A Cryptic Alternative for the Evolution of Hyphae

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A growing awareness of a subsurface fossil record of mostly hyphal fungi organisms stretching back through the Phanerozoic to ≈400 megaannum (Ma) and possibly earlier, provides an alternative view on hyphal development. Parallel with the emergence of hyphal fungi during Ordovician–Devonian times when plants colonized the land, which is the traditional notion of hyphal evolution, hypha-based fungi existed in the deep biosphere. New insights suggest that the fundamental functions of hyphae may have evolved in response to an ancient subsurface endolithic lifestyle and might have been in place before the colonization of land. To address the gaps in the current understanding of hyphal evolution a strategy based on research prospects involving investigations of uncharted geological material, new diagnostics, and comparisons to live species is proposed.

1. Introduction

Depending on their life cycle, metabolism, and habitat preference, microorganisms take on many morphological variations such as coccoids, rods, and filaments, or transitions between these.[1] Hyphae, the filamentous vegetative growth mode of fungi are exceptional in their nature and gives fungi many ecological advantages. While the basic function of most prokaryotic filaments (and those of eukaryotes such as filamentous algae) is compartmentalization and arrangement of cells, hyphae are highly advanced. Hyphae enable complex community building (mycelia), endo- and exo-symbiotic relationships (lichens, mycorrhizae, fungal-prokaryotic, fungal-animal), inter-cellular communication, and selective spatial exploration.[2]

Hyphae are the predominant filamentous form among higher fungi (Ascomycetes, Basidiomycetes), and they are frequent, but sometimes with variations (i.e., rhizoids), among lower fungi such as Chytridiomycota. Hyphal growth is not exclusive for fungi but also occurs among members of Actinobacteria, Oomycetes, and Stramenopila. Hyphoid cell types are found within both the animal and the plant kingdoms (e.g., neurons, pollen tubes).[3]

Although certain basic features, such as shape and growth pattern, can define hyphae, it is obvious that hyphae do not represent a uniform state but high variability and multiple evolutionary origins. Even filamentous fungi have hyphal morphologies that differ between clades (e.g., occurrence of septa and their associated pore complexes, anastomoses, and spindle pole bodies).[4,5]

The physiological functions of hyphae are many but predominantly trophic. Hyphae secrete enzymes across their cell wall to break down nearby complex organic compounds into simple, diffusible molecules that are absorbed into the cell to nourish growth. The morphology and directed growth also facilitate penetration of substrates and subsequent absorption of nutrients. Hyphae use hydrostatic pressure to penetrate tough substrates such as soil and plant tissue—enabling plant diseases, serving as mycorrhizal partners to plants, or decomposing litter and woody debris. Harder substrates, like many minerals and metals, dissolve by extraction of organic acids, chelates, and siderophores at the hyphal tip, sometimes leaving undulating tunnel-like structures in the penetrated substrates.[6,7]

The fundamental aspect that distinguishes hyphal growth from most other filamentous growth modes is the apical extension of the hyphae. This ensures that hyphae normally grow away from one another to form mycelium with an outwardly migrating growing front, a growth pattern that makes exploration and invasion the fundamental lifestyle of fungi.[8] The characteristic behavior of fungi is to explore the habitat with rapidly growing, sparsely branched hyphae. When some of these hyphae find a nutrient resource, the extension rate declines, rate of branching increases, and the mycelium captures and exploits the resource. Subsequently, the fungi send out a new generation of exploratory hyphae and/or populations of spores.[9–13] This lifestyle allows filamentous fungi to dominate their ecosystems because it gives them the tools they need to find and colonize new substrates rapidly, and grow away from nutrient-poor areas.
Branching of the hyphae enables substrates to be captured from the environment for absorption and efficient exploitation. Maintaining a high extension rate even under poor nutrient conditions allows fungi to maximize their chances of finding new food sources. The success of this growth habit is indicated by the extraordinary diversity of fungal species, their distribution in virtually every habitat on the planet and the parallel evolution of a similar growth strategy by other microorganisms among both bacteria and eukaryotes.\[2,3\]

The rigidity of fungal cell walls as well as the ability to anastomose and form interconnected filamentous networks further enable fungi to build stable and complex mycelial colonies. The interconnected nature of a mycelium on a cellular level enables translocation of molecules in the complex network including nuclei and organelles, nutrients, and water, and maintenance of general homeostasis;\[14,15\] crucial properties when engaging in an explorational lifestyle, invading and colonizing pristine, and sometimes spatially restricted, environments. It has been presumed that the colonization of land and subsequent development of plant symbioses has driven the specialization of fungal hyphae.\[16,17\] Recent discoveries of a rich fungal fossil record in deep, crystalline bedrock environments\[21\] allows us to consider an alternative history of hyphal evolution, one that occurred deep in Earth’s crust.

### 2. Early Fungal Evolution in Marine and/or Terrestrial Environments

The deep regions of the eukaryotic tree are far from resolved, and the exact order of divergence is under debate.\[19,20\] The nearest relatives of the fungal clades, including nucleoid amoebae and Microsporidia, are flagellated unicellular forms.\[21\] All the basal lineages are eukaryotes presumed to retain key characters of the last common ancestor of fungi and animals. These include a unicellular body bounded by a cell wall, which matures into a sporangium. Within the sporangium numerous uniflagellate zoospores develop, which are cleaved from the sporangial cytoplasm by fusion of vesicles produced by a Golgi apparatus, and swim with the aid of a flagellum to a fresh substrate. Attached to the substrate they retract the flagellum and secrete a cell wall to encyst. The cyst germinates to start the life cycle all over again. Most Chytridiomycota produce small, anucleate hypha-like filaments called rhizoids for attachment to substrates and penetration of food sources. It is generally believed that rhizoids served as precursors to hyphae, and some Chytridiomycota even form true hyphae. Thus, the transition from rhizoids to true hyphae presumably took place within the Chytridiomycota.\[21\]

The selective forces that pushed fungi to hyphal growth are presumed to be associated with the colonization of terrestrial habitats. Whereas Chytridiomycota are aquatic and display a variety of vegetative growth forms, including hyphae, their descendant lineages (previously named zygomycetes) are in general terrestrial and propagate as hyphae or yeasts (Figure 1). In higher fungi like Ascomycota and Basidiomycota yeasts occur but hyphae are the predominant vegetative growth stage. Thus, the deeper roots of the fungal tree under this scenario consist of non-hyphal fungi. It is currently uncertain what types of selection have favored hyphal growth. One attractive possibility may have been the association with plants as part of mycorrhizal symbioses.\[17\] It is widely accepted that the association of fungi with plants facilitated plant colonization of terrestrial habitats.\[16\] and it seems reasonable that elongated hyphae would be better suited to foraging for the minerals and nutrients that are sought by the autotrophic partner. It is also generally believed that the fungal-plant symbiosis lead to the unprecedented diversification of fungi in Devonian times.\[22\]

Morphological transitions in fungi are intriguing and far from understood. From an evolutionary point of view, based on morphology alone, it is easy to assume that the less complex unicellular yeast forms evolved into more complex multicellular hyphae. However, phylogenetic analyses point to hyphae as the ancestral growth form.\[8\] Many higher fungi are dimorphic, meaning that they can switch between the two predominant vegetative growth stages, hyphae and yeast. Such transitions are usually explained as adaptations to environmental changes.\[8,21\] For instance, filamentous fungi of the Onygenales grow as yeasts at elevated temperatures, but still as hyphae at lower temperatures, and numerous filamentous fungi of the Ascomycetes switch to yeast-like budding morphology when they produce conidia.\[23\] Filamentous soil fungi switch to yeast growth stages propagating by budding when inhaled by mammals;

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**Figure 1.** Phylogenetic tree showing introduction and losses of morphological traits within the fungal clades. Adapted with permission.\[21\] Copyright 2009, Cell Press.
a transition that probably reflects an ancestral response to predation by soil amoebae.\textsuperscript{25,26} There are also instances where respective hyphal and yeast growth is abandoned in favor of the other. Losses are explained by various environmental factors such as oxygen deficiency, nutritional requirements, pH, or desiccation. Many yeasts thrive in specialized ecological niches, sometimes classified as extreme, that might select against hyphal growth. Black yeasts, for instance, are often found in habitats such as salterns, polar deserts, and on rock surfaces.\textsuperscript{27} It is assumed that under these conditions, hyphal growth would be disadvantageous. Because of the severe stresses associated with these habitats, black yeasts exhibit numerous adaptations,\textsuperscript{28} including remarkably slow growth, thick melanized cell walls, and a meristematic morphology. Thus, environmental aspects seem to be the predominant trigger for morphological transitions within fungi.

The point in time for land colonization and evolution of terrestrial ecosystems is an interesting avenue of research. In the Cambrian time, and the lichen symbiosis was well established by the early Devonian,\textsuperscript{29,30} indicating that fungi are older. Fossil evidence for fungal interactions (such as cyanolichenization, mycorrhizas, and vesicular arbuscular mycorrhizal symbiosis) with other organisms comes from the \( \approx 400 \) million-year-old Rhynie chert in Scotland.\textsuperscript{31,32} The presence of fossil cryptospores suggests that photosynthetic organisms inhabited land already by the Cambrian.\textsuperscript{33} Regardless of when the earliest land plants appeared, it is most likely that they were preceded by heterotrophs preparing and shaping new niches in the terrestrial environment, which in turn must have been predated by cyanobacteria and heterotrophic bacteria.\textsuperscript{14} The fungal fossil record of the Precambrian is poor and ambiguous but suggests that marine filamentous fungi were present by the late Riphean (\( \approx 1.03–1.02 \) gigaannum (Ga)).\textsuperscript{35} A shallow-marine lichen-like symbiosis has been described from the Doushantuo Formation (\( \approx 635–551 \) Ma), South China.\textsuperscript{36} and some Ediacaran fossils (575–542 Ma) have been interpreted, on the basis of taphonomic observations, as fungi\textsuperscript{47} and lichens.\textsuperscript{38} Except for an ambiguous and highly disputed fungal/lichen interpretation of some Ediacaran fossils from alleged paleosols,\textsuperscript{39,40} the fossil record does not support a terrestrial presence of fungi prior to the Ordovician.\textsuperscript{32}

### 3. A Fungal Fossil Record in Deep Igneous Crust

The last decade has witnessed the discovery of a fungal fossil record in deep igneous rocks, both in the oceanic and the continental crust.\textsuperscript{41–43} Most of the fungal fossils consist of hyphae propagating from mineral surfaces into the open pore space of fractures and vugs where they form complex mycelium-like networks.\textsuperscript{18,44} Additional to hyphae, yeast-like growth stages and reproductive organs like sporophores, spores and spore sacs have been described.\textsuperscript{18,44,45} Based on morphological traits and the presence of chitin, some fungi fossils have been interpreted as Ascomycetes or stem-group Dikarya.\textsuperscript{46} Fossilized fungi from the Vesteris Seamount, Greenland Basin, were interpreted as Zygomycetes (or lineages previously named Zygomycetes) based on the identification of zygosporous and various morphological stages of the Zygomycetes reproduction cycle.\textsuperscript{47} Thus most major and higher clades of fungi are represented in the deep fungal fossil record. Studies of modern fungal communities in deep settings are in agreement with this and show a surprisingly high diversity represented by all major fungal phyla\textsuperscript{48,49} including several deep-branching novel lineages\textsuperscript{50} and adaptation to deep biosphere conditions.\textsuperscript{51}

Despite the recent discovery of this underexplored environment, a consistent fungal record stretches from the present to \( \approx 80 \) Ma in modern oceanic crust. Additionally, hypha-forming fungus-like fossils with close similarities to the fossilized fungi in the modern oceanic crust have been found in ophiolites of Cretaceous and Devonian ages.\textsuperscript{52,53,54} In continental crystalline basement fungal fossils ranging back to Devonian age (\( \approx 400 \) Ma) have been described from various sites in the Swedish igneous basement.\textsuperscript{55,56} Thus, hyphal fungi were present at great depths in the oceanic and continental crust contemporaneous with the presumed evolution of hyphae as plants and fungi colonized the terrestrial realm.\textsuperscript{18,57}

Preceding the Devonian fossils there is a two billion-year gap in the deep biosphere fossil record prior to the mycelium-forming hyphae of a fungus-like organism described in basalt from the 2.4 Ga Ongeluk Formation, South Africa.\textsuperscript{59} These fossils do not represent the remnants of rhizoids, considering size and morphology, but well developed hyphae that were able to anastomose. The striking similarity between the Paleoproterozoic Ongeluk fungi and Phanerozoic fungi with respect to morphology, dimensions, growth habit, and preservation is worth noting.\textsuperscript{18,53,54,59} The examples from Devonian pillow lavas are particularly significant because they show preservational features similar to those in the Ongeluk vesicles, with chlorite mineral encrustations of the filaments.\textsuperscript{53,54} Despite the similarities, the 2 Ga gap makes an evolutionary link between the Proterozoic and the Phanerozoic fossils difficult and we cannot exclude the possibility that the Ongeluk fossils represent a separate and/or extinct branch of fungus-like organisms.\textsuperscript{59}

### 4. Function of Hyphae in Cryptic Habitats

The presence of hyphal fungus-like fossils throughout the Phanerozoic and perhaps earlier suggests that hyphae represent a general morphologic state in deep igneous environments through time. Yeast morphology is also found in the fossil record but not as abundant as hyphae. This may be an effect of preservation, and other fungal tissues may not be as easily preserved as hyphae. Nevertheless, hyphae are abundant and found in all occurrences of deep fungal fossils. The advantage of the hyphal growth mode in these endolithic habitats is unknown. An obvious possibility would be attachment to rocky substrates. However, this does not seem to be the entire explanation because endolithic fungal colonization starts from a biofilm laid down on the fracture walls from which the hyphae grow out into the open pore space.\textsuperscript{18,43,44,60,61} The dominant function of hyphae among the endolithic fungi seems to be to conquer the open pore space rather than attachment. Numerous samples from the oceanic crust show how fungal hyphae fill the entire pore space by forming a three-dimensional network (Figure 2a–c). In most environments, hyphal growth is normally along a surface of a mineral grain or a root, while in cryptic pore space the general
Figure 2. ESEM images showing stepwise colonization of open pore space in subseafloor basalts. A) A few hyphae protrude perpendicularly from the biofilm that lines the inner wall of the vug. At the beginning of protrusion into the open pore space hyphae are already tending to branch and anastomose between branches (arrows), striving to build a complex mycelium. B) A complex mycelium protruding from an initial biofilm. C) A vug in basalt completely filled with fossil fungi mineralized by montmorillonite clay. D) A limited part of a carbonaceous fossilized fungal community in continental basement granite from the Lockne impact crater, drill core LOC1 (219.90 meters depth). The biofilm display a smooth surface while the nature of the hyphae varies between smooth and more irregular. bf: biofilm; hr: host rock; hy: hyphae.

direction of growth is predominantly perpendicular to a surface or substrate and away from other hyphae. Even though hyphal growth may follow mineral surfaces, the overall growth behavior of the mycelium results in propagation into the open pore space. Why is the invasion of open pore space so important in these environments? In an oligotrophic and space-limited environment, optimizing the surface for nutrition uptake may be crucial for survival. The source of subsurface dissolved organic carbon (DOC) is not well investigated but predominantly consists of prokaryotic (and eukaryotic?) biomass. Abiotically formed organic compounds can originate from fluid-mineral interactions but not in amounts sufficient for fungal metabolism, and certainly not at rates to sustain a continuously growing fungal community. DOC in deep mines in South Africa is predominantly derived from microbial metabolites and modified cell components (membrane lipids and proteins) ranging from 0.25 to 4.9 mg C/L.[62] Schäfer et al.[63] revealed the presence of sugars such as fucose in biofilm material filling the cracks within the Åspö Hard Rock Laboratory (HRL) aquifers. Leefman et al.[64] studied the formation of biofilms in the Åspö HRL and detected attachment (within 10 min) of amino acids, carbohydrates, and carboxylic acids. This suggests an abundance of these compounds in the water but also a more abundant and diverse microbial flora in shallow DOC-rich fluids compared to waters in deeper aquifers with less DOC.[65]

Maximizing the hyphal surface area for absorption of organic compounds will be key to survival and sustaining vital colonies. A simple calculation shows that a biofilm covering the surface of a spherical vug with a diameter of 1 mm has a surface area of 3141592 µm² (4π r² = 3141592 µm²). If we instead assume a mycelium in this particular vug and calculate the total surface area of such a mycelium with the average hyphal diameter of 10 µm and the average length of 200 µm (excluding any irregularities). The surface area of a cylinder with 10 µm diameter and 200 µm length would constitute an area of 6283 µm² (2πrh = 6283 µm²). If we assume that the mycelium consists of 1000 hyphae we get a total hyphal surface area of 6283000 µm². Producing a mycelium thus gives twice the surface area for uptake compared to a biofilm. Producing a three-dimensional mycelial network therefore increases the opportunities of contact between hyphal fluid and biomass particles compared to a biofilm limited to only the vesicle walls. Besides, biofilms lining host rocks are in general more smooth compared to hyphae that vary from smooth to irregular (Figure 2d). Thus, in an oligotrophic environment where habitable space is limited, the usage of all available space is crucial, and forming a three-dimensional (3D) network as a mycelium is the optimal strategy to produce maximized hyphal surface for nutrient uptake.

Another advantage with maximizing hyphal surface area is interaction with other microorganisms. Prokaryotes occur in symbiotic-like relationships with fungi, using their hyphal mycelium as a framework for growth.[43,44,60] In subseafloor basalts, fossil fungi are syngenetic with prokaryotes associated with iron- and manganese oxidation, whereas in continental basement, fossil fungi have been associated with bacterial sulfate reduction and methanogenesis.[56,57,61] A possibility is that hyphal growth into open pore space is a strategy, not only to reach open space, but also to act as substrate for prokaryotic growth,
enabling subsequent decomposition of the prokaryotic biomass by the fungal host. Thus, hyphal interaction with other organisms plays an important role in the subsurface realm. These above arguments would be applicable to other environments as well, like soils, but in subsurface endolithic habitats where habitable space is extremely limited they would be decisive.

The hyphal growth in subsurface pore space is in line with the fundamental behavior of fungi to explore their habitat.[2] The apical extension of the hyphae ensures that hyphae grow away from one another with the aim to find nutrients. With rapidly growing and sparsely branched hyphae, fungi can explore spatially restricted micro-cracks and fissures in rock (Figure 3). When hyphae find spatially more extensive pore space with better chances of mycelial growth and nutrient resource exploitation, the hyphal extension rate declines, rate of branching increases, and the mycelium growth is initiated. As the mycelium grows and available space as well as nutrient resources end, the fungi send out a new generation of exploratory hyphae and/or populations of spores to explore the interconnected network of microcracks in deep rocks[13-15] (Figure 4).

Despite preferred growth in open pore spaces, fungi are involved in dissolution and penetration of minerals in deep rock hosted environments. Fungal hyphae penetrate millimeters into secondary minerals like carbonates and zeolites, using physical force at mineral cleavages (Figure 5a) as well as chemical dissolution (Figure 5b).[44,60] This can be due either to trophic requirements, scavenging elements and metals like C, Ca, Na, P, Mg, Fe, Mn, or to the acquisition of new habitable space. It has also been shown that fungal hyphae have been “buried alive” by mineral growth but continue to grow by dissolving the mineral until the surface of the secondary mineral has been reached. Thus, boring in minerals may reflect survival strategies as well (Figure 5c).[60]

A similar cryptic behavior is seen among mycelial-fungi invading organismal carbonate tissues like bones, mollusc shells, and reef corals.[66-68] Exploratory fungal hyphae penetrate the substrates by dissolution or physical force and as they reach voids and open spaces within substrates they grow and form mycelia that partly or almost entirely fill the space in a similar manner as the endolithic fungi. These findings are exclusively Mesozoic and Cenozoic and thus later than the emergence of endolithic fungi. The growth pattern and opportunistic colonization of open space in an otherwise dense medium is identical to the behavior of endolithic fungi but whether or not the proliferation of hyphae in igneous rock micro-fractures tended to encourage fungal hyphal growth in other dense media is difficult to deduce at this point.

5. Endolithic Adaptations Enabled Terrestrial Take-Over

The development of hyphae and mycelia in rock-hosted environments is most likely a consequence of the nature of the habitats. The physical environment of microscale interconnected cracks and vesicles in deep rocks favors spatial exploration by apical...
extension of hyphae and subsequent growth of complex biological networks. It is reasonable to assume that marine fungi at one, or probably several, occasions were introduced to the sub-seafloor realm and were forced to adapt to the cryptic conditions. Marine fungi are flagellated but form hyphae when attaching to substrates, either in suspension in the water column or on the seafloor like whale carcasses, coral reefs, or rock substrates.[67] The microscale environment of interconnected fractures in igneous crust does not favor flagellated and motile fungi, but growth of hyphae or long-range dispersal of spores would be more effective. Therefore, an endolithic life style would result in loss of motile gametes and the development of hyphae and sporophores, as is seen in the deep biosphere fossil record including the 2.4 Ga fungus-like fossils. The basal lineages of fungi and most eukaryotes are flagellated unicellular microorganisms, and the hyphal nature of the endolithic deep biosphere fungus-like fossils including the 2.4 Ga Ongeluk fossils does not necessarily challenge the concept that unicellular fungi preceded hyphal fungi. However, it indicates that hyphal development was established in the subsurface realm at least 400 million years ago, probably earlier, and that the ability to form hyphae is latent among fungus-like organisms. Hyphal development could very well have been prompted by the physical nature of the deep endolithic habitats, and not by the colonization of terrestrial habitats on land.

The physical conditions of cryptic habitats enabled the development of hyphae and the exploring mode of life among early fungus-like organisms. The nature and requirements of hyphae in deep endolithic settings are not that different from the nature of fungal hyphae in surficial and soil environments, where fungi thrive by invading new habitats, penetrating substrates, decomposing organic matter, and engaging in symbiotic, or parasitic, relationships with other organisms. Thus, the early adaptations to an endolithic lifestyle in deep terrestrial settings involved development of the morphological forms necessary for colonization of the surficial terrestrial realm. The deep endolithic scenario is an equally plausible mechanism of hyphal evolution as the traditionally accepted one, but one that is favored by fossil evidence.

The presence of hyphal fungi in the deep subsurface and sub-seafloor crust contemporaneous with the fungal-plant colonization of land suggests that the fungal evolution may not be as constrained to the terrestrial realm as previous thought, and that early fungal diversification is more complex than the traditional “colonization-of-land-scenario”. The fossil record indicates that hyphal marine fungi were present prior to the colonization of land.[35–36] Marine fungi develop hyphae when attached to a substrate like marine debris or seafloor rocks, thus in agreement with a deep origin of hyphal fungi. The ambiguous and scarce fungal fossil record, however, eventually leads to the inevitable question: does the deep fungal fossil record merely reflect a downward migration of “surficial” fungi that (re)-colonized the deep subsurface or did fungi evolve at depth prior to the colonization of land and later migrated upwards to colonize the terrestrial environment? The fossil record is in favor of both concepts but we suggest the latter and less explored to be a legitimate alternative to the traditional surficial scenario that should be carefully tested and investigated.

In a “bottom-up” scenario specializations of hyphae would have been triggered by an endolithic lifestyle. It is likely such specializations also includes the ability of engaging in symbiotic relationships with other organisms. An intimate symbiotic-like fungal-prokaryotic partnership can be seen throughout the Phanerozoic and is established in the deep biosphere realm already at 400 Ma.[18,43,55–58] Such an interaction is probably a requirement for eukaryotic colonization of deep oligotrophic environments. If we assume a scenario where deep biosphere fungi predate the terrestrial diversification of fungi, early fungal/hyphal-prokaryote interactions could be seen as preadaptations to the subsequent connection to land plants.
Figure 5. A) ESEM image showing hyphae penetrating a calcite at crystal cleavages. B) Hyphae entering or exiting a zeolite crystal by chemical dissolution. Note the dissolution etch marks shown by white arrows. C) A 3D reconstruction of a zeolite produced by synchrotron based X-ray tomography (SRXTM). Immediate crystal formation “buried” fungal hyphae who continued to grow by dissolving the zeolite until they reached the mineral surface and as in (D) (isosurface rendering) crepted along the new surface. Black arrow marks the place of exit, white arrow the hyphae creeping along the new mineral surface.

6. Research Strategies

As a consequence of new insights of Phanerozoic (and possible Proterozoic) fungal fossils in deep settings we propose here the hypothesis that early (pre-Ordovician) fungal evolution took place among predominantly mycelial forms in the marine or cryptic crustal environment. Currently available data are insufficient for crucial tests of this hypothesis. There are obvious gaps in the deep fossil record that need to be addressed to understand fully the evolution of fungus-like fossils and especially hyphae as a morphological feature. To achieve a coherent deep fossil record by tracking the fungal record from the Ordovician backwards in time and even link the observations that are currently separated by 2 Ga we propose the following strategy:

- Explore ophiolites and continental crust of Paleozoic (especially Cambrian and Ordovician), Proterozoic, and Archaean age. For ancient oceanic crust, rocks that have undergone very low-grade metamorphism (e.g., sub-greenschist facies) are most likely to preserve fossil evidence. In continental crust the situation is slightly different and the fungi can be much younger than the rock. The age of the colonization and fossilization is then hard to attest. One approach we put forward is to correlate crust with thermochronology. With such records it is possible to identify when conditions were favorable for fungal growth, and also whether severe alteration may have played a part, leading to metamorphic overprint of fossils.\(^7\)\(^6\)\(^9\) For instance, eastern Finland has the oldest fission track ages as well as the lowest temperatures in the Fennoscandian shield, which gives the best potential for preservation of ancient fungi in the craton.\(^7\)\(^0\)
- Develop morphological diagnostic strategies for investigating fungi in deep igneous crust. SRXTM is already used for detailed morphological analysis at a resolution of a few micrometers but for more accurate determination of biological affinity based on morphology, imaging resolution needs to be improved even further. With instruments capable of higher resolution specific morphological diagnostics for hyphal fungi can be investigated and identified in more detail. Besides mycelium-forming filamentous hyphae with anastomosis, other characteristics like hyphal tip, Spitzenkörper and conidia should be carefully investigated.\(^4\)\(^5\) Such diagnostics can lead to more reliable determinations of hyphae but also enhance the fungal taxonomy of fossils.
- Development of morphological diagnostics should be accompanied by the development of more reliable chemical diagnostics. The use of biomarkers for fungi is restricted to degradation products of ergosterol and chitin but could probably be improved with careful investigations of live species. The goal should be to identify biomarkers for identification of fungi at phylum level at least. It is also preferable, when investigating paleontological material of such high ages, to use an array of microscopic (scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal laser scanning fluorescence microscopy) and spectroscopic (Raman, Fourier transform infrared (FTIR), X-ray absorption near edge

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structure (XANES)) techniques to ensure a robust analytical outcome and to optimize the detection of specific eukaryotic and fungal biomarkers. The differences in terms of cell structure and chemical composition between prokaryotic and eukaryotic cells result in different taphonomic behaviors, which can be used to discriminate between prokaryotic and eukaryotic fossils. Combining the Raman 1350–1600 cm\(^{-1}\) intensity ratio—\(I(1350)/I(1600)\) with the FTIR branching index of aliphatic chains, \(R3/2\) is one approach that has proven successful, and should be explored further.

- Develop techniques for more accurate dating of fossils in igneous crust. Radioisotopic dating of secondary minerals associated with fossils have enhanced the accuracy but dating of individual fossils would be preferable. However, such analyses rely on many factors, and certain requirements need to be fulfilled such as: 1) suitable mineralization of the organisms (e.g., U-rich minerals for U-Pb dating or pairs of Rb- and Sr-rich minerals for Rb/Sr dating); 2) closed system conditions to exclude mobilization of U; 3) the mineral assemblage having stayed below closure temperature for the isotope system; 4) absence in the precipitate of radiogenic lead, which could have been remobilized during previous transport; 5) control of mineral zonation. Identification of several generations and overgrowths so that dating represents the primary fungal hyphae fossilization event and not later overgrowths.

- Isolation of live fungi in deep igneous crust for morphological comparisons. This involves highly detailed morphological investigations of, for instance, growth features but also analysis of the community structure as a whole including microorganism interrelationships. Isolation and speciation by molecular approaches of strains are also of importance to understand the diversity and species richness of endolithic communities; knowing what species to expect makes it easier to relate morphologies of the fossil record to modern features. Even though the modern endolithic community structure are not comparable to the Proterozoic or even Paleozoic, it enhances the understanding of how to study these ecosystems. Using isolates it is also possible to perform laboratory experiments with strains on sterile rock pieces to observe initial colonization, growth, distribution, and preservation/mineralization. Such colonization experiments should also be carried out using free-living marine and terrestrial fungi.

- Refinement of molecular-clock models and their calibration against the fossil record to establish error ranges in the timing of early opisthokont evolution, with particular regard to the ages of the ancestral nodes of total-group and crown-group Opisthokonta, as well as the divergence times of major lineages within crown-group fungi.

7. Summary and Outlook

The long-standing view on the origin and evolution of fungal hyphae is that it developed in Ordovician–Devonian times when fungi, together with plants, colonized land. In the “colonization-of-land-scenario” the main drive for the development of hyphae was nutrient absorption from soils and facilitation of symbiotic and parasitic growth with, and off, plants. This is much like the fungal/plant interaction of mycorrhiza today, where a mutual exchange of nutrients favors the growth of both symbionts. Recently, the oceanic and continental igneous crusts were recognized as substantial fungal habitats in which mycelium-forming hyphae represent the predominant growth mode, according to the fossil record. The fossil record of mycelium-forming fungus-like organisms in the oceanic crust dates back to at least \(\approx 400\) Ma and maybe as much as 2.4 Ga. The emergence of hyphae in the deep subsurface realm contemporaneous with the diversification of fungi on land challenges the present notion of hyphal evolution. The evolutionary or environmental factors behind the early hypha development are still unknown but are crucial for understanding early fungal and eukaryotic evolution as well as habitat distribution. We suggest that most of the fundamental functions of hyphae evolved from an early subsurface endolithic life-style and were in place before the colonization of land. Here, we have presented a strategy for filling in the blanks of the evolution of fungal hyphae involving 1) the exploration of Paleozoic, Proterozoic, and Archaean rocks to establish a coherent fungal/hyphal fossil record, and emphasize the use of thermochronology to constrain the age of colonization; 2) the development of chemical and morphological diagnostics for fossil fungi; 3) the development of techniques for more accurate dating of fossils in deep crustal rocks; 4) isolation and culturing of live fungal species from endolithic settings for comparison with analogous fossils; and 5) refinement and calibration of molecular clocks to establish timing of early opisthokont evolution.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

deep biosphere, fungi, hyphal evolution

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