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1 Genomic and fossil windows into the secret lives of the most ancient fungi

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16

17

18 **Abstract**

19 Fungi have crucial roles in modern ecosystems as decomposers and pathogens, and they
20 engage in a variety of mutualistic associations with other organisms, especially plants. They
21 have a lengthy geological history, and there is an emerging understanding of their impact on
22 the evolution of Earth systems on a large scale. In this Review, we focus on the roles of fungi
23 in the establishment and early evolution of land and freshwater ecosystems. Today, questions
24 of evolution over deep time are informed by discoveries of new fossils and evolutionary
25 analysis of new genomes. Inferences can be drawn from evolutionary analysis by comparing
26 the genes and genomes of fungi with the biochemistry and development of their plant and
27 algal hosts. We then contrast this emerging picture against evidence from the fossil record to
28 develop a new, integrated perspective on the origin and early evolution of fungi.

29

30 **[H1] Introduction**

31 Fungi are crucial for thriving land plant communities as mycorrhizal symbionts^{1,2} and
32 decomposers³. For perhaps 500 million years^{4,5}, fungi have been supplying land plants with
33 phosphorus and other minerals, thereby speeding up photosynthesis and contributing to the
34 drawdown of atmospheric CO₂⁶⁻⁸. Yet phylogenomics tells us that fungi originated far earlier,

35 deep in the Precambrian⁹, perhaps a billion years ago^{2,10}. This information raises two
36 important questions: When did fungi emerge, and what were their early lifestyles?

37 How and when fungi diversified and adapted to their present roles in Earth systems
38 has long been hidden. Fossils representing simple filaments or spores of ancient fungi are
39 uncommon in the Precambrian fossil record, and difficult to recognize or to distinguish from
40 other organisms¹¹. Due largely to a lack of reliable fossil calibrations, comparisons of ages
41 depend on dated fungal and plant phylogenies^{2,12}, providing a stimulating framework for
42 thought, but these estimates are too imprecise to eliminate alternative hypotheses about
43 ancient ecological relationships between kingdoms.

44 Here, we review recent landmark studies that open new windows on the
45 understanding of the earliest fungi. Comparative analysis of genomes provides the most
46 direct available evidence of ancient interdependence between fungi and algae or land plants.
47 Fungi survive by secreting digestive enzymes and taking up the freed nutrients^{13,14}. The
48 record — at least the remaining record — of the origin of enzymes targeting algal or plant
49 cell walls is embedded in fungal genomes. As green algae evolved into land plants, and as the
50 polysaccharides in their cell walls diversified¹⁵, the evolution of fungal enzymes surely kept
51 pace^{3,16,17}. Comparative analysis of genomes of land plants¹⁸ and their closest algal
52 relatives^{19,20} reveal the evolutionary origins of constituents of plant immune systems that are
53 fundamental both for defense against fungal parasites and for the evolution of mycorrhizae²¹.
54 Green algae, and by extension, the fungi that they nourished may have begun to evolve in
55 freshwater environments, likely in the Precambrian, a billion years ago (1Ga), as highlighted
56 by the geology^{22,23} and recent analysis of the phylogenies of algae^{24,25}.

57 We address the puzzle of what could have nourished early fungi prior to the origin of
58 land plants by reviewing geological evidence of other life forms in shallow marine and
59 terrestrial environments^{22,23,26}. We go on to consider specific fossils and compare their dates
60 and traits to fungal phylogenies based on genomic analyses, asking which sorts of geological
61 and fossil data can be used to resolve conflicts between rocks and clocks. Recently, Loron et
62 al.²⁷ published evidence of 1 billion-year-old fungal fossils from Arctic Canada that they
63 named *Ourasphaira giraldae*. A 1 billion-year-old fungus is twice the age of land plants,
64 based on either fossils or molecular dating^{2,4}. This and other surprising fossil results invite
65 challenge. We review the evidence for interpreting Precambrian fossils as 'fungi'^{28,29}, discuss
66 the early occurrences of Paleozoic fungi, and highlight the potential contributions of new
67 tools toward resolving their identities, for example, confocal scanning laser microscopy³⁰,
68 synchrotron analyses^{27,29,31}, and chemical analyses^{27,29}. We document the tremendous strides

69 that have been made in characterizing fossils microscopically and chemically, but worry that
70 even greater ones will be needed when the specimens are hundreds of millions to billions of
71 years old and have endured pressures and temperatures far beyond those experienced by
72 living organisms.

73

74 **[H1] The first fungi: nutrition and ecology**

75 **[H2] Modern fungi evolved from phagotrophic, aerobic ancestors.** Within the eukaryotic tree
76 of life, Fungi, together with the nucleariid amoebas are the closest relatives of Holozoa³².
77 Over a billion years ago^{2,10}, the fungal lineage evolved as a grade of motile, unicellular,
78 amoeboid protists (FIG 1). The first amoeboid fungi may have been free living and, much
79 like modern nucleariids³³ (FIG 1) and aphelids³⁴, equipped with filose pseudopodia and a
80 phagotrophic mode of nutrition that involved engulfing and digesting organisms smaller than
81 themselves^{35,36}. Phagotrophs diversified, giving rise to obligate parasites such as *Rozella*,
82 which engulfs and digests the cytoplasm within a single host cell^{37,38}, and microsporidia,
83 which lost the capacity for phagotrophy while refining their adaptations as intracellular
84 parasites³⁹. In contrast to the ancestral phagotrophs, the vast majority of modern fungi, from
85 aquatic or semi-aquatic Chytridiomycota to the land-inhabiting yeasts, molds and mushrooms
86 of Dikarya, are osmotrophic^{13,14} (FIG 1). This mode of feeding involves the cytoplasmic
87 uptake of nutrients that diffuse across cell walls and often is coupled with the secretion by the
88 fungus of extracellular enzymes that release nutrients from substrates.

89 Fungi are almost all obligate aerobes or facultative anaerobes that require oxygen to
90 complete their life cycles. Oxygen is needed for oxidative phosphorylation and for sterol
91 biosynthesis. Additionally, genomes of early diverging fungi, including *Rozella allomyces*,
92 Chytridiomycota¹⁶, Zoopagomycota and Dikarya¹⁰ encode lytic polysaccharide
93 monooxygenases, enzymes that require oxygen to facilitate breakdown of polysaccharides⁴⁰.
94 Just two groups of obligate anaerobic fungi are known and both independently lost the
95 ancestral aerobic lifestyle. The microsporidia are one group; they live inside animal cells and
96 compensate for the loss of their mitochondria and absence of oxidative respiration by
97 absorbing nutrients and even ATP from their surrounding hosts⁴¹. The other group, the rumen
98 fungi (Neocallimastigomycotina in the Chytridiomycota) adapted to the anaerobic, cellulose-
99 rich environment in the digestive systems of herbivores. This adaptation has involved gain
100 from rumen bacteria, by horizontal transfer, of multiple genes in several pathways needed for

101 anaerobiosis⁴². Given the rarity of obligately anaerobic fungi, it seems safe to state that the
102 primary evolutionary radiation of their kingdom occurred in oxygenated environments.
103 [H2] *Plants are key to evolutionary success of osmotrophic fungi.* Modern land plants
104 provide fungi with a superb source of reliable energy. Much of the carbon from net annual
105 land-based productivity resulting from photosynthesis, currently about 56×10^{12} kg C/year⁴³,
106 passes through fungi. Mycorrhizal fungi symbiotic with roots take ~13% of plant primary
107 productivity⁴⁴. Saprotrophic fungi consume plant cell wall polymers, including the
108 polysaccharides cellulose and hemicellulose, and can degrade even lignin, a recalcitrant,
109 cross-linked, phenolic polymer. Annual production of cellulose, the most common organic
110 polymer, accounts for $\sim 23 \times 10^{12}$ kg C/year, or ~40% of the total net annual plant
111 productivity^{45,46}, and lignin, with $\sim 11 \times 10^{12}$ kg C/year, accounts for another 20% of the total
112 productivity⁴⁷. Wood-rotting fungi degrade lignin not as a direct source of nutrition, as far as
113 is known, but rather to expose the wood's cellulose and hemicellulose, which the fungus then
114 digests for energy^{46,48,49}.

115 Land plants and their closest green algal relatives, together constitute the
116 streptophytes (TABLE 1; FIG 1). The facts that follow lead us to infer that early osmotrophic
117 fungi adapted first to symbiosis or saprotrophy on freshwater streptophyte algae before
118 gaining access to carbon provided by land plants.

119 [H2] *Fungal enzyme systems tracked plant evolution from freshwater to land.* Genome-
120 based phylogenetic analysis of expansions of fungal enzyme-encoding genes offers insights
121 into the compounds that early phagotrophic, saprotrophic and symbiotic fungi could digest.
122 Cellulases are encoded in genomes of the vast majority of fungi from phagotrophic aphelids³⁶
123 to osmotrophs¹⁶, indicating that cellulose was an ancient source of nutrition for fungi.
124 Cellulose evolved before the earliest fungi; enzymes for the synthesis of cellulose appear in
125 cyanobacteria and are widely distributed across eukaryotic algae, protists and animals⁵⁰.
126 Cyanobacteria secrete a complex assortment of extracellular polysaccharides that may
127 include cellulose⁵¹ but this assortment can vary even within a species⁵². This variation, which
128 has been shown experimentally to contribute to defense against viruses⁵³ and fungi⁵⁴, could
129 have presented a barrier to digestion of cyanobacteria by early fungi. Cell walls of red and
130 green algae also vary in composition but they share cellulose as an important structural
131 component⁵⁰. Thus, cellulases retained in fungal genomes may represent a signature of
132 successful adaptation to digestion of eukaryotic algae.

133 Osmotrophic fungi share a rich array of additional enzymes that target other plant cell
134 wall components: hemicelluloses that include xyloglucans¹⁶, pectins¹⁷ and lignin⁴⁸.

135 Fortunately for evolutionary inferences, these components, and by extension, the fungal
136 enzymes needed to degrade them seem to have arisen much more recently than cellulose and
137 cellulases. Pectins and xyloglucans are known only in streptophytes^{15,55} and early diverging
138 streptophyte algae have few if any copies of genes for synthesis or modification of these
139 polysaccharides⁵⁶.

140 Phylogenetic reconstructions show that multiple gene copies of diverse pectinases are
141 retained in plant-saprotrophic species of fungi ranging from Chytridiomycota to Dikarya¹⁷. In
142 phylogenies, copies of the genes predicted to encode pectinases fall into diverse classes of
143 enzymes: glycoside hydrolases, polysaccharide lyases, and carbohydrate esterases. The
144 pectinase gene copies are inferred to have multiplied in number through five gene
145 duplications in the common ancestor of osmotrophic fungi¹⁷. Pectinase gene copies were lost
146 convergently from some lineages that adapted to nutrition sources other than streptophytes
147 and for example, few pectinase genes remain in *Batrachochytrium*, a genus that adapted to
148 parasitism on amphibians (Table 1). However, most osmotrophic fungi retained pectinases
149 throughout their evolution, implying that they have been under continuing selective pressure
150 to take their nutrition from enzymatic attack on the pectins of cell walls of living or dead
151 streptophyte algae and land plants¹⁷.

152 Although not analyzed in formal phylogenies, Chytridiomycota and Dikarya share
153 predicted xyloglucanases^{57,58}, further evidence that early osmotrophs digested the
154 streptophyte cell walls. Lignin is restricted to land plants, and an expansion of peroxidase
155 enzymes needed for lignin decomposition is credited with contributing to the diversification
156 of Basidiomycota³.

157 Fungal enzymes presumably diversified after their substrates became available. The
158 age of the first xyloglucan- and pectin-containing streptophyte is difficult to estimate.
159 Because few fossils are available, dates are poorly constrained leading to wide confidence
160 intervals within molecular clock studies, and to incongruence across studies. Assuming that
161 the pectins and xyloglucans evolved in streptophytes after the divergence of
162 Mesostigmatophyceae from other algal species (FIG 1, FIG 2), this would indicate an origin
163 sometime between 650 and 1326 million years ago (Ma; *i.e.*, either during the Neoproterozoic
164 (891–629 Ma⁴; ~850–650 Ma⁵⁹ or Mesoproterozoic (1326–920 Ma)² (FIG 2). Based on the
165 fossil record, wood evolved in early Devonian plants (407–397 Ma)^{60,61}, consistent with
166 estimates that Basidiomycota diversified beginning at 295 Ma (95% highest posterior density
167 interval (HPD) 399–195 Ma)³. Therefore, the age of pectin-and hemicellulose- containing

168 streptophytes provides a maximum age for the evolution of the osmotrophs, and the age of
169 lignin-containing plants provides a maximum age for the evolution of the Basidiomycota.
170 [H2] *Osmotrophic fungi evolved in freshwater habitats.* Streptophyte algae are inferred to
171 have diversified in freshwater settings^{24,25}, and the fungi that digested them must have shared
172 their habitat. Phylogenetic analysis points to a freshwater origin even for red algae, green
173 algae, and their shared ancestors²⁴, beginning at perhaps ~1.5 Ga⁵⁹ (billion years ago;
174 Mesoproterozoic) (FIG 2), followed by later radiations in the oceans. If these evolutionary
175 inferences are correct, then even the earliest phagotrophic fungi also had the opportunity to
176 establish nutritional relationships in a freshwater setting.

177 Phylogenies further support the evolutionary origin of osmotrophic fungi in
178 freshwater, including the modern fungi that now live in seawater. Freshwater habitats
179 predominate across early-diverging lineages including modern nucleariids and aquatic fungi
180 (FIG 1)⁶². Most of the extant fungi found in seawater appear in phylogenies to be nested
181 among isolates from land, a pattern that is consistent with many independent adaptations to
182 the marine environment^{32,63-65}. The few fungi currently considered to be novel marine species
183 may have close relatives on land that are as yet undiscovered. Strikingly, the fungi most
184 commonly recovered from deep ocean environments are also recovered from tap water, ice
185 cubes, and swimming pools⁶⁶. An estimated 95% or more of modern fungal species diversity
186 is still undocumented by science^{67,68}. The undescribed species from land, freshwater and
187 marine habitats, the ‘dark matter’ of the fungal world⁶⁹, may yet be the key to unlocking the
188 most ancient secrets of fungal origins.

189 [H2] *Streptophyte algae recognized fungi before land plants evolved fungal mutualisms.*
190 Documented alga-fungus interactions suggest that even before land plants arose, the genetic
191 machinery to recognize contact with fungi had evolved in freshwater streptophyte algae²¹. In
192 modern streptophyte algae, associations with multiple fungal species, including
193 Chytridiomycota, Basidiomycota and Ascomycota, are inferred from metagenomic 28S and
194 18S rDNA sequence data⁷⁰. Land plants recognize fungi via LysM-RLK receptors localized
195 to their plasma membranes, and streptophyte algae have proteins homologous to these
196 receptors²¹. In land plants, the signaling cascades initiated by receptor stimulation are
197 directed toward defensive, immunity-related pathways or to mutually beneficial mycorrhizal
198 symbioses⁷¹. The genes responsible for the perception of fungal signals and for the
199 downstream signaling pathway are inferred to have been present in the most recent common
200 ancestor of streptophytes²¹. From this ancient origin, these genes show differential patterns of
201 retention. Among streptophyte algae, the loss of the LysM-RLK receptor is inferred for

202 several species in Zygnematophyceae, whereas it is retained in Charales^{19,72}. Streptophyte
203 algae are missing other gene modules that land plants require for mycorrhizal association
204 with fungi, including those involved in the formation of arbuscules²¹. The presence of genes
205 encoding receptors known to trigger immune responses⁷¹, combined with the absence of the
206 downstream suite of symbiotic genes for mycorrhizal formation²¹, raises the possibility that
207 streptophyte algae were defending themselves against fungal parasites. By contrast, the land
208 plants including hornworts, liverworts and angiosperms share homologs of the necessary
209 symbiotic genes, indicating that the immediate common ancestor of land plants could already
210 form mycorrhizae^{20,21} (FIG 1). On balance, recent analysis is consistent with a hypothesis that
211 ancestral and modern streptophyte algae could recognize fungi but that intracellular
212 mycorrhizal symbiosis is a shared, derived character of land plants.

213 The abundance and broad phylogenetic distribution of arbuscular mycorrhizal fungi in
214 living land plants is consistent with ancient and enduring relationships between the kingdoms
215 in land-based ecosystems⁷³. Today, arbuscular mycorrhizal fungi are exclusively found
216 within the Mucoromycota, and the origin of (or perhaps predisposition for) modern
217 mutualisms was probably established in the last common ancestor of this fungal clade (FIG
218 1)⁷⁴. Glomeromycotina has been proposed as the original clade of arbuscular mycorrhizal
219 fungi (FIG 1)⁷⁴, because its species are extremely widespread among modern land plants and
220 because they evolved obligate biotrophy early and retained it throughout their diversification.
221 Recently, Bidartondo et al.⁷⁵ proposed that Endogonales (Mucoromycotina, FIG 1) could also
222 have been the earliest endomycorrhizal fungi, based on observation, and on phylogenetic and
223 physiological studies of liverworts, hornworts, lycophytes and ferns⁷⁵⁻⁸¹. Complicating
224 reconstruction of the ancestral states, mycorrhizae in Mucoromycotina, unlike those of
225 Glomeromycotina, evolved multiple times and their symbiotic networks do not form nested
226 subsets of species⁷⁷. The proposals are not mutually exclusive and proponents of both could
227 claim support from the evidence that Mucoromycotina and Glomeromycotina can
228 simultaneously colonize some hornworts, lycophytes and liverworts^{76,82}.

229 **[H1] Ecosystems of early fungi**

230 *[H2] [Au: Again, I've deleted the level two heading here.] Precambrian beginnings of*
231 *terrestrial, land and water ecosystems.* Our understanding of early fungi in freshwater or on
232 land is severely hampered by the rarity of well-preserved terrestrial sediments during the
233 Proterozoic (2600-541 Ma)^{22,24} and Early Paleozoic (541-419 Ma)^{83,84}. Due to the strong
234 sedimentary bias, many of the important and recently discovered Proterozoic eukaryotic

235 fossils are from tidal or shallow marine sediments⁸⁵⁻⁸⁷. Other geological lines of inquiry
236 indicate that primary production in terrestrial ecosystems underwent greater changes during
237 these periods than at any other time in Earth history. Decreases in the $^{13}\text{C}/^{12}\text{C}$ ratio in
238 Neoproterozoic carbonate rocks starting at 1 Ga have been interpreted as an indicator of a
239 substantial influx of carbon from widespread photosynthesizing communities on land that
240 preferentially incorporated $^{12}\text{CO}_2$ ²³. Although the earliest fossils strongly suggestive of
241 chlorophyte green algae are from coastal marine sediments of the Early Neoproterozoic, from
242 ~1.0 Ga⁸⁸, geochemical traces of green algal C₂₈ and C₂₉ sterols did not appear in marine
243 rocks until ~ 0.65 Ga, suggesting that the biomass of marine algae remained low for hundreds
244 of millions more years²⁶ (FIG 2). This scenario is consistent with the view that, in the early
245 Neoproterozoic, nutrition for fungi may have been more readily available in freshwater than
246 marine environments.

247 Although direct fossil evidence of eukaryotes living in freshwater or on land in the
248 Neoproterozoic is extremely limited, two geological sequences that show strong, although not
249 undisputed, characteristics of freshwater lake sequences, both approximately 1 Ga in age,
250 stand as exceptions to this general rule and corroborate the data from carbon isotope ratios.
251 Preliminary studies of microfossils from the Nonesuch Shale (Michigan, Wisconsin, USA)
252 suggest that primary production was maintained by planktonic cyanobacteria and benthic
253 cyanobacterial mats^{22,89,90}. Unicellular cyst-forming eukaryotes were present and might also
254 have contributed to primary production, although in freshwater settings there is no direct
255 evidence of morphologically specialized eukaryotic algal plankton beyond simple spherical
256 cells and cell clusters^{22,90}. In the Torridonian Group (Scotland, UK) there is some additional
257 evidence of minute (< 1 mm), possibly thalloid fossils that may have grown on emergent
258 surfaces²². Fossil fungi have not been recorded from land or freshwater sedimentary
259 sequences of this period of time. Patterned perforations in organic walled fossils look like
260 signs of damage by osmotrophic organisms, possibly bacteria, but possibly also fungi (FIG 3
261 B).

262 [H2] *Continental carbon sources diversify: Cambrian–Silurian Periods.* During the Early
263 Paleozoic, continental ecosystems underwent dramatic changes with the origin and
264 diversification of land plants and several groups of arthropods (Myriapoda, Hexapoda, and
265 Arachnida)⁸³. Recent calibrated phylogenies trace the divergence of the land plant lineage to
266 freshwater antecedents in the Ediacaran Period (Late Neoproterozoic at ~600 Ma) but place
267 the first diversification of the crown group to between 515 and 474 Ma (Mid Cambrian–Early
268 Ordovician)⁴ (FIG 2). Similarly, phylogenetic analyses of arthropods date land colonization
269 by Myriapoda probably to the Early Cambrian (~540 Ma) and slightly later for Hexapoda and
270 Arachnida, between Ordovician and Silurian (~500-440 Ma)⁹¹. Fungi on land, including
271 Mucoromycota, long predate these events, their first radiations taking place > 600 Ma as
272 judged by dated phylogenies^{2,10,17}. This dating is consistent with a fungal mediated
273 colonization of land by plants, perhaps as a mycorrhizal symbiosis^{21,72,92}.

274 As in the Neoproterozoic, terrestrial sediments are rare in Cambrian, Ordovician and
275 Silurian rocks, limiting our access to fossil evidence for life in freshwater or on land^{83,84}.
276 Terrestrially-derived organic remains are first found in the middle Cambrian (FIG 3, for
277 example in estuarine muds of the Bright Angel Shale, ~505 Ma in the eastern Grand Canyon
278 (Arizona, USA))⁹³. By the mid-Paleozoic Era (~449 Ma), evidence for abundant organic
279 carbon that could be consumed by fungi is known from the coalified remains of microbial
280 mats probably derived from exposed soil crusts⁹⁴. During the Ordovician through to the mid
281 Silurian (485-419 Ma), evidence for plant life on land also comes indirectly from spores
282 deposited in shallow estuarine marine sediments^{22,83,95}, and from shifts in the geological
283 carbon isotope record possibly attributable to land plant expansion⁹⁶. Taken together, these
284 data suggest that the Early Paleozoic starting at 541 Ma witnessed an extension and
285 diversification of the types of plant-based carbon that are targeted by enzyme systems of
286 osmotrophic fungi, including cellulose, hemicellulose, pectins, cutins, and sporopollenin.

287 [H1] **First fungi come into sight**

288 [H2] *Equivocal evidence of fossil Fungi from Proterozoic saline deposits.* Direct fossil
289 evidence of fungi during the Proterozoic is questionable and limited to a few examples from
290 deep-sea basalts or shallow marine sediments (FIG 2, TABLE 2). The oldest fossils attributed
291 to fungi are filaments found within vesicles and fissures in basalts of the Ongeluk Formation
292 (South Africa) that formed on a seafloor 2.4 billion years ago²⁸. If correctly attributed, these
293 fossils would imply that fungi are twice as old as estimates derived from calibrations of the
294 fungal tree of life (FIG 2)^{2,10,59}. A suitable source of nutrition for such ancient heterotrophs

295 would be difficult to infer. Furthermore, the deep biosphere is anaerobic, which is
296 inconsistent with the metabolic requirement for oxygen by most fungi. Although intriguing,
297 these filaments do not exhibit any features that would establish a compelling link to fungi,
298 and in our view, even their biological origin remains questionable.

299 Much more convincing in terms of biogenicity are filamentous fossils recovered from
300 1.01 to 0.89 Ga (Early Neoproterozoic) shallow estuarine marine deposits of the Grassy Bay
301 Formation Arctic Canada (Table 2)^{27,97}. Named *Ourasphaira giraldae*, these are branched,
302 apparently septate, and they develop conspicuous spheres (33–80 µm diameter) interpreted as
303 spores. By themselves, these features are not compelling evidence of an affinity with fungi as
304 they are also found in algae. Analysis of the organics by Fourier-transform infrared (FTIR)
305 spectroscopy yielded absorption bands that were interpreted as indicating chitin or chitosan
306 typical of fungi (BOX 1)²⁷. We contend however that controls would be needed to show that
307 FTIR could distinguish fossilized, partially degraded chitin from cellulose⁹⁸, and that lacking
308 these controls, the affinities of *O. giraldae* with fungi in general and Dikarya in particular
309 remain doubtful. Most recently, pseudo-septate filaments attributed to fungi were discovered
310 in 0.80 to 0.72 Ga (Early-Mid Neoproterozoic) sediments recovered from deep drill cores
311 made originally during the 1950s in the Mbuji-Mayi Supergroup (Democratic Republic of
312 Congo)²⁹ (TABLE 2). It should be noted that an alternative perspective on the dating of this
313 sequence would make these fossils very much older, perhaps as much as ~1Ga⁹⁹. The
314 sediments are a marine influenced dolomite that is thought to have been subject to aerial
315 exposure. Evidence of chitin was inferred using a combination of complementary analytical
316 techniques (TABLE 2, BOX 1). The common theme among these recent reports is a
317 filamentous structure with few morphological characteristics. Establishing a fungal affinity
318 relies on ruling out filamentous bacteria or archaea and photosynthetic eukaryotes by
319 establishing the presence of chitin residues in thermally altered and partly decomposed
320 organics using various chemical analytical techniques. Even if one accepts the evidence of
321 chitin, it is not possible to specify a more precise position for these fossils in the fungal tree
322 of life.

323 [H2] *Fossil evidence of Fungi in the Paleozoic.* Direct evidence of fossil fungi from the
324 Cambrian through Silurian Periods is sparse. Fossil hyphae and spores of *Palaeoglomus grayi*
325 from the Ordovician Guttenberg Formation (Wisconsin, USA) are frequently cited as the
326 earliest evidence of Glomeromycotina at ~460 Ma¹⁰⁰. Although resembling extant
327 Glomeromycotina, they are not associated with any plant fossils. Furthermore, these remains

328 could be more recent contaminants of the porous dolomitic sediment in which they were
329 found¹¹.

330 *Tortotubus protuberans* (also known as *Ornatifilum lornensis*), when first
331 documented from Silurian sediments, was interpreted as an ascomycete with what appeared
332 to be lateral, flask-shaped, spore-producing cells¹⁰¹. However, Smith¹⁰² compared material
333 from several Silurian and Devonian sites, and he showed that the flask-shaped cells were
334 broken bases of filaments instead of spore-producing structures, casting serious doubt on an
335 affinity with Ascomycota. Fossils of *T. protuberans* are plausibly of terrestrial origin and
336 their features include branched filaments with pseudo-septate pores. Smith's study illustrates
337 the value of basing interpretation on careful observations and a rich set of collections.

338 The beginning of the Devonian Period (419 Ma) saw a substantial change in the
339 nature of the geological record marked by the widespread development of terrestrially
340 deposited sediments on a global scale. The shift from dominantly marine to terrestrial
341 preservation resulted in the first appearance of many land-dwelling lineages in the fossil
342 record^{4,83,103}. Many fossil sites from across Europe, Asia, the Americas and Australia testify
343 to the diversity of life on land, but one site stands out because organisms and their
344 interactions were remarkably preserved. The Rhynie chert (407 Ma, FIG 3) preserves
345 elements of a geothermal wetland including seven species of small herbaceous vascular
346 plants (~ 20 cm maximum height), representatives of eight groups of arthropods, one
347 nematode, several groups of green algae including a charophycean streptophyte alga,
348 oomycetes, testate amoebae, cyanobacteria and numerous varied fungi, including
349 Chytridiomycota, Blastocladiomycota, Mucoromycota, and possibly Ascomycota^{11,104-108}.
350 Furthermore, Rhynie chert plants and fungi demonstrated saprotrophic^{11,30,109} (FIG 4) as well
351 as symbiotic (both parasitic¹⁰⁸⁻¹¹¹ and mutualistic^{76,112,113}) interactions (FIG 3). Mycorrhiza-
352 like associations in the Rhynie chert provide the earliest evidence of a relationship that can be
353 traced through the geological record up to the present time. Fossils document a
354 Mucoromycotina mycorrhizal association⁷⁶, in addition to two well-known fossils of
355 arbuscular mycorrhizal associations involving Glomeromycotina^{76,112,113}. In early fossil plants
356 (lacking roots or rhizomes) the development of arbuscular mycorrhizae was in prostrate and
357 aerial axes^{76,112,113}, a situation similar to that found in some modern bryophytes, ferns and
358 orchids¹¹⁴. The diverse and well-established mycorrhizal interactions between fungi and
359 plants in the Rhynie chert are consistent with molecular phylogenic and genomic inferences
360 that these interactions predate the Devonian Period by over 100 Ma.

361 [H2] *Puzzling fungi of the Devonian Period.* As the sedimentary window on to early
362 ecosystems opened during the Devonian Period, many new organisms came into view for the
363 first time. When interpreting their affinities and the nature of their interactions, it is tempting
364 to shoehorn them into modern groups or categories that might not have existed at the time.
365 Yet many taxa of the Devonian may now be extinct without descendants.

366 A prime example of the problem of identifying fossil fungi based on the morphology
367 of extant species is *Paleopyrenomycites devonicus* from the Rhynie chert. This important
368 fossil is widely used in dating the fungal tree of life (TABLE 2)^{115,116} and an affinity with
369 Ascomycota is generally accepted. However, its position within the clade—whether basal in
370 Ascomycota or nested among Pezizomycotina—is contentious^{2,12,117,118}. It is worth noting
371 that dispersed spores and other evidence of an Ascomycota radiation do not appear in the
372 sedimentary record until much later, during the Mesozoic Era, >250 Ma^{119,120}, providing
373 support for the idea that *Paleopyrenomycites devonicus* represents an extinct clade of early-
374 diverging Ascomycota.

375 Among the most unexpected fossils allied to fungi is *Prototaxites*, which is
376 encountered in Devonian rocks ~419-359 Ma¹²¹. The largest specimens resembled tree trunks
377 or logs, reaching over 8 meters in length although small specimens also occurred. Based on
378 its structure of interwoven filaments and tubes of various sizes, Hueber¹²² hypothesized that
379 *Prototaxites logani* was a giant sporophore of a species in the Basidiomycota. Recently, an
380 affinity with Ascomycota was suggested for the small *Prototaxites taiti* with the discovery of
381 a 1 mm-thick layer of meiotic cells, 'asci', each with more than 8 spores¹²¹. The ecological
382 role of *Prototaxites* remains enigmatic. Hobbie and Boyce¹²³ concluded that the carbon
383 isotope ratios of *Prototaxites* indicated that it was a saprotrophic fungus. Selosse argued that
384 the surrounding Devonian primary producers could not have supported such a large
385 saprotroph, and suggested that lichenization, which would have enabled carbon acquisition
386 from algal partners, was more likely¹²⁴.

387 Adding to the mysteries of *Prototaxites* is the recently described *Cyathinema*
388 *digermulense*¹²⁵. This organically-preserved fossil from the Nyborg formation in Arctic
389 Norway at 580 Ma resembles the charcoalfied body parts of *Prototaxites* sp.¹²¹, although
390 *Cyathinema* predates *Prototaxites* by 150 million years. In particular, the cups-and-tubes of
391 *Cyathinema* resemble the tubular threads in the hymeninal layer of *Prototaxites*. A more
392 prosaic interpretation of the phylogenetic relationships of *Cyathinema* would place it on the
393 red algal lineage, an interpretation consistent with its probable location in a shallow-marine
394 habitat, rather than in a terrestrial habitat as occupied by *Prototaxites*. This example

395 illustrates the implicit challenges in distinguishing fossil fungi from algae, while highlighting
396 the importance of finding more fossils to understand the diversity of early eukaryotes.

397 Lichens, mutualistic symbioses between fungi and cyanobacteria or chlorophyte
398 algae, are polyphyletic, arising from multiple lineages of Ascomycota and Basidiomycota.
399 Based on recent molecular clock analysis of multiple lichen fungi and their diverse,
400 polyphyletic algal associates, Nelsen et al.¹² concluded that extant lichen lineages originated
401 after, not before land plants. Lichenization probably enabled diversification of the partners
402 after the rise of multicellular land plants, by adaptation to niches unsuitable for the latter⁷³.
403 Cyanobacteria are found in ~ 15% of lichens and they have been available as potential
404 photosynthetic partners for ~2.4 Ga, yet there is no evidence for their involvement in lichens
405 until after the evolution of land plants¹².

406 The early fossil evidence for lichens is scant¹²⁶. The controversial interpretations of
407 *Dickinsonia* fossils from the Ediacaran (635 to 541 Ma) as lichens¹²⁷ or fungi¹²⁸ have been
408 widely criticized¹²⁶ and are further contradicted by recent geochemical evidence of
409 cholestane, which is a residue of cholesterol, a sterol that is more consistent with an animal
410 than fungal interpretation¹²⁹. Likewise, the lichen-like symbioses with cyanobacteria in the
411 marine Doushantuo Formation (551 to 635 Ma)¹³⁰ are less than compelling and alternative
412 hypotheses involving filamentous bacteria rather than fungi are possible. The lichen-like
413 *Winfrenatia reticulata* described in the terrestrial Rhynie chert (407 Ma)¹³¹⁻¹³³ bears no
414 morphological similarity to extant lichens, and, although it represents a loose consortium of
415 fungi and cyanobacteria (TABLE 2), a mutualistic relationship cannot be inferred from the
416 morphological clues that the fossil presents. The earliest, most compelling evidence of fossil
417 lichens comes from Early Devonian (415 Ma) sediments of the Ditton Group (England)^{134,135}.
418 Two types of thallus-like fossils preserved in charcoal (*Chlorolichenomycites salopensis* and
419 *Cyanolichenomycites devonicus*) have an internally stratified structure. Fungal hyphae are
420 exceptionally well-preserved; photobionts less so but plausibly represented by clusters of
421 pyrite crystals. The fungus was interpreted as a Pezizomycotina but it would be older than
422 estimates of the stem age of extant lichens (FIG 2)¹². Here again, the Devonian fossils might
423 be extinct lineages of lichens that are unrepresented in molecular phylogenetic studies of
424 living species.

425 [H1] Conclusions and future directions

426 We contend that fungi were early colonists of freshwater environments that were already
427 occupied by biofilms of cyanobacteria and photosynthetic eukaryotes, the forerunners of

428 green and red algae and land plants. The earliest fungi were protist-like with phagotrophic
429 nutrition. Osmotrophic nutrition and mycelial growth initially developed as responses to the
430 availability of abundant carbon sources produced by photosynthetic streptophytes. The
431 diversification of pectinase and xyloglucanase genes in fungi and the capacity for
432 streptophyte algae to recognize and respond to fungi indicate that fungal adaptations to land
433 plants as a source of carbon began with their freshwater algal ancestors. Another major step
434 enabled the rise of the current terrestrial flora and fungi: the mycorrhizal association. The rise
435 and spread of woody plants on land triggered a later diversification of oxidative fungal
436 enzymes capable of depolymerizing lignin to make available for fungal nutrition the vast
437 stores of cellulose and hemicellulose trapped in wood. Perhaps similar patterns could also be
438 observed for other complex organic molecules that appeared early in the geological record
439 (for example, sporopollenin and cutin).

440 Osmotrophic fungi that live in modern ocean sediments and in the deep biosphere are
441 most probably derived from freshwater or land species. Rare Precambrian freshwater
442 sediments do exist, and these perhaps hold the best prospects for the discovery of mutually
443 corroborating fossils of fungi. It would be fascinating to find new, deeply diverging extant
444 species from freshwater and marine habitats and then to put such fungi into a phylogenomic
445 perspective. Fungal diversity is still poorly known and bringing the fungal dark matter to
446 light will help to decipher the origins of their kingdom. A deeper understanding of the
447 modern diversity of fungi in soils, freshwater and marine systems is needed to understand
448 fungal roles in modern ecosystems and will be helpful to the interpretation of the fossils in
449 the early geological record.

450 We have reviewed the profound insights that are resulting as a flood of new
451 techniques are applied to the analysis of fungal genomes and to the analysis of fossil
452 chemistry and morphology. At present, dated phylogenies and genomic evidence for
453 interactions with streptophyte hosts indicate that fungi are older than the unambiguous
454 evidence for them provided by their 407 Ma Rhynie chert fossils. Fascinating, newly
455 discovered fossils backed by chemical analysis are far older, but their occurrences are
456 separated by gaps of over a hundred million years, and their identity as fungi is equivocal and
457 has yet to be corroborated by information about their habitats or ecology.

458 We anticipate that discoveries and interpretation of new fossils will close gaps to
459 reveal patterns of fungal continuity, radiation and extinction through the ages. The
460 sophistication of evolutionary inferences from genomes will be increased by taking into
461 account increasingly robust patterns recorded in the fossils. We hope that new researchers

462 and their funding agencies take note of the opportunities that are arising for the critical
463 challenge of fundamental ideas about ancient fungi and their ecology. Recognizing the fungi,
464 correlating them with habitat, and placing them into the tree of life, although never
465 straightforward, will pave the way to a better understanding of ancient ecosystems.

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469 **References**

470 **[Au: For references that are particularly worth reading (5-10% of the total), please**
471 **provide a single bold sentence that indicates the importance of the work.]**

472

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478 **Competing interests**

479 The authors declare no competing interests.

480 **Peer review information**

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482 for their contribution to the peer review of this work.

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486 **Table 1. Host organisms for fungi through geological time**

Host		Hosts and associated fungi through geological time
Cyanobacteria	NRMICRO-19-159-Berbee-t1.1	Cyanobacteria made up a large fraction of aquatic biomass through the Precambrian, but there is no convincing fossil evidence that early fungi fed upon them. The diversity of fungi associated with modern cyanobacteria is low.
Streptophyte	NRMICRO-19-	Before land plants, early saprotrophic or symbiotic

green algae	159-Berbee-t1.2	fungi may have used freshwater streptophyte algae as a source of nutrition. Genomes of both algae and fungi point to a relationship going back ~750 Ma. Ancestral streptophytes gave rise to some species such as <i>Nitella hyalina</i> ¹³⁶ , shown here, which remained aquatic; other descendants evolved into land plants, possibly aided by mutually beneficial relationships with fungi.
Land plants	NRMICRO-19-159-Berbee-t1.3	Since their earliest proposed origin ~500 Ma ⁴ , land plants including bryophytes and vascular plants have been the most important source of carbon for the vast majority of saprotrophic and symbiotic fungi. Land plants are also the main beneficiaries of fungal ecosystem services including mycorrhizal partnerships and decomposition.
Green algae or cyanobacteria in lichens	NRMICRO-19-159-Berbee-t1.4	. Lichens are stable, mutualistic symbiotic associations where eukaryotic algae and/or cyanobacteria provide carbon to their fungal partners. Dated phylogenies show that lichens arose convergently, and that the clades of algae, cyanobacteria, and fungi that gave rise to extant lichens did not radiate until ~400 Ma, after land plants had begun to diversify ¹² .
Animals	NRMICRO-19-159-Berbee-t1.5	. Modern fungi include the predominantly aquatic Chytridiomycota, some of which cause diseases (such as chytridiomycosis in amphibians). Fungal species that infect animals are derived from more general saprotrophs, for example ref ¹³⁷ ; fungal evolution did not depend primarily on animals.

487 Ma, million years ago. Images provided by (top to bottom): NASA, Kenneth Karol (New
488 York Botanical Garden), Daniel Mosquin (University of British Columbia Botanical Garden;
489 both liverwort and lichen) and REF.¹³⁸.

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Table 2. Interpretation of fossils: fungi or equivocal?

Fossil		Age and morphology (techniques)	Environmental conditions	Interpretation	Reason for the interpretation
Unnamed filament ²⁸	NRMICR O-19- 159- Berbee- t2.1	2.4 Ga; filament (Syn, LM and SEM)	Not organic, anaerobic, marine environment	Uncertain biogenicity; probably not fungal	Inconsistent with other data. Too old; molecular clock dates eukaryotes 1.9-1.7 Ga ⁵⁹ ; first complex walled (eukaryotic) acritarchs ~1.7 Ga ¹³⁹
<i>Ourasphaira giraldae</i> ^{27,97}	NRMICR O-19- 159- Berbee- t2.2;	1.0-0.89 Ga; filaments and spores (LM, SEM, TEM, μFTIR and Raman	Organic from estuarine environment	Possible fungal affinity, might be protist or alga	Unexpected but of great interest. No other bona fide fungal fossils are of similar age; fungal phylogeny predicts

		spectroscopy)			non-filamentous fungi this early ¹⁷ . Possible source of nutrition: consistent with fossil evidence of marine algae ^{88,140} ; predates geochemical evidence of expansion of marine eukaryotic algae ²⁶
Unnamed mycelium-like structures ²⁹³	NRMICR O-19-159-Berbee-t2.3	810 to 715 Ma; filaments (LM, SEM, TEM, μ FTIR, XANES, Raman spectroscopy and chitin staining)	Organic from coastal, lagoon, perennial lacustrine pond	Uncertain fungal affinity, might be alga	Consistent with fungal phylogeny ¹⁷ ; possible source of nutrition consistent with fossil evidence of marine algae ^{88,140} ; predates geochemical

					l evidence of expansion of marine eukaryotic algae ²⁶
<i>Cyathinema digermulense</i> ¹²⁵	NRMICR O-19-159-Berberet2.4;	580 Ma; filaments (LM, SEM and Raman spectroscopy)	Organic from shallow marine environment, a deltaic environment cannot be excluded	Uncertain affinity: share some resemblance with <i>Prototaxites</i> ; might be alga	Consistent with fungal phylogeny ¹⁷ ; possible source of nutrition consistent with fossil evidence of marine algae ^{88,140}
<i>Palaeoglomus grayi</i> ^{100,141}	NRMICR O-19-159-Berberet2.5	460 Ma; filaments and spores (LM)	Organic from shallow marine environment	Fungal (Glomeromycotina); possibly contaminant, found in dolomitic rock	Consistent with fungal and plant phylogenies ^{5,17} ; early plants, a possible source of nutrition, would have been available ¹⁴²
<i>Tortotubus</i> ^{102,143}	NRMICR O-19-159-Berberet	419-359 Ma; filaments and spores	Organic from shallow marine or	Possibly fungal but unlike any living taxon, leaving open a	Consistent with fungal and plant phylogeny ⁵ ;

	t2.6	(LM and SEM)	terrestrial environment	possible protist or algal affinity	early land plants, a possible source of nutrition were present ¹⁴² .
<i>Paleopyrenomycites</i> 115,116	NRMICR O-19- 159- Berbee- t2.7	407 Ma; filaments, sporophor es and spores (LM)	Organic from terrestrial environment ; sporulating structure in land plant axis	Fungal, likely Dikarya; possibly extinct clade of early- diverging Ascomycota	Unexpected : corroboratin g Ascomycot a fossils appear ~250 Ma; consistent with expectation s for Dikarya, the fossil is immersed in a plant stem that it used for nutrition; fossil widely used to calibrate fungal molecular clocks
<i>Winfrenatia</i> ^{131,133}	NRMICR	407 Ma;	Organic	Affinity	Consistent

	O-19-159-Berbee-t2.8	filaments (LM)	from terrestrial environment ; cyanobacteria associated with fungal filaments	unresolved; could be a lichen in the broad sense, but not in the narrower sense of a stable, spatially correlated arrangement of a fungus and a photobiont	with fungal phylogeny ¹⁷ , and with fossil evidence of fungi and cyanobacteria ¹²
<i>Prototaxites</i> ^{121,122}	NRMICR O-19-159-Berbee-t2.9	419-370 Ma; filaments, sporophores and spores (LM, SEM and CLSM)	Organic from terrestrial environments	Affinity unresolved; possibly fungal, but unlike any living taxon	Available sources of nutrition included algae ²⁴ , microbial mats ⁹⁴ , and early land plants ¹⁴² .

495 Syn: Synchrotron; LM: light microscopy; SEM: scanning electron microscopy; TEM:
496 transmission electron microscopy; μ FTIR: Fourier transform infrared microspectroscopy;
497 CLSM: confocal laser scanning laser microscopy; XANES: X-ray absorption near edge
498 structure. Images provided by (top to bottom): image reproduced with permission from
499 REF.²⁸; REF.⁹⁷; REF.²⁹; REF.¹²⁵; Dirk Redecker (Université de Bourgogne); Paul Strother;
500 Christine Strullu-Derrien; Hans Kerp (University of Münster); and Christine Strullu-Derrien.

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504 **Fig. 1. Evolution of early fungi.** Fungi and animals evolved in the Precambrian from
505 phagotrophic, amoeba-like ancestors that engulf nutrients into food vacuoles within their cells
506 and left no known fossil record. The dated phylogeny (top) shows that modern, multicellular

507 fungi descended from unicellular phagotrophs that evolved cell walls and became
508 osmotrophic, secreting digestive enzymes into the surrounding environment. Fungi originated
509 in freshwater and undertook several evolutionary transitions (bottom). (1) Predating fungi,
510 fossils of cyanobacteria are known from freshwater⁸⁹ and marine habitats (a). Stem
511 Viridiplantae are ancestors to all green algae²⁵(b). (2) Phagotrophic amoebae (c) gave rise to
512 fungi^{35,62}. (3) Unicellular streptophyte alga (d) arose. Early osmotrophic fungi (e) parasitized
513 a multicellular streptophyte alga and saprotrophic fungi (f) digested detritus including
514 streptophyte cell walls. (4) Cells of early vascular plants contain symbiotic fungal
515 arbuscules¹¹² and hyphal coils⁷⁶ (g). (5) Wood, substrate for radiation of decay fungi³,
516 evolved in the Devonian (407-397 Ma)^{60,61}. Dates of divergence of plants and chlorophyte
517 algae are from Morris⁴, of most fungi from Chang et al.^{17,19}, of microsporidia, aphelids and
518 *Nuclearia* are arbitrary, and all others from Parfrey et al.⁵⁹ Image (c) is adapted with
519 permission from REF.³³

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523 **Figure 2. Geological ages of fungi, host plants, and pivotal events in eukaryote**

524 **diversification.** Estimates from molecular clocks (dashed lines) are compared with direct
525 fossil or biomarker evidence (solid lines). Perplexing 'fungal' fossils are shown below the
526 geological timeline. These and other recently discovered fossils delight by inviting
527 speculation while confounding expectations. Permission to reproduce images is from: (a)
528 REF.²⁸; (b) REF.⁹⁷; (c) REF.²⁹; (d) REF.¹²⁵; (e) Dirk Redecker (Université de Bourgogne); (f)
529 Paul Strother; (g) Christine Strullu-Derrien; (h) Hans Kerp (University of Münster); (i)
530 Christine Strullu-Derrien..

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533 **Figure 3. Early Paleozoic fungi: landscapes, hosts and fossils.** The top row shows early
534 terrestrial life. Fossilized cyanobacteria (~530 Ma) accumulated around the edges of a former
535 vernal pool, sitting on oxidized Cambrian Tapeats Sandstone in the Grand Canyon (a).
536 Patterned perforations (arrows) in the vesicle walls indicate microbial, possibly actinomycete
537 or fungal damage to unicellular microfossil from 1 Ga lake sediments, from the Torridonian
538 Sequence in Scotland (b). Landscapes of cryptogamic crusts of fungi, algae, and bryophytes
539 in Neoproterozoic to Cambrian landscapes (850-485 Ma) may have looked like the basalt
540 fields in Iceland (c). Middle and bottom rows show Early Devonian 407 Ma Rhynie
541 environment and Rhynie chert fossils. Leafless plants surrounded silica-rich hot springs in
542 Rhynie, Scotland (d). Fossilized axes of early land plants were preserved (e). Bottom row
543 shows microscopic images of fossils from thin sections. Arrows indicate mycorrhizal fungal
544 arbuscules, fungal hyphae that branch into successively finer filaments, within cells of extinct
545 land plant *Aglaophyton major* (f). The streptophyte alga *Palaeonitella cranii* consists of an

546 axis with whorls of filaments (bars 100 μm) (g, h) Asterisks indicate successive internodes in
547 a healthy alga (left) (g); asterisks indicate successive internode cells enlarged in response to
548 infection by a parasitic fungus (right) (h), The zoosporangium of a Chytridiomycota fungus is
549 attached to a wavy-walled rounded structure by a rhizoid (arrow) (i).
550 Permission to reproduce images is from: Paul Strother (a, b); Anthony Ives (University of
551 Wisconsin-Madison) (c); Victor Leshyk (Victor Leshyk Illustration) (d); Christine Strullu-
552 Derrien (f); REF¹¹ (g, h); REF¹⁰⁸ (i).
553

554 **Figure 4. Rhynie chert sporangium.** Confocal laser scanning microscopy reveals internal
555 structural detail of a sporangium of *Retesporangicus lyonii* from the 407 Ma Rhynie chert.
556 Light microscopy (a), confocal laser microscopy (b-d), image processed (c, d³⁰ to show outer
557 surface of the sporangium (c) and closely packed interior spores (d). Bottom two images
558 reproduced with permission from REF.³⁰.

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560 **Box 1. New tools for old fossils**

561 **Molecular approaches**

- 562 • Molecular clocks estimate geological ages of nodes using fossil calibrations, and by
563 assuming that modeled molecular change is proportional to time. Reliable clocks
564 would be based on accurate phylogenies, correct interpretation and dating of
565 fossils¹⁴⁴, and on correct modeling of molecular evolutionary processes.
- 566 • Criteria for reliable clocks are hard to meet. The temptation is strong to constrain
567 clade ages closely by over-interpreting the few available fossil calibration points,
568 ignoring alternative interpretations that would more realistically reveal uncertainty.

569 **Microscopy**

- 570 • Confocal laser scanning microscopy (CLSM) yields 3D images of minute objects in
571 exquisite detail with a resolution of $<1 \mu\text{m}$ ¹⁴⁵. This method resolved spores encased in
572 fungal sporangia and embedded in Rhynie chert^{108,146}. The CLSM digital tomographic
573 datasets (sets of optical sections) are converted computationally into 3D visualizations
574 or animations of fungal structures³⁰. Similar to other techniques listed below, CLSM
575 is largely non-destructive.
- 576 • Scanning electron microscopic analysis of weathering patterns in mineral particles,
577 together with detection of high levels of iron by energy-dispersive X-ray spectroscopy
578 can help distinguish fungal fossils in paleosols³¹.
- 579 • Fluorescent stains characterize cell wall components, although without high
580 specificity. Wheat-germ agglutinin complexed with a fluorophore labels the N-
581 acetylglucosamine residues in chitin. However, it also binds other sugars, and N-

582 acetylglucosamine occurs in other organisms, for example, sheaths of some
583 cyanobacteria¹⁴⁷. Calcofluor white fluorescence labels chitin, cellulose and other
584 polysaccharides.

585 **Spectroscopic methods**

- 586 • Raman microspectroscopy can reveal the geological history of exposure to heat and
587 pressure of an organic fossil^{29,148,149}. This data can help distinguish *bona fide* fossils
588 from recent contaminants.
- 589 • Fourier transform-infrared (FITR) microspectroscopy involves infrared irradiation of
590 samples and it detects organic functional groups including the amino groups that
591 distinguish chitin from cellulose^{27,98}.
- 592 • Synchrotron X-ray absorption near edge structure (XANES) uses high energy
593 irradiation to quantify speciation and bonding in inorganic-rich fossils^{29,98}.

594 In summary, although new genomic, microscopic and spectroscopic techniques cannot
595 roll back the passage of time, they can, in combination, provide tantalizing new clues that
596 challenge our concepts of the age, morphology and ecology of the most ancient fungi.

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598

599 **Glossary**

600 **Mycorrhiza:** Soil fungi make close contact with plant roots or other organs to enter a
601 mutualistic symbiosis, providing mineral nutrients to the plant, and gaining carbon energy in
602 return.

603 **Symbionts/symbiosis:** Symbionts are unlike organisms that live together in a symbiosis, a
604 close relationship; used here in the historical sense of De Bary 1879, the relationship can be
605 mutualistic, benefiting both partners, or parasitic where one organism benefits at the expense
606 of another.

607 **Precambrian:** The geological interval beginning with the origin of the Earth at 4.6 Ga and
608 ending at 541 Ma; encompassing the Proterozoic Eon (2500-541 Ma).

609 **Terrestrial:** Non-marine geological environments; includes freshwater as well as land.

610 **Paleozoic:** The geological era extending from 541 to 252 Ma, after the Precambrian.

611 **Nucleariid amoebas:** Free-living, unicellular amoeboid protists on the fungal lineage that
612 entrap their prey with thread-like filose pseudopodia.

613 **Holozoa:** Multicellular animals (Metazoa) and their closest (less well known) unicellular
614 relatives, including *Sphaeroforma* and collar flagellates.

615 **Aphelids:** Unicellular parasites counted among fungi, the amoeba-like aphelids feed by
616 injecting themselves into an algal cell, and then engulfing and ingesting its contents.
617 **Arbuscules:** Symbiotic, tree-like, branched fungal filaments within a plant cell that function
618 as sites of plant-fungus nutrient exchange in arbuscular mycorrhizae.
619 **Biotrophy:** Nutrition from other living organisms; may refer to mutualists or parasites that
620 are not killing their host.
621 **Thalloid:** Resembling a plant-like body but lacking roots, stems, leaves.
622 **Testate amoebae:** Unicellular protists, polyphyletic, with an organic, mineral or agglutinated
623 shell (test) that partially encloses the cell.
624 **Prostrate axes:** Creeping stem-like branches of early fossil plants that can be surficial or
625 subterranean and that do not have all the characters of a root or a rhizome of living plants.
626 **Aerial axes:** Erect stem-like branches of early fossil plants that do not have all the characters
627 of a stem of living plants.
628 **Sporophore:** Spore-producing structure in fungi; a mushroom is a modern example.
629 **Acritarchs:** Organic-walled microfossils of biological affinity, some eukaryotic.

630

631 **ToC blurb**

632 Fungi originated in a freshwater environment and their evolution accompanied the rise of
633 algae and land plants. In this Review, Berbee and colleagues examine the fossil and genomic
634 record of ancient fungi and the inferences we can make about their lifestyle.

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644 **Thorough review of fossil and phylogenetic evidence for the geological first appearances**
645 **and diversification of mycorrhizae.**

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- 682 **Time-calibrated phylogenies show that the fungal and algal lineages that form lichens**
683 **have evolved after vascular plants; lichens in the modern sense could not have**
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