

# The Ecology of Fungal Entomopathogens

Helen Roy · Fernando Vega · Dave Chandler ·  
Mark Goettel · Judith Pell · Eric Wajnberg  
Editors

# The Ecology of Fungal Entomopathogens

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*Editors*

Helen E. Roy  
CEH Wallingford  
Biological Records Centre  
Crowmarsh Gifford  
Wallingford, Oxon  
United Kingdom OX 10 8BB  
hele@ceh.as.uk

Fernando E. Vega  
USDA - ARS  
Plant Sciences Institute &  
Invasive Insect Biocontrol  
Beltsville MD 20705  
Bldg. 011A, BARC-West  
USA

Dave Chandler  
Warwick HRI, University of Warwick  
Wellesbourne  
CV35 9EF Warwick  
United Kingdom

Mark S. Goettel  
Agriculture & Agri-Food Canada  
Lethbridge AB T1J 4B1  
Canada

Judith Pell  
Rothamsted Research  
AL5 2JQ Harpenden  
United Kingdom

Eric Wajnberg  
Institut National de la Recherche  
Agronomique (INRA)  
400 route des Chappes  
06903 Sophia Antipolis CX  
France  
wajnberg@sophia.inra.fr

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*Cover illustration:* Scatophaga – Photograph by Jørgen Eilenberg

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# Deep space and hidden depths: understanding the evolution and ecology of fungal entomopathogens

Helen E. Roy · Eoin L. Brodie · Dave Chandler ·  
Mark S. Goettel · Judith K. Pell ·  
Eric Wajnberg · Fernando E. Vega

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**Abstract** Entomopathogens are important natural enemies of many insect and mite species and as such have been recognised as providing an important ecosystem service. Indeed, fungal entomopathogens have been widely investigated as biological control agents of pest insects in attempts to improve the sustainability of crop protection. However, even though our understanding of the ecology of fungal entomopathogens has vastly increased since the early 1800s, we still require in-depth ecological research that can expand our scientific horizons in a manner that facilitates widespread adoption of these organisms as efficient biological control agents. Fungal entomopathogens have evolved some intricate interactions

with arthropods, plants and other microorganisms. The full importance and complexity of these relationships is only just becoming apparent. It is important to shift our thinking from conventional biological control, to an understanding of an as yet unknown “deep space”. The use of molecular techniques and phylogenetic analyses have helped us move in this direction, and have provided important insights on fungal relationships. Nevertheless, new techniques such as the PhyloChip and pyrosequencing might help us see beyond the familiar fields, into areas that could help us forge a new understanding of the ecology of fungal entomopathogens.

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H. E. Roy (✉)  
NERC Centre for Ecology & Hydrology, Wallingford,  
Oxfordshire OX10 8BB, UK  
e-mail: Hele@ceh.ac.uk

E. L. Brodie  
Ecology Department, Earth Sciences Division, Lawrence  
Berkeley National Laboratory, Berkeley, CA 94720, USA

D. Chandler  
Warwick HRI, University of Warwick, Wellesbourne,  
Warwick CV35 9EF, UK

M. S. Goettel  
Agriculture and Agri-Food Canada, Lethbridge Research  
Centre, 5403-1 Avenue South, P.O. Box 3000,  
Lethbridge, AB T1J 4B1, Canada

J. K. Pell  
Department of Plant and Invertebrate Ecology,  
Rothamsted Research, Harpenden, Hertfordshire AL5  
2JQ, UK

E. Wajnberg  
INRA, 400 Route des Chappes, BP 167, 06903 Sophia  
Antipolis Cedex, France

F. E. Vega  
Sustainable Perennial Crops Laboratory, United States  
Department of Agriculture, Agricultural Research  
Service, Building 001, BARC-West Beltsville, MD  
20705, USA

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*Fungi have a profound impact on global ecosystems. They modify our habitats and are essential for many ecosystem functions*  
Blackwell et al. (2006).

It has been estimated that the Kingdom Fungi consists of 1.5 million species (Hawksworth 2001; Mueller and Schmit 2007; Schmit and Mueller 2007), with approximately 110,000 described species (Kirk et al. 2008). Of these, 700 species in 90 genera are recognized as insect pathogens (Roberts and Humber 1981), and approximately 170 pest control products have been developed based on at least 12 species of fungal entomopathogens (de Faria and Wraight 2007). Undoubtedly, fungal entomopathogens are important natural enemies of many insect and mite species and as such, provide an important ecosystem service contributing to pest control with minimal detectable negative effects on the environment (Vestergaard et al. 2003). However, the small subset of fungi developed as biological control agents have had limited success. Our ability to employ them effectively and reliably for pest control in the field has not matched up to expectations (Vestergaard et al. 2003; Chandler et al. 2008; Vega et al. 2009). In part, this may be because of variable and unpredictable levels of efficacy compared to chemical pesticides (Waage 1997; Vega et al. 2009), but we also lack some basic understanding of their ecology and evolution (Vega et al. 2009).

The importance of basic knowledge, theory and predictive ability in the use of biological control agents has been recognised for some time (Gurr et al. 2000). However, the dearth of basic information on fungal entomopathogens is pronounced even though these organisms have historically dominated the field of microbial control (Lord 2005). Vega et al. (2009) have proposed the need for “a new paradigm for fungal entomopathogens that should refocus our efforts and hopefully lead to exciting new findings.” In this special issue of BioControl we report on some of the latest research, innovations and ideas relating to fungal entomopathogens within an ecological context.

One of the most significant challenges facing insect pathologists is to understand the evolutionary history and relationships amongst fungal entomopathogens. Intricate interactions with arthropods, plants and other microorganisms are evident, but the full importance and complexity of these relationships is just becoming apparent. The advent of new molecular tools over the last few decades has dramatically improved the resolution of fungal systematics and there have been huge advances in this field (Blackwell et al. 2006; Hibbett et al. 2007; Humber 2008; Blackwell 2009; Enkerli and Widmer 2009). The acquisition of a phylogeny enables us to examine evolutionary relationships and better understand and predict ecological interactions (Blackwell 2009). Molecular tools will provide methods for examining the host-pathogen dynamics in complex environments (Enkerli and Widmer 2009). Enkerli and Widmer (2009) comprehensively review the tools available within the context of population ecology studies.

Meyling and Hajek (2009) provide an excellent background to ecological interactions relevant to fungal entomopathogens from a community ecology perspective. An ecological context is important for increasing our empirical understanding of host-parasite interactions and improving the efficacy of these microbes as biological control agents. Fungal entomopathogens often exist as patches in a spatially heterogeneous matrix (Rodríguez and Torres-Sorando 2001) and metapopulation dynamics could be particularly pertinent to describing these spatially distinct populations that are connected by dispersal. Meyling and Hajek (2009) describe how insects and their fungal pathogens could be used as model species for exploring metapopulation theory using experimental and predictive models.

In recent years there have been intriguing advances in our appreciation of the role of fungal entomopathogens beyond their applied role as biological control agents of insects. Pathogens have traditionally been neglected in life history studies and often considered as having negligible impact (Hawkins et al. 1997). Hesketh et al. (2009) review the role of fungal entomopathogens as natural enemies of insects in semi-natural habitats, describing the theoretical host-pathogen models available to examine their role in population regulation. The need to consider the complexity, and particularly the heterogeneity, of semi-natural habitats within the context

of theoretical models and as a framework for empirical studies is highlighted. However, Hesketh et al. (2009) acknowledge that fundamental gaps in our understanding of fungal entomopathogens from an ecological perspective, coupled with a lack of empirical data to test theoretical predictions, is impeding progress.

Ecological understanding has never been more vital than in this period of unprecedented environmental change:

Changes in biodiversity due to human activities were more rapid in the past 50 years than at any time in human history, and the drivers of change that cause biodiversity loss and lead to changes in ecosystem services are either steady, show no evidence of declining over time, or are increasing in intensity (Millennium Ecosystem Assessment 2005).

Many studies on the effects of the major drivers of biodiversity loss (habitat destruction, invasive species, exploitation, climate change and pollution) involve just one trophic level and often just one species. Fungal entomopathogens provide an additional trophic level that should be included in such studies, particularly in relation to climate change and habitat destruction (Roy and Cottrell 2008; Roy et al. 2009). Cory and Ericsson (2009) review the literature on tri-trophic interactions encompassing fungal entomopathogens. The promising roles of plant volatiles and plant surface chemistry on ecological interactions between host insects and their pathogenic fungi are described. Although intriguing concepts such as the “bodyguard hypothesis” have been examined and demonstrated for natural enemies such as parasitoids and predators, there is a lack of empirical evidence for this in fungal entomopathogens. This is likely because there has simply been limited research in this field. Cory and Ericsson (2009) assess the relevance of plant-mediated effects on fungal entomopathogens and urge researchers to focus work on the considerable gaps in knowledge concerning fungal entomopathogens and tritrophic interactions.

Behavioural ecology will be critical in the exploration of tritrophic interactions. Baverstock et al. (2009) provide a review of fungal entomopathogens and insect behaviour. The behavioural response of an insect to a fungal pathogen will not only have a direct effect on the efficacy of the fungus as a biological

control agent but also provide us with a model system for understanding interactions within guilds. Simple laboratory bioassays can provide a measure of insect mortality in the presence of a pathogen but experiments designed to include elements of spatial complexity are critical to improving accuracy of predictions. The papers reviewed by Baverstock et al. (2009) demonstrate this and reveal manipulations of host behaviour induced by fungi and countermeasures employed by the host (Roy et al. 2006). The often complex interactions between fungus and host are being unravelled through eloquent research and the importance of these often subtle behavioural modifications in determining the success or failure of biological control cannot be underplayed.

The opportunities and challenges provided by the soil environment, and specifically the rhizosphere, have long been recognised (Vega et al. 2009) but only now are the subtleties slowly being revealed (Bruck 2009). There is no doubt that the ecology of fungal entomopathogens in the rhizosphere is a neglected area of research within insect pathology. A better understanding of their ecology in the rhizosphere will not only help in the development of successful microbial control strategies against root-feeding insect pests, but is also certain to reveal intriguing insights into the subterranean “hidden depths” of fungal entomopathogens.

Ownley et al. (2009) review the ecology and evolution of fungal entomopathogens as antagonists of plant pathogens. Simultaneous biological control of both insect pests and plant pathogens has been reported for the hypocrealean fungal entomopathogens, *Beauveria bassiana* and *Lecanicillium* spp. and accumulating evidence shows that *Beauveria* spp. can colonize a wide array of plant species endophytically. Furthermore, traits that are important for insect pathogenicity are also involved in pathogenicity to phytopathogens.

From 1845 to 1916, Elie Metchnikoff assessed an insect disease of wheatchafers later identified as the hypocrealean fungus *Metarhizium anisopliae* (Lord 2005). These early studies inspired many to focus their research on assessing the potential of fungal entomopathogens as microbial control agents. A series of papers in this special issue of BioControl explore advances in their use for biological control of pest insects. Jackson et al. (2009) eloquently describe the importance of linking ecology with formulation and

production of fungal entomopathogens for biological control. The commercial drivers of formulation (maximising yield, storage stability and ease of application) are often in conflict with ecological considerations. However, efficacy can be improved dramatically by considering ecological factors such as the importance of environmental conditions on the host-pathogen interaction (Jackson et al. 2009).

Biological control strategies include classical, inundative augmentation and conservation approaches. Hajek and Delalibera (2009) examine the use of fungal entomopathogens in classical biological control and conclude that they have been used more frequently than other types of pathogens and provide a sustainable avenue for controlling arthropod pests, especially the increasing numbers of invasive species. Inundative biological control strategies rely on the released organism exerting control without subsequent transmission and reproduction in a similar way to a synthetic pesticide; the chemical paradigm. Jaronski (2009) aptly demonstrates the drawbacks of taking this approach in isolation with fungal entomopathogens. In most cases, effective application of sufficient inoculum to rapidly reduce pest numbers to below economic threshold levels is financially and logistically prohibitive. Biotic, abiotic and economic realities certainly restrict such an approach in most field situations although there have been some notable successes in controlling pest insects in glasshouses. Through a better understanding of the ecology of fungal entomopathogens and the dynamics of the pest, crop and environment, it may be possible to employ inundative application of fungi within ecologically based integrated pest management systems. However, it will be imperative that such strategies encompass the complex and multifaceted interactions that the released organism must contend with. The review on conservation biological control by Pell et al. (2009) explores the novel ways in which fungal entomopathogens can be enhanced in the environment. Understanding the dynamics of fungal entomopathogens at the field and landscape scale is imperative for implementing conservation biological strategies. There have been a number of eloquent studies demonstrating the potential of such an approach and these are comprehensively reviewed by Pell et al. (2009).

The realm of ecology is vast and deciphering insect-fungal pathogen interactions within an

ecological context will take us on voyages beyond our imagination. New and innovative methods will provide the inspiration to explore the hidden depths and deep space of these interactions. The PhyloChip microarray hybridization technique might point at what the future holds for mycological research. At present, the PhyloChip allows for the identification of bacterial and archaeal organisms using 16S rRNA-targeted oligonucleotide microarrays (Brodie et al. 2007; DeSantis et al. 2007). The method takes advantage of the variation in the 16S rRNA gene to capture the broad range of microbial diversity that may be present in a given sample, without the need for microbial cultivation. This high-throughput technique makes it possible to identify overall microbial diversity, and combined with dissection of specific insect tissues (e.g., foregut, midgut, hindgut), determine microbial communities in these tissues. A version is currently being developed for the analysis of fungal community diversity. Similarly, sequencing technologies such as 454-pyrosequencing now permit large numbers of shorter sequences (pyrotags) to be obtained from a large number of samples by employing sequence barcoding techniques (Hamady et al. 2008). These approaches allow deeper profiling of complex microbial communities from the deep-sea (Sogin et al. 2006) to the gut microbiota of humans and 59 other mammals (Ley et al. 2008). Greif and Currah (2007) have shown that fungal entomopathogens are common components of the surface mycota of arthropods, and that they are not necessarily restricted to diseased insects. Once a microarray technique similar to the PhyloChip or pyrotag sequencing has been developed for fungal entomopathogens, what would their uses reveal in insects? Will fungal entomopathogens be found to be common inhabitants of the cuticle of uninfected insects? Could they also be common internal inhabitants of uninfected insects? Furthermore, using microarray techniques for sampling fungal entomopathogens as plant endophytes might reveal that they are much more common and globally distributed than is presently thought. Would the same situation occur in the rhizosphere? If the answer to any of these questions were positive, what would this imply for our understanding of fungal entomopathogens?

There might be a “deep space” that will only be revealed when we start to decipher the myriad fungal inhabitants in insects and plants, which at present



remain in “hidden depths”. The importance of these interactions has been superbly described by Berenbaum and Eisner (2008):

There is no limit to what remains to be discovered in that interactive zone between macroorganism and microbe, where so many biological mutualisms and antagonisms play out. Microbes blanket the planet, and in their infinite variety they must be involved in infinite interactions. Deciphering these could lead to a vast increase in ecological knowledge, as well as to the isolation of natural products of unforeseen function.

Let the adventure begin!

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## Author Biographies

**Helen E. Roy** leads zoological research in the Biological Records Centre at the NERC Centre for Ecology & Hydrology (UK). The focus of her research is insect community interactions with particular emphasis on the effects of environmental change. She is an associate editor of *BioControl*.

**Eoin L. Brodie** develops and applies culture independent approaches to analyze microbial communities and conducts research on climate change impacts on their structure and function.

**Dave Chandler** is an insect pathologist at the University of Warwick, UK. He has studied entomopathogenic fungi for just over 20 years. He has particular interests in entomopathogenic fungi as biocontrol agents of horticultural crops, fungal physiology and ecology, and the pathogens of honeybees.

**Mark S. Goettel** is an insect pathologist at the Lethbridge Research Centre of Agriculture & Agri-Food Canada, specializing in the development of entomopathogenic fungi as microbial control agents of insects. In addition to this research, he has been extensively involved in the review and revision of the regulations for registration of microbial control agents and has addressed regulatory and safety issues at the international level. He is currently President of the Society for Invertebrate Pathology and has been Editor-in-Chief of *Biocontrol Science & Technology* since 2000.

**Judith K. Pell** heads the Insect Pathology Group in the Department for Plant and Invertebrate Ecology at Rothamsted Research, UK. She leads research on the ecology of entomopathogenic fungi, to elucidate their role in population regulation and community structure and to inform biological control strategies. Specifically: intraguild interactions; the relationships between guild diversity, habitat diversity and ecosystem function; pathogen-induced host behavioural change.

**Eric Wajnberg** is a population biologist specialised in behavioural ecology, statistical modelling and population genetics. He is also an expert in biological control, with more than 20 years experience of working with insect parasitoids. He has been the Editor in Chief of *BioControl* since 2006.

**Fernando E. Vega** is an entomologist with the United States Department of Agriculture, Agricultural Research Service, in Beltsville, Maryland, USA. He conducts research on biological methods to control the coffee berry borer, the most important insect pest of coffee throughout the world. He is co-editor, with Meredith Blackwell, of *Insect-Fungal Associations: Ecology and Evolution*, published by Oxford University Press in 2005, and serves as an Editorial Board Member for *Fungal Ecology*.

# Fungal evolution and taxonomy

Meredith Blackwell

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**Abstract** Fungi and insects are closely associated in many terrestrial and some aquatic habitats. In addition to the pathogenic associations, many more interactions involve fungal spore dispersal. Recent advances in the study of insect-associated fungi have come from phylogenetic analyses with increased taxon sampling and additional DNA loci. In addition to providing stable phylogenies, some molecular studies have begun to unravel problems of dating of evolutionary events, convergent evolution and host switching. These studies also enlighten our understanding of fungal ecology and the development of organismal interactions. Mycologists continue to rely heavily, however, on identified specimens based on morphology to incorporate more of the estimated 1.5 million species of fungi in phylogenetic studies.

**Keywords** Insect fungi · Fungal phylogeny · Hypocreales

## Introduction

In the eighteenth century Otto von Munchhausen, a contemporary of Linnaeus, determined the nature of

fungi: his observations lead him to believe that fungi were the dwellings of animals (Findlay 1982). Mycologists now understand the organismal nature of an estimated 1.5 million species of fungi, and they also have learned much more about the associations between fungi and animals, especially insects, occurring in habitats they share. We have confirmed that some fungi are indeed the houses and sustenance of animals. We also have found that fungi sometimes live within insects and other arthropods. Insects also are essential to carry fungi from depleted substrate to a new home (Figs. 1, 2).

There are many interactions between fungi and insects ranging from transient to obligate associations, some of which kill insects, but a large number that benefit either the insect or the fungus or in which the benefit is reciprocal. Among basidiomycetes there are classic examples of farming interactions in which Old World termites cultivate a monophyletic group of fungi and New World leaf-cutting ants cultivate two distinct cultivar groups (Currie et al. 2003; Munkacsy et al. 2004; Little and Currie 2008). Other basidiomycetes (e.g., species of *Septobasidium*) parasitize scale insects, although most of the scales in the colony are protected from insect parasites within chambers of the fungal thallus (Henk and Vilgalys 2007). Many insects are adapted for living their entire lives within the fruiting bodies of basidiomycetes, where they ingest the tissue and reproduce, leaving only to find a fresh fungus when the old substrate is depleted. In addition various basidiomycetes are adapted for dispersal by insects.

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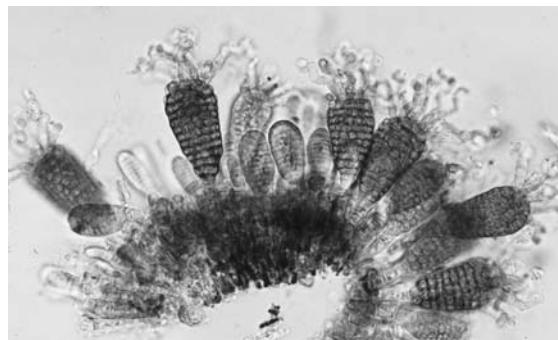
M. Blackwell (✉)  
Department of Biological Sciences, Louisiana State  
University, Baton Rouge, LA 70803, USA  
e-mail: mblackwell@lsu.edu



**Fig. 1** The capilliconidium of *Basidiobolus ranarum*, once placed in an unclassified genus, *Amphoromorpha*, is attached to an insect seta by a darkened attachment region. Although *B. ranarum* also has more obvious forcibly discharged spores, insect dispersal is important in the life cycle of the fungus, as evidenced by the development of the fungus from the excrement of many insect-eating amphibians. Stained with glycerol acid fuchsin. Source: Weir and Blackwell (2005)



**Fig. 2** Stinkhorns such as *Mutinus* sp. are adapted for insect dispersal. Their fetid odors attract flies and other insects to the slimy slurry of spores. Many spores adhere to the insect body and later are deposited in habitats favorable for germination. Photo: Nhu Nguyen

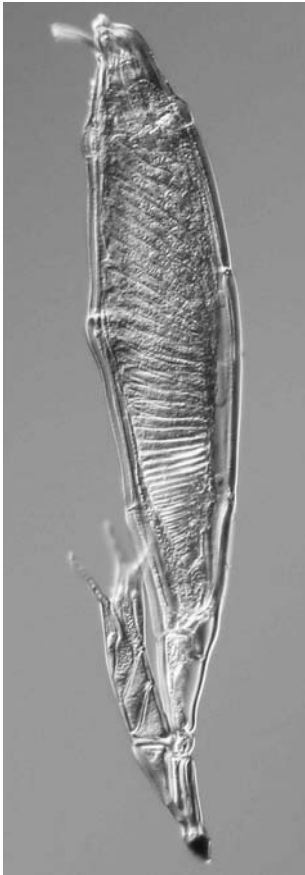


**Fig. 3** Insects may be hosts for small poorly known fungi such as *Muiogone medusae*, probably an asexual ascomycete. Source: Weir and Blackwell (2005)

Ascomycete associations with arthropods include numerous dispersal and fertilization interactions. There also are farming interactions between bark beetles and ascomycetes in several orders. Some ascomycetes parasitize insects and other arthropods (Figs. 3, 4), and some of the virulent pathogens are discussed throughout this special issue. Ascomycetes, especially yeasts, are found in the insect gut, sometimes as endosymbionts in special compartments, and the yeasts may detoxify plant materials or provide enzymes to attack plant cell walls that are intractable to the insects (Vega and Dowd 2005). This is a powerful interaction that allows insects to move into habitats they otherwise could not utilize at so little genetic expense.

In the past, mycologists relied heavily on morphological characters to suggest relationships among fungi, although in the case of insect-associated fungi, morphology often has been the result of convergent evolution. The use of DNA characters has helped to improve and stabilize our understanding of fungal relationships and to develop a phylogenetic classification to the level of order (Hibbett et al. 2007). This work has progressed from phylogenetic trees based on analyses of a partial gene to multiple genes to entire genomes. The new classification (Hibbett et al. 2007) is anticipated to remain stable because it is based on a multiple locus phylogeny and increased taxon sampling rather than subjective opinions based on few, often plastic, morphological characters. Figure 5 provides a skeletal outline of major taxonomic groups in the new classification. This is an ongoing process, and new lineages and taxa have already been added to the classification because fungi

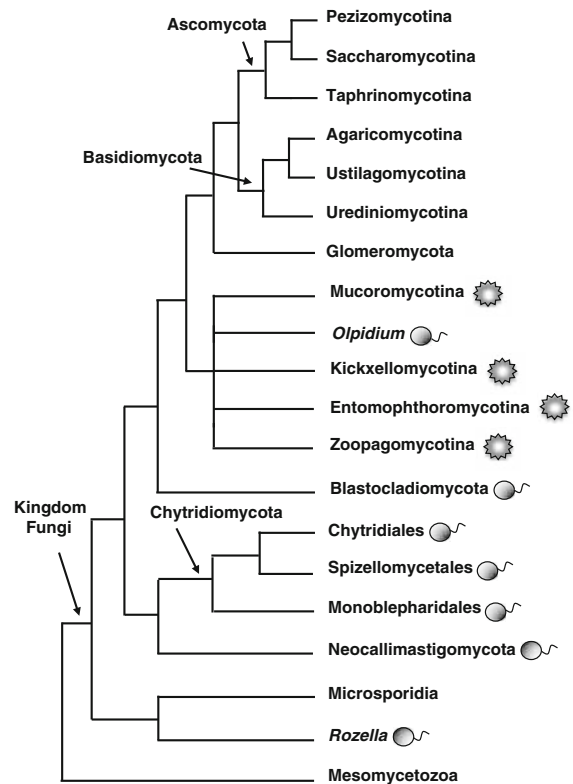




**Fig. 4** *Prolixandromyces triandrus* (Laboulbeniales) is one member of a speciose group of ascomycetes that are obligate biotrophic ectoparasites of arthropods. The lack of a germ tube and determinate growth of the thallus are derived characters that set the group apart from all other fungi. One species originally was described as a parasitic worm of nycterid bat flies. Photo: Alex Weir

remain under-sampled (Blackwell et al. 2006; Hibbett et al. 2007). For example, recently discovered fungal diversity includes a relatively large ascomycete lineage, Soil Clone Group 1, that has been identified only from environmental samples. This clade is known from widely spaced localities including several sites in northern Europe and North America (Schadt et al. 2003; Vandenkoornhuysen et al. 2002). It is now possible to detect fungi we have never seen as well as those that are not culturable.

Molecular techniques have revolutionized the study of insect-associated fungi. For example, asexual morphs of fungi, many of which are insect pathogens, previously were placed as form genera in groups such as deuteromycota or fungi imperfecti,



**Fig. 5** The tree presents the major groups of fungi in the new classification (Hibbett et al. 2007; White et al. 2006). The best-known fungal parasites of insects are classified in Pezizomycotina, sometimes called filamentous ascomycetes (see Fig. 6 and text for more detail). Note the greater diversity of zoosporic (indicated by flagellate cell diagram) and zygosporic (indicated by zygosporangium diagram) fungal groups than previously appreciated (e.g., Alexopoulos et al. 1996). The flagellum appears to have been lost on more than one occasion

based solely on artificial morphological characteristics. We now can place the asexual fungi among their nearest sexual relatives, and terms including deuteromycota have been abandoned completely (Blackwell et al. 2006; Hibbett et al. 2007). Just as exciting is the identification of some insect fungi that only recently have been determined for the first time (e.g., attine ant associates and Laboulbeniales). Identification of the species involved in the attine associations, in particular, has renewed interest in the system and has allowed for population studies, determination of the approximate age of the interactions, and new evolutionary perspectives. The new phylogenetic information on many fungi has revolutionized our understanding of the associations between fungi and other organisms. Now we not only recognize

individual fungi in all their guises, but we can track common evolutionary histories of all the participants in the associations.

### Past fungal-insect associations

In addition to fungal fossil evidence, molecular clock divergence rates provide estimates of the timing of historical interactions between insects and fungi. Because the fungal fossil record is scanty, the DNA estimates will give earlier dates than fossils, and these dates are dependent on the calibration points used in estimating ages of certain fungal lineages. Based on molecules, fungi are estimated to have at least a billion year history on earth (Matheny et al. 2009; Taylor and Berbee 2006). Fossils provide evidence only of more recent fungal activities because fungi are small and often ephemeral. It is possible, however, to discover fossil fungi, especially when the hosts and specialized substrates are targeted, and these serve as the essential reference points for calibration.

There is evidence that well-developed fungal communities were present in the Devonian (416–359 million years ago (Ma)) with assemblages comprised of several phyla already associated with vascular plants in the Rhynie Chert (400 Ma) of Scotland. The Rhynie fungi have been recognized as chytrids, Glomeromycota and Ascomycota. Fungal diversity grew rapidly with the increase in terrestrial environments. The rise of insects began somewhat later than that of fungi with the appearance of the first wingless insects in the Devonian. The Carboniferous Period (359–299 Ma) witnessed the diversification of plants and winged insects as well as fungi, and these primarily flourished on the land. The first mushroom fossil was relatively late in the fossil record, appearing only about 90 Ma in the Cretaceous (145.5–65.5 Ma). Winged insect fossils were found in Devonian deposits, and some groups such as cockroaches, associated with certain fungi today, were present in late Paleozoic (before 251 Ma) and into the early Mesozoic (after 251 Ma), when fossils of most modern insect orders appeared. Some of these insects, including roaches, termites, dung beetles and wood wasps are closely associated with fungi today (Blackwell 2000).

There is little early fossil evidence of fungus–arthropod associations, but several such associations

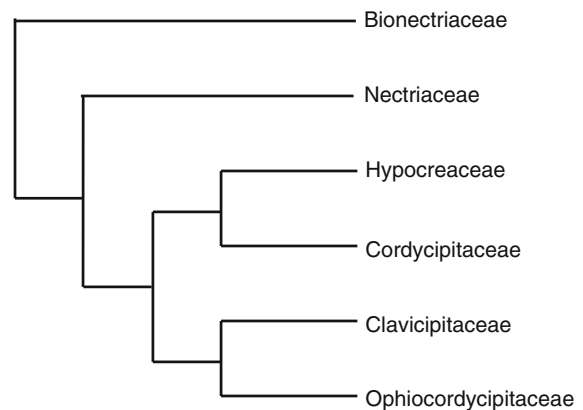
have been reported from the Carboniferous. White and Taylor (1989) reported an eccrinid trichomycete (eccrinids are now considered to be members of the Mesomycetozoa rather than fungi) in Late Carboniferous associated with what was assumed to be the gut lining of an arthropod. Trace fossils of millipedes, common hosts of eccrinids today, were first reported in Late Ordovician (488–444 Ma). This report, however, has not been confirmed. Many ephemeral fungi and associated insects have been preserved in amber. A coral fungus with a sand fly larva (Diptera: Psychodidae) preserved in early Cretaceous amber (ca. 100 Ma) suggests that the flies may have been feeding on the fungus (Poinar et al. 2006). Cretaceous amber also yielded an *Ophiocordyceps*-like anamorph associated with scale insects, corresponding well with a hypothesized Jurassic origin of *Cordyceps*-like fungi based on DNA divergence rates (Sung et al. 2008; Nikoh and Fukatsu 2000). Other insect-associated fungi, including a laboulbeniomyceete on a dipteran, have been discovered by targeting amber-preserved insects that are known hosts of extant fungi (Rossi et al. 2005). The laboulbeniomyceete is from Baltic amber (55–35 Ma) that was later redeposited (22 Ma). Younger Dominican Republic amber has yielded a number of insect pathogens such as Entomophthorales on a termite, *Beauveria* on a worker ant and a *Mucor*-like fungus on a walking stick (Poinar 1992).

In cases where there are good calibration points as in fossils of fungus-eating insects, phylogenetic studies can provide information on the history of insect–fungus associations. For example, many extant beetles are closely associated with fungi for habitats and nutritional resources. A molecular study aimed at discerning the history of lifestyle preferences of Coleoptera suggested that beetles evolved about 285 Ma (Hunt et al. 2007) with at least 15 independent origins of fungus feeding (e.g., certain clades of Staphylinidae, Scarabaeoidea, Leiodidae). A major shift to fungal feeding by speciose cucujoid beetles is estimated to have occurred about 236 Ma and represents a relatively derived condition with some reversals (e.g., Chrysomeloidea, Curculionoidea; Hunt et al. 2007). One might suspect that fossil mushrooms would have damage from browsing insects, but this is not the case for the few early mushroom fossils known (D. Hibbett, personal communication 2009).

## Phylogeny and phylogenetic classification

The simple morphology and heterotrophic nutrition of fungi was used for many years as the basic criteria for their identification. These traits, however, did not distinguish fungi from other groups of organisms with a similar ecology. It has taken several hundred years to refine a classification of fungi by searching out characters from life cycles, biochemical pathways and ultrastructural anatomy. Characters such as site of meiosis in the life cycle, flagellation, cell wall carbohydrate, mitochondrial structure and pathway of lysine synthesis were used to define a monophyletic kingdom *Fungi* (Alexopoulos et al. 1996). Although these characters served to separate fungi from unrelated organisms [e.g., slime molds (*Myxomycetes*) and water molds (*Oomycota*)], these “all or none” characters did not allow mycologists to group the organisms based on their similarities. Eventually, the use of rDNA overcame this deficit and brought the advantage of large numbers of characters that could be analyzed by phylogenetic methods to answer questions about evolutionary pathways. The non-photosynthetic *Oomycetes*, such as *Lagenidium giganteum*, important in attempts to control aquatic insect larvae (Kerwin and Petersen 1997), are now grouped as straminipiles with brown algae and other photosynthetic protists that contain chlorophylls *a* and *c*. Other organisms previously considered to be fungi also have been excluded from the kingdom on the basis of DNA analyses, and these include two of the groups of arthropod-associated “trichomycetes” in the orders *Amoebidiales* and *Ecrrinales* that now are placed in the *Mesomycetozoa*, a group basal to fungi.

Improved molecular techniques and analysis methods and a dynamic community of mycologists came together in an effort to improve taxon sampling and acquire trees based on multiple alleles [see articles in *Mycologia* 98(6)]. The phylogenetic studies were the basis of a phylogenetic classification (Fig. 6) that has been widely accepted and to which more taxa are being added (Hibbett et al. 2007). Many mycologists worked to achieve the partially resolved tree upon which a phylogenetic classification to the level of order could be established. The tree is based on the best data available, often multiple DNA loci [see *Mycologia* 98(6) and <http://aftol.org/>] and sometimes whole genomes (Robbertse et al. 2006). Work on structural and biochemical characters continues so



**Fig. 6** Tree showing the relationships of families classified in Hypocreales (after Sung et al. 2007; <http://cordyceps.us/>). The order contains many insect pathogens and has been the subject of studies of interkingdom host switching (Spatafora et al. 2007)

that these characters can be integrated into other phylogenetic information (<http://aftol.org/>, especially <http://aftol.umn.edu/>). A number of characters are known to be useful at certain taxonomic levels, and include flagellar apparatus in flagellated fungi, septal pore plugs of *Agaricomycotina*, and type of membrane sterol in certain zygomycetes and basidiomycetes. A short overview of the major higher taxa recognized using molecular characters follows. See Humber (2008) for a more detailed discussion of the phylogenetic placement of insect-associated fungi.

### Basal fungi

Current fungal trees (White et al. 2006; Hibbett et al. 2007) show a greater diversity of early diverging fungi than was previously known, especially when the derived nonflagellated anaerobic microsporidian parasites are included. Early in the use of small subunit ribosomal RNA gene (SSU rDNA) sequences for phylogenetic analyses, *Microsporidia* were considered to be basal pre-mitochondrial eukaryotes. More recently, however, based on protein-coding genes, these vertebrate and insect parasites appear to be among basal fungal groups or just basal to fungi in phylogenetic trees. The phylogenetic position of microsporidians, near or within *Fungi*, is supported by the traits that indicate the derived condition of the group. If microsporidians are included in *Fungi*, they will stand with *Entomophthorales* as one of the few basal groups of fungi that have widespread

associations with arthropods. In the past, zoosporic fungi known as chytrids were considered members of a single phylum, and it was assumed that flagellation was lost on only one occasion. Based on analyses with additional genes and increased taxon sampling, the flagellated phylum Blastocladiomycota does not form a monophyletic group with other flagellated phyla (Chytridiomycota and Neocallimastigomycota), and flagellation appears to have been lost on more than one occasion. The Blastocladiomycota contains some parasites of aquatic insects. These fungi differ from all other true fungi because meiosis is sporic, resulting in an alternation of generations (diplobiontic life cycle) between a diploid sporothallus and a haploid gametothallus. The *Coelomomyces* relies on two different aquatic arthropod hosts to complete its alternating life cycle. This discovery helped to explain why it had been so difficult to reinfect mosquitoes in lab experiments (Whisler et al. 1974). In addition, the phylogenetic position of specialized, flagellated, intracellular parasites in two genera (*Rozella* and *Olpidium*) are not well resolved, and they lie outside other flagellated clades in current trees (Fig. 5).

Zygosporic fungi are not resolved as a monophyletic group, but can be placed informally in three or more clades (White et al. 2006; Hibbett et al. 2007). “Zygomycota I” contains a core group of mucoralean fungi. A related group, Mortierellales, has species sometimes associated with insects. “Zygomycota II,” contains a monophyletic group, the DKH clade consisting of Dimargaritales, Harpellales, Kickxellales and Zoopagales. The species in the DHK clade have septate hyphae characterized by distinctive septal pore plugs. These fungi are often parasitic or predaceous on invertebrate animals including insects and in some cases other fungi. Members of Harpellales are well known as gut inhabitants of arthropods. “Zygomycota III” consists of Entomophthorales. *Basidiobolus* (Fig. 1), a traditional member of the order is not included and the position of the genus is still not clear (Fig. 5). Many of these species are insect pathogens (*Entomophaga* and *Entomophthora*), some with strict specificity (*Massospora* and *Strongwellisia*). *Basidiobolus* has dispersal interactions with insects, and infections of mammals may result (Blackwell and Malloch 1989). The insect pathogenic aspect of Entomophthorales was discussed by Humber (2008).

## Glomeromycota

Members of the Glomeromycota are obligate arbuscular mycorrhizal (AM) fungi that are widespread associates of the roots of many plants. AM fungi were once considered to be zygomycetes, although they do not produce zygospores. One small group of species placed in Endogonales once were considered close relatives of AM fungi, but are classified in a clade with Mucorales and other zygosporic fungi (Fig. 5).

## Ascomycota

Among the Ascomycota, many previously proposed evolutionary scenarios have not been supported. The phylum is divided into three subphyla (Taphrinomycotina, Saccharomycotina and Pezizomycotina), and recent phylogenetic analyses have revealed several surprising finds (Fig. 5). For example, discomycetes (apothecial ascomycetes) were assumed to be highly derived forms, but phylogenetic studies using DNA characters indicate that these ascomycetes are basal members of the large group of mainly filamentous apothecial ascomycetes that we now call Pezizomycotina. It is of interest that species of *Neoleecta* in one of the basal ascomycetes group, Taphrinomycotina, possess apothecial ascumata. Not only apothecia but also other ascumata are evolutionary labile and do not define monophyletic groups. Although there are no well-known associations between members of the Taphrinomycotina and insects, many members of Saccharomycotina and Pezizomycotina are insect associates (Suh et al. 2004; Humber 2008). Insects are important dispersers of plant pathogens, especially tree diseases caused by members of Pezizomycotina. It is within the Pezizomycotina that the most important insect pathogens are classified. These include members of the Hypocreales that have interactions not only with arthropods, but plants and other fungi as well. Recent work on the order revealed that the well-known insect parasite, *Cordyceps*, is not monophyletic, and species have been placed in three separate families (Fig. 6; Table 1). The phenomenon of host-switching in the Hypocreales is discussed below (See host switching). Other insect-associated members of Pezizomycotina include the bee parasites in the genus *Ascospaera* (Eurotiales) and *Podonectria* (Tubeufiaceae) on scale insects. The Laboulbeniomyces (Fig. 4) are all



**Table 1** Classification of arthropod parasites previously placed in Clavicipitaceae

Clavicipitaceae: *Conoideocrella*, *Hypocrella*, *Metacordyceps*, *Moelleriella*, *Orbiocrella*, *Regiocrella*, *Samuelisia*, *Shimizuomyces*, *Villosiclava* (Major anamorphs: *Metarhizium*, *Paecilomyces* s.l. *Pochonia*)

Cordycipitaceae: *Ascopolyporus*, *Cordyceps*, *Hyperdermium*, *Torrubiella* (Major anamorphs: *Akanthomyces*, *Beauveria*, *Isaria*, *Lecanicillium*, *Simplicillium*)

Ophiocordycipitaceae: *Cordyceps* s.l., *Elaphocordyceps*, *Ophiocordyceps*, *Torrubiella*, *Hirsutella*, *Hymenostilbe*)

These ascomycetes and their anamorphs are now classified in three lineages reflected in changes of family level taxa: Cordycipitaceae, Clavicipitaceae and Ophiocordycipitaceae. About 160 additional taxa originally classified as species of *Cordyceps* sensu Kobayasi and Mains require additional study in order to place them in the new phylogenetic classification and to determine their anamorphs. Several anamorph genera are probably polyphyletic (Sung et al. 2007; Spatafora et al. 2007; <http://cordyceps.us/>)

associated with insects and other arthropods, most as biotrophic parasites. No other group of ascomycetes except the Hypocreales, however, has so many associations with arthropods (Fig. 6; Table 1).

## Basidiomycota

Basidiomycetes are classified in three subphyla, Pucciniomycotina, Ustilaginomycotina and Agaricomycotina (Fig. 5). The basal clade, Pucciniomycotina, contains the rust fungi, important plant pathogens, some of which have insect associations, especially fertilization and dispersal by chrysomelid beetles, flies and butterflies. Also included in Pucciniomycotina are species of Septobasidiales, parasites of scale insects. Smut fungi, Ustilaginomycotina, are plant pathogens. Members of Agaricomycotina have many associations with insects, including providing habitat for insects and other invertebrate animals. Several lineages of fungi of this subphylum are cultivated by ants and termites, and many members are dispersed by insects, including wood decaying fungi injected into wood by siricid wood wasps.

Applications of molecular methods to the study of insect-associated fungi

### *Distinguishing convergent evolution*

In the past, mycologists were aware of the difficulties in detecting relationships among certain insect-associated fungi. For example, insect-associated fungi possess a suite of morphological characters involving spore-producing structures. The so-called ophiostomatoid fungi (e.g., *Ophiostoma*) are ascomycetes with long necked perithecia, evanescent asci, and adhesive spores collected in droplets at the perithecial tip, traits that promote ascospore dispersal by insects (Blackwell et al. 1993). The very characters used for defining and grouping ophiostomatoid taxa have proven deceptive, and DNA sequences were required to sort them into their independent lineages. For example, all species of several genera once were considered congeneric with the species placed in *Ceratocystis*. What is remarkable is that all of the genera now are placed in distinct orders: Ophiostomatales (*Ophiostoma*), Microascales (*Ceratocystis*, *Sphaeronaemella*), Laboulbeniales (*Pyxidiophora*), and *Kathistes*, in a separate unnamed order (Blackwell 1994; Blackwell

et al. 2003). In addition to convergence among ophiostomatoid sexual states, there are many examples of convergence of coniodigenous cells and conidia. These include species of *Chalara*, the anamorph of insect-associated fungi in *Ceratocystis* that also is an anamorph of at least five orders of ascomycetes (Nag Raj and Kendrick 1993). *Ophiostoma* and *Ceratocystis* have similar asexual relatives, all placed in *Ambrosiella*, implying that traits of both asexual and sexual states are being selected upon for insect associations (Cassar and Blackwell 1996).

Another example of a presumed convergent character among insect-associated ascomycetes is the hat-shaped (galeate) ascospore. This trait is found among a number of clades of insect-associated Saccharomycotina (especially previous members of the genus *Pichia*) and several clades of Pezizomycotina (Eurotiales, Ophiostomatales). Hat-shaped ascospores were once the basis for a taxonomic revision including yeasts and the galeate-spored Pezizomycotina in a common family (Redhead and Malloch 1977).

There also are examples of what appear to be rapid divergence. Obligate arthropod parasites (Laboulbeniomyces, Fig. 4) previously have been placed in four different fungal phyla as well as in floridean red algae. In addition, certain species also were considered to be insect setae or even parasitic worms (Blackwell 1994). Some insect-associated Basidiomycota such as *Septobasidium* are morphologically distinct from near relatives, and molecular characters were required to place these organisms among their rust fungus relatives.

### Host switching

Molecular techniques provide opportunities to trace changes in nutritional modes of fungi. Current patterns of fungal-insect parasitism may be explained by the “related host hypothesis” reported for certain attine ant-associated fungi in which cospeciation patterns are detected by congruence of species level phylogenetic trees of interacting ants and fungi (Little and Currie 2008). Far more often, however, fungi with close arthropod associations display a pattern of host switching, so that closely related fungi are not necessarily associated with closely related insects and vice versa (Nikoh and Fukatsu 2000). The related host hypothesis has been used to explain some of the “interkingdom host shifts” evident among clades of

the Hypocreales (Spatafora et al. 2007; Sung et al. 2007; <http://cordyceps.us/>). These studies provide an excellent understanding of widespread host shifts and were cited as well-designed studies to show such changes and to make the corresponding nomenclatural changes that so often lag behind the phylogenetic work (Spatafora et al. 2007; Sung et al. 2007; <http://cordyceps.us/>; Vega et al. 2009). One less well-known case involves not only host switching, but also a dramatic change in life histories. A small group of endosymbionts of plant hoppers arose from pathogenic members of Ophiocordycipitaceae to evolve into an obligate association for both fungi and insects (Suh et al. 2001).

The host habitat hypothesis (Nikoh and Fukatsu 2000) was proposed to explain the associations of distantly related hosts of Hypocreales, but other examples are found among other fungi and their hosts. The “host habitat hypothesis” also may explain distributions of obligate biotrophic parasites (Laboulbeniales, Septobasidiales, Pucciniomycotina), pathogens (Entomophthorales, Blastocladiomycota, Eurotiales) and perhaps even commensals (Harpellales and Asellariales). Other examples provide insight into the host habitat hypothesis. *Laboulbenia ecitonis* is a species that parasitizes unrelated hosts that are inhabitants of legionary ant nests. The hosts, including histrid and staphylinid beetles, two species of mites and the ants themselves, are relatively confined in a common habitat (Benjamin 1965). Removal of all hosts except a mite species could appear as a rapid host shift to an unrelated host, especially if the nearest fungal relative were determined to be restricted to related ant hosts.

Some species of Laboulbeniales have broad host distributions, while strict specificity is assumed for others. De Kesel’s (1996) experimental study provided insight into how host isolation and subsequent fungal specialization might occur after removal of some hosts. Assume that a generalist fungus is associated with a number of arthropod hosts; subsequently most of the potential host taxa disappear from the habitat, and the fungal parasite becomes geographically isolated on the single remaining host. Isolation followed by divergence of the fungus could lead to specialization on that particular host (Suh et al. 2005). For example, a single carabid species, the only one available, was the usual host for a laboulbenian fungus (De Kesel 1996). The fungus,

however, was able to infect some, but not all carabids. Beetles outside of Carabidae, however, never served as hosts. This study also indicates that there is sometimes a host genetic component in infection, and the absence of infection in some potential hosts may indicate that divergence and host switching are in progress.

### Future considerations

Progress in evolutionary understanding and phylogenetic classification continues to be made as more taxa are sampled and more genes and genomes become available for analysis. For some time there will be a great need for biologists who know the biology and ecology of the organisms, and can collect and identify them for molecular studies. Obtaining correctly identified fungi is of paramount importance to understanding the evolutionary relationships among fungi, which will help us understand their evolutionary history.

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### Author Biography

**Meredith Blackwell** is a fungal biologist, who is interested in arthropod associated fungi. Her current research deals with biodiversity of insect gut fungi. She has been involved in several community-wide projects, including the Research Coordination Network: A phylogeny for kingdom Fungi (Deep Hypha) and Assembling the Fungal Tree of Life II (AFToL II).