

Phylogeny of the Zygomycota based on nuclear ribosomal sequence data

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Abstract: The Zygomycota is an ecologically heterogeneous assemblage of nonzoosporic fungi comprising two classes, Zygomycetes and Trichomycetes. Phylogenetic analyses have suggested that the phylum is polyphyletic; two of four orders of Trichomycetes are related to the Mesomycetozoa (protists) that diverged near the fungal/animal split. Current circumscription of the Zygomycota includes only orders with representatives that produce zygospores. We present a molecular-based phylogeny including recognized representatives of the Zygomycetes and Trichomycetes with a combined dataset for nuclear rRNA 18S (SSU), 5.8S and 28S (LSU) genes. Tree reconstruction by Bayesian analyses suggests the Zygomycota is paraphyletic. Although 12 clades were identified only some of these correspond to the nine orders of Zygomycota currently recognized. A large superordinal clade, comprising the Dimargaritales, Harpellales, Kickxellales and Zoopagales, grouping together many symbiotic fungi, also is identified in part by a unique septal structure. Although Harpellales and Kickxellales are not monophyletic, these

lineages are distinct from the Mucorales, Endogonales and Mortierellales, which appear more closely related to the Ascomycota + Basidiomycota + Glomeromycota. The final major group, the insect-associated Entomophthorales, appears to be polyphyletic. In the present analyses *Basidiobolus* and *Neozygites* group within Zygomycota but not with the Entomophthorales. Clades are discussed with special reference to traditional classifications, mapping morphological characters and ecology, where possible, as a snapshot of our current phylogenetic perspective of the Zygomycota.

Key words: Asellariales, basal lineages, Chytridiomycota, Fungi, molecular systematics, opisthokont

INTRODUCTION

Most studies suggest that the phylum Zygomycota is not monophyletic and the classification of the entire phylum is in flux. The Zygomycota currently is divided into two classes, the Zygomycetes and Trichomycetes. However molecular phylogenies suggest that neither group is natural (i.e. monophyletic). Two orders previously classified as Trichomycetes, the Amoebidiales and Eccrinales, are not fungi (Benny and O'Donnell 2000, Cafaro 2005). Furthermore Microsporidia, animal pathogens previously considered to be protists, might have evolved within the Zygomycota (but see Tanabe et al 2005) (Keeling et al 2000, Forget et al 2002, Keeling 2003). One significant recent change has been the removal of the glomeralean arbuscular mycorrhizal (AM) fungi from Zygomycota and their reclassification as a separate phylum, the Glomeromycota (Schubler et al 2001).

Members of the Zygomycota have been considered a primitive and early diverging lineage of the Fungi because they lack complex fruiting structures and most representatives have coenocytic aseptate hyphae during all or part of their life cycle. Zygomycota often might be overlooked even by mycologists, but they can be encountered as near as the kitchen, disguised as moldy bread or fruit (James and O'Donnell 2004). Some are beneficial, such as *Rhizopus*, used in several traditional fermented foods, including tempeh and cassava. Most species of the Zygomycota are saprobic, but some species are facultative pathogens of plants, animals (including humans) or even other fungi. The Trichomycetes are obligate endosymbionts of arthropods. Indeed members of the Zygomycota are ubiquitous in nature, in a seemingly endless variety of interactions.

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This paper is dedicated to the great many students of the "lower Fungi"—past and present—who helped introduce us to this wonderfully eclectic and diverse group that we can only hope to highlight herein.

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For this paper the Zygomycota is divided into nine orders: Asellariales, Dimargaritales, Endogonales, Entomophthorales, Harpellales, Kickxellales, Mortierellales, Mucorales and Zoopagales. The clades defined by this ordinal classification correspond in large part with niche and habitat, as well as mode of asexual and sexual reproduction. Many species are saprophytic in soil and dung (Mucorales, Mortierellales and Kickxellales). Some (Mucorales) are extremely fast growing in agar culture while others (Zoopagales and Entomophthorales) may be slower growing reflecting their parasitic strategy on various small animals, including insects, rotifers or even amoebae. Members of Zoopagales, Dimargaritales and Mucorales may be parasitic on other fungi (including mushrooms, as well as other zygomycetes). Trichomycetes (Harpellales and Asellariales) have specialized for arthropod gut wall attachment. Some Harpellales are remarkable in their ability to shift from a commensalistic to parasitic mode for dispersal (White et al 2006). Other parasitic groups have invasive haustoria (Dimargaritales and Zoopagales) or grow as naked protoplasm inside the host cell wall where they are in direct contact with the invaginated host cytoplasm (Entomophthorales). The Endogonales are either saprophytic or ectomycorrhizal with plants.

Not only do these fungi display a variety of life histories but also the morphology of the Zygomycota is diverse with regard to reproduction and dispersal. Asexual spores are single-celled and include mitospore sporangiospores and true conidia (for review see Benny et al 2001). Most zygomycetes reproduce by sporangiospores, which are derived from the internal cleavage of sporangial cytoplasm. These spores are wind or animal dispersed after rupture of the sporangium wall (Ingold 1978). Asexual spores of the Entomophthorales appear to have a different ontogeny and are referred to as conidia. They are produced at the end of differentiated hyphae and lack a sporangial wall. Most are forcibly discharged and some can germinate and produce a secondary spore. Meiospores called zygospores, where known, are produced with or without the conjugation of compatible hyphae and are usually thick-walled and globose; however they are biconical in the Harpellales (Benny et al 2001, Lichtwardt et al 2001).

Whereas morphology formed the traditional foundation for taxonomy and classification, phylogenetic analyses of DNA sequence data were necessary to reveal major evolutionary trends among the basal fungi (Jensen et al 1998, O'Donnell et al 1998, Sugiyama 1998, James et al 2000, Tanabe et al 2000, Gottlieb and Lichtwardt 2001, O'Donnell et al 2001, White et al 2001, White 2002, 2006, Tanabe et al 2004, Cafaro 2005, Seif et al 2005, Tanabe et al 2005). Most

molecular phylogenetic studies have highlighted the nonmonophyly of the previously defined Zygomycota and recognized the need for additional contributions, especially with understudied and unculturable taxa. For example analyses to date have shown that the Entomophthorales is monophyletic with the exception of the inconsistent placement of *Basidiobolus*. Aside from the protistan Trichomycetes (Amoebiasales and Eccrinales) a third order (Harpellales) appears nested within the Zygomycetes (O'Donnell et al 1998, Lutzoni et al 2004, Taylor et al 2004, Cafaro 2005) whereas the remaining order (Asellariales) has not been included in phylogenetic studies to date.

This is the first phylogeny to incorporate nearly full length rDNA sequences (18S, 5.8S and 28S rRNA genes) for the Zygomycota. Taxa were selected to include representatives of as many recognized orders of this phylum as possible. Based on the results of this three-locus phylogeny, the monophyly of the Zygomycota, its two classes, the Zygomycetes and Trichomycetes, and eight orders are assessed. The results presented herein serve to highlight our current understanding of their evolutionary history and to punctuate areas where our knowledge is woefully inadequate.

MATERIALS AND METHODS

Sequences of 104 taxa representing all fungal phyla and including metazoan and Mesomycetozoa outgroups were obtained from the Assembling the Fungal Tree of Life (<http://ocid.nacse.org/research/aftol/>) and GenBank (<http://www.ncbi.nlm.nih.gov/>) databases and combined with sequences generated during this study. Many taxa ($n = 46$) contain nearly full length sequences for the rRNA operon (nearly complete 18S [SSU], 28S [LSU] and in most cases 5.8S). Both 18S and partial 28S sequences were available for 38 taxa; 20 are represented only by 18S sequences (see SUPPLEMENTARY TABLE I).

Data for the 18S, 28S and 5.8S ribosomal coding regions were manually aligned with GeneDoc v2.6 (Nicholas and Nicholas 1997) and combined into a single supermatrix (10 053 characters) in which ambiguous regions (exsets) were excluded from further analyses with MacClade v4.05 (Maddison and Maddison 2002). The final dataset contained 3832 aligned characters used for analyses (1395 parsimony informative and 1996 invariant). We used Modeltest v3.7 (Posada and Crandall 1998) to determine the most appropriate model of evolution for use in a maximum likelihood framework. This model (GTR+I+G) was used to estimate phylogenetic relationships and clade support with MrBayes v3.1 (Huelsenbeck and Ronquist 2001). Two independent runs were conducted, each with four chains for 1×10^7 generations, in which trees were sampled every 500 generations. Stationarity of MCMC

sampling and the appropriate burn-in values were assessed with Tracer v1.3 (Rambaut and Drummond 2003). Support for clades was determined with parsimony bootstrap (1000 replicates with heuristic searching) as implemented in PAUP v4.0b10 (Swofford 2002).

RESULTS AND DISCUSSION

The combined rDNA analysis represents the largest and most comprehensive phylogenetic sampling of the Zygomycota to date. We present an overview of the clades resolved (FIG. 1) with emphasis on monophyletic groups and discuss several problematic and/or unresolved lineages.

Mucorales.—With more than 300 described species, this is the largest order (roughly 30%) of known Zygomycota (Hawksworth et al 1995). Morphologically members are easily distinguished from other Fungi: Asexual reproduction is primarily via multi-spored and/or uni- to few-spored sporangia on mostly coenocytic, branched and rapidly growing mycelium (FIG. 2E); sporangiospores are always single-celled and nonmotile; sexual reproduction, where known, is by the formation of zygospores between opposed or apposed suspensors (FIG. 2A–D; O’Donnell 1979, Benny et al 2001, James and O’Donnell 2004). Self-sterility or heterothallism is much more common than homothallism within the Mucorales, and sexual reproduction has not been documented for a number of species (O’Donnell et al 2001). For this reason asexual structures are used almost exclusively to identify species. Multilocus DNA sequence data have not been employed to investigate species limits within this or any other order of the Zygomycota. However, given their simple morphology, it is reasonable to assume that phylogenetic species recognition will reveal cryptic speciation throughout this phylum (Taylor et al 2000).

As saprotrophs, mucoraleans are some of the most common microbes recovered from organic debris, soil, air and dung. All Mucorales are easily cultured. With the exception of the Umbelopsidaceae, which produce restricted, low-growing, ochraceous-to-red-dish colonies (Meyer and Gams 2003), almost all other Mucorales (Mucoraceae *sensu* Benny 2005 <http://www.zygomycetes.org/>) produce rapidly growing colonies (distinct from the Mortierellales, see below) and abundant aerial mycelium fill a 100 mm diam Petri dish in less than a week.

A few species are facultative biotrophic or necrotrophic mycoparasites (Jeffries 1985), plant or animal pathogens, including systemic infections of humans, especially in immuno-compromised or suppressed patients (de Hoog et al 2000, Ribes et al 2000, Rinaldi 1989). A notable plant disease, seedling blight of rice,

is caused by a species of *Rhizopus* that harbors an endosymbiotic bacterium, *Burkholderia*, that produces the phytotoxin rhizoxin (Partida-Martinez and Hertweck 2005). Other species cause postharvest storage rots of fruits such as the ubiquitous *Rhizopus stolonifer*, the agent of strawberry soft-rot (James and O’Donnell 2004). In contrast beneficial species are used in traditional fermented foods in Asia such as *R. oligosporus* in the coconut cake bongkrek and the soybean cake tempeh, a staple in Indonesia for more than 2000 y and now a commercial success in North America and Europe (Nout and Kiers 2005). *Actinomyces elegans* and *Mucor dispersus* are used in the soybean cake tofu in China (Hesseltine 1991).

Two mucoralean zygomycetes have been used as model organisms, *Pilobolus kleinii* for studying the rapid nonmuscular movement associated with explosive discharge of its sporangium and *Phycomyces blakesleeanus* for elucidating the biology of light and color (Cerdá-Olmedo 2001). The exquisite hydraulic system responsible for the explosive fracture of a *Pilobolus* sporangium, clocked at 0.1–10 μ s, represents one of the most rapid movements ever documented in the fungi (Skotheim and Mahadevan 2005). Whole genome sequencing projects have targeted *P. blakesleeanus* (JGI DOE Joint Genome Institute, <http://www.jgi.doe.gov/sequencing/why/CSP2006/Pblakesleeanus.html>) and *R. oryzae*, the most important etiological agent of human mucormycosis, (Broad Institute of Harvard and MIT, http://www.broad.mit.edu/annotation/fungi/rhizopus_oryzae/).

The available phylogenetic data support Benjamin’s (1979) narrower circumscription of the Mucorales, which was based on differences in nutritional mode, sexual and asexual reproductive morphology and septal structure. Compared to the broad circumscription of Hesseltine and Ellis (1973), Benjamin (1979) segregated the Kickxellales, Dimargaritales, Zoopagales and Endogonales. Although the Endogonales *sensu* Benjamin (1979) is polyphyletic with many members now recognized in a separate phylum, the Glomeromycota (Schüßler et al 2001, Redecker 2005), several of Benjamin’s hypotheses have been supported largely by molecular phylogenetic studies (Gehrig et al 1996, Jensen et al 1998, Tanabe et al 2000, Keeling 2003, Lutzoni et al 2004, Tanabe et al 2005), including the one presented here (FIG. 1).

Based on our analyses of combined ribosomal RNA loci, the Mucorales and Endogonales appear to be strongly supported as sister taxa (FIG. 1); the current report is the first to propose this novel phylogenetic hypothesis. Previous analyses of SSU rRNA gene sequence data have suggested the Mucorales was either a sister group of the Entomophthorales

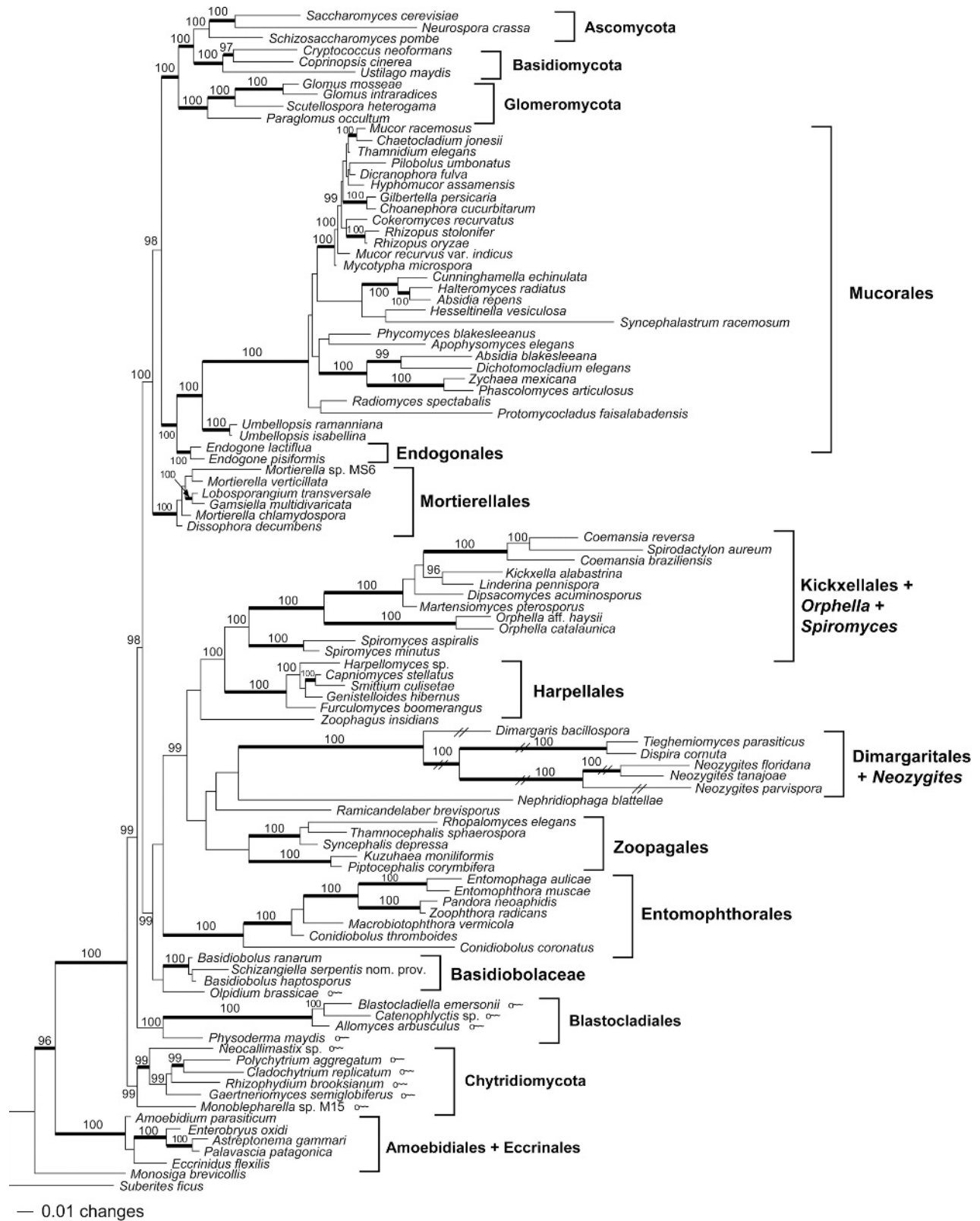


FIG. 1. Phylogeny of Zygomycota based on rRNA operon (18S+28S+5.8S genes). Shown is a majority-rule consensus phylogram computed from the set of 36 000 credible trees with branch lengths averaged over trees. Numbers above branches indicate nodes that were supported by $\geq 95\%$ Bayesian posterior probability. Branches supported by parsimony bootstrap $\geq 70\%$ are indicated by thickened lines. Hashed lines indicate long branches divided by 8 to improve visibility of branch lengths.

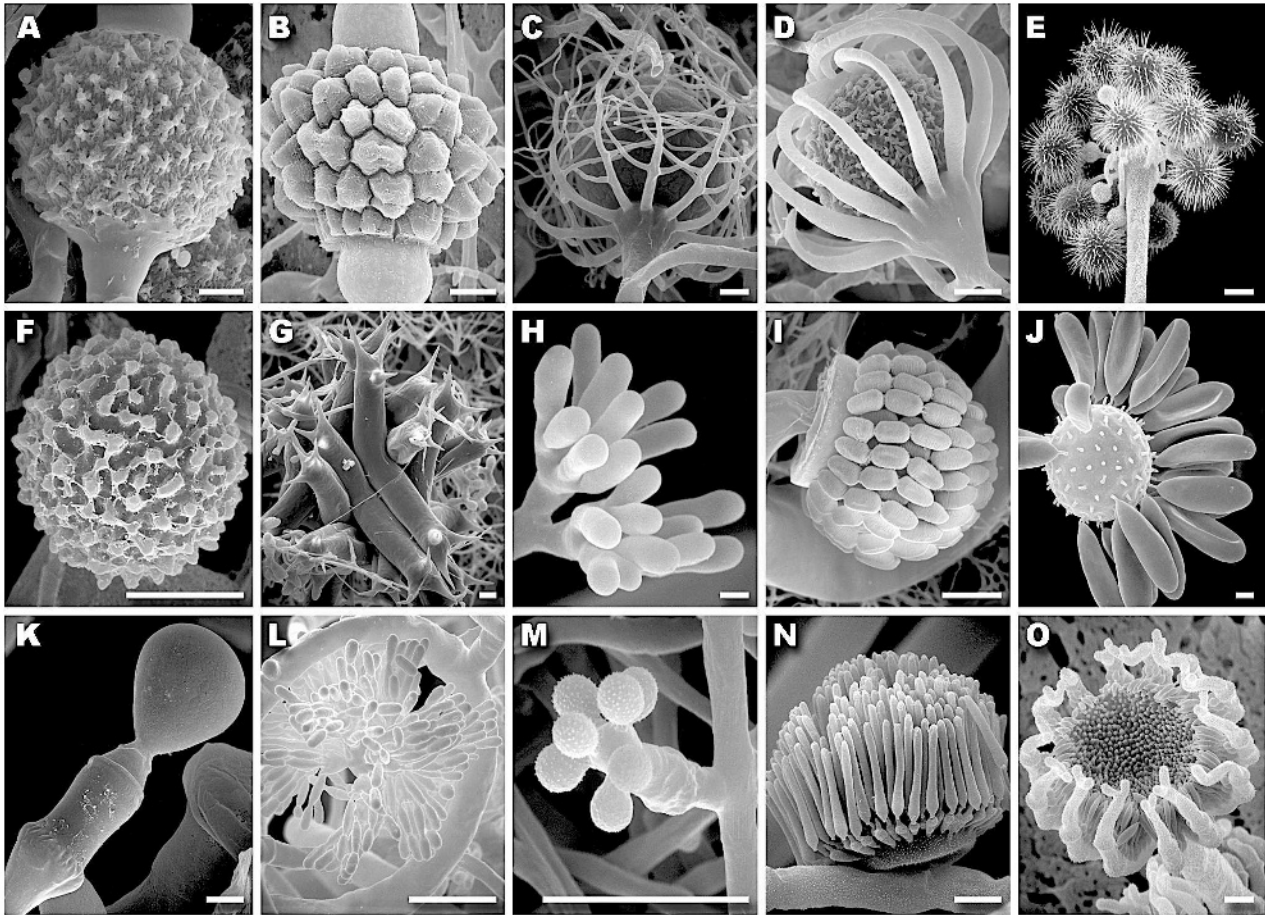


FIG. 2. Scanning electron micrographs of Zygomyces illustrating morphological diversity of sexual and asexual reproductive structures. A–E. Mucorales. Zygospores of A. *Cokeromyces recurvatus*, B. *Cunninghamella homothallicus*, C. *Radiomyces spectabilis* and D. *Absidia spinosa*. E. *Hesselтинella vesiculosa* sporangia. F–G. Mortierellales. F. *Mortierella (Gamsiella) multidivariata* chlamydo-spore. G. *Lobosporangium transversalis* sporangia borne on arachnoid mycelium. H–J. Zoopagales. H. *Piptocephalis cormbifera* immature sporangia. I. *Syncephalis cornu* sporophore bearing senescent sporangia with uniseriate sporangiospores. J. *Rhopalomyces elegans* fertile vesicle with monosporous sporangia. K. *Basidiobolus ranarum* monosporous sporangium. L. Dimargaritales. *Dispira cornuta* two-spored sporangia. M–O. Kickxellales. Monosporous sporangia of M. *Spiromyces minutus*, N. *Linderina pennispора* and O. *Kickxella alabastrina*. Bars: A–F, I–O = 10 μ m; G, H = 20 μ m.

(Gehrig et al 1996, Jensen et al 1998), the Ascomycota + Basidiomycota (Helgason et al 2003) or their relationships were unresolved (Bruns et al 1992, Tanabe et al 2000). Phylogenetic relationships of the Mucorales also were unresolved in a Bayesian SSU+LSU two-locus phylogeny (Lutzoni et al 2004). Similarly phylogenies inferred from amino acid sequence data from one or two loci have failed to resolve evolutionary relationships of the Mucorales (Keeling et al 2000, Keeling 2003, Helgason et al 2003), except for a possible sister group relationship with the Mortierellales (Tanabe et al 2004, Tanabe et al 2005) or the Entomophthorales (Keeling 2003). Sequences of the Endogonales however were

noticeably absent from all of the amino acid-based phylogenies. Phylogeny reconstructions based on β -tubulin weakly supported a Mucorales + Entomophthorales sister group relationship but did not include sequences of the Mortierellales (Keeling 2003).

The genera currently classified within the monophyletic Mucorales (Lutzoni et al 2004) have been classified in up to 16 families, based primarily on differences in sexual and asexual reproductive morphology (Benny et al 2001). However our study supports the results of another based on three genes that indicated large scale polyphyly at the family level (O'Donnell et al 2001); in that study four of seven

families containing two or more genera were inferred to be polyphyletic (i.e. Mucoraceae, Thamniaceae, Chaetocladiaceae and Radiomycetaceae). Given that these polyphyletic families comprise more than 80% of the Mucorales, the recent proposal to place all mucoralean families in synonymy with the Mucoraceae, with the exception of the Umbelopsidaceae, seems plausible (Benny 2005).

We anticipate that as whole genomes become available these resources will help to accelerate a broad array of basic studies such as elucidating the evolution of sexual reproductive mode and phylogenetic species recognition (Taylor et al 2000) within these ecologically diverse and economically important fungi.

Mortierellales.—This order (FIG. 2F, G) comprises approximately 10% of the known Zygomycota with close to 100 recognized species (Hawksworth et al 1995) distributed among six genera (Benny 2005). With the exception of *Mortierella*, at more than 90 species, the other genera are monotypic (*Aquamortierella*, *Lobosporangium* and *Gamsiella*) or bitypic (*Dissophora* and *Modicella*). Except for *Aquamortierella* and *Modicella*, which are thought to be saprobic but never have been cultured, all members of this order are characterized by thin, delicate arachnoid-like mycelium (FIG. 2G) and zonate colonies that often produce a garlic-like odor in pure culture. Asexual reproduction is by uni- or multi-spored sporangia that either lack a columella or possess a rudimentary one and often by the production of chlamydospores (FIG. 2F). Sexual reproduction, where known, is by zygospores which are formed between apposed suspensors (see FIG. 12.9e in Taylor et al 2004).

Although Hesselstine and Ellis (1973) and Benjamin (1979) retained the Mortierellaceae within the Mucorales, the Mortierellales, exclusive of the Umbelopsidaceae, was supported strongly as an independent evolutionary lineage in the combined rDNA phylogeny (FIG. 1). Likewise phylogenies inferred from amino acid and nucleotide sequence data (Gehrig et al 1996, Helgason et al 2003, Lutzoni et al 2004, Tanabe et al 2004) supported a monophyletic Mortierellales; however it has been resolved either as part of a paraphyletic zygomycotan grade (Seif et al 2005) or sister of the Endogonales (Gehrig et al 1996) or Mucorales (Tanabe et al 2004). In addition a preliminary two-locus phylogenetic analysis of complete SSU and partial LSU sequence data (O'Donnell unpubl) suggests that *Mortierella* might be paraphyletic with respect to *Dissophora*, *Lobosporangium* and *Gamsiella* (Benny and Blackwell 2004), so generic relationships deserve further study. Expanded

taxon sampling is needed before a robust hypothesis of the evolutionary relationships within the Mortierellales can be formulated.

Taxa formerly recognized as subgenus *Micromucor* or the *Mortierella isabellina*-group of *Mortierella* (Gams 1977) were excluded from Mortierellales and strongly supported (FIG. 1) as monophyletic Umbelopsidaceae (Meyer and Gams 2003). This position, as early diverging members of a monophyletic Mucorales, has been noted elsewhere (FIG. 3; O'Donnell et al 2001, Lutzoni et al 2004).

Endogonales.—Symbiotic association of mycorrhizal fungi with plant roots is recognized as economically and ecologically important (Taylor et al 2004, Redecker and Rabb this issue). With the elevation of the Glomales to the Glomeromycota (Schüßler et al 2001) the only known member of the Zygomycota *sensu stricto* that forms such symbioses are the facultatively saprotrophic Endogonales. Formerly treated as a family in the Mucorales (Hesselstine and Ellis 1973), Benjamin (1979) validated Moreau's (1953) Endogonales by elevating the family to ordinal status. Morton and Benny (1990) subsequently emended the Endogonales to include only the Endogonaceae and *Endogone* and moved the other genera to a new order, the Glomales (Morton and Benny 1990). The Glomales and Endogonales are distinguished easily in that they produce intracellular, arbuscule-forming endomycorrhizae (AM) and ectomycorrhizae, respectively (Morton and Benny 1990, Benny et al 2001). Another hallmark of the Endogonales is that some species produce hypogeous fruiting structures called sporocarps that contain zygospores with apposed suspensors (see FIG. 15 in Benny et al 2001).

The Endogonales, sometimes called pea-truffles because some species of *Endogone* produce macroscopic sporocarps that are pea-like in appearance (Yao et al 1996), now includes four genera (Benny et al 2001, Kirk et al 2001). Phylogenetic studies based on SSU rDNA sequence analysis have indicated a possible sister group relationship with the Mortierellales (Gehrig et al 1996). However in the present study the Endogonales and Mucorales formed a strongly supported clade that is sister of the Ascomycota + Basidiomycota + Glomeromycota clade (FIG. 1). A sister group relationship of the Endogonales and Mucorales is supported by morphological evidence because they both form zygospores, in contrast to the Glomeromycota in which zygospores never have been observed (Sanders 1999). Lastly, the novel Endogonales + Mucorales clade has been recovered also from multilocus analyses that included protein coding sequences (James et al 2006a).

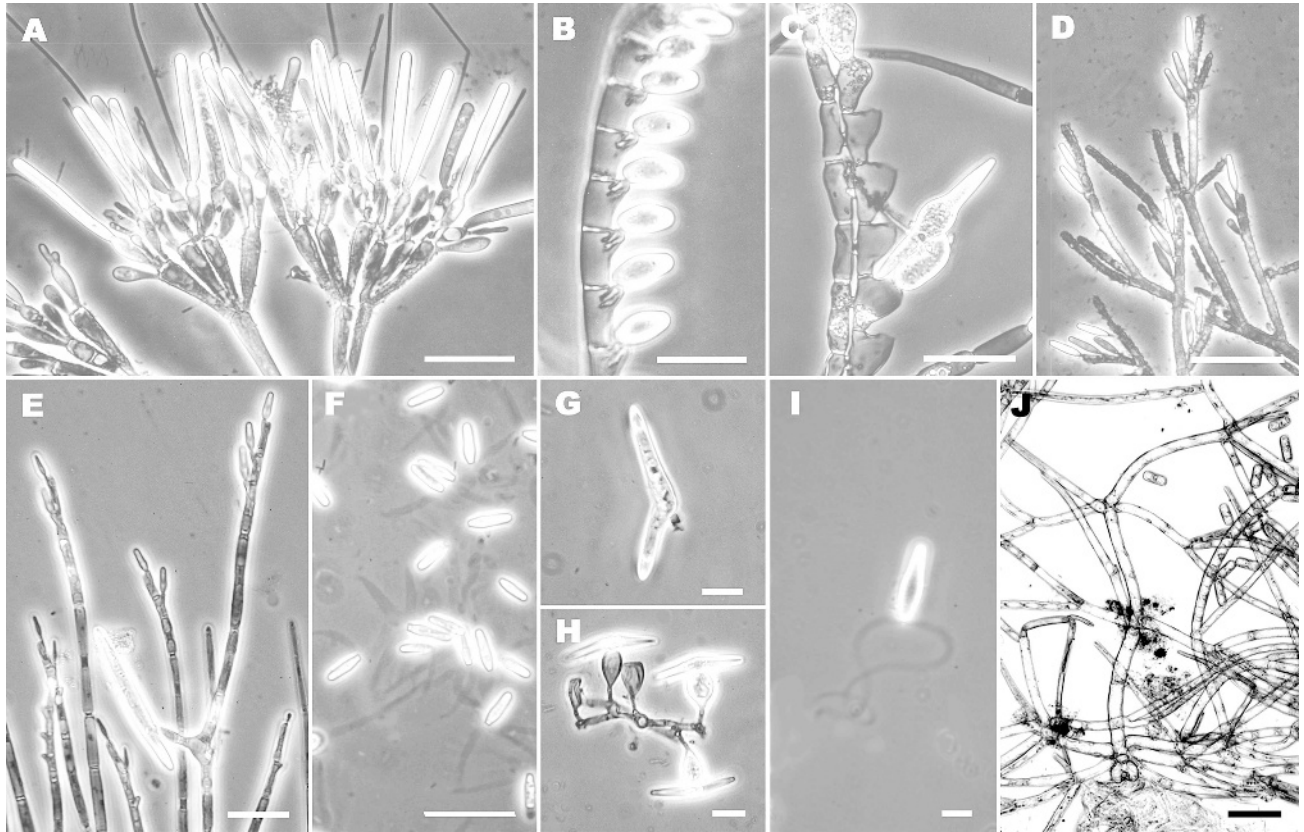


FIG. 3. Phase contrast photomicrographs of living (refractive) Trichomyces illustrating sexual and asexual reproductive structures. A–I. Harpellales from aquatic insect larvae. A. *Orphella catalaunica*. Sporulating heads with attached cylindrical asexual spores. B–C. *Harpellomyces eccentricus*. B. Trichospores with their appendages visible within generative cells. C. Zygospore (Type III) arising from conjugated cells. D. *Smittium culisetae*. Trichospores attached to fertile branchlets. E, F. *Capniomyces stellatus*. E. Attached, biconical zygospore (Type II) and immature trichospores. F. Released trichospores with multiple appendages. G. *Furculomyces boomerangus*. Released bent zygospore (Type II) with a short collar. H, I. *Genistelloides hibernus*. H. Biconical zygospores (Type I) with swollen zygosporophores (similar to suspensors in Zygomycetes) attached to two conjugated branches. I. Released trichospore with two appendages. J. Asellariales from a marine isopod, *Asellaria ligiae*. Thallus attached by a holdfast cell to the hindgut cuticle and releasing small cylindrical arthrospores. Bars: A–I = 20 μm ; J = 100 μm .

Harpellales + *Orphella* & *Kickxellales* + *Spiromyces*.—The Harpellales includes two families: Harpellaceae, all with unbranched thalli and having hosts that are lower dipterans, and Legeriomycetaceae, all with branched thalli associated with various nonpredaceous larval aquatic insect hosts with one exception in isopods (Lichtwardt 1986, Lichtwardt et al 1999, 2001, White 1999). Only the Harpellales possesses asexual spores called trichospores (specialized, deciduous, monosporous sporangia) that often bear nonmotile appendages (FIG. 3A, B, D, E, F and I). Four types (I–IV) of sexually produced biconical zygospores are recognized based on orientation of the zygospore on the zygosporophore (FIG. 3C, E, G and H) but Valle and Santamaria (2005) documented unique zygospores for *Orphella* (see this section,

below). Key morphological taxonomic characters among the Harpellales include the size and shape of the spores (FIG. 3A–I), the number of appendages per spore, number of spores per branchlet (FIG. 3A–E and H), shape or nature (with or without adhesive exudate or mucilage) of the gut anchoring holdfast and arthropod host type.

The Kickxellales was validated by Benjamin (1979) who also suggested that the Harpellales—itsself validated by Lichtwardt and Manier (1978)—be considered in the Zygomycetes, but the Harpellales has been accepted more broadly as Trichomyces (Benny et al 2001). Although seemingly rare members of Kickxellales are fairly common on dung and they also can be isolated from soil and insect cadavers (Benny et al 2001, Kurihara et al 2004). The Kickxellales have

grown from eight (Benjamin 1961, 1963, 1979) to 11 genera with the recent addition of *Myconymphaea*, *Mycoëmelia* and *Ramicandelaber* (Kurihara et al 2001, 2004), although *Ramicandelaber* did not cluster with other Kickxellales in the present study (see FIG. 1). Like Harpellales all members of Kickxellales have monosporous sporangia, usually borne on specialized fertile branches (sporocladia) (Benjamin 1979, Benny et al 2001, Kurihara et al 2004). Most Kickxellales have asexual spores that collect in a fluid droplet, except for *Spiromyces* and *Spirodactylon*, which are dry-spored at maturity (Ingold 1978); zygospores are hyaline and globose with two or three undifferentiated suspensors (Benjamin 1958, 1959, Benny et al 2001). Septa within Kickxellales are centrally perforate with biconvex plugs that persist in acid stains and weak base (Kurihara et al 2001). In addition a sac-like, labyrinthiform organelle or abscission vacuole is located just below the sporangial septum in *Kickxella* and *Linderina* (Benny et al 2001).

Several different relationships between the Kickxellales and Harpellales have been proposed (Benjamin 1979, Moss 1979, Lichtwardt et al 2001) in part due to the paucity of taxonomic characters (with unknown character states in many instances) and the challenge involved in working with unculturable microfungi (about 80% of Harpellales) (White 2006). The specialized, asexual trichospores of the Harpellales are produced laterally from generative cells with aseptate collars, and they are homologous to the monosporous sporangia (borne on pseudophialides) of the Kickxellales with the exception of *Spiromyces* (Benjamin 1966, Moss and Young 1978). Zygospore production, although different in the two orders (biconical versus globose), also has long been used as evidence for the close relationship of the two orders (Benjamin 1979). Other possible homologies include cell wall composition, septal ultrastructure, immunological affinities and sterol spectrum (Sangar et al 1972, Moss and Young 1978, Moss 1979, 1998, Benny and White 2001).

Molecular phylogenetic studies began with Walker's (1984) 5S rRNA analyses; however this locus lacked sufficient signal to infer natural relationships. O'Donnell et al (1998) used 18S rRNA data with additional support from morphological and physiological characters to infer a sister group relationship between the Harpellales and Kickxellales. Gottlieb and Lichtwardt (2001) demonstrated a similar pattern adding culturable members of Harpellales but also suggested that *Smittium* was polyphyletic. White (2006) used 18S and 28S rDNA to add various unculturable taxa and discovered that the Harpellales were monophyletic except for *Orphella*, a topology (FIG. 1) repeated here using two *Orphella* species and

a broadened dataset (all harpellids in the tree are represented in FIG. 3A–I). *Orphella* was thought to be one of the most derived genera of the Harpellales (Lichtwardt 1986) and species have distinctive specialized reproductive cells (Santamaria and Girbal 1998, Valle and Santamaria 2005) that at maturity protrude beyond the anus of their stonefly hosts (White and Lichtwardt 2004). As predicted by White (2002) the discovery of unusual coiled zygospores in *Orphella* (Valle and Santamaria 2005), in contrast to those found in both the Harpellales and Kickxellales, is consistent with its unique position in the combined rDNA phylogeny. Valle and Santamaria (2005) suggested that *Orphella* derived directly from a kickxellid ancestor, presumably from one resembling *Ramicandelaber brevisporus* (Kurihara et al 2004, see below).

Although the *Spiromyces* clade was resolved as either a sister of the Kickxellales or Harpellales in previous molecular phylogenetic analyses (O'Donnell et al 1998, Tanabe et al 2000, White 2002, 2006), *Spiromyces* was supported strongly as a sister of *Orphella* + Kickxellales in the current study (FIG. 1). The inconsistent placement of the *Spiromyces* clade might reflect the need (i) for more sequence data, (ii) to reconsider the genus or (iii) to consider the effect of long-branch attraction (hereafter = LBA, see next section).

Dimargaritales + *Neozygites*, *Zoopagales* and *uncertain lineages*.—Genes of some taxa of the weakly supported clade consisting of the Dimargaritales, Zoopagales and several other fungi might have undergone accelerated evolution and the cluster might not be natural due to LBA (FIG. 1). LBA is the phenomenon in which taxa that are evolving at different rates are artificially attracted to each other due to biases inherent in the method of tree reconstruction (Bergsten 2005). Notably the unexpected inclusion of entomophthoralean *Neozygites* with Dimargaritales on the longest branches observed results in the paraphyly of Dimargaritales. *Ramicandelaber*, originally classified within the Kickxellales (Ogawa et al 2001) and the cockroach parasite, *Nephridiophaga*, formerly considered a protozoan until an affinity to the Zygomycota was suggested with 18S rDNA molecular analysis (Wylezich et al 2004), appear as basal members of the clade but with weak support. The clade should be evaluated further with more slowly evolving protein coding genes. The monophyly of the Zoopagales is not supported because of the uncertain grouping of *Zoophagus insidians* with Harpellales + Kickxellales, consistent with a study based on 18S rDNA (Tanabe et al 2000). Given the diversity of ecology and morphology of

uncultured and uninvestigated zoopagalean fungi, the monophyly of the Zoopagales remains an open question.

In contrast to the present phylogenetic study, RPBI protein sequence-based analyses placed the Dimargaritales in a position basal to the Kickxellales-Harpellales (Tanabe et al 2004). A Dimargaritales + Kickxellales + Harpellales (i.e. the DKH clade) relationship receives morphological support as well because all three orders produce regularly formed septa with a lenticular cavity. Conversely, support for a Dimargaritales + Zoopagales sister group relationship may be reflected in the putative synapomorphic production of common parasitic organs such as haustoria. The possible significance of parasitism in the evolution of the Zoopagales with members of the "DKH" cluster requires further study.

Entomophthorales + *Basidiobolus*.—*Entomophthorales* literally translates to "insect destroyers", with the common housefly infected with *Entomophthora muscae* on a window pane being the most widely used example (see tree FIG. 1, also FIGS. 5–24 in Alexopoulos et al 1996). Many species are saprophytic; however some facultative or obligate pathogens show potential for the biological control of pest insects (Carruthers and Hural 1990). Some species of *Basidiobolus* and *Conidiobolus* cause serious mycoses in animals including humans (de Hoog et al 2000).

Thaxter (1888) produced the first monographic treatment of *Entomophthorales*, and Underwood (1899) was the first to adopt the ordinal name. Zygospores, coenocytic thalli and repeated conidial discharge distinguish the *Entomophthorales* (Benny et al 2001). *Entomophthoralean* asexual spores are different from other *Zygomycota* ontogenetically because they are holoblastic conidia that lack a sporangiospore wall. In addition to their commonly observed forcibly discharged conidia some species also form secondary capilliconidia (Benny et al 2001, FIGS. 32–35), similar to those formed by some members of the Zoopagales (Blackwell and Malloch 1991). Secondary capilliconidia (insect dispersed) provide an alternative dispersal mechanism (Ben-Ze'ev and Kenneth 1982, Humber 1981, King and Humber 1981). Genera of *Entomophthorales* have been distinguished phenotypically by conidial and conidiophore features, mode of discharge and general habit and host range (Benny et al 2001).

The *Entomophthorales* have been classified in up to six families and 22 genera (Kirk et al 2001). Humber's (1989) classification, which included six families (i.e. Ancylistaceae, Basidiobolaceae, Completoriaceae, Entomophthoraceae, Meristacraceae and Neozygiteaceae), will require further data to assess

its monophyly (FIG. 1). In the present rDNA-based phylogenetic analysis three clades of *Entomophthorales* were resolved: (i) *Basidiobolus* with an undescribed snake pathogen (listed in GenBank as *Schizangiella serpentis* sp. nov. Humber); (ii) the core *Entomophthorales* including *Conidiobolus*; and (iii) *Neozygites* with the Dimargaritales. Similarly the SSU phylogeny of Jensen et al (1998) recovered the "core" *Entomophthorales* as a monophyletic group, excluding *Basidiobolus*, which was nested within the Chytridiales, as noted initially by Nagahama et al (1995).

Conidial release of *Basidiobolus* is by a "rocket mechanism" with its conidium remaining intact and with the upper part of the conidiophore. The rocket mechanism contrasts with the core *Entomophthorales* described as having a "rounding-up" mechanism or "papillar eversion" (Jensen et al 1998). Although a separate order, the Basidiobolales, has been proposed for *Basidiobolus* spp. (Cavalier-Smith 1998) concerns about LBA (James et al 2000) suggest that this proposal needs to be evaluated critically based on multilocus analyses including slowly evolving protein coding sequences. Morphological characters, such as a large nucleus and spindle pole body with microtubular structure, however do distinguish *Basidiobolus* when compared to *Entomophthorales* (see below, Chytridiomycota). Careful evaluation also is suggested for species of *Neozygites*, obligate parasites of mites and insects (Ben-Ze'ev et al 1987, Humber 1989, Keller 1997) clustered in a putatively "fast evolving" Dimargaritales clade in this study (FIG. 1).

Missing Zygomycota.—*Asellariales*. This is a small order of arthropod gut fungi (Trichomycetes) with two recognized genera, *Asellaria* and *Orchesellaria* (in isopods and springtails, respectively), and a putative third member, *Baltomyces*, also in isopods (Cafaro 1999). The members of the order are characterized by branched thalli and arthrospores (FIG. 3J). Taxonomy hinges on basal cell morphology (Valle 2006). Ultrastructurally septa are incomplete with a lenticular cavity and a plug (Saikawa et al 1997), similar to those of the DKH clade. Sexual reproduction is unknown and none have been cultured. Sequences of *Asellariales* must be obtained to test the hypothesis of a close relationship between the *Asellariales* and Harpellales based on morphological data. Such an analysis also will be essential to help resolve the basal branches in the *Zygomycota* phylogeny.

Amoebidiales + *Eccrinales*.—Phylogenetic analyses of these orders (previously misclassified as Trichomycetes) including results of the present study (FIG. 1), indicate that both are monophyletic and are sister taxa (Benny and O'Donnell 2000, Cafaro 2005). They

belong to the protist group Mesomycetozoa (Adl et al 2005), recently established to accommodate a group of fish and shellfish parasites, human and anuran pathogens as well as the ectocommensal *Amoebidium parasiticum* (Mendoza et al 2002). The group, initially known by the acronym DRIPs, first was recognized by Ragan et al (1996) based on 18S sequence data that placed the group near the animal-fungal divergence. Amoebidiales and Eccrinales have convergent adaptations for life in the guts of arthropods. Our current understanding of these organisms is limited, but they warrant additional study and expanded taxon sampling because the group could provide answers to animal-fungal origins and divergence.

Chytridiomycota and early diverging Fungi.—Our analysis placed Chytridiomycetes on the basal-most branches of the fungal tree, consistent with idea that the common ancestor of the Fungi had a flagellated life stage (Cavalier-Smith 2001). The phylogeny based on combined rDNA recovers three groups of Chytridiomycetes: the Blastocladales, the “core Chytridiomycota” and *Olpidium*. These results suggest that the Chytridiomycota is paraphyletic, a result consistent with other phylogenetic studies (James et al 2006b, Forget et al 2002). The placement of *Basidiobolus* has been problematic because some earlier phylogenetic studies consistently grouped *Basidiobolus* with Chytridiomycetes (Nagahama et al 1995, James et al 2000). Despite morphological similarities to other Entomophthorales this grouping was interesting because *Basidiobolus* spp. possess a nucleus-associated organelle similar to a centriole, as found only in flagellated fungi (McKerracher and Heath 1985). In our study *Basidiobolus* spp. grouped in a novel position, separate from the majority of chytrids and Entomophthorales. Specifically they branched early among the Zygomycota with the chytrid *Olpidium brassicae*, although without statistical support. Previous studies using 18S (James et al 2000) and RPB1 (Tanabe et al 2005) suggested a relationship between the Blastocladales and Entomophthorales. In this study however the Blastocladales forms a lineage of Fungi separate from other Zygomycota and Chytridiomycota.

We purposefully did not include Microsporidia in these analyses, despite the hypothesis that they have diverged from among the Zygomycota (Keeling 2003), because of their notoriously rapid evolution and sensitivity to LBA artifacts in ribosomal RNA phylogenies (Keeling and Fast 2005). Further sampling of slow-evolving genes of diverse Zygomycota eventually might allow a definitive placement of these enigmatic organisms. It also is possible that other yet undiscovered or unrecognized extant taxa also will be

placed within the early diverging fungi. Clearly our phylogenetic understanding of the Zygomycota is enlightened by analyses of combined rDNA sequence data, but further studies with broader taxon and gene sampling are warranted as the next significant step toward resolving the natural relationships of the nonzoosporic basal fungi.

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SUPPLEMENTARY TABLE I. List of taxa used in phylogenetic analyses with source information for sequence data (where GenBank is indicated, sequences were downloaded from that database) whereas all others that are listed with a strain number or other code have been deposited in GenBank (accession numbers are indicated) as part of the Assembling the Tree of Life (AFToL) project or for this publication

Source/Strain	Species/Strain	Order/Clade	GenBank accession numbers		
			18S	28S	5.8S/ITS
GenBank	<i>Basidiobolus haptosporus</i> ARSEF 263	Entomophthorales?	AF368504	–	–
NRRL 34594	<i>Basidiobolus ranarum</i>	Entomophthorales	AY635841	DQ273807	AY997030
NRRL 28638	<i>Conidiobolus coronatus</i>	Entomophthorales	AF113418	AY546691	AY997041
GenBank	<i>Conidiobolus thromboides</i> ARSEF 115	Entomophthorales	AF052401	–	–
GenBank	<i>Entomophaga aulicae</i> FPMI 646	Entomophthorales	U35394	U35394	U35394
ARSEF 3074	<i>Entomophthora muscae</i>	Entomophthorales	AY635820	DQ273772	AY997047
GenBank	<i>Macrobotrophthora vermicola</i> ARSEF 650	Entomophthorales	AF052400	–	–
GenBank	<i>Neozygites floridana</i> ARSEF 662	Entomophthorales	AY233985	–	–
GenBank	<i>Neozygites parvispora</i> ARSEF 6276	Entomophthorales	AF296760	–	–
GenBank	<i>Neozygites tanajoae</i> BIN35G	Entomophthorales	AY233983	–	–
GenBank	<i>Pandora neoaphidis</i> KVL 633	Entomophthorales	AF052405	–	–
GenBank	<i>Schizangiella serpentis</i> nom. prov. [†] , ARSEF 203	Entomophthorales	AF368523	–	–
GenBank	<i>Zoophthora radicans</i> KVL 610	Entomophthorales	AF052404	–	–
GenBank	<i>Nephridiophaga blatellae</i>	?	AY603958	–	–
GenBank	<i>Coemansia braziliensis</i> NRRL 1566	Kickxellales	AF007532	AF031069	–
NRRL 1564	<i>Coemansia reversa</i>	Kickxellales	AF007533	AY546689	AY997039
GenBank	<i>Dipsacomyces acuminosporus</i> NRRL 2925	Kickxellales	AF007534	AF031065	–
GenBank	<i>Kickxella alabastrina</i> NRRL 2693	Kickxellales	AF007537	AF031064	–
GenBank	<i>Linderina pennispora</i> NRRL 3781	Kickxellales	AF007538	AF031063	–
GenBank	<i>Martensiomycetes pterosporus</i> NRRL 2642	Kickxellales	AF007539	AF031066	–
GenBank	<i>Ramicandelaber brevisporus</i> NBRC100469	Kickxellales	AB196323	–	–
GenBank	<i>Spirodactylon aureum</i> NRRL 2810	Kickxellales	AF007541	AF031068	–
NRRL 22631	<i>Spiromyces aspiralis</i>	Kickxellales?	AF007543	DQ273801	AY997090
NRRL 3067	<i>Spiromyces minutus</i>	Kickxellales?	AF007542	DQ273810	AY997091
NRRL 2808	<i>Dimargaris bacillispora</i>	Dimargaritales	AB016020	DQ273791	AY997043
GenBank	<i>Dispira cornuta</i> NRRL A-16 1010	Dimargaritales	AB016021	–	–
GenBank	<i>Tieghemiomyces parasiticus</i> NRRL 2924	Dimargaritales	AB016022	–	–
167-25-2*	<i>Capniomyces stellatus</i>	Harpellales	EF396191	EF396194	EF396189
AUS-42-7	<i>Furculomyces boomerangus</i>	Harpellales	AF007535	DQ273809	AY997050
GenBank	<i>Genistelloides hibernus</i> TN-11-1	Harpellales	AF007536	AF031062	–
081b-28-1*	<i>Harpellomyces</i> sp.	Harpellales	EF396192	EF396195	EF396190
576-19M-5*	<i>Orphella catalaunica</i>	Harpellales?	EF396193	EF396196	–
NS-35-W16	<i>Orphella</i> aff. <i>haysii</i> ^{††}	Harpellales?	DQ322626	DQ273830	AY997068
COL-18-3	<i>Smittium culisetae</i>	Harpellales	AF007540	DQ273773	AY997089
NRRL 13723	<i>Kuzuhaea moniliformis</i>	Zoopagales	AB016010	DQ273796	AY997057
NRRL 2385	<i>Piptocephalis corymbifera</i>	Zoopagales	AB016023	AY546690	AY997073
NRRL A-10835	<i>Rhopalomyces elegans</i>	Zoopagales	AY635834	DQ273795	–

SUPPLEMENTARY TABLE I. Continued

Source/Strain	Species/Strain	Order/Clade	GenBank accession numbers		
			18S	28S	5.8S/ITS
GenBank	<i>Syncephalis depressa</i> NRRL 22627	Zoopagales	AB016011	–	–
GenBank	<i>Thamnocephalis sphaerospora</i> NRRL 22810	Zoopagales	AB016013	–	–
GenBank	<i>Zoophagus insidians</i>	Zoopagales	AB016009	–	–
GenBank	<i>Absidia blakesleeana</i> NRRL 1304	Mucorales	AF157117	AF157171	–
GenBank	<i>Absidia repens</i> NRRL 1336	Mucorales	AF113410	AF113448	–
GenBank	<i>Apophysomyces elegans</i> NRRL 28632	Mucorales	AF113412	AF113450	–
GenBank	<i>Chaetocladium jonesii</i> NRRL 2343	Mucorales	AF157126	AF157180	–
GenBank	<i>Choanephora cucurbitarum</i> NRRL 2744	Mucorales	AF157127	AF157181	–
NRRL 2808-h	<i>Cokeromyces recurvatus</i>	Mucorales	AY635843	DQ273812	AY997040
GenBank	<i>Cunninghamella echinulata</i> NRRL 1382	Mucorales	AF157130	AF157184	–
GenBank	<i>Dichotomocladium elegans</i> NRRL 6236	Mucorales	AF157131	AF157185	–
GenBank	<i>Dicranophora fulva</i> NRRL 22204	Mucorales	AF157132	AF157186	–
GenBank	<i>Gilbertella persicaria</i> NRRL 2357	Mucorales	AF157136	AF157190	–
GenBank	<i>Halteromyces radiatus</i> NRRL 6197	Mucorales	AF157138	AF157192	–
GenBank	<i>Hesseltinella vesiculosa</i> NRRL 3301	Mucorales	AF157140	AF157194	–
GenBank	<i>Hyphomucor assamensis</i> NRRL 22324	Mucorales	AF157141	AF157195	–
GenBank	<i>Mucor racemosus</i> ATCC1216B	Mucorales	AJ271061	AJ271061	AJ271061
GenBank	<i>Mucor recurvus</i> var. <i>indicus</i> NRRL 3247	Mucorales	AF157146	AF157200	–
GenBank	<i>Mycotypha microspora</i> NRRL 1572	Mucorales	AF157148	AF157202	–
GenBank	<i>Phascolomyces articulatus</i> NRRL 2880	Mucorales	AF157150	AF157204	–
NRRL 1555	<i>Phycomyces blakesleeanus</i>	Mucorales	AY635837	DQ273800	AY997071
GenBank	<i>Pilobolus umbonatus</i> NRRL 6349	Mucorales	AF157153	AF157207	AY829473
GenBank	<i>Protomycocladus faisalabadensis</i> NRRL 22826	Mucorales	AF157156	AF157210	–
GenBank	<i>Radiomyces spectabilis</i> NRRL 2753	Mucorales	AF157157	AF157211	–
GenBank	<i>Rhizopus oryzae</i> NRRL 28631	Mucorales	AF113440	AF113481	–
DAOM225708	<i>Rhizopus stolonifer</i>	Mucorales	DQ536474	DQ273817	AY997085
GenBank	<i>Syncephalastrum racemosum</i> NRRL 2496	Mucorales	X89437	AF113484	–
GenBank	<i>Thamnidium elegans</i> NRRL 2467	Mucorales	AF157163	AF157217	–
GenBank	<i>Umbelopsis isabellina</i> NRRL 1757	Mucorales	AF157166	AF157220	–
NRRL5844	<i>Umbelopsis ramanniana</i>	Mucorales	DQ322627	DQ273797	AY997097
GenBank	<i>Zychaea mexicana</i> NRRL 6237	Mucorales	AF157169	AF157223	–
GenBank	<i>Dissophora decumbens</i> NRRL 22416	Mortierellales	AF157133	AF157187	–

SUPPLEMENTARY TABLE I. Continued

Source/Strain	Species/Strain	Order/Clade	GenBank accession numbers		
			18S	28S	5.8S/ITS
GenBank	<i>Gamsiella multidivariata</i> NRRL 6456	Mortierellales	AF157144	AF157198	–
GenBank	<i>Lobosporangium transversale</i> NRRL 3116	Mortierellales	AF113424	AF113462	–
GenBank	<i>Mortierella chlamydospora</i> NRRL 2769	Mortierellales	AF157143	AF157197	–
MS-6	<i>Mortierella</i> sp.	Mortierellales	AY635828	DQ273786	AY997062
NRRL6337	<i>Mortierella verticillata</i>	Mortierellales	AF157145	DQ273794	AY997063
OSC80932	<i>Endogone lactiflua</i>	Endogonales	DQ536471	DQ273788	AY997045
DAOM233144	<i>Endogone pisiformis</i>	Endogonales	DQ322628	DQ273811	AY997046
4695rac-11G2	<i>Glomus intraradices</i>	Glomales	DQ322630	DQ273828	AY997054
UT101	<i>Glomus mosseae</i>	Glomales	AY635833	DQ273793	AY997053
IA702	<i>Paraglomus occultum</i>	Glomales	DQ322629	DQ273827	AY997069
FL225	<i>Scutellospora heterogama</i>	Glomales	AY635832	DQ273792	AY997088
SS218	<i>Olpidium brassicae</i>	Olpidium	DQ322624	DQ273818	AY997067
JEL136	<i>Rhizophydium brooksonianum</i>	Chytridiales	AY601710	DQ273770	AY997079
JEL180	<i>Cladochytrium replicatum</i>	Chytridiales	AY546683	AY546688	AY997037
JEL109	<i>Polychytrium aggregatum</i>	Chytridiales	AY601711	AY546686	AY997074
UCB-91-10	<i>Gaertneriomyces semiglobiferus</i>	Spizellomycetales	AF164247	DQ273778	AY997051
M15	<i>Monoblepharella</i> sp.	Monoblepharidales	AY546682	AY546687	AY997060
GE13	<i>Neocallimastix</i> sp.	Neocallimastigales	DQ322625	DQ273822	AY997064
Brazil2	<i>Allomyces arbusculus</i>	Blastocladales	AY552524	AY552525	AY997028
UCB-49-1	<i>Blastocladia emersonii</i>	Blastocladales	AY635842	DQ273808	AY997032
JEL298	<i>Catenophlyctis</i> sp.	Blastocladales	AY635822	DQ273780	AY997034
Herb	<i>Physoderma maydis</i>	Blastocladales	AY601708	DQ273768	AY997072
GenBank	<i>Neurospora crassa</i>	Dikaryomycota	X04971	AF286411	AY046222
GenBank	<i>Ustilago maydis</i>	Dikaryomycota	X62396	AF453938	AF135431
GenBank	<i>Coprinopsis cinerea</i>	Dikaryomycota	M92991	AF041494+ AF041559+ AF041625+ AF041691	AB097562
GenBank	<i>Schizosaccharomyces pombe</i>	Dikaryomycota	AY251633	Z19136	V01361
GenBank	<i>Saccharomyces cerevisiae</i>	Dikaryomycota	V01335	J01355	AY130313
GenBank	<i>Cryptococcus neoformans</i>	Dikaryomycota	L05428	AF356652	AY227753
FRA-1-14	<i>Amoebidium parasiticum</i>	Amoebidiales	Y19155	DQ273802	AY997029
GenBank	<i>Astreptonema gammari</i>	Eccrinales	AY336709	–	–
GenBank	<i>Palavascia patagonica</i>	Eccrinales	AY682845	–	–
GenBank	<i>Enterobryus oxidi</i>	Eccrinales	AY336710	–	–
GenBank	<i>Eccrinidus flexilis</i>	Eccrinales	AY336700	–	–
GenBank	<i>Suberites ficus</i>	Metazoa	AF100947	AY026381	AJ627184
GenBank	<i>Monosiga brevicollis</i>	Choanoflagellate	AF100940	AY026374	–

* these are clone numbers, rather than strain numbers

† Listed in Genbank as *Schizangiella serpentis* sp. nov. Humber but not formally described at time of this publication.

†† Being described as a new species by Strongman and White.