PROJECT DESCRIPTION

Results of Prior NSF Support

Roy Halling has served as a PI on several prior NSF grants, the most recent of which is "Macrofungi of Costa Rica", a collaborative project between REH and G. M. Mueller (DEB 9972018, $165,800, Sept. 1999-Aug. 2003). This work began as a biotic survey of "The Agaricales of Costa Rican *Quercus* Forests" funded by NSF and U.S.A.I.D. (DEB 9300798 with subcontract to NYBG, $62,065, May 1993-April 1997). We and our collaborators have been documenting the diversity and ecology of boletes and other macrofungi associated with Neotropical oak forests. The project has focussed on six conservation areas in Costa Rica: Guanacaste, Tempisque, Arenal, Amistad Pacifico, Amistad Caribe, and Osa. We were primarily responsible for the initiation of Costa Rica's National Fungal Inventory. Collaborators in Costa Rica include the University of Costa Rica Biology Department and INBio (Costa Rica's National Institute of Biodiversity), which were targeted for infrastructure building and enhancement. Selected publications that are directly related to the proposed research are listed below. For a full list see: http://www.nybg.org/bsci/res/hall/sumry2.html.

1. Halling, R. E. 1996 (1997). Boletaceae (Agaricales): Latitudinal biodiversity and biological interactions in Costa Rica and Colombia. *Rev. Biol. Trop*. 44 (suppl. 4): 111-114.
2. Halling, R. E. and G. M. Mueller. 1999. New boletes from Costa Rica. *Mycologia* 91: 893-899.
3. Halling, R. E. 1999. New Leccinums from Costa Rica. *Kew Bull*. 54: 747-753.
4. Halling, R. E. and G. M. Mueller. 2001. *Tylopilus bulbosus* sp. nov. from Costa Rica. *Harvard Papers in Botany* 6: 109-112.
5. Halling, R. E. and G. M. Mueller. 2002. Agarics and boletes of Neotropical oakwoods. In: *Tropical Mycology*, Watling, R., J. C. Frankland, A. M. Ainsworth, S. Isaac, and C. H. Robinson, eds. CABI Publishing, UK, p. 1-10.
6. Amtoft, A., R. E. Halling, and G. M. Mueller. 2003. *Tylopilus alkalixanthus*, a new species of Boletaceae from Costa Rica. *Brittonia* (in press).
7. Halling, R. E. and G. M. Mueller. 2003. *Leccinum* (Boletaceae) in Costa Rica. *Mycologia* (in press).
8. Halling, R. E. and G. M. Mueller. 2003. Mushrooms of the Central Talamancas, Costa Rica. New York Botanical Garden Press (in revision), approx. 200 p.

The projects supported one post-doc (Ana E. Franco M. from Colombia), two Costa Rican undergraduates (Milagro Mata, Loengrin Umaña), one Costa Rican Master's candidate (Juan Luís Mata), two REU's (Anja Amtoft, Tracy Scanlan), and provided support for a weeklong workshop on Basidiomycetes held in Costa Rica and attended by 18 students from throughout Central America. A web site describes the work in progress (http://www.nybg.org/bsci/res/hall) and provides a link to searchable databases. Presently, 7149 records of Boletaceae (sensu stricto) are searchable (http://www.nybg.org/bsci/hcol/fung/Boletaceae.html) based on all specimens held in the New York Botanical Garden Mycology Herbarium.

Manfred Binder has not served as a PI on NSF-supported research, but is a Co-PI on an ongoing project, titled "Phylogenetic relationships of cyphelloid and aquatic homobasidiomycetes" (D. Hibbett, PI; DEB 0128925; $300,000; Sept. 2002-Aug. 2005). The first product of this new research project will be an analysis of phylogenetic relationships of cyphelloid and aquatic fungi and related taxa based on nuclear large subunit ribosomal DNA (nuc-lsu rDNA) sequences. Sequences of approximately 60 species of cyphelloid and aquatic forms have been obtained, including 3 mitosporic aquatic basidiomycetes. These data are being combined with sequences from exisiting nuc-lsu rDNA data sets (in collaboration with Dr. Jean-Marc Moncalvo). We anticipate that the first manuscript describing analyses of these data will be submitted in early 2003.

David Hibbett has served as PI on several NSF grants in the last five years, including the project "Collaborative Research: Assembling the Fungal Tree of Life (AFTOL)" (DEB 0228657; $550,606; Jan. 2003-Dec. 2006). The most relevant completed project on which DSH has served as PI is "Morphological and ecological diversification in the homobasidiomycetes: a molecular phylogenetic analysis" (DEB-9903835; $190,000; Sept. 1999-Aug. 2002). During the funding period, thirteen articles and one chapter were published or accepted, and two publications are still in review. A final publication from this project, which describes analyses of a 656-species data set of homobasidiomycetes, emphasizing corticioid taxa, is in preparation. Six selected publications are listed below. For a full list, see: http://www.clarku.edu/faculty/dhibbett/publications.htm.

1. Hibbett, D. S., Luz-Beatriz Gilbert, and Michael J. Donoghue. 2000. Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. *Nature* 407: 506-508.
2. Hibbett, D. S., and M. J. Donoghue. 2001. Analysis of correlations among wood decay mechanisms, mating systems, and substrate ranges in homobasidiomycetes. *Systematic Biology* 50: 215-242.
3. Binder, M., D. S. Hibbett, and H.-P. Molitoris. 2001. Phylogenetic relationships of the marine gasteromycete *Nia vibrissa*. *Mycologia* 93: 679-688.
4. Hibbett, D. S., and R. G. Thorn. 2001. Basidiomycota: Homobasidiomycetes. Pp. 121-168 *in*: The Mycota, vol. VII part B, Systematics and Evolution (D. J. McLaughlin, E. G. McLaughlin, and P. A. Lemke, eds.). Springer Verlag.
5. Binder, M., and D. S. Hibbett. 2002. Higher-level phylogenetic relationships of homobasidiomycetes (mushroom-forming fungi) inferred from four rDNA regions. *Molecular Phylogenetics and Evolution* 22: 76-90.
6. Hibbett, D. S., and M. Binder. 2002. Evolution of complex fruiting body morphologies in homobasidiomycetes. *Proc. Roy. Soc. London Ser. B.* 269: 1963-1969.

This project supported M. Binder as a post-doctoral fellow, as well as two female Clark University undergraduates (Amanda Little, Brooke Barbera) and one African-American high school student (Damian Ramsey), who were supported on REU and RAMHSS supplements.

Proposed Research

Introduction

The Boletales is a monophyletic order of homobasidiomycetes (Fungi) that includes approximately 1025 described species (Kirk et al. 2001) that are distributed worldwide. The Boletales are diverse in both morphology and ecology. The typical fruiting body form in the group is pileate-stipitate (with a cap and stalk), with a tubular hymenophore (spore-bearing surface). In addition, there are corticioid (crust-like resupinate), agaricoid (pileate-stipitate with lamellate hymenophore), and gasteroid (puffball-like, hymenium enclosed) forms. Most Boletales function as ectomycorrhizal partners of conifers and angiosperms. A smaller number of species are wood-decaying saprotrophs, and a few are suspected to be mycoparasites. Because of their ability to transfer mineral nutrients to plants and decompose organic matter, these organisms play key roles in nutrient cycling in many ecosystems.

The Boletales have been widely studied by fungal systematists, chemists, ecologists, and mycorrhizal biologists (e.g., Agerer 1987-1998, Arpin & Kühner 1977, Besl & Bresinsky 1997, Both 1993, Gill & Steglich 1987, Moser 1983, Singer 1986, Smith & Thiers, 1971, Watling 1970). However, investigations in all these areas have been hampered by lack of adequate identification tools, and the absence of a comprehensive phylogenetic classification for the group. We propose a collaborative project that will contribute toward a global classification of the Boletales. The proposed research involves a nested set of three subprojects: **1. Multi-gene phylogeny using exemplars of all major groups of Boletales and representatives of potential outgroups; 2) Survey of diverse Boletales, using nuclear large-subunit rDNA and ITS sequences; and**, **3) Monographic studies on selected genera and species-complexes in Boletaceae.**

Background information

Ecology and biogeography of Boletales:

Boletales are found in most forest ecosystems all over the world. Extrapolating from previous surveys of fungal diversity (Agerer 1987-1998, Hawksworth et al. 1995, Kirk et al. 2001, Smith & Read 1997), we estimate that roughly 90% of the species in this group are potentially ectomycorrhizal, and that the Boletales may represent 18-25% of all ectomycorrhizal fungi. The plant families Betulaceae, Caesalpiniaceae, Casuarinaceae, Dipterocarpaceae, Ericaceae, Fagaceae, Mimosaceae, Myrtaceae, Pinaceae, and Salicaceae have been proven or implicated to form ectomycorrhizal symbioses with Boletales (Newman & Reddell 1987, Halling, pers. obs.). The remaining Boletales are almost all wood-decaying saprotrophs. Among these is the infamous "dry rot" fungus (*Serpula lacrymans*), which is a destructive decomposer of wood in buildings (Jennings & Bravery 1991).

Despite the importance of the Boletales, no surveys for continental or global distribution patterns are available for this group, except for local geographic treatments (as is true for fungi in general). In fact, the ranges of even the most common species are poorly known (Redhead 1989), but evidence is accumulating that some are more widely distributed globally than previously thought (Halling 1996, 2001, Mueller & Halling 1995). Most species of the Boletales have been described from Europe and North America (e.g., Bessette et al. 2000, Earle 1905, Fries 1874, Frost 1874, Ginns 1982, Lannoy & Estades 1995, Murrill 1938, Peck 1889, Quelét 1888, Singer 1945, 1965, Smith & Thiers 1971). North America and south-east Asia appear to be the main centers of diversity in Boletales (Wu & Mueller 1997, Watling 2001). These regions share a surprisingly large number of taxa (roughly 40% fide Wu & Mueller 1997) that are not represented in Europe.

In the southern hemisphere, a few species of Boletales (less than 10) have distributions that overlap with *Nothofagus* forests in Australia, Papua New Guinea, New Caledonia, and New Zealand (Horak 1983). However, these are not the same species that are associated with *Nothofagus* along the Pacific coast of South America (five species, Horak 1977). Most Australian species of Boletales appear to be associated with Myrtaceae (Bougher & Syme 1998, Grgurinovic 1997, Halling pers. obs.). However, the Australian Boletales flora remains poorly known. Knowledge of the African Boletales flora is similarly quite fragmentary, and is based mostly on material collected in the former European colonies (e.g., Heinemann 1951-1966, Heinemann & Rameloo 1980-1989, Watling & Turnbull 1993, 1994).

Systematics and molecular phylogeny of Boletales:

Several molecular phylogenetic studies, using nuc-lsu rDNA, nuc-ssu rDNA, mitochondrial large subunit rDNA (mt-lsu), and ATP6 sequences, suggest that the Boletales is monophyletic (Binder & Bresinsky 2002a, Bresinsky et al. 1999, Bruns et al. 1998, Grubisha et al. 2001, Hughey et al. 2000, Kretzer & Bruns 1999, Jarosch 2001). A recent study using four rDNA regions (nuc-ssu, nuc-lsu, mt-ssu, mt-lsu) showed 100% bootstrap support for the Boletales (Binder & Hibbett 2002). The same study suggested that the Agaricales (euagarics clade) is the sister group to the Boletales (bootstrap=94%). However, another study based on nuc-lsu sequences and 5.8S rDNA sequences suggested that a group of "athelioid" species (resupinate forms) may be the sister group of the Boletales, although there was low bootstrap support (Larsson 2002). We have recently completed a major survey of resupinate homobasidiomycetes which suggests that a clade containing *Jaapia argillacea* and *Athelia* spp. may be the sister group of the Boletales, with the euagarics clade as the next most closely related group (Hibbett & Binder 2002; Binder & Hibbett, in prep.). However, our studies did not include the same "athelioid" taxa that were sampled by Larsson (2002). In summary, the monophyly of the Boletales is well supported, but its sister group is not resolved with confidence.

Five major groups, which have been classified as suborders (Agerer 1999, Binder & Bresinsky 2002a, Besl & Bresinsky 1997, Gilbert 1931), are currently recognized within the Boletales: **Boletineae** (including the families Boletaceae, Chamonixiaceae, Octavianinaceae,

Strobilomycetaceae), **Paxillineae** (Gyrodontaceae, Melanogastraceae, Paxillaceae), **Sclerodermatineae** (Astraeaceae, Boletinellaceae, Gyroporaceae, Calostomataceae, Pisolithaceae, Sclerodermataceae), **Suillineae** (Gomphidiaceae, Suillaceae, Truncocolumellaceae, Rhizopogonaceae), and **Coniophorineae** including Tapinellineae (Coniophoraceae, Serpulaceae, Tapinellaceae). Each of these suborders includes stipitate-pileate and gasteroid forms, and the Paxillineae and Coniophorineae also include resupinate forms. Thus, currently accepted taxonomy suggests that there has been much homoplasy in fruiting body evolution in Boletales (Binder & Bresinsky 2002a, Binder & Hibbett 2002, Jarosch & Besl 2001). There also appear to have been multiple shifts between saprotrophy and ectomycorrhizal symbiosis, especially within the Coniophorineae (Bresinsky et al. 1999, Jarosch 2001).

Of the five suborders of Boletales, four appear to be monophyletic based on molecular analyses (Binder & Bresinsky 2002a, Jarosch 2001), including unpublished analyses by M. Binder (see **Preliminary Results**), but the Coniophorineae appears to be non-monophyletic. Testing the monophyly of the suborders, especially the Coniophorineae, will be a focus of the proposed research. In addition to questions about the monophyly of the Coniophorineae, there is considerable uncertainty regarding the higher-level relationships among the suborders and basal relationships of the Boletales as a whole, which makes it difficult to infer patterns of character evolution and plesiomorphic conditions in the Boletales (Binder & Hibbett 2002, Jarosch 2001).

Nuc-lsu rDNA has been widely sampled in Boletales and appears to provide adequate resolution of terminal groups within the suborders (Binder 1999, Binder & Bresinsky 2002a, 2000b, Jarosch 2001). Many relevant taxa have yet to be investigated for nuc-lsu rDNA, however, which leaves many taxonomic issues unresolved. For example, the majority of nuc-lsu sequences for species in the Boletineae originates from European material, largely from the work of M. Binder and colleagues (Binder 1999, Jarosch 2001), but the North American Boletineae are largely uninvestigated in this regard, because major North American studies have focussed on the acquisition of ITS data (for members of the Suillineae) and on a broader sampling of mt-lsu sequences (Bruns et al. 1998, Grubisha et al. 2002, Kretzer et al. 1996, Wu et al. 2000). As a consequence, there is ongoing debate over generic concepts in the Boletineae (Watling 2001) and the phylogenetic interpretation of certain morphological and anatomical characters, such as spore ornamentation, hymenophoral trama type, spore print color, and stipe ornamentation (Bresinsky 1996, Pegler & Young 1981, Singer 1986, Smith & Thiers 1971). Many of these characters have been emphasized in generic delimitations, but now appear (on the basis of molecular phylogenies) to have evolved repeatedly (Binder 1999, Binder & Fischer 1997, Binder & Besl 2000, Bresinsky et al. 1999).

Finally, the ITS region has become a popular tool for molecular ecology of Boletales (Horton & Bruns 2001), and it has also been helpful in resolving relationships within terminal groups of *Suillus, Rhizopogon,* and *Leccinum* (Grubisha et al. 2002, Kretzer et al. 1996; see **Preliminary Results**). Again, extensive ITS data sets from across the Boletales are not yet available, which limits both molecular ecological studies and species-level systematics.

Taxonomic problems in selected genera and species-complexes:

Generic and species limits have been hotly debated in many groups of Boletales in the last 30 years (Smith & Thiers 1971, Corner 1972, Pegler &Young 1981, Singer 1986). The disagreements largely involve subjective opinions about which morphological features should be emphasized in taxonomy. What is needed now is to combine morphological and molecular approaches to develop modern monographs, which will provide models for future taxonomic studies of Boletales. Such studies will require intensive sampling and an integration of field and laboratory analyses (many characters can only be observed in fresh material). While there are numerous candidate groups, we have chosen to focus on two small genera, *Afroboletus* and *Heimiella*, and four widely distributed species (based on morphological criteria, see below), which may in fact represent multiple taxa.

Afroboletus (five species) occurs in Africa, and *Heimiella* (8 species) occurs in E and SE Asia and Central America (Pegler & Young 1981, Watling & Turnbull 1993). These taxa were selected

because they have been the subject of taxonomic debate, they have a manageable number of species, and we have access to many relevant specimens (our own and those of collaborators, see Letters of Support). Thus, we are well positioned to produce needed monographic treatments of these genera. Pegler and Young (1981) suggested that *Afroboletus* is distinct from *Strobilomyces*, and that *Heimiella* is distinct from *Boletellus*, but Singer (1986) recognized only *Strobilomyces* and *Boletellus*. However, Singer (1986) failed to recognize (ignored?) certain subtle, but easily observable, spore characters noted by Corner (1972) and Pegler and Young (1981). We will test the competing views, and assess the phylogenetic significance of morphological characters in these groups.

We have also pinpointed four species complexes that are reported to occur on all continents except Antarctica, including *Boletellus ananas*, *Pulveroboletus ravenelii* (not known in Europe), *Tylopilus chromapes*, and *T. eximius* (Corner 1972, Halling 2001, Singer 1986, Smith & Thiers 1971, Watling & Turnbull 1993, Halling pers. obs.). The near global occurrence of these groups suggests that they could have ancient relict distributions, and may harbor cryptic species. Subtle morphological variation within these groups also suggests that they could be more diverse than current taxonomy indicates. *Boletellus ananas, P. ravenelii*, and *T. eximius* are each currently classified as a single morphospecies. Wolfe and Bougher (1993) performed phenetic analyses of anatomical characters (using the NTSYS package) in herbarium materials of *T. chromapes*-like specimens. Based on their results, they recognized nine species in *Tylopilus* subg. *Roseoscabra*, including a restricted concept of *T. chromapes*. The distinction of these species has not been evaluated with fresh material or molecular characters, however. Moreover, Wolfe and Bougher (1993) did not include *T. nanas*, a member of subg. *Roseoscabra* that Halling has recently collected in Indonesia.

Research Plan

Proposed research in brief

The proposed research consists of a nested set of three sub-projects that will assess phylogenetic relationships and revise the taxonomy of Boletales from high to low taxonomic levels:

Part 1: Multi-gene phylogeny using exemplars of all major groups of Boletales and representatives of potential outgroups. A dataset of eight loci (nuc-ssu, nuc-lsu, mt-lsu, ATP6, RPB1, RPB2, EF-1α, and ITS) of 48 species from the major clades of Boletales, as well as potential outgroup taxa will be analyzed. The chosen genes are the same ones that are employed in the AFTOL project (plus mt-lsu rDNA). The goals of these studies are to 1) provide a "backbone" phylogeny of the Boletales; 2) test the monophyly of the Coniophorineae; 3) resolve the order of early branching events in the group; and 4) identify the sister group of the Boletales.

Part 2: Survey of diverse Boletales, using nuclear large-subunit rDNA and ITS sequences. An extensive data set of nuc-lsu rDNA and ITS sequences of approximately 800 species from all continents except Antarctica will be developed, with an emphasis on North American, European, and neotropical material. The goals of these studies are to 1) develop a phylogenetic framework within which generic-level diversity of Boletales can be addressed; 2) provide resources for species-level taxonomy and molecular ecology; and 3) understand the evolution of fruiting body form and nutritional modes in the Boletales.

Part 3: Monographic studies on selected genera and species-complexes in Boletaceae. Monographic studies will be performed in two small genera, *Afroboletus* and *Heimiella*, and four widely distributed species complexes, *Boletellus ananas*, *Pulveroboletus ravenelii*, *Tylopilus chromapes*, and *T. eximius*. These taxa will be studied intensively throughout their known distributions using existing collections and new collections to be made in Australia. Morphological studies and multigene phylogenetic analyses will be performed. The goals of these studies are to 1) resolve generic and species limits and provide identification tools; 2) reassess biogeographic patterns; and 3) provide a model for future monographic studies in Boletales.

Proposed research in detail.

Part 1: Multi-gene phylogeny using exemplars of all major groups of Boletales and representatives of potential outgroups.

Taxa to be sampled:

This part of the proposed research will sample exemplars of all the major groups of Boletales (28 spp.), as well as potential sister groups of the Boletales (7 spp.), and exemplars of other major groups of homobasidiomycetes sensu Hibbett and Thorn (2001) (12 spp.). The heterobasidiomycetes *Auricularia auricula-judae* and *Dacrymyces chrysospermus* will be included for rooting purposes (Swann & Taylor 1995, Weiss & Oberwinkler 2000). Taxon sampling will be coordinated with the ongoing AFTOL project; data for 28 of the 48 species to be sampled in this part of the proposed research will be obtained from AFTOL (Table 1). Boletales taxa will include representatives of each of the five currently recognized suborders, with an emphasis on the Coniophorineae, which may not be monophyletic (see **Background Information** and **Preliminary Results**). Taxa representing potential sister groups to the Boletales will include representatives of the euagarics clade, which Binder and Hibbett (2002) suggested is the sister group of the Boletales, as well as certain resupinate forms (e.g. *Athelia* spp., *Jaapia argillacea, Piloderma* spp., *Tylospora* spp.) that have appeared to be closely related to the Boletales in analyses by Larsson (2002) and Hibbett and Binder (2002). The euagarics clade includes over 8000 species, so exemplars will be selected based on previous analyses that have resolved major groups within the euagarics clade, including the most basal groups within the euagarics clade (Binder & Hibbett 2002; Moncalvo et al. 2000, 2002). Material of all of these taxa are in our possession or are readily available from established culture collections.

Table 1. Taxon sampling for Part 1.

Boletales
Boletineae (8): *Boletellus ananas, Boletus edulis*,* *******, Chalciporus piperatus, Leccinum aurantiacum, Phylloporus rhodoxanthus, Retiboletus ornatipes, Strobilomyces floccopus*, Tylopilus felleus**
Sclerodermatineae (4): *Boletinellus merulioides, Calostoma cinnabarina**, *******, Gyroporus cyanescens, Phlebopus portentosus**
Coniophorineae (8): *Austropaxillus infundibuliformis, Coniophora puteana*, Hygrophoropsis aurantiaca*, Leucogyrophana olivascens, Leucogyrophana pulverulenta, Pseudomerulius aureus, Serpula lacrymans*, Tapinella panuoides**
Paxillineae (3): *Hydnomerulius pinastri, Melanogaster tuberiformis, Paxillus involutus*,*
Suillineae (5): *Chroogomphus rutilus, Gomphidius glutinosus*, Rhizopogon subcaerulescens*, Suillus granulatus, Truncocolumella citrina*
Exemplars of potential sister groups of Boletales
Euagarics clade (3): *Hygrocybe conica*, Plicaturopsis crispa*, Schizophyllum commune**
Resupinate forms (4): *Athelia epiphylla*, *Jaapia argillacea, Piloderma byssinum, Tylospora asterophora*
Other homobasidiomycetes
Russuloid clade (2): *Amphinema byssoides*, Asterostroma andinum**
Thelephoroid clade (2): *Sarcodon imbricatum*, Thelephora palmata**
Cantharelloid clade (2): *Botryobasidium subcoronatum*, Hydnum albidum**
Hymenochaetoid clade (2): *Hydnochaete olivacea*, Repetobasidium mirificium**
Gomphoid-phalloid clade (2): *Gomphus floccosus*, Ramaricium alboflavescens**
Polyporoid clade (2): *Candelabrochaete africana*, Sparassis crispa**
Outgroups
Auriculariales: *Auricularia auricula-judae*;* **Dacrymycetales:** *Dacrymyces chrysospermus**

*Data for these species will be obtained from the AFTOL project.

Genes to be sampled and molecular techniques:

We will develop a multi-gene data set using the same seven loci as proposed in the AFTOL project: nuc-ssu rDNA (1.8 kb), 5' nuc-lsu rDNA (5' LR0R-LR5, 1.0 kb), ATP6 (0.64kb), RPB1 (2.5 kb), RPB2 (2.5 kb), EF-1α (1.4 kb), and ITS rDNA (0.6-1.5 kb), and mt-lsu rDNA (ML5-ML6, 0.35-0.47 kb). Datasets of nuc-lsu, mt-lsu, and ATP6 sequences of Boletales have been published (Binder & Bresinsky 2002a, Bresinsky et al. 1999, Bruns et al. 1998, Kretzer & Bruns 1999), but they overlap only to a minor extent and a combined phylogenetic analysis is only possible for a small number of species. Moreover, many basal nodes of the major clades in the Boletales are weakly supported in the single-gene analyses, and there is a lack of resolution in some terminal groups. It is therefore necessary to extend the existing data sets with additional genes, sampled in the same sets of taxa.

Laboratory techniques of fungal molecular systematics have become standard and will not be described in detail. Briefly, we will extract DNA from fruiting bodies or mycelia, and amplify target genes with the PCR. Whenever possible, we will sequence PCR products directly, but as necessary we will clone PCR products using TA cloning prior to sequencing. PCR products and clones will be cycle sequenced using ABI PRISM dye-terminator chemistry, and sequencing reactions will be run on ABI automated DNA sequencers. We have extensive experience with these techniques (e.g., Binder & Hibbett, 2002; Hibbett & Binder 2002).

Oligonucleotide primers for ribosomal genes (nuc-ssu, nuc-lsu, mt-lsu, ITS region) are well established (Gardes & Bruns 1993, Vilgalys & Hester 1990, White et al. 1990), have been used in numerous studies due to their universal nature (e.g., Binder & Hibbett 2002, Bruns & Szaro 1992, Bruns et al. 1989, 1998, Gardes et al. 1991, Hibbett et al. 1997, Humpert et al 2001, Kretzer et al. 1996, Moncalvo et al. 2002, Pine et al. 1999), and are routinely amplified in our laboratory. ATP6 primers originate from a study focussing on higher level relationships in the Boletales (Kretzer & Bruns 1999) and have been shown to enhance resolution of many clades. Fungal primers are also available for RPB1, RPB2, and EF-1α (Liu et al. 1999, Matheny et al. 2002, O'Donnell et al. 1998b, 2001, Rehner 2001; http://ocid.NACSE.ORG/research/deephyphae/EF1primer.pdf). Nevertheless, protein coding genes frequently exhibit degeneracy at second and third codon positions, so we expect to spend considerable effort on designing primers and optimizing PCR conditions.

Phylogenetic analyses:

Sequences will be aligned with a combination of algorithmic methods, implemented in CLUSTAL X (Thompson et al 1997), and manual adjustment, using the MacClade 4.0 (Maddison & Maddison 2000) data editor. For protein-coding genes, nucleotide sequences will be translated and amino acid sequences will be used to guide alignments.

It is widely appreciated that combining data from different loci may lower phylogenetic accuracy if the genes have different phylogenetic histories or evolve according to strongly differing models (Bull et al. 1993, Cunningham 1997a, 1997b, Dolphin et al. 2000, Dowton & Austin 2002). At present, however, there is no clear consensus about the best methods to detect significant conflict (i.e., that which is likely to reduce phylogenetic accuracy in combined analysis) or how to compensate for it. We will perform bootstrapped parsimony analyses and Bayesian analyses (see below) of the individual loci and examine their results for cases of strongly supported conflict (positively conflicting nodes with support over 70% bootstrap frequency or 95% posterior probability). If such conflict is detected, we will further explore the conflict using the Shimodaira-Hasegawa (SH, 1999) test with constrained topologies. If the SH test confirms that there is significant conflict, we will not combine the data in their entirety, but will prune taxa or sequences of individual genes from the data set. We will base our best estimate of the phylogeny on analyses of the most inclusive data set possible, as determined by results of the SH tests.

Phylogenetic analyses will be performed using parsimony and maximum likelihood (ML) methods, implemented in PAUP* 4.0 (Swofford 2001), and Bayesian methods, implemented in MrBayes 3.0 (Huelsenbeck & Ronquist 2001). Parsimony analyses will use heuristic methods with 1000 replicates, with starting trees generated by random taxon addition sequences, TBR branch

swapping, and MAXTREES set to autoincrease. Bootstrapped parsimony analyses will use 1000 replicates, with a single heuristic search per replicate. Prior to ML searches, we will select an optimal model of sequence evolution with the program ModelTest (Posada & Crandall 1998), using one of the most parsimonious trees to evaluate models. ML searches will use trees from parsimony analyses as starting trees for heuristic searches with TBR branch swapping. We do not anticipate that it will be practical to perform bootstrapped ML searches. However, Bayesian analyses will be performed (with the same models as in ML analyses). At least four Markov chains will be run in each Bayesian analysis, which will be started from random trees and will be run for $2\text{-}5\times10^6$ cycles, with trees sampled every 100 cycles. The initial 10% of trees obtained during the "burn in" period will be discarded prior to calculation of posterior probabilities. Support for clades will be based on bootstrap frequencies and posterior probabilities. In addition, we will test the monophyly of suborders of Boletales using topologically constrained parsimony analyses, coupled with the SH test of constrained trees. We will use both monophyly and "inverse monophyly" constraints, which will allow us to test how strongly the data support or reject monophyly of suborders of Boletales.

Part 2: Survey of diverse Boletales, using nuclear large-subunit rDNA and ITS sequences.
Taxa to be sampled:
This part of the proposed research will produce comprehensive phylogenetic trees that will form the basis of a taxonomic revision at the generic and familial levels. We aim to sample approximately 800 species (Table 2 and http://www.clarku.edu/faculty/dhibbett/Boletales.htm), including representatives of all 64 widely recognized genera of Boletales, as well as certain problematical taxa that have been unplaced in prior classifications (*Neopaxillus*, *Podoserpula, Pogisperma*, *Tylopilogaster*). Taxa that could be related to the focal groups in **Part 3** (i.e., potential sister taxa) are also targeted.

The list of target species is biased toward taxa that occur in Europe and North and Central America (approx. 640 spp.; Table 2), many of which have also been reported from other continents. Whenever possible, we will sample multiple collections (typically, 2-5) of individual species from Europe and the Americas, targeting isolates from different geographic regions. In total, we expect to sample roughly 1200 isolates. Our focus on species that occur in Europe and the Americas is warranted because the majority of species of Boletales, including the type species of most genera, have been described either in Europe or North America. Therefore, to provide the framework for a comprehensive, global classification of the Boletales it is necessary to understand the relationships of European and North American taxa. Most of the species that we have selected are widely distributed, and are well represented by recent collections from which we should be able to extract useable DNA.

DNA isolates of 270 species and herbarium specimens of another 230 species are already in our possession (these are over half of the target species). Many species that we have yet to sample will be provided by our collaborators (see Letters of Support). Additional material will be requested from the Harry D. Thiers Herbarium of San Francisco State University and the University of Michigan Herbarium, or will be obtained in fieldwork to be conducted in Australia (see below).

Table 2. Taxon sampling for Part 2. This list indicates only the number of species to be sampled in each genus. For complete information on species, geographical distribution, availability, and available sequences, see: http://www.clarku.edu/faculty/dhibbett/Boletales.htm.

Boletineae (507,323)*, *Afroboletus* (7,0), *Aureoboletus* (5,4), *Austroboletus* (16,7), *Boletellus* (29,14), *Boletochaete* (3,1), *Boletus* (166,115), *Buchwaldoboletus* (4,2), *Chalciporus* (11,7), *Chamonixia* (7,3), *Fistulinella* (8,1), *Gastroboletus* (9,7), *Gastroleccinum* (1,0), *Gastrotylopilus* (1,0), *Heimiella* (6,4), *Leccinum* (67,58), *Octavianina* (3,3), *Paxillogaster* (1,0), *Phylloboletellus* (1,0), *Phyllobolites* (1,0), *Phylloporus* (19,10), *Porphyrellus* (4,3), *Pseudoboletus* (1,1), *Pulveroboletus* (4,4), *Retiboletus* (5,5), *Setogyroporus* (1,0), *Sinoboletus* (7,7), *Strobilomyces* (16,4), *Tubosaeta* (6,0), *Tylopilus* (52,32), *Veloporphyrellus* (2,0), *Xanthoconium* (7,4), *Xerocomus* (37,23)

Sclerodermatineae (80,41), *Astraeus* (3,2), *Boletinellus* (3,3), *Calostoma* (11,4), *Gyroporus* (11,6), *Horakiella* (1,0), *Phlebopus* (12,5), *Pisolithus* (5,2), *Rubinoboletus* (10,1), *Scleroderma* (23,17), *Veligaster* (1,1)
Paxillineae (32,16), *Alpova* (8,2), *Gyrodon* (6,3), *Hydnomerulius* (1,1), *Melanogaster* (6,4), *Paragyrodon* (1,1), *Paxillus* (10,5)
Suillineae (134,86), *Boletinus* (5,2), *Brauniellula* (1,0), *Chroogomphus* (11,4), *Gomphidius* (8,4), *Rhizopogon* (58,26), *Suillus* (50,49), *Truncocolumella* (1,1)
Coniophorineae (40,30), *Austropaxillus* (8,8), *Coniophora* (7,6), *Gymnopaxillus* (3,2), *Hygrophoropsis* (4,2), *Leucogyrophana* (9,5), *Meiorganum* (1,0), *Pseudomerulius* (2,2), *Serpula* (4,3), *Tapinella* (2,2)
incertae sedis (8,6), *Neopaxillus* (1,0), *Podoserpula* (1,0), *Pogisperma* (3,3), *Tylopilogaster* (3,3)

*The first number is the number of target species in a taxon, the second number is the number of species that have already been sampled (see **Preliminary Results**).

Genes to be sampled:

This part of the proposed research will use the ITS1-2 regions (including 5.8S rDNA) and an approximately 1kb region at the 5' end of the nuc-lsu (25S) rDNA, bounded by primers LR0R and LR5, which have been widely sampled in Boletales, the euagarics clade, and other basidiomycetes (e.g., Moncalvo et al. 2002; Binder & Bresinsky 2002a, and **Preliminary Results**). The ITS is a rapidly-evolving region that is too variable for analyses across the entire Boletales, but it is very useful for analyses at low taxonomic levels, and it has become a popular tool for molecular ecology of mycorrhizae (Horton & Bruns 2001). In contrast, the nuc-lsu rDNA can be aligned across all groups of Boletales and can resolve major groups within the order (Binder & Bresinsky 2002a, Jarosch 2001). We will obtain ITS sequences from every isolate that is studied, and nuc-lsu rDNA sequences from every isolate that has a unique ITS sequence (or at minium, one per species). All of the nuc-lsu rDNA sequences will be combined into a single data set for a global analysis of the Boletales, while subsets of the ITS sequences will be analyzed to resolve relationships of terminal clades (see below). This approach will provide resolution at low taxonomic levels, while insuring that the nuc-lsu data set includes as many unique lineages as possible.

Phylogenetic and character evolution analyses:

We will perform analyses of many relatively small data sets of ITS sequences, and one large data set of nuc-lsu rDNA sequences. Alignment will be peformed as described above (**Part 1**). ITS regions frequently display length variation (e.g, Hibbett et al. 1995), which may complicate alignment. ITS sequences will only be aligned among taxa where length variation is not severe. ITS matrices will be analyzed using equally weighted parsimony and ML. For parsimony analyses, gapped positions will be recoded to provide additional characters, for which a variety of methods have been proposed (e.g., Bruns et al. 1992, Hibbett et al. 1995, Lutzoni et al. 2000). ITS data sets will be analyzed using parsimony with branch-and-bound or heuristic methods, as described previously, including bootstrapped parsimony analyses. ML analyses will use the best trees obtained in parsimony analyses as starting trees for heuristic analyses, as described previously.

In contrast to the ITS analyses, nuc-lsu rDNA analyses will involve data sets of up to around 800 species, with multiple accessions of some species. We will analyze the nuc-lsu rDNA data using the Parsimony Ratchet (PR) (Nixon 1998), which will be implemented in PAUP* 4.0b using the program PAUPRat (Sikes & Lewis 2001). PR analyses will use equally weighted parsimony and six-parameter weighted parsimony (Cunningham 1997a; Stanger-Hall & Cunningham 1998). Weights for the latter will be estimated from transformation rates estimated with ML, using an Excel spreadsheet available from C. Cunningham (http://www.biology.duke.edu/cunningham/Methods.html). Six-parameter weighting appears to improve phylogenetic accuracy relative to equally-weighted parsimony (Cunningham 1997a; Stanger-Hall & Cunningham 1998), but it has the disadvantage that it greatly increases run time. We will perform approximately ten "batches" of equally weighted PR analyses, with 200 iterations per batch, and one batch of six-parameter weighted PR analyses, with 200 iterations. In both types of analyses, 15% of the characters will be reweighted in each iteration. We have successfully implemented PR analyses using the methods described above on datasets of up

to 656 species of homobasidiomycetes, and have consistently found that it finds shorter trees than traditional heuristic analyses that use multiple starting trees (Binder & Hibbett, in prep.; and **Preliminary Results**). We will also perform bootstrap analyses using equally weighted parsimony (non-ratchet analyses), with 100-1000 replicates. Finally, we will perform constrained PR analyses, using backbone monophyly constraint trees that reflect the strongly supported aspects of both the higher-level analyses described in **Part 1**, as well as the analyses of ITS sequences described above.

We will use global phylogenies of the Boletales based on nuc-lsu rDNA sequences to study the evolution of fruiting body forms and saprotrophic vs. mycorrhizal nutritional modes in Boletales. We will code characters in discrete form and analyze them using equally weighted parsimony methods, in MacClade 4.0 (Maddison & Maddison 2000), and ML methods, in Discrete and Multistate (Pagel 1999). For the latter, we will use the "local" method of Pagel (1999) to estimate ancestral states. Specfic questions we will address are: 1) what was the ancestral form and nutritional mode of the Boletales? 2) are there evolutionary trends (i.e., asymmetries in rates of transformations) among fruiting body forms or nutritional modes? and, 3) is there a correlation between evolution of hypogeous fruiting bodies and mycorrhizal nutritional modes? To address the last question, we will evaluate models of evolution in which the rate of evolution of hypogeous forms is or is not independent of the nutritional mode. We have used these methods previously to study the evolution of ecological and morphological characters of homobasidiomycetes (Hibbett et al. 2000; Hibbett & Donoghue 2001; Hibbett & Binder 2002).

Part 3: Monographic studies on selected genera and species-complexes in Boletaceae.

Groups to be studied and availability of materials:

We will study all available material of the genera *Afroboletus* and *Heimiella*, and the species complexes *Boletellus ananas*, *Pulveroboletus ravenelii*, *Tylopilus chromapes*, and *T. eximius*. These will provide the basis for modern monographic work on Boletales. We have extensive collections at our disposal for the proposed studies, with our strongest material assets coming from Europe and the Americas. We also have access to specimens of all six proposed study groups from the Americas, SE Asia and NE Australia (including collections by Halling). In addition, some other recent material is currently being processed (some from under-collected sites no longer accessible because of current political instability), including material from Indonesia (including *T. nanas*) and Papua New Guinea from recent work by E. Horak and R. Halling. Material of *Tylopilus* subg. *Roseoscabra* studied by Wolfe and Bougher (1993) will be available from the Pennsylvania State University, Mont Alto, herbarium (which will soon be moving to the National Fungus Collection, USDA, Beltsville MD).

We have longstanding collaborative relationships with many overseas colleagues who have agreed to aid us with additional collections and logistical support (see Letters of Support). By example, Halling has provided recent materials to Verbeken and Buyck, and they have each offered to us their fine, recent collections of Boletales from Africa and Madagascar. In addition, a pre-proposal invitation to Halling for a visit to Australia in September 2003 (Lebel, pers. com.) has already been offered, which will facilitate the overseas field work and collaboration.

Field work:

Collections provided by our collaborators will be very valuable, but we maintain that specimens for morphological and genetic evaluation ideally need to be gathered by experienced individuals to insure that appropriate observations are made, and to enable development of useful identification aids. In addition, the vouchers need to be housed under regulated conditions in an institution that is dedicated to safeguarding these materials. Based on our past experiences, it is clear that the NYBG and Halling are able to preserve vouchers and conduct field expeditions in the manner needed to accomplish our goals. Field observations and collections by Halling and collaborators in both temperate zones as well as the paleo- and neotropics have been instrumental in the past for documentation and re-evaluation of taxa of Boletales (see papers by Halling in Prior Support).

The field work proposed here will target the six focal groups listed above, and will also provide materials that will be used in the broader phylogenetic studies proposed in **Part 2**. It is

anticipated that field observations will help suggest which taxa need re-alignment and/or revision, and may lead to the discovery of new taxa that we can include in our analyses.

We plan four field trips (May/June 2004; Feb/Mar 2005; June/Jul 2005, 2006) to Australia for two persons (Halling and a graduate student or post doc.). We have chosen Australia because its Boletales flora has been little documented, but appears to be very diverse, based on available data (Bougher & Syme 1998, Grgurinovic 1997, Halling pers. obs.). We have chosen to make multiple trips because estimation of diversity of macrofungi (which may produce fruiting bodies sporadically) in any locality requires repeat visits at different times of the year. Optimal collecting times will vary (±February in NE Australia, and ±May-July, SE Australia), and we will plan specific expeditions according to local expertise. T. Lebel, Melbourne, has agreed to work with us fully in arranging fieldwork (see Letters of Support). Of particular importance are forests of Myrtaceae, a hallmark of Australian vegetation, whose ectomycorrhizal formations are just beginning to receive attention (Claridge, Castellano, Trappe pers. coms.). Materials will be gathered according to established protocols outlined by Halling (1996). Especially important to this project will be notation of potential mycorrhizal associate(s) and gathering of material in CTAB buffer and silica gel for later DNA extraction. Adherence to all local regulations have been followed without provocation in the past and will be adhered to here. Halling has an MS Access database (now under refinement with authority files) that he has been using successfully for 10 years. All field data on fresh material will be entered on the computer (according to TDWG standards) the same day after returning from a collecting trip. Upon arrival at NYBG, all relevant data will be uploaded to the NYBG server and will be accessible to the public over the Internet.

Morphological characters and methods:

We will record diverse macromorphological and micromorphological characters (see below), which will be used primarily for purposes of description and will allow other workers to identify collections without recourse to molecular data. However, based on our experience and that of other workers in basidiomycete systematics (e.g., Hibbett & Vilgalys 1993), we do not expect that morphological characters alone will be particularly useful for phylogenetic reconstruction, because they are few in number and tend to display subtle, continuous variation (Stevens, 1998). For these reasons, we do not expect to construct and analyze matrices composed solely of morphological characters. Nevertheless, we will assess the impact of morphological characters on phylogenetic hypotheses, and we will test phylogenetic hypotheses suggsted by individual characters (see below for analytical methods).

Macromorphological characters useful in the field study of mushrooms have been outlined by Halling (1996). Characters that we will study include but are not limited to odor and taste; oxidative reactions to bruised or exposed tissues ("staining reactions"); qualitative reactions to particular chemicals (weak solutions of NH_4 and KOH); and the color of fresh spore deposits. These features can be instrumental in generic or species determinations (e.g., in *Phylloporus, Xerocomus, Xanthocomium, Leccinum* s.l. and *Boletus* s.l.), but most cannot be observed in herbarium specimens. Thus, it will be critical to record these characters from fresh material, further justifying the proposed fieldwork (see above).

Micromorphological features have been used since the mid 20th century with increasing frequency. Features that we will record include the anatomical orientation and cellular makeup of the surface layers of the fruitbodies; spore ornamentation, size, and shape; and types of cellular components in the spore-producing area. These characters are easily observed via the light microscope, but require material that has been well-preserved. For taxa with ornamented spores, we will use scanning electron microscopy to provide additional useful information. Careful analysis of micromorphological characters is time consuming, but this is essential for developing useful identification tools. We have extensive expertise using all of the characters discussed above in taxonomic studies of Boletales (e.g., Halling 1996, 1999, Halling & Mueller 1999, 2001, 2002; Halling et al. 1999).

Genes to be studied:

We will investigate at least two genes in each group of Boletales that we target for monographic studies. We will use the ITS, which is very well characterized and provides resolution at low taxonomic levels in Boletales (e.g., Grubisha et al. 2002, Jarosch and Bresinsky 1999, Kretzer et al. 1996, and M. Binder, unpublished), and a highly variable portion of the (unlinked, nuclear) RPB2 locus, bounded by conserved regions 6 and 7 (approx. 0.6Kb). RPB2 primers have been published (e.g., Liu et al. 1999), and have been modified and used in our laboratory to amplify and sequence this region (see **Preliminary Results**). Molecular techniques for this part of the proposed research are the same as those described previously (see **Part 1**).

Analytical methods:

The goals of analyses in this part of the proposed research will be to infer relationships within terminal groups (genera and species complexes) and resolve species limits. Analyses of each group will be rooted using the appropriate outgroup taxa, as identified in **Part 2**. ITS and RPB2 sequences will be analyzed independently, using parsimony, ML, and Bayesian methods, as described previously (**Part 1**). There is considerable debate regarding species concepts and recognition criteria in fungi (e.g., Hibbett et al. 1995, Petersen & Hughes 1999, Taylor et al. 2000). To estimate species limits, we will employ the criterion of "genealogical concordance" (Avise & Ball 1990; Baum & Shaw 1995; Taylor et al., 2000), in which reticulation of gene genealogies among individuals (of sexual species, such as Boletales) is interpreted to be evidence of conspecificity (hybridization is thought to be extremely rare in homobasidiomycetes). This method is potentially sensitive to lineage sorting, especially when divergences are recent and populations are large, but this is a general problem that applies to other methods of species recognition as well. In practice, the genealogical concordance approach has proven to be a powerful method for resolving fungal species (e.g., O'Donnell et al. 1998a, Kasuga et al. 1999, Koufopanou et al. 1998). Tests of gene tree conflict are critical to use of the genealogical concordance criterion, and will be performed using topologically constrained analyses and the SH test, as described in **Part 1**. Ideally, more than two loci would be used in such analyses, but in general this is beyond the scope of the proposed research. Nevertheless, as time and resources permit, we we will explore additional loci, such as EF-1α, to refine species limits. Ultimately, the different loci will be combined (after pruning taxa or individuals from the dataset to eliminate conflict) to estimate relationships within terminal groups of Boletales.

As noted above, we do not intend to perform analyses of morphological data on their own. We will, however, code certain characters that have been important in taxonomy of Boletales (e.g., spore ornamentation, spore print, oxidative reactions, hyphal anatomy) and infer their evolution in the context of trees derived from molecular data, using parsimony (Maddision & Maddison 2000). We will also add these morphological characters to matrices of molecular data, and test whether their inclusion affects the results of phylogenetic analyses using equally-weighted parsimony. Finally, we will perform constrained phylogenetic analyses of molecular data that reflect groupings implied by key morphological characters (see **Part 2**, above, for methods of evaluating constrained topologies). This approach will allow us to rigorously evaluate prior taxonomy and the phylogenetic significance of key morphological characters in Boletales.

Training and outreach

Training: The proposed research is labor-intensive. Two graduate students and two post-doctoral fellows will be supported, one each at Clark University and the New York Botanical Garden. REU funds will be sought to provide research experiences for one undergraduate per year at each institution (full-time during the summer, part-time during the academic year). An annual meeting of all associated personnel to review progress and plan research will be held once a year in August at either Clark University or the New York Botanical Garden (it is about a four-hour drive between institutions). These meetings will be announced in the Mycological Society of America newsletter and web site, and will be open to all Boletales researchers. In addition, while in Australia Halling will

offer mini-workshops on Boletales taxonomy for collaborators and students. These will be modelled on similar workshops taught by Halling during the last 10 years in Latin America.
Boletales web site: In addition to technical publications, a Boletales web site will be developed, which will provide descriptions of ongoing research, as well as a synthesis of the phylogeny, biodiversity, and ecology of the group. Images, literature guides, and other resources for researchers and educators will also be provided. Development of the web site will be based at the New York Botanical Garden, whose Science pages are under renovation for future updates, ease of navigation, and ease of data search (see: http://www.nybg.org/bsci/).

Rationale for the collaboration and roles of the PIs and Co-PI

Unique qualifications of the PIs and Co-PI: The PIs and Co-PI bring unique, complementary skills and resources to this collaborative project. Halling has nearly 30 years experience in working with the Boletales, including field and herbarium studies. He has collected or has access to much of the material that will be used in the project. Binder developed and analyzed large molecular data sets of Boletales as part of his Ph.D. dissertation, with an emphasis on European materials. The DNA bank from Binder's work is a valuable resource for the proposed research. Hibbett has a longstanding interest in homobasidiomycete molecular phylogenetics and the evolution of ecological and morphological characters in homobasidiomycetes. None of the PIs are expert in all aspects of the proposed research, which involves monographic studies, molecular phylogenetics, and analyses of character evolution. It is therefore essential that this project be executed as a collaboration.
Division of responsibilities: The proposed research will be divided as follows:
Part 1 (Multi-gene phylogeny using exemplars of all major groups of Boletales and representatives of potential outgroups) will be performed at Clark University, which has all the resources necessary for this part of the project. In addition, the ongoing AFTOL project at Clark will provide data and resources that will aid this part of the proposed research.
Part 2 (Survey of diverse Boletales, using nuc-lsu rDNA and ITS sequences) will be performed at Clark and NYBG. Each institution will generate nuc-lsu rDNA and ITS sequence data, and analyses will be performed at Clark. Publications will be prepared jointly between Clark and NYBG.
Part 3 (Monographic studies on selected genera and species-complexes in Boletaceae) will be performed at NYBG. Halling will perform or oversee all morphological and anatomical studies. Molecular data for this part of the proposed research will be generated at NYBG, but Binder and Hibbett will consult regarding phylogenetic analyses, including analyses combining molecular and morphological data. Halling will supervise all fieldwork.

Significance

Intellectual merit of the proposed activity: This project research will build on prior work in systematics of Boletales, including contributions by Halling and Binder, and will contribute to a phylogenetic classification of this ecologically important clade. Well-sampled phylogenetic trees will permit analyses of the evolution of fruiting body forms and other taxonomically important characters, as well as transitions between symbiotic and free-living lifestyles.
Broader impacts resulting from the proposed activity: This project will provide resources for fungal molecular ecologists (specifically ITS sequences in GenBank) and will promote the discovery of new species of Boletales. The Boletales web site will provide additional resources for researchers and educators. This project will provide training for graduate students, post-doctoral fellows, and undergraduates. It will also facilitate exchange and cooperation with international collaborators and programs (e.g., the Australian Fungimap project, see Letters of Support). Finally, this project will promote collaboration between Clark University, a small liberal-arts institution, and the New York Botanical Garden, one of the world's major herbaria (largest in the Western Hemisphere).

Preliminary results

Part 1: Multi-gene phylogeny using exemplars of all major groups of Boletales and representatives of potential outgroups. We constructed a 34-species nuc-lsu data set from our previous work and a pruned ATP6+mt-lsu data set from sequences of Kretzer and Bruns (1999) including the same species (Fig. 1) and performed bootstrap analyses to detect conflicts between the two data sets. The analysis of nuc-lsu data resolved five nodes with bootstrap values >95% (Fig. 1A), compared to five nodes with 100% support using the ATP6+mt-lsu data set (Fig. 1B). No strongly supported conflicts (bootstrap >90%) were observed between the partitions. A combined analysis of nuc-lsu+ATP6+mt-lsu sequences yielded nine nodes with 100% bootstrap values (Fig. 1C) and provided a highly resolved "backbone" phylogeny for the major clades in the Boletales. These results suggest that combined analyses of protein-coding genes and rDNA regions will provide resolution of major clades in the Boletales. However, the combined data set provided weak support for the Sclerodermatineae (bootstrap=50%) and the Coniophorineae remained unresolved, as in the separate analyses and other studies (Jarosch & Besl 2001, Jarosch 2001).

Part 2: Survey of diverse Boletales, using nuclear large-subunit rDNA and ITS sequences.

Analysis of nuc-lsu rDNA sequences: We constructed a data set including 339 nuc-lsu rDNA sequences, representing 257 species of Boletales and 75 outgroup species, using 166 sequences from Jarosch (2001) and Grushiba et al. (2001), 113 sequences from Binder and colleagues (1997a, 1997b, 2000, 2002a, 2002b), and 60 new sequences. We performed a series of analyses using parsimony. A traditional heuristic search with 1000 replicates yielded 16 trees of 7132 steps, whereas an equally weighted parsimony ratchet (PR) analysis yielded a tree of 7128 steps, and a six-parameter weighted PR analysis yielded 10 trees of 12976 steps. A schematic version of the tree is shown in Fig. 2A; the full tree can be viewed at http://www.clarku.edu/faculty/dhibbett/Boletales.htm.

We performed a bootstrap analysis with 1000 replicates (one heuristic search/replicate). Ninety-seven terminal nodes received bootstrap support greater than 70%. Many of the groups that were resolved suggest that there have been switches in morphology and nutritional mode, such as: 1) *Leucogyrophana mollusca* (resupinate, brown rot) and *Hygrophoropsis aurantiaca* (stipitate-pileate, brown rot); 2) *Coniophora* spp. (resupinate, brown rot) and *Paxillus gymnopus* and *P. chalybaeus*. (stipitate-pileate, brown rot assumed); 3) *Serpula* spp. (resupinate, brown rot) and *Austropaxillus* (stipitate-pileate, mycorrhizal) and *Gymnopaxillus* (gasteroid, mycorrhizal). These results indicate that the nuc-lsu rDNA provides resolution of many terminal groups in Boletales and that the PR is a useful tool for analyzing large datasets.

Analysis of ITS sequences: We generated 94 ITS1-5.8S-ITS2 sequences from eight genera in the Boletaceae, including *Aureoboletus*, *Boletellus*, *Boletus*, *Chalciporus*, *Leccinum*, *Porphyrellus*, *Tylopilus*, and *Xerocomus*. ITS amplicons ranged from 0.6 to 0.87 Kb, except in *Leccinum*, where they ranged from 1.05-1.5 Kb, due to numerous 19-64 bp repeated sequences (Binder, unpublished). Alignments within each genus were straightforward, and some genera could be aligned to each other as well. We aligned ITS sequences of 26 *Leccinum* spp. that are also present in the large nuc-lsu dataset (Fig. 2A., http://www.clarku.edu/faculty/dhibbett/Boletales.htm) and performed parsimony analyses (Fig.2B). The ITS sequences provided much greater resolution than the nuc-lsu rDNA sequences, suggesting that they will be useful for studying terminal groups of Boletales.

Part 3: Monographic studies on selected genera and species-complexes in Boletaceae.

This part of the proposed research will use ITS sequences, which are well documented (see above), and partial RPB2 sequences. To assess the utility of RPB2, we generated sequences from 9 species of Boletales, and compared them to nuc-lsu rDNA sequences (Table 3, only six species included). The RPB2 dataset included 529 aligned positions and 126 informative sites (23.8%), whereas the nuc-lsu data set included 925 aligned positions and 77 informative sites (8.3%). These results suggest that RPB2 has adequate variation at the nucleotide level to resolve terminal groups in Boletales.

Table 3. Pairwise distance comparisons of RPB2 and nuc-lsu sequences. Above diagonal: RPB2 distances; below diagonal: nuc-lsu distances.		1	2	3	4	5	6
	1 *Leccinum crocipodium*	---	0.104	0.260	0.229	0.250	0.256
	2 *Boletus satanas*	0.064	---	0.239	0.232	0.225	0.238
	3 *Suillus cavipes*	0.098	0.063	---	0.109	0.208	0.248
	4 *Suillus americanus*	0.104	0.067	0.033	---	0.209	0.240
	5 *Serpula himantioides*	0.110	0.071	0.074	0.075	---	0.251
	6 *Coniophora arida*	0.110	0.087	0.089	0.098	0.074	---

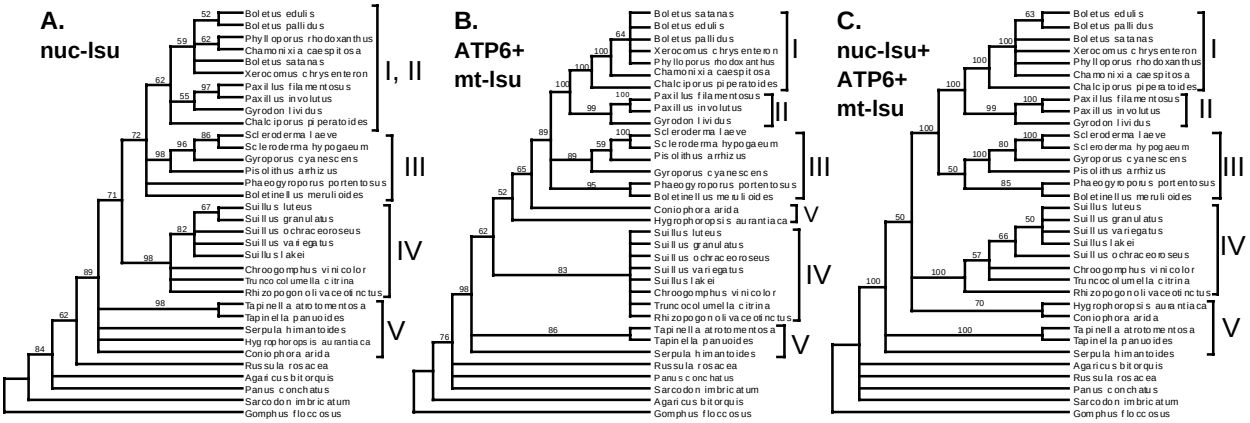

Figure 1. Higher-level relationships of the Boletales inferred from multiple genes using bootstrapped parsimony analyses (1000 replicates). **A.** nuc-lsu data set, **B.** mt-lsu data combined with ATP6 data from Kretzer and Bruns (1999), **C.** nuc-lsu, mt-lsu, and ATP6 data sets combined. I = Boletineae, II = Paxillineae, III = Sclerodermatineae, IV = Suillineae, V = Coniophorineae.

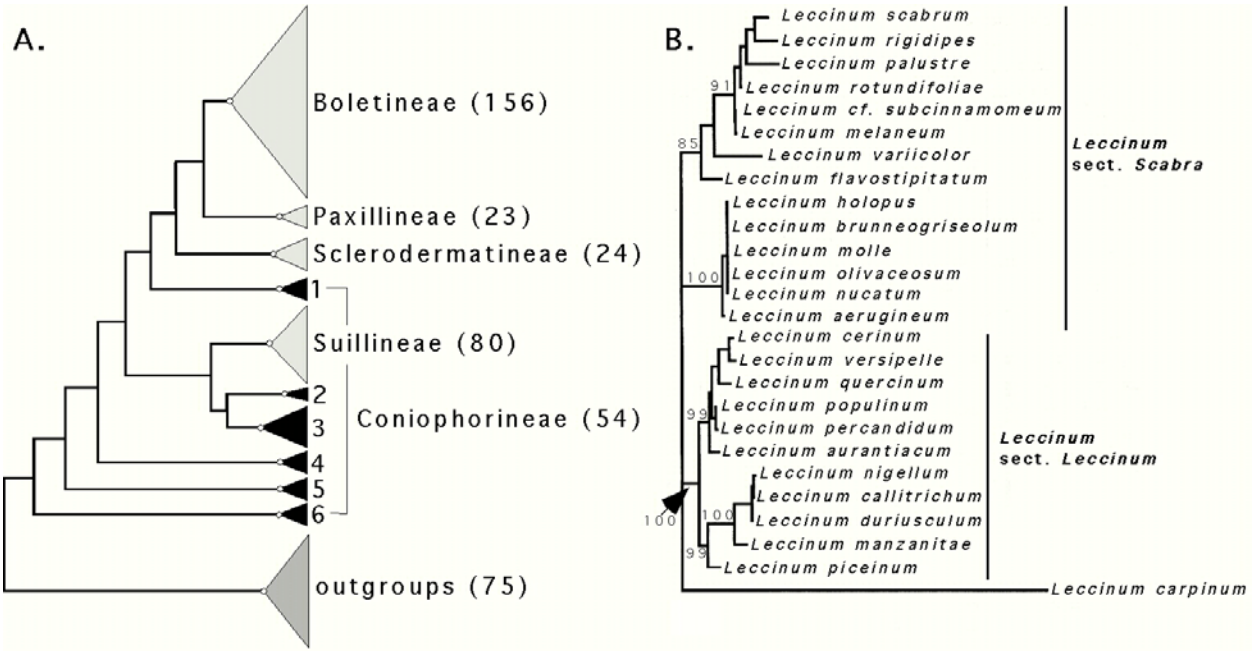

Figure 2. A: Phylogeny of Boletales based on weighted parsimony ratchet analysis of 339 nuc-lsu rDNA sequences; strict consensus of 10 trees, 12976 steps, CI=0.179, RI=0.718). **B:** Relationships of *Leccinum* spp. based on parsimony analyses of ITS sequences (one of 5 trees, 943 steps, CI=0.820, RI=0.896).