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# ASSEMBLING THE FUNGAL TREE OF LIFE: PROGRESS, CLASSIFICATION, AND EVOLUTION OF SUBCELLULAR TRAITS[1]

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Based on an overview of progress in molecular systematics of the true fungi (Fungi/Eumycota) since 1990, little overlap was found among single-locus data matrices, which explains why no large-scale multilocus phylogenetic analysis had been undertaken to reveal deep relationships among fungi. As part of the project ''Assembling the Fungal Tree of Life'' (AFTOL), results of four Bayesian analyses are reported with complementary bootstrap assessment of phylogenetic confidence based on (1) a combined two-locus data set (nucSSU and nucLSU rDNA) with 558 species representing all traditionally recognized fungal phyla (Ascomycota, Basidiomycota, Chytridiomycota, Zygomycota) and the Glomeromycota, (2) a combined three-locus data set (nucSSU, nucLSU, and mitSSU rDNA) with 236 species, (3) a combined three-locus data set (nucSSU, nucLSU rDNA, and *RPB2*) with 157 species, and (4) a combined four-locus data set (nucSSU, nucLSU, mitSSU rDNA, and *RPB2*) with 103 species. Because of the lack of complementarity among single-locus data sets, the last three analyses included only members of the Ascomycota and Basidiomycota. The four-locus analysis resolved multiple deep relationships within the Ascomycota and Basidiomycota that were not revealed previously or that received only weak support in previous studies. The impact of this newly discovered phylogenetic structure on supraordinal classifications is discussed. Based on these results and reanalysis of subcellular data, current knowledge of the evolution of septal features of fungal hyphae is synthesized, and a preliminary reassessment of ascomal evolution is presented. Based on previously unpublished data and sequences from GenBank, this study provides a phylogenetic synthesis for the Fungi and a framework for future phylogenetic studies on fungi.

**Key words:** fungal classification; fungal morphology and ultrastructure; fungal phylogenetics; fungal systematics; mitochondrial small subunit ribosomal DNA (mitSSU rDNA); nuclear small and large subunit ribosomal DNA (nucSSU and nucLSU rDNA); RNA polymerase subunit (*RPB2*).

Fungi make up one of the major clades of life. Roughly 80 000 species of fungi have been described, but the actual number of species has been estimated at approximately 1.5 million (Hawksworth, 1991, 2001; Hawksworth et al., 1995). This number may yet underestimate the true magnitude of fungal biodiversity (Hywel-Jones, 1993; Dreyfuss and Chapela, 1994; Blackwell and Jones, 1997; Frölich and Hyde, 1999; Arnold et al., 2000; Hyde, 2000a, b; Gilbert et al., 2002; Persoh, 2002; Persoh and Rambold, 2003). One major source of error in estimates of fungal diversity is the existence of many cryptic species within morphologically homogeneous groups, which has been repeatedly demonstrated using molecular data (e.g., Hibbett and Donoghue, 1996; O'Donnell et al., 2004).

Mycology has traditionally been a subdiscipline of botany, but phylogenetic analyses of both ribosomal DNA and protein-coding genes suggest that fungi are actually more closely related to animals than plants (Wainright et al., 1993; Baldauf and Palmer, 1993; Berbee and Taylor, 2001; Lang et al., 2002). Molecular analyses have also demonstrated that some heterotrophic eukaryotes that have been classified as Fungi, such as the plasmodial and cellular slime molds and the water molds (Myxomycota, Dictyosteliomycota, and Oomycota, respectively) are outside of the group. At the same time, some unicellular eukaryotes previously classified among the ''protists'' have been shown to be Fungi, including *Pneumocystis carinii*, which is a serious pathogen of immunocompromised humans, and the Microsporidia, which are amitochondriate intracellular parasites of animals (Edman et al., 1988; Keeling, 2003). The exact phylogenetic placements of several fungal lineages, such as Microsporidia and Asellariales, are uncertain, though they are included in the Fungi in a recent classification by Cavalier-Smith (2001). Throughout this manuscript, the term ''Fungi'' refers to the monophyletic ''true fungi'' (also considered as a Kingdom of Eukaryota). In contrast, we use the more general term ''fungi'' to encompass all organisms traditionally studied by mycologists (i.e., true fungi, slime molds, water molds).

The major groups (phyla) that have traditionally been recognized within the true Fungi are the Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota. Molecular evidence suggests that the Chytridiomycota and Zygomycota are not monophyletic. Collectively, the Zygomycota and Chytridiomycota form a paraphyletic assemblage representing the earliest diverging lineages of Fungi. Chytridiomycota include unicellular or filamentous forms that produce flagellated cells at some point in the life cycle and which occur in aquatic and terrestrial habitats. It is plausible that the unicellular, flagellated, aquatic form is plesiomorphic in the Fungi as a whole, although the lack of resolution at the base of the fungal phylogeny makes it difficult to resolve this point. Traditionally, the Zygomycota comprise a diverse assemblage of taxa that include soil saprobes (Mucorales), symbionts of arthropods (Trichomycetes), and the widespread arbuscular mycorrhizae of plants (Glomerales; now recognized as a separate phylum Glomeromycota; Schüßler et al., 2001). They are primarily filamentous and lack flagella; the latter condition is also true for all Ascomycota and Basidiomycota. Therefore, understanding the pattern of relationships between Zygomycota and Chytridiomycota is important to resolving the number of losses of flagella and transitions to land in the evolution of Fungi.

The Ascomycota and Basidiomycota are generally resolved as monophyletic and are sister taxa (Bruns et al., 1992). Both feature the production of a dikaryotic (binucleate, functionally diploid) stage in the life cycle, albeit expressed to significantly different extents. The clade that contains these groups has been called the Dicaryomycota (Schaffer, 1975). Ascomycota and Basidiomycota display remarkable diversity in morphology and life cycles, ranging from single-celled yeast to extensive mycelial forms. The latter include the ''humongous fungus'' *Armillaria gallica*, which is a basidiomycete forest pathogen whose mycelial networks may occupy areas as great as 15 hectares, and which may live for 1000 years or more (Smith et al., 1992). The most complex life cycles in Fungi are those of the plant pathogenic rusts (Uredinales), which are basidiomycetes that may have two separate hosts and produce as many as five different kinds of sporulating structures during their life cycle. Many Ascomycota and Basidiomycota produce complex macroscopic fruiting bodies, such as gilled mushrooms, cup fungi, coral fungi, and other forms. Thus, Fungi represent an independent origin of true multicellularity in the eukaryotes.

Fungi play pivotal ecological roles in virtually all ecosystems. Saprotrophic Fungi are important in the cycling of nutrients, especially the carbon that is sequestered in wood and other plant tissues. Pathogenic and parasitic Fungi attack virtually all groups of organisms, including bacteria, plants, other Fungi, and animals, including humans. The economic impact of such Fungi is massive. Other Fungi function as mutualistic symbionts, including mycangial associates of insects, mycorrhizae, lichens, and endophytes. Through these symbioses, Fungi have enabled a diversity of other organisms to exploit novel habitats and resources. Indeed, the establishment of mycorrhizal associations may be a key factor that enabled plants to make the transition from aquatic to terrestrial habitats (Pirozynski and Malloch, 1975). Interest in the evolution of ecosystems (as well as historical biogeography) has fueled attempts to estimate the timing of appearance of the major fungal groups. Minimum age estimates are provided by a limited number of fossils, including spores of Glomerales (Glomeromycota) from the Ordovician (460 million years ago [mya]; Redecker et al., 2000), Chytridiomycota and Ascomycota (including lichens) from the Devonian (400 mya; Taylor et al., 1992, 1995, 1999), hyphae with clamp connections (which are diagnostic for Basidiomycota) from the Pennsylvanian (290

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mya; Dennis, 1970), and fruiting bodies of Basidiomycota from the Cretaceous (Hibbett et al., 1995; Smith et al., 2004).

Fossils and other lines of evidence have been used for calibration purposes in molecular clock analyses aimed at providing absolute age estimates for the major fungal groups. Using genes for nuclear small subunit ribosomal RNA, Berbee and Taylor (2001) suggested that the earliest divergences in the Fungi occurred about 800 mya and the Ascomycota-Basidiomycota divergence occurred about 600 mya. In contrast, an analysis using multiple protein-coding genes in both Fungi and plants by Heckman et al. (2001) suggested that the Fungi originated as long as 1.5 billion years ago, and the Ascomycota-Basidiomycota divergence occurred about 1.2 billion years ago. Sanderson (2003; Sanderson et al., 2004, in this issue) performed an analysis of multiple plastid-encoded genes that suggested that the dates proposed by Heckman et al. (2001) for plant divergences may be too early. By extrapolation, this would be also true for the Fungi, but there has not been a corresponding reanalysis of the fungal age estimates.

One goal of the study presented here is to synthesize progress since 1990 in our continuing endeavor to reconstruct the fungal tree of life, and to analyze all available data for four of the five most commonly sequenced loci for the Fungi (nuclear small and large subunit ribosomal DNA [nucSSU rDNA, nucLSU rDNA], mitochondrial small subunit ribosomal DNA [mitSSU rDNA] and the second largest subunit of RNA polymerase II [*RPB2*]). A related objective of this study is to summarize and integrate current knowledge regarding fungal subcellular features within this new phylogenetic framework.

***Molecular phylogenetic studies of the Fungi***—Examination of fungal sequence data in GenBank for the five most commonly sequenced loci revealed that 21 075 ITS, 7990 nucSSU, 5373 nucLSU, 1991 mitSSU, and 349 *RPB2* sequences were available as of early January 2004. As impressive as these numbers are in terms of our collective effort to generate DNA sequence data for the Fungi, none of these loci alone can resolve the fungal tree of life with a satisfactory level of phylogenetic confidence (Kurtzman and Robnett, 1998; Tehler et al., 2000; Berbee, 2001; Binder and Hibbett, 2002; Moncalvo et al., 2002; Tehler et al., 2003). Combining sequence data from multiple loci is an integral part of large-scale phylogenetic inference and is central to assembling the fungal tree of life. Therefore, the utility of existing data can be better described by assessing the taxonomic overlap among single-locus data sets. Among the 8025 sequences of nucSSU and 5442 sequences of nucLSU available for this project, 3279 and 2781, respectively, were from taxa for which only that locus had been sequenced. Of the remaining sequences, only 1010 represented taxa for which both nucSSU and nucLSU data were available. Of these species, 573 had sequence lengths, or overlap, >600 bp for both loci and were identified at the species level. Of these 573 taxa, mitSSU sequences were also available for 253 taxa, and *RPB2* sequences were available for 161 taxa. NucSSU, nucLSU, mitSSU, and *RPB2* sequences were available for 107 taxa. Despite the very large number of ITS sequences available in GenBank, the low degree of overlap with taxa sequenced for other loci is even more pronounced: only 145 taxa also were available for both nucSSU and nucLSU. In part, the lack of overlap between taxa sequenced for ITS and those sequenced for other loci reflects the generation of many ITS sequences from environmental PCR studies, where it is not possible with most of the current methods to obtain a second amplicon from the same individual or species, and from survey data in which species names are not assigned. The disparity between taxa sequenced for ITS vs. other loci also reflects the popularity of this locus for population-level and single locus, species-level studies.

Together, these data suggest that most phylogenetic studies published to date have sought to maximize the number of fungal taxa by restricting their analyses to one locus. To quantify this observation, we surveyed 560 publications reporting fungal phylogenetic trees published from 1990 through 2003 (Fig. 1). Of the 595 trees considered in these studies, 489 (82.2%) were based on a single locus (Fig. 1A; see also Appendix 1, in Supplemental Data accompanying the online version of this article, for the complete list of papers used in this survey and the data extracted from each). Only 77 trees were based on two combined loci, 19 on three combined loci, and 10 on four or more combined loci (Appendix 1). Seven of the latter 10 studies were restricted to closely related species or strains within a species. Exceptions include Binder and Hibbett (2002), with 93 species representing most major clades of Homobasidiomycetes; Binder et al. (2001), with 15 species representing 10 orders; and Hibbett and Binder (2001), with 45 species representing nine orders.

Despite a striking increase in the number of trees published per year between 1990 and 2003, the proportion each year based on a single locus has remained relatively constant (Fig. 1A). Although the number of species included in published trees has generally increased over time, most studies have included fewer than 100 species (Fig. 1B), with an overall mean of 34.2 ± 2.3 species/study (range: 3–1155 species). The largest phylogenetic tree based on one locus included 1551 nucSSU sequences representing 60 orders (Tehler et al., 2003). The largest multilocus trees included 162 ITS + ß-tubulin sequences representing a single order of Fungi (Stenroos et al., 2002); 158 species representing 10 orders based on nucSSU, nucLSU, and mitSSU (Hibbett et al., 2000); 110 species in a single order sequenced for ITS and nucLSU (Peterson, 2000); and 108 nucSSU + nucLSU sequences representing 19 orders of Fungi (Miadlikowska and Lutzoni, 2004).

To our knowledge, phylogenetic studies including members from all four traditionally recognized phyla of Fungi (Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota) and the Glomeromycota, based on at least two combined loci and explicitly directed toward resolving the fungal tree of life, have not yet been published (but see Keeling et al., 2000). Although much effort has been invested in defining orders (compared to families, for example), few studies have focused on resolving relationships among orders of Fungi: 354 of 595 trees examined (59.5%) conveyed relationships within single orders (Fig. 1C, bottom panel). The largest number of orders considered in a single study ($N = 62$) resulted in a tree based only on nucSSU data (Tehler et al., 2000; Fig. 1C, top panel). The fungal trees based on combined data from multiple loci and encompassing the largest number of orders included 38 species representing 25 orders (Bhattacharya et al., 2000), 52 species representing 20 orders (Lutzoni et al., 2001), and 108 species representing 19 orders (Miadlikowska and Lutzoni, 2004). All of these studies focused on ascomycetes and were based on nucSSU and nucLSU rDNA. A study by Keeling (2003) is exceptional, covering 16 orders of fungi (34 species) using a combined analysis of two protein-coding genes (α- and ß-tubulin) to infer the phylogenetic placement of Microsporidia.

In part due to the recent proliferation of studies restricted to taxa within single orders, the mean number of orders per tree was significantly lower in studies published in 2001–2003 compared to those published in 1993–1995. Accordingly, there does not seem to be a correlation between improvements in technologies and progress toward resolving the deepest nodes in the fungal tree of life, reflecting the slow accumulation of studies combining multiple data partitions, multiple orders, and large numbers of species. This points to a lack of coordination in the past among mycology laboratories when sequencing different loci and various groups of fungi. As demonstrated by the results presented here, the recently funded (NSF) ''Deep Hypha'' coordination network and Assembling the Fungal Tree of Life (AFTOL) project have already contributed toward a more united effort in the choice of loci and taxa that are appropriate for small- and large-scale phylogenetic studies. However, the lack of overlap among existing data partitions just described also results from the fact that most phylogenetic studies have focused on closely related species. Many loci have been used by mycologists for evolutionary studies at that level, but few of these loci are appropriate to resolve relationships among the main lineages of the Fungi.

Even when trees are inferred using multiple loci, the phylogenetic signal may be limited strongly by the loci selected. Our survey data indicate that more than 83.9% of fungal phylogenies are based exclusively on sequences from the ribosomal RNA tandem repeats. The few protein-coding genes that have been sequenced for phylogenetic studies of fungi (e.g., *RPB2*; Liu et al., 1999) have demonstrated clearly that such genes can contribute greatly to resolving deep phylogenetic relationships with high support and/or increase support for topologies inferred using ribosomal RNA genes. To our knowledge, Matheny (2004), Reeb et al. (2004), and Wang et al. (2004) are the only studies to combine *RPB2* with other loci for inferring fungal relationships. In general, the use of protein-coding genes remains rare in fungal studies (but see Nam et al., 1997; Geiser et al., 1998; Kretzer and Bruns, 1999; Thon and Royse, 1999; Yun et al., 1999; Craven et al., 2001; Landvik et al., 2001; O'Donnell et al., 2001; Matheny et al., 2002; Myllys et al., 2002; Thell et al., 2002; Keeling, 2003; Liu and Hall, 2004; Tanabe et al., 2004). In general, there is a great need for housekeeping protein-coding genes to be sequenced and combined with other loci to assemble the fungal tree of life.

***Fungal subcellular characters***—Phylogenetic application of subcellular data in the Fungi became important in the early 1960s (Bracker, 1967), and improved chemical fixation techniques led to a subsequent outpouring of data (Beckett et al., 1974; Fuller, 1976). Since that time, continued improvements in cell preservation, especially freeze substitution (Hoch, 1986) and cytochemical analyses (Beckett, 1981; Read and Beckett, 1996; Müller et al., 1998), have made assessments of structural characters, such as membrane changes during nuclear division, reliable as phylogenetic markers. Nevertheless, structural aspects of fungal cells remain very incompletely known, as indicated by recent discoveries of new types of septa (Adams et al., 1995; Bauer et al., 1995), haustoria (Bauer et al., 1997), and nuclear division (Swann et al., 1999). Molecular sequence data are providing a clearer understanding of the diversity of the Fungi and of the many gaps in our knowledge of subcellular structure in unstudied and understudied groups. The phylogenetic significance of subcellular structure can be difficult to determine in the absence of an independent data set (Berbee and Taylor, 1995; McLaughlin et al., 1995a); however, guidance for their phylogenetic interpretation can be obtained from sequence data.

In conjunction with biochemical data (Bartnicki-Garcia, 1970, 1987), subcellular characters have provided insight into the phylum-level relationships of the Fungi and were used to distinguish Fungi from other organisms with fungal lifestyles before molecular sequence data were available. Biosynthetic pathways and cell wall composition not only separated Oomycota, Hyphochytriomycota, and Plasmodiophoromycota from the Chytridiomycota, but also supported modern phylum-level subdivision of the Fungi (Bartnicki-Garcia, 1970, 1987). Similarly, organization of the transition zone of the flagellar apparatus (i.e., the region lying between the flagellum proper and the kinetosome; Barr, 1992) and of the flagella rootlets (i.e., the microtubules and microfibrils associated with the kinetosome; Barr, 1981), clearly separate Chytridiomycota from other fungal groups with motile cells (Oomycota, Hyphochytriomycota, and Plasmodiophoromycota) that are more closely related to heterokont algae or other protists (Braselton, 2001; Cavalier-Smith, 2001; Dick, 2001; Fuller, 2001). Within the Chytridiomycota, the great diversity in flagella rootlet organization may indicate that this is a fungal group that diverged early during fungal evolution (Barr, 1981, 2001). These characters combined with the arrangement of other cellular components of motile cells, such as the microbody–lipid-globule complex (Powell, 1978), identify clades and orders within the phylum (Barr, 2001) and agree with subsequent molecular phylogenetic analysis (James et al., 2000).

Spindle pole body (SPB, an organelle that organizes microtubules during nuclear division; Alexopoulos et al., 1996) and nuclear division characters are diverse within the Fungi (Heath, 1980, 1986; McLaughlin et al., 1995b). In Chytridiomycota, centrioles are associated with SPBs. Except in *Basidiobolus*, which has a centriole-like structure (McKerracher and Heath, 1985), centrioles are absent from fungi that lack flagella. In the latter, SPB forms and behaviors typically become more elaborate. Nuclear division characters, including nuclear envelope changes, SPB–nuclear-envelope interactions, and chromatin and nucleolus behavior, along with SPB characters, have been used in phylogenetic analyses (Heath, 1986; Tehler, 1988; McLaughlin et al., 1995a; Swann et al., 1999), but the incompleteness of the data and problems with some earlier phylogenetic analyses (McLaughlin et al., 1995a) indicate the need for better and more complete data sets.

With the loss of motile cells, alternative methods of spore release evolved in Fungi (Alexopoulos et al., 1996; Cavalier-Smith, 2001). Sporangiospores and zygospores, both of which are internally formed, were retained in most Zygomycota (Alexopoulos et al., 1996; Benny et al., 2001). New mechanisms for conidium and meiospore formation and ballistosporic discharge have evolved in the Ascomycota and Basidiomycota. The substructure of the ascus wall, especially the ascus apex, has systematic value at higher taxonomic levels; however, dehiscence mechanisms are ecologically adaptive and probably of more restricted taxonomic significance (Bellemère, 1994). In the Basidiomycota, considerable progress has been made in understanding the ballistosporic discharge mechanism with its characteristic droplet (Money, 1998), but structural variations in basidiospore development and the hilar appendix (a small projection at the basidiospore base associated with droplet formation; McLaughlin et al., 1985; Yoon and McLaughlin, 1986;

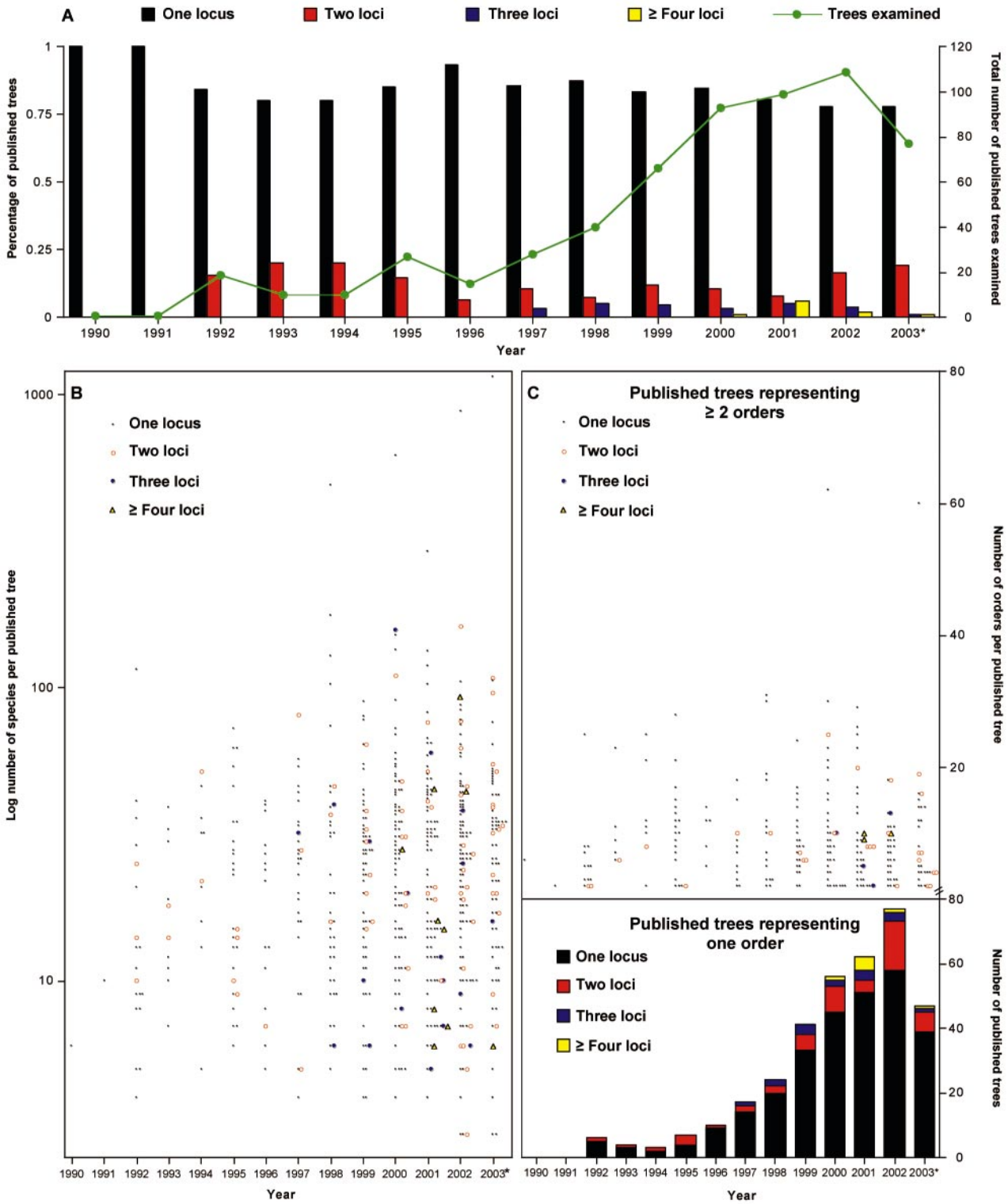


Fig. 1. Results of a survey of 595 phylogenetic trees representing relationships among fungi published during the period 1990–2003. See Appendix 1 (in Supplemental Data accompanying online version of this article) for the criteria used to select trees for this survey and for the complete list of cited papers. Data from six papers in press as of early January 2004 were combined with published works from 2003 and were included in this survey with the permission of the authors; accordingly, 2003 is marked with an asterisk (2003*) in each panel. (A) Percentage of phylogenetic trees for fungi per publication year based on one locus, or on multiple, combined loci (two, three, or four or more loci); and the total number of published trees examined in the present survey. Although the number of published studies has increased markedly since the early 1990s, the proportion based on one locus has remained largely unchanged over time. Studies based on combined data from multiple loci remain rare, and the majority of these are based on data from only two loci. (B) The number of species per tree, depicted on a $\log_{10}$ scale, and the number of loci (one locus, or combined data for two, three, or four or more loci) used to infer relationships among those species in each published tree. The five largest studies in terms of numbers of species are all based on single-locus data sets, whereas seven of 10 studies

Miller, 1988) are still too incompletely studied to assess their potential for phylogenetic analysis. The diversity of meiospore and meiosporangium characters and specialized cell types (e.g., sterile cells such as paraphyses and cystidia) are likely to be of systematic utility at lower taxonomic levels within these phyla (McLaughlin, 1982; Bellemère, 1994; Clémençon, 1997; Pfister and Kimbrough, 2001).

Yeasts are derived from filamentous taxa in three phyla (Benny et al., 2001; Fell et al., 2001; Kurtzman and Sugiyama, 2001). Ascomycetous and basidiomycetous yeasts may be differentiated using a number of phenotypic and molecular traits (Fell et al., 2001). In terms of cell division, these two phyla have been separated based on whether mitosis is initiated in the bud or parent, but both types of mitosis occur in basidiomycetous yeasts. However, other mitotic characters also separate these phyla (Frieders and McLaughlin, 1996; McLaughlin et al., 2004).

The subcellular structure of the septal pores has developmental and systematic significance but varies within major groups (Bracker, 1967; Beckett et al., 1974; McLaughlin et al., 2001). At the phylum level, Ascomycota generally have been thought to be separable from Basidiomycota based on differences in the uniperforate septal pore apparatus, but the possibility that a septal type may be plesiomorphic for these phyla has not been resolved.

***Objectives***—Despite the numerous technological advancements available to fungal systematists, progress in understanding the deepest nodes in the fungal tree of life will be limited without a new approach to conducting large-scale multilocus phylogenetic studies and phenotype-based comparative studies on Fungi. This novel approach will require concerted data acquisition by focusing sequencing efforts on specific loci and fungal taxa, by conducting phenotypic studies on specific fungal traits, by improving interaction among fungal systematists, and by the automation of data acquisition and analysis coupled with data bases accessible through the World Wide Web. These goals form the framework of AFTOL, which seeks to infer the phylogenetic relationships among 1500 species representing all fungal phyla based on eight loci (≈10 kb). Here, we report phylogenetic studies for the maximal number of species across all known fungal phyla for which DNA sequence data from two, three, and four loci are available. The resulting phylogenetic trees are based on sequences available in GenBank and unpublished sequences generated by various laboratories or by the AFTOL project. We then assess current knowledge regarding the evolution and potential phylogenetic signal of septal characters in Fungi.

## MATERIALS AND METHODS

***Taxon sampling***—*nucSSU + nucLSU*—Unique taxa, for which both nucSSU and nucLSU are available were mined from GenBank using the Python EUtils interface (http://www.dalkescientific.com/EUtils/) to the NCBI Entrez Programming Utilities (EPU) (http://www.ncbi.nlm.nih.gov/entrez/query/static/eutils_help.html). A total of 13 467 GenBank sequences were considered, of which 1010 unique taxa had both sequences available. Sequences that were selected incorrectly due to inconsistencies in the GenBank record "Definition Line" were discarded, as were sequences whose length was <600 base pairs or whose overlap with other taxa was <600 base pairs. Unpublished sequences available directly from the AFTOL project and laboratories associated with this project were combined with those available from GenBank and were included in preference to GenBank data. A total of 573 taxa formed the data set for analysis of the nucSSU + nucLSU data set. These taxa included members of all known major lineages of Fungi (Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, and Zygomycota). Our selection of four outgroup taxa from early diverging animal lineages (Choanoflagellida, Mesomycetozoa, Porifera, Anthozoa) was based on a phylogenetic study by Medina et al. (2001). A close relationship of these groups to the Fungi is also strongly suggested by 18S rDNA (Mendoza et al., 2002) and whole mitochondrial genome sequencing (Lang et al., 2002).

*nucSSU + nucLSU + mitSSU*—MitSSU sequences for 105 taxa were obtained from the AFTOL project. For each of the remaining taxa not available directly from AFTOL but present in the combined nucSSU + nucLSU data set, we queried GenBank for mitSSU using the EPU. One hundred forty-eight taxa were retrieved, such that the final nucSSU + nucLSU + mitSSU data set consisted of 253 unique taxa. In contrast to the nucSSU + nucLSU data set, sequences from these three loci were not available for any Chytridiomycota, Zygomycota, or Glomeromycota.

*nucSSU + nucLSU + RPB2—RPB2* sequences for 19 taxa were obtained from the AFTOL project and laboratories associated with this study. We queried GenBank using the EPU for *RPB2* data for each of the remaining taxa present in the combined nucSSU + nucLSU data set, but not available from AFTOL. One hundred forty-two taxa were retrieved from GenBank, such that the nucSSU + nucLSU + *RPB2* data set consisted of 161 taxa. Because sequences from these three loci were not available for taxa outside the Ascomycota and Basidiomycota, analyses were restricted to members of these two phyla.

*nucSSU + nucLSU + mitSSU + RPB2*—Taxa common to the three preceding data sets were combined, resulting in 107 unique taxa representing only the Ascomycota and Basidiomycota.

*Sources of sequences*—Voucher information and GenBank accession numbers for the new sequences deposited in GenBank as part of this study have been archived in Supplemental Data (Appendix 2) accompanying the online version of this article. Appendix 2 also contains GenBank identification numbers for all sequences used in our analyses, as well as accession numbers and general information for sequences obtained from genome centers (Duke Center for Genome Technology, Stanford Genome Technology Center, and The Institution for Genomic Research).

***Molecular data***—From a total of 1533 sequences included in this study, 283 (18%) are published here for the first time. Laboratory protocols used to generate these new sequences can be found in Hopple and Vilgalys (1999), Reeb et al. (2004), Schmitt et al. (2003), Sung et al. (2001), and Hofstetter et al. (2002). The five regions targeted for this study were ≈1.0 kb at the 5′ end of the nucSSU (NS17-nssu1088), ≈1.4 kb at the 5′ end of the nucLSU

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based on combined data from four or more loci contain no more than 28 species (range: 5–28 species). (C) The number of orders considered per tree with regard to the number of loci used to infer phylogenetic relationships. Lower panel: for published trees representing only single orders of fungi, the proportion of studies based on one locus or on combined data from two, three, or four or more loci. Upper panel: for studies representing two or more orders of fungi, the number of orders per tree and the number of loci used to infer phylogenetic relationships as a function of publication year. The 10 largest studies in terms of orders are based on single-locus data sets. To date, four studies based on combined data from three loci have considered representatives of two or more orders ($N$ = 2, 5, 10, and 13 orders). Only three trees based on combined data from four loci have been published for two or more orders ($N$ = 10, 10, and nine orders).

(LROR-LR7), ≈0.8 kb from universally conserved regions U2–U6 that form the minimal core secondary structure of mitSSU (Cummings et al., 1989; Zoller et al., 1999), and ≈2.1 kb from conserved regions 5–11 of *RPB2* (Liu et al., 1999; Reeb et al., 2004). Most primers used in this study can be found at these websites: http://www.biology.duke.edu/fungi/mycolab/primers.htm, http://www.lutzonilab.net/pages/primer.shtml, http://faculty.washington.edu/benhall/, http://plantbio.berkeley.edu/~bruns/primers.html, and http://ocid.nacse.org/research/aftol. Most sequences were subjected to BLAST searches for a first verification of their identities. They were assembled using Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and aligned manually with MacClade 4.06 (Maddison and Maddison, 2001) and SeaView (Galtier et al., 1996). Alignments of nucSSU, nucLSU, and mitSSU rDNA sequences and delimitation of ambiguously aligned regions were done accordingly to Lutzoni et al. (2000) and Reeb et al. (2004) using the secondary structure model (Kjer, 1995) of *Saccharomyces cerevisiae* (U53879, V00704, X07799, X07800, X14966) provided by Cannone et al. (2002) on the Comparative RNA Web Site (http://www.rna.icmb.utexas.edu/). The protein-coding gene *RPB2* was aligned with MacClade using the option nucleotides with amino acid colors to facilitate manual alignment. Ambiguously aligned regions were delimited manually (Lutzoni et al., 2000), taking into account the exchangeability of protein residues according to their chemical properties (Grantham, 1974). Sequences obtained from GenBank that could not be successfully aligned (i.e., those of doubtful homology or sequences that have diverged so much that they were virtually not alignable) were removed from the alignment (Appendix 3; see supplemental data accompanying the online version of this article).

***Phylogenetic analyses***—Bayesian Metropolis coupled Markov chain Monte Carlo (B-MCMCMC) analyses were conducted with MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001). All B-MCMCMC analyses were conducted using four chains, and a gamma distribution, if applicable, was approximated with four categories. In addition to posterior probabilities (PP), phylogenetic confidence was estimated with weighted maximum parsimony bootstrap proportions (MPBP), neighbor joining bootstrap proportions (NJBP) with maximum likelihood (ML) distance implemented using PAUP* 4.0b.10 (Swofford, 2002), and by analyzing bootstrapped data sets with B-MCMCMC (i.e., Bayesian bootstrap proportions, BBP; Douady et al., 2003). Step matrices for weighted parsimony analyses were generated using stepmatrix.py (written by F. Kauff and available upon request from FK or FL) as outlined in Gaya et al. (2003). Uninformative characters were excluded from all bootstrapped data sets analyzed with MP. Parsimony ratchet search strategies (PAUPRat; Nixon, 1999; Sikes and Lewis, 2001, http://www.ucalgary.ca/~dsikes/software2.htm) were implemented in PAUP*. Bootstrapped data sets subjected to B-MCMCMC analyses were generated with P4 0.78 (Foster, 2003). For each data partition and for the combined data set, a hierarchical likelihood ratio test (Modeltest 3.06; Posada and Crandall, 1998) was used to determine the appropriate model (nucleotide substitution and rate heterogeneity parameters). For each NJ analysis, parameter values were fixed to the optimal values calculated for the optimal model. For the *RPB2* data set, each codon position was subjected to a separate model in the B-MCMCMC analysis.

Following the recommendation in Reeb et al. (2004), we used NJBP (500 replicates) to detect topological conflicts among data partitions. A conflict was assumed to be significant if two different relationships (one monophyletic, the other nonmonophyletic) for the same set of taxa were both supported with bootstrap values ≥70% (Mason-Gamer and Kellogg, 1996). The program compat.py (written by F. Kauff and available upon request from FK or FL) was used to detect such topological incongruences. Taxa causing conflicts were removed (Appendix 3), and the test was reimplemented until no conflicts were detected. Each locus in the combined data sets was subjected to this incongruence test for all possible pairwise comparisons prior to inclusion.

Due to the poor level of resolution and support, single-gene trees are not presented here. The gene combinations (nucSSU + nucLSU, nucSSU + nucLSU + mitSSU, nucSSU + nucLSU + *RPB2*, and nucSSU + nucLSU + mitSSU + *RPB2*) were chosen to maximize the number of species, coverage of fungal diversity, as well as phylogenetic resolution and confidence. Because of the large size of the trees presented here and the amount of information associated with each tree, phylograms are only presented as archived supplementary material accompanying the online version of this article (see Appendices 4–6). For these three phylograms, lengths for each branch were averaged over all trees in the Bayesian posterior probability distribution after removal of the ''burn-in phase'' (sumt option in MrBayes v3.0b4).

*nucSSU + nucLSU*—Of 573 taxa, 15 had conflicting phylogenetic placements when the nucSSU and nucLSU NJ bootstrap trees were compared. Consequently, these species were excluded from further analyses (Appendix 3). The combined data set for the remaining 558 species was subjected to B-MCMCMC, and NJ bootstrap. For the B-MCMCMC analysis, we started six independent runs for 10 000 000 generations, sampling every 500th generation with starting trees obtained by randomly resolving dichotomies in the six best trees found by a weighted MP ratchet analysis with 200 iterations using PAUPRat. For both data partitions (nucLSU and nucSSU), we used a six-parameter model for the nucleotide substitution (GTR; Rodríguez et al., 1990) with a gamma shape distribution. A proportion of sites was assumed to be invariable. In the nucLSU partition, nucleotide frequencies were set to be equal. After verifying that all runs had converged on the same average likelihood level, the last 4000 trees (2 000 000 generations) of each run were used to calculate a 50% majority-rule consensus tree using PAUP* (Fig. 2). The NJ bootstrap was performed with 1000 replicates using ML distances, implementing a six-parameter model for the nucleotide substitution (GTR) with equal base frequencies, gamma shape distribution, and a proportion of sites assumed to be invariable.

*nucSSU + nucLSU + mitSSU*—Of 253 taxa, 17 were excluded from further analysis: five sequences were unalignable across the mitSSU partition, and 12 sequences demonstrated conflict among single-locus NJ bootstrap trees (Appendix 3). The combined data set for the remaining 236 taxa was subjected to B-MCMCMC and NJ bootstrap analyses. We are not presenting the resulting tree and associated support values, but we discuss the results that differ in comparison to other combinations of genes presented here. For the B-MCMCMC analysis, we ran six independent analyses of 5 000 000 generations, sampling every 500th generation, starting from random trees. For each of the three data partitions, we used a six-parameter model for the nucleotide substitution (GTR) with a gamma distribution. In the nucLSU and nucSSU partitions, nucleotide frequencies were set to be equal and a proportion of sites was assumed to be invariable. In the mitSSU partition, base frequencies were allowed to vary and all sites were assumed to be variable. Because the six runs did not converge at the same average likelihood level, they were extended for another 5 000 000 generations, using the last tree sampled in each previous run as the starting tree. For the run with the highest average likelihood score, the same starting tree was used to initiate two independent runs for a total of seven runs. At the end of these seven 10 000 000 generations, we extended the runs for another five million generations for a total of seven 15 000 000 generation runs. Only two runs (derived from the same starting tree, which was taken from the first set of five million generations with the highest average likelihood score) converged on the highest average likelihood level after 15 000 000 generations. After discarding the burn-in, we used the last 6000 and 8000 sampled trees from these two runs that converged, for a total of 14 000 trees, to calculate a 50% majority-rule consensus tree using PAUP*. The NJ bootstrap was performed with 1000 replicates using ML distances, implementing a six-parameter model (GTR) for the nucleotide substitution with unequal base frequencies, a gamma shape distribution, and a proportion of sites assumed to be invariable.

*nucSSU + nucLSU + RPB2*—Phylogenetic positions were incongruent among data partitions for four of the 161 taxa for which these sequence data were available (Appendix 3). This three-locus data set for the remaining 157 species was subjected to B-MCMCMC, NJ, and MP bootstrap analysis. For the B-MCMCMC analysis, we ran six independent analyses of 5 000 000 generations, sampling every 500th generation, with random starting trees. For each of the five data partitions (nucLSU, nucSSU, *RPB2* 1st, 2nd, 3rd position), we applied a six-parameter model for the nucleotide substitution (GTR) with a gamma shape distribution and a proportion of sites assumed to be

TABLE 1. Character states for characters in data matrix for morphological analysis of Basidiomycota.

1. Uniperforate septa
   Uniperforate septal pore absent, 0; simple with single central pore, 1; septal pore with elaborated margin, 2.
2. Uniperforate septal pore associated structures
   Uniperforate septal pore absent, 0; no associated structures, 1; microbodies, 2; septal pore cap, 3.
3. Septal pore cap basic structure
   Absent, 0; elaborated cap with adseptal or abseptal extensions, 1; simple cap, 2.
4. Septal pore cap detailed structure
   Cap absent, 0; smooth vesicular–tubular membranous abseptal extensions, 1; multiple saccules, 2; cap reticulate, 3; cap imperforate or uniperforate, 4; cap multiperforate, 5.
5. Zone of exclusion at pore
   Absent, 0; outside pore cap absent in vegetative phase, 1; outside pore cap present in vegetative phase, 2; pore cap enclosed by endoplasmic reticulum, 3; outside membranous plates present, 4; zone of exclusion bordered by microbodies (Uredinales type), 5.

invariable. For the nucLSU and nucSSU data sets, the nucleotide frequencies for the nucSSU were assumed to be equal. Five of the six initial runs converged at the same average likelihood level, and after discarding the specific burn-in for each of these five runs, we used a total of 20 000 trees to calculate a 50% majority-rule consensus tree using PAUP* (Fig. 4). The NJ bootstrap was performed with 1000 replicates using ML distances with a six-parameter model (GTR) for the nucleotide substitution, with unequal base frequencies, a gamma shape distribution, and a proportion of sites assumed to be invariable. For weighted MP bootstrap analyses, we analyzed 115 bootstrap replicates with 500 random addition sequences (RAS) per bootstrap replicate. This estimate of 500 RAS was based on the minimum number of RAS, of 1000, needed to find the most parsimonious tree(s) in the weighted MP search on the original data set. To this number, we added more RAS (up to 500) to maximize the probability of finding the most parsimonious tree(s) when analyzing bootstrapped data sets.

*nucSSU + nucLSU + mitSSU + RPB2*—Of 107 taxa, four demonstrated conflicts among partitions and were excluded from analyses of the four-locus data set (Appendix 3). This combined data set of the remaining 103 species was subjected to B-MCMCMC analysis, B-MCMCMC bootstrap, NJ bootstrap, and weighted MP bootstrap analysis. For each of the six data partitions in the B-MCMCMC analysis (nucLSU, nucSSU, mitSSU, *RPB2* 1st, 2nd, 3rd position), we applied a six-parameter model (GTR) for the nucleotide substitution with a gamma shape distribution and a proportion of sites assumed to be invariable. For the nucSSU, the nucleotide frequencies were assumed to be equal. We ran eight independent analyses of 5 000 000 generations each, which were initiated with random trees and sampled every 500th tree. All runs converged at the same average likelihood level, and after discarding the specific burn-in for each run, we used a total of 69 000 trees to calculate a 50% majority-rule consensus tree using PAUP* (Fig. 5). One hundred bootstrapped data sets were generated. Each of the six partitions was bootstrapped independently, maintaining the proportion of sites for each partition equal to the proportions found in the original combined data set. These 100 bootstrapped data sets were analyzed using the models described earlier with two separate runs of 2 000 000 generations starting from random trees. Each run was checked for convergence with the second run of the same replicate. After discarding the burn-in for each run, 1000 trees from each run were pooled to produce a 50% majority-rule consensus tree with Bayesian bootstrap proportions (BBP; Douady et al., 2003). The NJ bootstrap was performed with 1000 replicates using ML distances, implementing a six-parameter model (GTR) for the nucleotide substitution with unequal base frequencies, a gamma shape distribution, and a proportion of sites assumed to be invariable. Weighted MPBP were based on 102 bootstrap replicates with 500 random addition sequences per replicate.

*Subcellular data*—The cladogram in Fig. 6 was constructed based on the molecular analyses presented in this paper (Figs. 2 and 3) and was drawn using MacClade v4.03PPC (Maddison and Maddison, 2002). The cladogram in Fig. 7 is the result of phylogenetic analyses of morphological characters interpreted from published micrographs for selected taxa. Character states were evaluated for fixation methods and specimen quality and were scored according to a character set data base designed for the Assembling the Fungal Tree of Life project (http://aftol.umn.edu/). Taxa selected for the analysis include representatives of the Basidiomycota currently in the data base; these span the known major lineages within the phylum. Analyses were performed using PAUP* v4.0b10 (Swofford, 2002) with *Allomyces macrogynus* as the outgroup. All phylogenetic inferences were performed under the parsimony criterion. Branch and Bound searches were performed with default parsimony search parameters. Combinations of characters were evaluated iteratively for their ability to resolve expected relationships identified by molecular analyses. For character state descriptions, see Table 1, and for the final data set, Table 2. All characters were weighted equally. Searches for the most parsimonious trees, under the hypotheses that the Ustilaginomycetes and Urediniomycetes are monophyletic, were performed separately by using constrained trees constructed in MacClade. Constrained Branch and Bound searches were performed using the ''Enforce Topological Constraints'' function in PAUP*.

## RESULTS

***Alignments***—The alignment of 573 nucSSU sequences included 10 485 sites, of which 9563 were excluded, representing 26 ambiguously aligned regions, 16 spliceosomal introns, and 13 group I introns. The final size of the nucLSU alignment was 573 sequences by 7416 sites. A total of 6500 sites were excluded, representing 26 ambiguously aligned regions, 14 spliceosomal introns, and seven group I introns. The mitSSU alignment of 253 sequences was 3633 characters long, of which 3298 characters in 24 ambiguously aligned regions and one intron were excluded. The alignment for the *RPB2* included 161 sequences and had a total length of 3482 sites. Twenty-one ambiguously aligned regions and spliceosomal introns at eight splicing sites containing a total of 1688 sites were excluded from all analyses. All final alignments from which the trees in this article are derived can be obtained at http://www.lutzonilab.net/index.shtml.

*nucSSU + nucLSU*—Of 1838 characters included in the phylogenetic analyses of this combined data set, 442 were constant (180 nucSSU sites and 262 nucLSU) and 1396 were variable (742 nucSSU sites and 654 nucLSU). A total of 1073 were potentially parsimony informative (561 nucSSU and 512 nucLSU characters).

*nucSSU + nucLSU + mitSSU*—Of 2173 characters included in phylogenetic analyses of this combined data set, 968 were

TABLE 2. Data matrix used in the morphological analysis of the Basidiomycota.

| Taxon/Character | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| *Allomyces macrogynus* | 0 | 0 | 0 | 0 | 0 |
| *Exobasidium karstenii* | 1 | 1 | 0 | 0 | 4 |
| *Ustacystis waldsteiniae* | 1 | 1 | 0 | 0 | 4 |
| *Helicobasidium compactum* | 1 | 2 | 0 | 0 | 0 |
| *Melampsora lini* | 1 | 2 | 0 | 0 | 5 |
| *Eocronartium muscicola* | 1 | 2 | 0 | 0 | 5 |
| *Tilletia barclayana* | 2 | 1 | 0 | 0 | 4 |
| *Ditangifibulae dikaryotae* | 2 | 3 | 1 | 3 | — |
| *Trichosporon sporotrichoides* | 2 | 3 | 1 | 1 | 2 |
| *Tremella globospora* | 2 | 3 | 1 | 2 | 1 |
| *Tremellodendropsis tuberosa* | 2 | 3 | 2 | 4 | 3 |
| *Auricularia auricula-judae* | 2 | 3 | 2 | 4 | 1 |
| *Schizophyllum commune* | 2 | 3 | 2 | 5 | 1 |
| *Panellus stypticus* | 2 | 3 | 2 | 5 | 1 |
| *Laetisaria arvalis* | 2 | 3 | 2 | 5 | 1 |

constant (450 nucSSU, 448 nucLSU, and 70 mitSSU sites) and 1205 were variable (472 nucSSU, 468 nucLSU, and 265 mitSSU sites). A total of 830 sites were potentially parsimony informative (298 nucSSU characters, 329 nucLSU characters, and 203 mitSSU characters).

*nucSSU + nucLSU + RPB2*—Of 3632 characters included in phylogenetic analyses of this data set, 1459 were constant (469 nucSSU, 486 nucLSU and 504 *RPB2* sites) and 2173 were variable (453 nucSSU, 430 nucLSU, and 1290 *RPB2*). A total of 1748 characters were potentially parsimony informative (296 nucSSU, 322 nucLSU and 1130 *RPB2*).

*nucSSU + nucLSU + mitSSU + RPB2*—Of 3967 characters included in phylogenetic analyses of this combined data set, 1756 were constant (555 nucSSU, 529 nucLSU, 103 mitSSU, and 569 *RPB2* sites) and 2211 were variable (367 nucSSU, 387 nucLSU, 232 mitSSU, and 1225 *RPB2* sites). A total of 1574 sites were potentially parsimony informative (196 nucSSU, 260 nucLSU, 183 mitSSU, and 935 *RPB2* characters).

***Interpretation of support values***—Posterior probabilities provide complementary information to bootstrap proportions (Alfaro et al., 2003; Douady et al., 2003; Reeb et al., 2004). Bayesian MCMC methods are more efficient in recovering accurate support values (i.e., require fewer data to converge on the correct answer relative to parsimony and NJ nonparametric bootstrap [Alfaro et al., 2003; Wilcox et al., 2002; Hillis et al., 1994]), and high posterior probabilities can be obtained for wrong topological bipartitions with current programs implementing Bayesian MCMC, especially when internodes are very short (Alfaro et al., 2003; Buckley et al., 2002; Douady et al., 2003; Erixon et al., 2003; Kauff and Lutzoni, 2002; Leaché and Reeder, 2002; Suzuki et al., 2002; Whittingham et al., 2002; Wilcox et al., 2002; Reeb et al., 2004; Lewis et al., in press). For these reasons, we used a combination of both posterior probabilities and bootstrap proportions to assess the level of confidence for a specific node. Throughout this manuscript, we used the following scale: high (strong) support = PP $\geq$ 95% and at least one BP $\geq$ 70%; medium (moderate) support = PP $\geq$ 95% and 70% $>$ at least one BP $\geq$ 50%, or PP $<$ 95% and at least one BP $\geq$ 70%; low (poor or weak) support = PP $\geq$ 95% and all BPs $<$ 50%, or PP $<$ 95% and 70% $>$ at least one BP $\geq$ 50%; and no support = PP $<$ 95% and all BPs $<$ 50%. We also considered PP $\geq$ 95% to be statistically significant. Overall, our interpretation of various bootstrap proportions (MPBP, NJBP, and BBP) and posterior probabilities (PP) presented here follows Alfaro et al. (2003), Douady et al. (2003), and Reeb et al. (2004).

***Phylogenetic relationships among fungal phyla (Figs. 2 and 3)***—Of the five currently accepted phyla, the Chytridiomycota have been considered to have the most primitive traits because they are the only fungi that have retained reproduction with flagellated spores (zoospores). The Zygomycota are primarily coenocytic (lacking cell septa) and undergo sexual reproduction by formation of a thick-walled resting spore called a zygospore. The Glomeromycota (a recent segregate of the Zygomycota; Schüßler et al., 2001) form endomycorrhizae and reproduce with large, asexually produced spores. The Ascomycota and Basidiomycota are unified by possession of regularly septate hyphae and a dikaryotic life stage but differ in the structures involved in meiosis and sporulation. The Ascomycota contains the largest number of described species, including important model species (*Saccharomyces cerevisiae*, *Neurospora crassa*). Basidiomycetes include the conspicuous mushrooms and rust fungi.

The Fungi were resolved as a clade with a 100% Bayesian posterior probability (PP) with respect to the animalian outgroup taxa. Both the Ascomycota and Basidiomycota formed clades supported by a PP of 100% and NJBP of 67% and 93%, respectively, in the nucSSU + nucLSU analysis. Furthermore, a sister relationship between the Basidiomycota and Ascomycota (the ''Dikaryomycota'') received medium support (PP = 100% and NJBP = 54%). The Glomeromycota formed a clade (PP = 100% and NJBP = 98%) sister to the ''Dikaryomycota.'' This clade has often been recovered in nuclear rDNA phylogenetic analyses (Sugiyama, 1998; Schüßler et al., 2001; Tehler et al., 2003). It has recently been given the informal name ''Symbiomycota'' because most of its members form symbioses (Tehler et al., 2003), but statistical support for this clade in this (PP = 90%) and other studies has never been achieved.

Sampling of the Chytridiomycota and Zygomycota was scant with the exception of mucoralean Zygomycetes. The Chytridiomycota (minus *Allomyces arbusculus*) are part of the earliest known divergence within the Fungi and form an unsupported sister clade to the remaining fungi. The Zygomycota plus *Allomyces* comprise several lineages, roughly correspond-

ing to the ordinal level, that are part of a basal grade with respect to the ''Dikaryomycota'' + Glomeromycota. The nucSSU + nucLSU tree presented here indicates that both the Chytridiomycota and Zygomycota, as currently defined, are not monophyletic.

***Relationships among and within Chytridiomycota, Zygomycota, and Glomeromycota***—The nucSSU + nucLSU phylogeny (Figs. 2 and 3) resolves a chytrid clade sister to all remaining fungi and a paraphyletic assemblage of zygomycete lineages + *Allomyces*, which form a grade leading to the Glomeromycota + ''Dikaryomycota.'' Of the five orders of these groups represented by more than a single taxon, only one of these is monophyletic (Mortierellales).

The nucSSU + nucLSU analyses (Fig. 2) provide a conservative estimate, which is fully resolved (though not well supported), of the relationships among the earliest branching fungal lineages and provide new insight into a few critical branching events. The divergence of the two *Basidiobolus* spp. within the Zygomycota + *Allomyces* group is more consistent with the ecological and morphological traits of these fungi than the placement of the fungus within the chytrid lineage. *Monoblepharella* sp. (representing the Monoblepharidales) groups within the basalmost fungal clade of chytrid fungi (though this position is unsupported), that also includes the orders Chytridiales and Spizellomycetales. In contrast, the Blastocladiales, represented by *Allomyces arbusculus*, group with the remainder of the Fungi rather than with the other Chytridiomycetes.

***Phylogenetic relationships within Basidiomycota***—*nucSSU + nucLSU*—The nucSSU + nucLSU data set includes 203 species that represent all three major clades of Basidiomycota, the Urediniomycetes (represented by 15 species), Ustilaginomycetes (five species), and Hymenomycetes (183 species; Fig. 2). The Basidiomycota is strongly supported as monophyletic (PP = 100%; NJBP = 93%) as are the Urediniomycetes (PP = 100%, NJBP = 100%) and Ustilaginomycetes (PP = 100%, NJBP = 97%). The Hymenomycetes was found to be statistically significant as a monophyletic group; however, the overall support was not as strong as for the previous three groups (PP = 98%, NJBP < 50%). The Ustilaginomycetes and Hymenomycetes form an unsupported clade (PP = 68%, NJBP < 50%) that has the Urediniomycetes as its sister group (Figs. 2 and 3).

The classification for the Urediniomycetes adopted here follows Swann et al. (2001), who recognized six mutually exclusive clades, including five Linnaean taxa, the informal unranked ''*Erythrobasidium*, *Naohidea*, *Sakaguchia* clade'' (here, the *Naohidea* clade), and 10 genera classified as incertae sedis. The nucSSU + nucLSU data set includes representatives of the *Naohidea* clade and two orders of the Urediniomycetidae (Platygloeales and Uredinales), but does not include members of the Attractiellales, Mixiaceae, Microbotryomycetidae, Agaricostilbomycetidae, or any of the genera classified as incertae sedis.

Resolution and support within the Urediniomycetes is generally strong. *Naohidea sebacea* and three species of *Rhodotorula* form a strongly supported group (PP = 100%, NJBP = 100%) that corresponds to the *Naohidea* clade. *Rhodotorula*, however, is a highly polyphyletic group of yeasts that includes species outside of the *Naohidea* clade (Swann and Taylor, 1995; Fell et al., 2001). The remaining Urediniomycetes forms a strongly supported group (PP = 100%, NJBP = 100%) that is the sister group of the *Naohidea* clade, corresponding to the Urediniomycetidae sensu Swann et al. (2001) (Fig. 2). *Insolibasidium deformans*, representing the Platygloeales, is the sister group of the rest of the Urediniomycetidae, which here includes a strongly supported group (PP = 100%, NJBP = 100%) of nine species of Uredinales. The Uredinales is by far the clade of Urediniomycetes with the largest number of extant species, with about 7000 described species of plant pathogenic ''rusts'' (Kirk et al., 2001).

The classification of Ustilaginomycetes adopted here follows Bauer et al. (2001), who divided the group into 10 orders within three subclasses. The nucSSU + nucLSU data set includes representatives of two subclasses, the Ustilaginomycetidae, which is represented by two species of Ustilaginales (*Ustilago maydis*, *U. hordei*), and the Exobasidiomycetidae, which is represented by one species of Malasseziales (*Malassezia furfur*), one species of Tilletiales (*Tilletia caries*), and one species of Exobasidiales (*Exobasidium vaccinii*; Fig. 2). Groups not sampled in this study include the Entorhizomycetidae (containing one order), Urocystales (Ustilaginomycetidae), Georgefischeriales, Microstromatales, Entylomatales, Doassansiales (Exobasidiomycetidae), and several genera classified as incertae sedis.

*Ustilago hordei* and *U. maydis* (Ustilaginales) are strongly supported as sister taxa (100% PP and NJBP). *Malassezia furfur* (Malasseziales) received medium support as the sister group of the Ustilaginales (PP = 100%, NJBP = 69%). *Tilletia caries* (Tilletiales) is strongly supported as the sister group of *Exobasidium vaccinii* (Exobasidiales) (PP = 100%, NJBP = 83%). The *Tilletia-Exobasidium* clade is placed as the sister group of *Ustilago-Malassezia* clade, suggesting that Exobasidiomycetidae is paraphyletic (Fig. 2).

The classification for the Hymenomycetes adopted here primarily follows Hibbett and Thorn (2001), Larsson (2002), and Wells and Bandoni (2001). There has been much research on members of this clade, especially in the Homobasidiomycetes, and some recently discovered clades have not yet been given formal Linnaean names. Members of the Hymenomycetes have traditionally been divided into Homobasidiomycetes and heterobasidiomycetes pro parte (the ''heterobasidiomycetes'' in the broad sense includes taxa with septate basidia that are now placed as members of the Urediniomycetes or Ustilagi-

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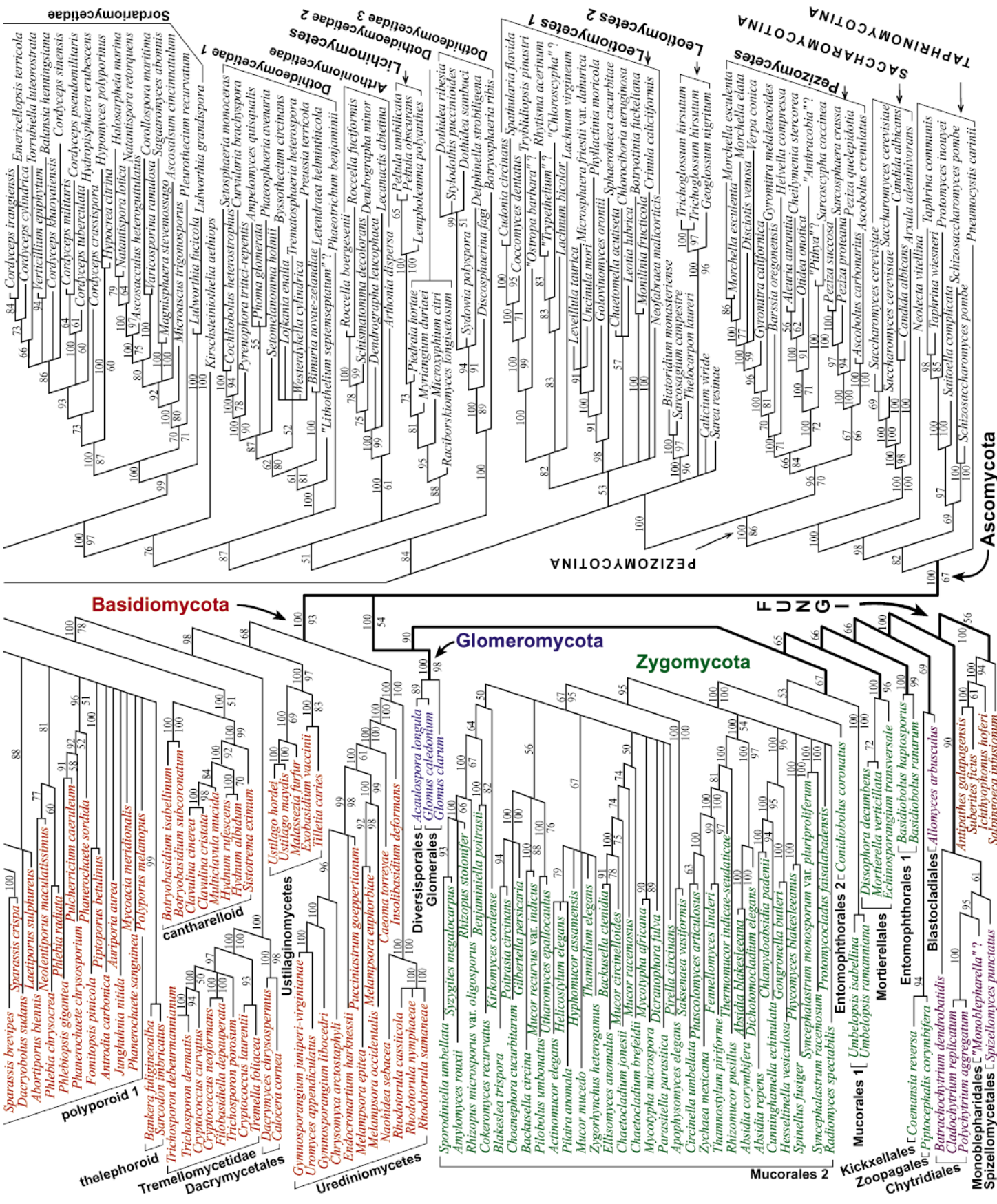
Fig. 2. Two-locus (nucSSU + nucLSU) Bayesian Metropolis coupled Markov chain Monte Carlo (MCMCMC) fungal tree depicting phylogenetic relationships among 558 taxa in 430 genera, 68 orders, and five phyla. This phylogeny resulted from a 50% majority rule consensus of 24 000 trees sampled with Bayesian MCMCMC. The resulting posterior probabilities (PP) are shown above internal branches. NJ bootstrap proportions (NJBP) with ML distances (1000 bootstrap replicates) are shown below internal branches. Species names are colored according to their respective phyla. Internal branches linking the five fungal phyla and their relationship to nonfungal outgroup taxa are represented by thicker lines. Branch lengths are not proportional to evolutionary rates or number of changes, but were instead adjusted for an optimal use of the graphic space. See Appendix 4 (in Supplemental Data accompanying the online version of this article) for a phylogram version of this tree with branch lengths proportional to the average number of substitutions per site.

Bayesian posterior probability
NJ bootstrap proportion
cantharelloid ?
euagarics
bolete
hymenochaetoid 2
polyporoid 2
hymenochaetoid 1
gomphoid-phalloid
Gloeophyllum
corticioid 2
russuloid
corticioid 1
polyporoid 1
auriculariales
Eurotiomycetes
Sordariomycetidae
Chaetothyriomycetidae
Eurotiomycetidae
Ostropomycetidae 1
Ostropomycetidae 2
Acarosporomycetidae
Lecanoromycetidae


Sordariomycetidae
Dothideomycetidae 1
Lichinomycetes
Arthoniomycetidae
Dothideomycetidae 2
Dothideomycetidae 3
Leotiomycetes 1
Leotiomycetes 2
PEZIZOMYCOTINA
Pezizomycetes
SACCHAROMYCOTINA
TAPHRINOMYCOTINA
Ascomycota
Basidiomycota
Glomeromycota
Zygomycota
FUNGI
polyporoid 1
thelephoroid
Tremellomycetidae
Dacrymycetales
Ustilaginomycetes
cantharelloid
Urediniomycetes
Diversisporales
Glomerales
Mucorales 1
Mucorales 2
Mortierellales
Entomophthorales 1
Entomophthorales 2
Kickxellales
Zoopagales
Blastocladiales
Chytridiales
Monoblepharidales
Spizellomycetales
Chytridiomycota
Outgroup

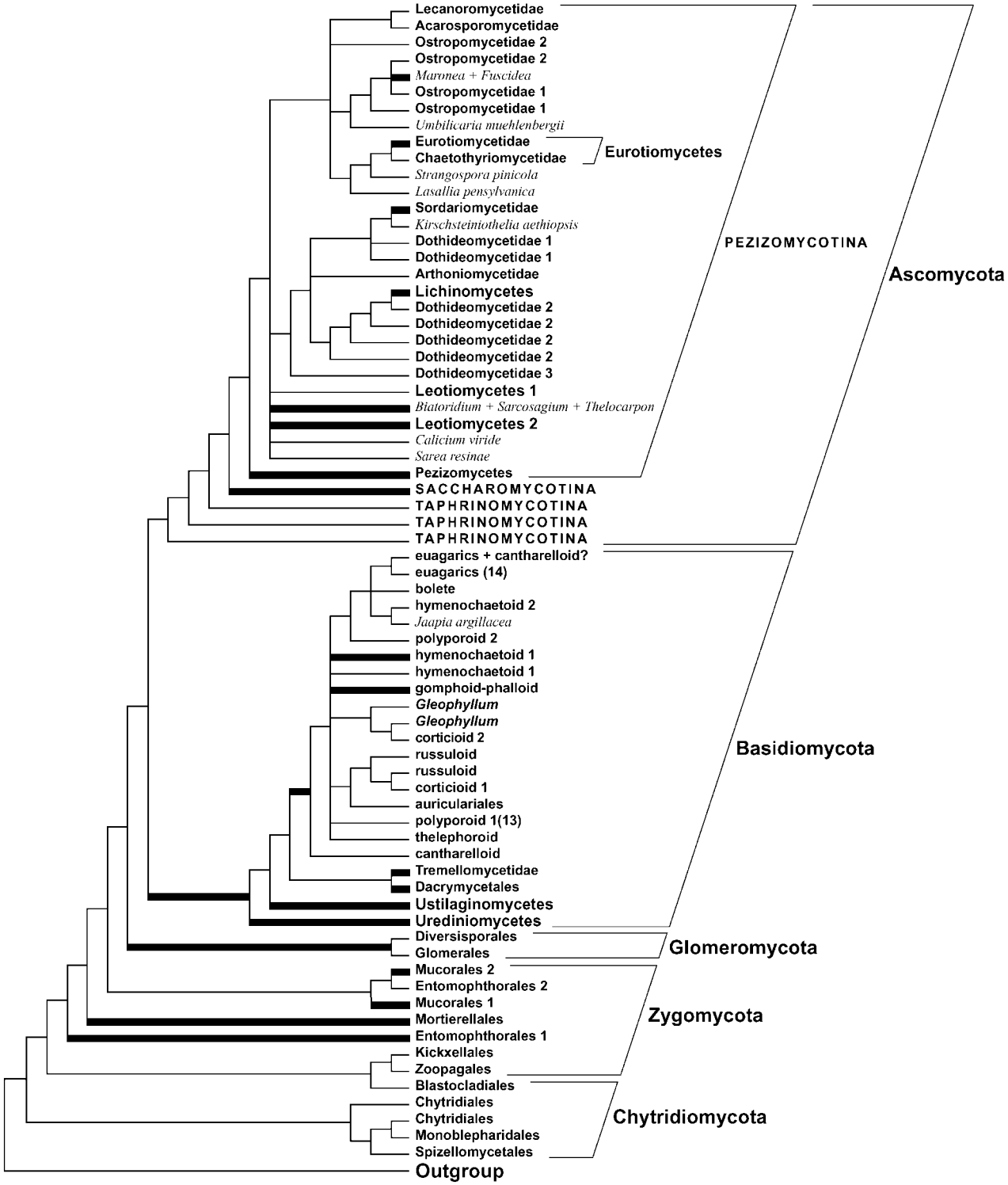



Fig. 3. Schematic summary of the two-gene tree presented in Fig. 2 for an easier visualization of relationships among major fungal lineages resolved by the nucSSU + nucLSU data set. All lineages of a nonmonophyletic taxon are shown as separate lineages, corresponding to multiple occurrences of certain taxon names. Thicker lines represent internodes in Fig. 2 that were associated with high support (i.e., PP $\geq$ 95% and NJBP $\geq$ 70%). Numbers in parentheses correspond to the number of branches stemming from the basal node of the corresponding clade in Fig. 2.

nomycetes). The Homobasidiomycetes has been divided into approximately 12 independent clades that have been given informal, unranked names (e.g., corticioid clade, athelioid clade; Hibbett and Thorn, 2001; Larsson, 2002). The remaining taxa of the Hymenomycetes are divided among the Tremellomycetidae, Dacrymycetales, and Auriculariales. The nucSSU + nucLSU data set includes representatives of 11 of the 12 independent clades of Homobasidiomycetes, as well as the Tremellomycetidae, Dacrymycetales, and Auriculariales; only the trechisporoid clade (Homobasidiomycetes; Larsson, 2002) is unrepresented.

The Hymenomycetes is resolved as monophyletic, but is supported only by Bayesian posterior probabilities (98%, NJBP $<$ 50%; Fig. 2). The Tremellomycetidae plus Dacrymycetales forms a clade with overall low support (PP = 100%, bootstrap $<$ 50%), placed as the sister group of the rest of the Hymenomycetes. The monophyly of the remaining Hymenomycetes (Homobasidiomycetes plus Auriculariales) is strongly supported (PP = 100%, NJBP = 78%).

The cantharelloid clade is associated with a significant posterior probability (100%) but virtually no bootstrap support (51%). In addition, the root endophyte *Piriformospora indica*, which has been placed in the cantharelloid clade in some analyses (M. Binder et al., Clark University, unpublished manuscript) is here placed in the euagarics clade (Fig. 2). The cantharelloid clade is placed as the sister group of an unsupported clade (PP = 54%, NJBP $<$ 50%) that includes the rest of the Homobasidiomycetes and the Auriculariales. The basal node in this large clade consists of a 20-way polytomy (Fig. 2).

Three of the 12 major independent clades of Homobasidiomycetes recognized by Hibbett and Thorn (2001) and Larsson (2002) are resolved as monophyletic, including the bolete clade, gomphoid-phalloid clade, and thelephoroid clade. Posterior probabilities for these groups are 100, 100, and 99%, respectively, but all bootstrap values are 50% or less, except for the gomphoid-phalloid clade with an NJBP of 100%. Three other major clades sensu Hibbett and Thorn (2001) and Larsson (2002) are paraphyletic because a single species that was expected to fall elsewhere is nested within them, including the euagarics clade (containing *Piriformospora indica*, putatively of the cantharelloid clade), russuloid clade (containing *Laetisaria fuciformis*, corticioid clade), and *Gloeophyllum* clade (containing *Dendrocorticium roseocarneum*, corticioid clade). Posterior probabilities for these clades are 100, 55, and 67%, respectively, and all bootstrap values are less than 50%.

The polyporoid clade (Hibbett and Thorn, 2001) comprises 13 lineages of Homobasidiomycetes that are here unresolved. In addition, *Lopharia mirabilis*, a putative member of the polyporoid clade, is placed as the sister group of a clade containing the bolete clade, euagarics clade, *Jaapia argillacea*, and *Repetobasidium mirificum*. The hymenochaetoid clade sensu Hibbett and Thorn (2001) is diphyletic.

In general, there is little resolution of higher-order structure within the Homobasidiomycetes. One notable exception is a clade containing the euagarics clade, bolete clade, and *Jaapia argillacea*, which received support from the Bayesian analysis only (PP = 98%, NJBP $<$ 50%). The inclusion in this clade of *Repetobasidium mirificum*, putatively a member of the hymenochaetoid clade, may be an artifact.

*nucSSU + nucLSU + RPB2*—This data set includes 55 species of Basidiomycota, which represent only the Hymenomycetes (Fig. 4). *Dacrymyces chrysospermus* represents the Dacrymycetales, but the remaining taxa are all Homobasidiomycetes. The Hymenomycetes is strongly supported as monophyletic by two of the three measures of confidence that were employed (PP = 100%, MPBP = 100%, NJBP $<$ 50%) and *Dacrymyces chrysospermus* is placed as the sister group of the Homobasidiomycetes (PP = 100%, MPBP = 73%, NJBP $<$ 50%).

Eight of the 12 major independent clades of Homobasidiomycetes (Hibbett and Thorn, 2001; Larsson, 2002) are represented in the nucSSU + nucLSU + *RPB2* data set. The clades (and their Bayesian posterior probabilities) include the cantharelloid clade (100%), gomphoid-phalloid clade (100%), hymenochaetoid clade (85%), russuloid clade (97%), bolete clade (100%), and euagarics clade (100%). *Sarcodon imbricatus*, a member of the thelephoroid clade, is nested within the polyporoid clade, and this grouping received 74% posterior probability. Bootstrap support for all of these groups is weak, however. In contrast to the two-gene nucSSU + nucLSU analysis, the backbone of the Homobasidiomycetes is well resolved, if not strongly supported. The cantharelloid clade is again the sister group of the remaining Homobasidiomycetes. Above this node, the gomphoid-phalloid clade is the next clade to branch off. The hymenochaetoid clade, russuloid clade, and a clade containing the remaining groups of Homobasidiomycetes form a trichotomy. The euagarics and bolete clades are resolved as sister groups, but with weaker support than in the two-gene analysis (PP = 86%, MPBP and NJBP $<$ 50%). The polyporoid clade (plus *Sarcodon*) is resolved as the sister group of the euagarics/bolete clade, but without support (PP = 51%, MPBP and NJBP $<$ 50%). Within the polyporoid clade, the core polyporoid clade (a group of species sharing characters of white-rot production and tetrapolar mating systems) is resolved with support only from the Bayesian analysis (PP = 99%; MPBP and NJBP $<$ 50%).

*nucSSU + nucLSU + mitSSU + RPB2*—This data set includes 39 species of Basidiomycota that represent seven clades of Homobasidiomycetes (Fig. 5), which were also present in the three-gene nucSSU + nucLSU + *RPB2* analysis (the thelephoroid clade, however, is not represented in this data set). The Homobasidiomycetes received 100% support values from three of the four measures of confidence that were employed (NJBP = 73%). Five of the seven major clades of Homobasidiomycetes received 100% posterior probabilities, but the polyporoid clade received 84% posterior probability, which is a slightly higher probability than this clade received in the three-gene analysis (74%). The hymenochaetoid clade is monotypic in the four-gene data set. The other measures of confidence (BBP, NJBP, MPBP) for these clades were more variable than the Bayesian posterior probabilities.

The four-gene tree differs from the three-gene tree (Fig. 4) in that the gomphoid-phalloid clade is placed as the sister group of the rest of the Homobasidiomycetes (in the three-gene tree the cantharelloid clade occupies this position). Again, there is a trichotomy above the earliest divergence in the Homobasidiomycetes, but this time it involves the cantharelloid clade, hymenochaetoid clade, and a clade that contains the remaining Homobasidiomycetes. Despite these differences, the relatively early branching position of the cantharelloid, gomphoid-phalloid, and hymenochaetoid clades are consistent in both the three-gene and four-gene analyses.

The sister-group relationship of the bolete clade and euagarics clade receives stronger support in this analysis than in

the three-gene analysis, as measured by Bayesian posterior probabilities (100% vs. 86%; Figs. 4, 5). The polyporoid clade is again resolved as the sister group of the euagarics/bolete clade, but in this analysis the node receives support, as measured by Bayesian posterior probabilities (98%; bootstrap proportions < 50%).

***Phylogenetic relationships within Ascomycota***—*nucSSU + nucLSU*—Of three subphyla recognized by Ericksson et al. (2004) within the Ascomycota, only the Taphrinomycotina are not monophyletic in our nucSSU + nucLSU Bayesian tree (Figs. 2 and 3). This result is congruent with previous broad phylogenetic studies of the Ascomycota (see Liu and Hall, 2004; Taylor et al., 2004; Reeb et al., 2004). However, only Neolectales (represented by *Neolecta vitellina*) forms a lineage distinct from the rest of the Taphrinomycotina (PP ≥ 95%). The Taphrinomycetes and Schizosaccharomycetes form a nonsupported monophyletic group (PP = 69%). *Pneumocystis carinii* (Pneumocystidomycetes) is shown here as part of the most basal divergence within the Ascomycota, but the phylogenetic placement of this species was not significant (PP = 82%). Liu and Hall (2004) reported *Pneumocystis* as being nested within Taphrinomycota, but *RPB2* did not provide sufficient signal to obtain a significant posterior probability (PP = 87%). Even if our sampling represents all five families within this subphylum (Eriksson et al., 2004), our taxon sampling and this combination of nucSSU and nucLSU is insufficient to propose changes to existing classifications. However, our results support the recognition of *Neolecta* at the highest taxonomic level within the Ascomycota as proposed by Kirk et al. (2001). Within the context of this study, this requires raising the Neolectomycetes to the subphylum rank.

Our nucSSU + nucLSU data set (Fig. 2) was sufficient to confirm the monophyly of the Saccharomycotina with highly significant support values (PP = 100%; NJBP = 100%). Only three genera are represented here (*Saccharomyces*, *Candida*, and *Arxula*), but to our knowledge there are no recent phylogenetic studies that have proposed that the group is not monophyletic (Taylor et al., 2004).

The Pezizomycotina (euascomycetes) was confirmed as monophyletic with high support values (PP = 100%; NJBP = 86%) despite our extensive taxon sampling within this subphylum when using nucSSU and nucLSU data together. These two genes combined also confirmed the monophyly of the operculate discomycetous Pezizomycetes (PP = 96%; NJBP = 70%) and their sister relationship to the rest of the Pezizomycotina (PP = 100%). Seven of 15 families recognized by Eriksson et al. (2004) within the Pezizomycetes are represented here by 20 species distributed across 15 genera (Fig. 2). Only one order (Pezizales) has been recognized within the Pezizomycetes (Eriksson et al., 2004) or Pezizomycetidae (Kirk et al., 2001). The nucSSU + nucLSU based tree presented here (Fig. 2) shows relationships among families of Pezizomycetes that could lead to the establishment of multiple orders to emphasize synapomorphies and diagnostic features uniting these families. The Morchellaceae (represented by *Disciotis*, *Morchella*, and *Verpa*), Helvellaceae (represented by *Barssia* and *Helvella*) and Discinaceae (represented by *Gyromitra*) form a strongly supported monophyletic group (PP = 100% and NJBP = 71%) as reported by O'Donnell et al. (1997). The same is true for the families Pyronemataceae (represented by *Aleuria*, *Anthracobia*, *Cheilymenia*, and *Otidea*) and Sarcoscyphaceae (represented by *Pithya* and *Sarcoscypha*) with a PP = 100% and NJBP of 72%. Except for the Helvellaceae, all families for which we had more than one genus included in our analyses (i.e., Morchellaceae, Pezizaceae, Pyronemataceae, and Sarcoscyphaceae) were found to be monophyletic with posterior probabilities = 100% and with NJBP ranging from 59 to 100%. The Helvellaceae forms an unsupported paraphyletic group that may be attributed to low taxon sampling. All genera represented by more than one species were also found to be strongly supported monophyletic groups, except for *Gyromitra* (paraphyletic) and *Peziza*, within which *Sarcosphaera* is nested.

After the divergence of the Pezizomycetes, the relationships among the remaining classes and subclasses within the Pezizomycotina (i.e., inoperculate euascomycetes) are unresolved or poorly supported for the most part. Other than the well-supported monophyly of the inoperculate euascomycetes, the only strongly supported supraordinal relationships within this group that have been revealed by nucSSU + nucLSU trees prior to this study (see Taylor et al., 2004, for a summary) are the Arthoniomycetidae-Dothideomycetidae-Sordariomycetidae clade (corresponding to the Sordariomycetes), the Acarosporomycetidae-Eurotiomycetes-Lecanoromycetidae-Ostropomycetidae clade and the Eurotiomycetidae-Chaetothyriomycetidae clade (corresponding to the Eurotiomycetes). Because of our extensive taxon sampling across all known fungal phyla, causing the exclusion of additional sites that are not ambiguously aligned within phyla, some of the resolution or support was not recovered in this study (Fig. 2). The Acarosporomycetidae-Eurotiomycetes-Lecanoromycetidae-Ostropomycetidae group and the Eurotiomycetes were recovered as monophyletic groups (PP = 91% and 64%, respectively). The Sordariomycetes was retrieved as monophyletic with the exception of the enigmatic placement of the Lichinomycetes in this two-locus phylogeny. Although the Sordariomycetes was not supported by these analyses (PP = 84%, NJBP < 50%), it received low and medium support in the three- and four-gene analyses, respectively, albeit with a major reduction in taxon sampling (Figs. 4 and 5).

The Sordariomycetidae, which comprises taxa possessing perithecial and cleistothecial ascomata, is resolved as a strongly supported clade (PP = 100%, NJBP = 97%) with more terminal clades corresponding to well-characterized orders. The sampling presented here represents eight of the 13 orders and 23 of the 45 families currently recognized in the Sordariomycetidae (= Sordariomycetes sensu Eriksson et al., 2004). Relationships among orders within the Sordariomycetidae are resolved with varying levels of support. The Hypocreales, Microascales, and Halosphaeriales (= Hypocreomycetidae sensu Eriksson et al., 2004) comprise a monophyletic group (PP = 100%), with the Lulworthiales as a sister group (PP = 99%). The second grouping of orders includes the Sordariales and Diaporthales (PP = 100%, NJBP = 98%) (= Sordariomycetidae sensu Eriksson et al., 2004) and the ((Sordariales, Diaporthales), Xylariales) (PP = 97%). The monophyletic groups of families and orders revealed by our nucSSU + nucLSU phylogeny are consistent with previous rDNA studies (Berbee and Taylor, 1992; Spatafora and Blackwell, 1993, 1994a; Spatafora et al., 1998) and are largely congruent with characters associated with ontogeny of the perithecial central cavity as being phylogenetically informative at the ordinal and supraordinal levels (Luttrell, 1951; Reynolds, 1981; Spatafora and Blackwell, 1994b). Numerous enigmatic taxa of the Sordariomycetidae were resolved in a manner inconsistent with their

current classification and will require further investigation. These include *Pleurothecium recurvatum* (anamorphic of the Sordariales), *Apiosporia sinensis* (Apiosporaceae, incertae sedis), and *Thyridium vestutum* (Thyridiaceae, incertae sedis).

The Dothideomycetidae was polyphyletic, comprising three lineages that are designated Dothideomycetidae 1 through 3. The sampling here includes representatives from three of the seven orders and 14 of the 68 families currently classified in the Dothideomycetes sensu lato (Eriksson et al., 2004). Dothideomycetidae 1 is unresolved and includes the Pleosporales as well as representatives from several incertae sedis families including *Lojkania* (Fenestellaceae), *Byssothecium* (Dacampiaceae), and *Ampelomyces* (anamorphic Ascomycota). Dothideomycetidae 2 is a weakly (low) supported paraphyletic group that includes the Myriangiales as well as representatives from several incertae sedis families including *Raciborskiomyces* (Pseudoperisporiaceae), *Microxyphium* (Coccoideaceae), and *Piedraia* (Piedraiaceae). Dothideomycetidae 3 is a poorly supported clade (PP = 100% and NJBP < 50%) that includes representatives of the Dothideales and Dothioraceae incertae sedis (*Discosphaeria*, *Sydowia*, *Delphinella*). The Dothideomycetidae and the Dothideales have been inconsistently resolved as monophyletic taxa in previous rDNA analyses with varying levels of support and remain one of the significant challenges in resolving the internal nodes of the Pezizomycotina (Berbee, 1996). Clearly, a considerable increase in sampling of taxa (especially type species) and data are needed to more confidently resolve the relationships of the Dothideomycetidae.

The Arthoniomycetidae includes more than 1000 species part of three families placed in one order—Arthoniales. The genera *Arthonia* and *Opegrapha* alone include approximately 400 and 300 species, respectively (Kirk et al., 2001). Most species form lichen symbioses with the green alga *Trentepohlia*. In contrast to their closest relatives (Dothideomycetidae and Sordariomycetidae), their ascomata are usually apothecial. The nucSSU and nucLSU have been sequenced for very few species within this order. Except for *Arthonia dispersa*, all species sampled are part of the Roccellaceae.The monophyly of the Roccellaceae (represented by *Dendrographa*, *Lecanactis*, *Roccella*, and *Schismatomma*) is strongly supported (PP = 100%, NJBP = 99%).

As recommended by Reeb et al. (2004) in their discussion of phylogenetic analyses based on a combined nucSSU + nucLSU + *RPB2* data set restricted to the Ascomycota, we recognize the Lichinales at the class level. This group of about 240 lichen-forming species, associated mostly with cyanobacteria other than *Nostoc*, is characterized by mature ascomata that are apothecial but more or less perithecial in early development. Our limited taxon sampling of three species representing two genera does not allow us to make any conclusions about relationships within this class (but see Schultz et al., 2001 and Schultz and Büdel, 2003 for the most complete phylogenetic study to date on this group of lichen-forming fungi). As in previous studies (Lutzoni et al., 2001; Kauff and Lutzoni, 2002; Miadlikowska and Lutzoni, 2004), the nucSSU in combination with nucLSU data is not sufficient to resolve the placement of the Lichinomycetes within the Ascomycota with high phylogenetic confidence.

The Leotiomycetes represents one of the more problematic taxa of the Ascomycota and consists of two lineages in these analyses. It includes all apothecial ascomycetes (cup fungi) that possess inoperculate asci, as well as the Erysiphales, which produces cleistothecial-like ascomata (gymnothecia). These analyses include three of the five orders and nine of the 21 families currently recognized in the Leotiomycetes sensu Eriksson et al. (2004). Leotiomycetes 1 is a poorly supported clade that includes taxa currently classified in the Erysiphales (*Sphaerotheca-Microsphaera* clade), the helotialean families Dermataceae (*Neofabraea*), Helotiaceae (*Crinula*, *Chlorociboria*, *Chloroscypha*), Hyaloscyphaceae (*Lachnum*), Leotiaceae (*Leotia*), and Sclerotiniaceae (*Botryotinia* and *Monilinia*), and the rhytismatalean families Cudoniaceae (*Cudonia*, *Spathularia*) and Rhytismataceae (*Coccomyces*, *Rhytisma*, *Tryblidiopsis*).

The Leotiomycetes 2 equates to the Geoglossaceae, one of the ''earth-tongue'' families, and includes the genera *Geoglossum* and *Trichoglossum*. The separation of the Geoglossaceae from other leotialean taxa has been observed in other rDNA-based phylogenies (Platt, 2000) and is consistent with differences in ascospore and paraphysis morphologies as compared to the other ''earth-tongue'' genera. Importantly, this finding supports the convergent evolution of the ''earth-tongue'' ascoma based on the placement of *Cudonia*/*Spathularia*, *Geoglossum*/*Trichoglossum*, and *Leotia*; however, additional sampling is needed to resolve this issue with greater confidence.

As far as we know, this is the most extensive taxon sampling ever analyzed phylogenetically for the Eurotiomycetes that is based on more than one locus. The monophyly of the Eurotiomycetidae is highly supported (PP = 100%, NJBP = 96%) despite the large-scale taxon sampling across the fungi. Eriksson et al. (2004) recognize two orders within this group—Eurotiales (three families) and Onygenales (five families). As currently circumscribed, these two groups are not monophyletic. The Ascosphaeraceae and Eremascaceae are closely related (PP = 100%, NJBP = 92%). However, these two onygenalean families are more closely related (PP = 100%, NJBP = 91%) to the Trichocomaceae (*Byssochlamys*, *Hamigera*, and *Penicillium*), classified within the Eurotiales, than to the Arthrodermataceae, Gymnoascaceae, and Onygenaceae, which form the second strongly supported monophyletic group (PP = 100%, NJBP = 71%) part of the Onygenales. Therefore, this strongly suggests that the Onygenales (sensu Eriksson et al., 2004) should be redefined as two orders. One order (''Ascosphaerales'') would include the Ascosphaeraceae and Eremascaceae, as well as the anamorph *Paracoccidioides*. The other order would include the Arthrodermataceae, Gymnoascaceae and Onygenaceae. The latter group would correspond to the Onygenales sensu stricto and would include the human pathogenic anamorph *Coccidioides*.

The other subclass we recognize within the Eurotiomycetes, the Chaetothyriomycetidae, is poorly supported (PP = 98%, NJBP < 50%) in this broad context of the fungi with only partial sequences from the nucSSU and nucLSU. However, the (Pyrenulales (Verrucariales, Chaetothyriales)) monophyletic groups that we refer to as the Chaetothyriomycetidae is consistent with previous studies that found the same set of relationships with high support values (Lutzoni et al., 2001; Kauff and Lutzoni, 2002; Miadlikowska and Lutzoni, 2004; Reeb et al., 2004) and the three- and four-gene phylogenies presented here. Therefore, the Pyrenulales should not be considered as an order of uncertain position (sensu Eriksson et al., 2003, 2004) or as a member of the Dothideomycetidae (sensu Kirk et al., 2001). As expected with such low sampling within each of these three orders, all were revealed to be monophyletic

and highly supported (PP = 100%, NJBP = 88–98%). This was also true for all genera for which we sampled more than one species. The first division at the base of the Chaetothyriales corresponds to the two families recognized by Eriksson et al. (2004) for this order—Chaetothyriaceae (represented by *Ceramothyrium*) and Herpotrichiellaceae (represented by *Capronia* and its anamorph *Exophiala*). The phylogenetic placement of *Glyphium* within the Chaetothyriales (black yeasts) was somewhat surprising. Kirk et al. (2001) classified this genus within the Mytillinidiaceae, which is part of the Hysteriales (Dothideomycetidae). The black yeasts used to be classified within the loculoascomycetes/Dothideales sensu lato and, therefore, this could be another case of a dothideomycetidioid representative of the Chaetothyriomycetidae. However, this needs to be confirmed with additional taxa.

Phylogenetic resolution and support within the predominantly lichen-forming Acarosporomycetidae, Lecanoromycetidae, and Ostropomycetidae are poor, demonstrating that the addition of more taxa within these groups, even when combining nucSSU and nucLSU data, is not sufficient. Restricting the analysis to members of the Pezizomycotina or a subset of this subphylum improves both components (Kauff and Lutzoni, 2002; Miadlikowska and Lutzoni, 2004; Reeb et al., 2004). However, we predict that the same decay of support values and resolution will happen as more taxa continue to be added to these three subclasses in the absence of additional genes and characters. Nevertheless, the monophyletic circumscription of the Acarosporomycetidae (PP = 100%), and the nonmonophyly of the genera *Acarospora* and *Sarcogyne*, are consistent with Reeb et al. (2004) and the nucSSU + nucLSU + *RPB2* based tree presented here. For a more comprehensive phylogenetic study of the Acarosporales and its consequences on their classification within the Ascomycota, see Reeb et al. (2004).

Based on the nucSSU + nucLSU phylogeny, the Lecanoromycetidae are monophyletic but received support only from the Bayesian analysis (PP = 100%). *Calicium viride* was unresolved in this two-gene phylogeny, but is clearly part of this subphylum based on previous studies (Kauff and Lutzoni, 2002; Wedin et al., 2002; Lücking et al., 2004; Lumbsch et al., 2004; Miadlikowska and Lutzoni, 2004; Reeb et al., 2004) and the three- and four-gene trees presented here (Figs. 4 and 5). With only a few exceptions, e.g., monophyly of the suborder Peltigerineae (Miadlikowska and Lutzoni, 2004), phylogenetic relationships within the Lecanoromycetidae are not resolved or received poor to no support. Phylogenetic placement of *Megalospora tuberculosa* sister to the Teloschistaceae (PP = 100%) is shown here for the first time. If this result is confirmed by additional *Megalospora* species, this would strongly suggest that this genus should be classified within the Teloschistales (sensu Miadlikowska and Lutzoni, 2004) rather than in the Lecanorineae (sensu Eriksson et al., 2004) or the Lecanorales (sensu Kirk et al., 2001). The phylogenetic placement of *Lopezaria versicolor* sister to *Scoliciosporum umbrinum* (PP = 99%) confirms Sipman's conclusion (1983) that *Lopezaria versicolor* is not a member of the genus *Megalospora*. The sister relationship of *Lecanora concolor* to these two genera (not significant, PP = 93%) suggests that *Lopezaria* might be a member of the Lecanoraceae. However, this hypothetical classification of *Lopezaria* needs to be confirmed with higher support values and more species from the Lecanoraceae. *Lopezaria* is considered to be a Lecanorales or Lecanoromycetes genera incertae sedis by Kirk et al. (2001) and Eriksson et al. (2004), respectively.

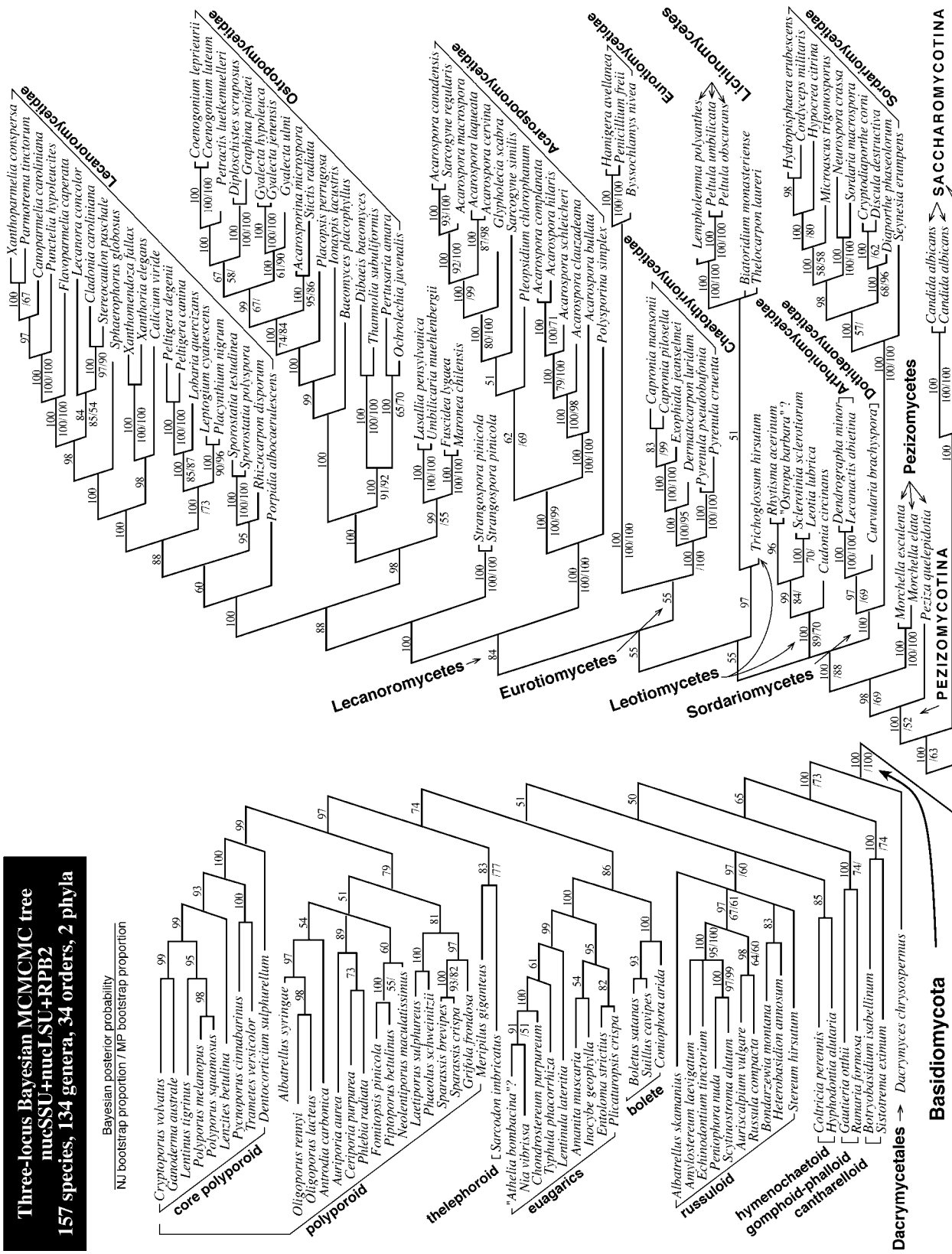
Members of the Fuscideaceae (represented by *Fuscidea* and *Maronea*) were nested within the Ostropomycetidae (Miadlikowska and Lutzoni, 2004; Reeb et al., 2004) and two *Gyalecta* species and *Trapeliopsis granulosa* are unresolved outside the *Umbilicaria* + Fuscideaceae + Ostropomycetidae clade, hence the Ostropomycetidae clade is not recovered in this two-gene phylogeny. These unusual relationships compared to Miadlikowska and Lutzoni (2004), Reeb et al. (2004), and the three- and four-locus trees presented here are due to the loss of many fast-evolving sites, associated with such a broad sampling across the fungi. Many of these sites become impossible to align unequivocally as more distant taxa are added to the alignment. This explains also why none of the reconstructed relationships among the putative members of the Ostropomycetidae are well supported in this broad two-gene phylogeny compared to other phylogenetic analyses of the nucSSU + nucLSU that were restricted to the Ascomycota (Lutzoni et al., 2001; Kauff and Lutzoni, 2002; Miadlikowska and Lutzoni, 2004; Reeb et al., 2004). Despite these nonsignificant topological discrepancies, the emended order Pertusariales, including Coccotremataceae, Pertusariaceae, and Icmadophilaceae (Miadlikowska and Lutzoni, 2004) is found to be monophyletic, but received support only from the Bayesian analysis in this two-gene based phylogeny (PP = 100%).

*nucSSU + nucLSU + RPB2*—Basal Ascomycota relationships in the nucSSU + nucLSU + *RPB2* Bayesian tree (Fig. 4) are similar to relationships revealed by our two-locus Bayesian tree (Figs. 2 and 3). The Taphrinomycotina was found to be paraphyletic; however, because *Neolecta* is absent from this three-locus phylogeny, this paraphyly is not significant in terms of posterior probabilities (PP = 69%; MPBP = 72%). The addition of *RPB2* to the nucSSU + nucLSU data set confirms the monophyly of Saccharomycotina, Pezizomycotina, and the inoperculate euascomycetes (i.e., all members of the Pezizomycotina except the Pezizomycetes) as was revealed by our two-locus tree. One difference lies in the Pezizomycetes forming a paraphyletic group (PP = 98%, MPBP = 69%) as for the nucSSU + nucLSU + *RPB2* phylogeny of Reeb et al. (2004) and *RPB2* phylogeny of Liu and Hall (2004).

Relationships among subclasses within the inoperculate euascomycetes are better resolved in our nucSSU + nucLSU + *RPB2* Bayesian tree compared to the nucSSU + nucLSU

→

Fig. 4. Phylogenetic relationships among 157 ascomycete and basidiomycete taxa based on combined evidence from nucSSU, nucLSU, and *RPB2*. This phylogeny resulted from a 50% majority rule consensus of 20 000 trees sampled with Bayesian Metropolis coupled Markov chain Monte Carlo. The resulting posterior probabilities (PP) are shown above internal branches. NJ bootstrap proportions (NJBP) with ML distance (1000 bootstrap replicates) are shown below internal branches before the slash sign, and weighted MP bootstrap proportions (MPBP) are shown below internal branches after the slash sign. Branch lengths are not proportional to evolutionary rates or number of changes, but were adjusted for optimal use of the graphic space. See Appendix 5 (in Supplemental Data with online version of this article) for a phylogram of this tree with branch lengths proportional to the average number of substitutions per site.


Three-locus Bayesian MCMCMC tree
nucSSU+nucLSU+RPB2
157 species, 134 genera, 34 orders, 2 phyla
Bayesian posterior probability
NJ bootstrap proportion / MP bootstrap proportion
Xanthoparmelia conspersa
Parmotrema tinctorum
Canoparmelia caroliniana
Punctelia hypoleucites
Flavoparmelia caperata
Lecanora concolor
Cladonia caraliniana
Stereocaulon paschale
Sphaerophorus globosus
Xanthomendoza fallax
Xanthoria elegans
Caloicium viride
Peltigera degenii
Peltigera canina
Lobaria quercizans
Leptogium cyanescens
Placynthium nigrum
Sporostatia testudinea
Rhizocarpon disporum
Porpidia albocaerulescens
Coenogonium leprieurii
Coenogonium luteum
Petractis luelkemuelleri
Diploschistes scruposus
Graphina paitiaei
Gyalecta hypoleuca
Gyalecta jenensis
Gyalecta ulmi
Acarosporina microspora
Stictis radiata
Placopsis pertusoa
Ionaspis lacustris
Baeomyces placophyllus
Dibaeis baeomyces
Thamnolia subuliformis
Pertusaria amara
Ochrolechia juvenalis
Lasallia pensylvanica
Umbilicaria muehlenbergii
Fuscidea lygaea
Maronea chilensis
Strangospora pinicola
Strangospora pinicola
Acarospora canadensis
Sarcogyne regularis
Acarospora macrospora
Acarospora laqueata
Acarospora cervina
Glypholecia scabra
Sarcogyne similis
Pleopsidium chlorophanum
Acarospora complanata
Acarospora hilaris
Acarospora schleicheri
Acarospora clauzadeana
Acarospora bullata
Polysporina simplex
Hamigera avellanea
Penicillium freii
Byssochlamys nivea
Capronia mansonii
Capronia pilosella
Exophiala jeanselmei
Dermatocarpon luridum
Pyrenula pseudobufonia
Pyrenula cruenta
Trichoglossum hirsutum
Rhytisma acerinum
"Ostropa barbara"?
Sclerotinia sclerotiorum
Leotia lubrica
Cudonia circinans
Dendrographa minor
Lecanactis abietina
Curvularia brachyspora
Morchella esculenta
Morchella elata
Peziza quelepidotia
Taphrina communis
Taphrina wiesneri
Protomyces inouyei
Saitoella complicata
Candida albicans
Candida albicans
Saccharomyces cerevisiae
Saccharomyces cerevisiae
Lecanoromycetes
Eurotiomycetes
Leotiomycetes
Sordariomycetes
Pezizomycetes
PEZIZOMYCOTINA
TAPHRINOMYCOTINA
SACCHAROMYCOTINA
Ascomycota
Outgroup: Chytridiomycota, Zygomycota, Glomeralean fungi
Lecanoromycetidae
Ostropomycetidae
Acarosporomycetidae
Eurotiomycetidae
Chaetothyriomycetidae
Lichinomycetes
Arthoniomycetidae
Dothideomycetidae
Sordariomycetidae
Cryptoporus volvatus
Ganoderma australe
Lentinus tigrinus
Polyporus melanopus
Polyporus squamosus
Lenzites betulina
Pycnoporus cinnabarinus
Trametes versicolor
Dentocorticium sulphurellum
core polyporoid
Albatrellus syringae
Oligoporus rennyi
Oligoporus lacteus
Antrodia carbonica
Auriporia aurea
Ceriporia purpurea
Phlebia radiata
Fomitopsis pinicola
Piptoporus betulinus
Neolentiporus maculatissimus
Laetiporus sulphureus
Phaeolus schweinitzii
Sparassis brevipes
Sparassis crispa
Grifola frondosa
Meripilus giganteus
polyporoid
Sarcodon imbricatus
"Athelia bombacina"?
Nia vibrissa
Chondrostereum purpureum
Typhula phacorrhiza
Lentinula lateritia
thelephoroid
Amanita muscaria
Inocybe geophylla
Entoloma strictius
Plicaturopsis crispa
euagarics
Boletus satanas
Suillus cavipes
Coniophora arida
bolete
Albatrellus skamanius
Amylostereum laevigatum
Echinodontium tinctorium
Peniophora nuda
Scytinostroma alutum
russuloid
Auriscalpium vulgare
Russula compacta
Bondarzewia montana
Heterobasidion annosum
Stereum hirsutum
Cotylicia perennis
Hyphodontia alutaria
Gautieria otthii
Ramaria formosa
Botryobasidium isabellinum
Sistotrema eximum
hymenochaetoid
gomphoid-phalloid
cantharelloid
Dacrymycetales
Dacrymyces chrysospermus
Basidiomycota
/Hymenomycetes

based phylogeny, probably due to a smaller taxon sampling combined with the addition of data from a 2.1-kb portion of the *RPB2* gene. The Sordariomycetes, polyphyletic in the two-gene tree, forms a monophyletic group in this three-gene phylogeny (PP = 100%; also in Lutzoni et al., 2001; Kauff and Lutzoni, 2002; Reeb et al., 2004). Combining *RPB2* with nucSSU and nucLSU revealed for the first time (see Taylor et al., 2004) that the Arthoniomycetidae are more closely related to the Dothideomycetidae with medium support (PP = 97%, MPBP = 69%) than to the Sordariomycetidae. However, taxon sampling of the Arthoniomycetidae and the Dothideomycetidae is very poor, and additional sequence data, especially from more typical members of the Dothideales, are needed to verify this relationship (Fig. 4). Relationships among orders within the Sordariomycetidae are consistent with the richer taxon sample of the nucSSU + nucLSU rDNA phylogeny with the exception of the paraphyletic relationship between Sordariales and Diaporthales, which in those analyses formed a monophyletic group (Fig. 2). A similar phenomenon was observed in a reduced taxon sampling of rDNA alone (Berbee and Taylor, 1992).

If we exclude *Ostropa barbara*, the Leotiomycetes are paraphyletic with *Trichoglossum* forming a distinct lineage sister to the Lichinomycetes-*Biatoridium*-*Thelocarpon* group (PP = 97%). As for previous molecular phylogenetic studies, the addition of *RPB2* data could not resolve the phylogenetic position of the remaining monophyletic members of the Leotiomycetes (PP = 100%, NJBP = 89%, MPBP = 70%), compared to the Sordariomycetes and the Lichinomycetes-Eurotiomycetes-Lecanoromycetes group.

The Lichinomycetes, which were nested within the Dothideomycetidae 2 group in the two-locus phylogeny (PP = 95%; Fig. 2), are now sister to the Eurotiomycetes-Lecanoromycetes group (without support), together with *Biatoridium* (Lecanorales), *Thelocarpon* (Pezizomycotina incertae sedis), and *Trichoglossum* (Leotiomycetes). Such a close relationship between the Lichinomycetes and the Eurotiomycetes-Lecanoromycetes was associated with medium support (PP = 100%, BBP = 67%) based on an analysis restricted to the Ascomycota by Reeb et al. (2004).

The sister relationship of the Eurotiomycetes to the Lecanoromycetes in Reeb et al. (2004) was also recovered here by the addition of the *RPB2* data to the nucSSU + nucLSU data set, but with high phylogenetic uncertainty (Fig. 4). The sister relationship of the Eurotiomycetidae with the Chaetothyriomycetidae, that has been shown with high confidence in several studies (see Taylor et al., 2004; Reeb et al., 2004) and shown in our two-locus tree with no support (Fig. 2), was recovered with this three-gene phylogeny but with no support. The high resolution and support for the monophyly of the Chaetothyriomycetidae confirms the inclusion, with high confidence, of the Pyrenulales (Ascomycota incertae sedis in Eriksson et al., 2003, 2004) within this subclass, as was shown previously (Lutzoni et al., 2001; Kauff and Lutzoni, 2002; Reeb et al., 2004), and in the two-gene phylogeny presented above (Fig. 2).

Within the Lecanoromycetes, three main groups can be distinguished: Acarosporomycetidae, Ostropomycetidae, and Lecanoromycetidae. The basal subclass Acarosporomycetidae forms a highly supported monophyletic group, and relationships within this subclass are identical to findings of Reeb et al. (2004). Both the Lecanoromycetidae and the newly established Ostropomycetidae (Reeb et al., 2004) form monophyletic groups with high posterior probabilities. However, the delimitation of these two subclasses is uncertain. This is only the second time that the sister relationship of the Pertusariales + Icmadophilaceae to the Ostropales + Baeomycetales (sensu Kauff and Lutzoni, 2002) is revealed (Reeb et al., 2004); both studies were based on a combined nucSSU + nucLSU + *RPB2* data set. The position of *Strangospora* is still uncertain (also see Reeb et al., 2004) and could represent an independent lineage within the Lecanoromycetes. The Fuscideaceae + Umbilicariaceae group could also be recognized as a separate subclass or subsumed within the Ostropomycetidae. The placement of the Umbilicariaceae has been highly unstable among past phylogenetic studies.

*nucSSU + nucLSU + mitSSU + RPB2*—In general, the main groups (subphylum to subclass levels) as outlined by Taylor et al. (2004) were revealed by this four-locus Bayesian MCMCMC analysis (Fig. 5). However, the addition of both the mitSSU and *RPB2* to the nucSSU and nucLSU data greatly improved the level of resolution and support for deep relationships within the Ascomycota compared to previous studies (compare our Figs. 5 and 8 to the schematic tree in Fig. 12.5 of Taylor et al., 2004). The Ostropomycetidae are shown here to share a most recent common ancestor with the Lecanoromycetidae, as supported by a significant posterior probability and moderate Bayesian bootstrap proportion. The same internode was resolved, albeit with insufficient support, by Miadlikowska and Lutzoni (2004), who used a two-gene combined nucSSU and nucLSU data set. By adding *RPB2* to nucSSU and nucLSU data, Reeb et al. (2004) resolved this phylogenetic relationship with a significant posterior probability (98%) and Bayesian bootstrap value of 70%. In addition, our four-locus phylogeny is the first to show a statistically significant (PP = 100%) sister relationship between the Acarosporomycetidae and the Ostropomycetidae-Lecanoromycetidae group.

The four-locus phylogeny inferred here allows us to restrict the Lecanoromycetes to include members of the Acarosporomycetidae, Lecanoromycetidae, and Ostropomycetidae, and to recircumscribe the Eurotiomycetes. The latter is now composed of two subclasses, the Chaetothyriomycetidae, which includes members of the Chaetothyriales, Pyrenulales, and Verrucariales; and the Eurotiomycetidae, which corresponds to the Plectomycetes as defined by Geiser and LoBuglio (2001), that is, including members of the Ascosphaerales, Onygenales, and Eurotiales. Except for *Stictis radiata* and *Acarosporina microspora*, which are members of the nonlichenized Stictidaceae, all species shown in the Lecanoromycetes in Fig. 5 are lichen-forming species. In contrast, the proportion of lichenized to nonlichenized species is more balanced in the Eurotiomycetes: all members of the Chaetothyriales and Eurotiales are believed to be nonlichenized, whereas most species of the Pyrenulales and Verrucariales exhibit the lichen habit.

Another relationship that was unknown based on previous molecular evidence as summarized by Taylor et al. (2004) was the phylogenetic placement of the Lichinomycetes relative to the Eurotiomycetes, Laboulbeniomycetes, Lecanoromycetes, Leotiomycetes, and Sordariomycetes. The combination of *RPB2* with nucSSU and nucLSU by Reeb et al. (2004) showed for the first time that the Lichinomycetes are sister to the Lecanoromycetes-Eurotiomycetes group (PP = 100%, BBP = 67%), along with members of the Thelocarpaceae represented by *Thelocarpon* (previously incertae sedis at the family level within the Ascomycota; Eriksson et al., 2004), *Biatoridium*

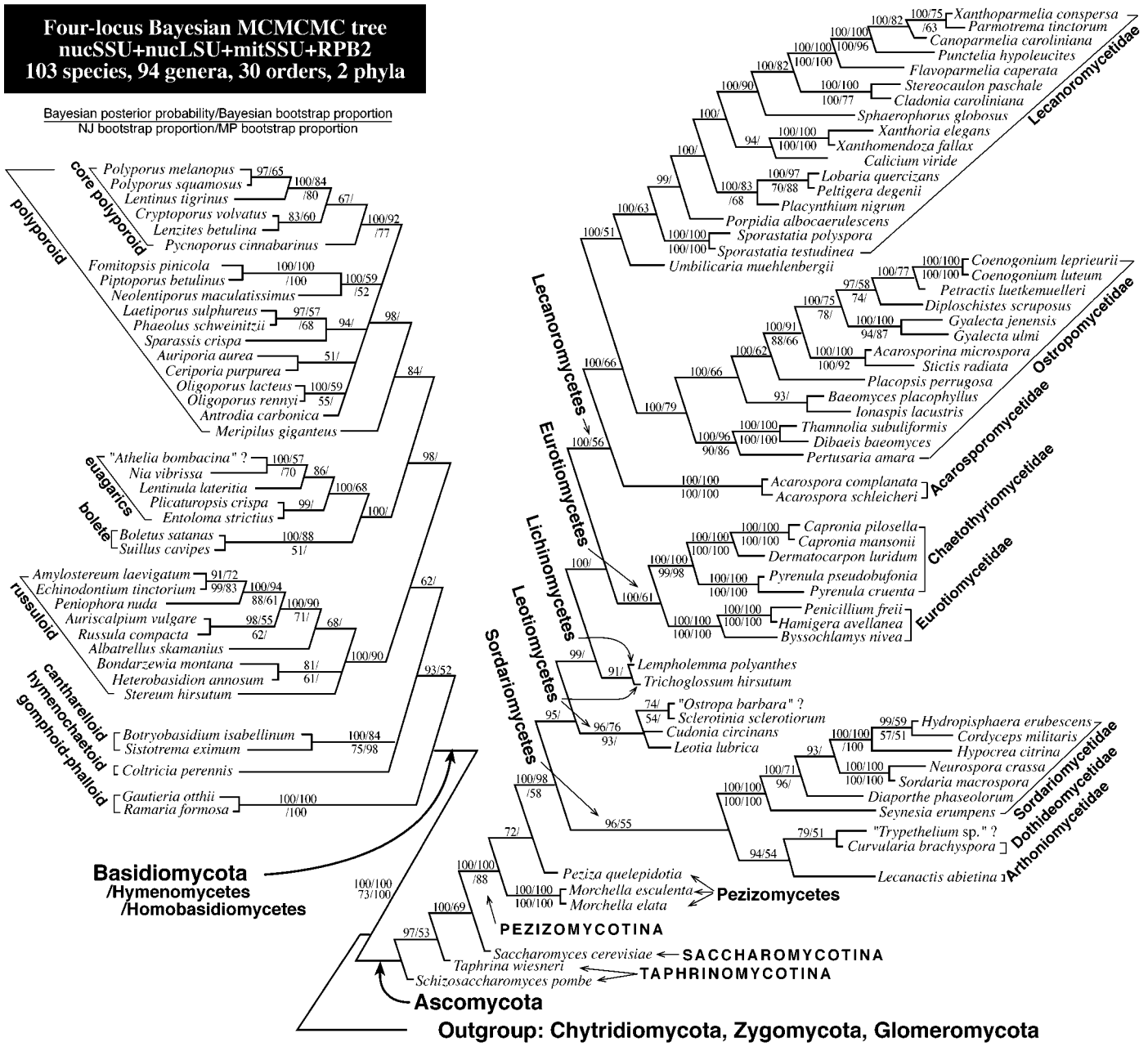



Fig. 5. Phylogenetic relationships among 103 ascomycete and basidiomycete species based on combined evidence from nucSSU, nucLSU, mitSSU rDNA, and *RPB2*. This phylogeny resulted from a 50% majority rule consensus of 69 000 trees sampled with Bayesian Metropolis coupled Markov chain Monte Carlo (MCMCMC). The resulting posterior probabilities (PP) are shown above internal branches before the slash sign. One hundred Bayesian MCMCMC analyses were conducted on bootstrapped versions of this four-locus data set. Bayesian bootstrap proportions (BBP) ≥50% are presented above internal branches after the slash sign. NJ bootstrap proportions (NJBP) with ML distance (1000 bootstrap replicates) are shown below internal branches before the slash sign. Weighted MP bootstrap proportions (MPBP) are shown below branches after the slash sign. Branch lengths are not proportional to evolutionary rates or number of changes, but were adjusted for optimal use of space. See Appendix 6 (in Supplemental Data accompanying the online version of this article) for a phylogram version of this tree with branch lengths proportional to the average number of substitutions per site.

(previously incertae sedis at the genus level within the Lecanorales; Eriksson et al., 2004), and a subgroup of the Leotiomycetes represented by *Trichoglossum hirsutum*. This phylogenetic placement of the Lichinomycetes and part of the Leotiomycetes is confirmed by our four-locus phylogeny but with less phylogenetic confidence (PP = 99%, BBP < 50%). This lower support in the present study compared to that of Reeb et al. (2004) reflects the restriction of that study to members of the Ascomycota. This observation is substantiated by a comparison of our three-locus tree (Fig. 4), which is based on the same loci (nucSSU + nucLSU + *RPB2*) and virtually the same taxon sampling within the Ascomycota as that of Reeb et al. (2004). Similarly, deep relationships within the Pezizomycotina (euascomycetes) are mostly poorly supported in the three-locus tree presented here (Fig. 4) compared to the Ascomycota-only tree presented by Reeb et al. (2004).

As we continue moving closer to the early evolution of the Pezizomycotina (euascomycetes), we enter a portion of the tree with the highest level of phylogenetic uncertainty. It is likely that most members of the Leotiomycetes will occupy this part of the tree, albeit in a nonmonophyletic manner, but their position has been very unstable in most previous studies. Reeb et al. (2004) were the first to report a moderately supported sister relationship between a subgroup of the Leotiomycetes (represented by *Cudonia* and *Rhytisma*) and the Sordariomycetes (PP = 97%, BBP = 68%). The four-locus phylogeny (Fig. 5) shows a subgroup of the Leotiomycetes, represented by *Sclerotinia*, *Cudonia*, and *Leotia* that corresponds to Leotiomycetes clade 1 in the nucSSU + nucLSU rDNA tree, forming a monophyletic group, but with significantly increased levels of support (PP = 96%, BBP = 76%, NJBP = 93%). The phylogenetic placement of *Trichoglossum hirsutum*, which is the only representative left of the Leotiomycetes clade 2 from Fig. 2, remains uncertain with four loci concatenated into one data set. As in the three-gene phylogeny (Fig. 4), the four-gene phylogeny shows *Trichoglossum* closely related to members of the Lichinomycetes. The nucSSU + nucLSU + *RPB2* phylogenetic tree of Reeb et al. (2004) differed by the inclusion of *Trichoglossum hirsutum* in a monophyletic group with the Lichinomycetes, Lecanoromycetes, and Eurotiomycetes (PP = 100%, BBP = 67%), and the Leotiomycetes group 1 forming a monophyletic group with the Sordariomycetes (PP = 97%, BBP = 68%) that is sister to the Lichinomycetes-Leotiomycetes 2-Eurotiomycetes-Lecanoromycetes group. The diphyly of the Leotiomycetes needs to be confirmed with additional taxa and characters. Liu and Hall (2004) reported the Leotiomycetes as being monophyletic, but their taxon sampling did not include *Trichoglossum* or any representatives of our Leotiomycetes clade 2.

The affiliation of the Arthoniomycetidae (mostly lichen-forming) within the Sordariomycetes has not been resolved with high confidence by any previous studies. This is still true for our four-gene phylogeny with a nearly significant posterior probability for recognizing the Arthoniomycetidae as sister to the Dothideomycetidae. Our three-locus analysis (nucLSU, nucSSU, *RPB2*) supports this result with a significant posterior probability of 97% and a MPBP of 69%.

Based on our four-locus analysis, we still cannot conclude whether the Pezizomycetes are mono- or paraphyletic (Fig. 5). Reeb et al. (2004) found the same paraphyletic relationship, such that *Peziza* represents a separate lineage from *Morchella*, but was supported by a posterior probability of 98%, BBP of 69% and ML bootstrap proportion of 76%. The four-gene tree supports a paraphyletic Taphrinomycotina, although a more extensive taxon sampling with a multilocus approach is needed.

***nucSSU + nucLSU + mitSSU***—When combining the nucSSU-nucLSU with the mitSSU data set, no major gains in terms of phylogenetic confidence were made compared to the addition of *RPB2* to the same two loci. This is perhaps due to the unusual behavior of the B-MCMCMC analysis associated with the addition of the mitSSU. Six Bayesian analyses of three times 5 000 000 generations were initiated with random starting trees. None of the independent runs converged on the same average likelihood level after 15 000 000 generations. The oscillation around the average likelihood was higher with the nucSSU + nucLSU + mitSSU data set than in any other Bayesian analyses of the combined data sets. In contrast, when combining the nucSSU + nucLSU data with the *RPB2* data, five of six Bayesian runs converged on the same average likelihood level after 4 000 000 generations. However, it is important to note that the nucSSU + nucLSU + *RPB2* data set included fewer taxa than the nucSSU + nucLSU + mitSSU data set (157 vs. 236, respectively), which could explain, in part, some of the differences in the efficiency of the respective searches. It is unclear which features of the mitSSU hindered the B-MCMCMC search process, but odd behaviors of the mitSSU have been noted previously (see Miadlikowska and Lutzoni, 2004).

***Revision of septal features of Fungi***—The septal data represent a selective survey only and are intended to provide a general overview of variation in the Fungi; see Bracker (1967), Beckett et al. (1974), Kimbrough (1994), Markham (1994), Wells (1994), McLaughlin et al. (1995b), and Bauer et al. (1997) for additional taxa and variation in septal pore organization. Septa, like many subcellular features, are dynamic structures that change as cells develop and age. Comparisons here are between septa that are assumed to be mature but not senescent. In the following account, two types of multipored septa are distinguished: multiperforate septa in which the pore diameter is usually comparable to that in most uniperforate septa, and plasmodesmata with narrow pores frequently containing desmotubules.

In the Chytridiomycota, both types of multipored septa are found (Fig. 6): plasmodesmata with desmotubules in *Powellomyces variabilis* (Spizellomycetales) and Chytridiales (Taylor and Fuller, 1980; Powell and Gillette, 1987), and multiperforate septa with large pores bordering the lateral hyphal wall and an occluded central pore in *Allomyces* (Blastocladiales). Plasmodesmata are present in some Zygomycota and Ascomycota (Fig. 6), while taxa in several classes of filamentous ascomycetes and one basidiomycete taxon possess multiperforate septa (not illustrated; Reichle and Alexander, 1965; Wetmore, 1973; Doublés and McLaughlin, 1991).

Uniperforate septa are found in some Zygomycota but are more common in Ascomycota and Basidiomycota (Figs. 6, 7). A septal pore with a lenticular cavity and nonmembrane-bound occlusion characterizes several orders of Zygomycota (Benny et al., 2001), but the adjacent nonmembrane-bound globules in *Dimargaris cristalligena* have a restricted distribution (Fig. 6). In filamentous ascomycetes, Woronin bodies are associated with septa in vegetative and nonascogenous hyphae, but they are generally absent from the dikaryotic ascogenous hyphae and asci. The septal pore in ascomycete dikaryons exhibits a wide variety of differentiated pore-occluding structures, which appear to have phylogenetic utility (Kimbrough, 1994). The structure of the septal pore changes during ascus development (Fig. 6). Thus, ascomycete septa may have three morphologies depending on the stage of development.

Septal structure is generally more uniform at different stages of development in Basidiomycota than in Ascomycota. However, septal forms show a range of variation from Urediniomycetes with ascomycete-like septa to Hymenomycetes with complex septal pore caps (Fig. 7). Transitions in the septal pore structure are seen in the classes of Basidiomycota, with simple septa in the Urediniomycetes, septa with and without septal swellings in the Ustilaginomycetes, and septal pore swellings with and without septal pore caps in the Hymenomycetes (not illustrated). The relationships in the morphological tree parallel those from molecular results (Figs. 2, 7).

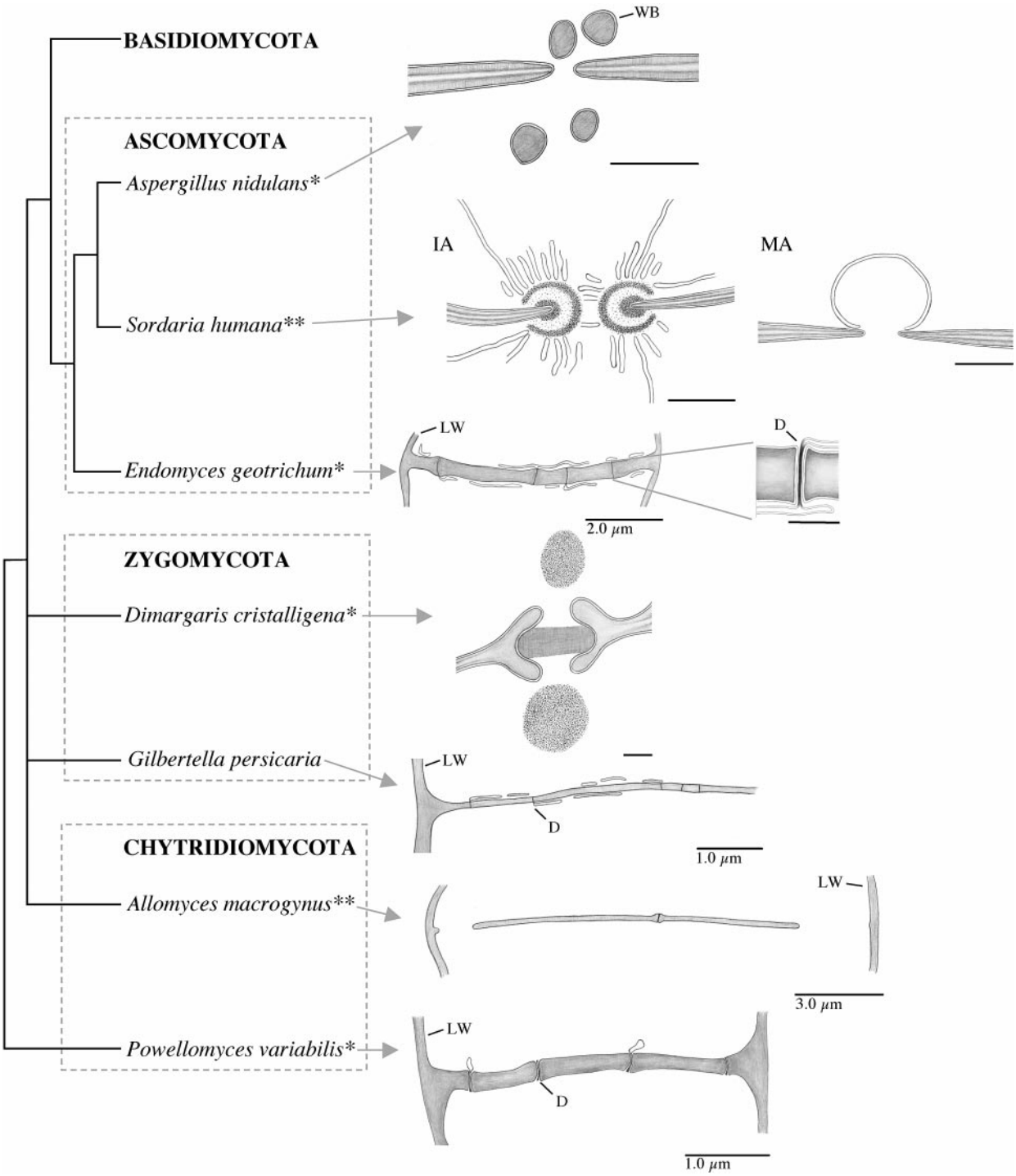



Fig. 6. Cladogram based on current molecular hypotheses of the relationships among the major lineages of Fungi illustrating septal pore variation in three phyla. Drawings are interpretations of published micrographs of vegetative septa, except for *Sordaria humana*, which are based on septa of the mature (MA) and immature (IA) ascus, and *Gilbertella persicaria*, which is based on the gametangial septum. An asterisk (*) indicates a taxon not present in Fig. 2; a double asterisk (**) indicates a different species of a monophyletic genus present in Fig. 2. Six variations on septal pore organization are illustrated: multiperforate septum with plasmodesmata and desmotubules (D; *Powellomyces variabilis*, Spizellomycetales; *Gilbertella persicaria*, *Endomyces geotrichum*, Saccharomycotina), multiperforate septum with peripheral pores and plugged central pore (*Allomyces macrogynus*), uniperforate septum with lenticular cavity, nonmembrane-bound pore occlusion, and associating nonmembrane-bound globules (*Dimargaris cristalligena*, Dimargaritales, possible sister group to Kickxellales), uniperforate septum with Woronin bodies (WB; *Aspergillus nidulans*, Eurotiomycetidae), and uniperforate septum with torus and radiating tubular cisternae or membranous subspherical pore cap (*Sordaria humana* IA and MA, respectively). LW, lateral wall of hypha; scale bars = 0.25 μm except where indicated. Illustrations from top to bottom interpreted from Momany et al. (2002), Beckett (1981), Kreger-van Rij and Veenhuis (1972), Jeffries and Young (1979), Hawker et al. (1966), Meyer and Fuller (1985), Powell (1974).

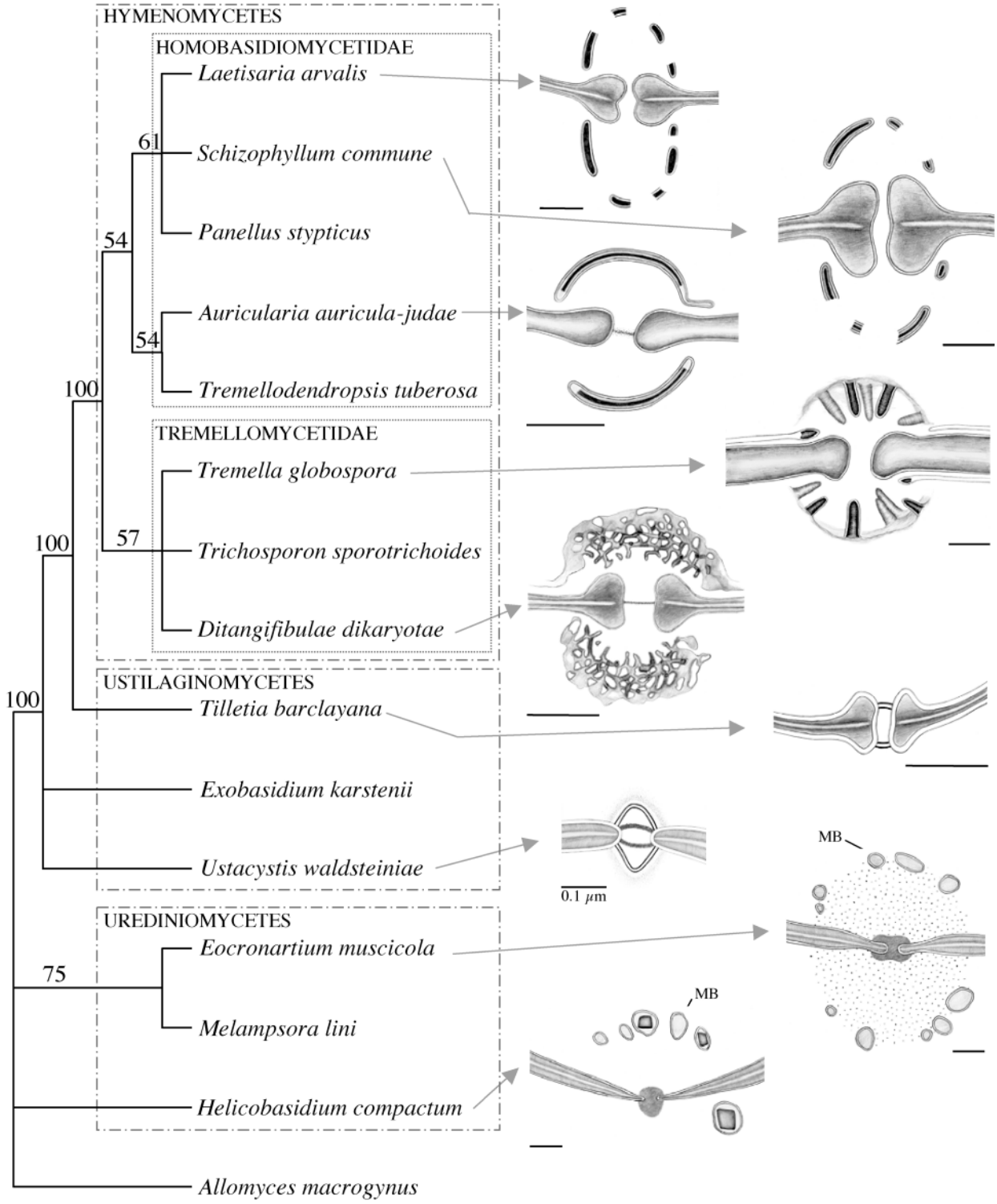



Fig. 7. Cladogram from morphological character analysis of selected taxa in the Basidiomycota illustrating variation in the septal pore apparatus of uniperforate septa in the three classes. The tree is a 50% majority-rule consensus of 336 equally parsimonious trees of 17 steps using a character matrix of equal weight and rooted using *Allomyces macrogynus*. Values above branches indicate frequency of branch recovery in all equally parsimonious trees. The following septal pore variations are illustrated: simple septum with membranous or nonmembranous pore occlusions and with (Urediniomycetes) or without (*Ustacystis waldsteiniae*) associated microbodies (MB), septal pore swelling without pore caps (*Tilletia barclayana*), septal pore swelling with two variations of elaborated septal pore caps (Tremellomycetidae), and septal pore swelling with simple pore cap with or without perforations (Homobasidiomycetidae). Scale bars = 0.25 μm except where indicated. Illustrations from top to bottom interpreted from Hoch and Howard (1981); Müller et al. (1998); Lü and McLaughlin (1991); Berbee and Wells (1988); Adams et al. (1995); Bauer et al. (1997); Bauer et al. (1995); Boehm and McLaughlin (1989); D. McLaughlin, University of Minnesota.

However, unconstrained analyses yielded 336 equally most parsimonious trees (L = 17; RI, CI, RCI = 1.0) and indicate that the Ustilaginomycetes and Urediniomycetes are not monophyletic (Fig. 7). In constrained analyses (not shown), 2388 equally parsimonious trees (L = 18; RI = 0.952, CI = 0.944, RCI = 0.899) that satisfy monophyly of the Ustilaginomycetes are only one step longer than those of the unconstrained analyses, while the 84 equally most parsimonious trees that satisfy monophyly of the Urediniomycetes are only a subset of the unconstrained trees.

Within the Urediniomycetes, two septal pore organizations are shown: the ascomycete-like type in *Helicobasidium compactum* and a more derived type in the rust *Melampsora lini* (Littlefield and Bracker, 1971) and its relative *Eocronartium muscicola*, with a zone of exclusion surrounding a pulley-wheel-shaped septal pore plug. Two divergent types of septal pore organization in the Ustilaginomycetes are illustrated, one with and the other without septal pore swellings, but both with membranous pore occlusions. In the Hymenomycetes, the basic structure of the pore cap distinguishes the two subclasses. When present, elaborated caps are characteristic of the Tremellomycetidae, while simple caps with variations in internal structure and size of the cap pores are characteristic of the Homobasidiomycetidae (McLaughlin et al., 1995b).

## DISCUSSION

***A need for balanced taxon sampling***—Multigene phylogenies using all sequences available clearly show that the overall sampling has been strongly biased toward the Pezizomycotina (euascomycetes) and the Homobasidiomycetes. None of the members of the Chytridiomycota, Zygomycota, or the Glomeromycota has had another locus (such as mitSSU and *RPB2*) sequenced in addition to nucSSU and nucLSU. The same is true for basal groups within the Basidiomycota (e.g., Urediniomycetes, Ustilaginomycetes, Tremellomycetidae, and the thelephoroid clade). The least sampled subphyla in multilocus phylogenetic studies of the Ascomycota also are part of the earliest divergences within this phylum—the Taphrinomycotina and Saccharomycotina. Priority should be given to all these early branching groups in future systematic studies of the Fungi, with a concerted effort to sequence at least both the nucSSU and nucLSU for each targeted species within these phyla. Within the earliest branching fungal lineages, further sampling of the nonmonophyletic orders Chytridiales, Blastocladiales, Zoopagales, and Entomophthorales will increase our knowledge of phylogenetic diversity in these poorly known groups. Within the Pezizomycotina, the Leotiomycetes, Dothideomycetes, Lichinomycetes, Arthoniomycetes, and Chaetothyriomycetidae should be the primary targets of future studies. Maximum gains toward assembling the fungal tree of life would be achieved by sequencing at least the four loci we have included in this study. All alignments generated by our study are available to the mycological community. We hope this will be an incentive to include the nucSSU, nucLSU, *RPB2* and mitSSU in future phylogenetic studies of the Fungi.

***Using phylogenetic tools to detect errors in GenBank and fungal culture collections***—AFTOL and multilocus phylogenetic studies in general provide an ideal opportunity to detect errors in GenBank and fungal culture collections. Conflicts among loci indicate that at least one of the single-locus phylogenies may be wrong in representing species phylogenies. Among many possible analytical artifacts and biological factors such as lineage sorting and recombination (see Bull et al., 1993; Lutzoni, 1997), incongruent results among single-locus phylogenies could result from an error in the lab or in the preparation of data sets. An unexpected phylogenetic placement, even if consistent across multiple loci, could also be a sign that the specimen used for the culture was misidentified or that the culture is from a contaminant fungus.

Throughout this article, taxon names in quotes followed by a question mark indicate cases in which we thought the results were unusual and needed to be verified. For example, two isolates in the nucSSU + nucLSU data set, ''*Athelia arachnoidea*'' and ''*Hyphoderma praetermissum*,'' are probably misidentified, based on results of analyses with much more extensive sampling (Larsson, 2002; M. Binder et al., Clark University, unpublished manuscript). The isolate labeled ''*A. arachnoidea*'' is a member of the polyporoid clade, and the isolate labeled ''*H. praetermissum*'' is a member of the athelioid clade, which was recognized by Larsson (2002). Rigorously identified isolates of *Hyphoderma praetermissum* and *A. arachnoidea* have been shown to belong to the hymenochaetoid clade and the athelioid clade, respectively (Larsson, 2002; M. Binder et al., unpublished manuscript). One species in the nucSSU + nucLSU + *RPB2* and four-gene data sets, ''*Athelia bombacina*,'' is of uncertain identity. The results of this analysis strongly suggest that this isolate is nested in the euagarics clade, which conflicts with its expected position in the athelioid clade (Larsson, 2002). If the isolate included here is correctly identified (see Appendix 2), then *Athelia* is polyphyletic.

*Ostropa barbara* by definition is a member of the Ostropales and, therefore, of the Ostropomycetidae. However, this taxon was consistently found in the Leotiomycetes across all of our phylogenetic analyses. No conflict was ever detected among the four loci for this species. A closer look at the source of the material showed that the four sequences were generated as part of AFTOL from a culture provided by a culture collection (Appendix 2). A reasonable explanation is that this culture is not from *Ostropa*, and these data must be verified by sequencing at least two more individuals or species from this genus. If this suspicion is confirmed, there should be mechanisms to inform the fungal culture collections to take the appropriate measures and to contact GenBank to inform them that sequences from this strain are misidentified. A strong case needs to be made for fungal phylogenetic studies to voucher cultures with specimens and to annotate every sequence appropriately in GenBank (see Blackwell and Chapman, 1993, as well as a series of letters on this topic in the *New Phytologist* 161: 1–21).

*Trypethelium* sp. is another interesting case. According to Kirk et al. (2001) and Eriksson et al. (2004), this genus should be part of the Pyrenulales, represented here by two *Pyrenula* species. In our study, sequences from this taxon were found to be significantly in conflict and, consequently, were removed from the two- and three-gene phylogenetic analyses (Figs. 2 and 4). However, the conflict was not found to be significant for the taxon sampling part of our four-gene phylogenetic analyses. Because the resulting tree places *Trypethelium* within the Dothideomycetidae and some of the sequences from this sample were in conflict in other pairwise tests among data partitions, this requires that at least two more species (or two individuals from distant populations) from the same genus be sequenced to confirm this result. Moreover, the source of the conflict should be identified for the existing sequences before

any conclusions are drawn. It is interesting to note that the use of different subpartitions of existing data sets could increase the accuracy of the available tests to detect conflicts among data partitions.

Detecting conflicts among data partitions and keeping track of problematic sequences is intrinsic to efficient large-scale multilocus phylogenetic studies. For this reason, we are reporting all taxa and the source of their sequences for which we detected a significant conflict or that were removed from our final phylogenetic analyses for other reasons (Appendix 3). These sequences in GenBank and strains should be excluded from future phylogenetic studies.

***Enigmatic fungi and the relationship between fungi and protozoa***—It has taken decades for mycologists to make the distinction between the Fungi and the fungus-like protists, and it is still a work-in-progress. Affinities between taxa formerly considered fungi and other eukaryote groups have been demonstrated using molecular phylogenies (Barr, 1992; Cavalier-Smith, 2001). Two groups with striking gross morphological similarity to Fungi, the oomycetes and hyphochytrids, have been removed from the Fungi and are now classified with brown algae and diatoms in the heterokonta (Gunderson et al., 1987; van der Auwera et al., 1995). The converse has also recently been demonstrated in molecular phylogenetic studies showing that taxa previously considered protozoa are actually Fungi (*Pneumocystis*, Microsporidia).

*Pneumocystis carinii* is found in lungs and is associated with pneumonia in a large variety of mammals. *Pneumocystis* was initially described as a trypanosome and shares with other protozoa the inability to be permanently cultured in vitro and resistance to the broad-spectrum antifungal drug amphotericin B (Stringer, 1996). The importance of *Pneumocystis* has grown due to rising incidence of HIV infections, because the pathogen is found primarily in immunocompromised individuals. Accepted as a protozoan for over 70 years, it was the sequencing of the 18S ribosomal RNA gene that suggested that *P. carinii* was a member of the Ascomycota (Edman et al., 1988). Increased sampling of the nucSSU and nucLSU rDNA sequences from Fungi supports *Pneumocystis* as a member of the Taphrinomycotina (Fig. 2), a position supported by analyses of ß-tubulin (Landvik et al., 2001) and *RPB2* sequences (Liu and Hall, 2004).

Microsporidia are widespread, highly reduced, obligately intracellular parasites that infect a variety of animals, but primarily arthropods and fish (Keeling and Fast, 2002). Intracellular growth takes the form of a cell-wall-less trophic form termed a meront, but reproduction is through a spore with an endospore wall composed partially of chitin. For a long time, Microsporidia were treated as a unique phylum of protozoa with uncertain affinities (Cavalier-Smith, 2001). The first nuc18S rDNA and EF-1α phylogenies indicated that Microsporidia were early-diverging eukaryotes, a fact consistent with their amitochondriate nature (Vossbrinck et al., 1987; Kamaishi et al., 1996). Instability of this placement and very long branches leading to Microsporidia made this position suspect. More recently, phylogenies generated using *RPB1* (Hirt et al., 1999), α- and ß-tubulin sequences (Keeling et al., 2000), and other protein-encoding genes (see Keeling and Fast, 2002) have challenged the early-diverging eukaryote hypothesis and instead indicate that Microsporidia are derived from within the Fungi. Microsporidia are currently hypothesized to be nested within the Fungi, possibly related to Zygomycota (Keeling, 2003), and have undergone extreme nuclear genome reduction (Katinka et al., 2001) and degeneration of the mitochondrion to a remnant genome-lacking organelle called the ''mitosome'' (Williams et al., 2002). No representatives of Microsporidia are included in this study. This is because of the extreme amount of divergence of their nucSSU and nucLSU, which would have jeopardized our analyses by extensively increasing regions where the alignment was judged to be ambiguous and would have led to the removal of a large number of sites. The phylogenetic placement of these highly specialized fungi needs to be the focus of studies and analyses that are specifically designed to address this issue.

Phylogenies of the crown eukaryotes have demonstrated the relationship of the animal and fungal kingdoms (Baldauf and Palmer, 1993; Baldauf et al., 2000; Lang et al., 2002). These two kingdoms are now known to be a part of a larger group that includes choanoflagellates and other protists (the Mesomycetozoa), termed the Opisthokonts (Cavalier-Smith and Chao, 1995; Ragan et al., 1996). Included among the choanoflagellates and Mesomycetozoa is *Amoebidium parasiticum*, which was once considered a trichomycete (Benny and O'Donnell, 2000; Ustinova et al., 2000). Trees based on concatenated mitochondrial proteins show the Mesomycetozoa and choanoflagelletes grouping with the animals rather than fungi (Lang et al., 2002). Microsporidia are also clearly part of this Opisthokont radiation, and if they did not diverge from within the Fungi, they may be the sister taxon (Keeling and Fast, 2002).

Because the majority of fungi are still undiscovered, a robust phylogeny of known taxonomic groups will be essential for placement of unknown species as these are discovered. As demonstrated for Bacteria and Archaea (Pace, 1997), the Fungi are likely to harbor many lineages whose discovery is dependent on phylogenetic analyses. Using DNA sequences cloned directly from a diverse variety of environments, novel lineages representing all of the major fungal phyla have recently been described (Lopez-Garcia et al., 2001; Edgcomb et al., 2002; Vandenkoornhuyse et al., 2002; Schadt et al., 2003).

***Current status of phylogenetic relationships among earliest diverging fungal lineages***—A new clarity of the relatedness among the earliest diverging fungal lineages is emerging from both analyses of nuclear and mitochondrial rDNA as well as protein-coding genes (O'Donnell et al., 2001; Forget et al., 2002; Bullerwell et al., 2003; Helgason et al., 2003; Keeling, 2003; Tanabe et al., 2004). The Chytridiomycota and Zygomycota are well demonstrated, using rDNA analyses, to be part of the earliest known divergence that took place during fungal evolution (Bruns et al., 1992; Berbee and Taylor, 1993; Tanabe et al., 2000). However, the Chytridiomycota, as it is currently circumscribed, appears polyphyletic because of placement of the blastocladialean chytrids with the Zygomycota (James et al., 2000; Forget et al., 2002; Tanabe et al., 2004). The Zygomycota sensu lato are also polyphyletic or minimally paraphyletic. The recent elevation of the Glomales to phylum status as the Glomeromycota (Schüßler et al., 2001) is supported by these and other rDNA analyses (James et al., 2000; Tehler et al., 2003). The relationship of the Glomeromycota to the various orders of Zygomycota needs to be clarified by the use of additional non-rDNA loci. Nonetheless, if we are to adopt a classification system based on phylogenetic criteria, other zygomycete lineages and the Blastocladiales

may need to undergo the same transition to a higher taxonomic rank because they form a paraphyletic assemblage.

Other relationships among the earliest diverging fungal lineages are being resolved through further study of protein-encoding genes. The results of the current two-gene analysis support the grouping of the Monoblepharidales with the Spizellomycetales and Chytridiales (Fig. 2), a result that is strongly supported by complete mitochondrial genome sequencing (Bullerwell et al., 2003). Although the entomophthoralean fungus *Basidiobolus* consistently groups with the Chytridiomycetes in nucSSU phylogenies (Nagahama et al., 1995; Jensen et al., 1998; James et al., 2000), more recent analyses suggest that *Basidiobolus* is a zygomycete-like fungus, only distantly related to other entomophthorales (Keeling, 2003; Tanabe et al., 2004). Another very promising recent result is the recovery of the clade of Zygomycota orders (Dimargaritales + Harpellales + Kickxellales) possessing a septal pore with a lenticular cavity using *RPB1* sequences (Tanabe et al., 2004). Unfortunately, it was not possible to include any Microsporidia in our phylogenetic analyses. Future studies hopefully will resolve the placement of the Microsporidia in the Metazoa/Mesomycetozoa/Fungi clade (Keeling and Fast, 2002).

***Current status of Basidiomycota phylogeny***—Inspection of Figs. 2–5 reveals that basidiomycete phylogenetics is still informed primarily by nuclear ribosomal genes. The most intensive sampling of these genes has been conducted within the Hymenomycetes, which contains about 68% of the known species of Basidiomycota (Kirk et al., 2001), but accounts for 90% of the Basidiomycota in the nucSSU + nucLSU data set (Fig. 2). The most commonly sampled region for higher-level analyses in Basidiomycota is the 5′ end of the nucLSU rDNA. Several large analyses of this gene have been published recently including one study with 877 species that focused on the euagarics clade (Moncalvo et al., 2002), and two others with 481 and 656 species, respectively, from across the Homobasidiomycetes (Hibbett and Binder, 2002; M. Binder et al., unpublished manuscript). Even these large analyses do not begin to synthesize all the available nucLSU data, however. As of this writing, there are approximately 4915 nucLSU sequences from Basidiomycota in GenBank, including 4056 sequences from Hymenomycetes and 3250 sequences from Homobasidiomycetes. The nucSSU rDNA also has been popular for phylogenetic studies in Basidiomycota, but it has not been as intensively sampled as the nucLSU rDNA. There are approximately 1639 sequences of the nucSSU rDNA from Basidiomycota in GenBank, including 1076 sequences from Hymenomycetes and 840 sequences from Homobasidiomycetes. A recent analysis of 1551 nucSSU sequences included more than 300 sequences of Basidiomycota (Tehler et al., 2003). The fact that there are only 203 species of Basidiomycota in the combined nucSSU + nucLSU data set in the present analysis indicates that sampling of these regions has proceeded with little coordination among research groups.

Much progress has been made in resolving clades within the Basidiomycota through the use of nucLSU and nucSSU sequences. Nevertheless, these regions on their own cannot resolve many of the deeper nodes within the Basidiomycota. This was shown by Binder and Hibbett (2002), who compared the resolving power of each of four different rDNA regions (including nuclear and mitochondrial large and small subunit rDNAs) to every possible two-, three-, and four-region combination in analyses of Homobasidiomycetes. Not surprisingly, considerable increases in resolving power were obtained by combining data. Similarly, the intensively sampled 877-species data set of Moncalvo et al. (2002) resolved ''one hundred and seventeen clades of euagarics''—a major advance by any measure—but was unable to resolve relationships among those clades.

The three major clades of Basidiomycota, the Urediniomycetes, Ustilaginomycetes, and Hymenomycetes, have been resolved in single-gene analyses of nucSSU rDNA (Swann and Taylor, 1993, 1995; Nishida et al., 1995; Swann et al., 1999) and nucLSU rDNA sequences (McLaughlin et al., 1995a; Begerow et al., 1997), with varying levels of bootstrap support. The order of branching among these groups has never been strongly resolved, however, and, as the present study shows (Fig. 7), ultrastructural characters also cannot resolve this problem. The nucSSU + nucLSU data set is the only data set in the present study that includes representatives of all three major groups of Basidiomycota. Each of the groups is strongly supported as monophyletic by Bayesian posterior probabilities, and the Urediniomycetes and Ustilaginomycetes also receive strong bootstrap support (Fig. 2). The order of branching among these groups is not strongly supported, however, which suggests that additional data will be necessary to resolve the earliest evolutionary events within the Basidiomycota.

The sampling of the Urediniomycetes in the present study is rather limited in comparison to previous single-gene analyses (Swann and Taylor, 1995; Swann et al., 1999; Fell, 2001). Nevertheless, the results obtained here are compatible with the classification of Swann et al. (2001), and most of the nodes within the Urediniomycetes received strong to moderate support (Fig. 2). The *Naohidea* clade and the Urediniomycetidae (Platygloeales and Uredinales) are each strongly supported, but, as noted previously, there are at least four other independent clades of Urediniomycetes that are not included in the present data set. Taxa that are not represented here include species with a broad range of nutritional modes, including saprotrophs and symbionts of insects, fungi, ferns, and mosses (Swann et al., 2001). Inclusion of these taxa will be necessary to understand the evolution of ecological associations in Urediniomycetes.

The Ustilaginomycetes is represented here by only five species, which represent two of three subclasses recognized by Bauer et al. (2001). A few Ustilaginomycetes have appeared in analyses using nucSSU rDNA (e.g., Nishida et al., 1995; Swann and Taylor, 1995; Swann et al., 1999), but by far the most extensive sampling in this group has been performed by Begerow et al. (1997, 2000, 2002) and Piepenbring et al. (1999), who examined nucLSU rDNA. Analyses by Begerow et al. supported the monophyly of the subclasses Ustilaginomycetidae and Entorhizomycetidae with strong bootstrap values, but the Exobasidiomycetidae received weak bootstrap support or was resolved as paraphyletic. The present analysis of nucSSU + nucLSU sequences suggests that the Exobasidiomycetidae is paraphyletic, and the critical node uniting *Malassezia furfur* (Malasseziales, Exobasidiomycetidae) and two *Ustilago* species (Ustilaginales, Ustilaginomycetidae) received moderate support (PP = 100%, NJBP = 69%). While the sampling here is quite limited, the results of this study agree with those of Begerow et al. (1997) in suggesting that the higher-level classification of Ustilaginomycetes may require revision.

The Hymenomycetes is well represented in all three data sets analyzed here, which permits a comparison of the resolv-

ing power afforded by different combinations of genes. The comparison is somewhat crude, because the taxa do not overlap perfectly among data sets, but the general picture is one of increasing robustness and resolution with increasing numbers of genes (Figs. 2–5). In the nucSSU + nucLSU data set, the monophyly of the Hymenomycetes is strongly supported (PP = 100%, NJBP = 78%). Within the Hymenomycetes, however, resolution is poor. There is a large polytomy at the base of the clade, and several of the major clades of Homobasidiomycetes that were strongly supported in analyses with four rDNA regions (Binder and Hibbett, 2002) are not resolved as monophyletic here (e.g., the hymenochaetoid clade), often because of the inclusion of ''oddball'' taxa that probably belong to other groups (e.g., *Piriformospora indica* in the euagarics clade, or *Laetisaria fuciformis* in the russuloid clade). The lack of resolution and robustness in the deeper nodes of the Hymenomycetes in the analysis of the nucSSU + nucLSU data set echoes earlier single-gene analyses of the Hymenomycetes (e.g., Gargas et al., 1995; Hibbett and Donoghue, 1995; Weiß and Oberwinkler, 2001).

Another problem that is evident in the analysis of the nucSSU + nucLSU data set is that of misidentified isolates. In this study, the isolates labeled ''*Athelia arachnoidea*'' and ''*Hyphoderma praetermissum*'' are probably misidentified, based on other analyses with more extensive sampling (Larsson, 2002; M. Binder et al., unpublished manuscript). ''*Athelia bombacina*,'' which is nested in the euagarics clade in the analyses of the three- and four-gene data sets, may also be misidentified. As a putative member of the athelioid clade (Larsson, 2002; M. Binder et al., unpublished manuscript), it was expected to cluster outside of the euagarics clade. The presence of misidentified sequences in GenBank (and the lack of an option for third-party annotation) is a troubling source of error. On the other hand, our growing ability to detect such errors through comparison with multiple sequences for many species reflects the maturation of fungal molecular systematics.

It is beyond the scope of this paper to provide detailed commentary regarding the relationships of Hymenomycetes inferred from the nucSSU + nucLSU data set. For this, the reader is directed to previous phylogenetic studies providing overviews of specific groups, including the Tremellomycetidae (Chen, 1998; Fell et al., 2001), Auriculariales (Weiß and Oberwinkler, 2001), and Homobasidiomycetes (Hibbett and Thorn, 2001; Larsson, 2002; Moncalvo et al., 2002; Larsson and Larsson, 2003; M. Binder et al., Clark University, unpublished manuscript).

Overall resolution of the major clades of the Hymenomycetes in the analyses of the three- and four-gene data sets is markedly superior to that in the analysis of the nucSSU + nucLSU data set (Figs. 2–5). However, the level of sampling in the three data sets is uneven, which complicates the comparison: there are 183 species of Hymenomycetes in the nucSSU + nucLSU data set, compared to 55 species in the nucSSU + nucLSU + *RPB2* data set, and 39 species in the nucSSU + nucLSU + mitSSU + *RPB2* data set. The clades that are resolved in the three- and four-gene analyses are a subset of the groups that Hibbett and Thorn (2001) and Binder and Hibbett (2002) recognized based on rDNA sequences. In the four-gene analysis, each of the clades receives 100% posterior probability in Bayesian analysis, except the hymenochaetoid clade, which is represented by a single species, and the polyporoid clade, which receives 84% posterior probability. NJ and MP bootstrap support for most clades is weak, however.

The polyporoid clade, with 18 species, is the most intensively sampled clade of Basidiomycota in the four-gene data set. Most species in this group have a poroid or smooth hymenophore, produce fruiting bodies on wood, and, as far as is known, are saprotrophic. In spite of this morphological and ecological consistency, the polyporoid clade has always been weakly supported or resolved as nonmonophyletic in previous analyses (e.g., Hibbett and Donoghue, 1995; Binder and Hibbett, 2002; Larsson, 2002), and in the nucSSU + nucLSU analysis in the present study, it collapses into a large polytomy near the base of the Hymenomycetes (Fig. 2). In the four-gene analysis, the polypore *Meripilus giganteus* is placed as the sister group of the remaining members of the polyporoid clade, which are supported as monophyletic with 98% posterior probability (Fig. 5). Similar results are obtained in the three-gene analysis. Here, *M. giganteus* and the toothed fungus *Sarcodon imbricatum* form a clade that is the sister group of the remaining members of the polyporoid clade, which are supported with 97% posterior probabilty (Fig. 4). *Sarcodon imbricatum* is a member of the thelephoroid clade (Thelephorales) and its placement as the sister group of *M. giganteus* is suspect. Nevertheless, the apparent support for the rest of the polyporoid clade is noteworthy. Another significant node is that uniting the euagarics clade, bolete clade, and polyporoid clade, which receives 98% posterior probability in the four-gene analysis (Fig. 5). Resolution of this node could be an important step toward reconstructing the ''backbone'' phylogeny of the Hymenomycetes, which has previously been difficult to resolve. The euagarics clade and bolete clade are significantly supported as sister groups in the four-gene analysis by Bayesian posterior probability (100%, bootstrap $<$50%), in agreement with analysis of four rDNA genes by Hibbett and Binder (2002).

In summary, the present study provides an overview of the current knowledge of Basidiomycota phylogeny and clearly reflects the activities of many individual researchers (Fig. 2). Missing from this picture are the highly detailed topologies for individual groups that have been produced with nucLSU and ITS data. Coordination among research groups will be needed to assure that the thousands of rDNA sequences of Basidiomycota now in GenBank can eventually be combined with sequences of protein-coding loci. Results of the three- and four-gene analyses are promising and encourage us to add representatives of the many major clades that are as yet unrepresented, including all Urediniomycetes and Ustilaginomycetes. Obviously, until members of those groups are included, we will not know if the combination of rDNA and *RPB2* loci will help resolve the deepest divergences within the Basidiomycota.

***Current status of Ascomycota phylogeny and a preliminary reassessment of ascomal evolution***—The addition of about 2.1 kb from *RPB2* to the nucSSU and nucLSU data (see also Reeb et al., 2004) and 0.8 kb from the mitSSU rDNA to this three-gene data set (Fig. 5; see also Lumbsch et al., 2002, 2004) revealed three main groups that were never found with high phylogenetic confidence when analyses were restricted to the nuclear rDNA or when the mitSSU was added to the nucSSU and nucLSU (i.e., as shown in Taylor et al., 2004). This enhanced resolution of deep relationships with high phylogenetic confidence within the Pezizomycotina is summarized

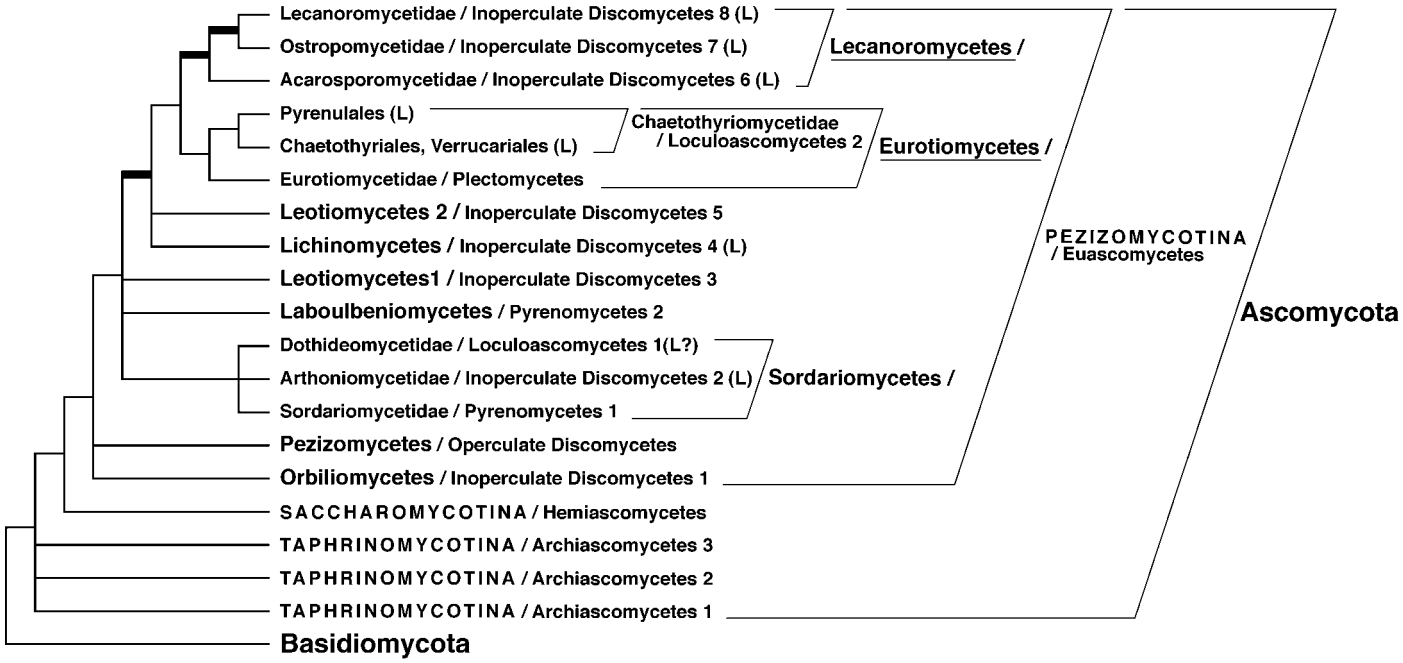



Fig. 8. Depiction of progress in our understanding of relationships among the Ascomycota resulting from Reeb et al. (2004) and this study, compared to Taylor et al. (2004). Thicker and darker internodes represent new relationships and underlined names correspond to re-circumscribed taxonomic entities when compared to Taylor et al. (2004). Supraordinal names and common names are shown on the tree before and after the slash sign, respectively. Taxa listed at the tips of terminal branches that include lichen-forming species are annotated with ''(L).'' Note the phylogenetic uncertainty among several lineages, including Taphrinomycotina (= Archiascomycetes), within the Pezizomycotina (= Euascomycetes) and within the Sordariomycetes (among Dothideomycetidae, Arthoniomycetidae, and Sordariomycetidae). ''Loculoascomycetes'' (e.g., Chaetothyriales, Dothideomycetidae, Verrucariales, and Pyrenulales) do not denote monophyletic groupings. Most cleistothecial fungi (''plectomycetes'') occur in a monophyletic lineage (Eurotiomycetidae; Geiser and LoBuglio, 2001), while others are derived members of other lineages such as the Sordariomycetes (''pyrenomycetes''). The vast majority of ''pyrenomycetes'' are members of the Sordariomycetes with a few unique and poorly known perithecial species among the Laboulbeniomycetes. Because of the enhanced level of support for relationships among lichen-forming ascomycetes revealed by this study, the Lecanoromycetes can now be restricted to the Acarosporomycetidae, Ostropomycetidae, and Lecanoromycetidae; and the monophyletic group including the Eurotiales and Onygenales (sensu Eriksson et al., 2004), Chaetothyriales, Verrucariales, and Pyrenulales can now be recognized at the class level (Eurotiomycetes) with two distinct subclasses (Chaetothyriomycetidae and Eurotiomycetidae).

by thicker and darker internodes in Fig. 8. The sister relationship of the Acarosporomycetidae to the Lecanoromycetidae-Ostropomycetidae group (Acarosporomycetidae (Lecanoromycetidae, Ostropomycetidae)), which can now be recognized as the Lecanoromycetes, and its sister relationship to the Eurotiomycetes are major advancements in our understanding of the lichen-forming discomycetes. Using nucSSU + nucLSU + *RPB2* in their study of the Ascomycota, Reeb et al. (2004) revealed that these three subclasses form a monophyletic group, but this three-locus data set was not sufficient to provide significant posterior probability (92%) or bootstrap proportions higher than 54%. However, these lower support values could also be due to the inclusion of *Strangospora*, which is absent from our four-gene tree (but see Fig. 4). Because of lack of support, Reeb et al. (2004) and Taylor et al. (2004) had no choice but to include the Eurotiomycetidae as part of the Lecanoromycetes, even if phenotypic traits of the former warrant its recognition as a separate class. Based on nucLSU + mitSSU, Lumbsch et al. (2004) also concluded that the Eurotiomycetes (as defined here) are sister to the Lecanoromycetes (PP = 95%).

An earlier origin of the lichen symbiosis than reported by Lutzoni et al. (2001) is suggested by the strong support for a close relationship of the lichen-forming Lichinomycetes, Thelocarpaceae, and *Biatoridium* to the Eurotiomycetes-Lecanoromycetes group.These relationships reveal perhaps the deepest internode where a transition to lichenization might have taken place. Except for the Arthoniomycetidae, the latter internode supports all sampled lichen-forming fungi as one clade of mostly lichenized fungi, thus supporting the hypothesis of a low number of lichen origins, especially in comparison to the high number of losses of the lichen symbiosis (Lutzoni et al., 2001). This is in contradiction with the conclusions by Liu and Hall (2004), which were based only on a *RPB2* Bayesian phylogeny that did not include representatives from the mostly lichen-forming Lichinomycetes, Acarosporomycetidae, Pyrenulales, Thelocarpaceae, *Biatoridium*, and Umbilicariaceae + Fuscideaceae groups, which are essential to assess the evolution of lichen symbiosis (Reeb et al., 2004). None of this additional phylogenetic structure, including the monophyly of the Dothideomycetidae-Arthoniomycetidae-Sordariomycetidae lineage that we refer to as the Sordariomycetes, is part of current classifications of the Ascomycota (Kirk et al., 2001; Eriksson et al., 2004).

The nonmonophyly of the Leotiomycetes, or inoperculate discomycetes, is not surprising, as it has long been recognized as a taxon of convenience (Gernandt et al., 2001). Furthermore, it is one of the more diverse classes of the Ascomycota and is grossly undersampled in these analyses. The resolution of the terminal clades of the Leotiomycetes and their relationships to the other clades of the Pezizomycotina is one of the more critical pieces required to resolve the more basal and internal nodes of the Pezizomycotina.

The necessity to advance towards multigene analyses does

not mean that additional analyses of the nucSSU and nucLSU are now irrelevant to our improvement of the Ascomycota phylogeny. To the contrary, when these two genes are combined and analyzed with a Bayesian MCMCMC approach, and NJBP for complementary information about phylogenetic confidence (Fig. 2), major progress can be accomplished at the ordinal, family, and genus level (e.g., within the Sordariomycetidae and Pezizomycetes; see also Miadlikowska and Lutzoni, 2004, for an example within the Lecanoromycetidae). The same is true for analyses of the nucSSU + nucLSU + mitSSU or the nucSSU + nucLSU + *RPB2,* especially when they are restricted to a portion of the ascomycetes (e.g., Lumbsch et al., 2002, 2004; and Reeb et al., 2004). The dual strategy of increasing the number of taxa for at least the nucSSU and LSU and continuing to add loci for a larger number of species will greatly improve the status of Ascomycota phylogeny. In agreement with Reeb et al. (2004), we did not detect significant topological conflicts between our *RPB2* and other gene trees when using the 70% criterion described in the Materials and Methods. It is possible that the inconsistencies between the *RPB2* tree of Liu and Hall (2004) and our three- and four-gene trees are due to artifacts resulting from current implementation of B-MCMCMC in MrBayes, the use of a single gene, and the preferential reliance of Liu and Hall on posterior probabilities (Reeb et al., 2004).

Except for ultrastructural features, we did not include phenotypical characters in this large-scale study. Therefore, a discussion of the circumscription of major emerging clades by nonmolecular characters would go beyond the scope of this paper. Yet, the analyses presented here are consistent with the apothecium being the ancestral ascomal morphology for the Pezizomycotina (Gernandt et al., 2001). Similarly, the operculate ascus arose early during the evolution of the Pezizomycotina; however, our failure to include members of the Orbiliomycetes complicates this interpretation (Pfister, 1997). Regardless, the phylogenies presented to date are consistent with the hypothesis that morphological complexity of ascomata and asci arose early during the evolution of the Pezizomycotina, and certain morphologically simple taxa (e.g., Eurotiales, Sordariales) likely represent derived morphologies via reduction in complexity (Suh and Blackwell, 1999). An interesting corollary to this pattern of evolution is that the vast majority of known ectomycorrhizal Ascomycota are members of the Pezizomycetes, consistent with an early origin of mycorrhizae within the Ascomycota. This point has been largely overlooked in considering the evolution of nutritional modes of the Ascomycota, because the majority of known mycorrhizal fungi are members of the Basidiomycota.

Until recently, characters such as ascoma ontogeny (Nannfeldt, 1932; Luttrell, 1955; Henssen and Jahns, 1973), hamathecium structure (Groenhart, 1965; Luttrell, 1965; Janex-Favre, 1971; Eriksson, 1981; Liew et al., 2000), and ascus type (Luttrell, 1951; Eriksson, 1981; Hafellner, 1984) were used to define major lineages. Current classifications of the Pezizomycotina and relationships within this subphylum presented here do not correlate with previous classifications. For example, the recognition of loculoascomycetes was considered one of the major advances in Ascomycota phylogeny. However, members of this group, which included taxa defined by their ascomata ontogeny (nongenerative tissue forming stromata), hamathecium structure (pseudoparaphyses), and/or their bitunicate asci (Nannfeldt, 1932; Luttrell, 1955, 1973; Eriksson, 1981; Barr, 1987, 1990), are in our analysis clearly demonstrated to fall within at least two distinct clades: the Sordariomycetes (Dothideomycetidae) and Eurotiomycetes (Chaetothyriales, Verrucariales, Pyrenulales). Also, the Sordariomycetes per se now includes three clades, one of which is traditionally considered loculoascomycetous (Dothideomycetes), one ascohymenial (Sordariomycetidae), and one intermediate (Arthoniomycetidae; see Henssen and Jahns, 1973). This might indicate that the relevant characters were either not sufficiently well studied in these groups or that these characters only partly correlate molecular phylogenies of the Ascomycota. Additional sampling to test the validity of the Sordariomycetes, as defined here, is essential in order to refine our hypotheses regarding the evolution of ascomata and ascus dehiscence and evolution of nutritional modes and symbioses. The Dothideomycetidae are assumed to form pseudothecia, which in many taxa superficially resemble the perithecia of the Sordariomycetidae, but unlike true perithecia, are interpreted as developing prior to and independent from fertilization of the ascogonium (Nannfeldt, 1932; Luttrell, 1955). The two taxa also differ by producing bitunicate and unitunicate asci, respectively, with the positive correlation between the pseudothecia and bitunicate asci being a long-accepted paradigm in ascomycete systematics, except for the Pyrenulales, Verrucariales, and Chaetothyriales, which are here shown to be closely related lineages in the Eurotiomycetes. However, Liu and Hall (2004) recovered the traditional delimitation of the loculoascomycetes as monophyletic in their *RPB2* phylogeny; see Reeb et al. (2004) for a discussion of putative discrepancies between *RPB2*- and rDNA-based phylogenies. Also, the Arthoniales with their apothecioid ascomata, and the Coryneliales, a rather poorly known order described as bearing true perithecia and bitunicate asci, do not fit the pseudothecium-bitunicate ascus correlation, and their sampling in future studies will be illuminating. The Sordariomycetes as defined here also comprise distinct lineages of lichenized (Arthoniomycetidae) and nonlichenized clades, with a more robust resolution needed to clarify the number and polarity of gains and losses of lichenization events.

Another striking example of conflict between morphological features and classifications based on DNA sequences is the Ostropomycetidae, and in particular the Ostropales. Sherwood (1977) restricted the Ostropales to nonlichenized fungi with hemiangiocarpous apothecia, paraphysal amyloid hymenium and chiefly filiform ascospores. However, according to this and other recent molecular studies (Kauff and Lutzoni, 2002; Lücking et al., 2004; Lumbsch et al., 2004; Grube et al., 2004), Ostropales sensu lato now includes an assembly of families with a wide array of ascoma, hamathecium, ascus, and ascospore types: either apothecia (most lineages) or genuine perithecia (Porinaceae, Protothelenellaceae); paraphysal (most lineages) or paraphysoid hamathecium (Gomphillaceae); and thick-walled, unitunicate (''annelasceous''), nonamyloid asci (Thelotremataceae, Graphidaceae), thin-walled unitunicate, partly amyloid asci (Coenogoniaceae, Gyalectaceae), and even asci previously believed to be fissitunicate (Protothelenellaceae, Gomphillaceae).

It is obvious from these considerations that one of the major challenges of the emerging fungal tree of life, especially for the Ascomycota, is to reevaluate virtually all characters that have been used until recently to classify and characterize major clades, to reconstruct their evolution, and to identify and characterize cases of homoplasy among traits believed to be homologous. Characters that have been considered diagnostic

in defining taxonomic groups include such characters as true (ascohymenial) perithecia vs. (ascolocular) pseudothecia, hamathecium structures, and in particular ascus structures. The inclusion of ultrastructural traits, as shown here for Basidiomycota, could shed new light in our understanding of the evolution of morphological and anatomical traits of the Ascomycota.

***Integration of ultrastructural features in phylogenetic studies of the fungi***—Molecular phylogenies are still too poorly resolved to determine the evolution of septal pore structure and organization in the Fungi, and the gaps in structural studies compound the problem. Multipored septa appear to be plesiomorphic, but there still is not enough subcellular data to determine whether the plasmodesmata or the multiperforate type is ancestral. Uniperforate septa may have been derived from a multipored type, but the number of times this has occurred is unclear. In the Ascomycota, and in a single taxon in the Basidiomycota, the multiperforate septum appears to be derived from a uniperforate type, and the scattered pores in these septa differ from the peripherally arranged pores in *Allomyces* (Chytridiomycota). Multiple septal types are reported from the Zygomycota: plasmodesmata in the Mucorales, uniperforate septa with a lenticular cavity in a group of related taxa (Dimargaritales, Kickxellales, and Harpellales), and continuous septa in *Basidiobolus ranarum* (Gull and Trinci, 1975) though images indicate a possible central plug. This diversity may reflect a polyphyletic Zygomycota.

In the Basidiomycota, the septal pore swelling is characteristic of the Hymenomycetes but also of some Ustilaginomycetes. The molecular evidence indicates that Ustilaginomycetes are monophyletic and sister to Hymenomycetes and that the septal pore swelling is plesiomorphic with subsequent loss in Ustilaginomycetes and conservation in Hymenomycetes. In unconstrained morphological analyses, the septal pore swelling of *Tilletia* is responsible for the lack of monophyly of Ustilaginomycetes. The *Tilletia* septal type also occurs in the Tremellomycetidae (Hymenomycetes), i.e., one with septal pore swelling and septal pore cap absent (see McLaughlin et al., 1995b). Whether the absence of the septal pore cap in some taxa in this subclass indicates that it has never been present or that it was subsequently lost is not yet clear. *Trichosporon sporotrichoides* typically lacks a septal pore cap, but it is sometimes present (Müller et al., 1998). The possibility that Woronin bodies are present in the Urediniomycetes as in the Ascomycota has been suggested (Markham, 1994; see *Helicobasidium compactum* in Figs. 6 and 7), but requires cytochemical evidence that the microbodies in Basidiomycota are truly comparable (Jedd and Chua, 2000). No class of the Basidiomycota is characterized by a single or identical septal pore apparatus. Whether this statement applies to all fungal classes awaits a much more complete subcellular data set.

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