Int. J. Plant Sci. 182(2):109–122. 2021.

1058-5893/2021/18202-0004$15.00 DOI: 10.1086/712250

# *RHIZOPHYDITES MATRYOSHKAE* GEN. ET SP. NOV. (FOSSIL CHYTRIDIOMYCOTA) ON SPORES OF THE EARLY LAND PLANT *HORNEOPHYTON LIGNIERI* FROM THE LOWER DEVONIAN RHYNIE CHERT

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*Editor: Patrick S. Herendeen*

*Premise of research*. As our understanding of the importance of fungi in ecosystems today increases, we are still in the early stages of appreciating their roles in the past. The famous Early Devonian Rhynie chert contains a remarkable diversity of fungal fossils and copious evidence of fungal interactions; however, only relatively few of them have been described.

*Methodology*. Thin sections of the chert were studied at high magnification in transmitted light. Images of fossils were captured digitally and processed in Adobe Photoshop.

*Pivotal results*. A distinct eucarpic, monocentric chytrid (Chytridiomycota), *Rhizophydites matryoshkae* gen. et sp. nov., occurs on partially degraded in situ spores of the early land plant *Horneophyton lignieri*. Zoosporangia are epibiotic, usually spheroidal and less than 30 $\mu$m in diameter, and inoperculate, and they possess one to four discharge papillae or short tubes. Small bodies present within and outside many of the zoosporangia are suggestive of encysted zoospores and germinating zoospore cysts. Several specimens comprise two or more successive generations of zoosporangia occurring one inside another, a feature that allows for a direct comparison of the fossils with modern *Rhizophydium* (Rhizophydiales).

*Conclusions*. The discovery of *R. matryoshkae* interacting with the spores of *H. lignieri* expands our knowledge of the biological relationships that define complexity in early continental ecosystems and provides a new calibration point that can be used to align molecular clock estimates with fossil evidence in the discussion of chytrid evolution.

*Keywords:* internal sporangial proliferation, life cycle, mycoloop, *Rhizophydium proliferum*, saprotrophism, zoosporangium.

## Introduction

The Lower Devonian Rhynie chert from Aberdeenshire, Scotland, contains a remarkable diversity of exquisitely preserved fossils of early nonmarine aquatic and terrestrial plants, animals, fungi, and other microorganisms (Trewin and Kerp 2017; Garwood et al. 2020). Many of these fossils also provide insights into the different levels of interrelationships that existed between organisms during this epoch of geologic time (e.g., Dotzler et al. 2009; Edwards et al. 2017; Strullu-Derrien 2018). Fungal interactions with other fungi (interfungal relationships) and with land plants represent the most frequently encountered organismal associations in the Rhynie chert (Boullard and Lemoigne 1971; Taylor et al. 2004, 2015; Krings et al. 2017).

Various types of micrometer-size spheroidal, pyriform, or clavate vesicle-like structures, some with pores or papillae in the wall, occur on intact and decaying land plant axes and spores in the Rhynie chert (e.g., Kidston and Lang 1921; Taylor et al. 1992*b*; Krings et al. 2017). They are mostly interpreted as chytrids (Chytridiomycota) or chytrid-like remains of uncertain affinity (sensu Krings et al. 2009*a*) on the basis of correspondences, in overall appearance, to the reproductive structures (zoosporangia, resting spore stages) of present-day chytrids growing on plant tissue, spores, and pollen grains and because rhizoids extending from the vesicle into the host are often also present. While scattered evidence of such associations can be found almost everywhere in the Rhynie chert, large sample sets of specimens are available primarily from areas containing silicified plant litter, that is, accumulations of decaying land plant axes and empty or partially discharged sporangia (e.g., Krings et al. 2016, 2017). Unfortunately, this evidence has received very little attention to date.

Plant-associated chytrids today are effective as decomposers and nutrient recyclers, parasites, and potent disease causative agents (Powell 2017; van de Vossenberg et al. 2019). Unraveling the biological and ecological versatility of interactions between chytrid-like organisms and land plants in the Rhynie paleoecosystem would

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*Manuscript received July 2020; revised manuscript received September 2020; electronically published January 25, 2021.*

therefore be important for more accurately understanding the dynamics within early land plant communities. However, the majority of the chytrid-like remains preserved in association with the early land plants in the Rhynie chert lack diagnostic characters that are consistent (or consistently recognizable) among several specimens, and they thus cannot be directly compared with modern equivalents.

This article describes a distinct monocentric chytrid with epibiotic zoosporangia that occurs in large numbers in some litter layers of the Rhynie chert as a colonizer of spores of the land plant *Horneophyton lignieri* (Kidst. et W.H. Lang) Barghoorn et Darrah. Several specimens show new zoosporangia developing within the old, empty zoosporangia, a peculiarity documented here for the first time in fossils that allows for a direct comparison with the modern genus *Rhizophydium* Schenk (Rhizophydiales). This discovery expands the inventory of organismal interactions in early nonmarine ecosystems and provides a new calibration point that can be used to align molecular clock estimates with fossil evidence in the discussion of the evolutionary history of the Chytridiomycota.

## Geological Setting

The Rhynie chert Lagerstätte is situated northwest of the village of Rhynie in Aberdeenshire, Scotland. The fossil-bearing layers occur in the Rhynie Cherts Unit of the Windyfield Shales Member, within the lower part of the Dryden Flags Formation (Rice and Ashcroft 2003; Parry et al. 2011). The Rhynie Cherts Unit is made up of chert interpreted as siliceous terrestrial hot spring sinters, carbonaceous sandstones that represent silicified paleosols, lacustrine shales with a freshwater biota, and volcanic (tuffaceous) debris from the basin margin (Rice et al. 2002). The paleoenvironment has been interpreted as a series of ephemeral freshwater pools within a hot spring environment with a semiarid climate affected by volcanic activity (Trewin and Rice 1992; Rice et al. 2002; Rice and Ashcroft 2003). Preserved in the chert are both aquatic (freshwater) facies from the pools and subaerial soil/litter horizons with in situ plants from the margins of the pools. Organisms preserved in the Rhynie chert include bacteria, cyanobacteria, peronosporomycetes, fungi, algae, vascular plants, and animals (Kerp and Hass 2004; Trewin and Kerp 2017; Edwards et al. 2018; Garwood et al. 2020). Fossil preservation is interpreted as a result of temporary flooding of silica-rich water or groundwater high in silica percolating to the surface (Powell et al. 2000; Trewin and Fayers 2016).

The Rhynie chert biota has been regarded as early (but not earliest) Pragian to earliest Emsian in age based on spore assemblages (Wellman 2006, 2017; Wellman et al. 2006). High-precision U-Pb dating of zircon and titanite (possibly of magmatic origin) from hydrothermally altered andesite indicates an absolute age of 411.5 ± 1.3 Ma for the Rhynie chert biota (Parry et al. 2011), while another age constraint using $^{40}Ar/^{39}Ar$ on K-feldspar from two quartz-feldspar veins that are part of the hydrothermal system responsible for the formation of the Rhynie chert yields a mean age (recalculated to be U-Pb comparable) of 407.1 ± 2.2 Ma (Mark et al. 2011) for the fossilized biota. However, the andesite cannot be fixed with certainty in the stratigraphic sequence because it appears to be linked to the magma chamber at depth and is certainly older than the hydrothermal alteration (Mark et al. 2013). As a result, the estimate in Mark et al. (2011) likely gives a more accurate age for the hydrothermal system and hence the age of the Rhynie chert biota.

## Material and Methods

Fifteen sporangia of *Horneophyton lignieri* in various stages of decay that contain a total of several thousand spores were identified in litter accumulations present in thin sections (80–100 μm thick) prepared from two different chert blocks by cementing wafers of chert to a glass slide and then grinding the wafer with silicon carbide powder until the section was thin enough to transmit light. Thin sections were analyzed with a Leica DM LB2 transmitted light microscope (illumination: 12 V, 100 W, halogen, stabilized) and the highest practicable total magnification, that is, ×400 (objective: HCX PL Fluotar; numerical aperture [NA] = 0.75) and ×1000 (objective: HCX PL APO; NA = 1.35; oil). No filters were used in the light path. Digital images were captured with a Leica DFC480 camera and optimized for color and white balance using the Leica Application Suite software before images were exported directly into Adobe Photoshop CS6 via the TWAIN interface (http://twain.org) as TIFF files with eight bits per channel. Images were processed minimally in Adobe Photoshop CS6 for brightness and contrast, and images of the same specimen were recorded at multiple focal planes and stacked to produce composite images. Measurements were taken using Adobe Photoshop CS6. Chert blocks and thin sections are deposited in the Bayerische Staatssammlung für Paläontologie und Geologie (SNSB-BSPG) in Munich, Germany, under acquisition numbers SNSB-BSPG 1964 XX and 2013 V. Slide numbers for all figured specimens are given in the figure captions.

## Results

### *Host Sporangia and Spores*

The fossils used in this study are present in thin sections of 15 different largely intact or partially degraded early land plant sporangia in orientations ranging from transverse to longitudinal (fig. 1*A*–1*C*). All sporangia are characterized by a central columella (denoted as "CO" in fig. 1) and contain in situ spores of the *Emphanisporites decoratus* type (see Bhutta 1973*a*; Eggert 1974; Wellman et al. 2004) in various states of degradation (fig. 1*D*, 1*E*), and they therefore can be assigned to *Horneophyton lignieri* with confidence (for details on *H. lignieri*, refer to Taylor et al. 2009; Kerp 2017; Cascales-Miñana et al. 2019 and references therein). The sporangia (or columellate sporangial lobes sensu Eggert 1974; Edwards 2004) are situated in silicified litter layers composed primarily of loosely to densely spaced axis fragments of *H. lignieri* and, to a lesser extent, *Aglaophyton majus* (Kidst. et W.H. Lang) D.S. Edwards, abundant dispersed spores of *H. lignieri* and *A. majus*, fungal hyphae, mycelial cords, scattered individuals and clusters of the enigmatic microfossil *Perexiflasca tayloriana* M. Krings et al. (2017), various fungal propagules, and reproductive units, along with sediment.

All 15 sporangia contain at least some evidence of colonization by a conspicuous fungus that predominantly occurs attached to the outer surface of the spores but sometimes may also be detached and floating freely between the spores. Overall colonization density of the spores is less than 10%. Evidence of the fungus is generally rare in largely intact sporangia with well-preserved in

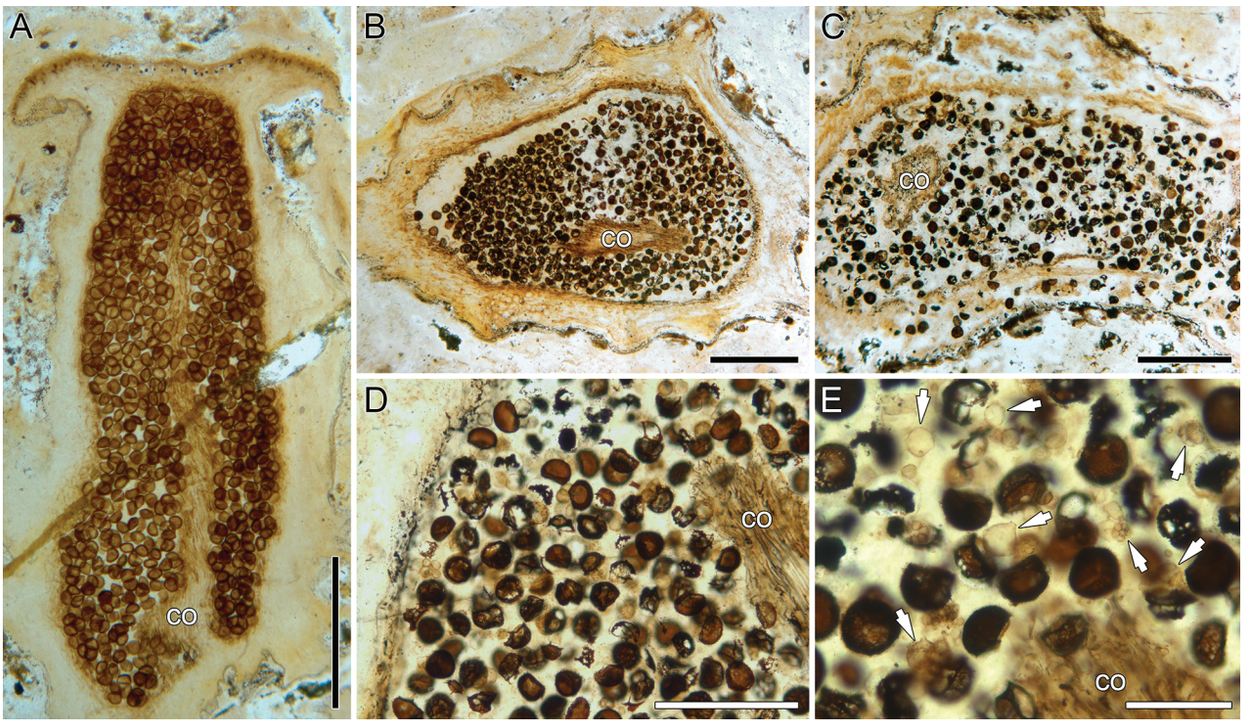


**Fig. 1** Sporangia containing in situ spores of *Horneophyton lignieri* in longitudinal and transverse section views. *A*, Nearly complete sporangial lobe in longitudinal section view. Slide SNSB-BSPG 1964 XX 24. *B*, *C*, Partially degraded sporangia in transverse section view. Slide SNSB-BSPG 1964 XX 99. *D*, Detail of *B* (rotated ~100° counterclockwise), showing partially degraded spores in situ colonized by *Rhizophydites matryoshkae*. *E*, Detail of *D*, focusing on infected spores (arrows). CO = columella. Scale bars = 500 μm (*A*–*C*), 250 μm (*D*), 100 μm (*E*).

situ spores (less than 2% of the spores colonized), whereas partially degraded in situ spore masses can show colonization densities of 25% or more (fig. 1*D*, arrows in 1*E*). More than 300 spores with attached, well-preserved fungal thalli have been identified in the 15 sporangia, in addition to a few (fewer than 10) among the dispersed *H. lignieri* spores that co-occur with the sporangia in the thin sections.

### *Fungal Remains*

The fungus occurs in the form of small, saclike, epibiotic vesicles (figs. 2, 3) that we interpret as zoosporangia of a monocentric chytrid (see "*Rhizophydites matryoshkae*: Affinities and Life Cycle"). They typically arise from the proximal surface of the host spore, where the suture (trilete mark) is also located, and they may occur singly (fig. 2*A*, 2*B*, 2*J*, 2*K*) or in multiples arising from a common penetration site in the host wall (fig. 2*C*, 2*H*). Zoosporangia in different phases of development sometimes also occur in compact clusters; some arise from the host spore, while others are attached to the outer surface of adjacent zoosporangia (fig. 2*D*–2*F*). Rhizoidal systems are not normally recognizable; if preserved, they appear as one or two sparsely branched rhizoidal axes that arise from a single site on the zoosporangium and extend into the lumen of the host spore (fig. 2*H*, 2*I*). Zoosporangia are highly variable in size and shape. Most are spheroidal and 20–30(–33) μm in diameter, but some may also be cubical or rhomboidal, somewhat elongate to spindle shaped, or (ob) pyriform and 25 μm at the widest point by 6 μm at the narrowest point; all possess a smooth wall between 0.2 and 1.5 μm thick. From one to four prominent discharge papillae or short tubes, 4–9 μm wide at the base and 4.8 μm tall, are present in the majority of the zoosporangia (arrows in fig. 2*A*, 2*B*, 2*F*, 2*J*, 2$K_1$); no opercula were found. Structures probably representing subsporangial swellings (7 μm × up to 9.5 μm in diameter) are sometimes present; however, they always occur on the outside of the host spore (figs. 2*G*, 3*G*, 3*P*).

While approximately 75% of the zoosporangia are empty, others contain one to several minute bodies of 2.8–4.7 μm in diameter (e.g., white arrow in fig. 2$K_2$) that most likely represent encysted zoospores on the basis of the fact that they are bounded on the outside by a delicate but distinct wall. Still others are partly or entirely filled with walled, spheroidal, or drop-shaped structures (fig. 3*D*–3*J*) that are 5.2–9.6 μm in diameter and that appear to represent miniature zoosporangia on the basis of the presence of a single distal discharge papilla or short tube in some of them (arrows in fig. 3*H*–3*J*). One large globous zoosporangium (fig. 3*G*) containing numerous miniature zoosporangia is subtended by a trapezoidal subsporangial swelling attached to the outer surface of the host spore (denoted as "S" in fig. 3*G*) and two rhizoidal axes extending from the swelling into the host spore lumen (white arrows in fig. 3*G*). What appears to be a discharge papilla is located apically in this zoosporangium (black arrow in fig. 3*G*). There are also several ruptured zoosporangia, as well as encysted zoospores and miniature zoosporangia that do not occur

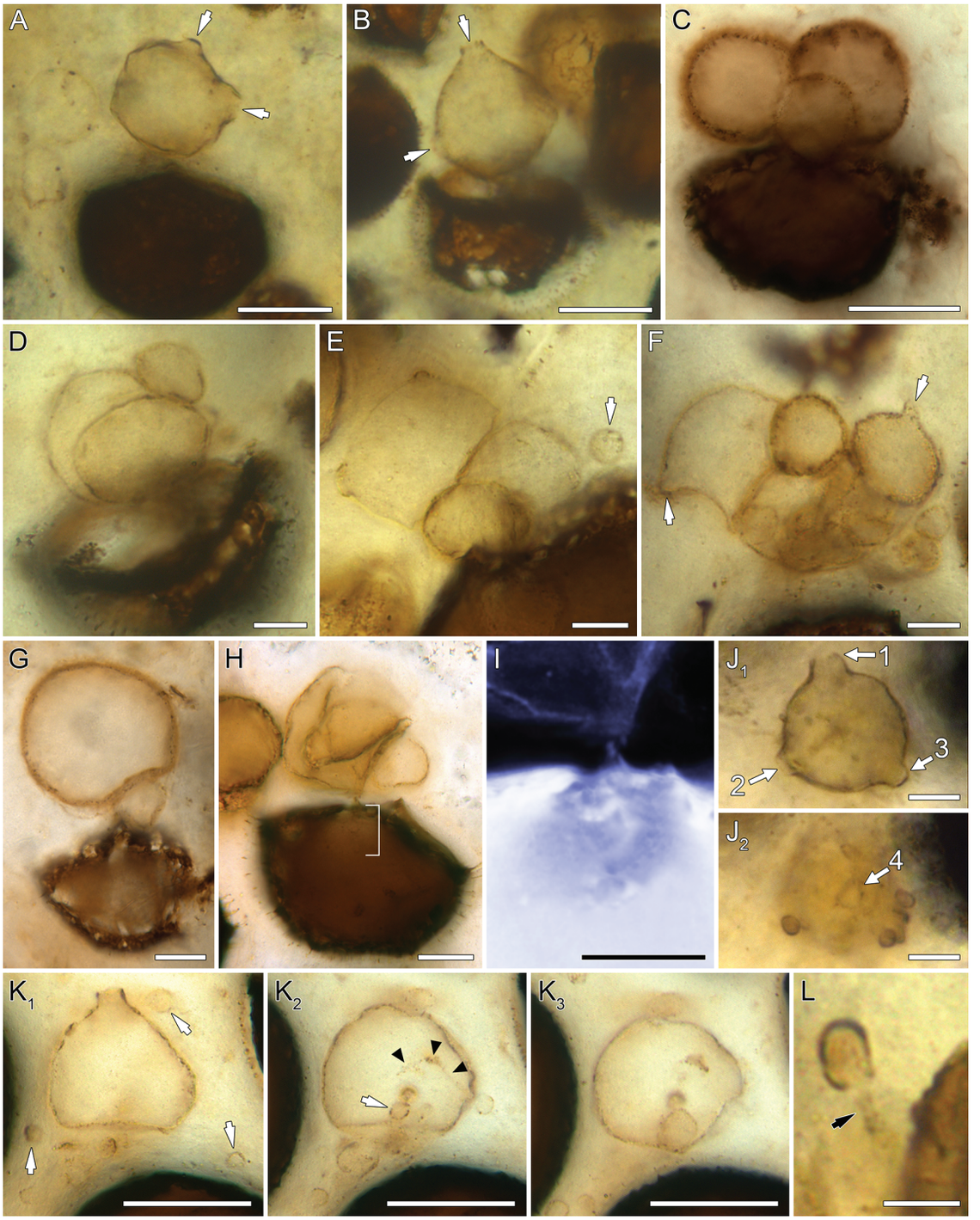


**Fig. 2** Morphology of *Rhizophydites matryoshkae*. All specimens are from slide SNSB-BSPG 1964 XX 99 unless indicated otherwise. *A*, *B*, Mature thalli with short discharge tubes (arrows). *C*, Spheroidal zoosporangia arising from a common site in the host wall. Slide SNSB-BSPG 2013 V 27. *D–F*, Zoosporangia in different phases of development, some arising from a host spore, others from the surfaces of adjacent zoosporangia; note the miniature zoosporangium attached via a rhizoidal axis in *E* (arrow) and the discharge papillae or short tubes (arrows) in *F*. *G*, Spheroidal zoosporangium with subsporangial swelling. Slide SNSB-BSPG 2013 V 27. *H*, Bundle of zoosporangia emerging from the host spore. Slide SNSB-BSPG 2013 V 27. *I*, Detail of *H* (bracketed area) in inverted light, focusing on the rhizoidal system. $J_1$, $J_2$, Zoosporangium with four discharge papillae (denoted as 1–4); note the several encysted zoospores on the outer zoosporangium surface in $J_2$. $K_1$–$K_3$, Different focal planes of a zoosporangium with one discharge tube. The arrows in $K_1$ indicate encysted zoospores in the immediate vicinity; the arrow in $K_2$ shows an encysted zoospore in the lumen, and the arrowheads point at a rhizoidal axis extending into the host lumen from an encysted zoospore attached to the outer surface. Note what appears to be a developing miniature zoosporangium inside the parent zoosporangium in $K_3$. *L*, Encysted zoospore with a developing rhizoidal axis (arrow). Scale bars = 25 μm (*A–C*, $K_1$–$K_3$), 10 μm (*D–*$J_2$), 5 μm (*L*).

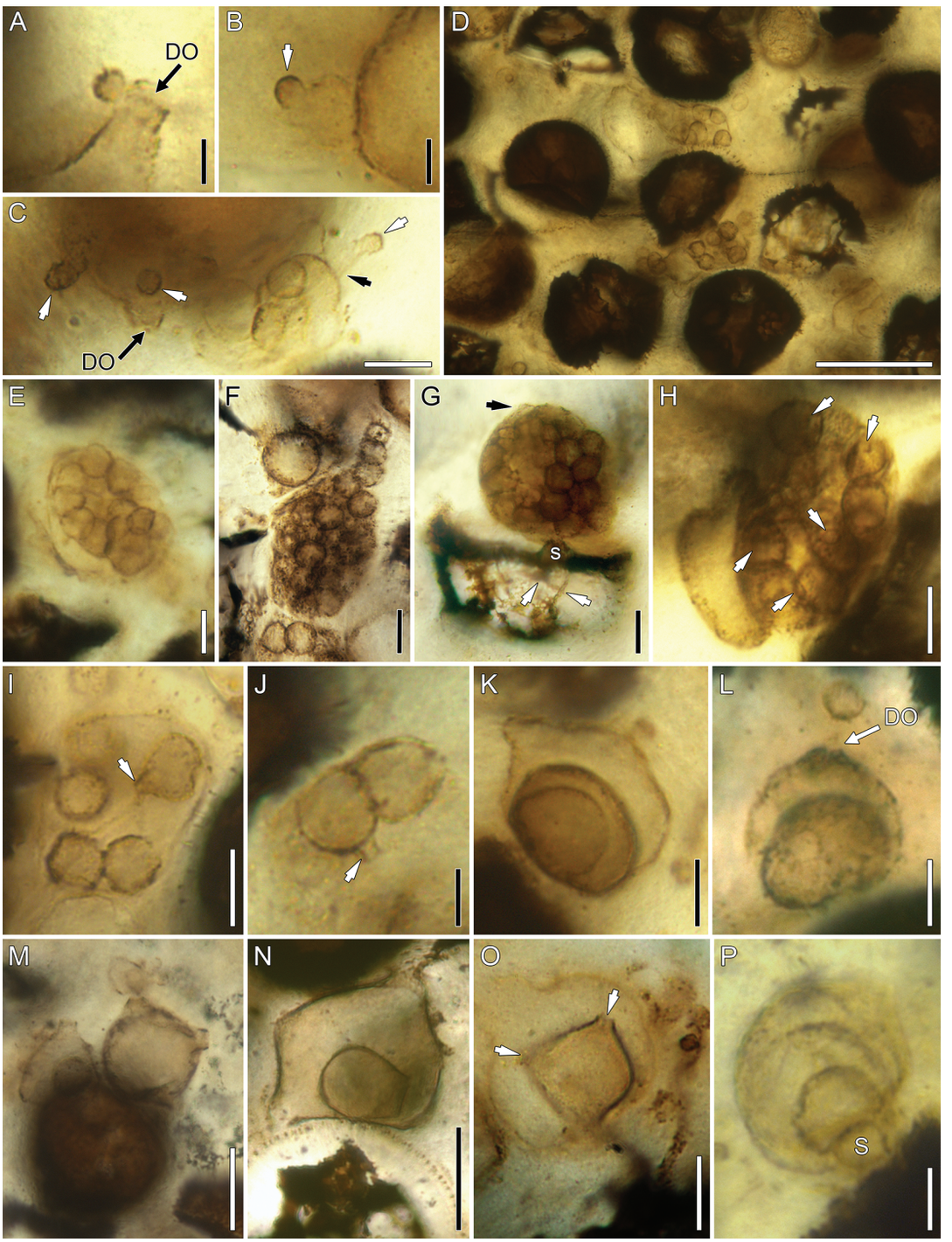


**Fig. 3** Morphology of *Rhizophydites matryoshkae*. All specimens are from slide SNSB-BSPG 1964 XX 99. *A*, Discharge tube (DO) with encysted zoospore. *B*, Encysted zoospore (arrow) and developing thallus. *C*, Encysted zoospores (white arrows), one with a rhizoidal axis (black arrow) on the outer surface of old, partially collapsed zoosporangia; note the miniature zoosporangia in one zoosporangium. DO = discharge tube of one of the old zoosporangia. *D*, Detail of figure 1*E*, showing zoosporangia containing miniature zoosporangia. *E–J*, Miniature zoosporangia inside normal-size zoosporangia ("false proliferation"). The white arrows in *G* indicate the rhizoidal system, and the black arrow shows what appears to be a discharge opening. The arrows in *H–J* indicate discharge papillae or tubes of miniature zoosporangia. S = subsporangial swelling. *K*, *L*, Internal sporangial proliferation. DO = discharge opening. *M*, Mature zoosporangia, the left one containing a miniature zoosporangium, the right one with miniature zoosporangia growing from a discharge tube. *N*, *O*, Internal sporangial proliferation; note the discharge papillae of the internal zoosporangium in *O* (arrows). *P*, Successive generations of zoosporangia placed one inside another. S = subsporangial swelling. Scale bars = 5 μm (*A*, *B*, *J*), 10 μm (*C*, *E–I*, *K*, *L*, *O*, *P*), 50 μm (*D*), 25 μm (*M*, *N*).

within zoosporangia but rather are located close to discharge openings (e.g., fig. 3*A*, 3*L*) or float freely within the confines of the *H. lignieri* sporangium (arrows in fig. 2$K_1$). Other encysted zoospores (fig. 2$J_2$; white arrows in fig. 3*B*, 3*C*), in addition to miniature or developing zoosporangia (arrow in fig. 2*E*), are attached to the outer surfaces of plant spores or large zoosporangia, sometimes via a short pedicel (black arrows in figs. 2*L*, 3*C*) or with what appears to be a rhizoid extending into the host (black arrowheads in fig. 2$K_2$). The most interesting structural feature of the zoosporangia concerns more than 25 specimens evidencing the development of new "normal" zoosporangia within old, empty zoosporangia (fig. 3*K*–3*P*). Zoosporangia occurring within other zoosporangia may also possess one to four discharge papillae or short tubes (e.g., arrows in fig. 3*O*). Most of the specimens display a single smaller zoosporangium inside a larger one, but some consist of up to four successive generations of zoosporangia of decreasing size placed one inside another (fig. 3*K*, 3*P*).

## Taxonomy

*Chytridiomycota (Chytrids)*

Rhizophydites *M. Krings et C.J. Harper, gen. nov.*

*MycoBank MB 834563*

*Diagnosis*. Simple eucarpic fossil, monocentric thalli; epibiotic zoosporangia, variable in size and shape, much less than 40 μm in diameter, with one or several discharge openings, inoperculate; endobiotic rhizoidal system, arising from a single site on the zoosporangium; subsporangial swelling giving off one or two rhizoidal axes may be present; thalli occur singly or in planar assemblages, sometimes also in multiples extending from a common site on substrate or in clusters comprising individuals in different phases of development, some growing from substrate and others from adjacent zoosporangia; new zoosporangia may develop inside the old zoosporangia.

*Type species*. *Rhizophydites matryoshkae* M. Krings et C.J. Harper (this article).

Rhizophydites matryoshkae *M. Krings et C.J. Harper, sp. nov.*

*MycoBank MB 834568*

*Diagnosis*. Zoosporangia smooth walled, sessile, usually spheroidal, less than 35 μm in diameter but may also be drop shaped, rhomboidal, obpyriform, or somewhat elongate to spindle shaped; one to several generations of zoosporangia of decreasing size may occur one inside another or with large numbers of spheroidal or pyriform miniature zoosporangia in the old zoosporangium; one to four discharge openings, prominent, papilla-like or short tube–like, up to 5 μm high; miniature zoosporangia usually spheroidal, less than 10 μm in diameter, with a single distal discharge papilla or short tube; encysted zoospores spheroidal to ovoid, 2.5–4.5 (–5.5) μm in diameter, sessile or pedicellate, often present on outer surfaces of zoosporangia; rhizoidal system originating from base of zoosporangium or, if present, subsporangial swelling, extending into host lumen; hosts are spores of early land plants *Horneophyton lignieri* and *Aglaophyton majus*.

*Holotype*. Specimen in figure 2*B* from slide SNSB-BSPG 1964 XX 99, SNSB-BSPG, Munich, Germany.

*Collection locality*. Rhynie, Aberdeenshire, Scotland, National Grid Reference NJ 494276 (lat. 57°20′09.97″N, long. 002° 50′31.83″W).

*Stratigraphic position*. Dryden Flags Formation.

*Age*. Early Devonian; Pragian-?earliest Emsian (see Wellman 2006, 2017; Wellman et al. 2006), 411.5 ± 1.3 Ma (Parry et al. 2011), 407.1 ± 2.2 Ma (Mark et al. 2011).

*Remarks*. *Rhizophydites matryoshkae* is based on considerable information on the morphology, together with specific developmental details, that renders the form distinct, readily recognizable, and distinguishable from other Rhynie chert fungi; makes the formulation of a diagnosis containing a sufficiently large set of diagnostic characters possible; and enables taxonomic assignment (see "Discussion" below). However, because of the geologic age and given the need for molecular data in fungal taxonomy today, we refrain from assigning the fossil to any one of the present-day chytrid genera and species but rather propose a new fossil taxon for the form. We also include in *R. matryoshkae* several unnamed specimens of *A. majus* figured by Taylor et al. (1992*b*: figs. 19, 23, 24) because they are not distinguishable morphologically from the fossils described in this study. All specimens are interpreted as belonging to a single fossil species, albeit with the caveat that they might represent several biological species that are impossible to distinguish on the basis of the material at hand.

*Etymology*. The genus name acknowledges the morphological congruence that exists between the fossil and the present-day genus *Rhizophydium* (Rhizophydiales; Chytridiomycota), in particular *Rhizophydium proliferum* J.S. Knox et R.A. Paterson; the ending *-ites* is used to designate a fossil taxon, as recommended by Pirozynski and Weresub (1979). The epithet is proposed because some of the specimens are reminiscent of Russian matryoshka dolls, which are the sets of wooden dolls of decreasing size placed one inside another.

## Discussion

The oldest fossils of chytrids and chytrid-like organisms preserved within the context of the environment in which they lived (often even in situ) come from the Lower Devonian Rhynie chert, and they include a variety of holocarpic and eucarpic forms, such as saprotrophs and parasites of land plants, charophytes, microscopic algae, and other fungi (Kidston and Lang 1921; Harvey et al. 1969; Illman 1984; Taylor et al. 1992*a*, 1992*b*; Hass et al. 1994; Krings et al. 2007, 2009*b*, 2016, 2017; Krings and Taylor 2014; Strullu-Derrien et al. 2016, 2017; Krings and Harper 2018, 2019*a*, 2019*b*, 2020; Harper and Krings 2019; Krings and Kerp 2019). Some of these fossils not only are morphologically very similar to modern chytrids but also enter into the same types of interactions with other organisms and even elicit the same host responses (e.g., Taylor et al. 1992*a*; Krings and Harper 2018).

### *Previous Records of Fungal Interactions with Land Plant Spores in the Rhynie Chert*

Plant spores and pollen grains today are attractive substrates and habitats for chytrids and other microscopic fungi because of their abundance and nutritional density (Sparrow 1960; Hutchison and Barron 1997; Czeczuga and Muszyńska 2001, 2004*a*,

2004*b*), so much so that mycologists can even use them to "bait" certain fungi (Couch 1939; Shearer et al. 2004; Powell 2017). It is therefore not surprising that the spores of the Rhynie chert early land plants also contain evidence of colonization by fungi, including chytrid-like forms. However, our knowledge of such relationships in the Rhynie paleoecosystem is limited because only a few examples have been documented, all based on small sample sets or single specimens, and none have been formally described and named. For example, spheroidal to ovoid structures, some with pores in the wall or discharge tubes to the outside (fig. 4*A*–4*D*), occur singly, in chains, or in clusters of up to eight in the lumens of *Aglaophyton majus* and *Horneophyton lignieri* spores (Illman 1984: figs. 1–5; Taylor et al. 1992*b*: fig. 22; Harper and Krings 2021: fig. 3*a*). They have been compared to present-day *Olpidium* (A. Braun) Rabenh. and *Entophlyctis* A. Fisch. (Chytridiomycota), *Olpidiopsis* Cornu (Oomycota), and *Hyphochytrium catenoides* Karling (Hyphochytridiomycota; Illman 1984); however, there is also evidence to suggest that some could be glomoid spores (e.g., fig. 4*E*; see Krings et al. 2015; Harper et al. 2017). Other documented examples of fungi associated with spores of *A. majus* are several different types of epibiotic putative chytrid zoosporangia, some with discharge pores or papillae and others containing large numbers of spherules interpreted as encysted zoospores (Taylor et al. 1992*b*: figs. 16–19, 23–34, 2004: fig. 5*j*, 5*k*, 2015: fig. 4.15; Taylor and Krings 2005: fig. 15).

## Rhizophydites matryoshkae: *Affinities and Life Cycle*

*Comparisons and affinities.* The fossil fungus described in this study is defined by the following structural traits: (i) a single vesicle-like structure interpreted as a zoosporangium that is epibiotic, is usually less than 30 $\mu$m in diameter (fig. 2*A*, 2*B*), and at maturity develops one to four discharge papillae or short tubes lacking opercula (fig. 2*J*), (ii) an endobiotic rhizoidal system that extends into the host lumen from a single site on the zoosporangium (fig. 2*I*) or, rarely, from a subsporangial swelling (fig. 3*G*), and (iii) the occasional development of new zoosporangia inside the old zoosporangia. On the basis of traits i and ii, we interpret the fossil as a eucarpic and monocentric member of the Chytridiomycota (chytrids) with an epibiotic inoperculate zoosporangium. Additional support for this interpretation is the small bodies present in the lumen, on the outer surface, and in the immediate vicinity of many of the vesicle-like structures (figs. 2*J*–2*L*, 3*A*–3*C*), which are suggestive of encysted zoospores and germinating zoospore cysts from which rhizoidal axes extend (for details, refer to fig. 6 and "Life cycle" in "Discussion").

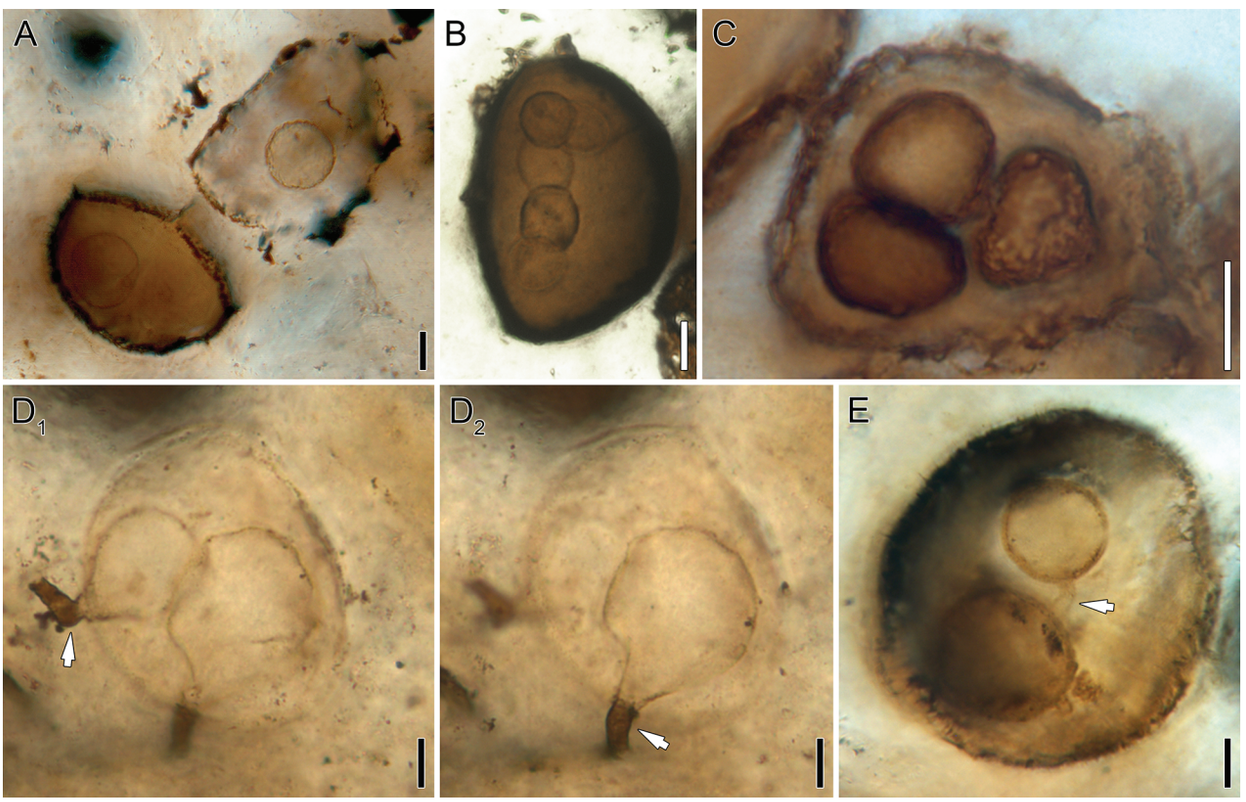


**Fig. 4** Fungal remains (reproductive units) in *Horneophyton lignieri* spores. *A*–*C*, Spheroidal and drop-shaped units occurring singly, in a chain-like arrangement, and in a small cluster. Slides SNSB-BSPG 2013 V 27 (*A*), 1964 XX 24 (*B*, *C*). $D_1$, $D_2$, Different focal planes of a spore containing drop-shaped thalli with prominent discharge tubes (arrows). Slide SNSB-BSPG 2017 XXIV 5. *E*, Reproductive units resembling glomoid spores, one with a portion of subtending hypha (arrow). Slide SNSB-BSPG 2013 V 27. Scale bars = 10 $\mu$m.

However, we cannot rule out the possibility of the affinity of the fossils to the Hyphochytridiomycota, a group of stramenopiles with chytrid-like thallus morphologies (Beakes et al. 2014).

The new fungus differs in size, morphology, and host preference from all chytrids and chytrid-like organisms previously described from the Rhynie chert, except for the specimens figured by Taylor et al. (1992*b*: figs. 19, 23, 24), which are addressed in the "Remarks" section of the protolog. Moreover, one Rhynie chert zoosporangium figured by Taylor et al. (1992*b*: fig. 43) corresponds in morphology to the bigger of the two zoosporangia shown in our figure 3*M*. However, this specimen is associated with a fungal spore. The new fossil is also somewhat similar to *Illmanomyces corniger* M. Krings et T.N. Taylor, which has an epibiotic zoosporangium and endobiotic rhizoidal system (Krings and Taylor 2014). However, the zoosporangia of *I. corniger* are distinctly larger (greater than 60 μm in diameter), and the form colonizes fungal (glomeromycotan) rather than land plant spores. Moreover, mature zoosporangia have four or five prominent discharge tubes, each up to 30 μm long, whereas the discharge apparatuses are up to only 5 μm long in the new form. Another Rhynie chert chytrid that is vaguely morphologically reminiscent but also, on average, bigger is *Cultoraquaticus trewinii* Strullu-Derr., which usually has two discharge papillae (Strullu-Derrien et al. 2016).

The best-suited modern morphological equivalents of the new fossil are species in the chytrid genus *Rhizophydium* (e.g., Karling 1946) and maybe other taxa in Rhizophydiales that comprise forms characterized by an inoperculate zoosporangium with one to several discharge openings and an endobiotic rhizoidal system extending from a single site on the zoosporangium (e.g., Barr 1969; Letcher et al. 2006, 2008, 2015; Letcher and Powell 2012; Seto and Degawa 2018; Jerônimo et al. 2019). On the other hand, fossil thalli with globose zoosporangia subtended by a prominent swelling (e.g., fig. 2*G*) are perhaps more comparable to those of *Gaertneriomyces* D.J.S. Barr or *Spizellomyces* D.J.S. Barr (both Spizellomycetales), which are monocentric chytrids with a spherical, inoperculate zoosporangium from which branched rhizoids are given off, usually via an apophysis (Barr 1980, 1984; Wakefield et al. 2010; Powell et al. 2018). However, the apophysis is part of the rhizoidal system and thus typically occurs endobiotically, not epibiotically as the subsporangial swelling in the fossils does.

Trait iii is a conspicuous feature of the new fungus that is documented here for the first time in fossils. It is present in some 20% of the specimens, where it occurs in two distinct manifestations (*m1*, *m2*). Some of the specimens showing this trait (*m1*) consist of two to several generations of normal zoosporangia of decreasing size placed one inside another (fig. 3*K*, 3*P*), whereas others (*m2*) represent normal-size zoosporangia that contain one or more miniature zoosporangia (fig. 3*D*–3*H*), each with a single discharge papilla or short tube (fig. 3*I*, 3*J*).

Manifestation *m1* resembles patterns resulting from internal sporangial proliferation (i.e., the successive growth of new zoosporangia inside the old zoosporangia), as it occurs widely in present-day Oomycota (Beakes and Thines 2017), marine fungoid protists of the genus *Thraustochytrium* Sparrow (Chen and Chien 2002; Fossier Marchan et al. 2018), and certain Hyphochytridiomycota (Karling 1939). It has also been reported in Chytridiomycota, including *Cladochytrium crassum* Hillegas and *Cladochytrium hyalinum* Berdan (both Cladochytriales; Berdan 1941: fig. 58; Hillegas 1941: fig. 18), *Polychytrium aggregatum* Ajello (Cladochytriales; Ajello 1942: figs. 1*p*, 10, 13; Chen et al. 2000), *Phlyctochytrium proliferum* Ingold (Chytridiales; Ingold 1941: text-figs. 1*B*–1*G*, 2*B*, fig. 1), *Chytridium proliferum* Karling (Chytridiales; Karling 1967*b*: plate XXIV, figs. 25, 26), *Rhizophydium novozeylandiense* (Karling) Karling (Rhizophydiales; Karling 1967*a*: plate XII, figs. 6, 7), and *Rhizophydium proliferum* (Rhizophydiales; Knox and Paterson 1973: fig. 10). None of these present-day chytrids are morphologically similar to the fossils, with the exception of *R. proliferum*, a species from Antarctica in which a sporangial proliferation pattern that closely resembles *m1* (cf. fig. 5*A* with 5*B*) frequently occurs and, therefore, is regarded as the most consistent and conspicuous characteristic of the species (Knox and Peterson 1973). These authors state that the rhizoidal system gives rise to the proliferations in *P. proliferum* because the internal zoosporangia always have the same point of attachment and apparently the same rhizoids as the parent, precisely as seems to be the case in the fossil (fig. 3*K*, 3*L*, 3*N*–3*P*). If this is accurate, then the development of successive zoosporangia inside one another in *R. proliferum* and presumably also in the fossil would correspond to "true internal sporangial proliferation" sensu Karling (1937, p. 70). This process involves either the penetration of an old zoosporangium by a portion of the thallus beneath (e.g., a nucleated rhizoid) and its subsequent development into a zoosporangium or an unequal and incomplete cleavage whereby a part of the nucleated protoplasm remains behind and later develops into a new zoosporangium. Unfortunately, no structural features that could be used to determine exactly how the *m1* specimens formed have been found.

Patterns resembling internal sporangial proliferation can also develop from the germination of zoospores in situ (i.e., within the parent zoosporangium; e.g., Karling 1937; Ingold 1941), a process that Karling (1963) calls "false proliferation." Documented evidence of zoospores that become trapped within the parent zoosporangium, proceed to encyst, and eventually develop into new zoosporangia within the confines of the old zoosporangium is not rare in present-day chytrids (e.g., Raitschenko 1902; Karling 1936, 1937, 1946, 1968; Sparrow 1936; Voos 1969; Olive 1980; Dayal 1997). We consider it very likely that the fossil *m2* specimens are also evidence of the development of small thalli from zoospores that, for some reason, remained in their zoosporangium after zoospore discharge and hence that these specimens represent the first record of false proliferation in fossil chytrids.

On the basis of the above comparisons and considerations, we conclude that the new fossil is best accommodated in the chytrid order Rhizophydiales and most likely represents a member of *Rhizophydium* because of the similarities between some of the fossil zoosporangia showing internal sporangial proliferation and the present-day *R. proliferum*. However, because molecular data and zoospore ultrastructure are necessary to safely assign organisms in Rhizophydiales to present-day genera and species (e.g., Letcher et al. 2006, 2008, 2012, 2015), the fossils are placed in a newly proposed fossil genus and species, *Rhizophydites matryoshkae*. We also acknowledge that the phylogenetic position of many of the "traditional" (i.e., morphology-based) species in *Rhizophydium*, including *R. proliferum*, continues to be unresolved because information on zoospore ultrastructure and molecular data is not available. It is therefore possible that *R.*

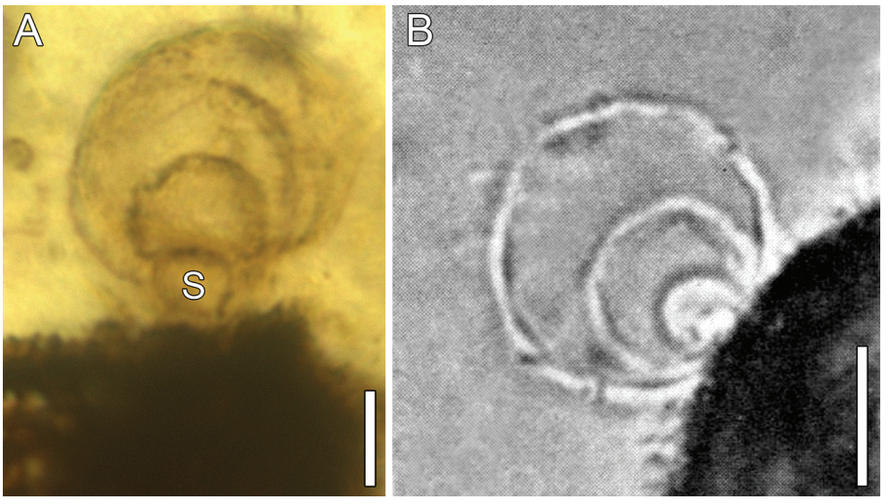


**Fig. 5** Side-by-side comparison of internal sporangial proliferation in *Rhizophydites matryoshkae* and *Rhizophydium proliferum*. *A*, *Rhizophydites matryoshkae* with four generations of zoosporangia placed one inside another. Slide SNSB-BSPG 1964 XX 99. S = subsporangial swelling. *B*, *Rhizophydium proliferum* on pine pollen, with three generations of zoosporangia placed one inside another. From Knox and Peterson (1973: fig. 10). Panel *B* has been modified from the original for clarity. Scale bars = 10 μm.

*proliferum*, which has been placed in *Rhizophydium* exclusively based on morphology (Knox and Peterson 1973), turns out to belong to a different lineage of the Chytridiomycota once zoospores and DNA have been analyzed. Owing to this caveat, we refrained from formally assigning *R. matryoshkae* to the Rhizophydiales or to any family within this order.

*Life cycle*. The sample set of more than 300 thalli allows us to propose a reconstruction of the life cycle of *R. matryoshkae* based on comparisons with the life cycle stages of present-day Rhizophydiales, including *R. proliferum* (Knox and Paterson 1973; Letcher and Powell 2012). The graphic representation of the life cycle of *R. matryoshkae* presented in figure 6 starts with a zoospore (fig. 6*A*) that is liberated from its zoosporangium, swims around, and eventually attaches to a host (fig. $6B_1$)—in the case of *R. matryoshkae*, either the surface of a land plant spore or another zoosporangium. On attachment, the zoospore encysts (figs. $2J_2$, 3*A*, 3*C*). The encysted zoospore eventually germinates and develops into a thallus by producing a rhizoidal axis into the host lumen (figs. $2K_2$ [arrowheads], 2*L*, $6B_2$) and an epibiotic zoosporangium (figs. 3*B*, $6C_1$). On maturation of the zoosporangium (fig. $6C_2$, $6C_3$), the zoospores are released through one to four prominent discharge openings (figs. 2*A*, 2*B*, 2*J*, 2*K*, 6*D*). This completes the normal life cycle of *R. matryoshkae*. However, it appears that, occasionally, a few—or even many—of the zoospores are not liberated (fig. 6*D*) but rather remain inside the parental zoosporangium (e.g., arrow in fig. $2K_2$), where they develop into thalli with miniature zoosporangia (figs. $2K_3$, 3*E*–3*J*, $6E_1$, $6E_2$). These miniature zoosporangia release their zoospore(s) through a single distal discharge papilla or tube (figs. 3*H*–3*J* [arrows], $6E_2$, $6E_3$). It remains uncertain how the zoospores then become liberated from the confines of the original zoosporangium, but there is some evidence to suggest that old zoosporangia rupture in the process of disintegration (fig. $6E_3$). Miniature zoosporangia can also develop from encysted zoospores attached to the outer surfaces of other zoosporangia or land plant spores (figs. 2*E*, 3*L*, $6F_1$, $6F_2$). Further complicating the life cycle of *R. matryoshkae* is internal sporangial proliferation in the form of new zoosporangia of decreasing size growing from the base of the old, empty zoosporangium (figs. 3*K*, 3*L*, 3*N*–3*P*, $6G_1$–$6G_6$). The presence of discharge tubes in many of the internal zoosporangia (e.g., arrows in fig. 3*L*, 3*O*) suggests that they were functional and produced zoospores.

## *Ecological Aspects*

*Nutritional mode*. Most present-day fungi thriving on plant spores and pollen grains are saprotrophs (Goldstein 1960; Krauss et al. 2011; Phuphumirat et al. 2011; Wurzbacher et al. 2014; Gleason et al. 2017), but there are also several parasites (e.g., Nair and Khan 1963; Classen et al. 2000). Characteristic degradation patterns in the walls of fossil spores and pollen grains indicate that saprotrophic fungi were also responsible (at least in part) for the decomposition of these structures in ancient ecosystems as early as the Carboniferous (Moore 1963; Elsik 1966, 1971). An interesting but difficult question therefore concerns the timing of when *R. matryoshkae* entered the *H. lignieri* sporangia and colonized the spores. In other words, were the host spores viable or nonviable when the colonization occurred? We cannot rule out the possibility that *R. matryoshkae* parasitized viable spores before germination and gametophyte development. However, present-day members of the Rhizophydiales are saprotrophs on spores and pollen grains (e.g., Skvarla and Anderegg 1972; Czeczuga and Muszyńska 2004*a*; Powell and Letcher 2014; Page and Flannery 2018). It should be noted, therefore, that the fossil fungus occurs in greater numbers only with in situ spore masses that show symptoms of degradation (fig. 1*D*, 1*E*), while it is very rare in sporangia containing intact spores. We hypothesize that the more extensively colonized spore masses were exposed to the activity of the fungus for a longer period

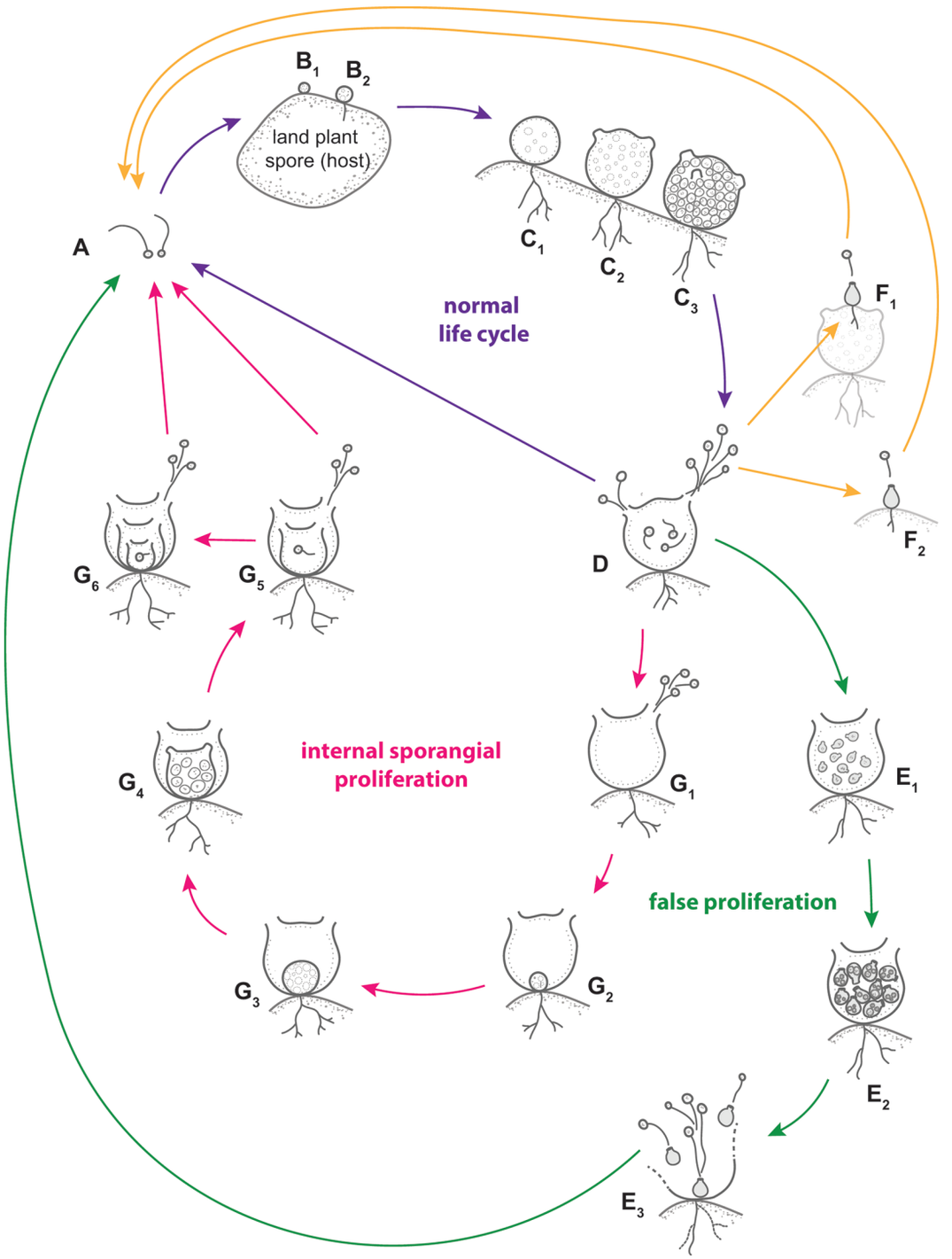


**Fig. 6** *Rhizophydites matryoshkae* life cycle reconstruction (for details, refer to "Life cycle" in "Discussion").

of time, during which the spores, along with the surrounding sporangium, partially degraded, and hence that *R. matryoshkae* was a saprotroph. The increased number of fungal thalli could result either from continuous colonization from the outside (e.g., through ruptures in the degrading sporangium wall) or, more likely, from reproduction of the fungus inside the *H. lignieri* sporangium. Unfortunately, no symptoms or patterns attributable specifically to fungal degradation (see Elsik 1966) have been found in any of the *H. lignieri* spores. Moreover, Wellman et al. (2004) believe that much of the spore degradation in *H.*

*lignieri* represents an artifact of preservation linked to the silicification process and thus may not be a good proxy indicator for spore viability. On the other hand, none of the intact or partially degraded spores included in this study show evidence of germination (see Bhutta 1973*a*), rendering it somewhat more likely that the host spores were nonviable when the fungal colonization occurred.

*Ecological role.* Pollen-degrading chytrids in ecosystems today provide a model for a possible ecological role for *R. matryoshkae* because the zoospores of these chytrids may be eaten by zooplankton and, in this way, transfer carbon and nutrients from inedible pollen grains up the food chain, a pathway (or energy "shunt") termed the "mycoloop" (Jephcott et al. 2017; Kagami et al. 2017). Chytrids in terrestrial food webs may play similar roles because they decompose detritus and dissolved organic matter and are subsequently grazed by protists and metazoans (Gleason et al. 2008, 2012). The existence of mycoloops in the Rhynie paleoecosystem has also been suggested by Strullu-Derrien et al. (2016), who speculated that nutrients obtained by the chytrid *C. trewinii* from its substrate were transferred to small crustaceans via the chytrid zoospores that served as food for the crustaceans. Spores of *H. lignieri* are remarkably abundant in some Rhynie chert horizons (Powell 1994; Powell et al. 2000; Wellman et al. 2004). It is, therefore, conceivable that zoospore production by *R. matryoshkae* dwelling on these spores could transfer significant amounts of carbon and nutrients to animals that fed on the zoospores and hence that the mycoloop dynamic was an important component of Rhynie food webs, especially at times when land plant spore deposition rates were high (see Powell et al. 2000; Masclaux et al. 2013).

### *Why* H. lignieri*?*

Numerous specimens of sporangia with in situ spores, in addition to dispersed spores, of the various Rhynie (and nearby contemporary Windyfield) chert land plants are figured in the literature (e.g., Kidston and Lang 1920; Bhutta 1973*a*, 1973*b*; Eggert 1974; El-Saadawy and Lacey 1979; Lyon and Edwards 1991; Powell et al. 1999; Wellman et al. 2004, 2006; Wellman 2006, 2017; Kerp et al. 2013). Screening of these images for evidence of spore-colonizing fungi shows that *R. matryoshkae* is associated with *H. lignieri* (this study) and, to a much lesser degree, *A. majus* (Taylor et al. 1992*b*), but apparently it does not occur on spores of any of the other plants. Spores and pollen grains of some plant groups today are known to be more attractive to fungi than others because of their morphology or biochemical makeup (Goldstein 1960). It is, therefore, possible that the spores of *H. lignieri* were particularly susceptible to colonization. However, the lack of evidence of *R. matryoshkae* on other spores could also reflect documentation bias resulting from the tendency to figure only nice-looking sporangia and spores, rather than ones tainted by fungi, in works not focusing on the fungi. Even the very detailed account of the spores of *H. lignieri* by Wellman et al. (2004) does not mention or figure spore-colonizing fungi. On the other hand, Powell (1994) and Wellman et al. (2004) note that spores of *H. lignieri* are particularly abundant and frequently occur in large clusters (spore masses) in some soil and litter horizons of the chert and that dissevered sporangia containing spores are also commonly located there. If these horizons represented environments that were conducive to the proliferation of chytrids (i.e., aquatic or wet to moist terrestrial; see Sparrow 1960), then there was perhaps a higher likelihood for *H. lignieri* spores to be colonized. *Horneophyton lignieri* has been characterized as a stress-tolerant plant thriving in unstable environments (e.g., as the initial colonizer on sinter surfaces; see Trewin and Kerp 2017), able to survive periodic submergence, and tolerant of water stress (Powell et al. 2000; Wellman 2017) and thus was most certainly exposed to diverse zoosporic fungi, including chytrids, not only in vivo but also during decay in autochthonous litter.

## Conclusions

Fungal associations with plant spores and pollen grains must have been common in ancient ecosystems. However, documented fossil evidence of such interactions is not common, due in part to bias introduced during the routine preparation and analysis of palynological samples (Aggarwal et al. 2015). Palynological studies typically focus on the identification of the palynomorphs and the information they provide on biodiversity, stratigraphy, and paleoecology. To be meaningful, such studies require complements of morphological features of the study objects that make identification consistent and reliable, and these typically involve shape, size, and the degree and type of ornamentation. None, however, involve the contents of the spores or pollen grains or ancillary structures variously attached to the outer surface. Such specimens are rarely described or illustrated and are otherwise discounted. The Early Devonian Rhynie chert offers a different approach. Because the land plant spores are embedded in a siliceous matrix and many occur in situ within sporangia, unusual spore contents and ancillary surface structures may be faithfully preserved and can be examined in detail on the basis of thin section preparations (Taylor et al. 2011). This offers a rare opportunity to specifically assess various types of spore-colonizing organisms, the evidence of which is typically lost during standard palynological preparations, with a few exceptions (e.g., see Elsik 1971). The spore-colonizing *Rhizophydites matryoshkae* described in this study is a fine example of this. Documentation of such fossils provides data that can be used to indirectly establish fungal diversity and ecosystem complexity through geologic time. A second approach relates to stages of the life cycle and how these structurally (and, by inference, functionally) may be similar to or differ from the life-history biology of other fossil forms and present-day equivalents. Finally, every new fossil fungus increases the number of calibration points that can be used to more accurately align molecular clocks with fossil evidence in discussions of fungal evolution.

## Acknowledgments

Part of this research served as an independent undergraduate research experience for S. M. Serbet and was supported by several University of Kansas funding programs: (i) Undergraduate Biology Program, (ii) College of Liberal Arts and Sciences Research Excellence Initiative Undergraduate Student Travel Award,

(iii) Drs. Lou and Gary McClelland Honors Research Endowment, (iv) Undergraduate Research Award, and (v) Undergraduate Research and Travel Grant. Moreover, the National Science Foundation provided funds for a research assistantship for S. M. Serbet (DBI-1561315 to Edith L. Taylor, Lawrence, KS). We gratefully acknowledge Peter M. Letcher (Tuscaloosa, AL) for helpful discussion on chytrid biology and John S. Knox (Lexington, VA) for granting permission to use a figure from his 1973 paper. We thank Stefan Sónyi and Helmut Martin (both Munich, Germany) for technical assistance. The manuscript benefited greatly from the constructive comments and suggestions of two anonymous referees.

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