa Clara, CA) and Qubit (Invitrogen, Carlsbad, CA). The final library was
193 sequenced on Illumina MiSeq (Illumina, Inc., San Diego, CA, USA) with a v3 reagent kit (600
194 cycles) to generate 300 bp paired-end reads. The sequencing was performed with >10% PhiX
195 spike-in. Sequence reads were demultiplexed by Zymo Research, Inc using the MiSeq Reporter.
196 The raw sequence reads generated for this ITS amplicon project were deposited at GenBank
197 under accession no. [PRJNA515720](https://www.ncbi.nlm.nih.gov/nuclink/PRJNA515720).

198 199 *Sequence processing*

200
201 Primers were removed using *cutadapt* (v. 1.14) (Martin, 2011). Forward and reverse reads were
202 then merged with *PEAR* (v. 0.9.5) (Zhang et al., 2014) using the script *run_pear.pl* (Comeau et
203 al., 2017) which implements *PEAR* in parallel.

204
205 The resulting merged fastq files were analyzed in R (v. 3.5.1) using *dada2* (v. 1.10.0), *decontam*
206 (v. 1.1.2), *phyloseq* (v. 1.26.0), *vegan* (v. 2.5.5), *FSA* (v. 0.8.25) and *ggplot2* (v. 3.1.0) (Callahan
207 et al., 2016; Davis et al., 2018; Dixon, 2003; McMurdie and Holmes, 2013; Ogle, 2016; R Core
208 Team, 2016; Wickham, 2009). For a detailed walkthrough of the following analysis using R, see
209 the R-markdown summary file (File S1).

210
211 Prior to denoising, reads were truncated at the first quality score of 2 and reads with an expected
212 error greater than 2 were removed. Since *PEAR* alters the quality scores during sequence
213 merging, we inflated the error profile used by *dada2* to denoise sequences by a factor of 3. Reads
214 were then denoised and merged using *dada2* to generate a table of amplicon sequence variants
215 (ASVs). Chimeric sequences were identified using *removeBimeraDenovo* and removed prior to
216 downstream analyses (~0.31% of sequences). Taxonomy was inferred using the RDP Naive
217 Bayesian Classifier algorithm with a modified UNITE (v. 8.0) database resulting in 2953 ASVs
218 (UNITE Community, 2019; Wang et al., 2007). The UNITE database was modified to include a
219 representative ITS amplicon sequence for the host plant, *Zostera marina* (KM051458.1). ASVs
220 were then named by giving each a unique number preceded by “SV” which stands for sequence
221 variant (e.g. SV1, SV2, etc).

222
223 Subsequently, *ITS-x* (v. 1.1b) was run on the unique ASVs (Bengtsson-Palme et al., 2013). This
224 program, which uses kingdom-specific hidden markov models to identify the ITS region of
225 sequences, could not detect a fungal ITS region in 1064 of the ASVs. These ASVs were thus
226 considered putatively non-fungal and were removed from downstream analysis.

227
228 *Decontam*’s prevalence method was used to identify possible contaminants with a threshold of
229 0.5 which will identify sequences that have a higher prevalence in negative controls than in true
230 samples. This threshold identified 38 possible contaminants which were then removed from the
231 dataset. Negative and positive controls were subsequently removed at this point in the analysis.
232 Remaining ASVs assigned as non-fungal at the domain level (e.g. ASVs assigned to the host
233 plant, *Z. marina*, or with no domain level classification) were removed from the dataset prior to
234 downstream analysis resulting in a final table of 1850 ASVs. Four samples were represented by
235 zero sequences after these filtering steps and were subsequently removed.

236
237 ***Sequence analysis and visualization***

238

239 For statistical analysis and visualization, the resulting data was then subset without replacement
240 to an even number of sequences per sample depending on the comparisons being made. As a
241 result, when investigating differences in epiphytes between bulk sample types a depth of 10,000
242 sequences was selected resulting in the inclusion of 49 samples: leaf ($n = 13$), root ($n = 14$),
243 rhizome ($n = 7$) and sediment ($n = 15$). When investigating differences across leaf length a
244 number of 5,000 sequences was chosen resulting in the inclusion of 50 samples: leaf epiphytes (n
245 $= 25$) and leaf endophytes ($n = 25$). These depths were selected after examining rarefaction
246 curves to balance maximizing the number of sequences per sample while also minimizing the
247 number of samples removed from downstream analysis.

248
249 To assess within-sample (i.e., alpha) diversity, the observed number of ASVs and the Shannon
250 index of samples were calculated using the `estimate_richness` function in `phyloseq`. Kruskal–
251 Wallis tests with 9,999 permutations were used to test for significant differences in alpha
252 diversity between bulk sample types (leaf, root, rhizome, sediment). For metrics in which the
253 Kruskal–Wallis test resulted in a rejected null hypothesis ($p < 0.05$), Bonferroni corrected post-
254 hoc Dunn tests were performed.

255
256 To assess between-sample (i.e., beta), diversity, Bray–Curtis (Bray et al., 1957) dissimilarities
257 were calculated using the `ordinate` function in `phyloseq` and visualized using principal
258 coordinates analysis. To test for significant differences in mean centroids between sample
259 categories (e.g., sample type), permutational manovas (PERMANOVAs) were performed with
260 9,999 permutations and to account for multiple comparisons, p-values were adjusted using the
261 Bonferroni correction (Anderson, 2001). PERMANOVA tests are known to be sensitive to
262 differences in dispersion when using abundance-based distance matrices like Bray–Curtis
263 (Warton et al., 2012), but are still more robust than other tests (Anderson and Walsh, 2013). To
264 control for this, we also tested for differences in mean dispersions between different sample
265 categories using the `betadisper` and `permutest` functions from the `vegan` package in R with 9,999
266 permutations. For `betadisper` results that resulted in a rejected null hypothesis ($p < 0.05$), the
267 post-hoc Tukey’s Honest Significant Difference (HSD) test was performed to identify which
268 categories had mean dispersions that were significantly different.

269
270 To compare fungal community composition, we collapsed ASVs into taxonomic orders using the
271 `tax_glom` function in `phyloseq` and then removed orders with a variance of less than one percent
272 when comparing between sample types and a variance of less than 0.1 percent when comparing
273 across leaf lengths. The average relative abundance of taxonomic orders was compared between
274 sample types using Bonferroni corrected Kruskal–Wallis tests in R. For orders where the
275 Kruskal–Wallis test resulted in a rejected null hypothesis, Bonferroni corrected post-hoc Dunn
276 tests were performed to identify which sample comparisons for each taxonomic order were
277 significantly different.

278

279 To examine the contribution of specific ASVs to fungal community composition across leaf
280 length, the dataset was filtered to include only ASVs with a mean abundance of greater than two
281 percent. The resulting ASVs were then compared to each other and those that shared greater than
282 99% sequence identity were grouped into what we refer to as “complexes”. Complexes were
283 given a name based on the the most abundant ASV in the group (e.g. SV8 complex). The
284 average relative abundance of ASVs and ASV complexes were compared between sample types
285 using Bonferroni corrected Kruskal–Wallis tests in R. For ASVs and ASV complexes where the
286 Kruskal–Wallis test resulted in a rejected null hypothesis, Bonferroni corrected post-hoc Dunn
287 tests were performed to identify which sample comparisons for each ASV or ASV complex were
288 significantly different.

289

290 *Sanger sequencing*

291

292 The most prevalent sequences associated with *Z. marina* leaf tissue all come from a single
293 complex (i.e., they all share > 99% sequence identity). This complex, which includes SV8,
294 SV11, SV16 and SV56, has been named the SV8 complex because SV8 is the most abundant
295 member of this group. None of the ASVs in the SV8 complex could be classified into a fungal
296 phylum using currently available databases. In order to attempt to improve taxonomic
297 classification of the SV8 complex, we used PCR to obtain sequence data for part of the 28S
298 rRNA gene from representatives of this complex found in our samples. To obtain this sequence,
299 a SV8-specific primer (5'-GGAGCATGTCTGTTTGAGAA-3') was designed for the ITS2
300 region using Primer-BLAST (Ye et al., 2012). This primer was then used in PCR along with the
301 reverse fungal 28S rRNA gene primer, LR3 (Vilgalys and Hester, 1990). PCR using this pair of
302 primers should at least in theory amplify the ITS2 and D1/D2 regions of the 28S rRNA gene for
303 the SV8 complex.

304

305 Using these primers, we performed PCR on DNA from two leaf epiphyte samples (sample IDs:
306 108A and 109A) using Taq DNA Polymerase (QIAGEN, Hilden, Germany) with the following
307 conditions: 95°C for 5 minutes, 35 cycles at 94°C for 30 seconds, 52°C for 15 seconds, 72°C for
308 1 minute, and a final extension at 72°C for 8 minutes (Kress and Erickson, 2012).

309

310 PCR products were purified using the Nucleospin Gel and PCR kit (QIAGEN, Hilden,
311 Germany). The resulting amplicon was sequenced using the Sanger method by the College of
312 Biological Sciences^{UC} DNA Sequencing Facility (<http://dnaseq.ucdavis.edu/>). The resulting ABI
313 files were viewed and a consensus sequence was produced using seqtrace following the Swabs to
314 Genomes workflow (Dunitz et al., 2015). Consensus sequences for the PCR products were
315 deposited to NCBI Genbank under accession no. [MK994004](https://www.ncbi.nlm.nih.gov/nuccore/MK994004), [MK994005](https://www.ncbi.nlm.nih.gov/nuccore/MK994005).

316

317 *Phylogenetic reconstruction*

318

319 Closely related sequences to the sequence from the PCR products above were identified using
320 NCBI's Standard Nucleotide BLAST's megablast option with default settings. These results
321 were then used to guide a literature search to identify additional sequences for inclusion during
322 phylogenetic reconstruction (Table S4) (Hassett et al., 2017; Karpov et al., 2014; Rad-Menéndez
323 et al., 2018; Simmons et al., 2009; Van den Wyngaert et al., 2018).

324
325 A sequence alignment of all sequences listed in Table S4 was generated using MAFFT (v. 7.402)
326 (Katoh, 2002) with default parameters on the CIPRES Science Gateway web server (Miller et al.,
327 2010). The alignment was trimmed using trimAl (v.1.2) with the -gappymethod method (Capella-
328 Gutiérrez et al., 2009). The resulting alignment included 22 sequences and 915 alignment
329 positions. JModelTest2 (v. 2.1.6) was run with default parameters on the CIPRES Science
330 Gateway web server to select a best-fit model of nucleotide substitution for use in phylogenetic
331 analyses (Darriba et al., 2012; Guindon and Gascuel, 2003). The best-fit model based on both the
332 Akaike Information Criterion and Bayesian Information Criterion values was the GTR + G + I
333 evolutionary model.

334
335 Using the CIPRES Science Gateway web server, phylogenetic trees were inferred from the
336 trimmed alignment using both Bayesian and maximum likelihood approaches and a GTR + G + I
337 model. Bayesian phylogenetic inference was performed using MrBayes (v. 3.2.2) with four
338 incrementally heated simultaneous Monte Carlo Markov Chains (MCMC) run over 40,000
339 generations, which was the optimal number of generations required to achieve a stop value of
340 0.01 or less for the convergence diagnostic (Huelsenbeck and Ronquist, 2001). The first 25% of
341 trees generated were discarded as burn-in and for the remaining trees, a majority rule consensus
342 tree was generated and used to calculate the Bayesian Posterior Probabilities. A maximum
343 likelihood approach was undertaken using RAxML (v. 8) using the -autoMRE option which
344 automatically determines the optimal number of bootstrap replicates (Stamatakis, 2014). The
345 resulting phylogenies were then visualized with FigTree (v. 1.4.2) and annotated in Adobe
346 Photoshop CS6 (Rambaut, 2009).

347

348 **Results**

349

350 ***Fungal alpha diversity differs between sample types***

351

352 Alpha diversity was significantly different between bulk sample types (K–W test; $p < 0.01$,
353 Figure S1, Table S5) for all diversity metrics. Post-hoc Dunn tests identified that the alpha
354 diversity for bulk root and rhizome tissues was consistently lower than the alpha diversity of the
355 sediment ($p < 0.05$, Table S6). This is consistent with previous sequence-based studies of
356 seagrass associated fungi (Hurtado-McCormick et al., 2019). The alpha diversity of the *Z.*
357 *marina* mycobiome was found to be much lower than that previously seen for the bacterial
358 communities associated with *Z. marina* (Ettinger et al., 2017).

359
360 Alpha diversity was not significantly different between root epiphytes and endophytes for any
361 metrics (K-W test, $p > 0.05$, Figure S2). However, alpha diversity was significantly different
362 between leaf epiphytes and endophytes when using the Shannon diversity index which
363 incorporates evenness (K-W test, $p < 0.05$), but was not significantly different when looking at
364 the number of observed ASVs ($p > 0.05$).

365 366 ***Fungal community structure differs between and within plant parts***

367
368 Fungal community structure differed significantly between different bulk sample types
369 (PERMANOVA, $p < 0.001$, Table S7, Figure S3). Subsequent pairwise PERMANOVA test
370 results found that community structure was significantly different for all sample type
371 comparisons ($p < 0.05$, Table S8) except sediment vs. roots and leaves vs. roots ($p > 0.05$).

372
373 We note, however, that within group variance, also known as dispersion, also differed
374 significantly between bulk sample types (betadisper, $p < 0.01$, Table S9), with rhizomes having
375 more dispersion than sediment or roots (Tukey HSD, $p < 0.01$, Table S10). PERMANOVA
376 results can be confounded by dispersion differences when not using a balanced design with equal
377 sample numbers for categories being compared. In such instances, a significant PERMANOVA
378 result is unable to distinguish between differences in mean dispersions and differences in mean
379 centroids. Thus, these results must be interpreted with circumspection.

380
381 Fungal community structure also differed significantly between leaf epiphyte and endophyte
382 communities (PERMANOVA, $p < 0.001$, Table S11, Figure 1) and between leaf length segments
383 ($p < 0.05$). Pair-wise PERMANOVA test results found that the first five inches of leaves had
384 significantly different community structure from the last fifteen inches ($p < 0.01$, Table S12). No
385 significant dispersion differences were detected between the community structure of epiphytes
386 and endophytes (betadisper, $p > 0.05$, Table S13) and the differences in mean dispersions
387 between leaf length segments was barely non-significant ($p = 0.052$). The first five inches of
388 leaves had dispersions that differed significantly from the last ten inches (Tukey HSD, $p < 0.05$,
389 Table S14).

390 391 ***Taxonomic composition of mycobiome***

392
393 The composition of the mycobiome of bulk samples types was generally comprised of members
394 of the taxonomic orders *Pleosporales*, *Helotiales*, *Saccharomycetales*, *Coniochaetales*,
395 *Glomerellales*, *Agaricales*, *Cystobasidiales* and *Malasseziales* (Table S15, Figure 2). However,
396 we note that the mycobiome of seagrass tissues was dominated by ASVs that were unable to be
397 classified to a specific fungal phylum using current databases.

398

399 The order *Agaricales* had a mean relative abundance that was significantly different between
400 sample types (K-W test, $p < 0.05$, Table S16), while the orders *Malasseziales* and *Glomerellales*
401 had mean relative abundances that were only marginally nonsignificant ($p = 0.06$). *Agaricales*
402 was enriched in the sediment relative to root and leaf tissues (Dunn, $p < 0.01$, Table S17).
403 Whereas *Malasseziales* had a higher mean relative abundance on the roots relative to the leaves
404 and sediment ($p < 0.01$) and *Glomerellales* had an increased abundance on the leaves relative to
405 all other sample types ($p < 0.05$).

406

407 ***Mycobiome variation in seagrass leaves***

408

409 There were no taxonomic orders with a mean relative abundance that was significantly different
410 between leaf segments (K-W test, $p > 0.05$, Table S18, Table S19, Figure 3). However given the
411 relatively high abundance of ASVs that were unable to be assigned confidently to a fungal order,
412 we investigated whether the mean relative abundance of specific ASVs varied across leaf
413 segments (Table S20, Figure 4). We found that the ASV, SV12, had a significantly higher
414 abundance in leaf segments further from the sediment (K-W test, $p < 0.001$, Table S21). This
415 was further supported by post-hoc Dunn test results ($p < 0.01$, Table S22). Based on megablast
416 searches at NCBI, SV12 most closely matches existing ITS2 data from fungal sequences
417 identified as coming from Aphelidomycota. In addition, we were interested in if there were
418 ASVs that had mean relative abundances that varied with epiphyte and endophyte status. We
419 found that the SV8 complex was enriched in epiphyte washes relative to endophyte samples (K-
420 W test, $p < 0.01$, Table S23, Figure S4).

421

422 ***Phylogenetic placement and identification of the SV8 complex***

423

424 Given the high relative abundance of the SV8 complex in the leaves (Figure 4) and its variable
425 status between epiphyte and endophyte samples, we sought to obtain the sequence of the 28S
426 rRNA gene region for the organism matching this ASV. We designed a primer specific to the
427 ITS2 ASV for the complex using the sequence of SV8 and used a universal fungal 28S rRNA
428 gene primer to obtain a linked ITS2-28S rRNA gene sequence associated with this complex. The
429 resulting ITS2 portion of the resulting PCR products align well with all ASVs from the SV8
430 complex. Therefore, we believe the 28S rRNA gene sequences obtained accurately represent the
431 flanking region to the taxonomic group represented by the SV8 complex. We then used these
432 28S rRNA gene sequences to place the organism represented by this ASV complex into a
433 phylogenetic context and assign it a taxonomic classification. This analysis revealed that the
434 SV8 complex (and thus also all ASVs within this complex) groups within the order
435 *Lobulomycetales* in the phylum Chytridiomycota (Figure 5). Specifically, the SV8 complex is
436 nested within the recently defined Novel Clade SW-I (Van den Wyngaert et al., 2018) and is
437 sister to several culture independent sequences obtained from the marine ecosystem (Hassett et
438 al., 2017).

439

440 Discussion

441

442 Here we offer an in depth survey of the fungi associated with *Z. marina*. We observed that the
443 mycobiome of *Z. marina* roots and rhizomes had lower alpha diversity than that of rhizosphere
444 sediment and that generally the *Z. marina* mycobiome had relatively low species diversity. Low
445 levels of fungal colonization as well as decreased alpha diversity compared to nearby sediment
446 have previously been made in culture and clone based studies of seagrasses (Devarajan and
447 Suryanarayanan, 2002; Ling et al., 2015; Sakayaroj et al., 2010; Shoemaker and Wyllie-
448 Echeverria, 2013; Van den Wyngaert et al., 2018; Venkatachalam et al., 2015). Possible reasons
449 for low abundance and/or infrequent colonization of fungi include indirect factors such as the
450 high salinity and low oxygen levels that are reflective of the marine ecosystem (Nielsen et al.,
451 1999) and direct factors such as seagrass-derived phenolic compounds which are known to act as
452 antimicrobials (Zapata and McMillan, 1979). Additionally, seasonal differences in fungal
453 colonization of seagrasses have been observed, adding an additional layer of complexity and thus
454 making fungal diversity and abundance comparisons difficult (Mata and Cebrián, 2013).

455

456 Previous sequence-based studies of seagrass associated fungi, observed high abundances of
457 sequences classified as *Pleosporales* (Hurtado-McCormick et al., 2019) or *Eurotiales*
458 (Wainwright et al., 2018), where as here the dominant community members appear to be from
459 understudied lineages like the Chytridiomycota. Additionally, Hurtado-McCormick et al. found
460 evidence of Glomeromycota (arbuscular mycorrhizal fungi) in their dataset, whereas we did not.
461 Possible reasons for these different observations include that these studies sampled different
462 seagrass species than we did, had different sampling schema and used different sequencing
463 methods and primer sets. The use of different sequencing primers specifically has been found to
464 have drastic effects on the results and conclusions of mycobiome studies (Frau et al., 2019).

465

466 We did see evidence of known DSE associating with *Z. marina*, including members of the
467 *Pleosporales* and *Glomerellales*, both of which include groups that are known to associate with
468 terrestrial plants (Arnold et al., 2007). We found that the *Glomerellales*, particularly a
469 *Colleotrichum* species (SV10), was abundant in the lower 10 inches of leaf tissue and enriched
470 on the leaves relative to other sample types. DSE, particularly members of the Pleosporales, have
471 previously been observed associating with seagrasses (Borovec and Vohník, 2018; Gnavi et al.,
472 2014; Panno et al., 2013; Torta et al., 2015; Vohník et al., 2015, 2016, 2017, 2019). In the
473 mediteranean seagrass, *Posidonea*, it has been observed that the abundance of the dominant root
474 associated fungi, a Pleosporales species (*Posidoniomyces atricolor* gen. et sp. nov.), is associated
475 with changes in root hair morphology and can form ecto-mycorrhizal-like structures suggesting a
476 close seagrass-fungi symbiosis (Borovec and Vohník, 2018; Vohník et al., 2016, 2017, 2019).

477

478 Although many of the ASVs observed here associated with *Z. marina* are still unidentified, we
479 were able to phylogenetically place the SV8 complex in Novel Clade SW-I in the
480 Lobulomycetales. Known members of Novel Clade SW-I are parasitic chytrids of freshwater
481 algae and diatoms (Frenken et al., 2017; Rad-Menéndez et al., 2018; Van den Wyngaert et al.,
482 2018). It is possible that the SV8 complex's high relative abundance both on and in *Z. marina*
483 leaves may indicate that it is directly infecting host plant tissues and suggests that seagrasses
484 may provide a novel niche for marine chytrids. However, we note that there is another possible
485 interpretation, which is that the SV8 complex is infecting algae or diatoms that are tightly
486 associated with the leaves of *Z. marina* which are themselves a part of the larger *Z. marina*
487 microbiome. This latter interpretation seems to be supported by the observation that the SV8
488 complex has a higher mean relative abundance in epiphyte washes relative to endophyte washes.
489 However, the closest cultivated relative of the SV8 complex forms sporangia outside of host
490 cells (Van den Wyngaert et al., 2018) and thus, we might expect to find more zoospores in the
491 epiphyte washes. Thus the higher relative abundance of the SV8 complex in the epiphyte washes
492 is consistent with both explanations.

493
494 Our observations here are not the first evidence of chytrid associations with seagrasses. A
495 rhizomycelial chytrid was previously observed as the most abundant fungus associating with
496 *Thalassia testudinum* leaves (Newell and Fell, 1980) and was hypothesized to be a possible
497 symbiont, or weak parasite, of the leaves. In a later study, the authors looked for this same
498 chytrid association in *Zostera* with little success (Newell, 1981). However the authors only
499 looked at one location at one time and we note that the SV8 complex was not prevalent during
500 our first two sampling time points. The high abundance of the SV8 complex during our third
501 sampling time point may be because we observed it during a bloom event. Members of the
502 Lobulomycetales have previously been seen to bloom in late summer, which is when we sampled
503 (Frenken et al., 2017).

504
505 Another ASV of interest, which was initially unable to be classified into a fungal phylum, is
506 SV12, which most closely matches existing ITS2 data from fungal sequences in the
507 Aphelidomycota. Known Aphelidomycota are intracellular parasites of green algae (Tedersoo et
508 al., 2018). The increased relative abundance of SV12 in the endophyte samples relative to the
509 epiphyte washes is consistent with the possibility that the SV12 containing organisms are
510 directly infecting *Z. marina* leaf tissue. However, as with the SV8 complex, it is also possible
511 that SV12 containing organisms could be associating with an algae that is itself tightly associated
512 with *Z. marina* leaves.

513
514 The prevalence of unclassifiable ASVs, many of which have closest matches in the
515 Chytridiomycota and Aphelidomycota, with *Z. marina* makes sense given that these lineages
516 have been previously observed to be the dominant fungal lineages in the marine and aquatic
517 ecosystems (Comeau et al., 2016; Grossart et al., 2019; Picard, 2017; Richards et al., 2012, 2015;

518 Rojas-Jimenez et al., 2019) and have life histories that include associations with green algae
519 (Letcher et al., 2013; Picard et al., 2013; Tedersoo et al., 2018). Thus, it is likely that seagrasses,
520 as marine plants, may be providing these fungal lineages with a new ecological niche.

521

522 **Conclusions**

523

524 We observed that *Z. marina* tissues harbor distinct fungal communities and present analyses and
525 speculation regarding the identity and possible functional roles of these species. For example,
526 we identified that the SV8 complex, which represents the most prevalent sequences associated
527 with *Z. marina* leaf tissue, is nested within Novel Clade SW-I in the order *Lobulomycetales* and
528 hypothesize that this chytrid may be directly infecting *Z. marina* leaf tissues. However, despite
529 their abundance and possible importance, as evidenced by the number of novel fungal sequences
530 observed here, we still know relatively little about the diversity and functional roles of marine fungi
531 in the seagrass ecosystem. We propose that seagrass beds may be hotspots of marine fungal
532 diversity, specifically the diversity of understudied lineages that are distantly related from well
533 studied fungi (e.g. the Dikarya). Additionally, seagrasses may be an interesting model with
534 which to look at terrestrial to marine transitions in the context of host-microbe interactions since
535 their evolutionary history (coevolution from a terrestrial ancestor) mirrors the evolutionary
536 history of many lineages of marine fungi, which are thought to have ancestors that secondarily
537 returned to the marine environment multiple times (Jones et al., 2015; Schoch et al., 2009;
538 Spatafora et al., 1998; Suetrong et al., 2009). This work helps lay a foundation for future
539 seagrass-fungal studies and highlights a need for future studies focusing on marine fungi and the
540 potential functional importance of these understudied communities to the larger seagrass
541 ecosystem.

542 **Author Contributions**

543 Cassandra L. Ettinger conceived and designed the experiments, performed sampling, analyzed
544 the data, prepared figures and/or tables, wrote and reviewed drafts of the paper.

545 Jonathan A. Eisen advised on data analysis, edited and reviewed drafts of the paper.

546 **DNA Deposition**

547 This ITS amplicon sequencing project has been deposited at GenBank under accession no.
548 [PRJNA515720](https://www.ncbi.nlm.nih.gov/nuclseq/PRJNA515720) and consensus sequences for the SV8 complex were deposited under accession
549 no. [MK994004](https://www.ncbi.nlm.nih.gov/nuclseq/MK994004), [MK994005](https://www.ncbi.nlm.nih.gov/nuclseq/MK994005).

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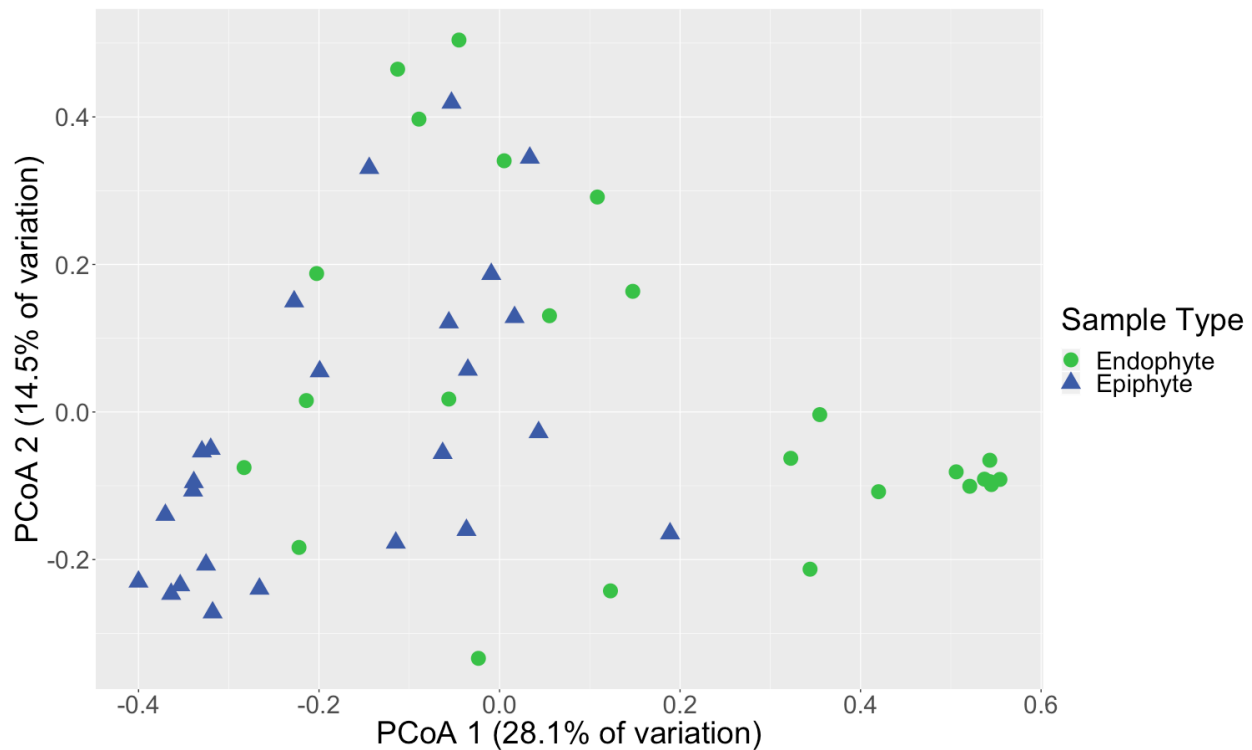
881 **Figures**

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883 **Figure 1: Leaf fungal community structure varies between epiphytes and endophytes.**

884 Principal Coordinates Analysis (PCoA) visualization of Bray-Curtis dissimilarities of fungal
885 communities associated with leaves. Points in the ordination are colored and represented by
886 shapes based on epiphyte (blue triangles; n = 25) or endophyte (green circles; n = 25) status. The
887 dataset was first subset to a depth of 5,000 ITS2 amplicon sequences per sample and then Bray-
888 Curtis dissimilarities were calculated using the ordinate function in phyloseq (McMurdie and
889 Holmes, 2013).

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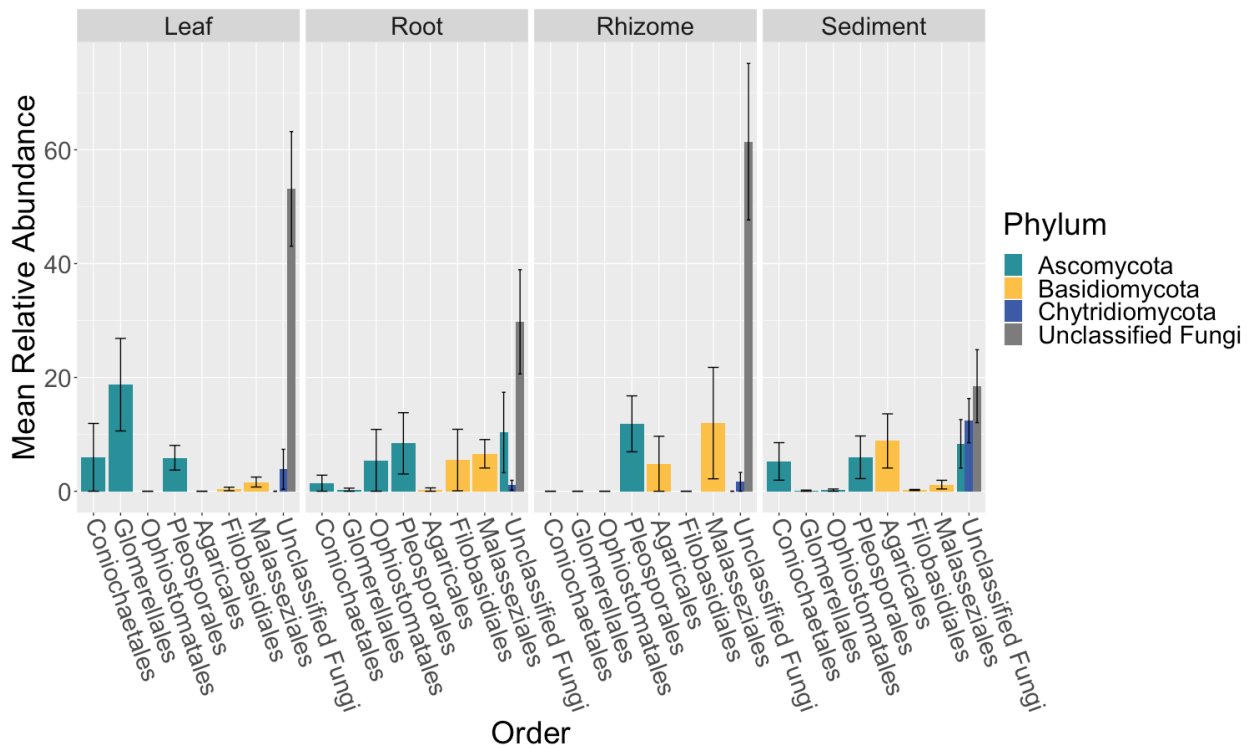
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905 **Figure 2: Fungal community composition differs between tissue types.**

906 The mean relative abundance of taxonomic orders with a variance of greater than one percent are
 907 shown across bulk samples types, leaf (n = 13), root (n = 14), rhizome (n = 7) and sediment (n =
 908 15), with the standard error of the mean represented by error bars and bars colored by taxonomic
 909 phylum. The dataset was first subset to a depth of 10,000 sequences per sample, collapsed at the
 910 order level using the tax_glom function in phyloseq (McMurdie and Holmes, 2013) and then
 911 converted into relative abundance values. Taxonomy was inferred for ITS2 amplicon sequence
 912 variants using the RDP Naive Bayesian Classifier algorithm with a modified UNITE (v. 8.0)
 913 database (UNITE Community, 2019; Wang et al., 2007).
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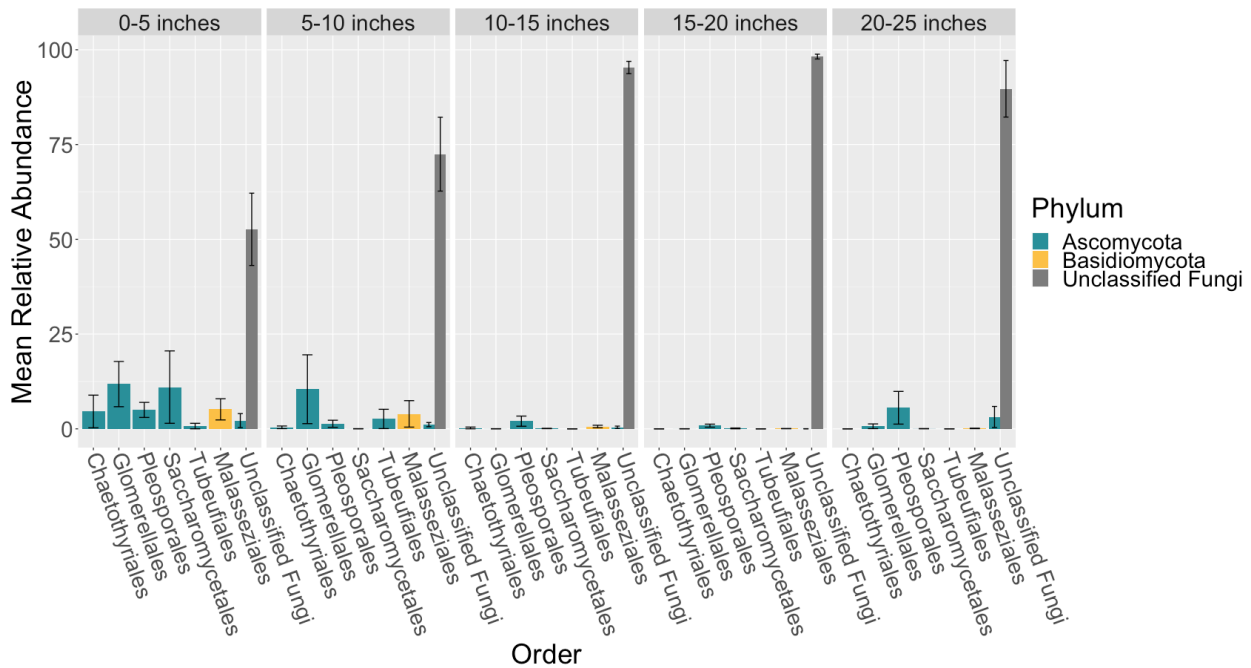


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929 **Figure 3: Mean relative abundance of unclassifiable fungi increases across leaf length**

930 The mean relative abundance of taxonomic orders with a variance of greater than 0.1 percent are
 931 shown across leaf segments, 0-5 inches (n = 10), 5-10 inches (n = 10), 10-15 inches (n = 10), 15-
 932 20 inches (n = 10) and 20-25 inches (n = 10), with the standard error of the mean represented by
 933 error bars and bars colored by taxonomic phylum. The dataset was first subset to a depth of
 934 5,000 sequences per sample, collapsed at the order level using the tax_glom function in phyloseq
 935 (McMurdie and Holmes, 2013) and then converted into relative abundance values. Taxonomy
 936 was inferred for ITS2 amplicon sequence variants using the RDP Naive Bayesian Classifier
 937 algorithm with a modified UNITE (v. 8.0) database (UNITE Community, 2019; Wang et al.,
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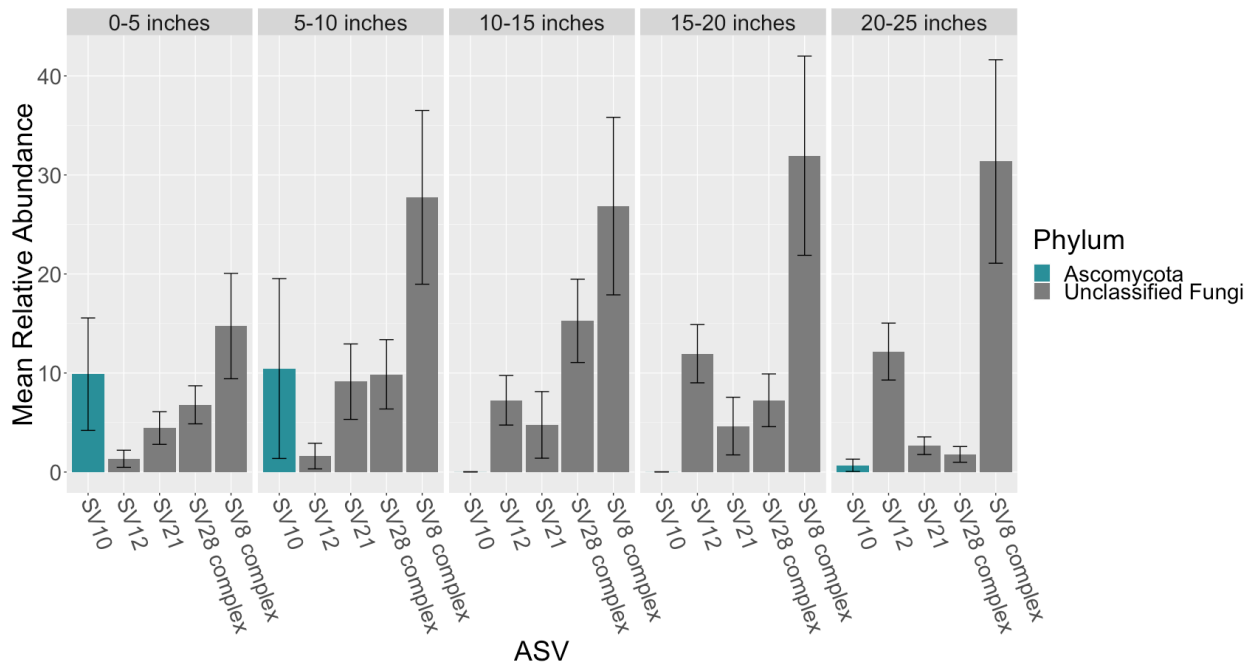
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954 **Figure 4: Mean relative abundance of amplicon sequence variants (ASVs) across leaf**
 955 **length**

956 The mean relative abundance of ASVs with a mean of greater than two percent are shown across
 957 leaf segments, 0-5 inches (n = 10), 5-10 inches (n = 10), 10-15 inches (n = 10), 15-20 inches (n =
 958 10) and 20-25 inches (n = 10), with the standard error of the mean represented by error bars and
 959 bars colored by taxonomic phylum. The dataset was first subset to a depth of 5,000 sequences
 960 per sample and then converted into relative abundance values. ASVs were grouped into
 961 complexes if ASVs shared greater than 99% sequence identity. Taxonomy was inferred for ITS2
 962 ASVs using the RDP Naive Bayesian Classifier algorithm with a modified UNITE (v. 8.0)
 963 database (UNITE Community, 2019; Wang et al., 2007).
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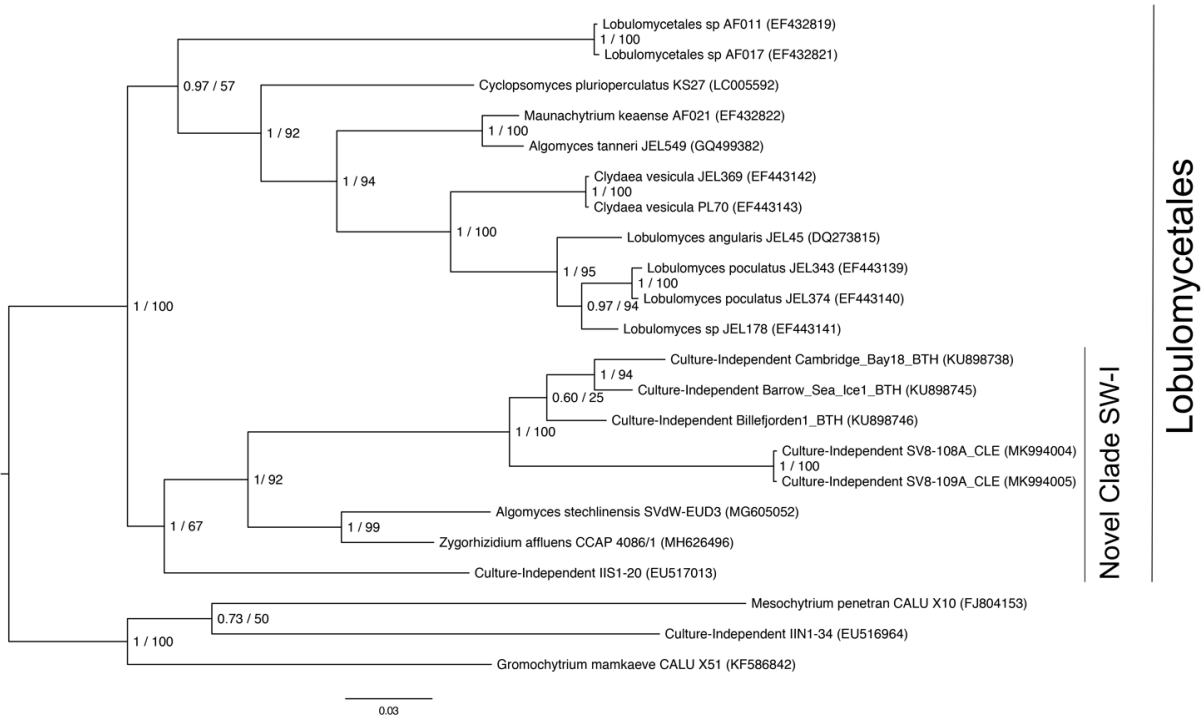


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980 **Figure 5: Phylogenetic placement of the SV8 complex in Chytridiomycota**

981 A molecular phylogeny of 28S rRNA genes was constructed using Bayesian inference. Sequence
 982 alignments were generated using MAFFT (v. 7.402) on the CIPRES Science Gateway web
 983 server, trimmed using trimAl (v.1.2) and a phylogenetic tree was inferred on the trimmed
 984 alignment with a GTR + G + I model using MrBayes (v. 3.2.2) (Huelsenbeck and Ronquist,
 985 2001; Katoh, 2002; Miller et al., 2010). Displayed at each node in the tree are the Bayesian
 986 posterior probabilities (first number) estimated with MrBayes (v. 3.2.2) and the maximum
 987 likelihood bootstrap values (second number) calculated with RAxML (v. 8) (Stamatakis, 2014).
 988 The SV8 complex represents a series of four abundant ASVs (SV8, SV11, SV16, SV56) that are
 989 greater than 99% identical. The 28S rRNA gene sequences for the SV8 complex are named SV8-
 990 108A_CLE and SV8-109A_CLE in this phylogeny and were amplified from two leaf epiphyte
 991 samples (sample IDs: 108A and 109A). The GenBank accession numbers of the sequences used
 992 to build this phylogeny can be found in Table S4.

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