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Broad geographical and ecological diversity from similar genomic toolkits in the ascomycete genus *Tetracladium*

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ABSTRACT

The ascomycete genus *Tetracladium* is best known for containing aquatic hyphomycetes, which are important decomposers in stream food webs. However, some species of *Tetracladium* are thought to be multifunctional and are also endobionts in plants. Surprisingly, *Tetracladium* sequences are increasingly being reported from metagenomics and metabarcoding studies of both plants and soils world-wide. It is not clear how these sequences are related to the described species and little is known about the non-aquatic biology of these fungi. Here, the genomes of 24 *Tetracladium* strains, including all described species, were sequenced and used to resolve relationships among taxa and to improve our understanding of ecological and genomic diversity in this group. All genome-sequenced *Tetracladium* fungi form a monophyletic group. Conspecific strains of *T. furcatum* from both aquatic saprotrophic and endobiont lifestyles and a putative cold-adapted clade are identified. Analysis of ITS sequences from water, soil, and plants from around the world reveals that multifunctionality may be widespread through the genus. Further, frequent reports of these fungi from extreme environments suggest they may have important but unknown roles in those ecosystems. Patterns of predicted carbohydrate active enzymes (CAZyme) and secondary metabolites in the *Tetracladium* genomes are more similar to each other than to other ascomycetes, regardless of ecology, suggesting a strong role for phylogeny shaping genome content in the genus. *Tetracladium* genomes are enriched for pectate lyase domains (including PL3-2), GH71 α -1,3-glucanase domains and CBM24 α -1,3-glucan/mutan binding modules, and both GH32 and CBM38, inulinase and inulin binding modules. These results indicate that these fungi are well-suited to digesting pectate and pectin in leaves when living as aquatic hyphomycetes, and inulin when living as root endobionts. Enrichment for α -1,3-glucanase domains may be associated with interactions with biofilm forming microorganisms in root and submerged leaf environments.

41 INTRODUCTION

42 The freshwater fungi traditionally called "aquatic hyphomycetes" are best known as ecologically
43 important decomposers in stream ecosystems. The group is polyphyletic, its members are classified
44 in various orders and families of Ascomycetes and Basidiomycetes. These fungi thrive on leaves and
45 other plant debris that enter streams and other water bodies from terrestrial plants. In the process,
46 carbon and nutrients from recalcitrant plant compounds, including cellulose and lignin, become
47 accessible to diverse consumers in the stream food web (reviewed in (1, 2)). Up to 99% of the carbon
48 in some streams enters as plant debris that is largely inaccessible to aquatic consumers without the
49 degradative activity of these fungi (3, 4). Aquatic hyphomycete adaptations to stream environments
50 include the production of asexual propagules (conidia) while under water. These spores are often
51 branched, typically tetradiate, or sigmoid, readily detachable from the fungus (*e.g.* from
52 conidiophores) and passively dispersed in water. The conidia strongly adhere to surfaces upon
53 contact. These features facilitate dispersal and colonization of new substrates (5-8). Less well known
54 is that some aquatic hyphomycetes are dual niche (9) or multifunctional (10) species that differ in
55 mode of nutrition or lifestyle in different environments, and are both decomposers of dead plant
56 material in water (saprotrophs) and endobionts in terrestrial or aquatic plants (6, 9, 11-15).
57 Although it has been known since at least 1939 (16) that some aquatic hyphomycetes could be
58 isolated from terrestrial sources, these fungi are still intensively studied in their aquatic context due
59 to their massive presence ecological importance, especially in streams.

60
61 The genus *Tetracladium* de Wild. (Ascomycetes, Leotiomyces) was described in 1893 as "*parmi les*
62 *algues, les diatomées et les débris de végétaux supérieurs dans les étangs, les fossés*" [among algae,
63 diatoms and higher plant debris in ponds, ditches] (17). Since that time, conidia of *Tetracladium*
64 have been recorded in aquatic habitats world-wide (18). The genus has grown to include eleven
65 species, most of which produce distinctive, characteristically shaped conidia (19) (Fig. 1). The three
66 most recent additions to the genus, *T. ellipsoideum*, *T. globosum*, and *T. psychrophilum* are the
67 exceptions (20). These species were described from glacial soil from the Tibet Plateau and have
68 elliptical and globose spores in the first two cases, and no reported spores in the third. These are the
69 first species in the genus described from soil. However, it has been known since 1970 that
70 *Tetracladium* could be isolated from terrestrial sources including sterilized roots (*T. marchalianum*
71 (21)) and one species, *T. nainitalense*, was described from endophytic isolates (11). *Tetracladium*-like
72 fungi, fungi identified as *Tetracladium* based on ITS similarity without morphological information,
73 are increasingly reported to be among the most frequently found and abundant taxa in culture and
74 metagenomics based studies of soil fungi, rhizosphere fungi, and endophytes world-wide. These

75 studies represent diverse ecosystems, soils, and associated plants, ranging from domesticated crops
76 (carrot (22) oilseed rape (23), ginseng (24), lettuce (25) and wheat (26, 27) to wild orchids (28) and
77 mosses (29), from agricultural fields (30) to glacial and subglacial soils (31, 32), from sea level (33) to
78 high altitudes (above 2600 m.a.s.l (34)), and spanning the globe from the Arctic (35) to the Antarctic
79 (29, 36). Three psychrophilic species have been described from glacial soils from the Tibet Plateau
80 (20)—one of the most extreme environments on earth; fungal extremophiles are of ecological and
81 industrial interest due to their roles in ecosystem functioning and the secondary metabolites they
82 produce (33, 37). Although hundreds of ITS sequences for *Tetracladium*-like fungi are available in
83 public databases, it is not yet known how the fungi from these diverse environments are related to
84 each other whether they fit within the genus *Tetracladium* as understood morphologically and
85 phylogenetically at present. The generic type species, *T. marchalianum*, was neotypified in 1989
86 (38), but lack of the neotype ITS sequence also hinders taxonomic efforts within the genus.

87
88 Compared to the aquatic lifestyle of *Tetracladium* species (2), little is known about the biology or
89 ecological importance of *Tetracladium* living outside of water. *In vitro*, some species are known to
90 degrade lignin (39), pectin (40), starch (41), solubilize phosphate (42), have antimicrobial activity
91 (43, 44), and to increase metal tolerance in host plants (45). Correlation studies have associated
92 *Tetracladium* with increased plant growth (46), however, clear patterns are not yet emerging from
93 which the roles or impact of *Tetracladium* as endophytes or in soil can be inferred (47). From studies
94 in other fungal functional groups, such as pathogens and non-aquatic hyphomycete endophytes,
95 there is increasing data available about secondary metabolite (SM, *e.g.* (48)) and carbohydrate
96 active enzyme (CAZymes, *e.g.* (49)) profiles, as predicted from genomes, that may be associated with
97 different lifestyles. By comparing the predicted molecular repertoires for *Tetracladium* both among
98 strains from different ecological niches within the genus and to those of better studied functional
99 groups it may be possible to gain insight into the ecology of *Tetracladium*. This reverse ecology
100 approach to understanding *Tetracladium* biology has not before been possible; to date there are no
101 publicly available *Tetracladium* genomes. Relatively few molecular tools exist for these species, they
102 include microsatellites (50), mass spectrometry profiles (51), and taxon-specific fluorescence in situ
103 hybridization probes (52).

104
105 The i) ecological importance of *Tetracladium* species in streams; ii) global reports of *Tetracladium*-
106 like species in soils and as endophytes; iii) paucity of information about the non-aquatic roles of
107 these fungi; iv) emerging reports of *Tetracladium* species from extreme environments; all signal a
108 need to better understand fungi callasified in the genus *Tetracladium*. Moreover, these features

109 raise an exciting suite of questions and opportunities for study. How have *Tetracladium* species
110 evolved to tolerate environmental extremes? What are the features of genomes and their regulation
111 that enable multifunctionality and broad environmental tolerance or adaptation in these species?
112 Can the same genotypes or species thrive as endophytes and aquatics? How is multifunctionality
113 distributed across the *Tetracladium* phylogeny? It is not possible to address all these questions in a
114 single study, but here the foundations are laid to address them and to support advances across all
115 aspects of *Tetracladium* biology. In this study, genomes of all known species of *Tetracladium* and
116 several *Tetracladium*-like species were sequenced, with more than one representative strain per
117 species when available. Phylogenomics was used to resolve evolutionary relationships within the
118 genus. To gain insight into the biology of these fungi from their genomes, secondary metabolites and
119 carbohydrate-active enzymes were predicted from the genomes to compare among species and
120 with profiles for plant pathogens and other functional groups. Further, analysis of the ITS sequences
121 of *Tetracladium*-like fungi from studies world-wide were analyzed together with the strains
122 sequenced herein to test and illustrate the geographic and ecological diversity of the genus.

123

124 **MATERIALS AND METHODS**

125 **Strains and sequencing**

126 Twenty-four fungal strains, including all described species in the genus *Tetracladium*, were obtained
127 for genome sequencing (see Table S1 for strain information and Nagoya Protocol status).

128 *Tetracladium marchalianum* JA005 (full designation JA005.1) and *T. setigerum* JA001 are new
129 collections from the Fyris River, Sweden. The remaining strains were obtained from the Czech
130 Collection of Microorganisms (CCM), CABI, China General Microbiological Culture Collection
131 (CGMCC), the Yeast Culture Collection at the Universidad de Chile, from Dai Hirose at Nihon
132 University, Japan, and from Magdalena Grudzinska-Sterno from the Swedish University of
133 Agricultural Sciences.

134

135 Cultures were maintained on potato dextrose agar (Fluka, cat #70139) at room temperature with
136 ambient light or at 4°C in the dark (CGMCC strains) with backup cultures frozen at -80°C in 18%
137 glycerol. Strains were grown in flasks with 50-200 mL of malt extract peptone medium (17 g/L malt
138 extract, 2.5 g/L bacto-peptone) at room temperature with shaking for up to one month to obtain
139 sufficient mycelium for DNA extraction. Mechanical disruption via grinding in liquid nitrogen or bead
140 beating (Mini-Beadbeater Biospec 25/s, for 3 s or less) was performed on fresh or freeze dried
141 mycelium before DNA extraction using the Zymo Quick DNA Fungal/Bacterial Miniprep Kit (D6005)
142 as directed.

143

144 Sequencing libraries were prepared at the SNP&SEQ Technology Platform in Uppsala, Sweden, from
145 1 µg DNA using the TruSeq PCRfree DNA sample preparation kit (FC-121-3001/3002, Illumina Inc.)
146 targeting an insert size of 350 bp as directed. Libraries were then sequenced paired-end with a 150
147 bp read length on an Illumina HiSEQX Ten, using v2.5 sequencing chemistry.

148

149 **Genome assembly and annotation**

150 Sequence reads were quality and adapter trimmed using Trimmomatic v0.36 (53). Bases with
151 quality scores below 3 were removed from the start and end of reads. Sequence falling below a
152 quality score of 20 in a sliding window of size 4 and adapter sequences were removed. Resulting
153 sequences greater than 36 bp were retained for genome assembly. Assembly was performed in in
154 Spades v3.11.1 (54) using the “careful” option to reduce mismatches and short indels. The resulting
155 scaffolds were used for further analysis. Assembled genomes were evaluated in QUAST V4.5.4 (55)
156 and BUSCO v3.0.2b (56) using the Pezizomycotina odb9 database to test for completeness. Average
157 depth of coverage was estimated in QualiMap v2.2.1 (57) after read mapping in BWA v0.7.17 (58).

158

159 Genomes were annotated using an iterative MAKER v3.01.2-beta (59) annotation pipeline. In the
160 initial run, protein and EST evidence from *Botrytis cinerea* B05.10 (ASM83294v1), *Glarea lozoyensis*
161 (ATCC 20868), *Phialocephala subalpina* (PAC v1), and *Sclerotinia sclerotiorum* (ASM14694v1),
162 accessed from Genbank (28-29 May 2018), were used as evidence to train the Hidden Markov
163 Model. Species specific repeat libraries generated using RepeatModeler v1.0.8 (60), using default
164 settings and the NCBI search engine, were used for repeat masking. Outputs from this run were used
165 to support *ab initio* gene predictions in GenMark-ES v4.33 (61) using the fungus algorithm option.
166 The GenMark outputs were then used in MAKER for gene annotation on scaffolds larger than 1000
167 bp.

168

169 Putative gene functions and protein domains for the annotated genes were identified using Blastp
170 (62) against the UniProtKB/Swiss-Prot annotated non-redundant database (63) (downloaded 29
171 October 2018) to return no more than one hit per gene with an evaluate threshold of 1×10^{-6} and
172 InterProScan v5.30-69.0 (64) with mapping to Gene Ontology and member database signatures
173 (without using the pre-calculated match lookup service). These data were used to update the
174 annotations in MAKER.

175

176

177 **Single copy orthologs and phylogenomics**

178 Single copy orthologs (SCO) present in all *Tetracladium* strains and 27 additional ascomycetes (Table
179 S2) were selected from ortholog groups identified by OrthoMCL v2.0.9 (65) (with inflation index 2).
180 Annotations for *Cadophora malorum* and *Articulospora tetracladia* are not available at NCBI and
181 were generated for use herein as described above. Before alignment, SCO sequences were
182 processed in PREQUAL v1.02 (66) using default settings to remove sequence stretches with no
183 evidence of homology. The SCO were then aligned (MAFFT v7.407 G-INS-I) with a variable scoring
184 matrix and $\alpha_{\max}=0.0.8$ (67) and high-entropy regions were trimmed (BMGE v1.12 (68) with the
185 BLOSUM95 similarity matrix). The resulting dataset (all-SCO) and two subsets of the data were used
186 for analyses. The two subsets contained SCOs from the all-SCO dataset that are also in the BUSCO
187 fungi (fungi-SCO) or ascomycete (asco-SCO) odb9 databases. Phylogenetic analyses were performed
188 with maximum likelihood (ML) in IQ-TREE v1.6.8 (69). For the all-SCO dataset, a guide tree inferred
189 under the X+G+C60 model (X = best-fit model inferred by BIC in ModelFinder (70); for +C60 see (71)).
190 The guide tree and best-fit model from that analysis were used to infer the final tree with the
191 posterior mean site frequency (PMSF) model (71) which better accounts for heterogeneity of amino
192 acid frequencies across sites. Branch support was evaluated using ultrafast bootstrap (UFBoot, (72))
193 and SH-like approximate likelihood ratio test (SH-aLRT), each with 1000 replicates. The fungi-SCO
194 and asco-SCO datasets were both analyzed using the best-fit models identified by ModelFinder and
195 support estimated using 1000 UFBoot and SH-aLRT replicates. The fungi-SCO dataset was
196 additionally analyzed using the FreeRate model (X+R5). Lifestyle, ecology and other designations for
197 the fungi in this study are based on the actual sample sequenced, this can differ from the
198 generalized biology of the species (Tables S1, S2). Trees were visualized and annotated using iTOL
199 (73) with *Tuber borchii* as the root.

200

201 **ITS phylogeny**

202 ITS sequences including the term *Tetracladium* were retrieved from GenBank and aligned in
203 Geneious 10.2.4 (<http://www.geneious.com>). Sequences containing data spanning the ITS region
204 (ITS1, 5.8s, and ITS2) were trimmed using BMGE (DNAPAM1 matrix). Using a distance matrix
205 produced in Geneious, sequences with identical bases and alignments were identified and removed
206 from the dataset. Sequences with excessive uncalled bases (>30) or poor alignments were also
207 removed. The strains sequenced herein may be doubly represented in the dataset if not identified as
208 duplicates in this process. Information about each sequence was manually searched to determine
209 the origin and lifestyle when possible. The ITS-region ML phylogeny was determined in IQTree with

210 the best-fit model and branch support estimated using 1000 non-parametric bootstrap replicates
211 and visualized with *Penicillium antarcticum* as the root.

212

213 **Carbohydrate active enzymes (CAZymes) and secondary metabolites**

214 To identify genes involved in recognition, metabolism, and synthesis of complex carbohydrates, the
215 proteins annotated in MAKER for each strain were compared against existing CAZyme-related
216 databases using the dbCAN2 meta server (74) (accessed online at <http://bcb.unl.edu/dbCAN2>,
217 September-October 2019) with HMMER, DIAMOND, and Hotpep and the default settings. Only
218 CAZymes predicted by at least two tools were used in further analyses. To count the domains
219 identified, each prediction identifier for multi-family/multi-domain genes were counted separately
220 not together as a new combined category. Secondary metabolite clusters were identified in
221 genomes using the antiSMASH 5.0 fungal version public web server (75) (accessed online at
222 <https://fungismash.secondarymetabolites.org>, August-September 2019) including use of the
223 *KnownClusterBlast* function. Cluster analysis and visualization were done in R (76) on scaled (*scale*)
224 count data using *heatmap*. Differences in numbers and types of predicted CAZymes and SM among
225 the genus *Tetracladium*, other Leotiomycetes, and the remaining taxa were evaluated using ANOVA
226 in R, with taxon-group as the main effect, and post hoc analyses with Tukey's HSD ($p \leq 0.05$) as
227 implemented in *agricolae*.

228

229 **RESULTS**

230 **Strains**

231 Four strains from this study have been deposited in the CCM culture collection and will be publicly
232 available after peer reviewed publication; JA005 (CCM 9013), JA001 (CCM 9012), AFCN889 (CCM
233 9011), and AFCN900 (CCM 9010). *Tetracladium sp.* 82A210 failed to revive from preserved and long
234 term cultures at the time the cultures were deposited. Efforts to revive this strain are ongoing and it
235 will be deposited in the CCM if successful.

236

237 **Genomes**

238 Twenty-four *Tetracladium* and *Tetracladium*-like strains, including all described species, were
239 sequenced and the resulting raw data deposited as ENA Study PRJEB36440 (available after peer
240 review). Data for each genome, including contigs, trimming, assembly, and coverage statistics as well
241 as sample and read number details are provided in Table S3. The genomes assembled into 266 –
242 6,765 scaffolds (median = 378). Scaffold N50 values ranged from 185,943 – 2,082,751 bp (median =
243 894,004 bp). Total assembled genome sizes ranged from 34.3 – 43.6 Gb. Genome-wide average GC

244 content in the assemblies was 46.13%. These values exclude scaffolds smaller than 500 bp. The
245 *Tetracladium sp.* T11Df assembly had the lowest depth of coverage (69X) and had more than 5-times
246 more scaffolds than the next highest, *T. maxilliforme* CCM F-529 with 1032. Depth of coverage
247 ranged from 69 – 178X (average = 128X). These high depths of coverage were not targeted, rather
248 they are the by-product of small genome size and high sequencer output; the 24 barcoded genomes
249 were pooled and sequenced in one lane on an Illumina HiSeqX Ten. All genomes are highly complete
250 as assessed in BUSCO. Of the 3,156 genes included in the Pezizomycotina odb9 database, 20 - 34
251 were missing from the genome of any strain. The genomes had 98.3 – 98.7% complete BUSCOs, and
252 97.8 – 98.5% were complete and single copy.

253

254 **Annotations and Orthologs**

255 The number of annotated gene models ranged from 7769 in *T. sp.* T11Df to 9799 in *T. sp.* 82A210
256 (mean 8768, median 8694; Table S3). To predict gene function, the annotated genes for each strain
257 were compared to the UniProtKB/Swiss-Prot annotated non-redundant protein database. These
258 analyses returned similarity-based information for an average of 6810 (median 6759) predicted
259 genes per strain. An average of 8681 protein signatures per strain (median 8613) were identified by
260 comparing the annotated gene models to the InterProScan database. The gff files containing all
261 annotation data will be available after peer review. A total of 1820 SCO present in all 51 genomes
262 (all-SCO) included in this study were identified from the output of OrthoMCL.

263

264 **Phylogeny**

265 The all-SCO dataset contained 794,205 aligned amino acid sites, 434,869 were parsimony-
266 informative (Table S4). Analysis using the PMSF model starting with the best fit model LG+F+I+G4
267 and a guide tree (Fig. S1) as input, resulted in a tree with all branches fully supported (100% UFBoot
268 and SH-aLRT)(Fig 2). All known and putative *Tetracladium* strains in this analysis form a
269 monophyletic clade with three distinct groups. Group A contains all *Tetracladium* species with
270 typical *Tetracladium* conidia, including *T. sp.* F-10008 which also produces conidia typical for
271 *Tetracladium* when submerged (19). *Tetracladium sp.* 82A210, conidia yet unknown, was isolated as
272 an endophyte of wheat and is resolved within *T. furcatum*. Group B includes the moss endobionts
273 from the Antarctic, *T. sp.* AFCN889 and *T. sp.* AFCN900, which appear to be conspecific and the three
274 soil species from the Tibet Plateau, *T. ellipsoideum*, *T. psychrophilum*, and *T. globosum*. Group C is
275 monotypic, containing only the Antarctic yeast *T. sp.* T11Df. Only two branches in the initial guide
276 tree were not supported (UFBoot values <95% and SH-aLRT <80%), one within Group A, separating *T.*

277 *furcatum* from *T. maxilliforme*, and one within the Leotiomyces separating *Phialocephala*
278 *scopiformis* and *Articulospora tetracladia* (Fig. S1).

279

280 To confirm the robustness of the phylogenetic relationships inferred from analyses of the large all-
281 SCO dataset additional analyses were performed using subsets of the data with more complex
282 substitution models than were computationally feasible for full dataset. The asco-SCO dataset
283 contained 182,311 parsimony informative amino acid sites (339,460 total) from 709 SCOs (Table S4).
284 ModelFinder identified LG+F+R6 as the best fit model. The resulting tree had a highly similar
285 topology to the all-SCO result, with uncertain resolution for *T. furcatum*/*T. maxilliforme* and *P.*
286 *scopiformis*/*A. tetracladia* (Fig. S2).

287

288 The fungi-SCO dataset was the smallest, with 35,673 parsimony informative sites (72,086 total) from
289 164 SCOs (Table S4). The best-fit model for the data was LG+F+R4. When analyzed using this model
290 there was again poor resolution for *T. furcatum*/*T. maxilliforme* and *P. scopiformis*/*A. tetracladia*
291 (Fig. S3). There was also insufficient support for the branch to *T. palmatum* and *T. setigerum*. Using
292 the empirical mixture model (C60), all branches that were unsupported with the simpler LG+F+R4
293 model met the threshold for support, but the branch separating *Coleophoma cylindrospora* from
294 *Scytalidium lignicola* was not supported (Fig. S3). Overall the phylogenies obtained in these analyses
295 were highly consistent. Within the *Tetracladium* and *Tetracladium*-like clade, only the relationships
296 of *T. furcatum* and *T. maxilliforme* to each other and the rest of Group A varied among analyses.

297

298 **ITS Phylogeny**

299 To evaluate how ecological diversity is distributed within the genus, a phylogeny including 198 ITS
300 DNA was produced and visualized in with information about the sequence (Fig. 3, Table S5). To
301 further highlight diversity in the genus, orchid and challenging/extreme-environment associated
302 sequences are also indicated. The later, identified as “cold” in Fig. 3, are from high latitude, high
303 altitude, alpine, and glacier associated locales. The analysis included 229 sequences, with seven
304 outgroups and the 24 strains sequenced herein. Of 402 total sites, 144 were parsimony informative
305 and the best fit model for the data was TIM3e+R3. Branch support was determined using standard
306 non-parametric bootstrapping (not UFBoot); bootstrap values >75% are considered well-supported
307 using this approach. Conservatively, only branches with bootstrap values >85% are highlighted in the
308 resulting figure (Fig. 3). As expected, this ITS tree does not reflect relationships between the species
309 of *Tetracladium*, but similar ITS sequences from different sources and lifestyles are clustered. Only
310 one *Tetracladium*-like sequence, JX630692, is not similar to species in Groups A, B, and C. When

311 compared to other ascomycetes in GenBank, this sequence does not return hits to any culture
312 identified *Tetracladium* species. Thus, the “*Tetracladium*” identity of this sequence is suspect.

313

314 **CAZymes**

315 Cluster analysis based on the predicted number of carbohydrate-binding modules (CBM) and
316 catalytic domains of each CAZyme class by species did not reveal any discernable ecological grouping
317 (Fig. 4, Table S6). Clustering of the majority of the *Tetracladium* species reflects phylogeny, not
318 ecology, because the only non-*Tetracladium* aquatic hyphomycete in the study does not cluster with
319 *Tetracladium* species and the *Tetracladium* species cluster together regardless of ecology. The
320 exception is the Antarctic yeast, *T. sp.* T11Df, which clusters with taxonomically and ecologically
321 diverse species.

322

323 Like other Leotiomycetes (490.8 ± 161), *Tetracladium* genomes (487.6 ± 47.9) contain more CAZyme
324 and CBM domains than other ascomycetes (329.1 ± 102.6 ; Fig. 5A; $F_2 = 6.97$, $p < 0.002$; Tukey’s HSD,
325 $p \leq 0.05$). Likewise, Leotiomycetes and *Tetracladium* genomes have more predicted carbohydrate
326 esterase (CE), glycoside hydrolase (GH), and glycosyltransferase (GT) domains than other
327 ascomycetes (Fig. 5C; CE: $F_2 = 8.5$, $p < 0.0001$; GH: $F_2 = 5.9$, $p = 0.005$; GT: $F_2 = 6.3$, $p = 0.004$; Tukey’s
328 HSD, $p \leq 0.05$). However, *Tetracladium* genomes have fewer predicted Auxiliary Activities (AA)
329 domains than other Leotiomycetes, and so are in line with other ascomycetes ($F_2 = 6.7$, $p = 0.0003$;
330 Tukey’s HSD, $p \leq 0.05$). Note that the taxon-groups differ in sample size (*Tetracladium* = 24,
331 Leotiomycetes = 18, Other ascomycetes = 8) and represent diversity at different taxonomic levels.
332 Also, only domains predicted by HMMER are presented here, summaries of all HMMER results
333 (Table S6) and results from Hotpep and DIAMOND (Table S7) are provided. These numbers are
334 influenced by the contents of the databases and the genome assemblies and are thus “predicted”
335 values.

336

337 *Tetracladium* genomes are specifically enriched for polysaccharide lyase (PL) domains relative to
338 other Leotiomycetes and ascomycetes (Fig. 5C; PL: $F_2 = 81.78$, $p < 0.0001$; Tukey’s HSD, $p \leq 0.05$).
339 *Tetracladium* genomes have 34 ± 4.98 (mean ± 1 standard deviation) PL domains per genome, which
340 is about 3 times the number in other Leotiomycetes (9.95 ± 9.97), and 6 times as many as the other
341 ascomycetes (5.5 ± 3.8). This difference in part reflects the higher copy number of pectate lyase (77)
342 PL3-2 in the *Tetracladium* genomes (9.3 ± 1.4) than the Leotiomycetes (1.8 ± 2.2) and other
343 ascomycetes (0.9 ± 0.8 ; $F_2 = 134.8$, $p < 2e-16$; Tukey’s HSD, $p \leq 0.05$; Table S6).

344

345 *Tetracladium* genomes also contain more CBMs (24.1 ± 6.2), than other Leotiomycetes (15.9 ± 9.1)
346 and ascomycetes (10.6 ± 5.2 ; CBM: $F_2 = 12.89$, $p < 0.0001$; Tukey's HSD, $p \leq 0.05$). Most CBMs (75-
347 91%) were associated with glycoside-hydrolases (GH). Up to three CBMs were found to co-occur
348 flanked by GH32 (GH32+CBM38+CBM38+CBM38+GH32). *Tetracladium* genomes contain more
349 copies of GH32 (4.3 ± 1.4) and CBM38 (2.5 ± 1.6) than the other taxon-groups (Leotiomycetes: GH32
350 2.6 ± 1.7 , CBM38 0.8 ± 1.1 ; ascomycetes: GH32 2.3 ± 1.8 , CBM38 0.3 ± 0.5 ; $F_2 = 8.4$, $p = 0.0007$;
351 Tukey's HSD, $p \leq 0.05$). GH32 family enzymes can function as invertases that convert sucrose into
352 fructose and glucose and act on inulin and fructose (77) and CBM38 has inulin-binding function (77).
353 The GH32+CBM38+CBM38+CBM38+GH32 conformation is unique within the genus *Tetracladium* in
354 this study. Versions of this CAZyme with one and two CBM38 between the flanking GH32s were also
355 predicted for some Leotiomycetes (Table S8). All *Tetracladium* genomes except *T. ellipsoideum* and
356 *T. sp.* T11Df had at least one GH32+CMB38 predicted CAZyme.

357
358 Copy number of CBM24 also contributes to the difference in CBM among taxon groups; there are 1-
359 15 copies (7.6 ± 4.7) in each *Tetracladium* genome, while the Leotiomycetes (0-11, 3.4 ± 4.0) and
360 ascomycete genomes (0-5, 2 ± 1.8) contain fewer (Table S6). CBM24 has α -1,3-glucan/mutan binding
361 function (77). In *Tetracladium*, CBM24 was almost always predicted in 1-3 copies in association with
362 GH71 an α -1,3-glucanase (77). GH71 is enriched in *Tetracladium* genomes (7.7 ± 3.1) relative to the
363 other Letiomycetes (4 ± 2.4) and ascomycetes (3.9 ± 2.5 ; $F_2 = 10.39$, $p = 0.0002$, Tukey's HSD, p
364 ≤ 0.05). The high copy number of GH71, and its association with 1-3 copies of CBM24 per predicted
365 CAZyme, explains the abundance of CBM24 in the *Tetracladium* genomes.

366
367 Strains of the same species have similar predicted CAZyme repertoires overall (Table S9). The same
368 copy number was predicted for 63-93% of the predicted CAZyme domains in the genomes of the the
369 six species represented by more than one strain. This includes *T. sp.* 82A210 within *T. furcatum*, and
370 *T.sp.* AFCN889 and *T. sp.* AFCN900 as one species. *Tetracladium furcatum* had the lowest similarity
371 among strains for copy number, which is consistent with the phylogenetics results; F11883 and
372 82A210 are more similar to each other than to F06983. F11883 and 82A210 are 91% identical for
373 copy number. Chromosome level assemblies are required to determine absolute numbers present in
374 the genomes. However, consistent counts between related genomes can aid interpretation overall
375 and give a first estimate of variation within species. In most cases where counts differed between
376 strains within species the count differed by ± 1 (75%, Table S9). Larger differences in copy numbers
377 can be seen for specific CAZymes within species in some cases. For example, GH71 (above) is
378 predicted in 4 copies in *T. marchalianum* F26399 and in 13 copies in *T. marchalianum* JA005 (Table

379 S6). As expected, CBM24 copy number also differed between these two strains (3 and 10
380 respectively).

381

382 **Secondary Metabolites**

383 *Tetracladium* genomes contain 24.7 ± 3.6 SM clusters (Fig. 5B) detected by antiSMASH which is
384 fewer than other Leotiomyces (43 ± 17.5), but in line with other ascomycetes (31.8 ± 15 ; $F_2 =$
385 11.53 , $p < 0.0001$; Tukey's HSD $p < 0.05$). All SM data are available in Table S9. *Tetracladium*
386 genomes each contain one betalactone cluster (1.0 ± 0.2), except *T. sp.* T11Df with none, setting
387 them apart from the other taxon-groups (Fig. 5D; Leotiomyces 0.4 ± 0.5 ; ascomycetes 0.5 ± 0.8 ; $F_2 =$
388 9.3 , $p < 0.001$; Tukey's HSD $p < 0.05$). Indole clusters are predicted in only 50% of *Tetracladium*
389 genomes and never more than 1 per genome (0.5 ± 0.5), which is fewer than in Leotiomyces
390 genomes (1.3 ± 1.1) but not different from other ascomycetes (1.3 ± 1.2 ; $F_2 = 5.1$, $p < 0.01$; Tukey's
391 HSD $p < 0.05$). More nonribosomal peptides (NRPS) are found in Leotiomyces (8.2 ± 4.6) and other
392 ascomycete (6.5 ± 4.1) genomes than in *Tetracladium* genomes (3.2 ± 0.8 ; $F_2 = 12.7$, $p < 0.0001$;
393 Tukey's HSD $p < 0.05$). All Leotiomyces genomes (9.2 ± 3.7), including *Tetracladium* (8.4 ± 1.2),
394 contain more NRPS-like SM clusters than the other ascomycetes (5.3 ± 2.8 ; $F_2 = 6.4$, $p = 0.004$;
395 Tukey's HSD $p < 0.05$). Fewer Type 1 polyketide synthase (T1PKS) are predicted in *Tetracladium*
396 genomes (6.8 ± 2.0) than in leotiomyces (17.2 ± 9.1), but neither group differs from the
397 ascomycetes (11.6 ± 5.1 ; $F_2 = 15.66$, $p < 0.0001$; Tukey's HSD $p < 0.05$). Both Leotiomyces (5.1 ± 2.0)
398 and other ascomycete genomes (5.5 ± 2.7) contain more terpene SM clusters than *Tetracladium*
399 genomes (3.4 ± 0.5 ; $F_2 = 7.7$, $p = 0.001$; Tukey's HSD $p < 0.05$). The frequencies of ribosomally
400 synthesized and posttranslationally modified peptides (fungal-RIPPs), phosphonates, siderophores
401 and Type III polyketide synthases (T3PKS) clusters were low and did not differ among taxon groups
402 (Table S10).

403

404 While *Tetracladium* genomes each contain around 24 SM clusters detectable by antiSMASH only
405 13% returned a BLAST match for most similar known cluster in the MiBIG database(78). In total 18
406 SM clusters were identified to type (Fig. S4, Table S10). A nonribosomal peptide synthetase (NPS),
407 Dimethylcoprogen, was the only SM predicted in all *Tetracladium* genomes (100% similarity (75)).
408 This cluster is found in only 2 of 18 (11%) of the other Leotiomyces genomes and 3 of 8 (38%) other
409 ascomycetes. All other identified SM clusters were Group or taxon specific within *Tetracladium*
410 (Table S10). Unique to some members of Group A: Solanapyrone, Hexadecydro-astechrome,
411 Cytochalasin, Phyllostictine A/phyllostictine B, Citreoviridin, Clavric acid, Aureobasidin A1 and
412 Chaetoglobosins. Unique to Group some members of Group B: Clapurines, Naphthopyrone,

413 Shearinine D, PR toxin, Pyranonigrin E, Azanigerone. Unique to Group C: Duclauxin. Depudecin
414 (Groups A and B), Brefeldin (B and C) were also predicted.

415

416 **DISCUSSION**

417 **Relationships among *Tetracladium* and *Tetracladium*-like fungi**

418 This study presents the first phylogeny with all species of *Tetracladium* described to date. The eleven
419 described species and all newly sequenced *Tetracladium*-like (putative) strains/species form a
420 monophyletic group with three partitions (Fig 2, Groups A, B, and C). Whereas interspecific
421 relationships within *Tetracladium* were unresolved in analyses using 18S (79), 28S (20), and ITS+28S
422 (80) data and few taxa, relationships within the genus are largely stable across analyses and datasets
423 herein. Within the *Tetracladium* and *Tetracladium*-like group only the branching of *T. furcatum* and
424 *T. maxilliforme* varied between analyses. *Tetracladium furcatum* and *T. maxilliforme* are well
425 supported separate species, but it is unclear whether they are sister species or whether *T. furcatum*
426 alone is sister to the rest of Group A.

427

428 The majority of species and strains in Group A originate from submerged plant debris or foam that
429 forms on rivers and produce stereotypical *Tetracladium*-shaped conidia which are typically
430 distinctive between species (19, 38). Although conidia have not been observed for the strain isolated
431 as an endophyte of wheat (Sweden, 2007), *T. sp.* 82A210, this strain falls within the species *T.*
432 *furcatum*, and is highly similar to strain F11883 (Czech Republic, 1983) which was isolated from foam
433 as a typical aquatic hyphomycete (Table S1). This result supports the idea that *T. furcatum* is
434 multifunctional, as opposed to having different ecologies for morphologically similar species.
435 *Tetracladium furcatum* was first reported as a root endophyte in 1996, based on the morphological
436 identification of conidia (81). It has also been associated with the endophytic lifestyle in
437 metabarcoding studies, including studies of terrestrial plants from the high Arctic (35) and
438 submerged aquatic plants in Norway (15). *Tetracladium nainitalense* which was isolated as
439 endophyte of *Eupatorium adenophorum* (11), is most closely related to species isolated as aquatic
440 hyphomycetes and has itself been isolated from foam in a stream (morphological identification (82)).
441 It should be noted that *Tetracladium* species are found as endophytes even in non-stream, non-
442 riparian habitats. This study also confirms the phylogenetic position of *T. sp.* F10008, collected as an
443 aquatic hyphomycete from Malaysia in 2008, as the sister species of *T. apiense* (19).

444

445 In the majority of cases when more than one strain per species was sequenced the strains are
446 resolved together as expected. Most strains in Group A were isolated by experts in aquatic

447 hyphomycetes (Table S1), who will have relied on spore morphology for initial identification. This
448 suggests that morphological identification of most Group A species from field samples can be
449 reliable. However, two species are potentially problematic. Strains historically identified as *T.*
450 *marchalianum*, F12812 (called *T. sp.* F12812 herein) and F26399, are not conspecific. The same is
451 true of two strains of *T. breve* (F12505 and F10501). Rather, the *T. marchalianum*-like strain F12812
452 is the sister species of the *T. breve*-like F12505, and *T. marchalianum*-like F26399 is sister to *T. breve*-
453 like F10501, and the two pairs are divergent. This finding is consistent with previous studies based
454 on one or few genes (19, 83). The *T. marchalianum* and *T. breve*-like strains also differ in predicted
455 secondary metabolite profiles (Table S10), suggesting that secondary metabolite profiles might be
456 valuable tools for species identification in the same way that protein fingerprinting is being
457 developed (51). *Tetracladium marchalianum*-like strains can readily be categorized as F12812 or
458 JA005/F26399-like using beta-tubulin sequence clustering (SI Fig. X), based on a preliminary analysis
459 of strains identified as *T. marchalianum* from a population genetics study (84). Note, the strains from
460 that study all cluster with JA005/F26399(Fig. S5). Both *T. marchalianum* and *T. breve* require further
461 study and taxonomic revision. Taxonomic revision is beyond the scope of the work presented here,
462 but is ongoing

463 As more fungi related to Groups B and C are discovered it is probable that these groups will be
464 described as separate genera. In addition to being divergent from Group A in the phylogeny, the
465 fungi in Group B have not been observed to produce the conidia typical of the genus. *Tetracladium*
466 *elipsoideum* and *T. globosum* are named for their ellipsoid and globose conidia. *T. psychrophilum*,
467 although described without conidia, does produce floating multiseptate elongated allantoid-lunate
468 conidia when submerged (Anderson personal observation). Sporulation in the two endobryophytic
469 strains (*T. sp.* AFCN889 and AFCN900) has not been observed. Based on strains included here,
470 including the ITS phylogeny below, it appears that Group B may predominantly contain psychrophilic
471 or psychrotolerant fungi. The sole representative of Group C, *T. sp.* T11Df, is particularly unusual in
472 that it grows as a yeast, producing short pseudohyphae in culture. All other species in the genus are
473 known in filamentous forms. Spores have not yet been observed in this species.

474 The family level phylogenetic relationships resolved in this study are in line with the results from
475 previous studies (85-87). Within the Leotiomyces, all clades identified by Johnston and colleagues
476 (88) and were represented here, are recovered. The family containing *Tetracladium*,
477 Vandijckellaceae (88, 89), was included in an analysis based on 15 concatenated sequences (88). The
478 tree herein differs from that result in the branching order of the Vandijckellaceae and helotioid taxa.
479 However, the corresponding nodes are not well supported in the Johnston tree where they receive

480 maximum 93% UFBoot, but are fully supported herein (100% UFBoot). The minimum support
481 considered reliable in ultrafast bootstrapping (UFBoot) in IQ-TREE is 95% (90, 91).

482 **Broad ecological and geographical diversity in *Tetracladium***

483 *Tetracladium*-like fungi are increasingly being reported from aquatic and terrestrial sources,
484 including from soils and as endobionts of plants, from around the world. Identification as
485 “*Tetracladium*-like” is frequently based only on ITS sequence data and ITS data can be useful to
486 identify species of *Tetracladium* in at least some cases (19). Here, from analysis of the *Tetracladium*-
487 like ITS sequences available in GenBank, it is clear that both ecological and geographical diversity are
488 wide-spread within the genus (Fig. 3). These sequences represent diversity in *Tetracladium* from 32
489 countries, regions, and territories, from sea level to high elevation, and from the Arctic to the
490 Antarctic (Table S5). It is striking how many *Tetracladium* sequences are coming from polar and
491 alpine regions, high elevations, or are glacier associated (Fig. 3). Frequently, fungi from these
492 extreme environments that were sequenced from soil, water or non-orchid plants are similar in ITS
493 to those from orchids from more temperate climates. Overall, patterns of strains from different
494 sources or lifestyles being distinct from each other are not observed. Rather, in most cases, the
495 ecologies and lifestyles are mixed among the groups of sequences most similar based on ITS. These
496 results suggest that multifunctionality is widespread across the genus. Further, *Tetracladium* fungi
497 are found in association with a broad diversity of plants (Table S5).

499 **Ecological clues from CAZymes and secondary metabolites**

500 The CAZyme and secondary metabolite profiles of *Tetracladium* species are more similar to each
501 other than to other taxa, regardless of ecology (Fig. 4). This suggests a strong role for phylogeny in
502 shaping the CAZyme and SM content of genomes within the genus. In comparison to genomes of
503 other Leotiomycetes and more distantly related ascomycetes, *Tetracladium* genomes are enriched
504 for PL domains which are associated with degradation of pectin and pectate. *Tetracladium* genomes
505 contain around 3-6 times more PL domains than the other fungi in the study which may be related
506 to their ecological role as aquatic hyphomycetes. Aquatic hyphomycetes are major decomposers of
507 leaves in streams and pectin and pectate are complex polysaccharides that are abundant in leaves.
508 In contrast, the genome of *Xylona heveae*, a horizontally transmitted endophyte of sapwood in
509 rubber trees, contains no predicted PL (92).

510
511 *Tetracladium* genomes contain a particularly large number of pectate lyase PL3-2 (EC 4.2.2.2) genes,
512 which is a feature they share with *A. tetracladia*, the only non-*Tetracladium* aquatic hyphomycete in
513 the study (Table S6). The PL repertoires of *Tetracladium* species and *A. tetracladia* are very similar

514 overall, however they are also qualitatively similar to the two *Cadophora* species in the study (Table
515 S6). The sequenced strain of *C. malorum*, was isolated from a deep sea shrimp from a depth of 2300
516 m below sea level at a hydrothermal vent along the Mid Atlantic Ridge (93), the other *Cadophora sp.*
517 was isolated as an endophyte of *Salix rosmarinifolia*; neither is expected to decompose leaves. Thus,
518 determining relative importance of ecology and phylogeny in shaping the PL content of these
519 genomes requires genomes from additional taxonomically and ecologically diverse fungi.

520

521 Fungal saprotrophs (of plants), endophytes, and plant pathogens are typically able to degrade plant
522 cell walls in order to enter a plant host or grow through a plant substrate and to obtain nutrition
523 from plants. Thus, some overlap in the CAZyme and SM content in the genomes of fungi that exhibit
524 these ecologies should be expected especially among taxa that are multifunctional (94). PL3-2, the
525 most abundant PL in *Tetracladium* genomes (above), is best known as part of the molecular arsenal
526 of plant pathogens including *Botrytis cinerea* and is highly expressed in developing infections (*e.g.*
527 tomato (95)). Also, pectic enzymes can elicit defense responses in plants (96). In *Tetracladium*,
528 expression of PL3-2, and other PLs, may require tight context dependent control, with high
529 expression during saprotrophy to degrade leaves, but no-to-low expression when living as
530 endobionts to avoid triggering plant defenses. The Dimethylcoprogens, nonribosomal peptide
531 synthetases involved in synthesis of siderophores (97) which have iron uptake and storage functions,
532 have also been reported as common among taxa within the Pleosporales, Dothideomycetes (98) an
533 order that includes many plant pathogens. Dimethylcoprogen, and siderophores generally, have
534 been associated with pathogenicity for some fungi including the corn pathogen *Cochliobolus*
535 *heterostrophus* and wheat pathogen *Fusarium graminearum* (99). However, iron homeostasis and
536 storage have other important roles in fungi, including resistance to reactive oxygen species (97, 99)
537 and the maintenance of mutualism in endophytic fungus–plant interactions (100). Going forward,
538 comparisons expression patterns associated with these PLs and SM clusters can help dissect how
539 organisms with similar molecular “toolboxes” selectively wield these tools appropriately for
540 saprotrophs, endobionts, and pathogens.

541

542 *Tetracladium* genomes are also rich in GH71 domains which are α -1,3-glucanases and their
543 associated non-catalytic CBM24 α -1,3-glucan/mutan binding modules, suggesting that these fungi
544 are well-suited to breaking down fungal cell walls; is an important component of the cell walls of
545 filamentous fungi and dimorphic yeast. As was the case for *S. sclerotiorum* and *B. cinerea* when
546 enrichment for GH71 in these fungi was first reported, it is unknown whether the abundant α -1,3-
547 glucanases in these genomes is associated degradation of the fungi’s own cell walls or those of

548 antagonistic fungi(101), or whether these enzymes play other roles entirely. Interestingly, α -1,3-
549 glucan is important in the matrix of fungal and bacterial biofilms (102) which can be disrupted by
550 glucanases (103, 104). Decomposing leaves in streams and the roots of plants are covered by
551 microbial biofilms and it is possible that enrichment for α -1,3-glucanase in *Tetracladium* genomes is
552 associated with interactions with those biofilms. Further, there is some evidence that α -1,3-
553 glucanases prevent plant detection of β -1,3-glucan in invading pathogens, negatively impacting
554 plant defensive responses. Transgenic rice plants expressing bacterial (105) or fungal (106) α -1,3-
555 glucanases demonstrate protection against fungal pathogens. Thus, it is also possible that these
556 enzymes are important in plant-fungal mutualism and beneficial to plants (107, 108).

557
558 *Tetracladium* genomes also contain more GH32 catalytic domains and associated CBM38 modules
559 than other taxon groups studied here. GH32 enzymes can function as invertases and also act on
560 inulin and fructose (77). Given the inulin-binding function of CBM28 the inulinase function may be
561 most enriched in *Tetracladium*. Inulin is a reserve carbohydrate in some plants that is stored in
562 roots, taproots, and bulbs. Some strains/species of *Tetracladium* were found to have these
563 components in a GH32+CBM38+CBM38+CBM38+GH32 conformation; unique among the taxa in this
564 study. These observations suggest that *Tetracladium* species obtain nutrition from inulin when living
565 as endobionts of plants. Studies of *Tetracladium* species as root endophytes are needed to test this
566 hypothesis.

567

568 **CONCLUSIONS**

569 The genomes of 24 *Tetracladium* and *Tetracladium*-like fungi, including representatives of all
570 described species, were sequenced and used to resolve relationships among the taxa and to improve
571 our understanding of ecological and genomic diversity in this group of ecologically important,
572 multifunctional fungi. All genome-sequenced *Tetracladium* and *Tetracladium*-like fungi in this study
573 form a monophyletic group, which may in time be subdivided into separate genera. From analysis of
574 ITS sequences from water, soil, and plants from around the world, it emerges that multifunctionality
575 may be widespread throughout the genus, that many species have multifunctional lifestyles.

576 Further, *Tetracladium* is frequently sampled from extreme and cold environments, suggesting that
577 these fungi may have important roles in those ecosystems and also may produce secondary
578 metabolites or enzymes of interest for industrial applications. Studies are needed to investigate the
579 terrestrial and endiobiont roles of *Tetracladium* fungi, including of where in plant roots these fungi
580 are found, whether they utilize inulin as a source of nutrition as endobionts, and how PL expression
581 is controlled in saprotroph and endiobiont contexts. Lastly, these fungi are more similar to each

582 other in genome content for SMs and CAZymes than they are to other taxa, regardless of variation in
583 the ecology of the fungi, suggesting that within *Tetracladium*, broad ecological diversity and
584 multifunctionality can be achieved among taxa using highly similar genomic toolkits.

585

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593

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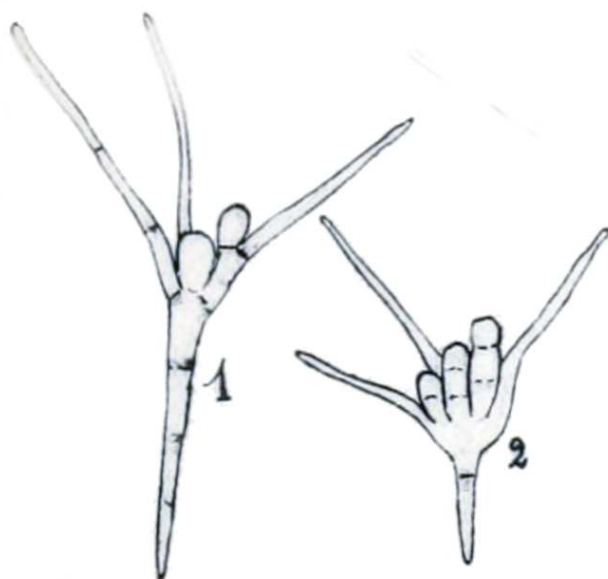
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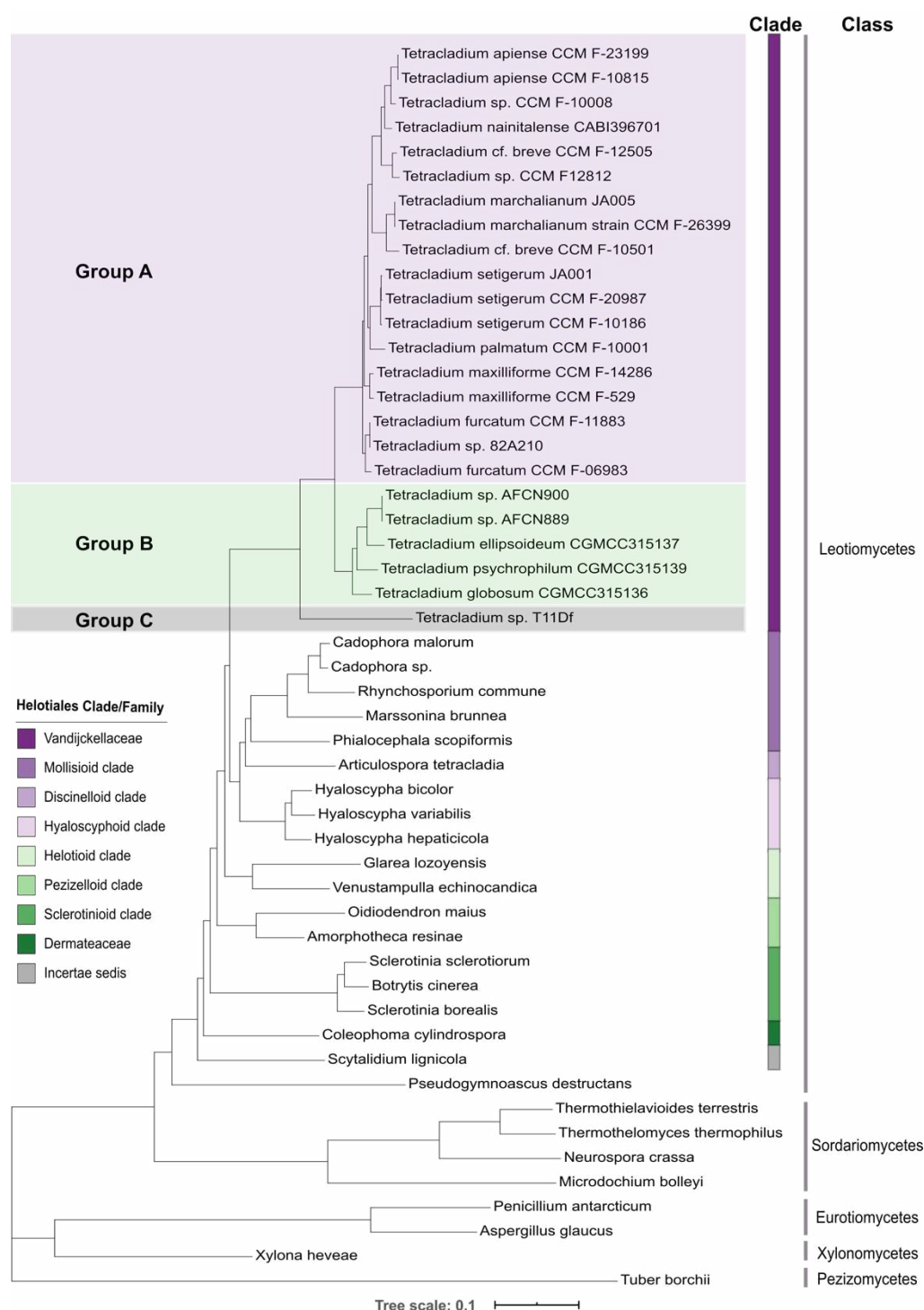
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856 **FIGURE 1**
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863 **Figure 1:** Conidia of *Tetracladium marchalianum* (1) and *T. cf. breve*(2), both originally described as
864 *T. marchalianum*. Illustrations by de Wildeman (17)(b.1866-d.1947). Modified from plate IV(17). For
865 reference, from the base of conidia 1 to the rounded terminus is around 35 μm .
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867 **FIGURE 2**

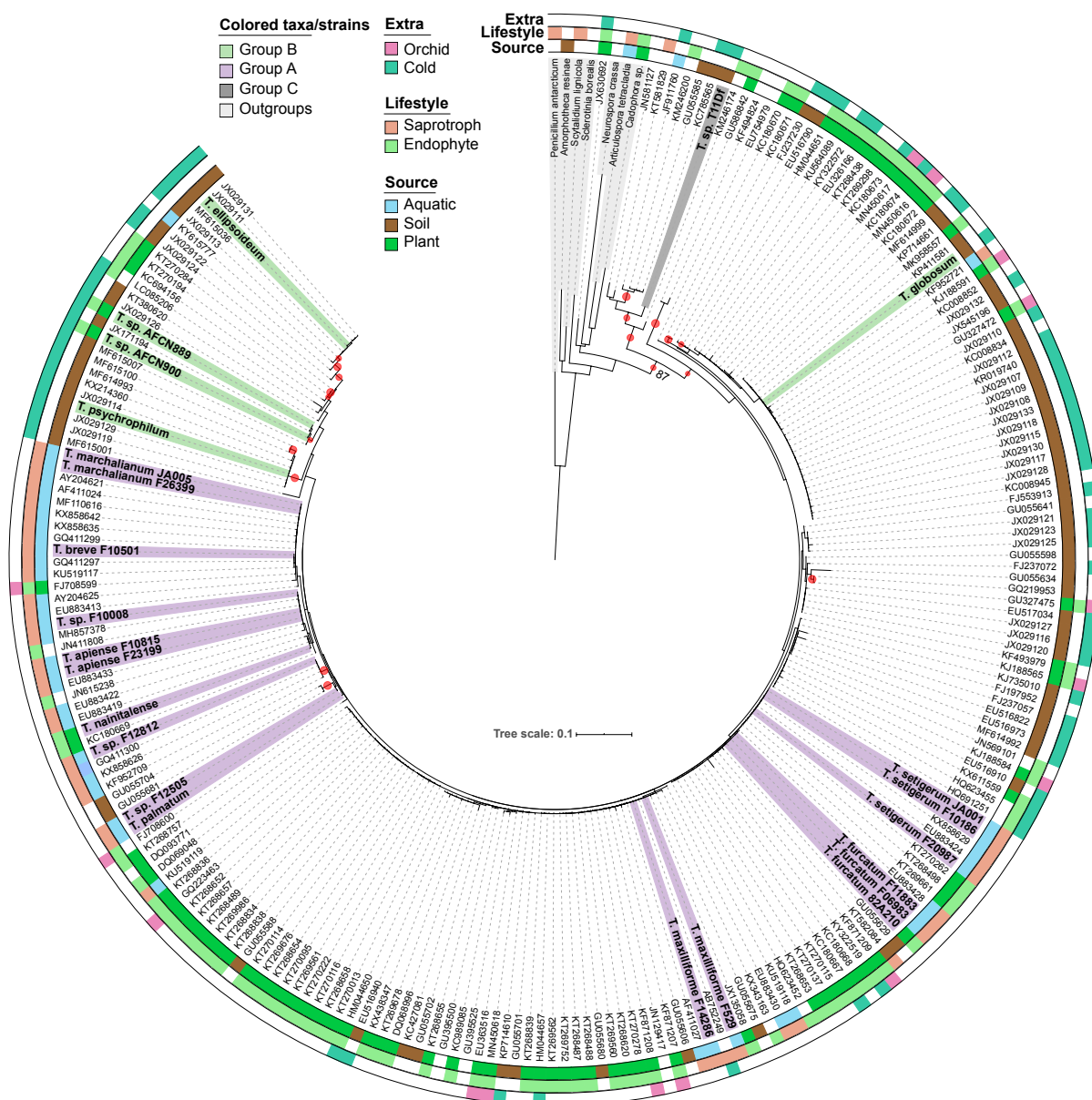


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Figure 2: Phylogeny of *Tetracladium* and *Tetracladium*-like taxa (Groups A, B, and C) within the Leotiomyces. Maximum likelihood phylogram inferred from 1820 single copy orthologs present in all 51 taxa in this analysis using the PMSF model in IQ-Tree. For fungi within the order Helotiales the color bar indicates the clade or family in which they belong, after Johnston *et al.* 2019 (details Table S2). All branches in this tree are fully supported (100% UFBoot and SH-aLRT). The tree scale represents the expected number of nucleotide substitutions per site. Phylogram drawn with *Tuber borchii* as the root.

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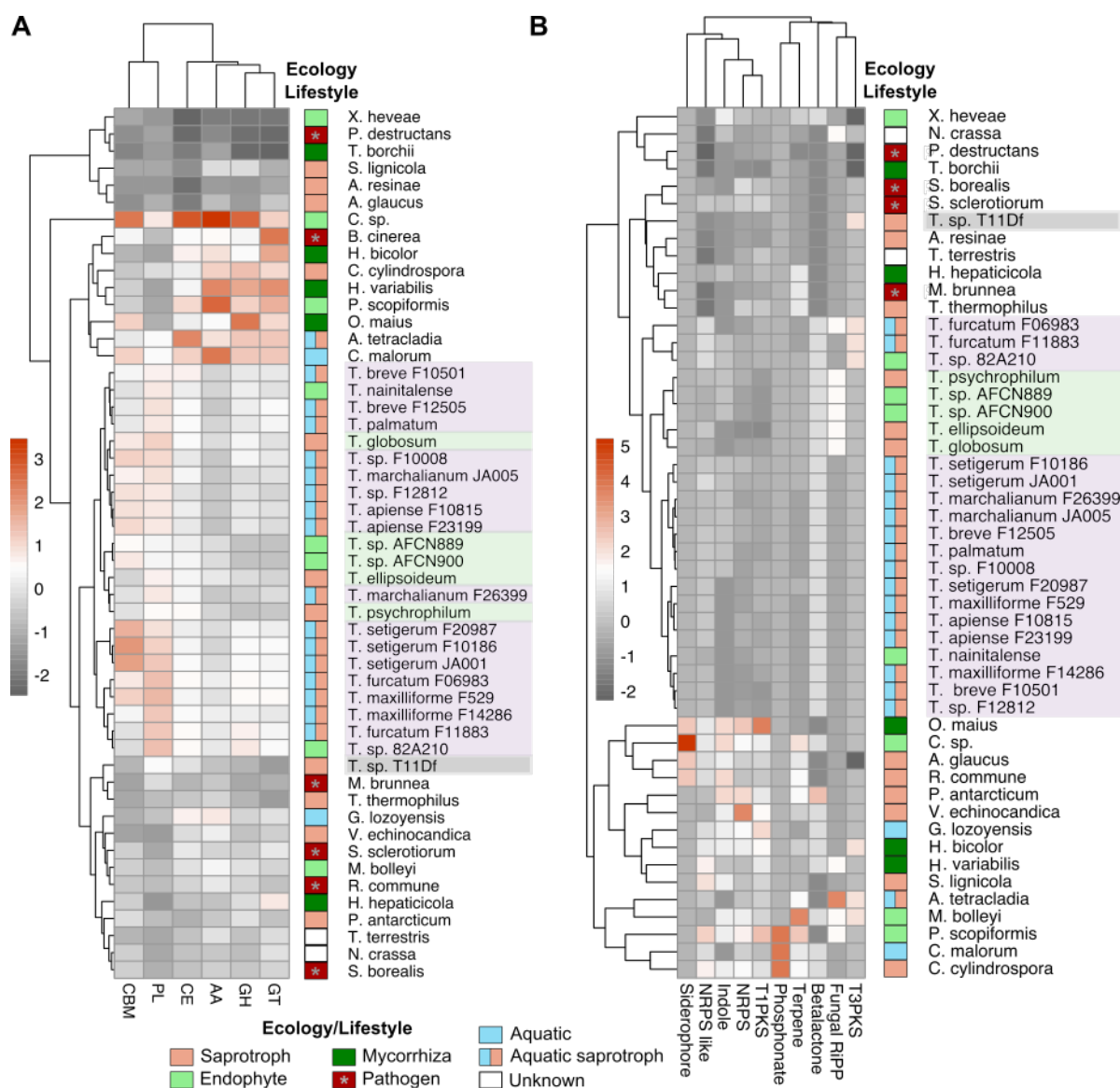
FIGURE 3



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Figure 3: ITS and ecological diversity within the *Tetracladium* and *Tetracladium*-like fungi based on ITS region data and sample information for 198 accessions from GenBank, the 24 newly sequenced genomes herein (Groups A, B, and C colored as in Fig. 2), and outgroups (grey). Maximum likelihood phylogram inferred using the model TIM3e+R3. Note: Only branches receiving non-parametric bootstrap support > 85% are highlighted in this figure. Sequences identified as “cold” in the Extra category originate from high latitude, high altitude, alpine, or glacier associated locales. The tree scale represents the expected number of nucleotide substitutions per site. Phylogram drawn with *Penicillium antarcticum* as the root.

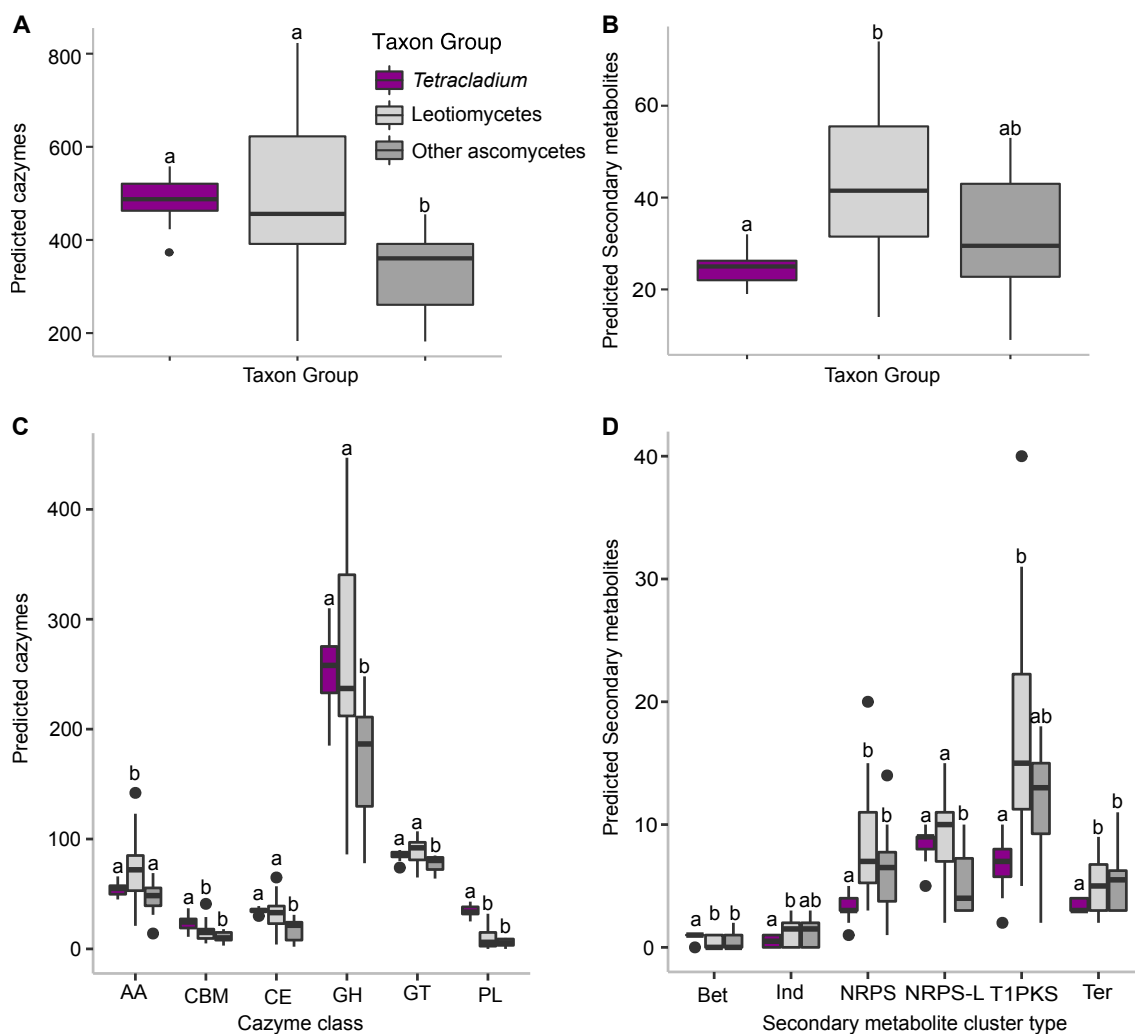
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Figure 4: *Tetracladium* and *Tetracladium*-like strains cluster together in analyses of the number of CBM and CAZyme domains (A) and secondary metabolite clusters (B) of each type regardless of ecology. Heat map colors are relative to scaled datasets with orange colors representing high abundances and grey colors representing lower abundances of the domain/cluster in each genome. Note: *P. destructans* is an animal pathogen, all other pathogens infect plants. CAZyme classes and associated modules: Auxiliary Activities (AA), carbohydrate binding modules (CBM), carbohydrate esterase (CE), glycoside hydrolase (GH), glycosyltransferase (GT), and polysaccharide lyase (PL).

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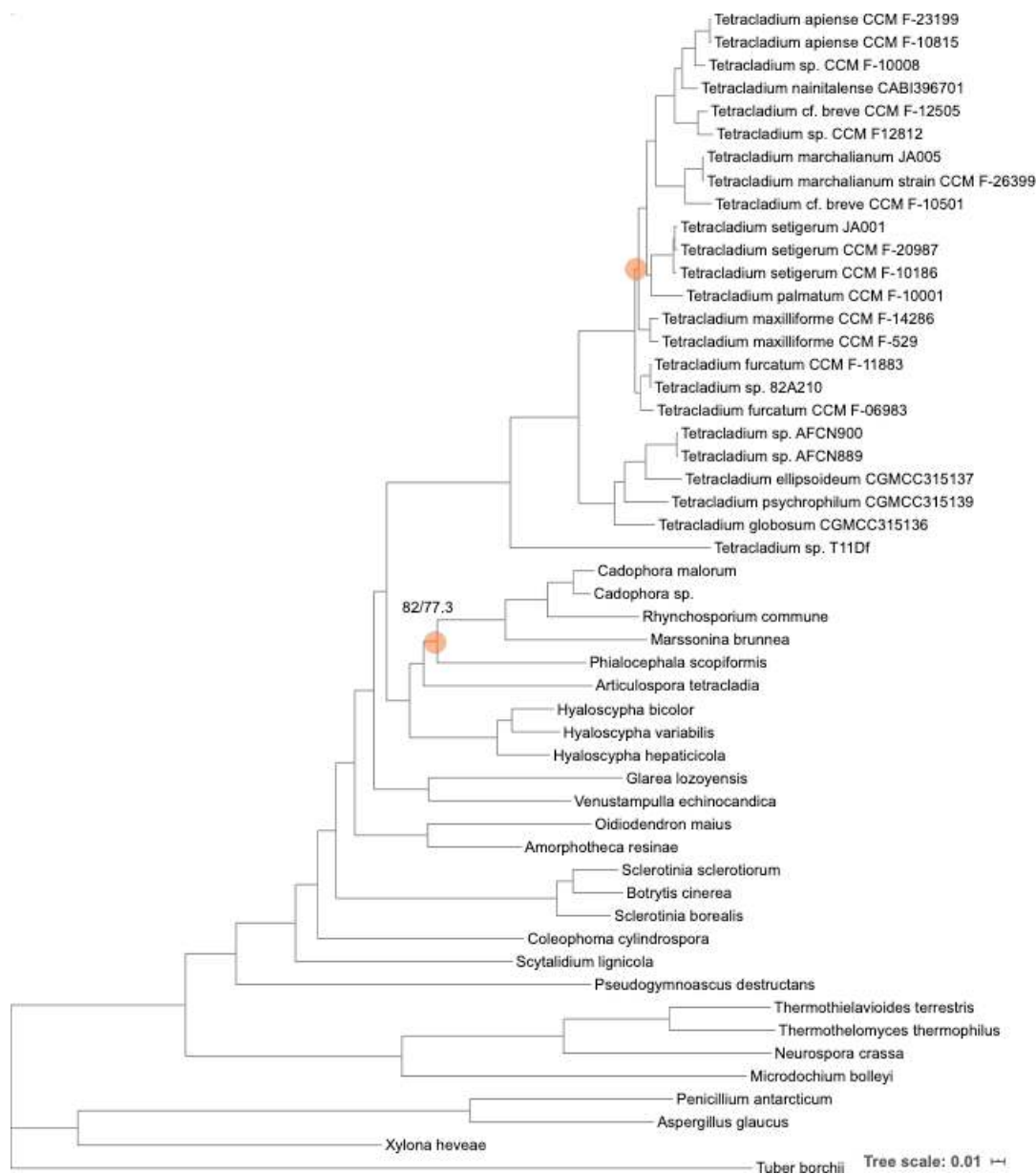


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Figure 5: Variation in total number of CAZyme and CBM domains (A), and SM clusters (B) identified in taxon groups containing *Tetracladium* and *Tetracladium*-like genomes (N=24), and the genomes of the other Leotiomycete (N=18) or other ascomycetes (N=8) in the study. Results were analyzed using analysis of variance with Tukey's HSD for comparisons among taxon-groups. In C and D, separate analyses were performed for each CAZyme or SM type. Taxon-groups sharing the same letter are not statistically different ($p > 0.05$). CAZyme classes and associated modules: Auxiliary Activities (AA), carbohydrate binding modules (CBM), carbohydrate esterase (CE), glycoside hydrolase (GH), glycosyltransferase (GT), and polysaccharide lyase (PL). SM cluster types: betalactone (Bet), indole (Ind), nonribosomal peptides (NRPS), NRPS-Like (NRPS-L) and terpene (ter). Other SM categories did not differ by taxon group (see Table S10).

921 FIGURE S1

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Figure S1. ML tree from analysis of the all-SCO dataset with model LG+F+I+G4. All branches received UFBoot values > 95% and SH-aLRT > 80% unless indicated (UFBoot%/ SH-aLRT%). The tree scale represents the expected number of nucleotide substitutions per site.

933 FIGURE S2

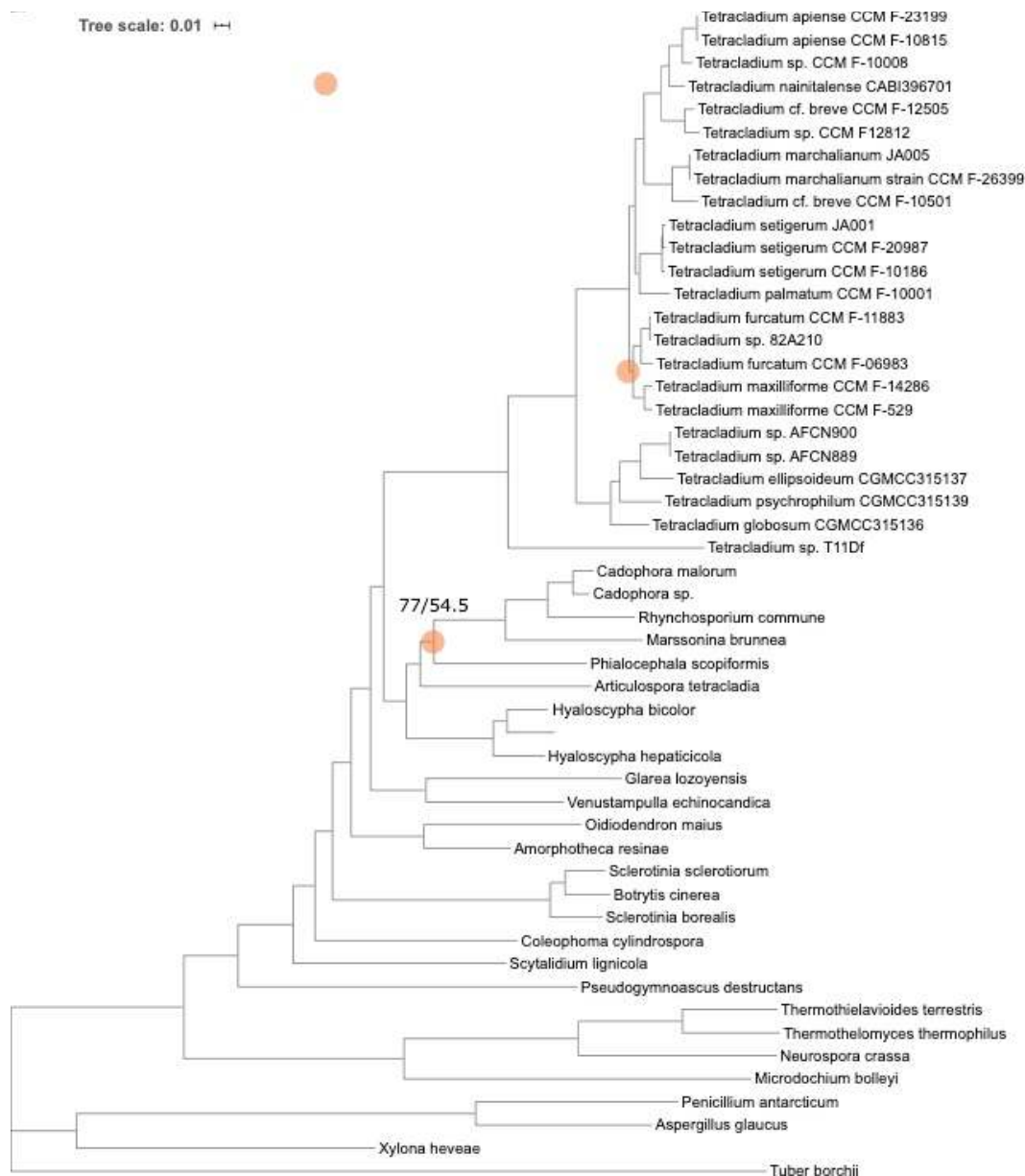
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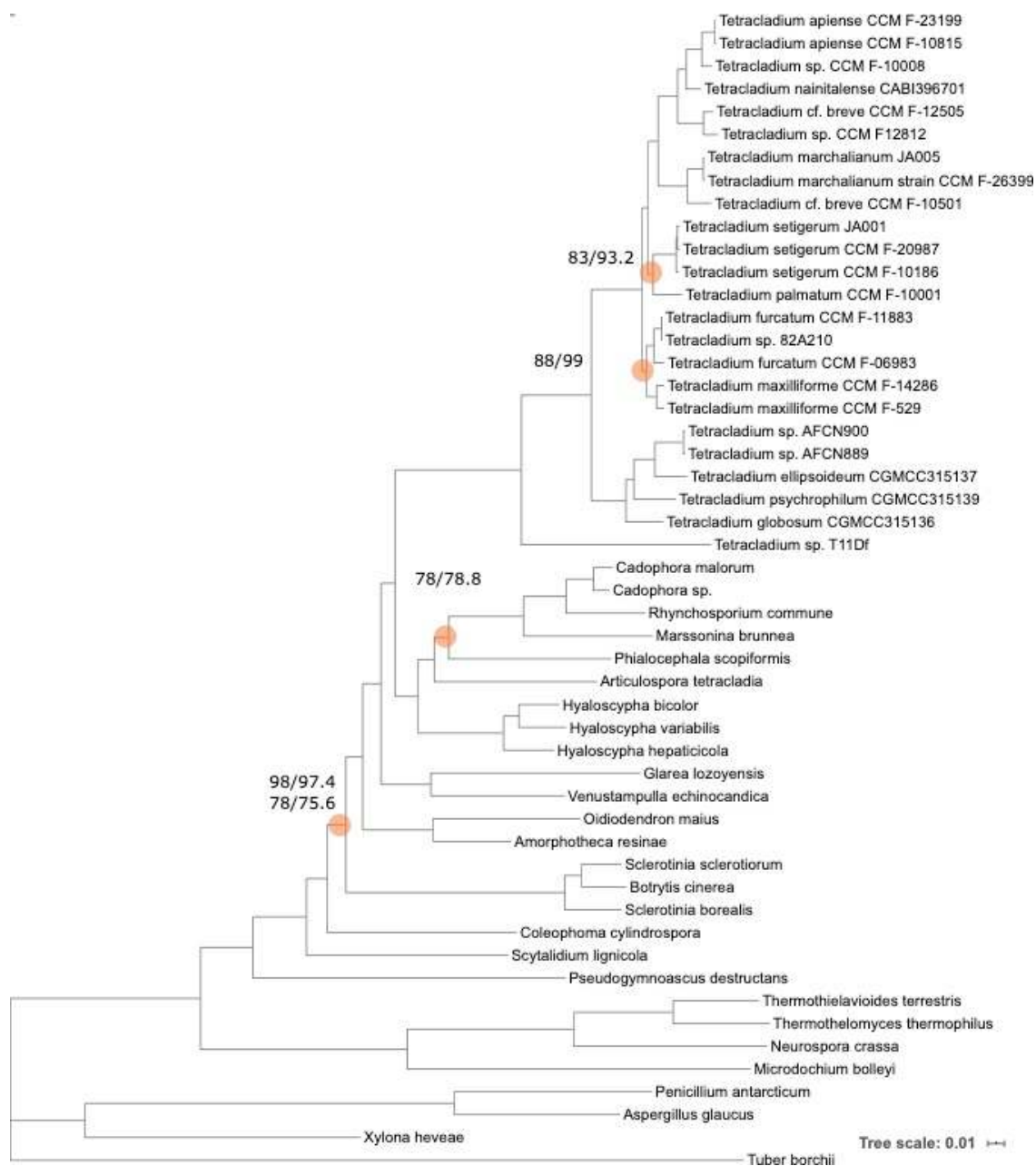
941 Figure S2. ML tree from analysis of the asco-SCO dataset with model LG+F+R6. All branches received

942 UFBoot values > 95% and SH-aLRT > 80% unless indicated (UFBoot%/ SH-aLRT%). The tree scale

943 represents the expected number of nucleotide substitutions per site.

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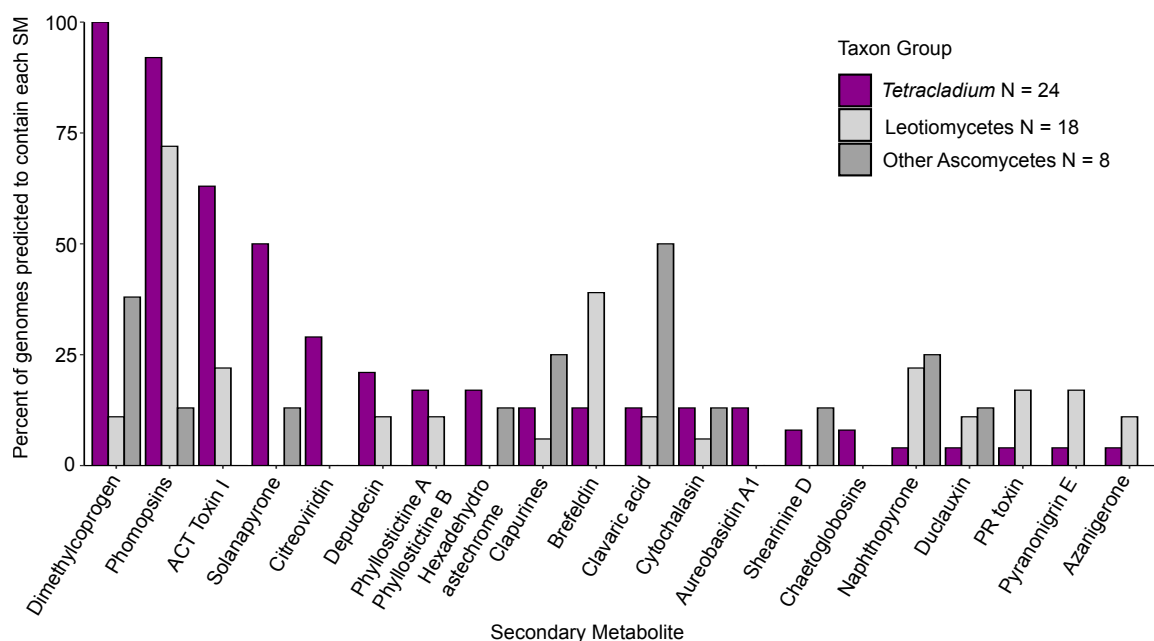
945 FIGURE S3
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Figure S4. Summary of ML trees from analysis of the fungi-SCO dataset model LG+F+I+G4 and using the FreeRate model. All branches received UFBoot values > 95% and SH-aLRT > 80% unless indicated. Support values are presented as UFBoot%/ SH-aLRT% for LG+F+I+G4 above, the FreeRate model below. Tree scale refers to the FreeRate model analysis. The tree scale represents the expected number of nucleotide substitutions per site.

957 FIGURE S4
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Figure S4. The 18 secondary metabolite clusters in *Tetracladium* genomes that were identifiable to type and the percent of genomes in each taxon-group where each SM type was also identified. Note the difference in sample size among taxon-groups.

968 FIGURE S5

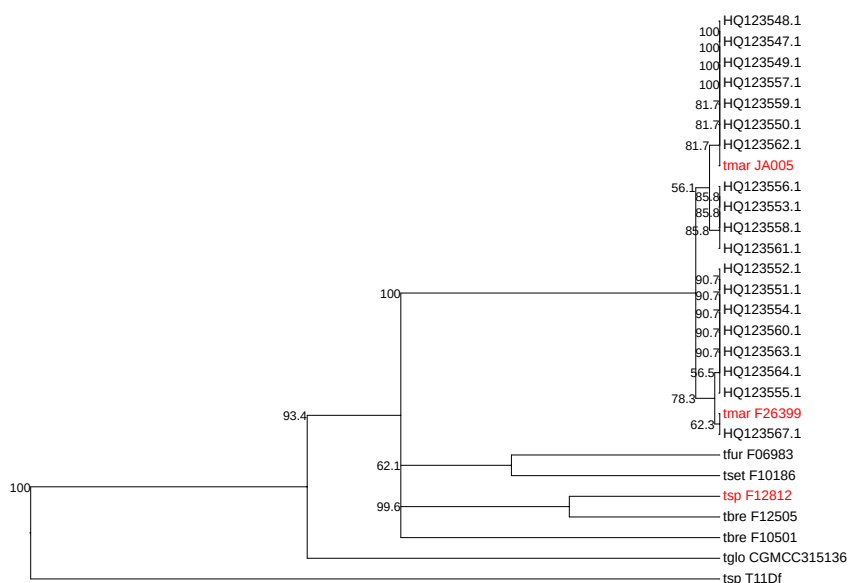
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Tree scale: 0.001



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976 Figure S5. UPGMA clustering of 46 beta-tubulin sequences. Analysis performed in Geneious 10.2.4

977 using a Jukes-Cantor genetic distance model branch support estimated using 1000 bootstraps with

978 resampling. All sequences with GenBank accessions starting with HQ come from the population

979 genetics study of Anderson and Shearer (84).

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