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

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

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Fungi are crucial for thriving land plant communities as mycorrhizal symbionts[1,2] and decomposers[3]. For perhaps 500 million years[4,5], fungi have been supplying land plants with phosphorus and other minerals, thereby speeding up photosynthesis and contributing to the drawdown of atmospheric carbon dioxide[6–8]. Yet phylogenomics tells us that fungi originated far earlier, deep in the Precambrian[9], perhaps a billion years ago[2,10]. This information raises two important questions: when did fungi emerge, and what were their early lifestyles?

How and when fungi diversified and adapted to their present roles in Earth systems have long been hidden. Fossils representing simple filaments or spores of ancient fungi are uncommon in the Precambrian fossil record, and difficult to recognize or to distinguish from other organisms[11]. Due largely to a lack of reliable fossil calibrations, comparisons of ages depend on dated fungal and plant phylogenies[2,12], providing a stimulating framework for thought, but these estimates are too imprecise to eliminate alternative hypotheses about ancient ecological relationships between kingdoms.

Here, we review recent landmark studies that open new windows on the understanding of the earliest fungi. Comparative analysis of genomes provides the most direct available evidence of ancient interdependence between fungi and algae or land plants. Fungi survive by secreting digestive enzymes and taking up the freed nutrients[13,14]. The record — at least the remaining record — of the origin of enzymes targeting algal or plant cell walls is embedded in fungal genomes. As green algae evolved into land plants, and as the polysaccharides in their cell walls diversified[15], the evolution of fungal enzymes surely kept pace[3,16,17]. Comparative analysis of genomes of land plants[18] and their closest algal relatives[19,20] reveals the evolutionary origins of constituents of plant immune systems that are fundamental both for defence against fungal parasites and for the evolution of mycorrhizae[21]. Green algae and, by extension, the fungi that they nourished may have begun to evolve in freshwater environments, likely in the Precambrian, a billion years ago, as highlighted by the geology[22,23] and recent analysis of the phylogenies of algae[24,25].

We address the puzzle of what could have nourished early fungi prior to the origin of land plants by reviewing geological evidence of other life forms in shallow marine and terrestrial environments[22,23,26]. We go on to consider specific fossils and compare their dates and traits with fungal phylogenies based on genomic analyses, asking which sorts of geological and fossil data can be used to resolve conflicts between rocks and clocks. Recently, Loron et al.[27] published evidence of 1 billion-year-old fungal fossils from Arctic Canada that they named *Ourasphaira giraldae*. A 1 billion-year-old fungus is twice the age of land plants, based on either fossils or molecular dating[2,4]. This and other surprising fossil results invite challenge. We review the evidence for interpreting Precambrian fossils as 'fungi'[28,29], discuss the early occurrences of Palaeozoic fungi and highlight the potential contributions of new tools towards resolving their identities, for example, confocal scanning laser microscopy[30], synchrotron analyses[27,29,31] and chemical analyses[27,29]. We document the tremendous strides that have been made in characterizing fossils microscopically and chemically, but worry that even greater ones

will be needed when the specimens are hundreds of millions to billions of years old and have endured pressures and temperatures far beyond those experienced by living organisms.

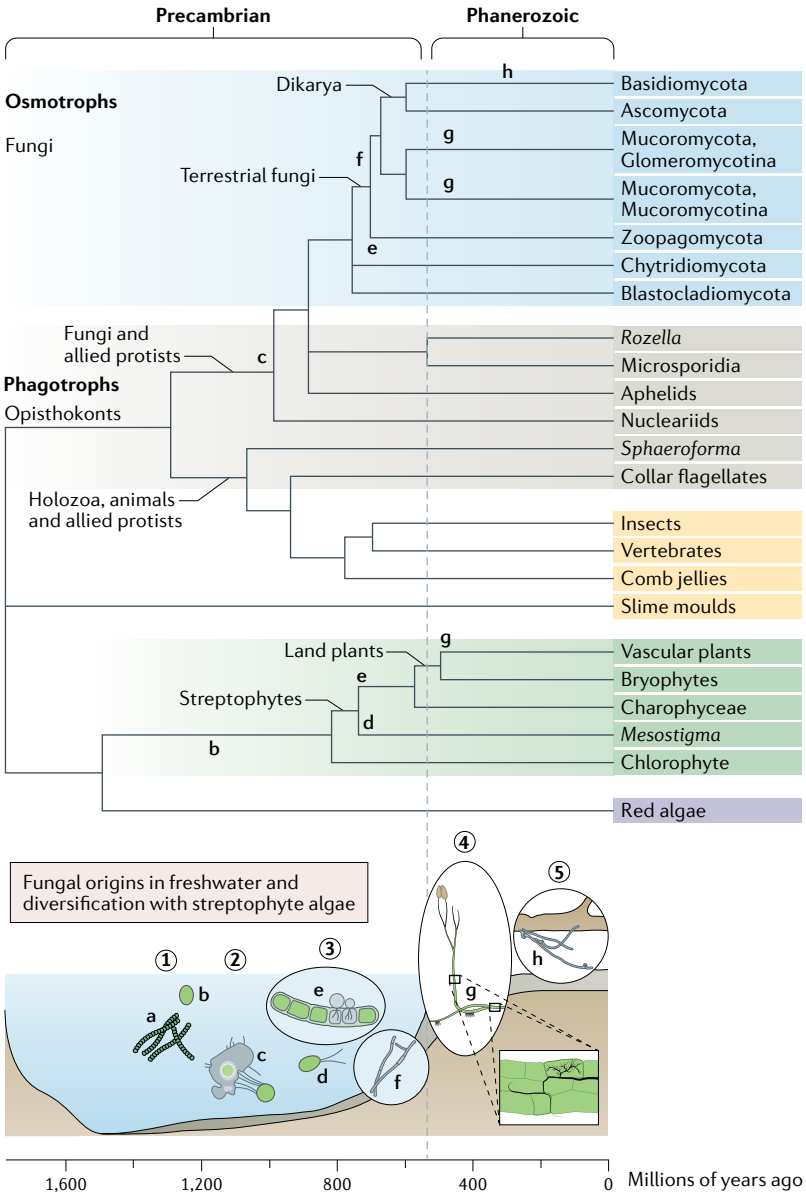


Fig. 1 | **Evolution of early fungi.** Fungi and animals evolved in the Precambrian from phagotrophic, amoeba-like ancestors that engulf nutrients into food vacuoles within their cells and left no known fossil record. The dated phylogeny (top) shows that modern, multicellular fungi descended from unicellular phagotrophs that evolved cell walls and became osmotrophic, secreting digestive enzymes into the surrounding environment. Fungi originated in freshwater and undertook several evolutionary transitions (bottom). Stage 1: Predating fungi, fossils of cyanobacteria are known from freshwater[89] and marine habitats (image **a**). Stem Viridiplantae are ancestors to all green algae[25] (image **b**). Stage 2: Phagotrophic amoebae (image **c**) gave rise to fungi[35,62]. Stage 3: Unicellular streptophyte alga (image **d**) arose. Early osmotrophic fungi (image **e**) parasitized a multicellular streptophyte alga and saprotrophic fungi (image **f**) digested detritus, including streptophyte cell walls. Stage 4: Cells of early vascular plants contain symbiotic fungal arbuscules[112] and hyphal coils[76] (image **g**). Stage 5: Wood, the substrate for radiation of decay fungi[3], evolved in the Devonian (407–397 million years ago)[60,61] (image **h**). Dates for divergence of plants and chlorophyte algae are from Morris[4]; for most fungi, dates for divergence are from Chang et al.[17] and Cheng et al.[19]; for microsporidia, aphelids and *Nuclearia*, they are arbitrary; and for all others, they are from Parfrey et al.[59]. Image **c** is adapted with permission from REF.[33], Elsevier.

The first fungi: nutrition and ecology

Modern fungi evolved from phagotrophic, aerobic ancestors. Within the eukaryotic tree of life, fungi together with the nucleariid amoebas are the closest relatives of Holozoa[32]. Over a billion years ago[2,10], the fungal lineage evolved as a grade of motile, unicellular, amoeboid protists (FIG. 1). The first amoeboid fungi may have been free living and, much like modern nucleariids[33] (FIG. 1) and aphelids[34], equipped with filose pseudopodia and a phagotrophic mode of nutrition that involved engulfing and digesting organisms smaller than themselves[35,36]. Phagotrophs diversified, giving rise to obligate parasites such as *Rozella*, which engulf and digest the cytoplasm within a single host cell[37,38], and microsporidia, which lost the capacity for phagotrophy while refining their adaptations as intracellular parasites[39]. In contrast to the ancestral phagotrophs, the vast majority of modern fungi, from aquatic or semi-aquatic Chytridiomycota to the land-inhabiting yeasts, moulds and mushrooms of Dikarya, are osmotrophic[13,14] (FIG. 1). This mode of feeding involves the cytoplasmic uptake of nutrients that diffuse across cell walls and often is coupled with the secretion by the fungus of extracellular enzymes that release nutrients from substrates.

Fungi are almost all obligate aerobes or facultative anaerobes that require oxygen to complete their life cycles. Oxygen is needed for oxidative phosphorylation and for sterol biosynthesis. Additionally, genomes of early diverging fungi, including *Rozella allomycis*, Chytridiomycota[16], Zoopagomycota and Dikarya[10], encode lytic polysaccharide monooxygenases, enzymes that require oxygen to facilitate breakdown of polysaccharides[40]. Just two groups of obligate anaerobic fungi are known and both independently lost the ancestral aerobic lifestyle. Microsporidia are one group; they live inside animal cells and compensate for the loss of their mitochondria and absence of oxidative respiration by absorbing nutrients and even ATP from their surrounding hosts[41]. The other group, the rumen fungi (Neocallimastigomycotina in Chytridiomycota), adapted to the anaerobic, cellulose-rich environment in the digestive systems of herbivores. This adaptation has involved gain from rumen bacteria, by horizontal transfer, of multiple genes in several pathways needed for anaerobiosis[42]. Given the rarity of obligately anaerobic fungi, it seems safe to state that the primary evolutionary radiation of their kingdom occurred in oxygenated environments.

Plants are key to evolutionary success of osmotrophic fungi. Modern land plants provide fungi with a superb source of reliable energy. Much of the carbon from net annual land-based productivity resulting from photosynthesis, currently about 56×10^{12} kg C/year[43], passes through fungi. Mycorrhizal fungi symbiotic with roots take ~13% of plant primary productivity[44]. Saprotrophic fungi consume plant cell wall polymers, including the polysaccharides cellulose and hemicellulose, and can degrade even lignin, a recalcitrant, cross-linked, phenolic polymer. Annual production of cellulose, the most common organic polymer, accounts for ~23×10^{12} kg C/year, or ~40% of the total net annual plant

Precambrian
The geological interval beginning with the origin of the Earth 4.6 billion years ago and ending 541 million years ago (Ma), encompassing the Proterozoic Eon (2,500–541 Ma).

Mycorrhizae
Soil fungi in close contact with plant roots or other organs in a mutualistic symbiosis, providing mineral nutrients to the plant, and gaining carbon energy in return.

Terrestrial
Non-marine geological environments, including freshwater as well as land.

Palaeozoic
The geological era extending from 541 to 252 million years ago, after the Precambrian.

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

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

Aphelids
Unicellular parasites counted among fungi, the amoeba-like aphelids feed by injecting themselves into an algal cell and then engulfing and ingesting its contents.

Symbiosis
A close relationship in which symbionts that are separate organisms live together. We use the term here in the historical sense of De Bary 1879; the relationship can be mutualistic, benefiting both partners, or parasitic, in which case one organism benefits at the expense of another.

Table 1 | **Host organisms for fungi through geological time**

Host		Hosts and associated fungi through geological time
Cyanobacteria		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 green algae		Before land plants, early saprotrophic or symbiotic 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 *Nitella hyalina*[136] shown here, which remained aquatic; other descendants evolved into land plants, possibly aided by mutually beneficial relationships with fungi
Land plants		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		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		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[137]; fungal evolution did not depend primarily on animals

Ma, million years ago. Images courtesy of (top to bottom) NASA; Kenneth Karol (New York Botanical Garden); Daniel Mosquin (University of British Columbia Botanical Garden; both liverwort and lichen); and Forrest Brem (Indiana University–Purdue University).

productivity[45,46], and lignin, with $\sim 11 \times 10^{12}$ kg C/year, accounts for another 20% of the total productivity[47]. Wood-rotting fungi degrade lignin not as a direct source of nutrition, as far as is known, but rather to expose the wood's cellulose and hemicellulose, which the fungus then digests for energy[46,48,49].

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

Fungal enzyme systems tracked plant evolution from freshwater to land. Genome-based phylogenetic analysis of expansions of fungal enzyme-encoding genes offers insights into the compounds that early phagotrophic, saprotrophic and symbiotic fungi could digest. Cellulases are encoded in genomes of the vast majority of fungi from phagotrophic aphelids[36] to osmotrophs[16], indicating that cellulose was an ancient source of nutrition for fungi. Cellulose evolved before the earliest fungi; enzymes for the synthesis of cellulose appear in cyanobacteria and are widely distributed across eukaryotic algae, protists and animals[50]. Cyanobacteria secrete a

complex assortment of extracellular polysaccharides that may include cellulose[51] but this assortment can vary even within a species[52]. This variation, which has been shown experimentally to contribute to defence against viruses[53] and fungi[54], could have presented a barrier to digestion of cyanobacteria by early fungi. Cell walls of red and green algae also vary in composition but they share cellulose as an important structural component[50]. Thus, cellulases retained in fungal genomes may represent a signature of successful adaptation to digestion of eukaryotic algae.

Osmotrophic fungi share a rich array of additional enzymes that target other plant cell wall components: hemicelluloses that include xyloglucans[16], pectins[17] and lignin[48]. Fortunately for evolutionary inferences, these components and, by extension, the fungal enzymes needed to degrade them seem to have arisen much more recently than cellulose and cellulases. Pectins and xyloglucans are known only in streptophytes[15,55] and early diverging streptophyte algae have few, if any, copies of genes for synthesis or modification of these polysaccharides[56].

Phylogenetic reconstructions show that multiple gene copies of diverse pectinases are retained in plant-saprotrophic species of fungi ranging from Chytridiomycota to Dikarya[17]. In phylogenies, copies of the genes predicted to encode pectinases fall into diverse classes of enzymes: glycoside hydrolases, polysaccharide lyases and carbohydrate esterases. The pectinase gene copies are inferred to have multiplied in number through five gene duplications in the common ancestor of osmotrophic fungi[17]. Pectinase gene copies were lost convergently from some lineages that adapted to nutrition sources other than streptophytes, and, for example, few pectinase genes remain in *Batrachochytrium*, a genus that adapted to parasitism on amphibians (TABLE 1). However, most osmotrophic fungi retained pectinases throughout their evolution, implying that they have been under continuing selective pressure to take their nutrition from enzymatic attack on the pectins of cell walls of living or dead streptophyte algae and land plants[17].

Although not analysed in formal phylogenies, Chytridiomycota and Dikarya share predicted xyloglucanases[57,58], further evidence that early osmotrophs digested the streptophyte cell walls. Lignin is restricted to land plants, and an expansion of peroxidase enzymes needed for lignin decomposition is credited with contributing to the diversification of Basidiomycota[3].

Fungal enzymes presumably diversified after their substrates became available. The age of the first xyloglucan- and pectin-containing streptophyte is difficult to estimate. Because few fossils are available, dates are poorly constrained, leading to wide confidence intervals within molecular clock studies, and to incongruence across studies. Assuming that the pectins and xyloglucans evolved in streptophytes after the divergence of Mesostigmatophyceae from other algal species (FIGS 1,2), this would indicate an origin sometime between 650 and 1,326 million years ago (Ma); that is, either during the Neoproterozoic (891–629 Ma (REF. [4]), ~850–650 Ma (REF. [59])) or Mesoproterozic (1,326–920 Ma)[2] (FIG. 2). Based on the fossil record, wood evolved in early Devonian plants (407–397 Ma)[60,61], consistent with estimates that Basidiomycota diversified beginning at 295 Ma (95% highest posterior density interval 399–195 Ma)[3]. Therefore, the age of pectin- and hemicellulose-containing streptophytes provides a maximum age for the evolution of the osmotrophs, and the age of lignin-containing plants provides a maximum age for the evolution of Basidiomycota.

Osmotrophic fungi evolved in freshwater habitats. Streptophyte algae are inferred to have diversified in freshwater settings[24,25], and the fungi that digested them must have shared their habitat. Phylogenetic analysis points to a freshwater origin even for red algae, green algae and their shared ancestors[24], beginning perhaps ~1.5 billion years ago (Ga; Mesoproterozoic)[59] (FIG. 2), followed by later radiations in the oceans. If these evolutionary inferences are correct, then even the earliest phagotrophic fungi also had the opportunity to establish nutritional relationships in a freshwater setting.

Phylogenies further support the evolutionary origin of osmotrophic fungi in freshwater, including the modern fungi that now live in seawater. Freshwater habitats predominate across early-diverging lineages, including modern nucleariids and aquatic fungi[62] (FIG. 1). Most of the extant fungi found in seawater appear in phylogenies to be nested among isolates from land, a pattern that is consistent with many independent adaptations to the marine environment[32,63–65]. The few fungi currently considered to be novel marine species may have close relatives on land that are as yet undiscovered. Strikingly, the fungi most commonly recovered from deep ocean environments are also recovered from tap water, ice cubes and swimming pools[66]. An estimated 95% or more of modern fungal species diversity is still undocumented by science[67,68]. The undescribed species from land, freshwater and marine habitats, the 'dark matter' of the fungal world[69], may yet be the key to unlocking the most ancient secrets of fungal origins.

Streptophyte algae recognized fungi before land plants evolved fungal mutualisms. Documented alga–fungus interactions suggest that even before land plants arose, the genetic machinery to recognize contact with fungi had evolved in freshwater streptophyte algae[21]. In modern streptophyte algae, associations with multiple fungal species, including Chytridiomycota, Basidiomycota and Ascomycota, are inferred from metagenomic 28S and 18S rDNA sequence data[70]. Land plants recognize fungi via LysM-RLK receptors localized to their plasma membranes, and streptophyte algae have proteins homologous to these receptors[21]. In land plants, the signalling cascades initiated by receptor stimulation are directed towards defensive, immunity-related pathways or to mutually beneficial mycorrhizal symbioses[71]. The genes responsible for the perception of fungal signals and for the downstream signalling pathway are inferred to have been present in the most recent common ancestor of streptophytes[21]. From this ancient origin, these genes show differential patterns of retention. Among streptophyte algae, the loss of the LysM-RLK receptor

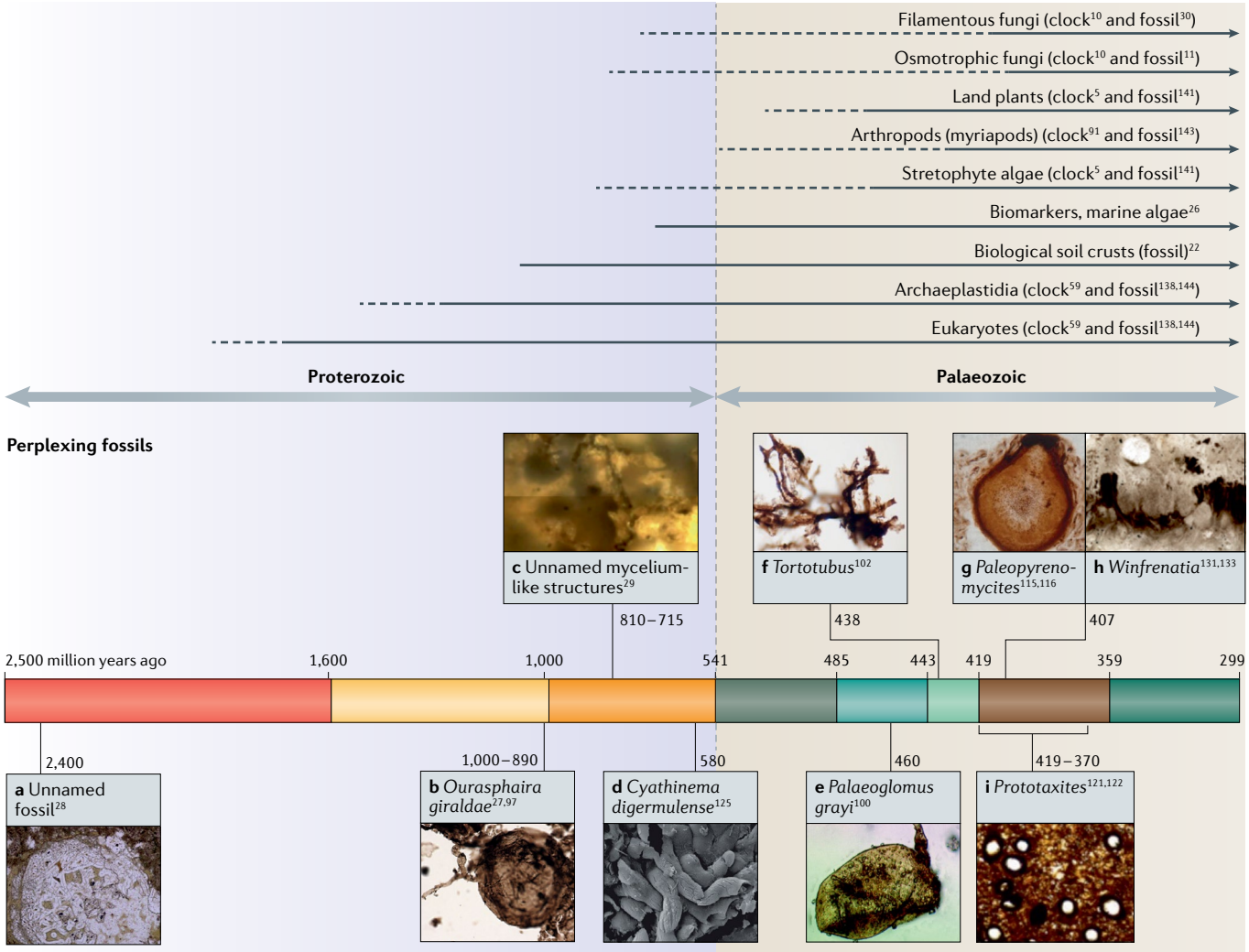


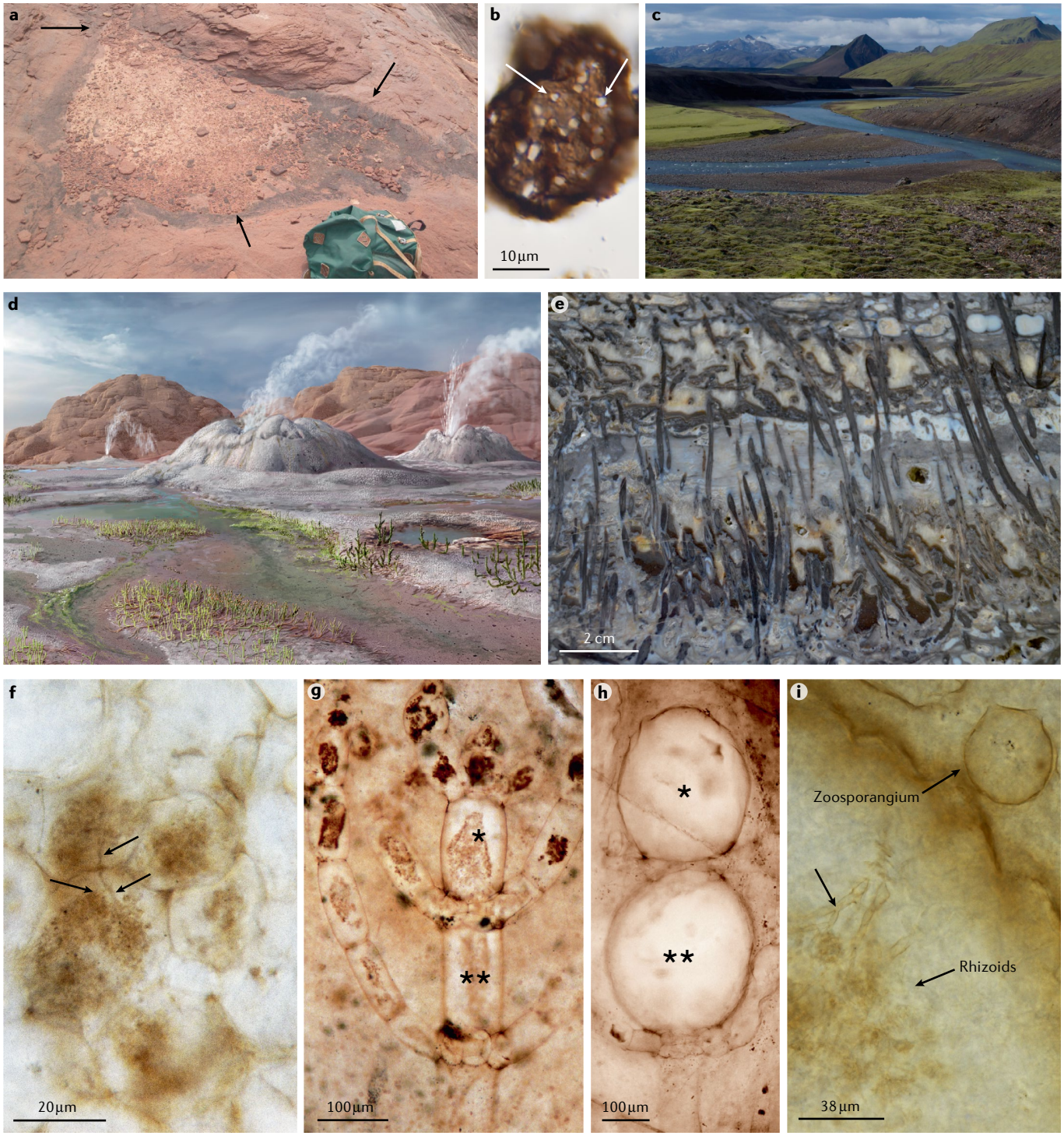
Fig. 2 | **Geological ages of fungi, host plants and pivotal events in eukaryote diversification.** Estimates from molecular clocks (dashed lines) are compared with direct fossil or biomarker evidence (solid lines). Perplexing 'fungal' fossils are shown below the geological timeline. These and other recently discovered fossils delight by inviting speculation while confounding expectations. Part **a** reprinted from REF.[28], Springer Nature Limited; part **b** reprinted with permission from REF.[97], Elsevier; part **c** reprinted with permission from REF.[29], AAAS; part **d** reprinted from REF.[125], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/); part **e** reprinted with permission from REF.[100], AAAS; part **f**, image courtesy of Paul Strother; part **g**, image courtesy of Christine Strullu-Derrien; part **h**, image courtesy of Hans Kerp (University of Münster); and part **i**, image courtesy of Christine Strullu-Derrien.

is inferred for several species in Zygnematophyceae, whereas it is retained in Charales[19,72]. Streptophyte algae are missing other gene modules that land plants require for mycorrhizal association with fungi, including those involved in the formation of arbuscules[21]. The presence of genes encoding receptors known to trigger immune responses[71], combined with the absence of the downstream suite of symbiotic genes for mycorrhizal formation[21], raises the possibility that streptophyte algae were defending themselves against fungal parasites. By contrast, land plants including hornworts, liverworts and angiosperms share homologues of the necessary symbiotic genes, indicating that the immediate common ancestor of land plants could already form mycorrhizae[20,21] (FIG. 1). On balance, recent analysis is consistent with a hypothesis that ancestral and modern streptophyte algae could recognize fungi but that intracellular mycorrhizal symbiosis is a shared, derived character of land plants.

The abundance and broad phylogenetic distribution of arbuscular mycorrhizal fungi in living land plants is consistent with ancient and enduring relationships between the kingdoms in land-based ecosystems[73]. Today, arbuscular mycorrhizal fungi are exclusively found within the Mucoromycota, and the origin of (or perhaps predisposition for) modern mutualisms was probably established in the last common ancestor of this fungal clade[74] (FIG. 1). Glomeromycotina has been proposed as the original clade of arbuscular mycorrhizal fungi[74] (FIG. 1), because its species are extremely widespread among modern land plants and because they evolved obligate biotrophy early and retained it throughout their diversification. Recently, Bidartondo et al.[75] proposed that Endogonales (Mucoromycotina; FIG. 1) could also have been the

Arbuscules
Symbiotic, tree-like, branched fungal filaments within a plant cell that function as sites of plant–fungus nutrient exchange in arbuscular mycorrhizae.

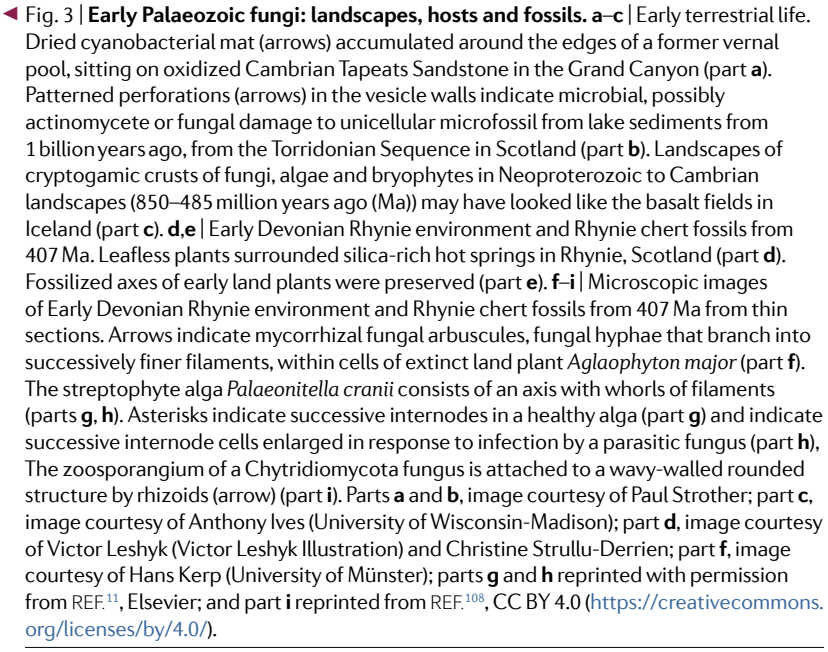
Biotrophy
Nutrition from other living organisms; may refer to mutualists or parasites that are not killing their host.



earliest endomycorrhizal fungi, based on observation and on phylogenetic and physiological studies of liverworts, hornworts, lycophytes and ferns[75–81]. Complicating reconstruction of the ancestral states, mycorrhizae in Mucoromycotina, unlike those of Glomeromycotina, evolved multiple times and their symbiotic networks do not form nested subsets of species[77]. The proposals are not mutually exclusive and proponents of both could claim support from the evidence that Mucoromycotina and Glomeromycotina can simultaneously colonize some hornworts, lycophytes and liverworts[76,82].

Ecosystems of early fungi

Precambrian beginnings of terrestrial, land and water ecosystems. Our understanding of early fungi in freshwater or on land is severely hampered by the rarity of well-preserved terrestrial sediments during the Proterozoic (2,600–541 Ma)[22,24] and Early Palaeozoic (541–419 Ma)[83,84]. Due to the strong sedimentary bias, many of the important and recently discovered Proterozoic eukaryotic fossils are from tidal or shallow marine sediments[85–87]. Other geological lines of inquiry indicate that primary production in



◀ Fig. 3 | **Early Palaeozoic fungi: landscapes, hosts and fossils. a–c** | Early terrestrial life. Dried cyanobacterial mat (arrows) accumulated around the edges of a former vernal pool, sitting on oxidized Cambrian Tapeats Sandstone in the Grand Canyon (part **a**). Patterned perforations (arrows) in the vesicle walls indicate microbial, possibly actinomycete or fungal damage to unicellular microfossil from lake sediments from 1 billion years ago, from the Torridonian Sequence in Scotland (part **b**). Landscapes of cryptogamic crusts of fungi, algae and bryophytes in Neoproterozoic to Cambrian landscapes (850–485 million years ago (Ma)) may have looked like the basalt fields in Iceland (part **c**). **d**,**e** | Early Devonian Rhynie environment and Rhynie chert fossils from 407 Ma. Leafless plants surrounded silica-rich hot springs in Rhynie, Scotland (part **d**). Fossilized axes of early land plants were preserved (part **e**). **f–i** | Microscopic images of Early Devonian Rhynie environment and Rhynie chert fossils from 407 Ma from thin sections. Arrows indicate mycorrhizal fungal arbuscules, fungal hyphae that branch into successively finer filaments, within cells of extinct land plant *Aglaophyton major* (part **f**). The streptophyte alga *Palaeonitella cranii* consists of an axis with whorls of filaments (parts **g**, **h**). Asterisks indicate successive internodes in a healthy alga (part **g**) and indicate successive internode cells enlarged in response to infection by a parasitic fungus (part **h**), The zoosporangium of a Chytridiomycota fungus is attached to a wavy-walled rounded structure by rhizoids (arrow) (part **i**). Parts **a** and **b**, image courtesy of Paul Strother; part **c**, image courtesy of Anthony Ives (University of Wisconsin-Madison); part **d**, image courtesy of Victor Leshyk (Victor Leshyk Illustration) and Christine Strullu-Derrien; part **f**, image courtesy of Hans Kerp (University of Münster); parts **g** and **h** reprinted with permission from REF.[11], Elsevier; and part **i** reprinted from REF.[108], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

terrestrial ecosystems underwent greater changes during these periods than at any other time in Earth history. Decreases in the $^{13}C/^{12}C$ ratio in Neoproterozoic carbonate rocks starting 1 Ga have been interpreted as an indicator of a substantial influx of carbon from widespread photosynthesizing communities on land that preferentially incorporated $^{12}CO_2$ (REF.[23]). Although the earliest fossils strongly suggestive of chlorophyte green algae are from coastal marine sediments of the Early Neoproterozoic, ~1.0 Ga[88], geochemical traces of green algal C_{28} and C_{29} sterols did not appear in marine rocks until ~0.65 Ga, suggesting that the biomass of marine algae remained low for hundreds of millions more years[26] (FIG. 2). This scenario is consistent with the view that, in the early Neoproterozoic, nutrition for fungi may have been more readily available in freshwater than in marine environments.

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

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

As in the Neoproterozoic, terrestrial sediments are rare in Cambrian, Ordovician and Silurian rocks, limiting our access to fossil evidence for life in freshwater or on land[83,84]. Terrestrially derived organic remains are first found in the middle Cambrian (FIG. 3; for example, in estuarine muds of the Bright Angel Shale ~505 Ma in the eastern Grand Canyon, AZ, USA)[93]. By the mid-Palaeozoic Era (~449 Ma), evidence for abundant organic carbon that could be consumed by fungi is known from the coalified remains of microbial mats probably derived from exposed soil crusts[94]. During the Ordovician through to the mid Silurian (485–419 Ma), evidence for plant life on land also comes indirectly from spores deposited in shallow estuarine marine sediments[22,83,95], and from shifts in the geological carbon isotope record possibly attributable to land plant expansion[96]. Taken together, these data suggest that the Early Palaeozoic starting at 541 Ma witnessed an extension and diversification of the types of plant-based carbon that are targeted by enzyme systems of osmotrophic fungi, including cellulose, hemicellulose, pectins, cutins and sporopollenin.

First fungi come into sight

Equivocal evidence of fossil Fungi from Proterozoic saline deposits. Direct fossil evidence of fungi during the Proterozoic is questionable and limited to a few examples from deep-sea basalts or shallow marine sediments (FIG. 2; TABLE 2). The oldest fossils attributed to fungi are filaments found within vesicles and fissures in basalts of the Ongeluk Formation (South Africa) that formed on a seafloor 2.4 Ga[28]. If correctly attributed, these fossils would imply that fungi are twice as old as estimates derived from calibrations of the fungal tree of life[2,10,59] (FIG. 2). A suitable source of nutrition for such ancient heterotrophs would be difficult to infer. Furthermore, the deep biosphere is anaerobic, which is inconsistent with the metabolic requirement for oxygen by most fungi. Although intriguing, these filaments do

Thalloid
Resembling a plant-like body but lacking roots, stems and leaves.

Table 2 | **Interpretation of fossils: fungi or equivocal?**

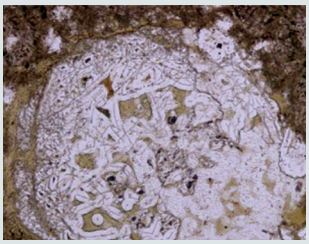
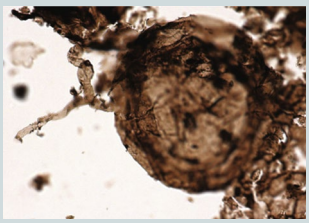
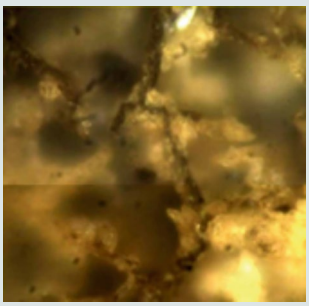
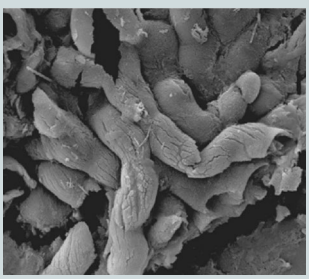
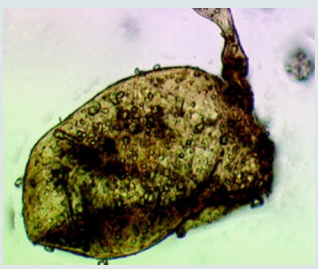
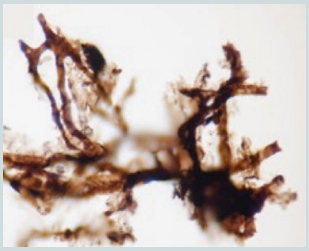
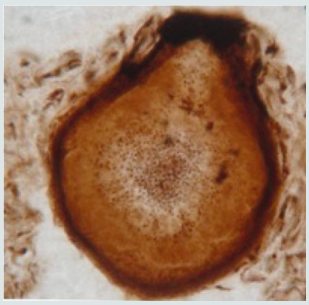
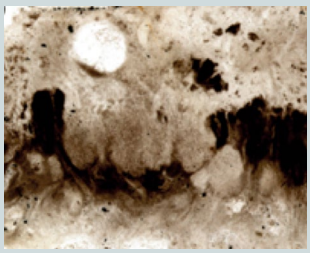
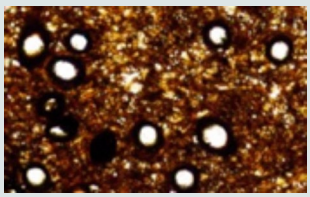
Fossil		Age and morphology (techniques)	Environmental conditions	Interpretation	Reason for the interpretation
Unnamed filament[28]		2.4 Ga; filament (Syn, LM and SEM)	Not organic, anaerobic, marine environment	Uncertain biogenicity; probably not fungal	Inconsistent with other data. Too old; molecular clock dates eukaryotes at 1.9–1.7 Ga (REF.[59]); first complex walled (eukaryotic) acritarchs ~1.7 Ga (REF.[138])
Ourasphaira giraldae[27,97]		1.0–0.89 Ga; filaments and spores (LM, SEM, TEM, μFTIR and Raman spectroscopy)	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 non-filamentous fungi this early[17]. Possible source of nutrition: consistent with fossil evidence of marine algae[88,139]; predates geochemical evidence of expansion of marine eukaryotic algae[26]
Unnamed mycelium-like structures[29]		810–715 Ma; filaments (LM, SEM, TEM, μFTIR, 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[88,139]; predates geochemical evidence of expansion of marine eukaryotic algae[26]
Cyathinema digermulense[125]		580 Ma; filaments (LM, SEM and Raman spectroscopy)	Organic from shallow marine environment, a deltaic environment cannot be excluded	Uncertain affinity: shares some resemblance with *Prototaxites*; might be alga	Consistent with fungal phylogeny[17]; possible source of nutrition consistent with fossil evidence of marine algae[88,139]
Palaeoglomus greyi[100,140]		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[141]
Tortotubus[102,142]		419–359 Ma; filaments and spores (LM and SEM)	Organic from shallow marine or terrestrial environment	Possibly fungal but unlike any living taxon, leaving open a possible protist or algal affinity	Consistent with fungal and plant phylogeny[5]; early land plants, a possible source of nutrition, were present[141]

Table 2 (cont.) | **Interpretation of fossils: fungi or equivocal?**

Fossil		Age and morphology (techniques)	Environmental conditions	Interpretation	Reason for the interpretation
Paleopyrenomycites[115,116]		407 Ma; filaments, sporophores and spores (LM)	Organic from terrestrial environment; sporulating structure in land–plant axis	Fungal, likely Dikarya; possibly extinct clade of early-diverging Ascomycota	Unexpected: corroborating Ascomycota fossils appear ~250 Ma; consistent with expectations for Dikarya, the fossil is immersed in a plant stem that it used for nutrition; fossil widely used to calibrate fungal molecular clocks
Winfrenatia[131,133]		407 Ma; filaments (LM)	Organic from terrestrial environment; cyanobacteria associated with fungal filaments	Affinity 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	Consistent with fungal phylogeny[17] and with fossil evidence of fungi and cyanobacteria[12]
Prototaxites[121,122]		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[141]

μFTIR, Fourier-transform infrared microspectroscopy; CLSM, confocal laser scanning laser microscopy; Ga, billion years ago; LM, light microscopy; Ma, million years ago; SEM, scanning electron microscopy; Syn, synchrotron; TEM, transmission electron microscopy; XANES, X-ray absorption near edge structure. Images (top to bottom) reprinted from REF.[28], Springer Nature Limited; reprinted with permission from REF.[97], Elsevier; reprinted with permission from REF.[29], AAAS; reprinted from REF.[125], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/); reprinted with permission from REF.[100], AAAS; image courtesy of Paul Strother; image courtesy of Christine Strullu-Derrien; image courtesy of Hans Kerp (University of Münster); and image courtesy of Christine Strullu-Derrien.

not exhibit any features that would establish a compelling link to fungi, and, in our view, even their biological origin remains questionable.

Much more convincing in terms of biogenicity are filamentous fossils from 1.01–0.89 Ga (Early Neoproterozoic) recovered from shallow estuarine marine deposits of the Grassy Bay Formation (Arctic Canada)[27,97] (TABLE 2). These *O. giraldae* are branched, apparently septate and develop conspicuous spheres (33–80 μm diameter) interpreted as spores. By themselves, these features are not compelling evidence of an affinity with fungi as they are also found in algae. Analysis of the organics by Fourier-transform infrared spectroscopy yielded absorption bands that were interpreted as indicating chitin or chitosan typical of fungi[27] (BOX 1). We contend, however, that controls would be needed to show that Fourier-transform infrared spectroscopy could distinguish fossilized, partially degraded chitin from cellulose[98], and that, lacking these controls, the affinities of *O. giraldae* with fungi in general, and Dikarya in particular, remain doubtful. Most recently, pseudo-septate filaments attributed to fungi were discovered in sediments from 0.80–0.72 Ga (Early–Mid Neoproterozoic) recovered from deep drill cores made originally during the 1950s in the Mbuji-Mayi Supergroup (Democratic Republic of Congo)[29] (TABLE 2). It should be noted that an alternative perspective on the dating of this sequence would make these fossils very much older, perhaps as much as ~1 Ga (REF.[99]). The sediments are a marine-influenced dolomite that is thought to have been subject to aerial exposure. Evidence of chitin was inferred using a combination of complementary analytical techniques (BOX 1; TABLE 2). The common theme among these recent reports is a filamentous structure with few morphological characteristics. Establishing a fungal affinity relies on ruling out filamentous bacteria or archaea and photosynthetic eukaryotes by establishing the presence of chitin residues in thermally altered and partly decomposed organics using various chemical analytical techniques. Even if one accepts the evidence of chitin, it is not possible to specify a more precise position for these fossils in the fungal tree of life.

Fossil evidence of fungi in the Palaeozoic. Direct evidence of fossil fungi from the Cambrian through Silurian Periods is sparse. Fossil hyphae and spores of *Palaeoglomus greyi* from the Ordovician Guttenberg Formation (Wisconsin, USA) are frequently cited as the earliest evidence of Glomeromycotina at ~460 Ma (REF.[100]). Although resembling extant Glomeromycotina, they are not associated with any plant fossils. Furthermore, these remains could be more recent contaminants of the porous dolomitic sediment in which they were found[11].

Acritarchs
Organic-walled microfossils of biological affinity, primarily eukaryotic.

Box 1 | **New tools for old fossils**

Molecular approaches

- Molecular clocks estimate geological ages of nodes using fossil calibrations, and by assuming that modelled molecular change is proportional to time. Reliable clocks would be based on accurate phylogenies, correct interpretation and dating of fossils[145], and correct modelling of molecular evolutionary processes.
- Criteria for reliable clocks are hard to meet. The temptation is strong to constrain clade ages closely by over-interpreting the few available fossil calibration points, ignoring alternative interpretations that would more realistically reveal uncertainty.

Microscopy

- Confocal laser scanning microscopy (CLSM) yields 3D images of minute objects in exquisite detail with a resolution of <1 µm (REF.[146]). This method resolved spores encased in fungal sporangia and embedded in Rhynie chert[108,147]. The CLSM digital tomographic data sets (sets of optical sections) are converted computationally into 3D visualizations or animations of fungal structures[30]. Similar to other techniques listed below, CLSM is largely non-destructive.
- Scanning electron microscopic analysis of weathering patterns in mineral particles, together with detection of high levels of iron by energy-dispersive X-ray spectroscopy, can help distinguish fungal fossils in paleosols[31].
- Fluorescent stains characterize cell wall components, although without high specificity. Wheat-germ agglutinin complexed with a fluorophore labels the *N*-acetylglucosamine residues in chitin. However, it also binds other sugars, and *N*-acetylglucosamine occurs in other organisms, for example, sheaths of some cyanobacteria[148]. Calcofluor white fluorescence labels chitin, cellulose and other polysaccharides.

Spectroscopic methods

- Raman microspectroscopy can reveal the geological history of exposure to heat and pressure of an organic fossil[29,149,150]. These data can help distinguish bona fide fossils from recent contaminants.
- Fourier-transform infrared microspectroscopy involves infrared irradiation of samples and detects organic functional groups, including the amino groups that distinguish chitin from cellulose[27,98].
- Synchrotron X-ray absorption near edge structure uses high-energy irradiation to quantify speciation and bonding in inorganic-rich fossils[29,98].

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

Tortotubus protuberans (also known as *Ornatifilum lornensis*), when first documented from Silurian sediments, was interpreted as an ascomycete with what appeared to be lateral, flask-shaped, spore-producing cells[101]. However, Smith[102] compared material from several Silurian and Devonian sites, and showed that the flask-shaped cells were broken bases of filaments instead of spore-producing structures, casting serious doubt on an affinity with Ascomycota. Fossils of *T. protuberans* are plausibly of terrestrial origin and their features include branched filaments with pseudo-septate pores. Smith's study illustrates the value of basing interpretation on careful observations and a rich set of collections.

The beginning of the Devonian Period (419 Ma) saw a substantial change in the nature of the geological record marked by the widespread development of terrestrially deposited sediments on a global scale. The shift from dominantly marine to terrestrial preservation resulted in the first appearance of many land-dwelling lineages in the fossil record[4,83,103]. Many fossil sites from across Europe, Asia, the Americas and Australia testify to the diversity of life on land, but one site stands out because organisms and their interactions were remarkably preserved. The Rhynie chert (407 Ma; FIG. 3) preserves elements of a geothermal wetland including seven species of small herbaceous vascular plants (~20 cm maximum height), representatives of eight groups of arthropods, one nematode, several groups of green algae, including a charophycean streptophyte alga, oomycetes, testate amoebae, cyanobacteria and numerous varied fungi, including Chytridiomycota, Blastocladiomycota, Mucoromycota and, possibly, Ascomycota[11,104–108]. Furthermore, Rhynie chert plants and fungi demonstrated saprotrophic[11,30,109] (FIG. 4) as well as symbiotic (both parasitic[108–111] and mutualistic[76,112,113]) interactions (FIG. 3). Mycorrhizal associations in the Rhynie chert provide the earliest evidence of a relationship that can be traced through the geological record up to the present time. Fossils document a Mucoromycotina mycorrhizal association[76], in addition to two well-known fossils of arbuscular mycorrhizal associations involving Glomeromycotina[76,112,113]. In early fossil plants (lacking roots or rhizomes), the development of arbuscular mycorrhizae was in prostrate axes and aerial axes[76,112,113], a situation similar to that found in some modern bryophytes, ferns and orchids[114]. The diverse and well-established mycorrhizal interactions between fungi and plants in the Rhynie chert are consistent with molecular phylogenic and genomic inferences that these interactions predate the Devonian Period by over 100 Ma.

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

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

Among the most unexpected fossils allied to fungi is *Prototaxites*, which is encountered in Devonian rocks ~419–359 Ma[121]. The largest specimens resembled tree trunks or logs, reaching over 8 m in length, although small specimens also occurred. Based on its structure of interwoven filaments and tubes of various sizes, Hueber[122] hypothesized that *Prototaxites logani* was a giant sporophore of a species in the Basidiomycota. Recently, an affinity with Ascomycota was suggested for the small *Prototaxites taiti* with the discovery of a 1-mm-thick layer of meiotic cells, 'asci', each with more than eight spores[121]. The ecological role of *Prototaxites*

Testate amoebae
Unicellular protists, polyphyletic, with an organic, mineral or agglutinated shell (test) that partially encloses the cell.

Prostrate axes
Creeping stem-like branches of early fossil plants that can be surficial or subterranean and that do not have all of the characteristics of a root or a rhizome of living plants.

Aerial axes
Erect stem-like branches of early fossil plants that do not have all the characteristics of the stem of living plants.

Sporophore
Spore-producing structure in fungi; a mushroom is a modern example.

remains enigmatic. Hobbie and Boyce[123] concluded that the carbon isotope ratios of *Prototaxites* indicated that it was a saprotrophic fungus. Selosse argued that the surrounding Devonian primary producers could not have supported such a large saprotroph, and suggested that lichenization, which would have enabled carbon acquisition from algal partners, was more likely[124].

Adding to the mysteries of *Prototaxites* is the recently described *Cyathinema digermulense*[125]. This organically preserved fossil from the Nyborg formation in Arctic Norway at 580 Ma resembles the charcoalified body parts of *Prototaxites* sp.[121], although *Cyathinema* predates *Prototaxites* by 150 million years. In particular, the cups and tubes of *Cyathinema* resemble the tubular threads in the hymeninal layer of *Prototaxites*. A more prosaic interpretation of the phylogenetic relationships of *Cyathinema* would place it on the red algal lineage, an interpretation consistent with its probable location in a shallow marine habitat, rather than in a terrestrial habitat as occupied by *Prototaxites*. This example illustrates the implicit challenges in distinguishing fossil fungi from algae, and highlights the importance of finding more fossils to understand the diversity of early eukaryotes.

Lichens, mutualistic symbioses between fungi and cyanobacteria or chlorophyte algae, are polyphyletic, arising from multiple lineages of Ascomycota and Basidiomycota. Based on recent molecular clock analysis of multiple lichen fungi and their diverse, polyphyletic algal associates, Nelsen et al.[12] concluded that extant lichen lineages originated after, not before, land plants. Lichenization probably enabled diversification of the partners after the rise of multicellular land plants, by adaptation to niches unsuitable for the latter[73]. Cyanobacteria are found in ~15% of lichens and they have been available as potential photosynthetic partners for ~2.4 Ga, yet there is no evidence for their involvement in lichens until after the evolution of land plants[12].

The early fossil evidence for lichens is scant[126]. The controversial interpretations of *Dickinsonia* fossils from the Ediacaran (635–541 Ma) as lichens[127] or fungi[128] have been widely criticized[126] and are further contradicted by recent geochemical evidence of cholestane, which is a residue of cholesterol, a sterol that is more consistent with an animal than fungal interpretation[129]. Likewise, the lichen-like symbioses with cyanobacteria in the marine Doushantuo Formation (551–635 Ma)[130] are less than compelling, and alternative hypotheses involving filamentous bacteria rather than fungi are possible. The lichen-like *Winfrenatia reticulata* described in the terrestrial Rhynie chert (407 Ma)[131–133] bears no morphological similarity to extant lichens, and although it represents a loose consortium of fungi and cyanobacteria (TABLE 2), a mutualistic relationship cannot be inferred from the morphological clues that the fossil presents. The earliest, most compelling evidence of fossil lichens comes from Early Devonian (415 Ma) sediments of the Ditton Group (England)[134,135]. Two types of thallus-like fossils preserved in charcoal (*Chlorolichenomycites salopensis* and *Cyanolichenomycites devonicus*) have an internally stratified structure. Fungal hyphae are exceptionally well preserved; photobionts less so, but plausibly represented by clusters of pyrite crystals. The fungus was interpreted as a Pezizomycotina but it would be older than estimates of the stem age of extant lichens[12] (FIG. 2). Here, again, the Devonian fossils might be extinct lineages of lichens that are unrepresented in molecular phylogenetic studies of living species.

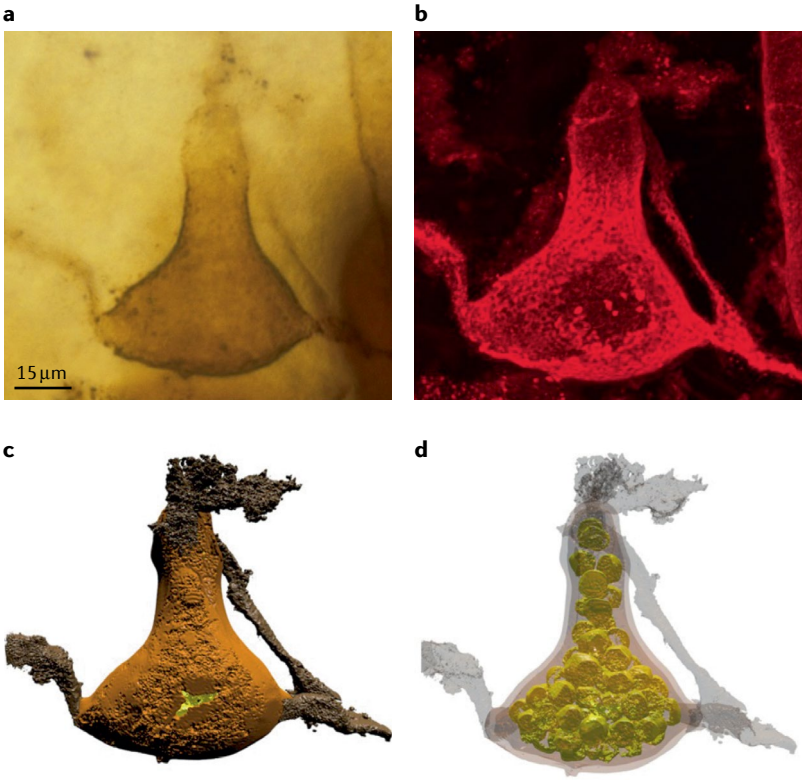


Fig. 4 | **Rhynie chert sporangium.** Confocal laser scanning microscopy reveals internal structural detail of a sporangium of *Retesporangicus lyonii* from the Rhynie chert from 407 million years ago. **a** | Light microscopy. **b**–**d** | Confocal laser microscopy. Images processed[30] to show outer surface of the sporangium (part **c**) and closely packed interior spores (part **d**). Parts **c** and **d** reprinted with permission from REF.[30], Royal Society of Publishing. © 2017 The Author(s).

Conclusions and future directions

We contend that fungi were early colonists of freshwater environments that were already occupied by biofilms of cyanobacteria and photosynthetic eukaryotes, the forerunners of green and red algae and land plants. The earliest fungi were protist-like with phagotrophic nutrition. Osmotrophic nutrition and mycelial growth initially developed as responses to the availability of abundant carbon sources produced by photosynthetic streptophytes. The diversification of pectinase and xyloglucanase genes in fungi and the capacity for streptophyte algae to recognize and respond to fungi indicate that fungal adaptations to land plants as a source of carbon began with their freshwater algal ancestors. Another major step enabled the rise of the current terrestrial flora and fungi: the mycorrhizal association. The rise and spread of woody plants on land triggered a later diversification of oxidative fungal enzymes capable of depolymerizing lignin to make available for fungal nutrition the vast stores of cellulose and hemicellulose trapped in

wood. Perhaps similar patterns could also be observed for other complex organic molecules that appeared early in the geological record (for example, sporopollenin and cutin).

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

We have reviewed the profound insights that are resulting as a flood of new techniques are applied to the analysis of fungal genomes and to the analysis of fossil chemistry and morphology. At present, dated phylogenies and genomic evidence for interactions with streptophyte hosts indicate that fungi are older than the unambiguous evidence for them provided by their Rhynie chert fossils of 407 Ma. Fascinating, newly discovered fossils backed by chemical analysis are far older, but their occurrences are separated by gaps of over 100 hundred million years, and their identity as fungi is equivocal and has yet to be corroborated by information about their habitats or ecology.

We anticipate that discoveries and interpretation of new fossils will close gaps to reveal patterns of fungal continuity, radiation and extinction through the ages. The sophistication of evolutionary inferences from genomes will be increased by taking into account increasingly robust patterns recorded in the fossils. We hope that new researchers and their funding agencies take note of the opportunities that are arising for the critical challenge of fundamental ideas about ancient fungi and their ecology. Recognizing the fungi, correlating them with habitat and placing them into the tree of life, although never straightforward, will pave the way to a better understanding of ancient ecosystems.

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