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Oldest fossil basidiomycete clamp connections

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Abstract A rachis of the fossil filicalean fern *Botryopteris antiqua* containing abundant septate hyphae with clamp connections is preserved in a late Visean (Mississippian; ~330 Ma) chert from Esnost (Autun Basin) in central France. Largely unbranched tubular hyphae pass from cell to cell, but may sometimes produce a branch from a clamp connection. Other clamp-bearing hyphae occur clustered in individual cells or small groups of adjacent host cells. These hyphae may be tubular, catenulate with numerous hyphal swellings, or they may display a combination of both. The Visean hyphae with clamp connections predate *Palaeancistrus martinii*, the heretofore oldest direct fossil evidence of Basidiomycota, by some 25 Ma.

Keywords Autun Basin (France) · Basidiomycota · Chert · Fossil fungi · Mississippian

Introduction

Molecular clock estimates have been used to suggest that the first Basidiomycota occurred during the Paleozoic, some 500 million years (Ma) ago (Berbee and Taylor 2001); other hypotheses suggest an even earlier occurrence (Heckman et al. 2001) depending on the calibration points used (see Taylor and Berbee 2006). However, the fossil record of these fungi has remained scanty and the identity of the few documented fossils (especially those from the Paleozoic) largely equivocal. For example, the enigmatic nematophyte *Prototaxites* Dawson (Silurian/Devonian) has been suggested as a basidiomycete (Hueber 2001), but there is no compelling evidence to date to demonstrate typical basidiomycete features. There have also been a number of reports from the nineteenth and early twentieth centuries of Carboniferous fossils that were initially interpreted as basidiomycetes based on superficial resemblance to basidiocarps of modern polypores (reports surveyed in Fleischmann et al. 2007). However, all these reports were later reevaluated and the specimens reinterpreted as being non-fungal (Pirozynski 1976).

Cell wall alterations in the progymnosperm wood *Calliclydon newberryi* (Dawson) Elkins et Wieland from the Upper Devonian (~360 Ma) of Indiana (USA) represent the oldest indirect fossil evidence for the existence of Basidiomycota (Stubblefield et al. 1985). Secondary xylem tracheids, decayed in varying degrees, show erosion troughs, cavities, and extensive lysis of the tracheid walls that are similar to symptoms caused by modern-day white rot fungi. However, in this case it is the disease symptoms, rather than evidence from the actual fungus, that have been used to assess the systematic affinities of the fungus.

One structural feature that can be used to positively identify a fossil basidiomycete in the absence of sexual

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reproductive structures is the clamp connection, a hyphal protrusion that develops at cell division to maintain the binucleate (dikaryon) condition. The first generally accepted basidiomycete body fossils with this feature occur in a structurally preserved *Zygopteris illinoensis* (Andrews) Baxter rhizome from the Middle Pennsylvanian (~305 Ma) of North America and are described as *Palaeancistrus martinii* R.L. Dennis (Dennis 1970). This fungus consists of branching, septate hyphae with clamp connections and hyphal swellings suggested as representing chlamydospores.

In this article, we describe septate hyphae with clamp connections in a structurally preserved rachis of the filicalean fern *Botryopteris antiqua* Kidston. The hyphae and clamp connections are somewhat similar to those described by Dennis (1969, 1970), but are Middle Mississippian (~330 Ma) in age, and thus approximately 25 Ma older than the oldest direct fossil evidence of Basidiomycota.

Materials and methods

The chert containing the infected *Botryopteris antiqua* rachis comes from the upper Visean [Mississippian (=Lower Carboniferous)] of the Autun Basin at the locality of Esnost, which is located about 10 km north of the city of Autun in the northern part of the Massif Central, central France. Details regarding the geological setting can be found in Scott et al. (1984); for information on the preservation of fossils and a paleoecological reconstruction of the Visean ecosystem, see Rex (1986). The Visean landscape at this site is interpreted as a series of pools and small lakes surrounded by swamp forest vegetation; the environment was dominated by active volcanism. The chert, which originally formed in the pools and lakes (Rex 1986), occurs as loose blocks within rhyolitic tuffs and can be collected in cultivated fields.

The genus *Botryopteris* is one of the most common and intensively studied genera of late Paleozoic filicalean ferns (Taylor et al. 2009). These ferns were relatively small, creeping or scrambling plants, trunk epiphytes on large trees, or formed compound systems of false trunks (e.g. Rößler and Galtier 2003). *Botryopteris antiqua* plants consist of scrambling foliar members (primary rachides) with erect pinnae; epiphyllous shoots may extend from the axils of the pinnae (Galtier 1970, fig. 36). The rachis (Fig. 1a) comprises an oval xylem bundle and phloem surrounded by an inner cortex of cells containing opaque materials and an outer cortex in which cell size decreases toward the periphery. All cortical cells are relatively thick walled.

The rachis (in transverse section) containing the fungus was identified in a thin section that was prepared by cementing a piece of chert to a glass slide and then

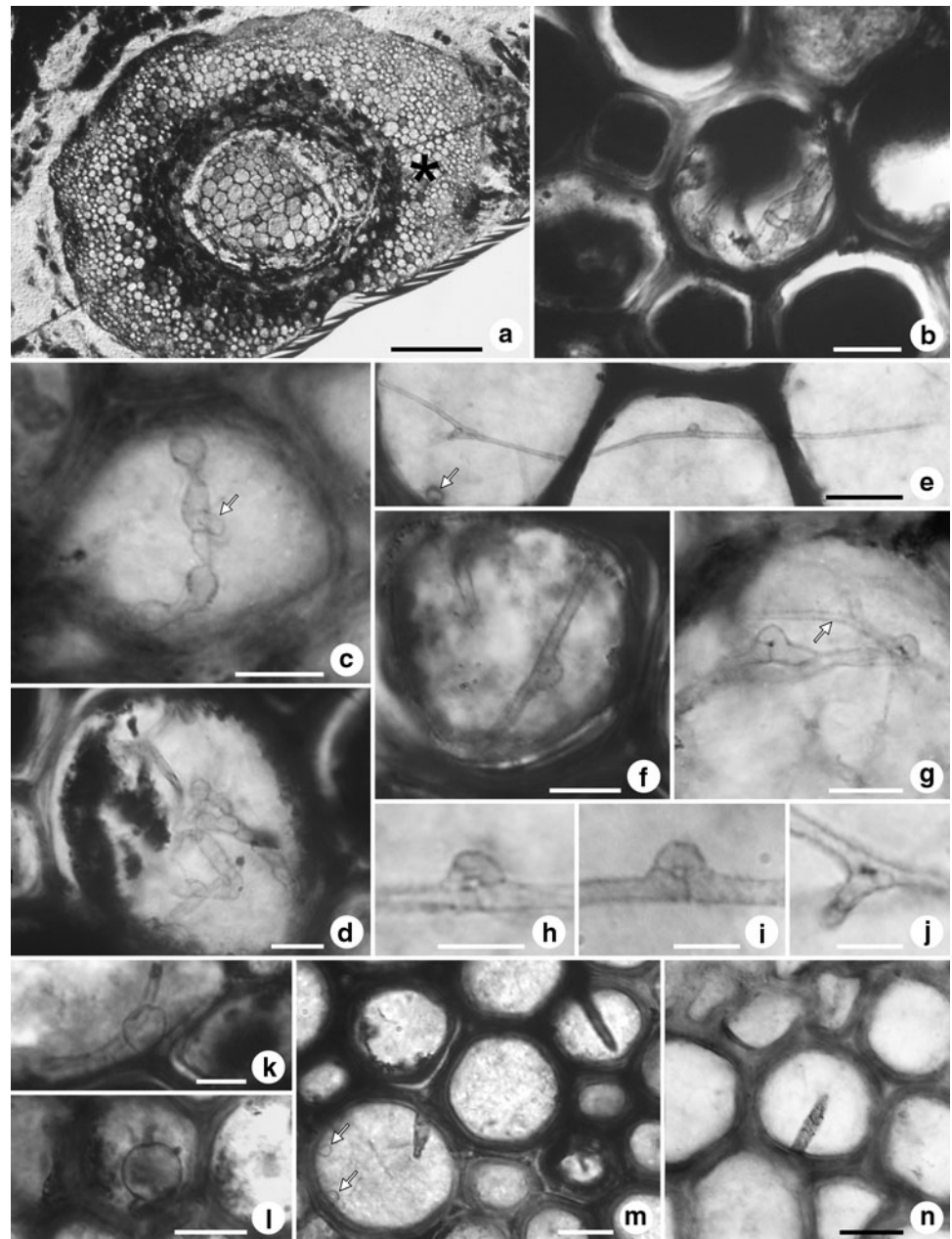
grinding the wafer until it was thin enough to be examined in transmitted light. The section was prepared during the late nineteenth century and is today housed in the Muséum National d'Histoire Naturelle (Laboratoire de Paléobotanique-Paléontologie, collection Renault) in Paris (France) under accession number REN-106/2268. The section was analyzed using normal transmitted light microscopy equipment (Leica); digital images were taken with a Leica DFC-480 camera.

Results

Hyphae with clamp connections are common in the large and relatively thick-walled cells of the inner portion of the outer cortex of the *Botryopteris antiqua* rachis (note asterisk in Fig. 1a), but have also been found in the inner cortex and xylem, as well as in the peripheral cortical layers. Hyphae of this type have not been detected in the chert matrix surrounding the rachis, nor have they been found in any other plant tissue present in the thin section.

Within the host tissues, clamp-bearing hyphae display two different modes of growth. They may grow as straight, slightly bent, or sinuous tubular running hyphae (up to 2 μm wide) that pass from cell to cell largely unbranched (Fig. 1e–g). Clamp connections occur at irregular intervals; the distance between two clamps ranges from 50 to 180 μm . If branching occurs, it is usually (but not always) by hyphal proliferation through clamp formation (Fig. 1j). The hyphal branch extending from a clamp may be as wide as the parental hypha (Fig. 1j) or narrower (arrow in Fig. 1g). Other hyphae with clamp connections branch repeatedly and form more or less dense clusters that are confined to individual host cells or, in rare instances, to small groups of adjacent cells (Fig. 1b–d). The hyphae comprising these clusters may be tubular and up to 2 μm wide (Fig. 1b), or catenulate and characterized by numerous hyphal swellings (Fig. 1c), or they may display a combination of both characteristics (Fig. 1d). Hyphal swellings are short-elliptical, spindle-shaped, or subcylindrical, up to 10 μm long and 2.5–4.5 μm wide, and alternate with more or less pronounced constrictions in which the hyphae range from 0.5 to 1 μm wide. If the hyphae are catenulate, septa and clamp connections typically occur on less pronounced constrictions (arrow in Fig. 1c). The clamp connections formed on all fossil hyphae (Fig. 1h,i) correspond in structure to ordinary clamp connections seen in living Basidiomycota. In the tubular running hyphae, septa are consistently associated with a clamp (Fig. 1e), whereas septum formation is not always associated with a clamp in the catenulate hyphae (Fig. 1d). Incomplete clamps have not been observed in any of the fossil hyphae.

Fig. 1 Hyphae with clamp connections in cortical cells of a *Botryopteris antiqua* rachis from the Middle Mississippian of France. **a** Transverse section through the rachis; *asterisk* indicates inner portion of outer cortex where clamp-bearing hyphae are most abundant. **b** Cluster of tubular hyphae in cortical cell. **c** Hypha with catenulate swellings; *arrow* indicates clamp connection (in surface view). **d** Cluster of hyphae showing hyphal swellings. **e** Tubular running hypha; *arrow* indicates large fungal hypha in cross section. **f, g** Running hyphae with clamp connections; *arrow* in **g** indicates narrower branch hypha. **h, i** Details of clamp connections. **j** Detail of **e**, showing development of a branch hypha from a clamp connection. **k, l** Spherical/ovoid structures in host cells. **m, n** Callosities in cortical cells of *B. antiqua*; *arrows* in **m** indicate large fungal hyphae in cross section. *Bars* **a** 500 μm ; **b–d, f, g, l, m** 10 μm ; **e** 50 μm ; **h–k** 5 μm ; **n** 20 μm



Co-occurring with the clamp-bearing hyphae in some of the host cells are spherical to ovoid structures (up to 8 μm in diameter) that appear to be attached to the host cell wall (Fig. 1k,l). Also present in many cortical cells is a second, considerably larger type of hypha (up to 10 μm wide), which typically extends parallel to the long axis of the rachis (arrows in Fig. 1e,m). Several cortical cells of the rachis suggest some degree of host reaction in the form of inwardly directed conical callosities, each up to 20 (–25) μm long and 6 μm wide (Fig. 1m,n). Callosities and clamp-bearing hyphae occur in close proximity but have not been observed physically connected.

Discussion

Basidiomycota today are important contributors to multiple levels of ecosystem functioning. For example, they are effective as degraders of different components in wood, including lignin, and as parasites and causative agents of diseases in plants. Moreover, they form mutualistic associations with a variety of other organisms; distinctive among these are ectomycorrhizae (Rinaldi et al. 2008). Provided that the estimate by Berbee and Taylor (2001) of 500 Ma as a minimum age for Basidiomycota as a distinct lineage is accurate, it is reasonable to assume that these

fungi were also important elements in ancient continental ecosystems, at least from the Early Devonian onward. However, fossil evidence for this hypothesis has been exceedingly slow to accumulate, and is largely restricted to records from the Mesozoic (Osborn et al. 1989; Hibbett et al. 1997; Krassilov and Makulbekov 2003; Poinar and Brown 2003; Smith et al. 2004) and Cenozoic (Poinar and Singer 1990; Magallón-Puebla and Cevallos-Ferriz 1993; LePage et al. 1997; Hibbett et al. 2003; Fleischmann et al. 2007). To date the oldest undisputed basidiomycete body fossil is *Palaeancistrus martinii* from the Middle Pennsylvanian (Dennis 1969, 1970).

The clamp-bearing hyphae from the upper Visean of France described in this article extend the oldest evidence for unequivocal basidiomycete body fossils back in time by some 25 Ma. The fossils resemble the Pennsylvanian *Palaeancistrus martinii* with regard to basic clamp connection morphology. Moreover, development of hyphal branches from clamp connections, which is a common feature among certain modern basidiomycetes (Routien 1948), has been documented for both fossil forms (Fig. 1j; and see Dennis 1970, fig. 7). Intercalary and terminal hyphal swellings, suggested as representing chlamydospores by Dennis (1970), also occur in the Visean hyphae, but are less prominent than in *P. martinii*. Prominent ovoid or nearly globose swellings like those figured by Dennis (1970, fig. 4) do not occur physically associated with the Visean hyphae, but in several host cells there are isolated spherical structures of approximately the same diameter as what are interpreted as chlamydospores in *P. martinii*. However, it is also possible that these spheres represent chytrid zoosporangia, or the propagules of some other fungus that lived in the rachis. In addition to these similarities between the Visean clamp-bearing hyphae and *P. martinii*, there are also several differences. For example, the Visean hyphae are distinctly narrower (i.e., up to 2 μm wide) than those of *P. martinii*, which may be up to 4.8 μm wide. Moreover, serial arrangement of hyphal swellings alternating with more or less pronounced constrictions (Fig. 1c,d) has not been described for *P. martinii*. Additional differences between the two fungi involve the presence of septa and clamps. Although we have detected septa lacking a clamp only in the catenulate hyphae, the hyphae illustrated by Dennis (1970, figs. 6, 8) suggest that clamps generally are not consistently associated with septum formation. In addition, Dennis (1970) describes and illustrates incomplete clamps, which we have not found in the Visean hyphae.

We are uncertain how the second, distinctly wider type of fungal hypha in the *Botryopteris antiqua* rachis (e.g., arrow in Fig. 1e) may be associated with the type bearing clamps because both types have not been observed in physical connection. It is possible that both types of hyphae

were produced by one fungus, and that the wide hyphae represent the principal means of spreading the fungus within the plant. Dennis (1970) compared *Palaeancistrus martinii* with hyphae of the extant *Panus tigrinus* (Bull.) Singer [= *Lentinus tigrinus* (Bull.) Fr.], a wood-decaying basidiomycete of the family Polyporaceae (Hibbett et al. 1994), and suggested that the fossil was a saprotroph. The biological nature of the relationship between *Botryopteris antiqua* and the Visean clamp-bearing hyphae is difficult to determine because of the incompleteness of both the host plant and fungus. The fungus could have been biotrophic or saprotrophic. If the conical callosities found in several of the cortical cells (Fig. 1m,n) formed in response to invading clamp-bearing hyphae, then this host response would favor evidence of a parasitic infection. Callosities (also called appositions, lignotubers, or papillae, among other terms; see Stubblefield et al. 1984) assume a variety of shapes, from wart-like to elongate conical, and represent inwardly directed projections consisting of newly synthesized wall material that are formed by living plant cells (but also by certain fungal spores) in response to invading fungi. Callosities encase the invading fungal hypha or filament, and it is widely interpreted that they are effective in preventing or retarding penetration by the parasite (Aist 1977). This type of host reaction has been observed in cells of numerous extant seed plants (Young 1926; Rioux and Biggs 1994) but is also known to occur in ferns (Archer and Cole 1986). It has also been recorded for several fossils, including the xylem and periderm of a lycophyte from the upper Visean of France (Krings et al. 2009) and a staminate gymnosperm strobilus from the Upper Pennsylvanian (~300 Ma) of North America (Stubblefield et al. 1984), but we are not aware of any previous record in fossil ferns.

The Basidiomycota is a monophyletic group of fungi that is composed of approximately 30,000 described species (Kirk et al. 2001; Blair 2009), but molecular and genetic studies indicate that there is an extraordinary diversity within this group yet to be discovered (Arnold et al. 2007; Kemler et al. 2009). This idea is in marked contrast to our current understanding of fungal paleobiodiversity, especially within the Basidiomycota. The preservation bias (or lack thereof) of fungi has certainly been a major influence in any attempts to document the paleodiversity and evolution of these organisms based on the fossil record. One of the unique features of the Basidiomycota is the clamp connection, a structure that is known only from this phylum and occurs in all subphyla. Despite the fact that the divergence between the Ascomycota and Basidiomycota is thought to be ancient, well-documented early basidiomycete fossils are not known. This lack is especially interesting since the famous Early Devonian (~400 Ma) Rhynie chert ecosystem has provided

abundant evidence of several fungal phyla, including the Chytridiomycota, Blastocladiomycota, Glomeromycota, and Ascomycota (Taylor et al. 2004). Although the presence of Ascomycota in the Rhynie chert (Taylor et al. 2005) indirectly indicates that the Basidiomycota had already evolved by that time, because the two are sister groups, any convincing fossil evidence of Basidiomycota is absent to date from this paleoecosystem. There may be several reasons for this, including the presence of basidiomycetes that simply do not look like any modern form, or, as we believe, examples simply have not been identified to date.

There is little doubt that multiple lineages of fungi and fungus-like microorganisms predate their documented existence in the fossil record, and that at least some of the genetic, biochemical, and structural features defining modern groups diverged early and appear to have remained relatively unchanged in modern forms (Bonfante and Genre 2008; Dotzler et al. 2009). The Visean clamp connections add support to this hypothesis. Other structural features of the Basidiomycota such as the morphology of the basidium and various types of hyphal septa also have preservation potential, and their discovery in the fossil record from the Paleozoic would undoubtedly greatly increase our understanding of the evolution and past diversity of this group of fungi. It is in this context that the fossil hyphae with clamp connections reported here can be used to increase the data points to further subsequent systematic and phylogenetic investigations of the Basidiomycota.

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