Horizontal gene cluster transfer increased hallucinogenic mushroom diversity

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Abstract:

Secondary metabolites are heterogeneous natural products that often mediate interactions between species. The tryptophan-derived secondary metabolite, psilocin, is a serotonin receptor agonist that induces altered states of consciousness. A phylogenetically disjunct group of mushroom-forming fungi in the Agaricales produce the psilocin prodrug, psilocybin. Spotty phylogenetic distributions of fungal compounds are sometimes explained by horizontal transfer of metabolic gene clusters among unrelated fungi with overlapping niches. We report the discovery of a psilocybin gene cluster in three hallucinogenic mushroom genomes, and evidence for its horizontal transfer between fungal lineages. Patterns of gene distribution and transmission suggest that psilocybin provides a fitness advantage in the dung and late wood-decay niches, which may be reservoirs of fungal indole-based metabolites that alter behavior of mycophagous and wood-eating invertebrates. These hallucinogenic mushroom genomes will serve as models in neurochemical ecology, advancing the prospecting and synthetic biology of novel neuropharmaceuticals.

16 Secondary metabolites (SMs) are small molecules that are widely employed in
17 defense, competition, and signaling among organisms (Raguso et al. 2015). Due to
18 their physiological activities, SMs have been adopted by both ancient and modern
19 human societies as medical, spiritual, or recreational drugs. Psilocin is a
20 psychoactive agonist of the serotonin (5-hydroxytryptamine, **5-HT**) -2a receptor
21 (Halberstadt and Geyer 2011) and is produced as the phosphorylated prodrug
22 psilocybin by a restricted number of distantly related mushroom forming families of
23 the Agaricales (Bolbitiaceae, Inocybaceae, Hymenogastraceae, Pluteaceae) (Allen
24 2010 May 19; Dinis-Oliveira 2017). Hallucinogenic mushrooms have a long history
25 of religious use, particularly in Mesoamerica, and were a catalyst of cultural
26 revolution in the West in the mid 20th century (Nyberg 1992; Letcher 2006).
27 Psilocybin was structurally described and synthesized in 1958 by Albert Hoffman
28 (Hofmann et al. 1958), and a biosynthetic pathway (Fig. 1B) was later proposed
29 based on the transformation of precursor molecules by *Psilocybe cubensis* (Agurell
30 and Nilsson 1968). However, prohibition since the 1970s (21 U.S. Code § 812 -
31 Schedules of controlled substances) has limited advances in psilocybin genetics,
32 ecology, and evolution. There has been a recent resurgence of the hallucinogen
33 research in the clinical setting. Brain state imaging studies of psilocin exposure have
34 identified changes in neural activity and interconnectivity that underlie subjective
35 experiences, and therapeutic trials have investigated psilocybin's potential for
36 treating major depression and addictive disorders (Griffiths et al. 2011; Carhart-
37 Harris et al. 2012; Petri et al. 2014; Carhart-Harris et al. 2016; Johnson et al. 2017).
38 While the ecological roles of psilocybin, like most SMs, remain unknown, psilocin's
39 mechanism of action suggests metazoans may be its principal targets.
40
41 A common feature of fungal SM biosynthesis is the organization of most or all
42 required anabolic, transport, and regulatory elements in gene clusters (GCs). GCs
43 are often discontinuously distributed among fungal taxa, partly due to horizontal
44 transfer (HT) among species with overlapping ecological niches (Gluck-Thaler and
45 Slot 2015). The sparse phylogenetic distribution of psilocybin, coupled with the
46 requirement for multiple enzymatic steps for its biosynthesis (tryptophan-

decarboxylation, N-methylation, indole-4-hydroxylation, and O-phosphorylation), suggest the psilocybin pathway might have dispersed via horizontal GC transfer, and therefore the genetic mechanism for psilocybin biosynthesis might be identified in searches for GCs with a common phylogenetic history and distribution among psilocybin producing (PS+) mushrooms. The pattern of HT events may further suggest ecological pressures that have driven the pathway's persistence (Baquero 2004).

We identified candidate psilocybin genes by sequencing three diverse PS+ mushroom monokaryon genomes -- *Psilocybe cyanescens*, *Panaeolus* (=*Copelandia*) *cyanescens*, and *Gymnopilus dilepis* (Table 1), and comparing them to three related mushrooms not known to produce psilocybin (PS-): *Galerina marginata*, Agaricales sp. 9 , and *Hypholoma sublateritium*. Of 37 gene homolog groups (HGs) consistent with a PS+ distribution among these taxa, only five were clustered, all in PS+ genomes (Fig. S1-pipeline and HGs). We retroactively designated *Gy. chrysopellus*, potentially PS+ because it possesses a cluster identical to *Gy. dilepis*, which is not a surprising oversight given inconsistent identifications, and geographical variation among *Gymnopilus* spp. phenotypes. Predicted functions of these five genes were also consistent with psilocybin biosynthesis and metabolite transport, and were putatively designated tryptophan decarboxylase (TDC), tryptamine N-methyltransferase (TMT), dimethyltryptamine-4-hydroxylase (D4H), psilocin phosphotransferase (PPT), and psilocybin transporter (PST). As SM GCs are infrequently identified in Basidiomycota compared with Ascomycota, this is a notable discovery (Quin et al. 2014).

To confirm GC function, we profiled the enzymology of heterologously expressed TDC and PPT, and assayed by LC-MS/MS analyses. We determined that TDC, the first committed step in the reaction and the only one not producing a drug-scheduled compound, has specific decarboxylase activity on tryptophan. TDC reactions produced tryptamine, identified at the characteristic m/z 144.1 [M+H]+, (Fig. S2, Supplemental data1). TDC did not decarboxylate phenylalanine, tyrosine or 5-

78 hydroxy-L-tryptophan (5-HTP) under the same conditions. We note that TDC is
79 similar to type II phosphatidylserine decarboxylases (PSDs), but has no significant
80 sequence similarity with a pyridoxal-5'-phosphate-dependent decarboxylase
81 recently characterized in *Ceriporiopsis subvermispora* as specific for L-tryptophan
82 and 5-HTP (Kalb et al. 2016). A unique GGSS sequence in a conserved C-terminal
83 motif (Fig. S3), suggests tryptophan decarboxylation is a previously unknown
84 derived function among PSDs (Wriessnegger et al. 2009; Choi et al. 2015). We
85 detected no activity of PPT on 5-HT or 4-Hydroxyindole (4-HI) as alternatives to the
86 psilocin substrate, possibly due to requirements for the 4-hydroxyl and the
87 methylated amine groups of psilocin, but further characterization of PPT and other
88 enzymes was prevented by the regulatory status of substrates and products.
89
90 Phylogenetic analyses of PS homologs from a local database of 618 fungal
91 proteomes yielded congruent gene tree topologies with respect to PS^+ taxa, and
92 clades of clustered PS genes from all gene trees excluded the PS^- taxa in the
93 database, suggesting the clustered genes are coordinately-inherited (Fig. 1, Fig. S4
94 A-F).The gene trees also suggest HT of the cluster from *Psilocybe* to *Panaeolus* and
95 HT of most PS genes between Atheliaceae and Agaricaceae when compared to a
96 phylogenomic tree of related Agaricales (Fig. 1). The direction of the latter HT is
97 ambiguous, and not strongly supported by all five genes. Analyses with TDC and PPT
98 amplicon sequences retrieved by degenerate PCR of unsequenced *Psilocybe* and
99 *Conocybe* genomes (Supplemental data1) suggest the dung fungus *Ps. cubensis*
100 vertically inherited the cluster, and *Pa. cyanescens* acquired the cluster from
101 *Psilocybe* sp., and possibly from a dung-associated lineage. Alternative hypotheses of
102 vertical inheritance in these lineages were rejected; exclusion of *Pa. cyanescens* and
103 *C. cyanopus* (AU test, $p = 0.004$) or *Pa. cyanescens* alone ($p = 0.036$) were
104 significantly worse constrained topologies (Supplemental data1). Furthermore, a
105 TDC gene tree-species tree reconciliation model allowing duplication, HT, and loss
106 (6 events: D=1, HT=3, L=2) is more parsimonious than a model that only allows
107 duplication and loss (28 events: D=3, L=25)(Fig. S5). PS gene orthologs were not
108 detected in *Ps. fuscofulva*, a PS^- species representing an early branch in *Psilocybe*

109 diversification (Borovička et al. 2015). Conservation of synteny flanking the *Ps.*
110 *cyanescens* PS cluster (Fig. 1) suggests it may have been recently acquired in
111 *Psilocybe* as well, or lost as a unit in close relatives. A genome wide scan did not
112 identify additional HT genes or clusters between *Psilocybe* and *Panaeolus* (Fig. S6).
113 HT is comparatively rare in the Basidiomycetes, suggesting the transfer of the PS
114 cluster may have special significance (Wisecaver et al. 2014), and is to our
115 knowledge, the first report of HT of a SM GC between lineages of mushroom-forming
116 fungi (Agaricomycotina).
117
118 Recent studies suggest that ecology can select for both genome content (Ma et al.
119 2010; de Jonge et al. 2013) and organization in eukaryotes through both vertical and
120 horizontal patterns of inheritance (Holliday et al. 2015; Kakioka et al. 2015).
121 Ordination of 10,998 HGs identified two principal components (PCs) that describe
122 22% of the variation in gene content among 16 Agaricales genomes (Fig. 2B).
123 Discrimination of genome composition along PC1 appears to reflect phylogenetic
124 differences, while discrimination along PC2 parallels ecological differences between
125 plant mutualists and other fungi. However, PC2 does not discriminate between dung
126 and wood-decay fungi. The functions of HGs most associated with each PC are
127 consistent with this interpretation. All eight metabolism-related processes in the
128 COG classification system are overrepresented in PC2, but only one is
129 overrepresented in PC1 HGs (Supplemental data1). The grouping of several
130 divergent lineages of wood and dung decay fungi to the exclusion of close
131 ectomycorrhizal relatives along PC2 may reflect similar selective pressures in the
132 decayed wood and dung environments, from recalcitrant plant polymers like lignin,
133 and invertebrate predation (Rouland-Lefèvre 2000). However, a small number of
134 HGs exclusive to either wood or dung associated fungi (Fig. S7, Supplemental data1)
135 are consistent with ecological specialization within each guild. Wood-specific genes
136 include functions in lignin degradation (e.g., peroxidase, isoamyl alcohol oxidase)
137 and carbohydrate transport, while dung-specific genes have functions in bacterial
138 cell wall degradation (e.g., lysozyme), hemicellulose degradation (e.g., Endo-1,4-
139 beta-xylanase, Alpha-L-arabinofuranosidase), and inorganic phosphate transport.

140 Niche-specific genes are largely consistent with vertical inheritance; however,
141 analyses support HT of a single ferric-reductase-like gene (pfam01794, pfam00175)
142 likely involved in iron uptake, to Coprinopsis and Panaeolus from dung-associated
143 Ascomycota (Supplemental data).
144
145 In addition to similar ecological pressures, similar genome content among wood and
146 dung-decaying fungi may also reflect the ecological diversification of
147 Agaricomycetes that accompanied major geological transformations (Fig. 2B). For
148 example, the emergence of true wood opened a massive saprotrophy niche space in
149 the upper Devonian (380 Mya), in which the Agaricomycetes diversified with the aid
150 of key enzymatic innovations (Floudas et al. 2012). The subsequent radiation of
151 herbivorous megafauna during the Eocene approximately 50 MYA (MacFadden
152 2000) and the spread of grasslands 40 MYA (Retallack 2001) expanded the
153 mammalian dung niche space in which invertebrates and fungi competed. These
154 changes parallel the repeated emergence of dung-specialization from plant-decay
155 ancestors in the radiation of *Psilocybe* and other Agaricales lineages (Ramirez-Cruz
156 et al. 2013; Tóth et al. 2013). Late stage wood decay fungi like *Psilocybe* spp. likely
157 harbor genetic exaptations for lignin tolerance/degradation, and competition with
158 invertebrates and prokaryotes, and acquisition of particularly adaptive functions by
159 other fungi (e.g. *Panaeolus*) through HT may have facilitated additional transitions
160 to dung saprotrophy.
161
162 The evolution of PS genes suggest they originally served roles in the wood-decay
163 niche, and more recently emerged through both vertical and horizontal transfer in
164 dung-decay fungi (Fig. 2). HT and retention of PS clusters are evidence of selection
165 on the PS pathway in the recipient lineage, as SM clusters are inherently unstable in
166 fungal genomes (Reynolds et al. 2017 Apr 28). Psilocybin neurological activity,
167 coupled with HT and retention in lineages that colonize dung and/or decayed wood,
168 which are rich in both mycophagous and competitor invertebrates (Rouland-Lefevre
169 2000), suggest that psilocybin may be a modulator of insect behavior. Psilocybin
170 and/or the related aeruginascin have also been identified in the lichenized agaric,

171 *Dictyonema huaorani*, and in the ectomycorrhizal genus *Inocybe* (Kosentka et al.
172 2013; Schmull et al. 2014). PS distribution in *Inocybe* is complementary to that of
173 the acetylcholine mimic, muscarine, which could suggest alternative strategies and
174 pressures to manipulate animal behavior beyond the dung and wood decay niches.
175 Neurotransmitter mimics may provide advantages to fungi by interfering with the
176 behavior of invertebrate competitors for woody resources (Hunt et al. 2007),
177 especially social insects, like termites, which emerged ~ 137 Mya, because they rely
178 on the coordinated activities of multiple castes (Genise 2017). It is thus intriguing
179 that D4H and PST have experienced massive gene family expansion through
180 duplication in *Fibulorhizoctonia* sp., which produce termite egg-mimicking sclerotia
181 in an ancient mutualistic relationship with *Reticulitermes* termites (Matsuura 2005).
182 While neurotransmitter agonists are not known to mediate this symbiosis, insect
183 predatory fungi (i.e. *Cordyceps* spp.) use neurotransmitter analogs to influence the
184 behavior of infected insects (de Bekker et al. 2014), and a number of repellents and
185 toxins in wood-decay fungi inhibit xylophagy and mycophagy by termites (Rouland-
186 Lefèvre 2000).
187
188 The identification of genes underlying PS biosynthesis is an important advance in
189 the field of neurochemical ecology, with both social and medical applications. The
190 sequences of the first *Psilocybe* and *Panaeolus* genomes presented here will be
191 important resources for the prospecting of novel neurotropic natural products
192 (Rutledge and Challis 2015). The discovery that a psilocybin cluster has been
193 horizontally transferred and subsequently maintained among the invertebrate-
194 challenged environments of dung and late wood-decay suggests these niches may be
195 reservoirs not only of new antibiotics (Bills et al. 2013 Aug 23), but also novel
196 neuroactive pharmaceuticals.
197

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203 resources. Genome assemblies are deposited in GenBank under accessions
204 SAMN07166449, SAMN07169033, and SAMN07169108.
205

206 **Table 1.** Genome assembly and annotation of psilocybin-producing mushrooms.

	length (nt)	scaffolds	contigs	av. depth of coverage (x)	N50 (nt)	Complete BUSCOs (%)	total proteins	decarboxylases / PSD [a]	P450's[a]	methyltransferases / DUF890 domain-proteins[a]	kinases / phosphotransferases / OG term 0PNAW[a]	MFS[a]
G. dilepis[b] TENN071165	47,177,497	8,423	10,681	16.5	33,540	73.43	16,257	28 / 9	151	89 / 4	275 / 29 / 1	37
Pa. cyanescens	44,965,162	9,521	11,850	25.7	32,751	75.66	13,420	28 / 8	148	91 / 2	267 / 16 / 2	34
Ps. cyanescens	53,483,841	18,721	38,006	44.7	46,250	72.18	15,973	38 /17	178	102 / 2	298 / 23 / 1	44

207
208
209 [a]Functional category of PS genes as annotated in Eggnog. [b]*G. dilepis* (= *G. aeruginosus* sensu L.R. Hesler) was isolated from oak
210 sawdust in Knoxville, Tennessee 4-Oct-2013. *Pa. cyanescens* and *Ps. cyanescens* basidiospores were supplied by The Spore Works,
211 Knoxville.

Figure Legends:

Figure 1. Psilocybin evolution. A. The PS cluster consists of tryptophan decarboxylase (TDC), 1-2 P450 monooxygenases (D4H), methyltransferase (TMT), phosphotransferase (PPT), and 1-2 MFS transporters (PST). B. Phylogenomic tree of Agaricales (tan) with Atheliales (blue) outgroup. Support values = (internode certainty, tree certainty). Clades 1-5 are as in Fig. 2B. C. PS locus synteny relative to *Ps. cyanescens* scaffold 5617 and *G. marginata* scaffold 9. D. RAxML phylogeny of TDC indicating putative HT branches; *Eutypa lata* is in Xylariales (Ascomycota, lavendar), an order correlated with absence of termites in coarse woody debris (Kirker et al. 2012), with members that produce a white rot of wood. Entomophthoromycotina sp. 1, rose) is commonly associated with amphibian dung and arthropods. Grey taxon names = PCR sequences, black = whole genome. Support is percent of 100 ML bootstraps. **54 similar D4H homologs not shown.

Figure 2. Patterns of ecological diversification of PS genes and Agaricales genomes. A. Ultrametric representation of PPT phylogeny, with root age hypothetically set to align ecological transitions with Earth history events. HT = horizontal transfer, VT = vertical transmission. B. Ordination of Agaricales genome content. Numerals correspond to clades in Fig. 1A.

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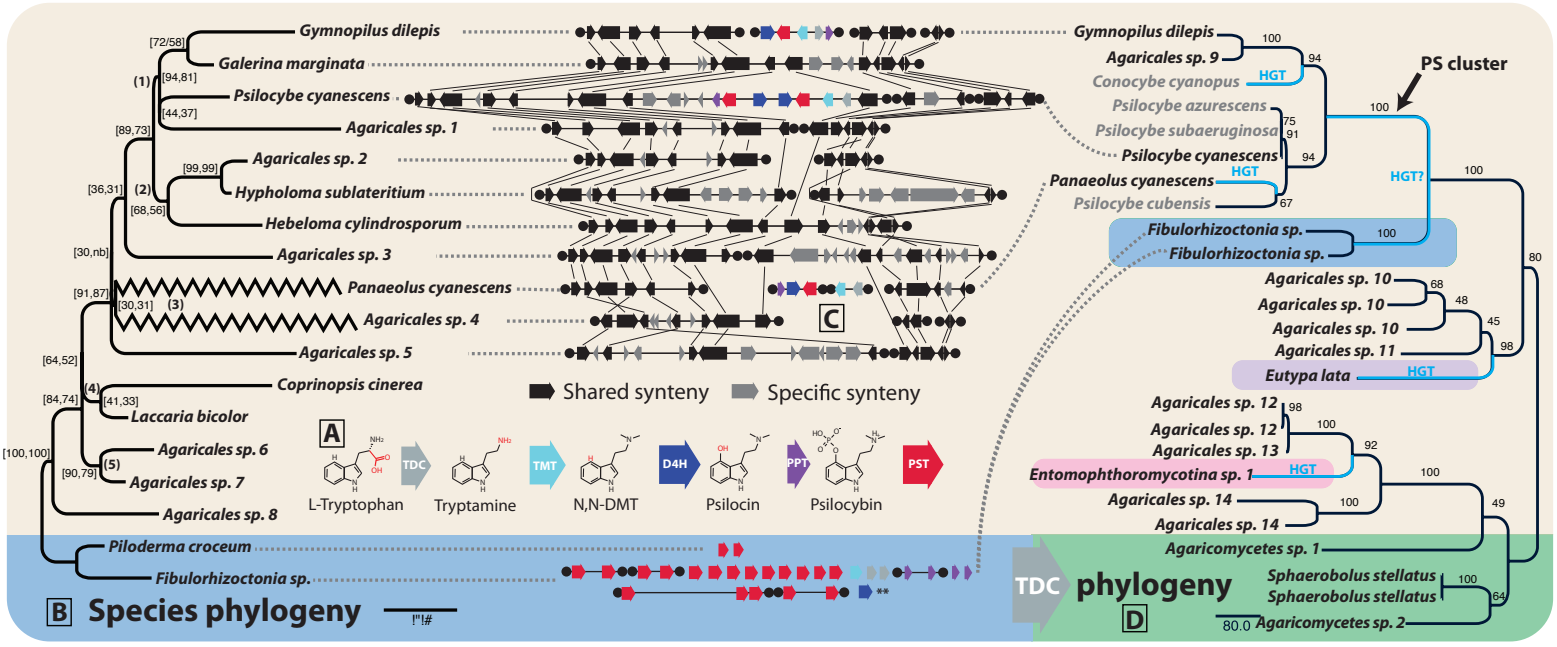
Gymnopilus dilepis
Galerina marginata
Psilocybe cyanescens
Agaricales sp. 1
Agaricales sp. 2
Hypholoma sublateritium
Hebeloma cylindrosporum
Agaricales sp. 3
Panaeolus cyanescens
Agaricales sp. 4
Agaricales sp. 5
Coprinopsis cinerea
Laccaria bicolor
Agaricales sp. 6
Agaricales sp. 7
Agaricales sp. 8
Piloderma croceum
Fibulorhizoctonia sp.
Shared synteny
Specific synteny
A
L-Tryptophan
TDC
Tryptamine
TMT
N,N-DMT
D4H
Psilocin
PPT
Psilocybin
PST
B
Species phylogeny
C
PS cluster
HGT
HGT?
Gymnopilus dilepis
Agaricales sp. 9
Conocybe cyanopus
Psilocybe azurescens
Psilocybe subaeruginosa
Psilocybe cyanescens
Panaeolus cyanescens
Psilocybe cubensis
Fibulorhizoctonia sp.
Fibulorhizoctonia sp.
Agaricales sp. 10
Agaricales sp. 10
Agaricales sp. 10
Agaricales sp. 11
Eutypa lata
Agaricales sp. 12
Agaricales sp. 12
Agaricales sp. 13
Entomophthoromycotina sp. 1
Agaricales sp. 14
Agaricales sp. 14
Agaricomycetes sp. 1
Sphaerobolus stellatus
Sphaerobolus stellatus
Agaricomycetes sp. 2
TDC phylogeny
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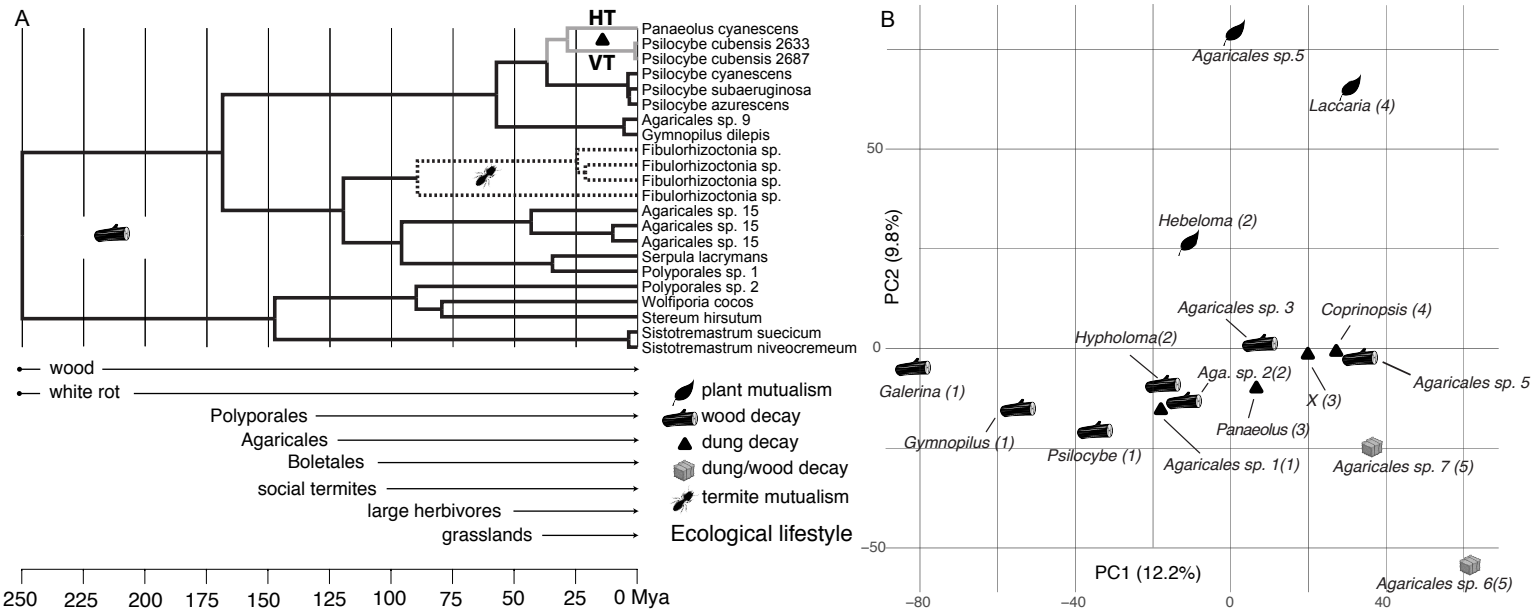
A
HT
VT
Panaeolus cyanescens
Psilocybe cubensis 2633
Psilocybe cubensis 2687
Psilocybe cyanescens
Psilocybe subaeruginosa
Psilocybe azurescens
Agaricales sp. 9
Gymnopilus dilepis
Fibulorhizoctonia sp.
Fibulorhizoctonia sp.
Fibulorhizoctonia sp.
Fibulorhizoctonia sp.
Agaricales sp. 15
Agaricales sp. 15
Agaricales sp. 15
Serpula lacrymans
Polyporales sp. 1
Polyporales sp. 2
Wolfiporia cocos
Stereum hirsutum
Sistotremastrum suecicum
Sistotremastrum niveocremeum
wood
white rot
Polyporales
Agaricales
Boletales
social termites
large herbivores
grasslands
plant mutualism
wood decay
dung decay
dung/wood decay
termite mutualism
Ecological lifestyle
250 225 200 175 150 125 100 75 50 25 0 Mya
B
Agaricales sp.5
Laccaria (4)
Hebeloma (2)
Agaricales sp. 3
Coprinopsis (4)
Hypholoma(2)
Aga. sp. 2(2)
Agaricales sp. 5
Galerina (1)
X (3)
Panaeolus (3)
Gymnopilus (1)
Psilocybe (1)
Agaricales sp. 1(1)
Agaricales sp. 7 (5)
Agaricales sp. 6(5)
PC2 (9.8%)
PC1 (12.2%)
50
0
−50
−80
−40
0
40