

## Molecular systematics and biological diversification of Boletales

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**Abstract:** Historical patterns of morphological evolution and ecology in the Boletales are largely unresolved but appear to involve extensive convergence. We studied phylogenetic relationships of Boletales based on two datasets. The nuc-lsu dataset is broadly sampled and includes roughly 30% of the described species of Boletales and 51 outgroup taxa across the Hymenomycetes. The multigene dataset (nuc-ssu, nuc-lsu, 5.8S, mt-lsu, *atp6*) sampled 42 key species of Boletales in a framework of 14 representative Hymenomycetes. The Boletales are strongly supported as monophyletic in our analyses on both datasets with parsimony, maximum likelihood and Bayesian approaches. Six major lineages of Boletales that currently are recognized on subordinal level, Boletineae, Paxillineae, Sclerodermatineae, Suillineae, Tapinellineae, Coniophorineae, received varied support. The backbone of the Boletales was moderately resolved in the analyses with the nuc-lsu dataset, but support was strong for most major groups. Nevertheless, most brown-rot producing forms were placed as a paraphyletic grade at the base of the Boletales. Analyses on the multigene dataset confirm sister group relationships among Boletales, Agaricales and Atheliales. Boletineae and Suillineae received the highest support values; Paxillineae and Sclerodermatineae were not consistently resolved as monophyletic groups. The Coniophorineae were not monophyletic in any analyses. The Tapinellineae consisting of morphologically diverse brown-rotting fungi forms the basal group in the Boletales. We performed ancestral state reconstruction with BayesMultiState, which suggested that the ancestor of the Boletales was a resupinate or polyporoid saprotrophic fungus, producing a brown-rot.

**Key words:** evolution, morphology, MRCA, multigene analyses, nuc-lsu rDNA, nutritional modes

### INTRODUCTION

The Boletales (Agaricomycetidae) is one of the major groups of mushroom-forming fungi that is represented in most forest ecosystems worldwide. This order contains approximately 1000 described species (Kirk et al 2001), which might be an underestimate considering that larger parts of the neotropics and the paleotropics are still understudied, where Boletales have reached most notable divergence. The Boletales includes conspicuous stipitate-pileate forms that mainly have tubular and sometimes lamellate hymenophores or intermediates that show transitions between the two types of hymenophores (Gilbert 1931). The Boletales also includes gasteromycetes (puffball-like forms), resupinate or crust-like fungi that produce smooth, meruloid (wrinkled to warted), or hydroid (toothed) hymenophores, and a single polypore-like species, *Bondarcevomyces taxi* (e.g. Besl and Bresinsky 1997, Jarosch 2001, Larsson et al 2004). In view of the existing diversity of fruiting body forms, there has been extensive homoplasy in the evolution of Boletales and there is no obvious morphological character that unites the group (FIG. 1). Species in Boletales pursue diverse habits, but unlike in their sister clades (Agaricales and Atheliales) white-rot saprotrophy is absent in the group (Binder et al 1997). Instead, saprotrophs among Boletales have developed a unique mode of brown-rot called “Coniophoraceae-rot” (Kämmerer et al 1985, Besl et al 1986) that primarily is aimed at decaying wood of conifers. Mycorrhizal associations are established by the majority of Boletales, and host plants include Betulaceae, Casuarinaceae, Dipterocarpaceae, Ericaceae, Fabaceae, Fagaceae, Mimosaceae, Myrtaceae, Pinaceae and Salicaceae (Newman and Reddell 1987). Some Boletales are mycoparasites, a deviation of either the saprotrophic or ectomycorrhizal mode that is limited to some species in Boletaceae and in Gomphidiaceae (Agerer 1991, Raidl 1997).

In recent years Boletales have been studied widely by fungal systematists, chemists, ecologists and mycorrhizal biologists (e.g. Agerer 1987–1998, Arpin and Kühner 1977, Besl and Bresinsky 1997, Both 1993, Gill and Steglich 1987, Moser 1983, Singer 1986, Smith and Thiers 1971, Watling 1970). Six suborders have been established that are thought to represent the major lineages of Boletales: Boletineae, Paxillineae, Sclerodermatineae, Suillineae, Coniophorineae, and Tapinellineae. Remarkably, phylogenetic inferences were used rarely to improve higher-level



FIG. 1. Morphological diversity in Boletales. a. *Bondarcevomyces taxi*; b. *B. taxi*, pores; c. *Coniophora puteana*; d. *Leucogyrophana mollusca*; e. *Hygrophoropsis aurantiaca*; f. *Suillus granulatus*; g. *Chroogomphus vinicolor*; h. *Boletinus merulioides*, hymenophore; i. *Calostoma cinnabarinum*; j. *Scleroderma septentrionale*; k. *Meiorganum neocaledonicum*, young hymenophore; l. “*Tylopilus*” *chromapes*; m. *Phylloporus centroamericanus*; n. *Xerocomus* sp. Pictures a and b courtesy Y.-C. Dai; m courtesy M.-A. Neves.

classifications in Boletales but had a synergistic effect on traditional and interdisciplinary methods in general. For example, the well established study of chemistry of Boletales pigments and other colorless secondary metabolites helped the application of chemotaxonomy to separate the Suillineae from Boletineae (Besl and Bresinsky 1997), in which the large preponderance of species with tubular hy-

menophores were placed at that time. Innovative methods shifted the focus from fruiting body morphology to below ground characters and the recognition of rhizomorphs and substrate hyphae as morphologically conserved characters led to the description of Tapinellineae and Coniophorineae in the study of Agerer (1999). Early phylogenetic studies on Boletales examined relationships between stipi-



tate-pileate and gasteroid forms and explored rate differences in base substitutions between nuclear and mitochondrial genes (Bruns et al 1989, Bruns and Szaro 1992). Several phylogenetic analyses with focus on systematics, using nuclear and mitochondrial rDNA, suggest that the Boletales is monophyletic (Binder and Bresinsky 2002a, Binder et al 2005, Bruns et al 1998, Grubisha et al 2001, Jarosch 2001, Kretzer and Bruns 1999) and show that Agaricales and Atheliales are sister groups of the Boletales (Hibbett and Binder 2002, Larsson et al 2004, Binder et al 2005).

This study combines the efforts from previous studies and provides 134 new sequences for 60 species. One objective was to assemble a multigene dataset (nuc-ssu, nuc-lsu, 5.8S, *atp6*, mt-lsu) to resolve sister-group relationships among Boletales, Agaricales and Atheliales and to test the monophyly of major groups in the Boletales with maximum likelihood and Bayesian methods. The second objective was to generate a most inclusive nuc-lsu dataset and to analyze it with Bayesian methods. The results of this analysis were used to estimate probabilities of ancestral states of morphology and nutritional mode for supported nodes with BayesMultiState (Pagel et al 2004).

#### MATERIALS AND METHODS

*Taxon sampling and molecular datasets.*—One hundred thirty-four sequences that were newly generated for this study included three nuclear rDNA regions (nuc-ssu, nuc-lsu, ITS) and two mitochondrial genes (*atp6*, mt-lsu). The sequences have been deposited in GenBank (DQ534563–DQ534696, SUPPLEMENTARY TABLE I, II, SUPPLEMENT). For DNA extraction protocols, PCR, cloning, sequencing and sequence alignment, refer to APPENDIX I and to the studies of Bruns et al (1998), Kretzer and Bruns (1999), Binder et al (2005) and references therein. Two datasets were assembled, a multigene dataset (nuc-ssu, nuc-lsu, 5.8S, *atp6*, mt-lsu) with 56 terminals and a broadly sampled nuc-lsu dataset with 485 terminals. All analyses were performed on a Linux Pro 9.2 Opteron AMD 246 cluster (Microway) unless otherwise noted. Both alignments were submitted to TreeBASE (SN2858). More information on alignment procedures and command blocks running MrBayes v3.1.1 (Ronquist and Huelsenbeck 2003) and PAUP\* 4.0b10 (Swofford 2002) are available (APPENDIX 1).

The multigene dataset included 42 species sampled across the six suborders of Boletales and 14 outgroup species. The studies by Bruns et al (1998) and Kretzer and Bruns (1999) provided the core data for the multigene dataset, which was expanded with 73 new sequences. Several genes were not amplified successfully in these species: *atp6* for *Athelia arachnoidea*, *Austropaxillus* sp., *Coniophora marmorata*, *Leucogyrophana mollusca*, *Pseudomerulius aureus*; mt-lsu for *Fomitiporia mediterranea*, *Melanogaster var-*

*iegatus*, *C. marmorata*, *Suillus spraguei*, *Porphyrellus porphyrosporus*; ITS for *Dendrocorticium roseocarneum*; and nuc-ssu for *Scleroderma hypogaeum* and *Suillus ochraceoroseus*.

The nuc-lsu dataset included 301 species of Boletales, which is roughly 30% of the described species in this order. In addition, some of these species were represented by multiple sequences (133 in total) and 51 outgroup species were selected to represent the major clades of homobasidiomycetes. Sequence data of outgroup species were gathered largely from the studies of Moncalvo et al (2002), Hibbett and Binder (2002) and Larsson et al (2004). Three hundred twenty-four sequences of Boletales were drawn from published studies (Binder et al 1997; Binder and Besl 2000; Binder and Bresinsky 2002a, b; Bresinsky et al 1999; Hughey et al 2000; Grubisha et al 2001; Jarosch 2001; Jarosch and Besl 2001; Peintner et al 2003). Fifty sequences that originate from unpublished studies (James et al, Carlier et al) are available from GenBank. We generated new sequences of 61 species for the nuc-lsu dataset.

*Phylogenetic analyses of the multigene dataset.*—To test for congruence among nuclear (nuc) and mitochondrial (mt) genes, a preliminary series of parsimony bootstrap analyses was performed in PAUP\*. Five separately estimated gene phylogenies using nucleotide data were obtained running 1000 replicates, all characters equally weighted, 10 random taxon addition sequences, tree bisection reconnection (TBR) branch swapping, with MAXTREES set to 10 000. None of the positively conflicting nodes between partitions received bootstrap support >63%, and the data were combined to a single dataset encompassing 3939 aligned positions. A bootstrap analysis then was performed on the concatenated dataset with the previously described settings.

The multigene dataset was analyzed further with maximum likelihood (ML) and a Bayesian approach (Metropolis-coupled MCMC or MC<sup>3</sup>). Six-parameter models were estimated as the best-fit likelihood models with Modeltest 3.06 (Posada and Crandall 2001) for all five partitions (GTR+ $\Gamma$ +I for nuc partitions, TVM+ $\Gamma$  for *atp6*, and TVM+ $\Gamma$ +I for mt-lsu), while there was considerable variation among model parameters between nuc and mt partitions. To perform the ML analysis the GTR+ $\Gamma$ +I model was specified with proportion of invariable sites and distribution of rates at variable sites modeled on a discrete gamma distribution ( $\alpha = 0.4$ ) with four rate classes. The substitution rate matrix was set to empirical frequencies and the proportion of invariable sites was estimated during the run. The ML analysis was started with a user-defined starting tree obtained with neighbor joining.

The GTR+ $\Gamma$ +I model also was specified in the MC<sup>3</sup> analysis as prior for both nuc and mt partitions, assuming equal probability for all trees and unconstrained branch length. The substitution rate matrix, transition/transversion rate ratio, character state frequencies, gamma shape parameter  $\alpha$  and proportion of invariant sites were unlinked across nuc and mt partitions and calculated independently by MrBayes. Posterior probabilities were determined twice by running one cold and three heated chains for  $10 \times 10^6$  generations in parallel mode, saving trees every 100th

generation. A 50% majority rule consensus tree was used to calculate posterior probabilities including the proportion of trees gathered after the convergence of likelihood scores was reached.

*Phylogenetic analyses of the nuc-lsu dataset (1071 positions).*—They were performed with a Bayesian MC<sup>3</sup> approach. Two parallel MC<sup>3</sup> analyses were run under the GTR+ $\Gamma$ +I model using four chains and an extended run time employing  $50 \times 10^6$  generations, saving trees every 100th generation. Posterior probabilities were calculated as previously described.

Ancestral state reconstruction was performed with most recent common ancestor (MRCA) analysis implemented in BayesMultiState v1.0.2 in maximum likelihood mode (Pagel et al 2004). Ancestral state reconstructions, based on either parsimony or maximum likelihood, are performed frequently with a single input tree, which implies that the phylogeny is known with certainty (e.g. Hibbett 2004). Such an assumption usually is not warranted. In contrast, the Bayesian approach combines probability estimates of ancestral traits across a statistically justified sample of trees, which effectively factors out phylogenetic uncertainty (e.g. Lutzoni et al 2001). BayesMultiState was used to estimate the probabilities of ancestral character states for morphology and nutritional mode at eight nodes, including the root node of the Boletales and seven nodes within the Boletales. Each of the eight nodes supported a group that was resolved as monophyletic in all trees recovered from the MC<sup>3</sup> analyses, except the Sclerodermatineae, which was resolved as monophyletic in 89% of the trees.

To reduce the computational burden, ancestral states were estimated with a sample of 250 trees recovered from the stationary tree distribution of the MC<sup>3</sup> analysis. These 250 trees represent all the unique topologies present in the set of trees sampled in the MC<sup>3</sup> analyses and were obtained with the tree filtering functions in PAUP\*. MRCA analysis accommodates compositional heterogeneity of clades, such as that exhibited by the Sclerodermatineae, by estimating ancestral states across trees at the shallowest node that subtends all the species assigned to the clade. In other words, if a group of species assigned to a clade at the outset of the analysis is not monophyletic on one of the trees analyzed, the MRCA approach estimates the state at the most recent common ancestor of those species in all of the trees (Pagel et al 2004). Two trait input files were constructed in the PAUP\* editor that coded for morphology and nutritional mode (five states each) of all species in the dataset (see SUPPLEMENTARY FIGURE 1 for the coding regime). Morphology was coded as: 0 = stipitate-pileate with tubes; 1 = stipitate-pileate with gills; 2 = gasteroid; 3 = resupinate; 4 = polyporoid. Intermediate states were accommodated with a combined state identifier (e.g. the stipitate-pileate hydroid fungus *Sarcodon imbricatus* was coded as 01). Coding for the nutritional mode included: 5 = potentially ectomycorrhizal; 6 = brown-rot saprotroph; 7 = white-rot saprotroph; 8 = mycoparasitic; – = uncertain state (which is treated as if the trait can be any of the other states 5–8). The MRCA analyses were set up to reconstruct eight specified nodes (TABLE I) and were run separately for

both trait files under the ML criterion performing 10 independent optimizations per tree. All MRCA analyses were run on a Powermac G5 via Darwin in OS  $\times$  10.4.3.

## RESULTS AND DISCUSSION

*Higher-level relationships of Boletales.*—Recent phylogenies using increased taxon sampling and multiple gene loci (Binder et al 2005, Hibbett and Binder 2002, Larsson et al 2004, Matheny et al 2006) consistently resolve a large clade that contains Agaricales, Atheliales and Boletales—the Agaricomycetidae. As in previous studies, sister relationships within the Agaricomycetidae remain ambiguous and receive varied support. The Atheliales is resolved as sister group of the Boletales in our multigene analyses (FIG. 2). This relationship is supported strongly by posterior probabilities (PP), however bootstrap support (BS) is weak (<50%). A sister relationship of Atheliales and Boletales also was inferred from combined nuc-lsu and 5.8S rDNA data by Larsson et al (2004) without receiving statistical support. Phylogenetic analyses of the nuc-lsu dataset in the present study (SUPPLEMENTARY FIG. 1) place the Agaricales as sister group of the Atheliales, again supported by high posterior probabilities. It therefore is important to identify the basal groups in all three orders and to generate comprehensive multigene data to resolve relationships within the Agaricomycetidae.

*Resolving the major clades of Boletales.*—Both nuc-lsu and multigene phylogenies support the Boletales as a monophyletic order and this result is consistent with previous studies (Binder and Bresinsky 2002a, Bruns et al 1998, Grubisha et al 2001, Jarosch 2001, Kretzer and Bruns 1999). Our approach to present two disparate datasets needs to be seen as a transitional stage between using the most inclusive taxon sampling and a steadily increasing availability of multiple genes sampled for the same set of species. Brown-rot producing saprotrophs were recovered as the earliest branching groups in this study and in most aforementioned analyses, however, assessing branching order among these clades proves to be difficult. The study by Kretzer and Bruns (1999) combined two mitochondrial loci (*atp6* and *mt-lsu*) for 23 members of the Boletales for the first time and resolved the Tapinellineae as basal lineage, but the position of other brown-rotting fungi was not well supported. Inferences of the multigene data in this study (FIG. 2) show that extended taxon sampling and the addition of three rDNA genes improves the overall resolution of major groups in Boletales but still is not answering all questions about sister relationships. Our results suggest that there might be as many as eight monophyletic lineages in the Boletales and that the

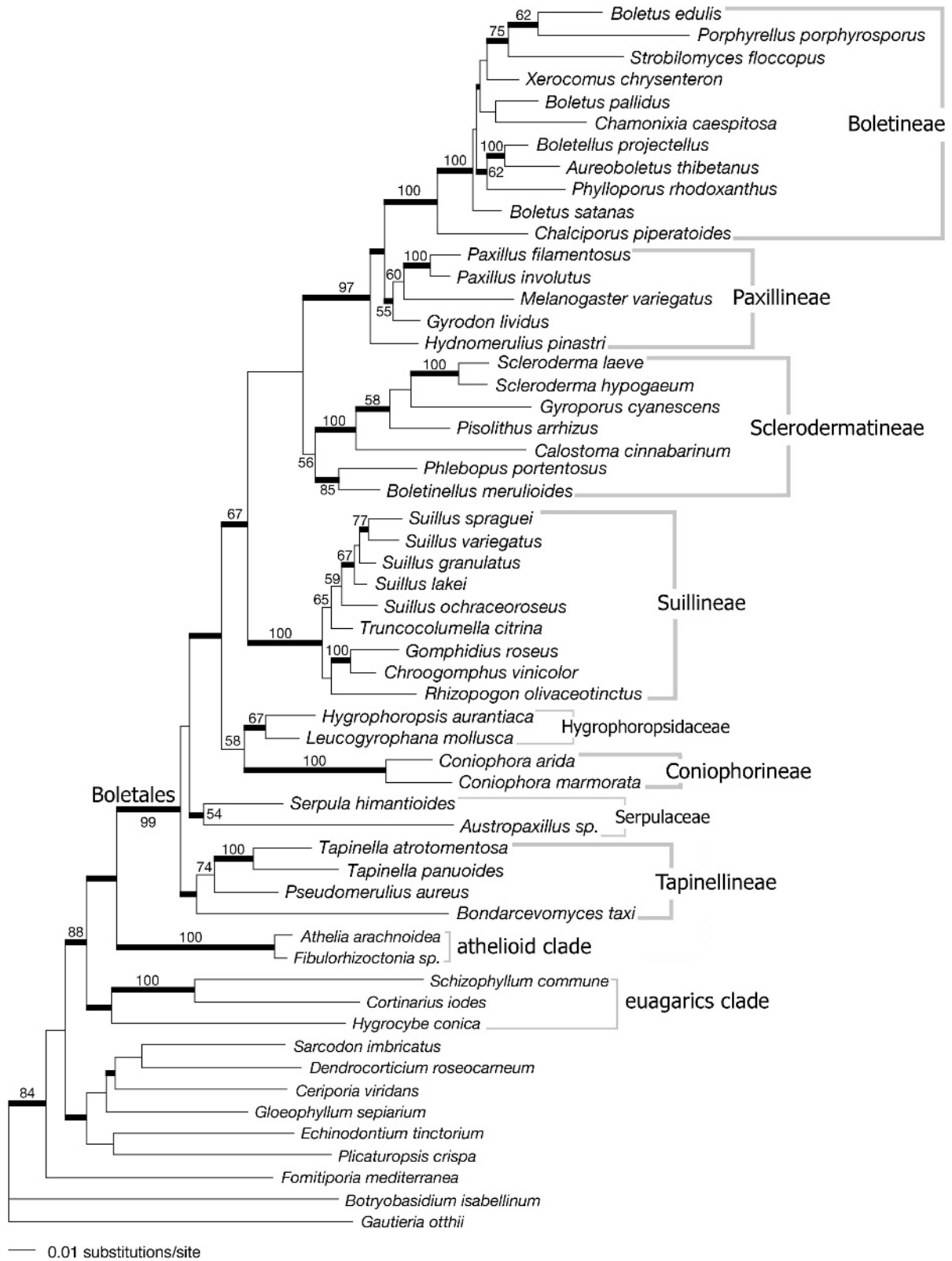


FIG. 2. Phylogenetic relationships of Boletales inferred from the multigene dataset (nuc-ssu, nuc-lsu, 5.8S, *atp6*, mt-lsu) under the ML criterion ( $-\ln L = 38227.648$ ). The dataset included 3791 characters after the exclusion of ambiguously aligned 148 characters. Bootstrap frequencies  $>50\%$  are shown at supported branches. Posterior probabilities 0.98–1.0 obtained in the Bayesian analyses are indicated by bold nodes. The major clades of Boletales and the sister groups of Boletales, Agaricales and Atheliales are indicated with brackets.



Tapinellineae and Coniophorineae form the basal clades. The Coniophorineae including Coniophoraceae, Serpulaceae and Hygrophoropsidaceae is not monophyletic and forms three independent groups. A sister relationship of Coniophoraceae and Hygrophoropsidaceae is weakly supported (BS = 58%) in the multigene analysis, however, additional *Leucogyrophana* spp. break up this relationship in the trees inferred from the nuc-lsu dataset (SUPPLEMENTARY FIG. 1). This result is consistent with the study of Jarosch and Besl (2001), showing that a morphologically well characterized genus *Leucogyrophana* is polyphyletic and accordingly should be divided into several new genera.

The more derived groups in the Boletales comprising Suillineae, Sclerodermatineae, Paxillineae and Boletineae form a clade with varying statistical support (PP = 0.98, BS = 67%). These four suborders include the majority of stipitate-pileate mushrooms with lamellate hymenophores (= agaricoid) and tubular hymenophores (= boletoid), false-truffles and earthballs, and also the majority of ectomycorrhizal forms. The Suillineae receives high support values (PP = 1.0, BS = 100%), although branching order within the clade is not resolved with confidence. The group includes gasteromycetes (*Rhizopogon*, *Truncocolumella*), agaricoid forms (*Gomphidius*, *Chroogomphus*) and boletoid fungi (*Suillus*). The Sclerodermatineae received weak support in the multigene analyses (BS = 56%) and strong support in the analyses on the nuc-lsu dataset (PP = 1.0). The group includes a few boletoid forms (*Boletinellus*, *Phlebopus*, *Gyroporus*) and an overwhelming diversity of gasteromycetes (Fischer 1899–1900). For example, *Scleroderma* spp. have compact peridia enclosing the gleba, *Pisolithus* spp. produce spore containing peridioles that resemble those in the Nidulariaceae (Agaricales), *Calostoma* spp. produce gelatinous-stalked fruiting bodies with multiple peridial layers, *Astraeus* spp. resemble the earthstars in the Geastrales, *Diplocystis wrightii* produces individual fruiting bodies congregated on shared stromata and *Tremello-gaster surinamensis* produces heavy fruiting bodies with strongly gelatinized peridial layers that superficially are similar to those of species in the Phallales. The genus *Gyroporus* is nested within the gasteromycetes. The Boletinellaceae (*Boletinellus*, *Phlebopus*) usually is resolved as basal group (Binder and Bresinsky 2002a, Hughey et al 2000) but sometimes forms an independent sister clade of the remaining Sclerodermatineae (e.g. Kretzer and Bruns 1999).

Family concepts in the Paxillineae are still in flux as reflected by a proposal to adopt the Paxillaceae in a wider sense by including Gyrodontaceae and Melanogastraceae (Bresinsky et al 1999). *Gyrodon*

spp. are morphologically similar to *Boletinellus* spp. in the Sclerodermatineae, which contributed much to taxonomic uncertainty (Binder and Bresinsky 2002a). Another question regards the extent and monophyly of Melanogastraceae. This family includes the ectomycorrhizal false-truffles *Alpova* and *Melanogaster* (Trappe 1975), which form two independent clades in this study. The Paxillineae is resolved as sister group of the Boletineae (PP = 1.0, BS = 97%) in the multigene analyses. In contrast to previous studies (Bresinsky et al 1999, Jarosch 2001) the Paxillineae formed either a paraphyletic or a polyphyletic group in our analyses. Without receiving a strong phylogenetic signal, the Paxillineae is sustained possibly as natural group by the production of secondary metabolites that are unique in the Boletales (Besl et al 1996).

The Boletineae is the most species-rich group of stipitate-pileate fungi with tubular hymenophores in the Boletales and also includes a few species with lamellate hymenophores and gasteroid forms. Support values for this suborder are high in the multigene analyses (PP = 1.0, BS = 100%) and *Chalciporus* occupies the basal position in the clade. The analyses on the nuc-lsu dataset (SUPPLEMENTARY FIG. 1) with increased taxon sampling show that relationships among genera are poorly resolved and moreover that most of the larger genera (e.g. *Boletus*, *Tylopilus*, *Xerocomus*) are not monophyletic.

*Morphological and ecological evolution.*—Important but also challenging key questions in the evolution of mushroom-forming fungi concern the directionality of morphological and ecological traits and their potential reversibility (Bruns and Shefferson 2004, Hibbett 2004, Hibbett et al 2000). The majority of species in the Boletales are thought to enter ectomycorrhizal symbioses, even though this assumption is based largely on observations in the field that need to be confirmed by additional evidence. Fortunately, there is increasing interest in documenting ectomycorrhizal fungi and collaborative projects, such as DEEMY (<http://www.deemy.de>) and UNITE (<http://unite.zbi.ee>; Kõljalg et al 2005), have become valuable resources for systematists and ecologists. Other species in the Boletales are brown-rot saprotrophs, especially on coniferous trees, and a few species are host specific mycoparasites that attack other Boletales. The Boletales also includes a great diversity of fruiting body forms (FIG. 1, clavarioid and coralloid fungi are absent in this group however) and therefore provides an excellent model to study character evolution on a relatively manageable scale. This study used multistate coding combined with a ML approach to estimate probabilities of ancestral states

optimized on eight nodes (SUPPLEMENTARY FIG. 1, TABLE I) that were resolved in the analyses of the nuc-1su dataset: Tapinellineae, Coniophorineae, Serpulaceae, Hygrophoropsidaceae, Suillineae, Sclerodermatineae, Boletineae and Boletales.

*Fruiting body evolution.* The ancestral morphological form of the Boletales was estimated as either resupinate ( $P = 0.545$ ) or polyporoid ( $P = 0.366$ ) and we interpret this result as inconclusive. Similarly, the ancestral fruiting body form of the Tapinellineae, which is placed at the base of the Boletales, was estimated as either resupinate or polyporoid, although the polyporoid condition received a higher probability. *Bondarcevomyces taxi* (FIG. 1a, b) is the only known polypore in the Boletales and its placement next to the resupinate fungus *Pseudomerulius aureus* in the Tapinellineae, which recently was discovered in the study by Larsson et al (2004), is a startling finding that challenges previous views of the morphological evolution in this group. Morphological transformations from resupinate to polyporoid fruiting bodies or vice versa are not uncommon in other fungal groups (Binder et al 2005), however the directionality of events appears to be nonuniform. The Hymenochaetales (Hymenochaetales) might serve as a good example because the family includes several genera (e.g. *Phellinus*, *Fomitiporia*) in which both fruiting body forms occur side by side and often represent cryptic species complexes (Fischer and Binder 2004). Taken together, our results suggest that at least five independent transformations from resupinate forms to stipitate-pileate forms with lamellate hymenophores have occurred in the basal lineages of Boletales. *Leucogyrophana olivascens* and *L. romellii* represent a clade entirely composed of resupinate fungi and they obviously are not closer related to stipitate-pileate forms. The Paxillineae were not reconstructed in the MRCA analyses but include another resupinate form, *Hydnomerulius pinastri*.

Moving up the tree, the results of the MRCA analyses strongly suggest that the most recent common ancestors of Serpulaceae, Hygrophoropsidaceae and Coniophorineae were resupinate forms. Extant resupinate forms (FIG. 1c, d) in these taxa include fungi with smooth hymenophores (*Coniophora*, *Leucogyrophana*) and meruloid hymenophores (*Serpula*, *Leucogyrophana*) and multiple transitions from resupinate fruiting bodies to stipitate-pileate fruiting bodies with lamellate hymenophores can be inferred in all three groups. For example, "*Paxillus*" *gymnopus* and "*P.*" *chalybaeus* are nested within *Coniophora*, *Austropaxillus* is the sister group of *Serpula* and *Leucogyrophana mollusca* (FIG. 1d) forms a clade with the false cantharelle *Hygrophoropsis aurantiaca* (FIG. 1e). All these relationships have

been suggested by Besl et al (1986) using the pigment chemistry of secondary metabolites as a comparative marker, and their findings found strong support in recent phylogenetic studies (Bresinsky et al 1999, Jarosch 2001, Jarosch and Besl 2001).

The tubular hymenophore type that is symptomatic for Boletales occurs in Suillineae, Sclerodermatineae, Paxillineae and Boletineae. The stipitate-pileate form with a tubular hymenophore is resolved as the ancestral state of the Boletineae and Sclerodermatineae. The Paxillineae also includes such typical boletoid forms, but its ancestral state was not estimated because this group was not resolved as monophyletic (FIG. 2, SUPPLEMENTARY FIG. 1; TABLE I). The clade including Boletineae, Paxillineae and Sclerodermatineae is not strongly supported. Nevertheless, it is most parsimonious to infer that the common ancestor of these groups had a boletoid form. If so, then the gasteroid taxa in all three suborders, and the lamellate taxa in the Paxillineae and Boletineae, must have been derived ultimately from boletoid forms.

Gasteromycetation occurs in most lineages of Boletales except Tapinellineae, Coniophorineae and Hygrophoropsidaceae. In most cases the ancestral states of clades containing gasteroid forms were resolved as nongasteroid. However, we obtained a surprising result in the Suillineae, which includes boletoid, agaricoid and gasteroid forms (TABLE I). The ancestral state of the Suillineae was supported strongly as gasteroid, implying parallel evolution of boletoid and lamellate forms and secondary evolution of ballistospory via reversals of gasteromycetation. This contradicts the generally accepted view that the loss of ballistospory is irreversible (Hibbett et al 1997, Savile 1955, Thiers 1984) as well as the specific findings of Bruns et al (1989), who suggested that *Rhizopogon* species are derived from within the suilloid clade by selection for animal dispersal and reduction of water loss.

Our finding that the ancestor of the Suillineae was gasteroid could be due to an error in phylogenetic reconstruction. Paraphyly of *Rhizopogon* has been suggested by several other phylogenetic studies (Binder and Bresinsky 2002a, Grubisha et al 2001, Jarosch 2001, Kretzer and Bruns 1999). The placement of *Rhizopogon* in our multigene analyses (FIG. 2), forming an unsupported sister group of the remaining Suillineae together with Gomphidiaceae, might be an artifact caused by asymmetric nuclear and mitochondrial base-substitution rates in different branches of the Suillineae (Bruns and Szaro 1992). Nevertheless, the analyses on the nuc-1su dataset with 25 *Rhizopogon* species produce a similar topology with a paraphyletic *Rhizopogon* at the base of

TABLE I. Probabilities of ancestral morphological and nutritional states at eight nodes in the Boletales estimated using MRCA

Node	1	2	3	4	5	6	*	7	8
Trait	Tapinellineae	Serpulaceae	Coniophorineae	Hygrophoropsidaceae	Suillineae	Sclerodermatineae	Paxillineae	Boletineae	Boletales
stipitate-pileate, tubes	—	—	—	—	√	√ 0.908	√	√ 0.709	√
stipitate-pileate, gills	√	√	√	√ 0.144	√ 0.990	—	√	√	√
gasteroid	—	√ 0.907	√ 0.955	√ 0.854	—	—	√	√ 0.245	√
resupinate	√ 0.226	—	—	—	—	—	—	—	√ 0.545
polyporoid	√ 0.672	—	—	—	—	—	—	—	√ 0.366
ectomycorrhizal	—	—	—	—	√ 0.983	—	—	—	—
mycoparasitic	—	—	—	—	—	√ 0.697	—	√ 0.938	—
brown-rot saprotrophic	√ 0.999	√ 0.999	√ 0.999	√ 0.999	—	—0.196	—	—	√ 0.936
white-rot saprotrophic	—	—	—	—	—	—	—	—	—

√ = character present, — = character absent; probabilities ( $P = 0-1$ ) are represented as arithmetic means across 250 input trees. Non-significant probabilities ( $P < 0.05$ ) are not shown. \*Paxillineae were either paraphyletic or polyphyletic and the node was therefore not reconstructed.

Suillineae (SUPPLEMENTARY FIG. 1). Of course, the position of *Rhizopogon* in our trees might be correct and the ancestral state of the Suillineae that was estimated with a ML approach might be an artifact caused by the use of an inappropriate model for fruiting body evolution (for a discussion of the use of Markov models to understand morphological evolution see Felsenstein 2004).

*Ecological evolution.* The results of the MRCA analyses suggest that brown-rot is the ancestral state for Boletales. Brown-rot also is estimated as the ancestral nutritional mode for Tapinellineae, Coniophorineae, Serpulaceae, Hygrophoropsidaceae and therefore might have evolved a single time in the basal clades of Boletales. A single switch from brown-rot to ectomycorrhiza emerges in the Serpulaceae leading from resupinate *Serpula* spp. saprotrophs to agaricoid *Austropaxillus* spp. and gasteroid *Gymnopaxillus* spp., which are associated with *Nothofagus* and *Eucalyptus* (Claridge et al 2001). Because *Tapinella* and *Hygrophoropsis* have maintained a saprotrophic survival system, changing the nutritional mode to ectomycorrhizal evidently is not correlated with the gain of agaricoid morphology. *Buchwaldoboletus lignicola*, reportedly a brown-rot fungus, is described growing on stumps of conifers, woody debris or needle litter (Pilát 1969). At first view this lifestyle is exceptional in the Boletineae and appears to be a prime example of a reversal from mutualism to saprotrophism. The results in the study of Szczepka and Sokól (1984) suggest that *B. lignicola* is able to grow only on wood that was decayed by the polypore *Phaeolus schweinitzii* and thus contradict that *B. lignicola* actually causes the brown-rot. These findings show evidence of an ecological specialization and close association between both fungi, however, we question whether the ecology of *B. lignicola* has been researched sufficiently.

Our ancestral state reconstructions suggest that mycoparasitism in Boletales is derived from ectomycorrhizal forms. An example from the Suillineae indicates that competition for food is most likely a crucial factor that triggers parasitic interactions among closely related groups. Species in Suillineae associate with Pinaceae and frequently produce their fruiting bodies nearby. *Chroogomphus* and *Gomphidium* spp. are penetrating already established ectomycorrhizae of *Suillus* and *Rhizopogon* spp. and access nutrition by sending haustoria into the rhizomorph hyphae of the fungal host or even into the cortical cells of the plant partner (Agerer 1987–1998, 1990, 1991; Miller 1964; Olsson et al 2000). The parasites produce clamydospores (asexual spores) inside the ectomycorrhiza and initiate fruiting body formation from primordia that develop on the rhizomorphs of



the exploited fungal host (Agerer 1990, 1991). The nutritional mode in the Boletinellaceae is still somewhat elusive and appears to include generalists, root parasites and ectomycorrhizal fungi (Brundrett and Kendrick 1987, Singer 1986). For example *Phlebobius tropicus* forms lethal symbioses with scale insects (*Pseudococcus comstockii*) that attack the roots of *Citrus* trees (Singer 1986 p 744). This relationship is even more complex because it involves ants dispersing the scale insects close to the roots (Singer 1986). The Boletineae contains a parasitic fungus that attacks the common earthball *Scleroderma citrinum*. *Pseudoboletus parasiticus* is capable of forming ectomycorrhizal associations, but this fungus is not efficient in nutrition uptake (Raidl 1997). *P. parasiticus* enters *S. citrinum* rhizomorphs to exhaust the host fruiting body and obtains ample nutrition to produce its own fruiting bodies (Raidl 1997). The sister group of *P. parasiticus* in our nuc-lsu analyses is the agaricoid species *Phylloboletellus chloephorus*, a rare fungus from South America and Mexico (Singer 1986). Bandala et al (2004) consider remnants of tropical deciduous forests as potential mycorrhizal partners in plantations where *P. chloephorus* occurs. If it can be demonstrated that *P. chloephorus* parasitizes another Boletales species in a similar way as *P. parasiticus* does, then this would be a nice example of a phylogenetic inference predicting the ecology of a fungus.

**Conclusions.**—The Boletales is a monophyletic group of fungi and there is growing evidence that the Atheliales is the sister group of Boletales. We resolve in our analyses eight major lineages of Boletales on suborder or family level. The Coniophorineae are not monophyletic and need larger taxonomical revision. The Paxillineae also are not monophyletic but form a strongly supported clade with Boletineae. We therefore suggest merging both groups (Taxonomy, SUPPLEMENT). The results of the MRCA analyses show that the diversification of brown-rotting fungi poses critical events in the evolution of early Boletales, including multiple transformations from resupinate to stipitate-pileate fungi. If *Hydnomerulius pinastri* is derived from other brown-rot producing species, then this suggests that brown-rot has a single origin in Boletales. In addition, if this hypothesis is correct, ectomycorrhizae have evolved at least twice in Boletales. Mycoparasites in the Boletales represent transitions from ectomycorrhizal lifestyles. The ancestral state of the nutritional mode of the Boletales was estimated as brown-rot and the ancestral morphology appears to be either polyporoid or resupinate. The analyses of the nuc-lsu dataset demonstrate the importance of this locus to identify phylogenetic key species.

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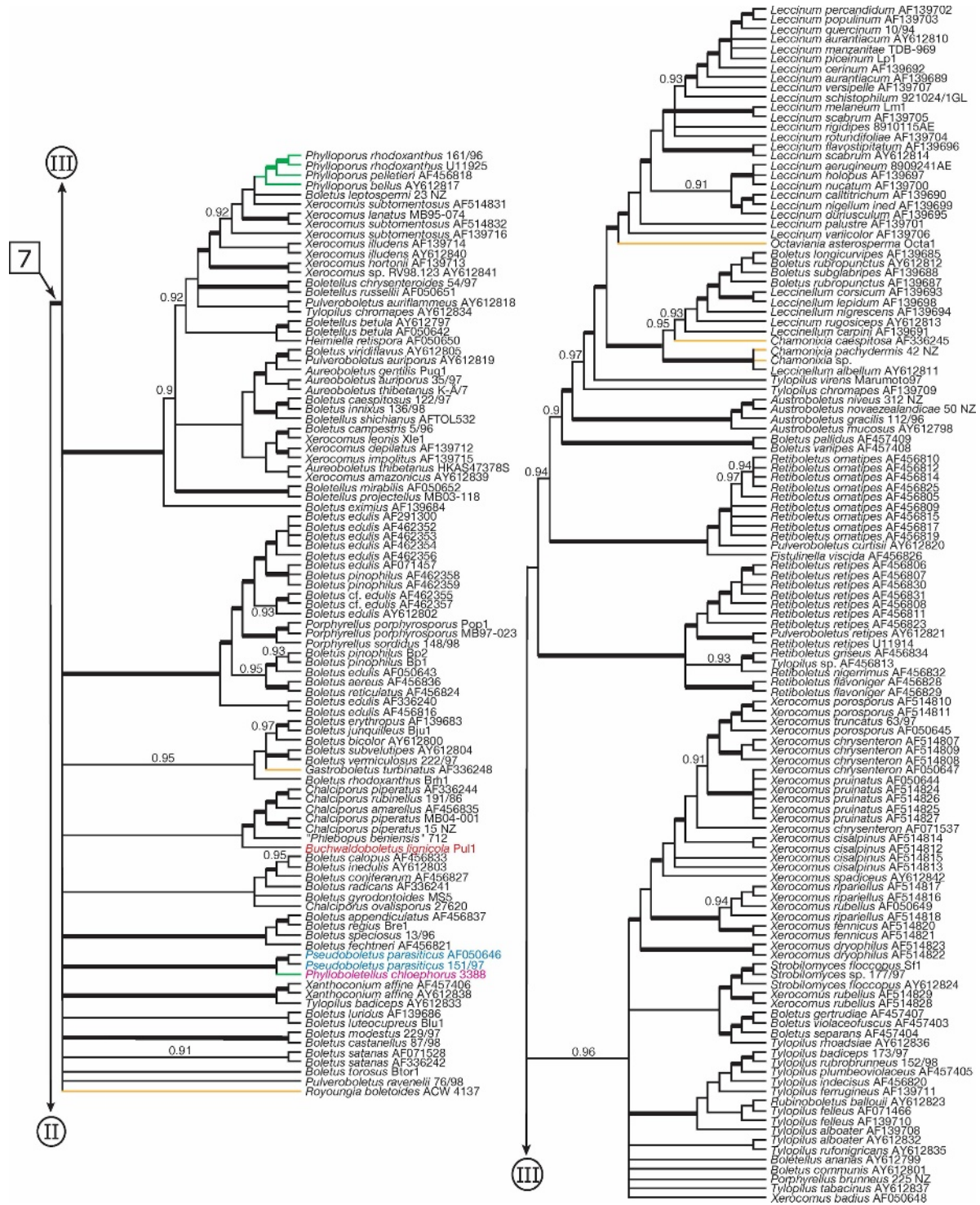
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SUPPLEMENTARY FIG. 1. Phylogenetic analyses of the nuc-18S data set. Shown is a majority rule consensus tree of 575300 trees sampled from stationary tree distributions of two independent MC3 analyses. Likelihood scores range from  $\ln L = 36197.381$  to  $36134.785$ . Branches printed in bold type indicate posterior probability ranges from 0.98–1.0, regardless of the color. Lower PP values (0.9–0.97) are written along branches. Character coding for ancestral state reconstructions is shown by branch shading (morphology) and shading of species names (nutritional mode). The eight nodes that were reconstructed in the MRCA analyses are indicated in boxes. Roman numerals connect the partitioned parts of the tree. GenBank accession numbers are provided for published sequences and strain numbers of taxa are provided, for which new sequences were generated in this study. Terminals marked with REG only are unpublished sequences originating from the study of Jarosch (2001).



## TAXONOMY

*Taxonomical implications.*—We have adopted a conservative approach to accommodate findings from recent phylogenies and propose a revised classification that reflects changes based on substantial evidence. The following outline adds no additional suborders, families or genera to the Boletales, however, excludes Serpulaceae and Hygrophoropsidaceae from the otherwise polyphyletic suborder Coniophorineae. Major changes on family level concern the Boletineae including Paxillaceae (incl. Melanogastraceae) as an additional family. The Strobilomycetaceae E.-J. Gilbert is here synonymized with Boletaceae in absence of characters or molecular evidence that would suggest maintaining two separate families. Chamonixiaceae Jülich, Octavianiaceae Loq. ex Pegler & T. W. K. Young, and Astraeaceae Zeller ex Jülich are already recognized as invalid names by the Index Fungorum ([www.indexfungorum.com](http://www.indexfungorum.com)). In addition, Boletinellaceae Binder & Bresinsky is a homonym of Boletinellaceae P. M. Kirk, P. F. Cannon & J. C. David. The current classification of Boletales is tentative and includes 16 families and 75 genera. For 16 genera (marked with asterisks) are no sequences available. Several taxa listed in the current GenBank classification ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) or in the 9th edition of the Dictionary of the Fungi (Kirk et al 2001) are excluded from the Boletales based on evidence from recent phylogenies (e. g. Binder et al 2005, Peintner et al 2001).

### Synopsis of the Boletales:

**Boletineae** Rea emend. E.-J. Gilbert

**Boletaceae** Chevall. (*Afroboletus*\* Pegler & T.W.K. Young, *Aureoboletus* Pouzar, *Austroboletus* (Corner) Wolfe, *Boletellus* Murrill, *Boletochaete*\* Singer, *Boletus* Dill. ex Fr., *Buchwaldoboletus* Pilát, *Chalciporus* Bataille, *Chamonixia* Rolland, *Fistulinella* Henn., *Gastroboletus* Lohwag, *Gastroleccinum*\* Thiers, *Gastrotylopilus*\* T.H. Li & Watling, *Heimiella* Boedijn, *Heimioporus*\* E. Horak, *Leccinellum* Bresinsky & Binder, *Leccinum* S.F. Gray, *Mycoamaranthus* Castellano, Trappe & Malajczuk, *Octaviania* O. Kuntze, *Paxillogaster*\* E. Horak, *Phylloboletellus* Singer, *Phyllobolites*\* Singer, *Porphyrellus* E.-J. Gilbert, *Pseudoboletus* Sutara, *Pulveroboletus* Murrill, *Retiboletus* Binder & Bresinsky, *Rhodactina* Pegler & T.W.K. Young, *Royoungia* Castellano, Trappe & Malajczuk, *Rubinoboletus* Pilát & Dermek, *Setogyroporus*\* Heinem. & Rammeloo, *Singeromyces*\* M.M. Moser, *Sinoboletus* M. Zang, *Strobilomyces* Berk., *Tubosaeta*\* E. Horak, *Tylopilus* P. Karst., *Velopor-*

*phyrellus*\* L.D. Gómez & Singer, *Xanthoconium* Singer, *Xerocomus* Quél.)

**Paxillaceae** Lotsy (*Alpova* C. W. Dodge, *Austrogaster*\* Singer, *Gyrodon* Opat., *Meiorganum*\* Heim, *Melanogaster* Corda, *Paragyrodon*, (Singer) Singer, *Paxillus* Fr.)

**Boletineae incertae sedis:** *Hydnomerulius* Jarosch & Besl

**Sclerodermatineae** Binder & Bresinsky

**Sclerodermataceae** E. Fisch. (*Chlorogaster*\* Laessøe & Jalink, *Horakiella*\* Castellano & Trappe, *Scleroderma* Pers, *Veligaster* Guzman)

**Boletinellaceae** P. M. Kirk, P. F. Cannon & J. C. David (*Boletinellus* Murrill, *Phlebopus* (R. Heim) Singer)

**Calostomataceae** E. Fisch. (*Calostoma* Desv.)

**Diplocystaceae** Kreisel (*Astraeus* Morgan, *Diplocystis* Berk. & M.A. Curtis, *Tremelloogaster* E. Fisch.)

**Gyroporaceae** (Singer) Binder & Bresinsky (*Gyroporus* Quél.)

**Pisolithaceae** Ulbr. (*Pisolithus* Alb. & Schwein.)

**Suillineae** Besl & Bresinsky

**Suillaceae** (Singer) Besl & Bresinsky (*Suillus* S.F. Gray)

**Gomphidiaceae** R. Maire ex Jülich (*Brauniellula* A.H. Smith & Singer, *Chroogomphus* (Singer) O.K. Mill., *Gomphidius* Fr., *Gomphogaster*\* O.K. Mill.)

**Truncocolumellaceae** Agerer (*Truncocolumella* Zeller)

**Rhizopogonaceae** Gäum. & C. W. Dodge (*Rhizopogon* Fr. & Nordholm)

**Coniophorineae** Agerer & Ch. Hahn

**Coniophoraceae** Ulbr. (*Coniophora* DC., *Gyrodontium* Pat., *Leucogyrophana arizonica* Ginns, *Paxillus chalybaeus* E. Horak, *P. gymnopus* Ch. Hahn)

**Tapinellineae** Agerer

**Tapinellaceae** Ch. Hahn (*Bondarceomyces* Parmasto, *Pseudomerulius* Jülich, *Tapinella* E.-J. Gilbert)

### Without subordinal placement:

**Hygrophoropsidaceae** Kühner (*Hygrophoropsis* (J. Schröt.) R. Maire ex Martin-Sans, *Leucogyrophana* Pouzar)

**Serpulaceae** Jarosch & Bresinsky (*Austropaxillus* Bresinsky & Jarosch, *Gymnopaxillus* Horak emend. Claridge, Trappe & Castellano, *Neopaxillus* Singer ###, *Serpula* (Pers.) S. F. Gray)

**Boletales incertae sedis:** *Leucogyrophana olivascens* (Berk. & M.A. Curtis) Ginns & Weresub, *L. romellii* Ginns



**Taxa excluded:** Gastrosporiaceae Pilát (Phallales), Hymenogasteraceae Vittad. (Agaricales), Leucogasteraceae Moreau ex Fogel (Russulales), *Stephanosporaceae* Oberw. & E. Horak (Agaricales).

[### Note on the placement of *Neopaxillus*. Blast searches using the ITS sequence of *Neopaxillus echinospermus* (Speg.) Singer (AJ419194; Martin and Raidl 2002) retrieve distinct hits in the Agaricales].

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## MATERIALS AND METHODS, SUPPLEMENT

*DNA extraction, cloning, sequencing, and sequence alignment.*—DNA was extracted from herbarium specimens and cultures using a phenol/chloroform extraction protocol (Lee and Taylor, 1990). The crude extracts were purified using GeneClean (Q-BIOgene, Irvine, California). DNA was diluted up to 100-fold with deionized water for use as PCR template. PCR reactions were performed for three nuclear and two mitochondrial rDNA regions using the primer combinations ITS1-F-ITS4 (ITS region including the 5.8S gene), LR0R-LR5 (nuc-lsu), PNS1-NS41 and NS19b-NS8 (nuc-ssu), ML5-ML6 (mt-lsu), and ATP6-1 – ATP6-2 (*atp6*). Sequences of primers used in this study have been described elsewhere (Vilgalys and Hester, 1990; White et al., 1990; Hibbett, 1996; Moncalvo et al., 2000). The amplifications were run in 35 cycles on a PTC-200 thermal cycler (MJ Research, Waltham, Massachusetts) using the following parameters: denaturation 94°C (1 min), annealing 50°C (45 sec), extension 72°C (1.5 min). PCR products were purified with Pellet Paint (Novagen, EMB Biosciences, San Diego, California). *Atp6* products were amplified using the protocol in Kretzer and Bruns (1999). In addition, some *atp6* products were cloned using TOPO TA cloning (Invitrogen, Carlsbad, California). Cleaned PCR products were inserted into the pCR 2.1-TOPO vector and transformed using the One Shot competent cell kit (Invitrogen). The cells were plated and incubated overnight on LB medium containing 50 µg/mL kanamycin, which was saturated with 50 µL X-gal. Three positive transformants each were directly analyzed with PCR using M13 Forward (-20) and M13 Reverse primers.

All PCR products were sequenced using BigDye terminator sequencing chemistry (Applied Biosystems, Foster City, California), purified with Pellet Paint, and run on an Applied Biosystems 3730 automated DNA sequencer. Contiguous sequences were assembled and edited using Sequencher 4.1 (GeneCodes Corp., Ann Arbor, Michigan). Automated alignments obtained by using ClustalX (Thompson et al 1997) were manually adjusted in MacClade 4.0 (Maddison and Maddison 2000).

*Phylogenetic analyses of the nuc-lsu data set and the multi-gene dataset.*—The following section describes how maximum parsimony analyses, maximum likelihood analyses, and Bayesian MC<sup>3</sup> analyses were run in this study:

```
[-----NUC-LSU DATA SET, MAXIMUM PARSIMONY ANALYSIS-----]
```

```
BEGIN ASSUMPTIONS;
```

```
EXSET * stored_2 = 9 13 39 44 49 63 70-72 79 80 89 115 116 119 126 131
140 144 153 160 196 200 203 205 211 212 235 264 296 301 347 356 362 372 378 391
406 413 420 435 444-460 485-487 494 498 505 506 516 517 553-559 613-637 651-702
710 711 774 775 802-804 834-836 857 858 885-895;
END;
```

```
BEGIN PAUP;
```

```
set criterion=parsimony;
```

```
delete/only;
outgroup Botryobasidium_isabellinum Gautieria_otthii/only;
assume ancstates=standard;
set maxtrees=2000 increase=no;
log file=allboletaleshs.log;
```

```
hsearch addseq=random nreps=10000 nchuck=2 chuckscore=1000;
```

```
rootTrees;
```

```
savetrees brlens=yes File=allboletaleshs.tre replace=no;
```

```
end;
```

```
[-----NUC-LSU DATA SET, BAYESIAN ANALYSIS-----]
```

```
BEGIN mrbayes;
```

```
charset all = 1-1071;
set partition = all;
```

```
exclude 9 13 39 44 49 63 70-72 79 80 89 115 116 119 126 131 140 144 153
160 196 200 203 205 211 212 235 264 296 301 347 356 362 372 378 391 406
413 420 435 444-460 485-487 494 498 505 506 516 517 553-559 613-637 651-
702 710 711 774 775 802-804 834-836 857 858 885-895;
```

```
outgroup Botryobasidium_isabellinum;
```

```
lset nst=6 rates=invgamma;
```

```
set autoclose = yes;
```

```
mcmc ngen=50000000 printfreq=1000 samplefreq=100 nchains=4
savebrlens=yes filename=allboletales_bay;
```

```
mcmc;
sumt filename=allboletales_bay burnin=150000 contype=halfcompat;
```

```
[The final burnin proportion of trees was estimated plotting likelihood
scores as a function of the number of generations in Excel and sumt was
run again using the accurate value]
```

```
END;
```

```
[*****END*****NUC-LSU DATA SET*****]
[-----MULTIGENE DATA SET, PARSIMONY BOOTSTRAP-----]
```

```
BEGIN ASSUMPTIONS;
```

```
OPTIONS DEFTYPE=unord PolyTcount=MINSTEPS;
```

```
charset ATOL_BOLETALES = 1-3939;
```



```

charset atp6 = 1-705;
charset mt_lsu = 706-1021;
charset 28S = 1022-1977;
charset ITS = 1978-2162;
charset 18S = 2163-3939;
charset ambiguous_alignment = 106-108 250-255 828-836 1090 1091 1094-1098
1163 1167 1389 1413 1414 1417 1418 1430-1444 1459 1465 1481 1497-1500 1512 1529-
1531 1555-1557 1570 1582 1596-1626 1724 1727 1728 1729 1756-1759 1767 1776 1781
1804 1824 1825 2145-2147 2198 2207 2796 2802-2806 2842-2845 3135 3190 3421 3534-
3538 3640-3642 3926-3939;

```

```

Taxset no_atp6 = Athelia_arachnoidea Austropaxillus_sp.
Coniophora_marmorata Leucogyrophana_mollusca Pseudomerulius_aureus;
Taxset no_mt_lsu = Fomitiporia_mediterranea Melanogaster_variegatus
Coniophora_marmorata Suillus_pictus Porphyrellus_porphyrosporus;
Taxset no_ITS = Dendrocorticium_roseocarneum;
Taxset no_18S = Scleroderma_hypogaeum Suillus_ochraceoroseus;

```

```

EXSET * stored_2 = 106-108 250-255 828-836 1090 1091 1094-1098 1163
1167 1389 1413 1414 1417 1418 1430-1444 1459 1465 1481 1497-1500 1512 1529-1531
1555-1557 1570 1582 1596-1626 1724 1727 1728 1729 1756-1759 1767 1776 1781 1804
1824 1825 2145-2147 2198 2207 2796 2802-2806 2842-2845 3135 3190 3421 3534-3538
3640-3642 3926-3939;

```

END;

BEGIN PAUP;

set criterion=parsimony;

```

delete/only;
outgroup Botryobasidium_isabellinum Gautieria_otthii/only;
assume ancstates=standard;
set maxtrees=10000 increase=no;
log file=atolboletales2.log;
bootstrap treefile=atolboletales2.out nreps=1000 conlevel=50
search=heuristic/ addseq=random nreps=100;
rootTrees;
savetrees from=1 to=1 file=atolboletales2.tre;

```

end;

[-----MULTIGENE DATA SET, MAXIMUM LIKELIHOOD ANALYSIS-----]

Begin trees;

```

tree usertree = [&U]
(Botryobasidium_isabellinum,(Gautieria_otthii,((((Ceriporia_viridans,Gloeophyllum_sepiarium),((Echinodontium_tinctorium,Plicaturopsis_crispa),(Sarcodon_imbricatus,Dendrocorticium_roseocarneum))),((Schizophyllum_commune,Cortinarius_iodes),Hygrocybe_conica),(Athelia_arachnoidea,Fibulorhizactonia_sp.),((((((((Calostoma_cinnabarinum,Gyroporus_cyanescens),(Scleroderma_laeve,Scleroderma_hypogaeum)),Pisolithus_arrhizus),(Phlebopus_portentosus,Boletinellus_merulioides)),((((((((Boletellus_projectellus,Aureoboletus_thibetanus),Phylloporus_rhodoxanthus),Xerocomus_chrysenteron),((Boletus_edulis,(Strobilomyces_floccopus,Porphyrellus_porphyrosporus)),Boletus_pallidus)),Boletus_satanas),Chamonixia_caespitosa),Chalciporus_piperatoides),(Hydnomerulius_pinastri,((Paxillus_filamentosus,Paxillus_involutus),Melanogaster_variegatus),Gyrodon_lividus))),((Hygrophoropsis_aurantiaca,Leucogyrophana_mollusca),(Coniophora_arida,Coniophora_marmorata)),((((Suillus_pictus,Suillus_variegatus),(Suillus_granulatus,Suillus_lakei)),Suillus_ochraceoroseus),Truncocolumella_citrina),Rhizopogon_olivaceotinctus),(Gomphidius_roseus,Chroogomphus_vinicolor)),((Tapinella_atrotentosa,Tapinella_panuoides),Pseudomerulius_aureus),Bondarcevomyces_taxi)),Austropaxillus_sp.),Serpula_himantioides))),Fomitiporia_mediterranea));
end;

```

BEGIN PAUP;

set criterion=likelihood;

set autoclose=yes warnreset=no;

```

delete/only;
outgroup Botryobasidium_isabellinum Gautieria_otthii/only;
set maxtrees=1000 increase=no;

lset nst=6 rates=gamma ncat=4 shape=0.4 basefreq=empirical;
lset pinvar=estimate;
lset tratio=estimate;

hsearch start = 1;

savetrees brlens=yes file=output.ml.trees replace=no;
end;

[-----MULTIGENE DATA SET, BAYESIAN ANALYSIS-----]

Begin mrbayes;

charset all = 1-3939;
charset atp6 = 1-705;
charset mt_lsu = 706-1021;
charset 28S = 1022-1977;
charset ITS = 1978-2162;
charset 18S = 2163-3939;

partition all = 5: atp6,mt_lsu,28S,ITS,18S;

set partition = all;

outgroup 1;

exclude 106-108 250-255 828-836 1090 1091 1094-1098 1163 1167 1389 1413 1414
1417 1418 1430-1444 1459 1465 1481 1497-1500 1512 1529-1531 1555-1557 1570 1582
1596-1626 1724 1727 1728 1729 1756-1759 1767 1776 1781 1804 1824 1825 2145-2147
2198 2207 2796 2802-2806 2842-2845 3135 3190 3421 3534-3538 3640-3642 3926-3939;

unlink revmat = (all);
unlink Tratio = (all);
unlink statefreq = (all);
unlink shape = (all);
unlink pinvar = (all);

lset nst=6 rates=invgamma;

set autoclose = yes;

mcmc ngen=10000000 printfreq=1000 samplefreq=100 nchains=4 savebrlens=yes
filename=Boletales_bay;

mcmc;
sumt filename=Boletales_bay.t burnin=15000;

[The final burnin proportion of trees was estimated plotting likelihood scores
as a function of the number of generations in Excel and sumt was run again using
the accurate value]

end;

[=====MODELS=====]

[-----MULTIGENE ANALYSES, MODELS ESTIMATED WITH MRMODELTEST 3.06-----]

[atp6 model:

Model selected: TVM+G
-lnL = 11664.4287

```

AIC = 23344.8574

Base frequencies:

freqA = 0.3640  
freqC = 0.0865  
freqG = 0.0715  
freqT = 0.4780

Substitution model:

Rate matrix

R(a) [A-C] = 1.1314  
R(b) [A-G] = 3.4024  
R(c) [A-T] = 1.1990  
R(d) [C-G] = 5.5481  
R(e) [C-T] = 3.4024  
R(f) [G-T] = 1.0000

Among-site rate variation

Proportion of invariable sites = 0

Variable sites (G)

Gamma distribution shape parameter = 0.3907

Likelihood settings from best-fit model (TVM+G) selected by AIC in Modeltest Version 3.06

BEGIN PAUP;

Lset Base=(0.3640 0.0865 0.0715) Nst=6 Rmat=(1.1314 3.4024 1.1990 5.5481 3.4024) Rates=gamma Shape=0.3907 Pinvar=0;  
END;]

[mt-lsu model:

Model selected: TVM+I+G

-lnL = 2548.5554  
AIC = 5115.1108

Base frequencies:

freqA = 0.3183  
freqC = 0.1805  
freqG = 0.2293  
freqT = 0.2719

Substitution model:

Rate matrix

R(a) [A-C] = 1.2879  
R(b) [A-G] = 3.0849  
R(c) [A-T] = 1.6795  
R(d) [C-G] = 0.6866  
R(e) [C-T] = 3.0849  
R(f) [G-T] = 1.0000

Among-site rate variation

Proportion of invariable sites (I) = 0.4408

Variable sites (G)

Gamma distribution shape parameter = 1.2415

BEGIN PAUP;

Lset Base=(0.3183 0.1805 0.2293) Nst=6 Rmat=(1.2879 3.0849 1.6795 0.6866 3.0849) Rates=gamma Shape=1.2415 Pinvar=0.4408;  
END;]

[nuc-lsu model:

Model selected: GTR+I+G

-lnL = 9461.9609  
AIC = 18943.9219

Base frequencies:

freqA = 0.2470  
freqC = 0.2279  
freqG = 0.2897  
freqT = 0.2354

Substitution model:

Rate matrix



```

R(a) [A-C] = 1.0873
R(b) [A-G] = 3.4091
R(c) [A-T] = 1.0670
R(d) [C-G] = 0.5938
R(e) [C-T] = 9.3781
R(f) [G-T] = 1.0000
Among-site rate variation
Proportion of invariable sites (I) = 0.4128
Variable sites (G)
Gamma distribution shape parameter = 0.6052

BEGIN PAUP;
Lset Base=(0.2470 0.2279 0.2897) Nst=6 Rmat=(1.0873 3.4091 1.0670 0.5938
9.3781) Rates=gamma Shape=0.6052 Pinvar=0.4128;
END;]

```

[ITS 5.8S model:

```

Model selected: GTR+I+G
-lnL = 9461.9609
AIC = 18943.9219

Base frequencies:
freqA = 0.2470
freqC = 0.2279
freqG = 0.2897
freqT = 0.2354
Substitution model:
Rate matrix
R(a) [A-C] = 1.0873
R(b) [A-G] = 3.4091
R(c) [A-T] = 1.0670
R(d) [C-G] = 0.5938
R(e) [C-T] = 9.3781
R(f) [G-T] = 1.0000
Among-site rate variation
Proportion of invariable sites (I) = 0.4128
Variable sites (G)
Gamma distribution shape parameter = 0.6052

```

```

BEGIN PAUP;
Lset Base=(0.2470 0.2279 0.2897) Nst=6 Rmat=(1.0873 3.4091 1.0670 0.5938
9.3781) Rates=gamma Shape=0.6052 Pinvar=0.4128;
END;]

```

[18S model:

```

Model selected: GTR+I+G
-lnL = 9461.9609
AIC = 18943.9219

Base frequencies:
freqA = 0.2470
freqC = 0.2279
freqG = 0.2897
freqT = 0.2354
Substitution model:
Rate matrix
R(a) [A-C] = 1.0873
R(b) [A-G] = 3.4091
R(c) [A-T] = 1.0670
R(d) [C-G] = 0.5938
R(e) [C-T] = 9.3781
R(f) [G-T] = 1.0000
Among-site rate variation
Proportion of invariable sites (I) = 0.4128
Variable sites (G)
Gamma distribution shape parameter = 0.6052

```

```

BEGIN PAUP;

```

```
Lset Base=(0.2470 0.2279 0.2897) Nst=6 Rmat=(1.0873 3.4091 1.0670 0.5938  
9.3781) Rates=gamma Shape=0.6052 Pinvar=0.4128;  
END;]
```