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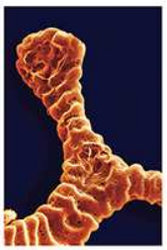

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Comparative genomics and phylogenomic investigation of the class Geoglossomycetes provide insights into ecological specialization and the systematics of Pezizomycotina

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ABSTRACT

Despite their global presence and ubiquity, members of the class Geoglossomycetes (Pezizomycotina, Ascomycota) are understudied systematically and ecologically. These fungi have long been presumed saprobic due to their occurrence in or near leaf litter and soils. Additionally, they lack an apparent association with other organisms, reinforcing this perception. However, observations of sporocarps near ericaceous shrubs have given rise to an alternative hypothesis that members of Geoglossomycetes may form ericoid mycorrhizae or ectomycorrhizae. This claim, however, has yet to be confirmed via microscopy or amplicon-based studies examining root communities. As a result, our current understanding of their ecology is based on cursory observations. This study presents a comparative analysis of genomic signatures related to ecological niche to investigate the hypothesis of an ericoid mycorrhizal or ectomycorrhizal ecology in the class. We compared the carbohydrate-active enzyme (CAZyme) and secondary metabolite contents of six newly sequenced Geoglossomycetes genomes with those of fungi representing specific ecologies across Pezizomycotina. Our analysis reveals CAZyme and secondary metabolite content patterns consistent with ectomycorrhizal (EcM) members of Pezizomycotina. Specifically, we found a reduction in CAZyme-encoding genes and secondary metabolite clusters that suggests a mutualistic ecology. Our work includes the broadest taxon sampling for a phylogenomic study of Pezizomycotina to date. It represents the first functional genomic and genome-scale phylogenetic study of the class Geoglossomycetes and improves the foundational knowledge of the ecology and evolution of these understudied fungi.

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Ascomycota; concordance; gene tree conflict; Geoglossaceae; metagenome; mutualism; mycorrhizae

INTRODUCTION

Geoglossomycetes is a small class of globally distributed earth-tongue-producing fungi in Pezizomycotina (Ascomycota). Members of the class have historically been the subjects of taxonomic and systematic dispute (Hustad and Miller 2015b) since their initial descriptions dating back to 1794 (Persoon 1794). The morphology of their club-shaped, darkly pigmented sporocarps is one of the relevant taxonomic features unifying the class (Hustad et al. 2013). Multiple occurrences of earth-tongue morphology within Ascomycota have led to uncertainty around the classification of Geoglossomycetes and species membership (Verkley 1994; Wang et al. 2006). Although once believed to ally with other earth-tongue-producing taxa of Leotiomycetes, Geoglossomycetes is now recognized as an independent and phylogenetically distinct lineage. Molecular phylogenetic analysis using a combination of nuclear and mitochondrial ribosomal genes and protein-coding genes (*nSSU, nLSU, mtSSU, TEF1, RPB1*, and *RPB2*) and broad sampling of taxa from Pezizomycotina led to the erection of the class Geoglossomycetes and the order Geoglossales (Schoch et al. 2009). Since its recognition as a class, studies have continued to clarify and improve the understanding of intraclass relationships within Geoglossomycetes (Hustad et al. 2011, 2013). Within the class's single family, Geoglossaceae, nine genera are currently recognized: *Geoglossum, Trichoglossum, Hemileucoglossum, Leucoglossum, Maasoglossum, Glutinoglossum, Sabuloglossum, Nothomitra*, and *Sarcoleotia*. The number of species divided among the genera remains unclear but is currently estimated at around 48 (Hustad and Miller 2015a).

Although multigene phylogenies have supported the monophyly of Geoglossomycetes and elucidated relationships between its genera, our understanding of the placement of the class within Pezizomycotina

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remains unclear. Whole-genome-scale data across the breadth of Pezizomycotina is limited yet necessary to resolve its class relationships. An absence of available genomes rom several classes, including Geoglossomycetes, Arthoniomycetes, Lichinomycetes, and Coniocybomycetes, has limited the inference of a robust and informative phylogeny of the subphylum until recently (Spatafora et al. 2017). Specifically, the inability to culture fungi from these lineages has impeded the generation of representative genomic data. Sequencing DNA directly from environmentally collected sporocarp material can lead to genomic assemblies contaminated with bacterial and plant sequences, obscuring the analysis of these data. However, in silico techniques now facilitate the extraction of targeted genomes from these types of metagenomic data (Quandt et al. 2015).

As with our systematic understanding, knowledge of the ecology of Geoglossomycetes is similarly lacking (Hustad et al. 2013). Studies were limited to cursory observations made by researchers, such as a presence in certain habitats or proximity to specific plant species. These fungi have long been presumed to be saprobic (Mchugh et al. 2001; Mleczko 2004; Richard et al. 2004), a hypothesis reinforced by their occurrence in habitats such as forests and pastures and their proximity to decaying wood and leaf litter (Griffith et al. 2013; Jordal et al. 2016; Kumar et al. 2013; Nannfeldt 1942; Ohenoja 1995). However, historical observations provide evidence that members of the class form mutualistic relationships with plants, challenging this idea and giving weight to a possible mycorrhizal, specifically ectomycorrhizal or ericoid mycorrhizal, ecology (Nitare 1982; Ohenoja 2000; Ohenoja et al. 2010).

An ectomycorrhizal (EcM) association is a symbiosis between a plant root and fungus in which the fungal partner forms a Hartig net surrounding root cells without penetrating the plant cell wall. This structure acts as the primary nutrient exchange site between the two organisms (Smith and Read 2010). This derivation of nutrients from the plant host reduces the need for associated fungi to produce expansive secretomes to degrade their substrate (Pellegrin et al. 2015). Within class Geoglossomycetes, an EcM ecology has been repeatedly hypothesized (Agerer 2006; Bougher 1995; Morris et al. 2008; Thoen et al. 2019; Wang et al. 2011), although never thoroughly tested or directly observed. Some ecological studies have, however, provided support for this hypothesis. A survey of stable isotopes occurring within fungi found extreme accumulation values in Geoglossomycetes, indicating an ectomycorrhizal ecology (Tedersoo et al. 2010). Members of Geoglossomycetes and many EcM fungi both lack the ability to grow in culture (Hustad et al. 2013). Although a lack of culturability is not a trait that uniquely identifies fungi as EcM, there is a hypothesis that the failure to meet specific nutritional requirements typically provided by the plant host makes axenic culturing of EcM fungi difficult or impossible. Members of Geoglossomycetes have been found in studies of root tip and soil communities through polymerase chain reaction (PCR) quantification assays and amplicon and sequencing studies (Bergemann and Garbelotto 2006; Gao and Yang 2016; Malysheva et al. 2018; Morris et al. 2008), providing further evidence of a mutualistic association.

In addition to EcM, an alternative ecology proposed for Geoglossomycetes is ericoid mycorrhizal (ErM). ErM associations are formed between the mycorrhizae and the plants in the heath family, Ericaceae. Whereas the hyphae of EcM fungi grow around the host cortex or epidermal cells, hyphae of ErM fungi penetrate the cell wall via hyphal coils, which act as sites for nutrient exchange (Smith and Read 2010). Although both are mycorrhizal symbioses, ErM and EcM differ in their biochemical and gene contents, and thus their ability to degrade plant material (Cairney and Meharg 2003; Read and Perez-Moreno 2003). ErM fungi possess the ability to grow as saprobes in addition to their mycorrhizal habit, which may explain the observed genetic and biochemical differences and ability to grow readily in axenic culture (Read and Perez-Moreno 2003). Nitare (1982) first proposed a parasitic or mycorrhizal relationship for *Geoglossum* after observing a co-occurrence with *Empetrum nigrum*, an ericaceous shrub. Species of *Sabuloglossum* have been observed in habitats alongside the ericaceous genera *Calluna* and *Vaccinium* (Beenken and Horn 2008; Hallgrímsson 1987; Nitare 1982; Ohenoja 1995; Tejklová et al. 2015), further supporting a possible ErM symbiosis. However, this co-occurrence could result from other factors such as a shared habitat or preferred conditions, including acidic and low-humus soils (Cairney and Meharg 2003; Read 1991). As with an EcM ecology or other mutualistic ecology, the hypothesis of an ErM ecology has a long historical standing in the study of Geoglossomycetes. This claim was recently tested by Baba et al. (2021) whose findings demonstrated the successful colonization of *Vaccinium* roots by *Sarcoleotia globosa* in vitro. The authors concluded that the association with *S. globosa* may extend to other nonericaceous plants, as the fungal DNA has been found in the roots of plants outside of Ericaceae. Although there is evidence for the capacity to form ErM, these claims have yet to be confirmed microscopically or via targeted amplicon-based environmental sequencing studies examining root communities.

Fingerprints of these ecological niches can be identified with comparative genomic methods as data become readily available. These approaches identify genes coding for a range of secretomes that may be synthesized as an adaptation to a specific habitat or substrate. One such group of genes can be annotated as carbohydrate-active enzymes (CAZymes). CAZymes are responsible for the metabolism of complex carbohydrates for nutrient acquisition (Lombard et al. 2014). Quantities of CAZyme-coding genes tend to show distinct patterns associated with specific ecological niches in fungi (Floudas et al. 2012; Kohler et al. 2015) due to their role in the degradation of plant cell wall material. An overall expanded repertoire of genes coding for CAZymes in fungi known to be plant-pathogenic or saprobic (Kohler et al. 2015). Due to their ecological life strategies, plant-pathogenic and saprobic fungi require the ability to degrade complex plant carbohydrates such as cellulose. Conversely, contractions in CAZyme families are common in mutualisms such as EcM, where the fungi are instead supplied with simple sugars from a plant partner. The CAZyme signature of ErM fungi has not been well characterized, as very few of these fungi have been examined from a comparative perspective (Grelet et al. 2016; Peter et al. 2016). However, initial studies have shown large numbers of genes encoding CAZymes in ErM fungi that may equal or even outnumber those found in saprobes (Grelet et al. 2016; Martino et al. 2018).

In addition to genes encoding for CAZymes, clues regarding a species' ecology can be found in genes for secondary metabolites. Secondary metabolites are bioactive compounds with low molecular weight responsible for a spectrum of biological functions and may reflect the organism's occupied ecological niche (Keller 2019). The role of secondary metabolites in the context of fungal ecology has not been categorized fully. As a result, a specific profile based on an organism's ecology cannot be predicted; however, we anticipate shared patterns reflected in fungi of a shared ecology.

Given the historical observations outlined and data collected in preliminary studies, we hypothesize that Geoglossomycetes possess a mutualistic ecology (EcM or ErM). We anticipate that members of Geoglossomycetes will show genomic signatures in genes coding for secondary metabolites that are more similar to those of EcM or ErM fungi than to those of saprobic fungi. By analyzing these specific genomic signatures, this study tests the proposed ecological life strategies historically assumed for members of Geoglossomycetes. As part of this work, we present the first whole draft genome sequences of six members of class Geoglossomycetes, including *Geoglossum cookeanum, Geoglossum glabrum, Glutinoglossum americanum, Nothomitra cinnamomea, Sabuloglossum arenarium*, and *Trichoglossum hirsutum*. We also present the first genome-scale phylogeny of Pezizomycotina to include Geoglossomycetes with the broadest taxon sampling of the subphylum to date. This research aims to add to our knowledge of both systematics and ecology of the class Geoglossomycetes.

MATERIALS AND METHODS

Collection, accessioning, and preservation.— *Trichoglossum hirsutum* (MICH-F-339092) was collected from Tyrone Township, Michigan, USA (43.259447, −85.688690). *Geoglossum cookeanum* (ILLS00171035) was collected from Great Smoky Mountains National Park, Swain, North Carolina, USA (35.5852, −83.3587). *Glutinoglossum americanum* (ILLS00171034) and *Geoglossum glabrum* (ILLS00121439) were collected from Great Smoky Mountains National Park, Sevier County, Tennessee, USA (35.707556, −83.381694 and 35.6667, −83.5833, respectively). *Nothomitra cinnamomea* (ILLS00171038) was collected from Bellafontaine, France (46.5595, 6.0645). *Sabuloglossum arenarium* (ILLS00171037) was collected from Veluwemeer, the Netherlands (52.368008, 5.631876).

After collection, *Trichoglossum hirsutum* was frozen (−20 C). All other specimens were air-dried or dried in a food dehydrator at ~40 C and accessioned with specimen voucher metadata into the ILLS Fungarium at the University of Illinois Urbana-Champaign (TABLE S2). Specimen metadata are freely available on the MyCoPortal (Miller and Bates 2017; MyCoPortal 2022).

DNA extraction and sequencing.— To extract genomic DNA, a mortar and pestle was used to grind sporocarp tissue. The Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was then used following the manufacturer's protocol with the following modifications: 8 µL of RNase A was added, and DNA was eluted into molecular-grade water. Sequencing libraries were constructed with the Illumina TruSeq DNA Library Prep Kit (Illumina, San Diego, California), and libraries were pooled. Sequencing of *T. hirsutum* was performed at the University of Michigan DNA Sequencing Core on the Illumina NextSeq platform, generating 150-bp paired-end reads. All other genomes were sequenced using the high-throughput Illumina HiSeq X Ten system. The initial sequence quality of individual libraries was assessed with FastQC 0.11.8 (Andrews 2010).

Genome assembly.—Raw FASTQ reads were trimmed of adapters and low-quality reads with Trimmomatic 0.39 (Bolger et al. 2014) using the following parameters: LEADING:3 TRAILING:15 SLIDINGWINDOW:4:10 MINLEN:36. De novo assembly of genomes into scaffolds was carried out using MEGAHIT 1.2.9 (Li et al. 2015) using the "meta-large" flag. Assemblies were filtered of small contigs ranging from 500 to 1000 bp. The minimum contig size for each assembly was determined by assessing the complexity of the metagenomic data. (TABLE S1).

Extracting target genomes from metagenomic data.—Sequencing from sporocarps resulted in metagenomic data composed of genomic data of the target fungus, nontarget bacteria, and other environmental contaminants. To determine a quick taxonomic annotation, filtered contigs were first assigned taxonomy by querying against the National Center for Biotechnology Information (NCBI) BLAST nucleotide database (Altschul et al. 1990). Hits with an e-value lower than $1e^{-5}$ were excluded from these results. Contigs were binned based on tetramer frequencies to train an emergent self-organizing map with Somoclu 1.7.5 (Wittek et al. 2013) to extract the target contigs. The resulting map was visualized with Databionic ESOM (Ultsch and Mörchen 2005), and taxonomic annotation of tetramers was overlaid onto the ESOM map. Based on the topology and taxonomic annotation, supervised binning of sequences was performed to extract contigs of the target genome. Assembly statistics were calculated with the Assemblathon script (Earl et al. 2011). Genome completion of the filtered assemblies was assessed by the presence of highly conserved proteins and genomic elements specific to fungi with FGMP 1.0.2 (Cissé and Stajich 2019) (TABLE 1).

Data retrieval and sampling.—To understand the placement of Geoglossomycetes within Pezizomycotina, we implemented a broad sampling approach. Additional genomes were acquired from the Joint Genome Institute's MycoCosm (Grigoriev et al. 2014) and NCBI GenBank (Benson et al. 2012) for comparative and phylogenetic analyses. In sampling for phylogenomic analysis, 108 additional taxa were selected to represent all families across each class within Pezizomycotina where available. Combined with the six new Geoglossomycetes genomes, this provided us with 114 total tips for phylogenetic inference. For the comparative analyses of genomic content, a subset of 53 taxa were selected to represent the diversity of ecologies from among the available genomes from Pezizomycotina (TABLE S2). This information was derived from literature and studies of the ecologies of these fungi.

Gene prediction and annotation.—Assembly sorting, gene prediction, functional annotation, and preparation for submission into GenBank were performed using Funannotate 1.8.5 (Palmer and Stajich 2017). Genomes of the additional taxa were functionally annotated alongside the Geoglossomycetes genomes. Secondary metabolite clusters were identified using antiSMASH 5.0 (Blin et al. 2019). The presence of carbohydrate-active enzymes (CAZymes) was detected in the annotated genomes using dbCAN2 (Zhang et al. 2018).

Phylogenetic reconstruction and analysis.—Genomes of the input 114 taxa were translated into protein data using the EMBOSS Transeq function (Madeira et al. 2022). Orthologous proteins were detected and clustered with Proteinortho 6 (Lechner et al. 2011). Eighty-four single-copy orthologous clusters present in 100% of all analyzed genomes were identified, then FASTA files of the clusters were obtained using the grab_proteins.pl script included with Proteinortho 6. Each cluster was then aligned individually using MAFFT 7.490 (Katoh and Standley 2013). Alignments were back translated with RevTrans 1.4 (Wernersson and Pedersen 2003) before trimming and gap removal with trimAl 1.2 (Capella-Gutiérrez et al. 2009) using the "gappyout" parameter. From each of the alignments, gene trees were estimated with RAxML-NG (Kozlov et al. 2019) using the GTR +GAMMA model and 100 bootstrap replicates. The resulting gene trees were run through ASTRAL 5.7.1 (Mirarab et al. 2014) to produce a coalescent-based species tree estimate. A conflict analysis was mapped onto the ASTRAL tree with PhyParts (Smith et al. 2015) using gene trees with bootstrap support of at least 50% (Hou et al. 2022; Koenen et al. 2019) and visualized with PhyPartsPieCharts (https://github.com/mossmatters/phyloscripts/tree/master/phypartspiecharts).

For comparison with the species tree, we also estimated a maximum-likelihood phylogeny with the combined 84-gene data set. The trimmed and aligned gene cluster FASTA files were concatenated using the catfasta2phyml.pl script (https://github.com/nylander/catfasta2phyml). The resulting concatenated alignment was composed of 186 606 alignment sites with 160 710 distinct alignment patterns. It was run through RAxML-NG using the GTR +GAMMA model and 300 bootstrap replicates.

Maximum-likelihood and coalescent trees were visualized with the Interactive Tree Of Life 4 (Letunic and Bork 2019).

Comparative genomic analysis.—Heatmaps and dendrograms were generated with the R package ComplexHeatmap (Gu et al. 2016). Values of CAZyme counts were centered before mapping, and unit variance scaling was applied to rows. Columns were clustered with Manhattan distance and Ward linkage method.

In order to visualize the variation in ecologies and CAZyme content across the phylogeny, we created a mirror tree in Mesquite 3.70 (Maddison et al. 2008) using the species tree topology. We used a parsimony criterion for trait mapping, as maximum likelihood is limited to two-character states.

Scripts and intermediate files used in this study are available on GitHub (https://github.com/tinamelie/Geoglossomycetes-genomics-workflow).

RESULTS

Genome size and annotated proteins.—Geoglossomycetes genomes in this study ranged from 25.7 to 36.2 Mb in size after assembly and removal of metagenomic contamination (TABLE 1; TABLE S2). The number of proteins found in each genome ranged from 7841 to 9562 (TABLE 1). *Nothomitra cinnamomea*, sister to the remaining sampled Geoglossomycetes (FIG. 1), stood out as the largest genome (36.2 Mb) while also possessing the most proteins (9562) and the lowest GC content (47.28%). FGMP-assessed completion ranged between 98.0% and 99.5% for all Geoglossomycetes assemblies.

Phylogenetic reconstruction of Pezizomycotina.—Across all 114 genomes, we recovered 84 single-copy orthologous clusters, which were used to construct the input gene trees for our phylogenetic analysis. Our ASTRAL coalescent tree supports Geoglossomycetes as a monophyletic group with 100% local posterior probability (FIG. 1). This topology agrees with our maximum-likelihood phylogeny (FIG. S1), where Geoglossomycetes was recovered as a clade with 100% bootstrap support. All relationships within the classes were resolved with high support. Lichinomycetes plus Coniocybomycetes were recovered as the sister group of Geoglossomycetes, with Xylonomycetes closely related to these three (FIG. 1). Our PhyParts concordance analysis found that most gene trees were concordant with respect to the intraclass topology, although many other portions of the species trees show extensive gene tree conflict (FIG. 2).

Comparative genomic analysis of CAZymes and secondary metabolite clusters.—Overall, we saw a contraction of genes coding for CAZymes in the six Geoglossomycetes genomes. Hierarchical clustering resulted in Geoglossomycetes grouping with both EcM and lichenized fungi due to shared contractions in many CAZyme families (FIG. 3). The mean number of CAZymes per genome in the Geoglossomycetes was lower than for any of the ecological groupings but most similar to that of the EcM fungi (FIG. S3). The pattern of variation across the phylogeny supports the idea that the low number of CAZyme-encoding genes in Geoglossomycetes represents a reduction compared with the ancestral state (FIG. S2). In addition to the contraction in CAZymes, these genomes show a contracted repertoire of genes coding for secondary metabolites (FIG. 4). This profile is shared with EcM fungi while diverging from lichenized fungi and all other ecological groups tested in our comparative genomic framework.

DISCUSSION

Consistent with previous work, members of Geoglossomycetes form a well-supported monophyletic group. We also recover the same relationships

Table 1. Genomic content and statistics of genomes generated in this study.

Genome	Assembly size (Mb)	Protein-coding genes	GC content (%)	Contigs	Longest contig (bp)	N_{50} length (bp)	FGMP completion (%)
Geoglossum cookeanum	27.9	7841	49.9	752	263 159	59 167	98.0
Geoglossum glabrum	28.3	8143	49.71	1875	133 613	29 050	99.2
Glutinoglossum americanum	25.7	7996	49.91	1319	342 060	75 377	98.0
Nothomitra cinnamomea	36.2	9562	47.28	772	440 952	110 326	99.5
Sabuloglossum arenarium	27.2	8689	48.64	1087	219 638	45 306	99.5
Trichoglossum hirsutum	28.8	8519	51.17	4948	81 679	11 852	98.7

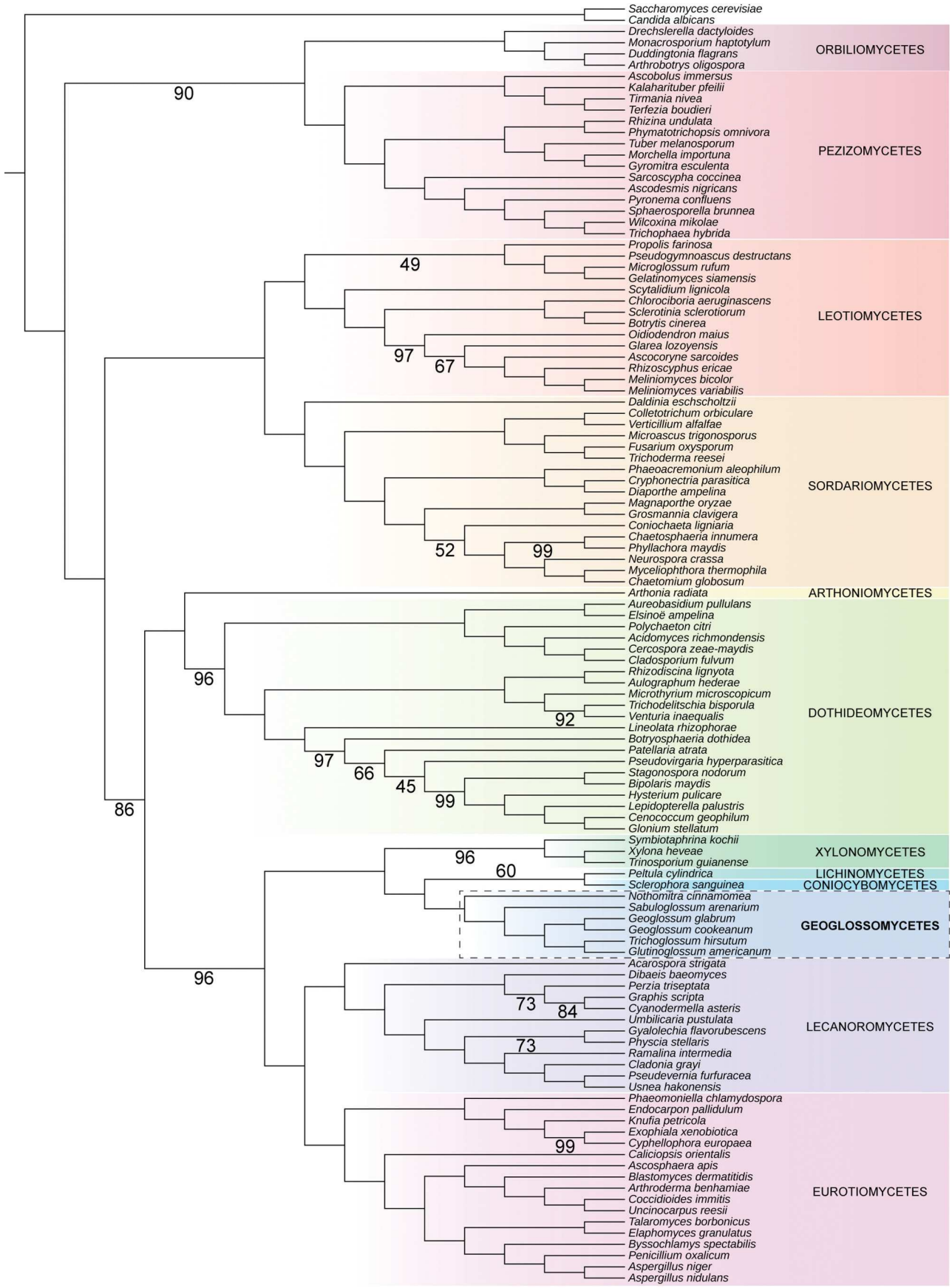

Figure 1. Multispecies coalescent tree of Pezizomycotina estimated with ASTRAL based on 84 single-copy gene trees. All branches have 100% local posterior probability except where noted.

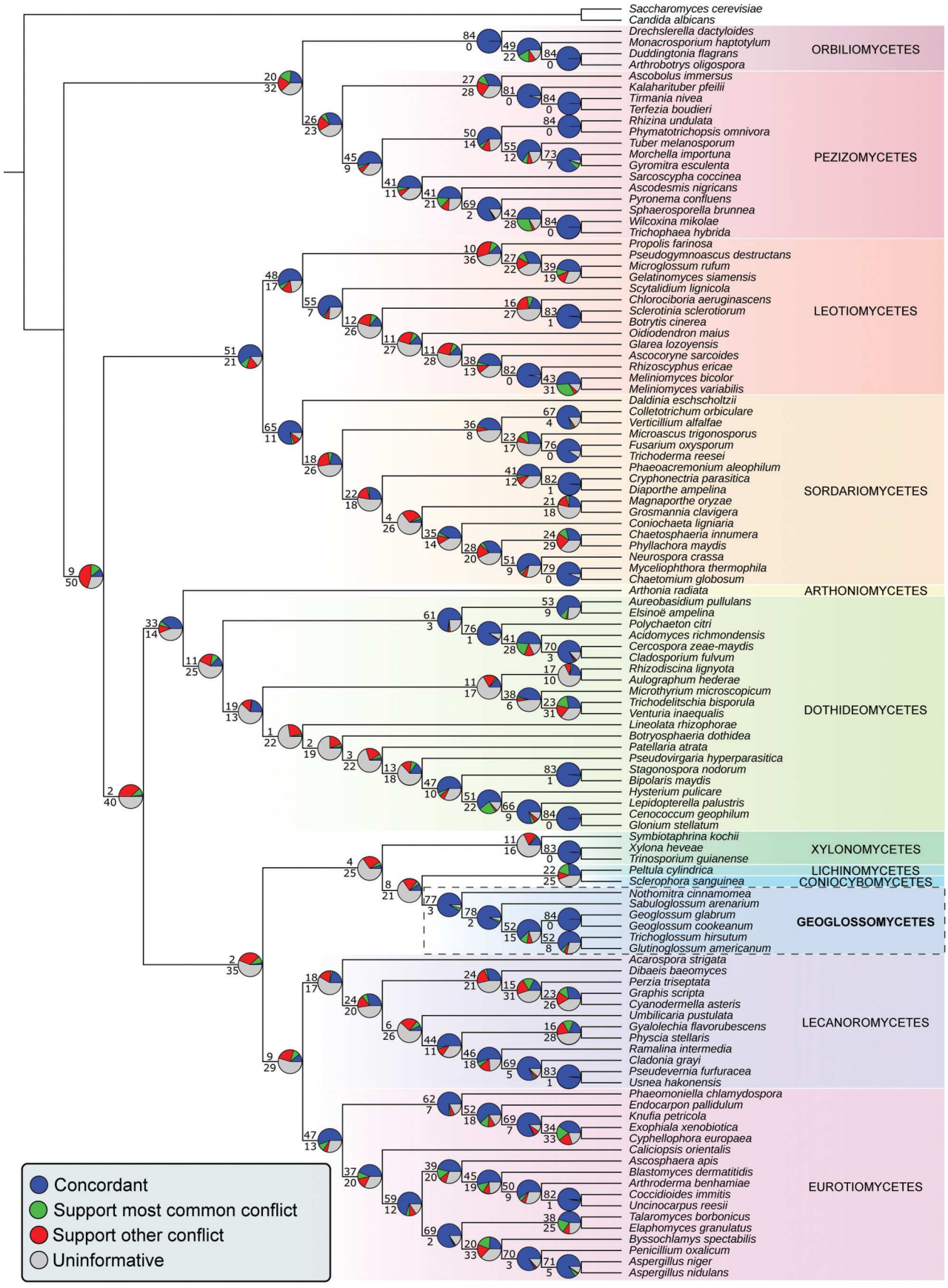

Figure 2. Summary of concordance among gene trees on the ASTRAL topology. The numbers on each branch represent concordant gene trees out of 84 (top) and the number of conflicting gene trees out of 84 (bottom). The pie charts show the proportion of gene trees in concordance (blue), supporting the dominant conflicting topology (green), supporting other conflicting topologies (red), and uninformative or have less than 50% bootstrap support (gray).

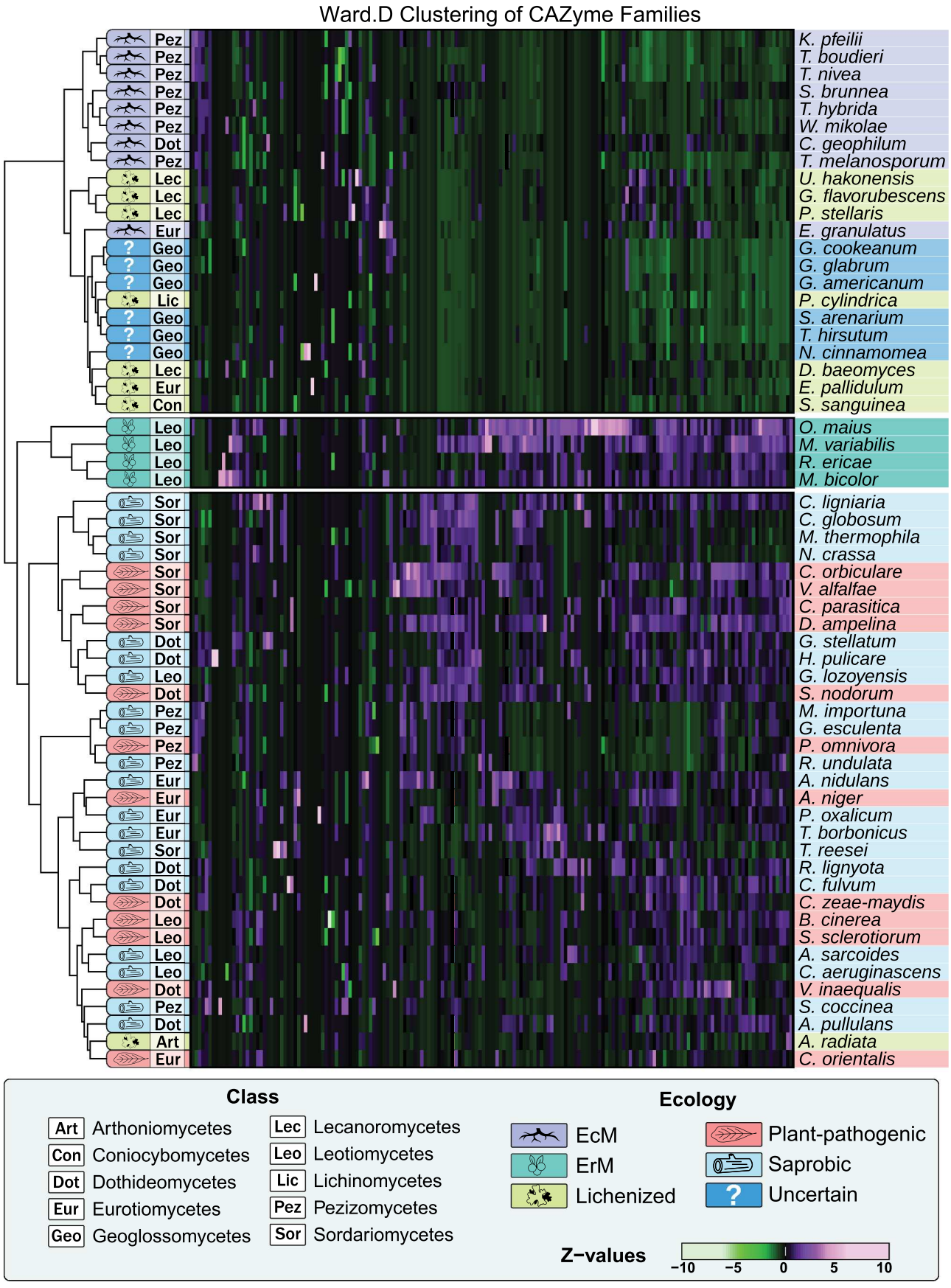

Figure 3. Members of Geoglossomycetes cluster with symbiotic fungi from Pezizomycotina based on CAZyme content. Heatmap and dendrogram were generated from 20 216 detected CAZyme-coding genes comprising 195 detected CAZyme families from 59 taxa. Values of CAZyme counts were centered prior to mapping, and unit variance scaling was applied to rows. Columns were clustered with Manhattan distance and Ward linkage.

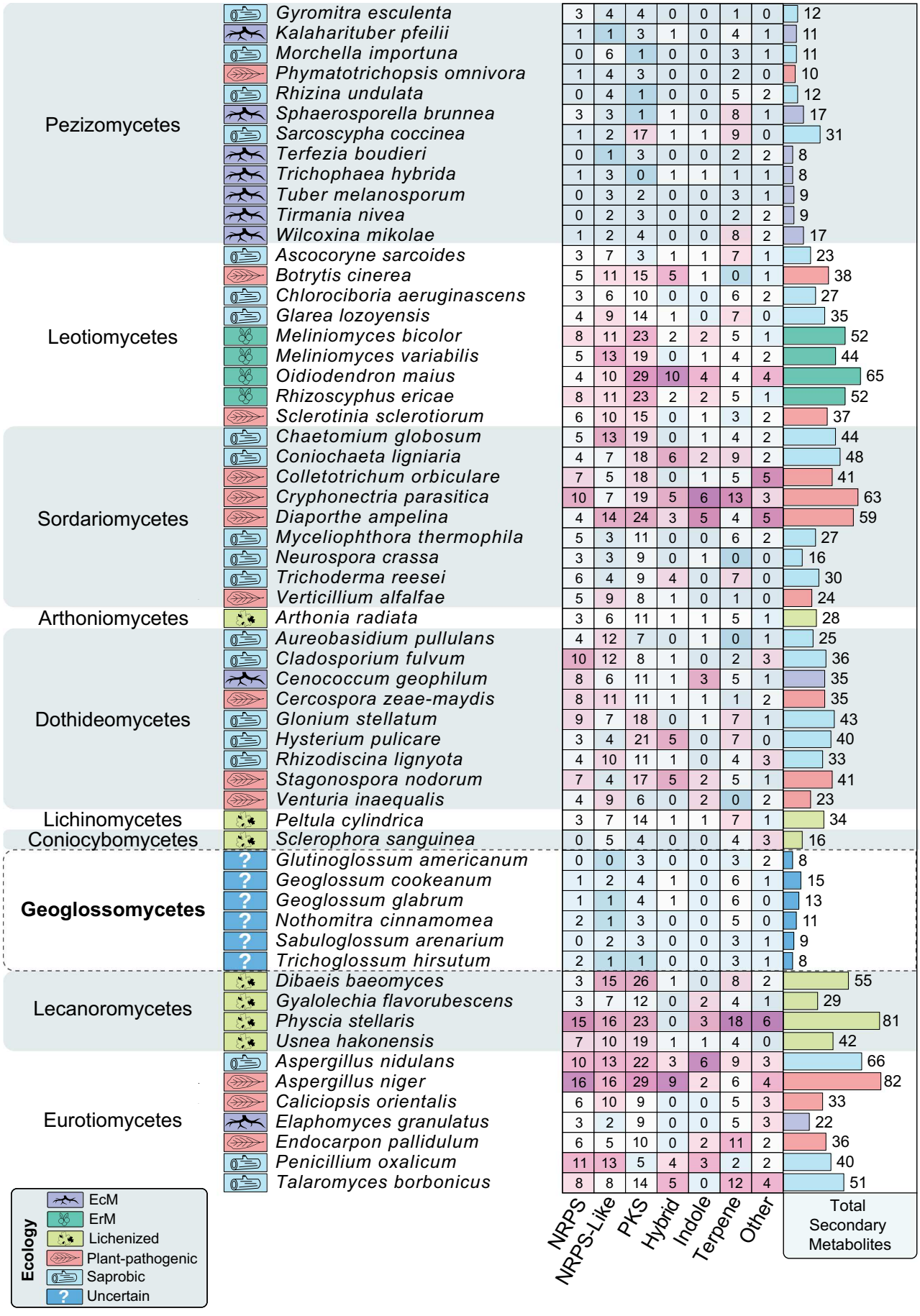

Figure 4. Fungi from class Geoglossomycetes share a reduced repertoire of secondary metabolite–encoding genes with EcM fungi. Secondary metabolite clusters were predicted by antiSMASH from taxa representing various ecologies across Pezizomycotina. Cells are colored by their centered and scaled column values, with lower values in blue and higher values in red.

among the genera as Hustad et al. (2013), albeit with higher support from our genome-wide data set. In addition, we find strong agreement among the gene trees for these intraclass relationships (FIG. 2). Nevertheless, our study includes only five of the nine described genera of Geoglossomycetes. Future work would benefit from the inclusion of representatives from the remaining genera, *Hemileucoglossum, Leucoglossum, Maasoglossum*, and *Sarcoleotia*, as well as expansion of sampling within the genera to test their monophyly.

We found the class placement well supported and mostly congruent with what is currently proposed for Pezizomycotina from analyses of genome-scale and multigene phylogenies (Spatafora et al. 2017). It does, however, differ from what was proposed when Geoglossomycetes was described (Schoch et al. 2009). Our use of whole genomes provided more data markers (84 vs. 6) than previous multigene studies of the group, which likely contributed to the shift in topology. In addition, our tree included the classes Coniocybomycetes and Lichinomycetes, which we found to be sister to Geoglossomycetes. There is clear conflict across the gene trees at the class-level nodes, as demonstrated by our concordance analysis. Therefore, it is unsurprising to estimate differing relationships with different sets of gene markers, especially in parts of the tree where genes disagree more than they agree.

Our sampling was limited by the availability of genomes from other classes within Pezizomycotina. We specifically lacked genomes from Laboulbeniomycetes, and only a single genus represented each of the classes Arthoniomycetes, Coniocybomycetes, and Lichinomycetes. Additionally, the availability of genomes in Orbiliomycetes and Xylonomycetes did not allow for a more thorough sampling of these classes. Future whole-genome studies examining the topology of Pezizomycotina would benefit from expanded sampling in these classes and the inclusion of members of Laboulbeniomycetes.

CAZymes and secondary metabolite contents.— Based on the comparative CAZyme and secondary metabolite analysis, it is unlikely that Geoglossomycetes have a saprobic ecology. The sampled members of Geoglossomycetes showed significantly lower CAZyme content compared with the selected fungi representing this ecological niche (FIG. S3). Members of Geoglossomycetes did not group with saprobes in the hierarchical clustering analysis of CAZyme family counts (FIG. 2). We specifically do not see a profile of enzymes necessary to facilitate plant matter degradation in a saprobic ecology. The overall contraction in CAZyme content shared by genomes of Geoglossomycetes, lichenized fungi, and EcM fungi supports a mutualistic ecology. The observed reduction of CAZymes in Geoglossomycetes suggests that they cannot independently break down plant material and implies the facilitation of a host for nutrient acquisition.

Our ancestral state reconstruction suggests multiple independent shifts in the number of CAZyme-encoding genes across the evolutionary history of Pezizomycotina (FIG. S2). It traces an evolutionary history of low CAZymes in Geoglossomycetes to the node it shares with its sister taxa, the lichenized classes of Lichinomycetes and Coniocybomycetes. A lichenized ancestry for these classes has been hypothesized for many fungal lineages across Pezizomycotina (Lutzoni et al. 2001) and appears to be the case for the large clade including Geoglossomycetes, Lecanoromycetes, and Eurotiomycetes, at least with the taxa sampled (FIG. S2). This ancestral state would have reduced the need for plant matter degradation, resulting in a contraction of the genes coding for CAZymes. The resulting reduction may have contributed to the class's shift to a mutualistic ecology, as its enzymatic ability to facilitate nutrient acquisition was diminished. Of course, this study has a small sample of the true extant diversity of this group; accurate state reconstruction requires thorough sampling, so further study is necessary.

Despite this shared evolutionary history, it is unlikely that Geoglossomycetes form a lichenized symbiosis, as there have been no previous observations or morphological indications of lichenization (such as the presence of a photobiont) in any of these taxa. In particular, the Geoglossomycetes cluster with the EcM taxa in the hierarchical analysis of CAZyme family counts, suggesting that it is a more likely ecology than ErM fungi. In addition, Geoglossomycetes also share a profile of a reduced number of gene clusters involved in secondary metabolites with other tested EcM fungi, a trait previously observed in fungi of this ecology (Martin et al. 2010; Peter et al. 2016; Quandt et al. 2015). By contrast, lichenized fungi have relatively large numbers of gene copies in these secondary metabolite clusters (FIG. S3).

Although our analyses show the most similarities between Geoglossomycetes and EcM compared with the other ecologies tested, we cannot confidently establish class members as EcM. We observed a notable difference between Geoglossomycetes and the EcM taxa we analyzed: Geoglossomycetes lack genome size expansion, whereas most other EcM fungi in Ascomycota exhibit such expansion. The genome size found in the sequenced genomes of our target Geoglossomycetes fell within the average range of

non-EcM fungi within Pezizomycotina. Most EcM fungi in Ascomycota, however, have significant expansions in genome size (Martin et al. 2010; Murat et al. 2018; Peter et al. 2016; Quandt et al. 2015).

Despite the various lines of evidence that align with an EcM ecology for Geoglossomycetes in our study, it is notable that one member of the clade (*Sarcoleotia globosa*) has been shown to form ErM in the laboratory (Baba et al. 2021). This species does not have a genome assembly and thus was not included here. Other phylogenetic studies, however, indicate that *Sarcoleotia* together with *Nothomitra* may be the sister group to the rest of the Geoglossomycetes (Hustad et al. 2013). We also recovered *Nothomitra* as the sister group, and if *Sarcoleotia* is confirmed to fall in this clade, it may represent a shift from an EcM ecology (as our study suggests for most Geoglossomycetes) to an ErM ecology. Still, a much-expanded taxon sampling together with ecological studies will be needed to elucidate the evolutionary history of mycorrhizal associations in Geoglossomycetes and any shifts between hosts.

If indeed most Geoglossomycetes are EcM, the question remains as to their most likely host or suite of hosts in natural populations. Previous researchers have hypothesized that *Geoglossum glabrum, Trichoglossum hirsutum*, and *Sarcoleotia globosa* are moss-associated (Kučera et al. 2008; Ohenoja 1995). Additionally, there are examples of many historical observations of the co-occurrence of Geoglossomycetes with bryophytes (Durand 1908; Hustad et al. 2014; Jumpponen et al. 1997; Schumacher and Sivertsen 1987; Tejklová et al. 2015). In addition to co-occurrence with bryophytes, members of Geoglossomycetes have been observed in habitats alongside the fungal families Clavariaceae, Entolomataceae, and Hygrophoraceae (Beenken and Horn 2008; Evans 2004; Mchugh et al. 2001; Mitchel 2010; Newton et al. 2003). Although there could be a complex mutualism at play, the shared habitat could be merely a by-product of overlap in suitable growing conditions.

An association with bryophytes would be consistent with the observed reduction of enzymes and secondary metabolites in the more permissive composition in the cell walls of bryophytes (Carella and Schornack 2018). It appears that limited digestion is required for symbiosis with bryophytes, which reduces the need for cell wall–degrading enzymes in the fungal partner (Pressel et al. 2010). Ideally, a comparative analysis of genomes of bryophyte-associated members of Pezizomycotina would have been beneficial to test this hypothesis. However, there are currently no available genomes representing bryophilous fungi and, therefore, no genomic characterization of the bryophilous ecology. There is also a general lack of research regarding the nature of the interaction between mosses and the fungi with which they associate (mutualism vs. parasitism) (Davey and Currah 2006). Future work examining a possible connection between Geoglossomycetes and mosses would be valuable in understanding the ecology of this class and potential mechanisms of mutualism. Comparative studies that include genomic data from the genus *Sarcoleotia* will also benefit studies of ecological diversity across the class.

Although our study of Geoglossomycetes is relevant from an ecological perspective, conservation should also be considered. Unfortunately, members of Geoglossomycetes appear on the IUCN Red List (International Union for Conservation of Nature Red List of Threatened Species) as near-threatened or vulnerable (Jordal 2019a, 2019b, 2019c). Their sensitivity to changing conditions has contributed to the threat of their disappearance (Hustad et al. 2013). In addition to their value as indicator species in grassland health studies (Mchugh et al. 2001), this study has shown their potential value as mutualists in their environments. Furthering our understanding will allow informed conservation efforts in the future.

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DATA AVAILABILITY STATEMENT

Workflows and scripts used to generate these data are publicly available on Tina Melie's GitHub page: https://github.com/tinamelie/Geoglossomycetes-genomics-workflow. Assembled and annotated genomes were deposited in NCBI GenBank (TABLE S2).

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