*Mycologia*, 86(1), 1994, pp. 1–17.

Issued 6 April 1994

# Minute mycological mysteries: the influence of arthropods on the lives of fungi[1]

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"The most beautiful thing we can experience is the mysterious. It is the source of all true art and science."
Albert Einstein, *What I Believe* (1930).

***Abstract:*** Fungi are commonly associated with arthropods in a variety of habitats. Many species of ascomycetes have morphological features, including evanescent asci and passively discharged ascospores, that facilitate ascospore dispersal by arthropods. Based on small subunit ribosomal DNA sequence analysis several lineages appear to have been derived independently by convergence. One monophyletic lineage includes *Pyxidiophora* and *Rickia* (Laboulbeniales). Evolution of Laboulbeniales from a *Pyxidiophora*-like ancestor is viewed perhaps as life cycle simplification to reduce excessive fine-tuning of the life cycle to two disparate hosts and to reduce habitat patchiness. The minute arthropod-borne fungi, *Thaxteriola, Endosporella, Laboulbeniopsis, Coreomycetopsis, Amphoropsis, Myriopodophila,* and *Entomocosma,* are included in the *Pyxidiophora–Rickia* lineage as part of the *Pyxidiophora* life cycle or as independent organisms on the basis of morphology and life history studies.

***Key Words:*** ascomycete systematics, dispersal, fungus-arthropod associations, Laboulbeniales, life history, patchy habitat, *Pyxidiophora,* 18S rRNA gene

The story I tell begins in the mid-eighteenth century, still a time of superstition and spontaneous generation, when secretive saprobes hidden in humus were linked with the devil. It was then that Otto von Münchhausen investigated the essential nature of fungi and concluded that these mysterious objects were the houses of animals (Ramsbottom, 1953). Although Münchhausen's observations were superficial, they serve to emphasize the common phenomenon of associations of fungi with insects. We now know that fungi not only house animals, but also feed them and clothe them in camouflage. We know that fungi lure insects with wafted scents, detoxify their food, and perform other feats of alchemy only now being recognized (Dowd, 1992). But fungi make use of animals as well (Batra, 1979; Wheeler and Blackwell, 1984; Anderson et al., 1984; Wilding et al., 1989), and it is some of these fungi that have captured my interest.

This story will dwell on fungi that fly through the air as excrescences on elytra (the wing covers of beetles) and flocculence of flies; in fact, purposeful phoronts (hitchhikers) all making use of the wings and energy of insects. These are fraudulent fungi, masters of deceit, perjurers in our attempts to solve evolutionary enigmas. The trail left by several genera of minute fungi will lead us first to mycelial forms, past a zygomycete, then finally to an even more mysterious group of obligate parasites of arthropods, the Laboulbeniales (FIGS. 1–12). The evidence used to follow the evolutionary path of these fungi includes morphology, life history studies, and, in the end, analysis of sequences of nucleic acids. For me the story has a happy ending, replete with resolution.

## MINUTE FUNGI

Roland Thaxter (1914, 1920) and Carlos Spegazzini (1918) took a detour from their better known mycological pursuits when they described a number of minute fungi found in the habitats of the larger Laboulbeniales that they studied. The fungi they described include such ill-known, long-named genera as *Amphoromorpha* Thaxter (FIGS. 1, 2), *Endosporella* Thaxter (FIG. 4), *Coreomycetopsis* Thaxter (FIG. 5), *Laboulbeniopsis* Thaxter, *Amphoropsis* Speg., *Myriopodophila* Speg., *Thaxteriola* Speg. (FIG. 6), and *Entomocosma* Speg. Most of these minute forms are characterized by having few cells (1–15) usually linearly superposed, being lightly pigmented, and as is their wont, all stick by glutinous globs to the outer parts of unsuspecting arthropods.

When many more of these minimal forms were yet to be described, Spegazzini died (1926) and Thaxter (1920) tired, remarking as he quit: "Although the examination of mycological novelties possesses a certain fascination, it may have its drawbacks, since in the present . . . their interest may be neutralized . . . by

[1] Presidential address to the Mycological Society of America presented 20 June 1993 at Athens, Georgia.

Meredith Blackwell, President, Mycological Society of America, August 1992–June 1993. Pictured with Farley (left) and Mosham (right), contributors to her research. Photograph by Ray Neyland.

their very novelty, which may be of such a nature as to make it impossible to assign them a satisfactory position among their fellows, or to arrive at any reasonable conclusion as to the true significance of their characteristics." Thaxter's Proustian sentence has a special meaning for me, because just as he felt fascination and frustration over the tiny fungi, so later did I. Thaxter (1920) recommended that the "inter-

lopers in the scheme of organic life" be catalogued so that eventually they might be satisfactorily distributed in "mycological pigeonholes" when other forms were found to fill the gaps.

For nearly 70 yr these fungi went to earth in the haunts of termites, beetles, flies, and mites, resurfacing occasionally to be glimpsed by only a very few (Povah, 1931; Benjamin, pers. comm.; Rossi and Balazuc, 1977; Rossi and Cesare Rossi, 1979, 1982; Majewski and Wiśniewski, 1978a, b; Blackwell, 1980; Blackwell and Rossi, 1986; Simpson and Stone, 1987; Levieux et al., 1989; Moser et al., 1989).

Special attention is called to the work of James Kimbrough, University of Florida, and his colleagues, who rediscovered myriad minute fungi, especially those found on termites, and not seen since the time of Thaxter (1914, 1920). This work provided information on morphology and life histories of species of *Antennopsis* Heim (FIG. 7) (Gouger and Kimbrough, 1969), *Termitaria* Thaxter (FIG. 8) (Khan and Kimbrough, 1974; Kimbrough and Lenz, 1982; Lenz and Kimbrough, 1982), and *Mattriolella* Colla (Kimbrough and Thorne, 1982; Thorne and Kimbrough, 1982), and led to the discovery of *Hormiscioideus* M. Blackwell & Kimbrough (Blackwell and Kimbrough, 1978). But, these fungi are darkly pigmented, filamentous in habit, and most likely unrelated to those with a few pale superposed cells. However, *Laboulbeniopsis* and *Coreomycetopsis,* studied by Kimbrough and Gouger (1970) and Blackwell and Kimbrough (1976a, b), are part of this story. It is clear that none of these fungi is really rare, only rarely sought.

Kimbrough suggested that some of the fungi might have unknown stages occurring within the termite galleries. Although we found no other stages in the substrate, this prophecy was eventually fulfilled, but for other fungi as you shall see. However, we were able to obtain life cycle and ultrastructural information on the all-but-impenetrable thalli of *Laboulbeniopsis termitarius* Thaxter and *Coreomycetopsis oedipus* Thaxter. These two species have similar morphologies at the light microscope level. At the ultrastructural level the spores produced by *C. oedipus* appear to be conidia; *L. termitarius* was interpreted as having ascospores. But, it is the cell attached to the termite that is remarkable with its secretory channels leading to an external attachment apparatus in both species, melanized only in *L. termitarius.* The similarity of the morphologically complex attachment region suggests a relationship, rather than convergence, for *L. termitarius* and *C. oedipus.* Could the two species have a teleomorph–anamorph relationship? If so, the conidial form, *Coreomycetopsis,* is far less common (Blackwell and Rossi, 1986). Furthermore, what could be pieced together of the life cycles did not indicate that they are obligate or even occasional stages of the same life cycle (Blackwell and Kimbrough, 1976a, b). Now, the perspective of almost 20 yr and the comparison of ultrastructural features of the attachment apparatus of *Coreomycetopsis, Laboulbeniopsis,* and *Pyxidiophora* (FIG. 13), the mycelial ascomycete to be discussed in more detail later, suggest that species of the three genera may have a close relationship. If this is so, and if the life cycles of *Laboulbeniopsis* and *Coreomycetopsis* are correct, an explanation would call for divergence of the ascospore of an ancestral *Pyxidiophora*-like form and loss of life cycle stages.

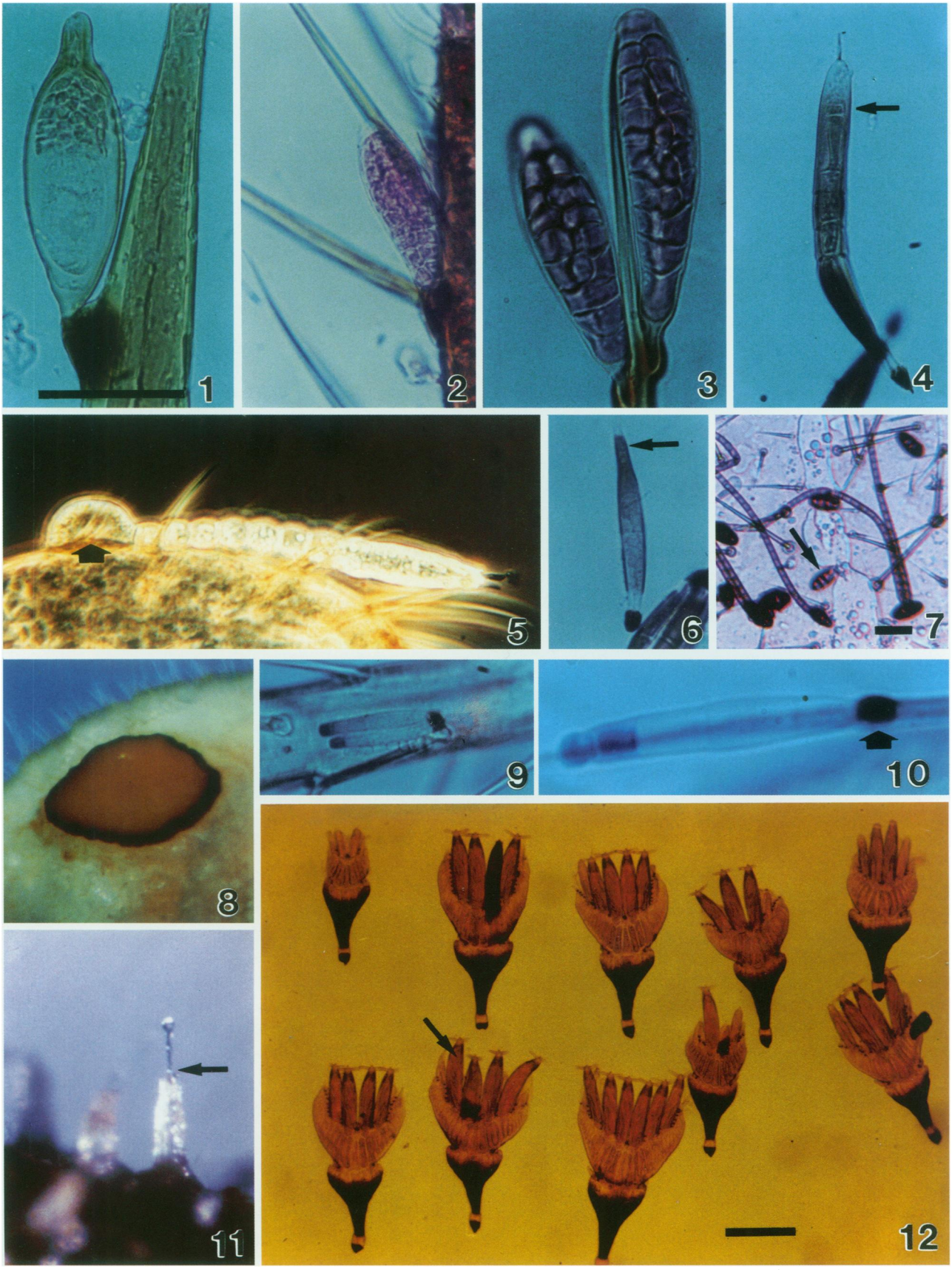
There is nothing more that can be said of the relationships of *Laboulbeniopsis* and *Coreomycetopsis* without further study, so I return to the six remaining minute fungi with thalli of few superposed cells: *Endosporella, Amphoropsis, Myriopodophila, Entomocosma, Thaxteriola,* and *Amphoromorpha.* There appears to be enough evidence now to place them in Thaxter's "pigeonholes." In a way the evidence comes from the missing forms Thaxter (1920) postulated, but the "forms" are not new species but rather fungi we already know.

If one looks through a keyhole, the restricted view reveals *Thaxteriola.* This is the sight that both Thaxter (1914, 1920) and Spegazzini (1918) saw (FIG. 14). Spegazzini (1909) looked through a second keyhole on two occasions and saw *Pyxidiophora* (as *Copranophilus* Speg. and *Treleasia* Speg.) (FIG. 15). But when the door is opened (FIG. 16) we are afforded new insight. The natural habitat is essential for making observations of fungi and their many associates (FIG. 17). It is now clear that *Thaxteriola* and *Pyxidiophora* are different stages in the life cycle of the same fungus.

There was little early speculation on the relationships of these fungi. Thaxter (1914, 1920) had not overlooked the morphological similarity of *Thaxteriola* and *Endosporella* to the male thallus of some dioecious species of Laboulbeniales, but he held the view that this was most likely only a superficial similarity. However, Gäumann and Dodge (1928) believed the similarity was real and that the two genera were actually conidium-producing members of the Laboulbeniales. I will return to this idea later.

### *PYXIDIOPHORA*

The observations of Thelma Perry, John Moser, and Robert Bridges, Southern Forest Experiment Station, USDA Forest Service, Pineville, Louisiana, provided the clues that led to the discovery that *Thaxteriola* developed from the ascospore of *Pyxidiophora,* an ascospore disguised by its production of conidia, rather than germination by germ tubes (FIGS. 9, 10)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(Blackwell et al., 1986a, b). Lundqvist (1980) already had recognized the ascospores of *Pyxidiophora* attached to phoretic mites of bark beetles. However, he had seen only immature specimens that had not yet produced conidia. *Acariniola* Majewski & Wiśniewski (1978a), a form close to *Thaxteriola,* was based on such an ascospore. In his study, Lundqvist (1980) recognized not only *Acariniola,* but also *Mycorhynchus* Sacc., *Treleasia, Copranophilus, Rhynchomyces* (Sacc.) Marchal ex Marchal, and *Ascolanthanus* Caill., as aliases of *Pyxidiophora.* The protean, slowly maturing ascospores have many guises to trick an unsuspecting systematist. However, given time the ascospore takes on a life of its own and becomes a nonmycelial *Thaxteriola* producing its own conidia on the arthropod disperser.

David Malloch, University of Toronto, and I were able to find species of *Pyxidiophora* on moose dung in New Brunswick (FIG. 11) and Ontario at a time when species of the genus on dung were poorly known in North America. We now find them almost any place we look, including my own departmental chairman's backyard in Baton Rouge. The dung habitat was much more amenable to life history studies than was that of bark beetles, where many more species of *Pyxidiophora* go undescribed for lack of life history information (Blackwell et al., 1989a). One important difference is that rather than collecting bark beetles in pheromone traps as they emerge from pine trees, the dung beetles easily can be baited and intercepted as they arrive at the new substrate bearing ascospores with conidia ready to begin their next generation. Dung heaps also are easier to observe for all their life forms than is the interior of a pine tree. We were able to delve more deeply into the mystery by following Pyxidiophoras from spore germination through spore dispersal (a too-often ignored part of the life history) to spore germination beginning the next generation (Blackwell and Malloch, 1989a). We watched as conidia budded yeast cells and then developed germ tubes to grow into mycelia bearing additional unexpected conidial stages. We also were able to observe the complex interactions between the fungus and its arthropod dispersers and to discover that at least some species of *Pyxidiophora* are mycoparasites (Malloch and Blackwell, 1993b). Thus, the application of careful life history studies in the field and in the laboratory with moist chamber cultures of dung pro-

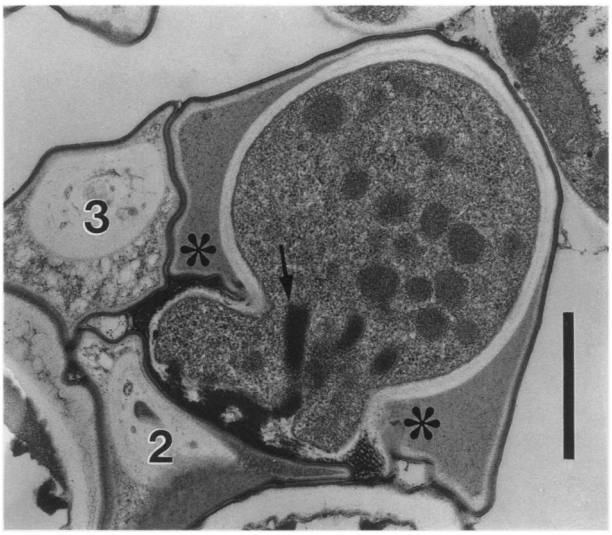


FIG. 13. The attachment apparatus of an ascospore of *Pyxidiophora* sp. resembles that of *Laboulbeniopsis termitarius* and *Coreomycetopsis oedipus.* Asterisks indicate thickened spore wall at eventual arthropod attachment region. Dense material in channels (arrow) accumulates outside the spore wall, but details of the process are unknown. The dense material is presumed to be an adhesive by which other ascospores (2, 3) may adhere to each other in the extruded spore mass before arthropod contact.

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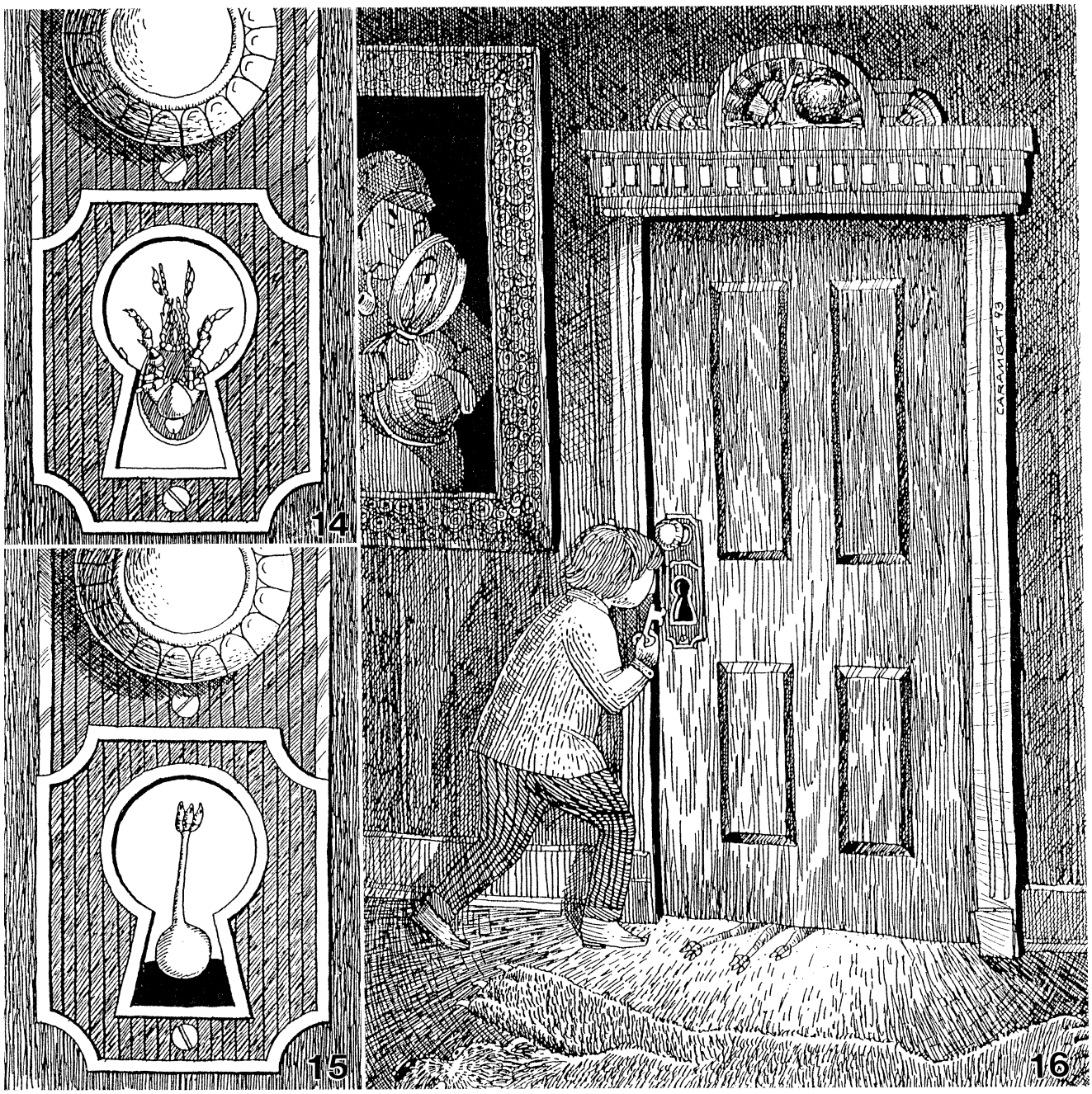
FIGS. 1–12. Fungi commonly found on the exoskeletion of arthropods. 1. *Amphoromorpha entomophila* Thaxter attached to an insect spine. Holotype, FH 8910. Bar = 50 μm. 2. *A. blattina* Thaxter attached to a roach. Holotype, FH 8907. Bar (FIG. 1) = 50 μm. 3. Capilliconidia of *Basidiobolus ranarum* Eidam on mites placed in the fungus cultures (after 2 days). Bar (FIG. 1) = 20 μm. 4. *Endosporella diapsis* Thaxter removed from insect. Holotype, FH 8997. Arrow indicates conidium. Bar (FIG. 1) = 50 μm. 5. *Coreomycetopsis oedipus* Thaxter on termite, Gainesville, Florida. Arrow at attachment to termite. Bar (FIG. 1) = 40 μm. 6. *Thaxteriola nigromarginata* Thaxter. Holotype, FH 9021. Arrow indicates conidium. Bar (FIG. 1) = 50 μm. 7. *Antennopsis gallica* Heim & Buchli. Baton Rouge, Louisiana. Four-celled basal attachment structure (arrow) prior to filament growth. Bar = 25 μm. 8. *Termitaria coronata* Thaxter on termite. Gainesville, Florida. Bar (FIG. 1) = 400 μm. 9, 10. *Thaxteriola* thalli with conidia. Developed on mites in moist chamber directly from ascospores of *Pyxidiophora* sp. Algonquin Park, Ontario. For FIG. 9, bar (FIG. 1) = 50 μm. In FIG. 10, arrow indicates attachment to mite spine. Bar (FIG. 1) = 25 μm. 11. Perithecium of an undescribed species of *Pyxidiophora.* Meredith Station, New Brunswick. Ascospores (above level of arrow) await an arthropod disperser. Bar (FIG. 1) = 200 μm. 12. *Dichomyces biformis* Thaxter. Arrow indicates one of many perithecia. Slide mounted by Roland Thaxter at FH. Bar = 200 μm.




FIGS. 14–16. Many fungal life cycles are imperfectly known. Pen and ink drawings by John Carambat. 14. This is the view of *Thaxteriola* that both Thaxter and Spegazzini saw. 15. Another restricted view shows *Pyxidiophora,* seen twice by Spegazzini. 16. The door is opened.

vided the clues necessary to reveal the true identity of not only *Thaxteriola* and *Acariniola,* but almost certainly *Endosporella, Entomocosma, Myriopodophila,* and *Amphoropsis* as well. Although we have not seen Spegazzini's types, his descriptions and illustrations are very similar to the developing ascospores of one species of *Pyxidiophora* that we studied (Blackwell and Malloch, 1989a).

*BASIDIOBOLUS*

The *Pyxidiophora–Thaxteriola* model involving highly specialized spores for arthropod dispersal suggested the identity of yet another fungus with a dispersal propagule, *Amphoromorpha.* However, without the exquisite drawings and detailed observations of Drechsler (1956) we would not have made the connection

between *Amphoromorpha* and *Basidiobolus* (Blackwell and Malloch, 1989c). Although many mycologists have focused on the forcibly discharged conidia of *Basidiobolus,* it was Drechsler who described the passively released capilliconidia of *B. ranarum* Eidam adhering to culture mites. We were able to synthesize *Amphoromorpha* by placing termites and mites in cultures of *B. ranarum* to observe the attachment and gradual darkening of the adhesive haptor material and, over time, protoplast cleavage (FIG. 3). However, this story is not yet complete, because the two species of *Amphoromorpha* described by Thaxter, *A. entomophila* (FIG. 1) and *A. blattina* (FIG. 2), are not exactly like the capilliconidia of *Basidiobolus ranarum* (FIG. 3) and probably represent undescribed species of *Basidiobolus.*

So, we find that convergent evolution has led to a similar method of dispersal both in zygomycetes and ascomycetes. In fact the similarity was so great that Thaxter (1914) had suggested that *Thaxteriola* (undescribed at the time) was perhaps congeneric with *Amphoromorpha.* Some of his beautifully preserved slides of *Thaxteriola* bear the label in his hand "Euamphoromorpha." It is ironic that Thaxter, who described *Amphoromorpha* (1914, 1920), had studied *Basidiobolus ranarum* earlier (1888); however, he did not culture it, and apparently never observed capilliconidia.

At this point I want to emphasize that life history studies provided the evidence needed for solving the mystery of the minute arthropod-borne *Amphoromorpha.* Thaxter was correct in predicting that forms eventually would be found to reveal its identity. The "forms" this time are the greater part of the life cycle of *Basidiobolus.*

## LABOULBENIALES

The *Pyxidiophora-Thaxteriola* model also was the key to the development of a hypothesis that offered a solution to a more widely publicized mystery. The Floridean (or red algal) hypothesis of the origin of ascomycetes through the Laboulbeniales was proposed by Sachs (1874). The hypothesis was an attempt to explain the unique morphological features of the distinctive group of obligate arthropod parasites (FIG. 12). Sexual reproductive structures, including a highly differentiated trichogyne and the absence of mycelium, are unusual ascomycete characteristics and superficially resemble features of floridean algae. Periodically, additional morphological and physiological data have been used to resurrect the hypothesis (Denison and Carroll, 1966; Kohlmeyer, 1973, 1975; Demoulin, 1974, 1985). However, others have viewed the Laboulbeniales as a mainline ascomycete group,

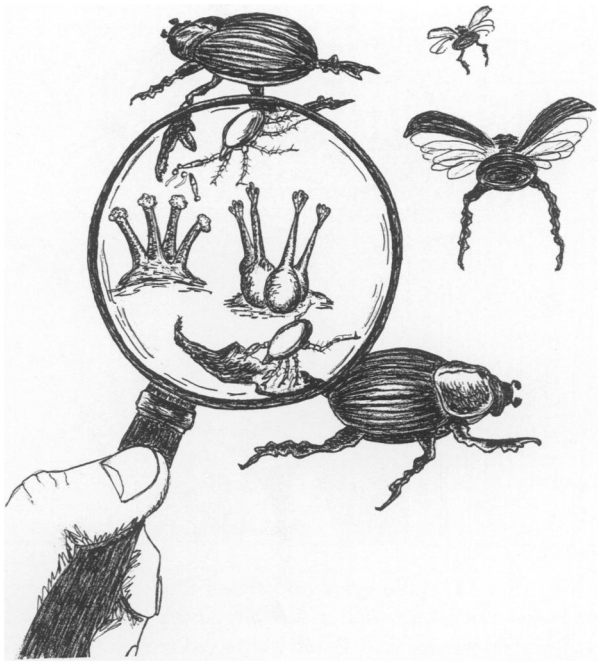


FIG. 17. Pyxidiophoras are intimately associated with their dispersers. Not only do phoretic mites and dung beetles disperse ascospores of some species, but conidia are dispersed locally on the substrate by other invertebrates. Pencil drawing by Ty Keller.

but with the relationships unspecified (de Bary, 1887; Hill, 1977; Barr, 1983, 1990; Tehler, 1988; Nakagiri, 1993).

There is now overwhelming molecular evidence to show that red algae are outside of the fungal lineage. Kwok and her colleagues (1986) used DNA–DNA hybridization to suggest that red algae and higher fungi are not closely related. The matter should have been buried by the DNA sequencing studies that included red algae in an analysis with ascomycetes, but that work was slow to become known to mycologists because it was hidden in the nonmycological literature (Bhattacharya et al., 1990; Hendriks et al., 1991). However, the more specific problem of the ascomycete lineage of the Laboulbeniales still was not addressed.

At the International Mycological Congress in Tampa, Florida, David Malloch (1976) had first suggested the relationship of *Pyxidiophora* and the Laboulbeniales on the basis of ascospore morphology. I wish I could remember the discussion, but I don't because I had never even heard of the genus then. However, later I was to become quite taken with these ascomycetes, and, later still, our comparison of the more complete life history of *Pyxidiophora* with that of the Laboulbeniales provided additional evidence for this hypothesis (Blackwell and Malloch, 1989b). We emphasized the similarity of ascospore development, development of an anamorph thallus from an asco-

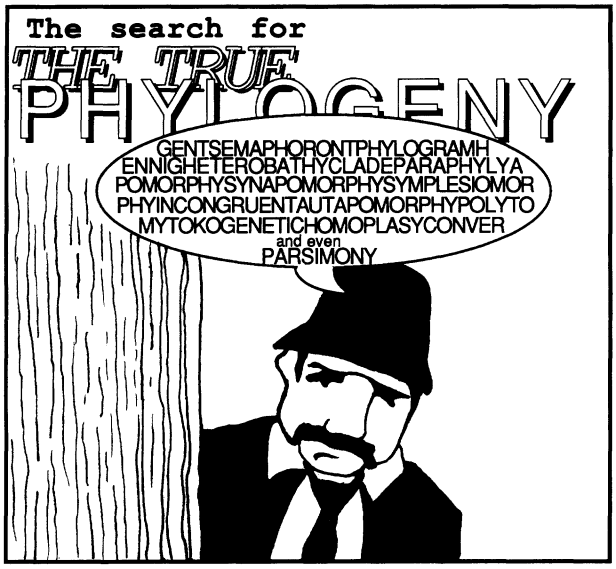



FIG. 18. "It is the great mystery of life itself which is at the bottom of all the mysterious language we are obliged to employ concerning it." Peter Mere Latham (1789–1875), *Collected works.*

spore, and dispersal by arthropods. The shift to an arthropod host and loss of a mycelial stage were viewed as an example of heterochrony. Furthermore, a terrestrial origin in arthropod habitats was more attractive than the marine one. It is interesting at this point to recall that Gäumann and Dodge (1928), without the knowledge of the complete life history of *Pyxidiophora,* actually had regarded *Thaxteriola* as an asexual form of the Laboulbeniales.

However, the answer to a more perplexing problem, the relationship of *Pyxidiophora* to other ascomycetes, could not be suggested on the basis of our morphological studies (Blackwell and Malloch, 1989b). Although *Pyxidiophora* shares features of early evanescent asci and long-necked perithecia with other ascomycetes, these characters almost certainly are due to parallel or convergent evolution, selected to facilitate arthropod dispersal. In addition, *Pyxidiophora* has a number of unique derived features that are unknown in other mycelial ascomycetes. The elaborate attachment apparatus at the distal end of the extruded ascospore (FIG. 13) is unlike anything known in other ascospores, although, as I have mentioned already, it is similar to those of *Laboulbeniopsis* and *Coreomycetopsis.* In addition, *Pyxidiophora* has only a single layer of perithecium wall cells. This condition also is rare among ascomycetes with a perithecium.

So, for the first time in this story, morphology and life history studies failed. They failed for several reasons that have caused recurring problems for fungal systematists. The few morphological characters available are not present in all the species of interest, they are probably the result of parallel or convergent evolution, and they cannot be polarized, resulting in an inability to determine evolutionary direction.

At this point it was necessary to take the longer road in a seeming detour to solving this mystery, a mystery that now has led from *Pyxidiophora* to the Laboulbeniales. By this time two new tools were available for mycological mystery solving. In 1966 the English translation of Willi Hennig's *Phylogenetic systematics* was published. Hennig's system provides an empirical method to arrive at phylogenetic conclusions. Phylogenetic analysis or cladistics has caused a revolution in systematics. Its simple, but rigorous philosophy of character analysis is compelling (Hennig, 1966), but its unique terminology presents a minor barrier to the uninitiated (FIG. 18). The second innovation was the development of procedures to amplify DNA from small quantities of template by the polymerase chain reaction (PCR). The revolutionary influence of this technique has just been recognized by no less than a Nobel Prize in chemistry for Kary Mullis. It is a technique that helped to satisfy my 20-yr curiosity about these fungi when I did not think it would be possible in my lifetime. Just after

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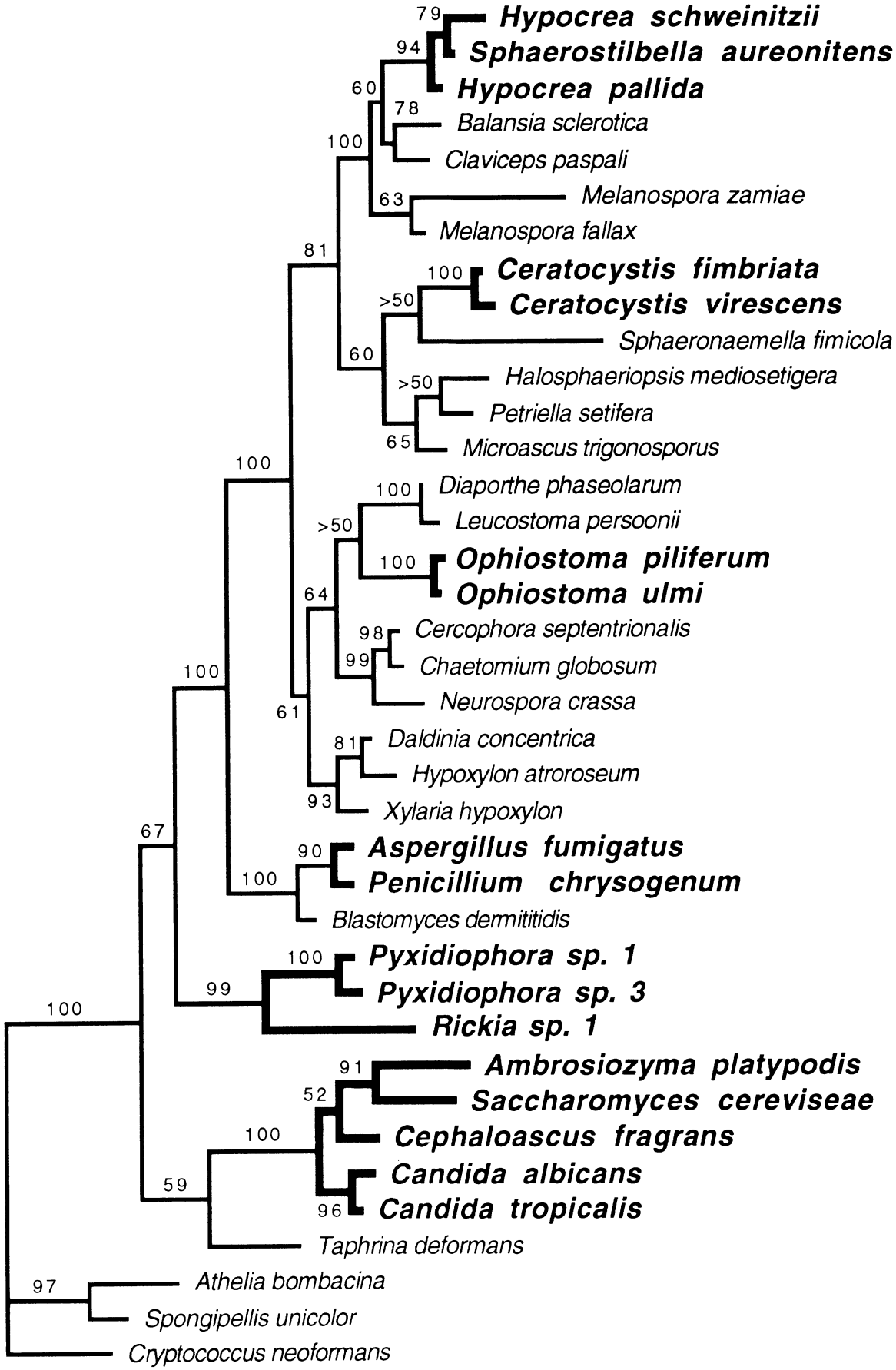
FIG. 19. Hypothesis of phylogenetic relationships of certain ascomycetes based on maximum parsimony analysis of partial sequences of nuclear-encoded small subunit rDNA. The highlighted regions of the tree are discussed in the text and summarized in FIG. 20. Of particular interest to the present discussion are the sister group relationship of Pyxidiophoras and the laboulbenialean *Rickia* sp. and the well supported exclusion of these taxa from the major group of perithecial ascomycetes. Near relatives of Pyxidiophoras and *Rickia* sp. still are unknown because of inadequate taxon sampling in this region of the tree. A single most parsimonious tree (781 steps) was obtained using PAUP, version 3.1.1 (Swofford, 1993). Tree statistics include consensus index = 0.474, retension index = 0.692, and rescaled consensus index = 0.328. The percentage of support (50% and higher) from 500 bootstrap replications is indicated on the branches. Information on included taxa and sequences is found in Spatafora and Blackwell (1993a, b, 1994) and Blackwell and Spatafora (1994), except for new sequence derived from *Rickia* sp. from laboratory colony of *Odontotaenius disjunctus* Illiger (Passalidae) collected in the vicinity of Baton Rouge, Louisiana.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Hypocrea schweinitzii
94
Sphaerostilbella aureonitens
Hypocrea pallida
60
78
Balansia sclerotica
100
Claviceps paspali
63
Melanospora zamiae
Melanospora fallax
81
100
Ceratocystis fimbriata
>50
Ceratocystis virescens
Sphaeronaemella fimicola
60
>50
Halosphaeriopsis mediosetigera
Petriella setifera
65
Microascus trigonosporus
100
100
Diaporthe phaseolarum
>50
Leucostoma persoonii
100
Ophiostoma piliferum
64
Ophiostoma ulmi
98
Cercophora septentrionalis
100
99
Chaetomium globosum
Neurospora crassa
61
81
Daldinia concentrica
Hypoxylon atroroseum
93
Xylaria hypoxylon
67
90
Aspergillus fumigatus
100
Penicillium chrysogenum
Blastomyces dermititidis
100
Pyxidiophora sp. 1
99
Pyxidiophora sp. 3
100
Rickia sp. 1
91
Ambrosiozyma platypodis
52
Saccharomyces cereviseae
100
Cephaloascus fragrans
Candida albicans
59
96
Candida tropicalis
Taphrina deformans
97
Athelia bombacina
Spongipellis unicolor
Cryptococcus neoformans

Tom White, Tom Bruns, and Steve Lee designed the primers and perfected the methods for fungus PCR in John Taylor's laboratory, they all gave freely of their unpublished information. The road to solving the mystery had taken me to Berkeley.

The methods, combined with the arrival of Joey Spatafora as a graduate student in my laboratory, led to the acquisition of new clues in the form of DNA sequences. Base positions of aligned sequences are easily analyzed with the techniques of cladistics and can be polarized by the outgroup method. In fact, one is actually forced into the use of explicit data analysis because of the large number of characters that can be obtained (Blackwell, 1993). Because we were interested in ordinal level relationships of *Pyxidiophora,* we chose a slowly evolving molecule, nuclear-encoded small subunit ribosomal DNA, that would avoid the problems of convergence encountered with strong selection pressure for the morphological features of dispersal. Furthermore, the characters extend across all taxa of interest and can be derived from any stage in a life cycle.

Most of the results of the study of ancestor–descendent relationships among perithecial ascomycetes with Spatafora have been published elsewhere (Spatafora and Blackwell, 1993a, b, 1994; Blackwell and Spatafora, 1994). Several hypotheses are evident both from our work and similar studies by others (FIG. 19):

1) The Ophiostomatales is not a monophyletic group (Spatafora and Blackwell, 1993b). In fact, several others have shown that not only members of the Ophistomatales, but other taxa with evanescent asci as well, occur in different lineages (Berbee and Taylor, 1992b; Hausner et al., 1992, 1993). Blackwell and Spatafora (1994) presented a hypothesis of six distinct lineages with evanescent asci and known or suspected arthropod dispersal (Malloch and Blackwell, 1992, 1993a).

2) Yeasts of the Endomycetales occur in a basal position of the tree and none appears to have a close relationship with extant mycelial forms as had been suggested (Redhead and Malloch, 1977; von Arx and van der Walt, 1987). Because many arthropod-dispersed mycelial ascomycetes actually are dimorphic with yeast stages occurring during times of arthropod association, the derivation of the yeasts by reduction had been an appealing idea. This analysis (FIG. 19) supports the similar conclusions of an earlier study, also designed to address the specific question as it applies to arthropod-associated fungi (Hausner et al., 1992).

3) *Pyxidiophora* is not in a hypocrealean lineage where it often has been placed (e.g., Lundqvist, 1980; Barr, 1990), nor is it a member of the Ophiostomatales (von Arx and van der Walt, 1987; Eriksson and Hawksworth, 1989; see Blackwell and Spatafora, 1994). On the basis of the conclusions drawn earlier (Blackwell and Malloch, 1989b), Eriksson and Hawksworth (1993) placed *Pyxidiophora* in the Laboulbeniales. However, the question of the closest mycelial relatives remains unanswered, because *Pyxidiophora* lies outside the other perithecial ascomycetes among loculoascomycetes and discomycetes where taxon sampling is still incomplete.

4) An increasing number of studies include asexual fungi in analyses not only to determine the close sexual relatives, but also to gain insight into the long-term success of asexual species (Guadet et al., 1989; Bowman et al., 1992; Berbee and Taylor, 1992a, 1993; Gordon, 1993; LoBuglio and Taylor, 1993; LoBuglio et al., 1993; Reynolds and Taylor, 1993). *Aspergillus fumigatus* Fresenius and *Penicillium chrysogenum* Thom are the only asexual species included in the analysis presented here. However, we also have included several asexual ascomycetes in our own studies. When we suspected a conidial isolate was in reality an asexual strain of *Pyxidiophora,* the DNA sequence analysis confirmed its genetic identity (Blackwell et al., 1993). In addition, Steven Cassar (1993) has used this method to great advantage, showing that species of the conidial genus *Ambrosiella* do not constitute a monophyletic group and placing them among their disparate sexual relatives. This is possible because, unlike morphological characters, nucleic acid sequences can provide characters that extend across all the life cycle stages of an organism. There is now no operational reason to exclude these forms from phylogenetic studies of sexual fungi and critical evolutionary insight is obtained by their inclusion (see Reynolds and Taylor, 1993). We can now aim for inclusion of all conidial fungi in phylogenies with their closest relatives.

Finally, this story nears its end with the addition of the sequence of *Rickia* sp., a member of the Laboulbeniales, and the last hypothesis: 5) the sister group relationship for *Pyxidiophora* spp. and *Rickia* sp. supports the view suggested by morphological and life history studies (Blackwell and Malloch, 1989b). Although it is always risky to include only a single representative of any group, especially when the risk of DNA contamination is great, we do have additional confirming sequence data from two independent *Rickia* extractions and from another laboulbenialean species that will be published as part of another study.

It is important to point out the usefulness at some level of all the characters that my colleagues and I have used. The characters include morphology (ultrastructural features as well), descriptive life history studies of fungi and their natural associates, and aligned DNA base positions. All have their place and

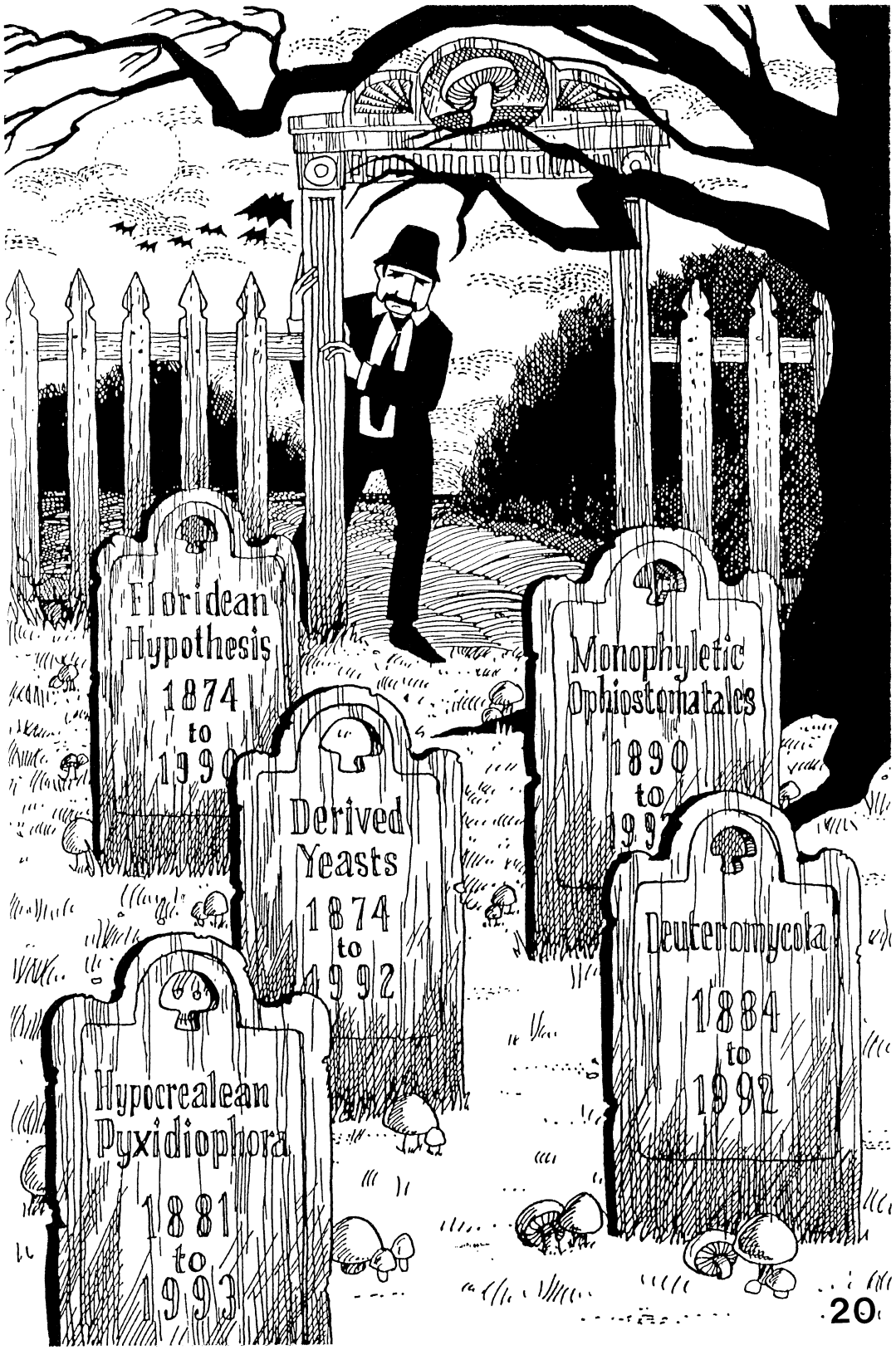


FIG. 20. A number of phylogenetic hypotheses concerning ascomycetes are not supported by rDNA analysis. Pen and ink drawing by John Carambat.

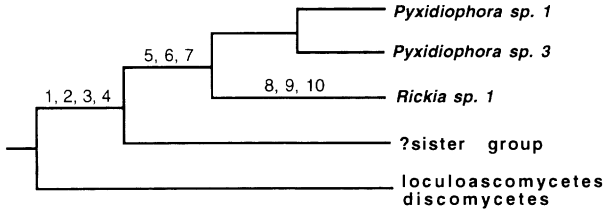


FIG. 21. Model proposed for the evolution of the Laboulbeniales. The *Pyxidiophora–Rickia* lineage is placed outside the main body of perithecial ascomycetes (FIG. 19). This lineage arises somewhere among discomycetes and loculoascomycetes (not shown in FIG. 19), but the sister group has not been identified with certainty because taxon sampling is incomplete in this region of the tree. However, events occuring in the older divergence of *Pyxidiophora–Rickia* and the sister group are postulated: 1) Phoretic ascospore dispersal was selected for with a reduction of the ascocarp and loss of forcible ascospore discharge. 2) Conidia were no longer wind-dispersed and assumed a restricted local dispersal function. 3) A mycoparasitic nutritional mode arose with 4) loss of cellulase production. Divergence of the *Pyxidiophora–Rickia* branch involved: 5) evolution of the attachment region of the ascospore for arthropod dispersal and as a preadaptation for 6) arthropod parasitism. 7) Repetitive ascospore germination arose with eventual complete loss of germ tube germination. In the *Rickia* lineage 8) the complete shift to the arthropod host resulted in loss of all mycelial stages which were on the fungal host. 9) The conidia produced in the distal cell (primary appendage) assumed a spermatial function, and 10) the perithecium developed from the proximal cell of the attached ascospore. This scenario invokes strong selection for ascospore dispersal, life cycle simplification to lessen excessive fine-tuning to the life cycles of two disparate hosts, and the reduction of habitat patchiness. See Benjamin (1971) and Tavares (1985) for a discussion of the development of laboulbenialean thalli. Evolution of the Laboulbeniales in a lineage with *Pyxidiophora* was discussed by Blackwell and Malloch (1989b).

independent data sets help to corroborate or question hypotheses; however, intractable problems caused by convergence, parallelism, unknown polarity, and a dearth of morphological characters across an entire group of taxa due to heterochrony and rapid divergence require molecular solutions (FIG. 20). Have these characters given us a true tree, one that reflects the manner in which evolution actually proceeded? We have no way of knowing now. Although the tree fits the data used in its construction, more taxa remain to be sampled, especially in the region where *Pyxidiophora* and *Rickia* occur. I only can hope that in 120 years the major points I have made will fare better than Sach's (1874).

Now that we have begun to recognize a pattern of monophyletic lineages and ancestor–descendent relationships, we can fill in additional data and continue attempts at falsification of the hypothesis depicted in the tree (FIG. 19). With a robust hypothesis we also can begin to use the evolutionary pattern to consider the processes that may have produced such a pattern, in other words, the underlying motives, means, and evolutionary opportunity.

## MOTIVE, MEANS, AND OPPORTUNITY

The *motive* for association of some fungi with insects appears to be dispersal, in this case ascospore dispersal (FIG. 21). *Pyxidiophora* is characteristic of rapidly decomposing substrates where its dispersers also are common (Blackwell and Malloch, 1989a, b); how better to escape a desert island of degraded resource than to have an island-hopping arthropod upon which to rely (Malloch and Blackwell, 1992). Species of the genus arrive at the fresh substrate with multiple conidia already formed and set to germinate quickly. Rapid production of large numbers of rather sticky conidia from the mycelium ensures that the scurrying, slithering cohabitants of the substrate will spread this additional inoculum throughout. The key life cycle events of the fungi and the height of their elevated ascospore mass from the substrate surface correlate well with the timing of activities and size of the dispersers; dispersers usually can be relied upon to find a fresh new substrate, even a very small one. *Pyxidiophora,* by being early, avoids competition for resources not only from some fungi but from invertebrate animals as well (Malloch and Blackwell, 1993a). There is another thing that we know about not only *Pyxidiophora,* but also about several other ascomycetes with well developed arthropod dispersal: their inability to degrade cellulose (as determined by the cellulose–azure blue method; unpublished data). Although from the tree (FIG. 19) we assume that lineages including *Ceratocystis* spp. and *Ophiostoma* spp. may have evolved from cellulase-producing ancestors, they, as well as *Pyxidiophora,* appear to have lost the ability. Does this mean that increasing reliance on insects for dependable fast, direct dispersal to ever-fresh substrates allows the fungi to move into patchy substrates exclusively and win the competition for ephemeral carbon sources, thereby eliminating the need for some costly enzyme systems? Are unexpressed genes present, or could nonfunctional pseudogenes be found to confirm an ongoing process of gene loss? However, *Pyxidiophora* confounds the question because most of the species are probably mycoparasites (Blackwell and Malloch, 1989b; Malloch and Blackwell, 1993b), and we suspect that in the *Thaxteriola* stage some of them can sink a haustorium into a mite as well. The fact is that *Pyxidiophora*

gets to the substrate very soon to find its hosts in the gut passage mycota found in dung or in the fungi present in its other substrates.

Could the next proposed evolutionary step displayed by a laboulbenialean ancestor have been the switch from a two-host system to a single host, the insect host as is suggested by the tree (FIG. 19)? There are two factors that are important to consider in this regard, the advantage of life cycle simplification and the hazards of a patchy habitat. Wulff (1985) suggested that there are advantages to the simplification of life cycles of mutualistic organisms beneficial to both associates that extend beyond the postulated value of loss of sexuality for maintaining a stable successful phenotype. Although the two-host system we envision for *Pyxidiophora* appears to be successful, it requires constant fine-tuning to, not only one, but two hosts. A less complex life cycle would seem to be an advantage, and, in fact, David Malloch (pers. comm.) pointed out that this change is analagous to that of rust fungi thought to be derived from heteroecious species by a shift to the nonsexual stage host. This is the phenomenon known as Tranzschel's Law. Although in some fungus–arthropod relationships asexual reproduction is retained with the loss of sexual reproduction, in these cases the fungus usually serves as a source of nutrition or overcomes defenses of a plant host for the arthropod. However, in *Pyxidiophora* and the Laboulbeniales the strong selection maintaining the sexual, dispersal stage is at the expense of an asexual stage that is not known to be of benefit to any of the associated organisms.

In addition to the fine-tuning that must be maintained with a two-host system, a patchy habitat (the quickly decomposing substrate) is increased in patchiness because an appropiate host fungus must be present in the substrate. The complete shift to the arthropod host would certainly free a *Pyxidiophora*-like fungus to accompany a straying disperser into uncharted territory where the fungal host might not be available. In fact some of the arthropods associated with *Pyxidiophora* today are known to stray from the usual substrates on occasion. Once confined to the arthropod host the sexually reproducing fungus could become isolated with its host to diverge with far fewer constraints. An arthropod surface might be seen as a patchy substrate, but as long as it is alive, rather long-lived past its last molt, and maintains contact with others of the same or even different species, the habitat becomes more or less continuous. These criteria are met by many hosts of the Laboulbeniales. Although about 1800 laboulbenialean species have been described, there are certainly many more to be discovered, as such a model would predict.

Because *Pyxidiophora* and *Rickia* unexpectedly occur outside of the large clade of perithecial ascomycetes, we erred in our prediction of their close relatives and this factor has affected the taxon sampling for DNA sequencing. As a result the nearest relatives of the fungi of greatest interest are not known, presenting an obstacle to providing a better model of evolution (FIG. 21). But with the development of a robust phylogenetic hypothesis we eventually can begin to look more earnestly for evidence to support these ideas or new ones about the modes and mechanisms of fungal evolution.

It is difficult always to exclude *means* from the motives attributed to the model just discussed above and the analogy becomes a little forced. However, the means by which the events occurred may be due to the action of selection on the particular fungal genome for dispersal characteristics. In the case of the minute thalli, *Pyxidiophora,* and the Laboulbeniales, the attachment organ is unique. Few other arthropod-borne fungi have the high degree of morphological differentiation for dispersal known in these species. It is possible that arthropod behavior and physiology select the fungus phenotype leading to cooccurrence and the possibility of isolation. Cassar (1993) suggested that ambrosia beetles may play a role in the selection and isolation of their *Ambrosiella* mycangial associates for their own benefit; these symbioses probably began with advantage primarily for the beetle. No benefit to the arthropods from *Pyxidiophora* or the Laboulbeniales is known; however, the outcome may be similar because of strong selection for dependable dispersal.

And, finally we come to *opportunity.* At the end of this story it would seem that the red algae in the sea were the red herrings.[2] Not only are secretive fungal saprobes hidden in humus, but the arthropods are there as well. There is a Silurian report of ascomycetes associated with microarthropod frass (Sherwood-Pike and Gray, 1985) that would suggest that the two groups of organisms have interacted in the same habitats for a very long time. However, Berbee and Taylor (1993) have used DNA sequence divergence to suggest that ascomycetes appeared later than the Silurian period. Although the time of ascomycete appearance is not settled, the abundance of both fungi and arthropods and their intimate contact in almost any habitat known to mycologists is evidence that whatever the length of time, there was ample opportunity for these organisms to exert a profound influence on each other. In some cases the influence rivals that of pollinators upon flowering plants.

[2] "The man in the wilderness asked of me/How many strawberries grow in the sea./I answered him as I thought good,/'As many as red herrings grow in the wood.'" Anonymous nursery rhyme.

ACKNOWLEDGMENTS

My list of acknowledgments is long because I am fortunate to have had much help and support as a fungal systematist. My first lessons in taxonomy came from my father, Robert E. May, who molded my taxonomic eye at an early age on the Bayou Vermillion. Not only did I learn the survival skills to distinguish poisonous snakes and poison ivy, but species of oak as well. From him I also heard the story of Louisiana iris introgression based on his collecting experience, even before Edgar Anderson was to publish his work. C. J. Alexopoulos introduced me to slime molds and fungi. In addition, he gave his students lessons on the importance of broad training, use of new techniques, and hard work (by example). He also gave us a great deal of freedom so that we could function later independently. James W. Kimbrough, my post-doctoral advisor, first introduced me to the wonderful world of arthropod-associated fungi and gave me guidance as well as independence.

From Robert L. Gilbertson I have had a continuing education on the ways of fungi. He stressed the importance of field observation and thorough knowledge of the complete fungus in nature and in culture. Some of my younger mentors have been John W. Taylor and Joseph W. Spatafora, who taught me new techniques that have provided additional ways of pursuing important questions about fungi. Steven C. Cassar and Kevin G. Jones continue this phase of my education, and I am forever indebted to them for helping to obtain the sequence of *Rickia* used in the analysis presented here. Still younger are the undergraduates in my lab, Brandye E. Sawyer and Hester Johnson, who also teach me as they, perhaps, become the newest generation of mycologists.

A number of other people have been helpful especially in the work that has been summarized here. They are those with many years of experience in ascomycetes and arthropod-associated fungi: G. Arnold, Margaret Barr, Richard K. Benjamin, Thomas Harrington, D. L. Hawksworth, Robert W. Lichtwardt, N. Lundqvist, E. S. Luttrell, Walter Rossi, Isabel I. Tavares, and J. Webster. J.R. Bridges, J.C. Moser, and T.J. Perry literally opened the door for me (FIG. 16). All have given freely of the greatest resource of all, their broad experience. However, the person who has helped most in this study is David Malloch with whom I have shared the excitement of revelation as each secret in the lives of these fungi was divulged.

I want to draw attention to the importance of collections such as the National History Museum (London), that provides me with arthropod collections, and the Farlow Herbarium, that Donald Pfister has made available for my use on numerous occasions. Also maintained at great expense are the culture collections of the world with their catalogues now available on my lab computer. Field research stations with adequate facilities are essential for studying organisms. The Huntsman Marine Centre, St. Andrews, New Brunswick, and the Wildlife Research Station, Algonquin Provincial Park, Ontario, were sites of much of the field work reported here. These are all expensive resources and we must be vigilant to ensure that they are maintained.

An important aspect of scientific life is the close camaraderie and friendly competition of colleagues. My fellow graduate students in Dr. Alexopoulos' lab fulfilled this requirement as the members of the Department of Botany and the Systematics and Evolutionary Biology Group at Louisiana State University do today. I owe a special debt to the Mycological Society of America. It is a society that welcomed me as a student member, provided me with colleagues and friends, gave me a place to publish results, and encouraged me to be of service. For this I thank you all. My activities in the MSA have given me pleasure as well as help in receiving academic tenure twice and promotions three times! It is a society I can heartily recommend to those beginning a career.

And finally, I have been fortunate to have had financial support for much of the work discussed here from the National Science Foundation (BSR-8918157 and DEB-9208027) and the Louisiana Education Quality Support Fund (NSF-LASER-EPSCoR 92-96-ADP-02). While the money is important, it is also the views of the stringent reviewers who help to expand my horizons to encompass a wider view of systematics.

I thank Thomas D. Bruns, Steven C. Cassar, Russell L. Chapman, Kevin G. Jones, and David J. McLaughlin for their many helpful comments on the manuscript.

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