A molecular phylogeny of the flagellated fungi (Chytridiomycota) and description of a new phylum (Blastocladiomycota)

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Abstract: Chytridiomycota (chytrids) is the only phylum of true Fungi that reproduces with motile spores (zoospores). Chytrids currently are classified into five orders based on habitat, zoospore characters and life cycles. In this paper we estimate the phylogeny of the chytrids with DNA sequences from the ribosomal RNA operon (18S+5.8S+28S subunits). To our surprise the morphologically reduced parasites *Olpidium* and *Rozella* comprise two entirely new, and separate, lineages on the fungal tree. *Olpidium brassicae* groups among the Zygomycota, and *Rozella* spp. are the earliest branch to diverge in the fungal kingdom. The phylogeny also suggests that Chytridiomycota is not monophyletic and there are four major lineages of chytrids: *Rozella* spp., *Olpidium brassicae*, the Blastocladiales and a ''core chytrid clade'' containing the remaining orders and families and the majority of flagellated fungi. Within the core chytrid group 11 subclades can be identified, each of which correlates well with zoospore ultrastructure or morphology. We provide a synopsis of each clade and

its morphological circumscription. The Blastocladiales appears to be the sister taxon of most nonflagellated fungi. Based on molecular phylogenetic and ultrastructural characters this order is elevated to a phylum, the Blastocladiomycota.

INTRODUCTION

The Chytridiomycota is a phylum of Fungi that reproduces through the production of motile spores (zoospores), typically propelled by a single, posteriorly directed flagellum. These organisms, often referred to as chytrid fungi or chytrids, have a global distribution with approximately 1000 described species. Based on biochemical characteristics, including chitin in cell walls, the α-aminoadipic acid lysine synthetic pathway and storage carbohydrates as glycogen, Bartnicki-Garcia (1970) classified the Chytridiomycota as true Fungi. Others considered chytrids as a transitional group between protists and Fungi because of their production of motile zoospores (Barr 1990). Phylogenetic studies with ribosomal RNA genes unified these views and conclusively demonstrated that chytrids were true Fungi that occupied a basal position in the fungal tree (e.g. Förster et al 1990).

Chytrids are essentially ubiquitous, occurring in diverse and unique habitats from the tropics to the arctic regions (Powell 1993). They are found in aquatic systems such as streams, ponds, and estuarine and marine ecosystems primarily as parasites of algae and plankton components. Perhaps the majority of chytrid species occur in terrestrial habitats (Barr 2001) such as forest, agricultural and desert soils, and acidic bogs as saprotrophs of refractory substrata including pollen, chitin, keratin and cellulose. In soil chytrids are obligate parasites of a wide variety of vascular plants, including potatoes (*Synchytrium*) and curcurbits (*Olpidium*). The only known chytrid parasite of vertebrates is *Batrachochytrium dendrobatidis* (Longcore et al 1999), considered the causative agent of die-offs and population declines of amphibian species (Berger et al 1998). In most habitats a few chytrid species are relatively frequent and abundant (i.e. *Chytriomyces hyalinus* in freshwater and *Rhizophlyctis rosea* in agricultural and perturbed soils),

whereas most species are infrequent and scarce to rare (Letcher and Powell 2001, Letcher et al 2004a).

The most prominent morphological feature of the chytrid body, or thallus, is the sporangium (FIG. 1). The sporangium is a sac-like structure in which internal divisions of the protoplasm result in production of zoospores. These zoospore-producing sporangia (zoosporangia) are thin-walled (FIG. 1k) whereas resting spores are thick-walled structures (FIG. 1a, l) that may germinate to produce a sporangium after a dormant period. Zoosporangia always are produced asexually, but resting spores may be sexually or asexually formed. Eucarpic chytrids are those that consist of a sporangium and filamentous rhizoids (FIG. 1k). In contrast holocarpic chytrids produce thalli that are entirely converted to sporangia during reproduction. Chytrid thalli can be either monocentric, in which an individual produces only a single sporangium (FIG. 1g), or polycentric, in which an individual is composed of multiple sporangia produced on a network of rhizoids termed a rhizomycelium (FIG. 1j). Classically chytrids also were described on the basis of whether they grow on (epibiotic, FIG. 1h) or within (endobiotic, FIG. 1a) their substrate. Other characteristics historically used for taxonomy include the presence of a lid-like operculum (FIG. 1e), which opens to allow zoospore release from a sporangium (Sparrow 1960), and the apophysis (FIG. 1i), which is a subsporangial swelling.

Zoospores are unwalled cells, usually 2–10 µm diam, which contain a single nucleus and, with the exception of some genera of the Neocallimastigales, are propelled by a single posteriorly oriented whiplash flagellum (FIG. 1b, m). Considerable effort has been placed on understanding the ultrastructure of the zoospore with electron microscopy; these studies have produced a wealth of information about the complexity of the internal contents of the spores. Two character-rich components of the zoospores can be identified: the flagellar apparatus, basal bodies and associated structures (Barr 1981), and the microbody-lipid globule complex (MLC, Powell 1978).

The Chytridiomycota is divided into five orders based primarily on the mode of reproduction and ultrastructure of the zoospore. Three groups can be distinguished largely on the basis of life cycle and gross morphology: the oogamous Monoblepharidales; the Blastocladiales displaying sporic meiosis and the alternation of sporophytic and gametophytic generations; and the Chytridiales, a group characterized by zygotic meiosis. A fourth order, Spizellomycetales, was separated from the Chytridiales on the basis of distinctive ultrastructural character states (Barr 1980), and a fifth order, Neocallimastigales, occurs exclusively as anaerobic symbionts of the rumen.

Chytridiales is the largest of the orders (more than 75 genera) and the classification of this group has been variously approached using developmental, sporangial and more recently ultrastructural characters. Sparrow (1960) considered the operculum to be a defining characteristic and created two series of families based on whether sporangia were operculate. In contrast Whiffen (1944), Roane and Paterson (1974) and Barr (1978) suggested development could be used to divide the Chytridiales into families. Systems of classification based on morphology and those based on development of the thallus and sporangium both have been shown by molecular phylogenetics to be inaccurate in defining genera and families of Chytridiales (James et al 2000, Letcher et al 2004b).

Molecular phylogenies with 18S rDNA have suggested that both the Chytridiomycota and Chytridiales might not be monophyletic (James et al 2000). Phylogenies based on data from entire mitochondrial genomes (Seif et al 2005) have suggested strongly that two main lineages exist within the Chytridiomycota—the Blastocladiales and the remaining four orders (the ''core chytrid clade''). In this paper we present a new dataset that includes an analysis of most chytrid 18S rDNA data in conjunction with sequence data for the entire rRNA operon (18S, 28S and 5.8S). These data provide a comprehensive view of chytrid phylogeny and define four major lineages of flagellated Fungi.

MATERIALS AND METHODS

Our dataset consists of taxa for which most of the rRNA operon was sequenced (18S+28S+5.8S subunits, n = 54) as well as chytrid taxa represented only by 18S data (n = 49) and additional fungal, animal and Mesomycetozoae taxa (n = 21; complete operon with one exception, *Diaphanoeca grandis*). GenBank accession numbers and strain/voucher data are provided (SUPPLEMENTARY TABLE I). Data gathered for the full operon were obtained by PCR, primarily with primers SR1R and LR12 (Vilgalys and Hester 1990) using LATaq (TaKaRa), with these thermocycling conditions: 94 C for 1 min followed by 35 cycles of denaturing at 94 C for 30 s, annealing at 55 C for 30 s, extension at 72 C for 5 min and a 10 min final extension at 72 C. Amplicons generally were cloned into pCR2.1-TOPO (Invitrogen).

The three gene regions were aligned by eye with GeneDoc v2.6 (http://www.cris.com/~Ketchup/genedoc.shtml) and combined into one supermatrix with MacClade 4.05 (Maddison and Maddison 2002). Regions of ambiguous alignment were excluded from further analysis leaving 4109 included characters, 1388 of which are parsimony informative. We estimated the phylogeny with MrBayes v3.1 (Huelsenbeck and Ronquist 2001) by four independent runs with the GTR+I+Γ model of evolution, sampling trees every 500 generations for 10×10^6 generations. We also assessed
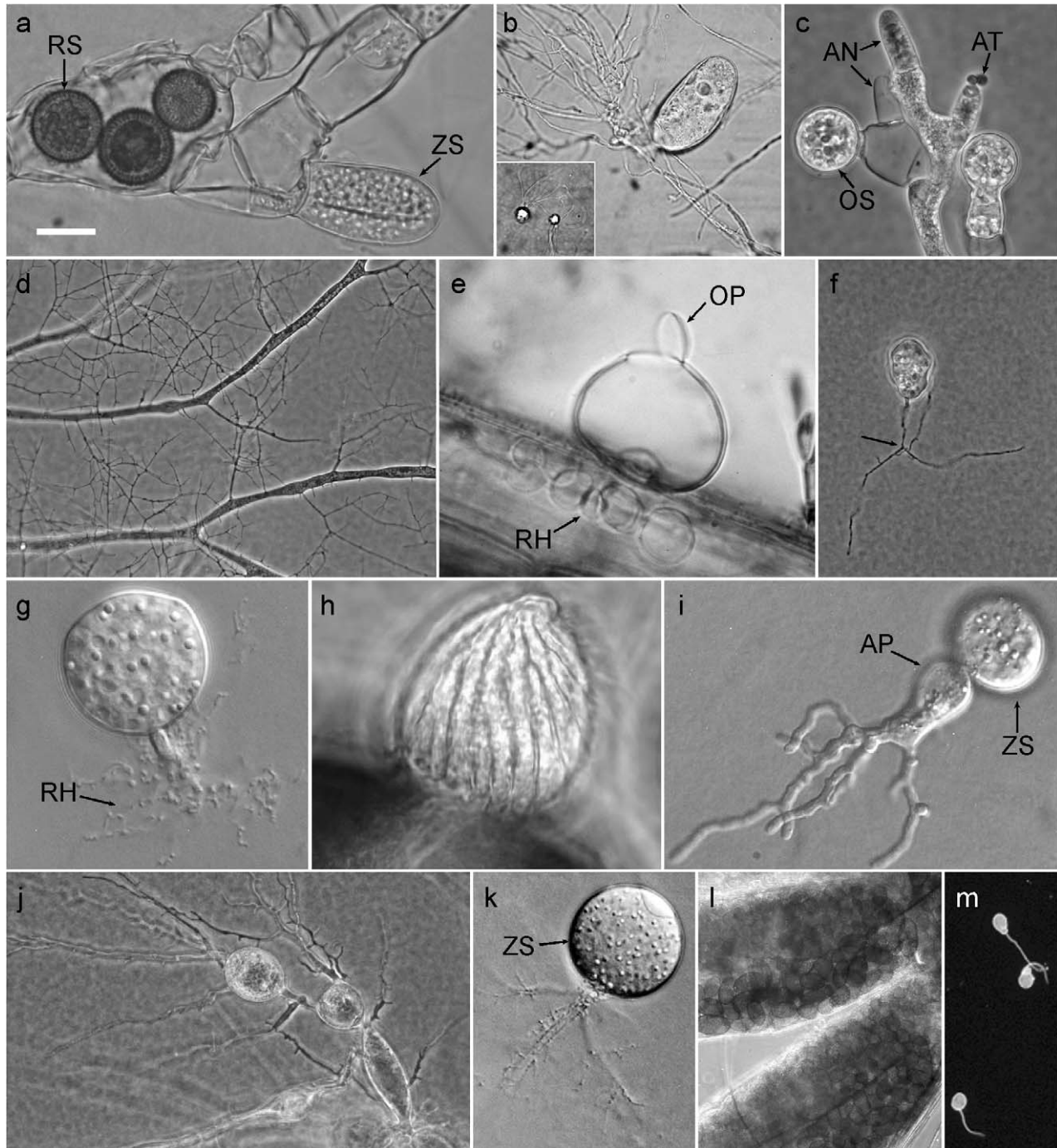
FIG. 1. Light micrographs of representative chytrids. a. *Rozella allomycis* isolate UCB-47-54 (Clade 1) parasitizing hyphae of *Allomyces.* The parasite grows inside the host and causes it to produce hypertrophied, highly septate cells within which the parasite may form thick-walled resting spores (RS) or unwalled zoosporangia (ZS) that use the host's cell wall as its own. b. *Neocallimastix* sp. (Clade 2A) monocentric thallus with profusely branched rhizoids, inset: multiflagellate zoospores (by Gary Easton). c. *Monoblepharella polymorpha* (Clade 2B) mature zygote or oospore (OS), empty and mature antheridia (AN) and antherozoids (AT) or male gametes emerging from antheridium; photo by Marilyn M. N. Mollicone. d. *Polychytrium aggregatum* isolate JEL109 (Clade 2C) finely branched rhizomycelium. e. *Catenochytridium* sp. (Clade 2D) monocentric, operculate (OP) zoosporangium with catenulate rhizoids (RH). f. *Chytriomyces angularis* isolate JEL45 (Clade 2F) monocentric, operculate sporangium with thread-like rhizoids that branch several µm from the sporangial base (arrow). g. *Terramyces subangulosum* isolate PL 076 (Clade 2G) monocentric, inoperculate sporangium with a thick rhizoidal axis, and densely branched rhizoids (RH) that taper to ∼0.5 µm at tips. h. *Blyttiomyces helicus* (Clade 2H) forming epibiotic,

support for nodes with parsimony bootstrap as implemented in PAUP v4.0b10 (Swofford 2002) and maximum likelihood bootstrap as implemented in RAxML-VI-HPC v2.0 (Stamatakis et al 2005).

RESULTS AND DISCUSSION

The phylogeny (FIG. 2) is the consensus of credible trees derived from the Bayesian analysis of rDNA genes from Chytridiomycota. These analyses confirm the basal position of this group among the Fungi and provide support for at least four major clades of Chytridiomycota. Clade 1 is the earliest diverging lineage in the Fungi and comprises *Rozella* spp. The largest, Clade 2 or the ''core chytrid clade,'' comprises Chytridiales, Spizellomycetales pro parte, Monoblepharidales and Neocallimastigales and can be subdivided further into 11 clades (clades 2A–2K). Clade 3 represents the Blastocladiales, grouping sister of the Dikarya+Glomeromycota+Zygomycota, and Clade 4 is represented solely by *Olpidium brassicae*, which groups among the Zygomycota. These results further suggest that Chytridiomycota is polyphyletic or at least paraphyletic. As suggested by a phylogenetic study of Opisthokonts, the amoeba *Nuclearia simplex* appears to be the closest known sister taxon of the Fungi (Steenkamp et al 2005). Below we discuss the membership of each clade, providing a brief statement on the systematics of each group in light of our phylogenetic findings.

Clade 1. Rozella.—Two isolates of *Rozella* are supported strongly as the earliest diverging lineage in the fungi. *Rozella* is a holocarpic parasite, primarily of chytrids and Oomycetes, that grows inside its host as a walless trophic form (Held 1981) until it produces thick-walled resting spores or zoosporangia that fill the host cell (FIG. 1a). The two strains in the rDNA phylogeny are parasites on other chytrids (*Allomyces* and *Rhizoclosmatium*). The ultrastructure of *R. allomycis* zoospores presents a unique combination of characters including a striated rhizoplast connecting the kinetosome (the basal body of the flagellum) to a large, spheroidal mitochondrion, which abuts a helmet-shaped nucleus (Held 1975). Phylogenetic analyses using the two largest subunits

of RNA polymerase II suggest that microsporidia might belong to this lineage (T.Y. James and R. Vilgalys, unpublished data).

Clade 2A. Neocallimastigales.—It is supported as the most basal group of the core chytrid clade (Clade 2) by Bayesian posterior probability. Members of this clade are characterized by obligate anaerobic growth and are found in the rumen and hindgut of many larger mammalian herbivores. They are morphologically similar to other chytrid orders and may be monocentric or polycentric. The rumen chytrids possess an organelle called a hydrogenosome that generates ATP and appears to be a degenerate mitochondrion lacking a genome (van der Giezen 2002). Rumen chytrid zoospores may be multiflagellate or uniflagellate (FIG. 1b). The zoospore of rumen chytrids lacks the nonflagellated centrioles (nfc) and lipid globules that are found in most other members of Clade 2 but possesses a microtubulular root emanating from a kinetosome associated spur (Heath et al 1983). Six described genera are circumscribed on the basis of number of flagella and sporangial characters (Ho and Barr 1995, Ozkose et al 2001).

Clade 2B. Monoblepharidales.—It is monophyletic and sister of the clade of Chytridiales+Spizellomycetales. Monoblepharidales is a small order consisting only of six genera. The earliest diverging lineage is the species *Hyaloraphidium curvatum*, once believed to be a colorless, unicellular green alga (Ustinova et al 2000). The planktonic *H. curvatum* has a lunate thallus superficially similar to that of some *Harpochytrium* species, but zoospores have not been observed in *H. curvatum*. The genera *Oedogoniomyces*, *Hyaloraphidium* and *Harpochytrium* all grow vegetatively as simple, unbranched thalli lacking rhizoids, whereas the remaining three genera, *Gonapodya*, *Monoblepharis* and *Monoblepharella*, are polycentric species with a well developed coenocytic mycelium. In polycentric genera oogamous reproduction occurs in which a motile male gamete (antherozoid) fuses with a nonmotile female gamete (egg, FIG. 1c). Hyphae of Monoblepharidales have a foamy or reticulate appearance. Zoospores of Monoblepharidales have a fenestrated cisterna called a rumposome (Fuller

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inoperculate sporangium with distinct helical pattern on pollen grain. i. *Spizellomyces plurigibbosus* isolate SW 001 (Clade 2I) monocentric, inoperculate zoosporangium (ZS), with swollen, apophysate rhizoidal axis (AP) and branched rhizoids that are blunt at the tips. j. *Catenomyces persicinus* (Clade 2J) polycentric thallus with intercalary zoosporangia. k. *Chytriomyces hyalinus* isolate MP 004 (Clade 2K) monocentric, operculate zoosporangium (ZS) with long branched rhizoids that taper to < 0.5 μm at the tips. l. *Coelomomyces stegomyiae* (Clade 3) elliptical resting spores inside the anal gills of mosquito host. m. *Olpidium bornovanus* (Clade 4) zoospores labeled with a FITC/Conconavalin A conjugate; photo by D'Ann Rochon. Approximate sizes relative to scale bar in FIG. 1a = 10 μm (e, g, h), 15 μm (a, i, m), 20 μm (b, f), 25 μm (c, k), 45 μm (d), 50 μm (j), and 150 μm (l).

Saccharomyces cerevisiae
Schizosaccharomyces pombe
Cryptococcus neoformans
Glomus intraradices
Scutellospora heterogama
Phycomyces blakesleeanus
Umbelopsis ramanniana
Olpidium brassicae SS218
Coemansia reversa
Smittium culisetae
Basidiobolus ranarum
Entomophthora muscae
Conidiobolus coronatus
Physoderma dulichii
Physoderma maculare
Physoderma maydis
Allomyces arbusculus Brazil2
Allomyces macrogynus ATCC46923
Microallomyces sp. CR74
Blastocladiella emersonii UCB 49-1
Catenaria anguillulae JEL194
Catenophlyctis variabilis JEL298
Coelomomyces stegomyiae
Chytriomyces confervae Barr097
Chytriomyces hyalinus UCB 81-1
Chytriomyces hyalinus MP4
Rhizoclosmatium globosum JEL06
Rhizoclosmatium sp. JEL347h
Chytriomyces clade (Miller's dentate) JEL103
Obelidium mucronatum JEL57
Phlyctorhiza endogena JEL80
Chytriomyces sp. JEL378
Siphonaria petersenii JEL102
Podochytrium sp. JEL161
Podochytrium dentatum JEL30
Entophlyctis luteolus JEL129
Chytriomyces clade JEL187
Physocladia obscura JEL137
Chytriomyces spinosus JEL59
Chytriomyces clade WB 235A
Chytriomyces clade JEL155
Asterophlyctis sarcoptoides JEL186
Rhizidium endosporangiatum JEL221
Chytriomyces clade JEL341
Chytriomyces annulatus JEL114
Chytriomyces clade JEL354
Phlyctochytrium planicorne JEL47
Entophlyctis sp. DU DC1
Catenomyces persicinus JEL342
Rhizophlyctis rosea UCB 47-07
Rhizophlyctis rosea JEL318
Rhizophlyctis rosea UCB 57-5
Gaertneriomyces semiglobiferus UCB 91-10
Triparticalcar arcticum BR59
Powellomyces hirtus UGA F18
Powellomyces sp. JEL95
Powellomyces variabilis UCB 85-1
Spizellomyces acuminatus 62A
Spizellomyces punctatus ATCC48900
Spizellomyces clade JEL355
Spizellomyces clade JEL371
Blyttiomyces helicus
Rhizophydium clade JEL08
Rhizophydium macroporosum PLAUS21
Rhizophydium elyensis PLAUS9
Rhizophydium chaetiferum JEL39
Kappamyces laurelensis PL98
Rhizophydium clade UGA F16
Rhizophydium brooksianum JEL136
Rhizophydium clade (operculate) JEL223
Rhizophydium clade JEL316
Rhizophlyctis harderi JEL171
Rhizophydium clade MP8
Entophlyctis sp. JEL174
Rhizophydium sphaerotheca JEL299
Rhizophydium clade JEL142
Batrachochytrium dendrobatidis JEL197
Entophlyctis helioformis JEL326
Chytridium polysiphoniae
Chytriomyces angularis JEL45
Synchytrium decipiens
Synchytrium ex. Otholobium
Synchytrium macrosporum
Synchytrium endobioticum
Allochytridium expandens UCB 69-3
Catenochytrium sp. JEL145
Septochytrium variabile JEL191
Endochytrium sp. JEL324
Endochytrium sp. JEL49
Chytridium? lagenarium JEL72
Nowakowskiella hemisphaerospora UCB 85-6
Nowakowskiella sp. JEL127
Nephrochytrium sp. JEL125
Cladochytrium replicatum JEL180
Polychytrium aggregatum JEL109
Lacustromyces hiemalis JEL31
Polychytrium clade JEL207
Karlingiomyces sp. JEL93
Monoblepharis insignis UCB 59-7
Monoblepharis hypogyna UCB 70-1
Monoblepharella mexicana UCB 78-1
Monoblepharella elongata CR91
Monoblepharella sp. M15
Gonapodya sp. JEL183
Harpochytrium JEL94
Oedogoniomyces sp. CR84
Hyaloraphidium curvatum SAG235-1
Caecomyces communis FG
Cyllamyces aberensis EO14
Piromyces communis FL
Orpinomyces joyonii NJ1
Neocallimastix frontalis MCH3
Neocallimastix sp. GE13
Rozella allomycis UCB 47-54
Rozella ex. Rhizoclosmatium JEL347
Nuclearia simplex
Capsaspora owczarzaki
Salpingoeca infusionum
Monosiga brevicollis
Diaphanoeca grandis
Ichthyophonus hoferi
Amoebidium parasiticum
Antipathes galapagensis
Suberites ficus

Dikarya
Glomeromycota
Zygomycota I
4. *Olpidium*
Zygomycota II
3. Blastocladiomycota
2K. Chytridiales: *Chytriomyces* clade
2J. Spizellomycetales: *Rhizophlyctis* clade
2I. Spizellomycetales: *Spizellomyces* clade
2H. Chytridiales: *Blyttiomyces*
2G. Chytridiales: *Rhizophydium* clade
2F. Chytridiales: *C. angularis* clade
2E. Chytridiales: *Synchytrium* clade
2D. Chytridiales: *Cladochytrium* clade
2C. Chytridiales: *Polychytrium* clade
2B. Monoblepharidales
2A. Neocallimastigales
1. *Rozella*
Choanoflagellida
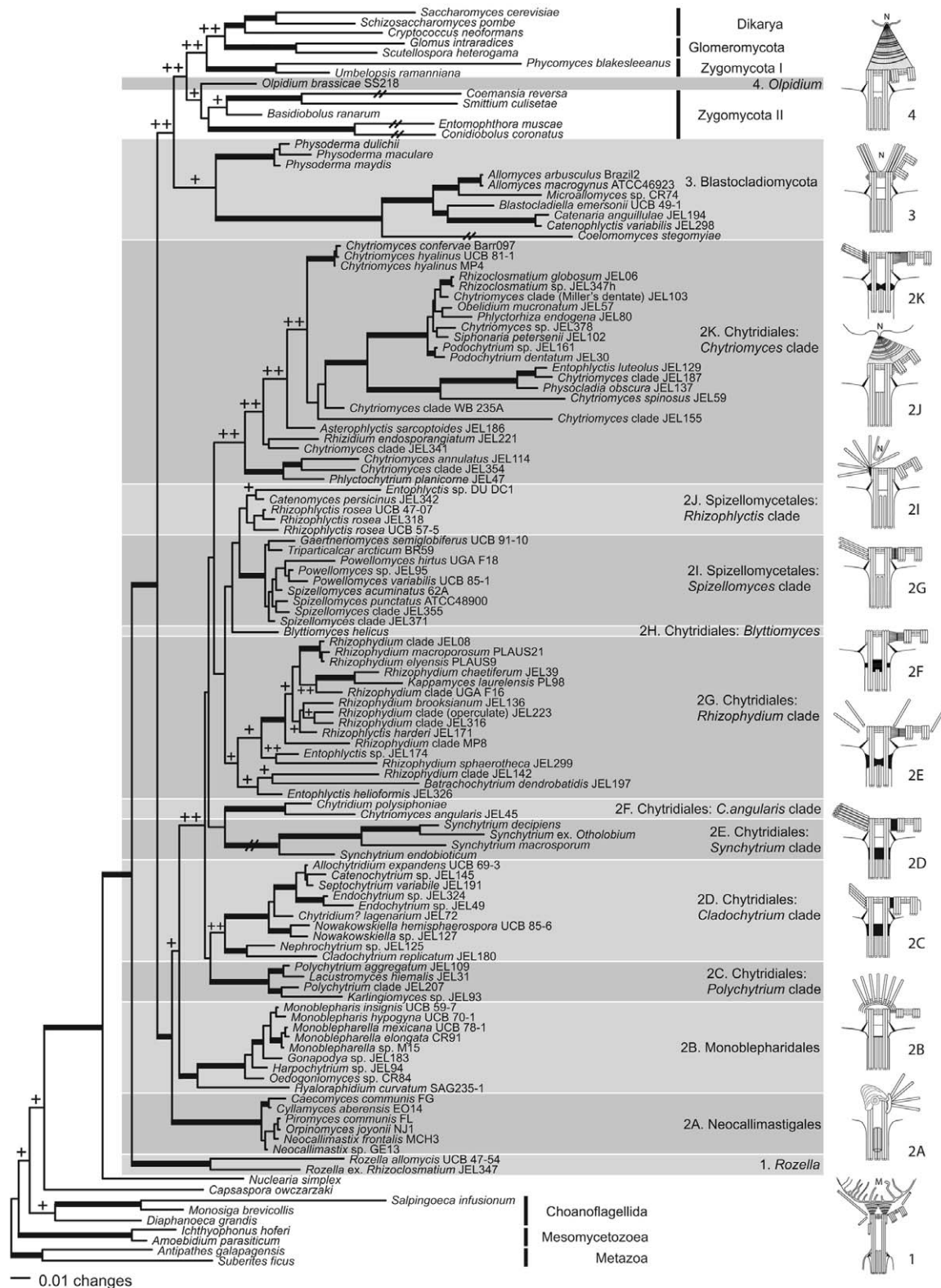Mesomycetozoea
Metazoa

— 0.01 changes

FIG. 2. Phylogeny of chytrid fungi based on rDNA operon. Shown is a majority-rule consensus phylogram computed from the set of 36 000 credible trees. A ''+'' above a node indicates branch was supported by ≥ 95% Bayesian posterior probability (BPP); ''++'' indicates branch supported by ≥ 95% BPP and maximum likelihood bootstrap (MLBS) ≥ 70%. Branches supported by BPP, MLBS and parsimony bootstrap ≥ 70% are indicated by thickened lines. Branches with double diagonal hash marks have been shortened four times to aid viewing. The schematic illustrations diagram the ultrastructural details of

1966) and most have an electron opaque core in the transitional zone of the axoneme similar to the one found in some members of the Chytridiales.

Clade 2C. Chytridiales.—Polychytrium *clade.* The two monotypic, polycentric genera *Polychytrium* and *Lacustromyces* and several monocentric species of *Karlingiomyces* make up the *Polychytrium* clade (formerly termed the *Lacustromyces* clade, James et al 2000). *Polychytrium* and *Lacustromyces* possess a rhizomycelium that lacks swellings containing nuclei (FIG. 1d). *Polychytrium aggregatum* is found in acidic lakes and bogs; *Lacustromyces hiemalis* occurs in lakes or commonly wet areas and *Karlingiomyces* can be isolated from aquatic or terrestrial habitats. Both the monocentric and polycentric species of this clade grow on chitin baits. Zoospores are spherical when in motion and usually larger than 4 μm diam. The unique ultrastructural feature of the clade is the composition of the kinetosome and its associated structures (Longcore 1993 unpublished). The nfc is as long as the kinetosome and the connection of the nfc to the kinetosome is distinctive. Densely staining material extends about 0.1 μm into the zoospore from kinetosome triplets 1 and 9; the primary microtubule root arises between these extensions. At the same level within the kinetosome is a scalloped ring (Longcore 1993).

Clade 2D. Chytridiales.—Cladochytrium. This clade (formerly *Nowakowskiella*) (James et al 2000) is a well supported lineage with a zoospore ultrastructure similar to *Nowakowskiella elegans* and *Cladochytrium replicatum* (Lucarotti 1981). It includes eight genera: *Allochytridium, Catenochytridium, Chytridium* (?) pro parte, *Cladochytrium, Endochytrium, Nephrochytrium, Nowakowskiella* and *Septochytrium*. Species in this group may be monocentric or polycentric and often are characterized by the presence of swellings in the rhizoids or rhizomycelium (FIG. 1e). They occur primarily on cellulose-rich substrates in aquatic and soil habitats. Members of the *Cladochytrium* clade have a zoospore that is typical for Chytridiales and most similar to that of the *Chytriomyces* clade (2K). One distinctive feature of the zoospore is that the microtubular root arising from the kinetosome is a cord-like bundle interconnected by fine fibrillar bridges (Lucarotti 1981, Barr 1986). Two major subclades were recovered in our phylogenetic analysis. *Nephrochytrium* and *Cladochytrium* form a clade that is sister of a clade containing the remaining genera.

Clade 2E. Chytridiales.—Synchytrium. This genus contains more than 200 species that are obligate parasites of green plants, particularly angiosperms. The four species of *Synchytrium* are monophyletic in the rDNA phylogeny (FIG. 2). The distinguishing feature of *Synchytrium* is colonial asexual reproduction by a sorus, a cluster of sporangia. The sorus of *Synchytrium* is derived by the internal cleavage of the common membrane of a single thallus into multiple sporangia. *Synchytrium* is included among Chytridiales, and the zoospore ultrastructure of two of the species generally conforms to the chytridialean type (Lange and Olson 1978, Montecillo et al 1980). The *S. endobioticum* (Lange and Olson 1978) zoospore contains an electron opaque plug at the base of the flagellum but lacks a fenestrated cisterna; in contrast the *S. macrosporum* zoospore has a fenestrated cisterna but lacks the electron opaque plug (Montecillo et al 1980). Both species lack aggregated ribosomes observed in most other Chytridiales. *Synchytrium* spp. host ranges vary from broad (Karling 1964, *S. macrosporum* can be inoculated onto 165 different plant families) to narrow (*S. decipiens* is restricted to the genus *Amphicarpaea*).

Clade 2F. Chytridiales.—C. angularise. *Chytriomyces angularis* is a monocentric, epibiotic, operculate fungus distinguished by a gibbous zoosporangium with thread-like rhizoids (FIG. 1f). Secondary rhizoids usually form several micrometers from the sporangial base and branch perpendicularly from the initial rhizoidal axis (FIG. 1f). In the literature fungi with this description were considered possibly to be an alternate form of *C. poculatus*. These fungi are recovered on pollen and snakeskin baits from soils or boggy areas and seem to be more abundant in acidic environments. This clade also includes *Chytridium polysiphoniae*, a parasite of the marine brown alga *Pylaiella littoralis*. Although *C. angularis* was classified a member of *Chytriomyces* based on its morphology and development, its zoospore (Longcore 1992) lacks features found in the Chytridiaceae (Letcher et al 2005); instead it is characterized by a unique connection of the kinetosome and nfc (Longcore 1992).

Clade 2G. Chytridiales.—Rhizophydium. This clade is composed primarily of members of the morphogenus *Rhizophydium*, which is characterized by a monocentric, epibiotic, inoperculate sporangium, an endobiotic rhizoidal axis that branches (FIG. 1g) and an epibiotic resting spore. Molecular studies with 28S

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the kinetosome, nonflagellated centriole and associated structures. ''N'' indicates nucleus; ''M'' indicates mitochondrion. Ultrastructures are indicated for all clades except 2H (*B. helicus*) for which information is not available.

rDNA indicate that the genus *Rhizophydium* is more variable than previously understood and actually represents multiple genera (Letcher and Powell 2005b). The genus *Kappamyces* was circumscribed on the bases of molecular monophyly and zoospore ultrastructure (Letcher and Powell 2005b). Species showing none of the usual *Rhizophydium* generic characters also are in this clade (FIG. 2), including endobiotic species (*Batrachochytrium dendrobatidis* and *Entophlyctis helioformis*), species with multiple rhizoidal axes (JEL142), species with swollen basal rhizoids (e.g. *Rhizophydium brooksianum*) and operculate species (e.g. JEL223).

Although thallus morphology among most members of the *Rhizophydium* clade is relatively conserved, zoospore ultrastructure is divergent. A key feature of the zoospore is the absence of an electron opaque plug in the base of the flagellum, which distinguishes it from most members of the Chytridiales (Letcher et al 2006). Other zoospore ultrastructural features that may be present include a kinetosome-associated structure (KAS) as a spur or shield (Letcher et al 2004b), a microtubular root in which four to five microtubules are stacked and a vesiculated, cup-shaped, invaginated region of the endoplasmic reticulum that surrounds the ribosomal aggregation, adjacent to the proximal end of the kinetosome. Character states of zoospore ultrastructural features are correlated with well supported clades of isolates within the *Rhizophydium* clade. The diversity of the *Rhizophydium* clade, as revealed by 18S, 28S, and ITS1–5.8S–ITS2 rDNA molecular analyses and zoospore ultrastructural analyses (Letcher et al 2004b), support naming the clade a new order Rhizophydiales, which contains many families and genera (Letcher et al 2006).

Clade 2H. Chytridiales.—Blyttiomyces helicus. This is a distinctive species that has a sporangium traversed by spiral thickenings. It grows saprotrophically on pollen (FIG. 1h) but has not been isolated into pure culture (J.E. Longcore unpublished). The rDNA sequencing for the species was from an enriched unifungal culture on pollen. The placement of *B. helicus* in our analysis is uncertain, but additional data suggest a grouping with *Catenomyces persicinus* (see *Clade 2J. Spizellomycetales.*—Rhizophlyctis *clade*, below, T.Y. James unpublished).

Clade 2I. Spizellomycetales.—Spizellomyces. This is a well supported lineage that appears to have diverged from among the lineages of Chytridiales. The clade contains the majority of the genera in the Spizellomycetales: *Gaertneriomyces, Kochiomyces, Powellomyces, Spizellomyces* and *Triparticalcar.* Most members of this clade are saprophytic in soil. All these genera have

counterparts in the Chytridiales with similar morphology and development (e.g. *Spizellomyces* has *Phlyctochytrium* morphology and development [FIG. 1i] but each possesses the distinctive Spizellomycetalean zoospore). At the ultrastructural level the nucleus is spatially or structurally associated with the kinetosome, the nfc lies at an angle to the kinetosome, organelles of the MLC are loosely associated and ribosomes are not aggregated around the nucleus (Barr 1980). Under a light microscope spizellomycetalean zoospores are recognizable because they can be amoeboid while swimming, are irregularly shaped and the flagellar insertion may move to a lateral position. The rDNA phylogeny (FIG. 2) suggests two groupings within this clade, one in which the nucleus possesses a heel that extends to the kinetosome (*Powellomyces* and *Spizellomyces*) and another in which this character is absent (*Gaertner-Gaertneriomyces* and *Triparticalcar*).

Clade 2J. Spizellomycetales.—Rhizophlyctis *clade.* This group is a heterogenous assemblage of five strains that is not well supported as a clade but possesses some morphological similarities. *Rhizophlyctis* is a diverse and polyphyletic group based on zoospore ultrastructure (Barr and Désaulniers 1986) and 18S rDNA phylogeny (James et al 2000). In *Rhizophlyctis rosea* the kinetosome is connected to the nucleus with a striated rhizoplast (Barr and Désaulniers 1986). The *R. rosea* zoospore shares similarities with other members of the Spizellomycetales, and Barr (1980) placed *Rhizophlyctis* with *Entophlyctis, Olpidium* and *Rozella* in Spizellomycetales based on the shared characters of association of the nucleus with the basal body and dispersed ribosomes. The *Rhizophlyctis* clade groups with the *Spizellomyces* clade (2I), albeit with no statistical support. The genera *Rozella* and *Olpidium*, which also have a striated rhizoplast associated with their kinetosomes, are unrelated to the *Rhizophlyctis* clade, indicating that this feature is homoplasious. *Catenomyces persicinus*, currently assigned to the Blastocladiales, is in this clade (FIG. 1j). It resembles *R. rosea* and also degrades cellulose; however, rather than being a soil organism like *R. rosea*, we have found it from aquatic habitats.

Clade 2K. Chytridiales.—Chytriomyces. It is composed of members of 13 studied genera (*Asterophlyctis, Asterophlyctis, Chytridium, Chytriomyces, Entophlyctis, Obelidium, Phlyctochytrium, Phlyctorhiza, Physocladia, Podochytrium, Polyphlyctis, Rhizidium, Rhizoclosmatium* and *Siphonaria*). These genera exhibit diverse thallus forms that include inoperculate or operculate sporangia (FIG. 1k), often apophysate rhizoidal axes, variation in sporangial ornamentation and monocentric or polycentric thalli. Because of this morphological diversity many species are identifiable with light

microscopy. Species identified as being in the genus *Chytriomyces* occur in several separate, well supported clades indicating that *Chytriomyces* as currently defined is polyphyletic (FIG. 2).

Although thallus morphology is divergent zoospore ultrastructure is conserved among the members of this clade. Most isolates from the *Chytriomyces* clade possess a Group I-type zoospore (Barr 1980) and form a clade that has been delineated as the Chytridiaceae (Letcher et al 2005). Features of that zoospore include a fenestrated cisterna, a lateral microtubular root composed of a bundle of approximately seven microtubules in a cord-like arrangement, and a pair of three stacked, flat, electron opaque plates adjacent to the kinetosome, a paracrystalline inclusion in the peripheral cytoplasm and an electron opaque plug in the base of the flagellum. *Phlyctochytrium planicorne* (Letcher and Powell 2005a) and *Polyphlyctis unispina* (Letcher et al 2005) have a Group II-type zoospore (Barr 1980), distinguishable from the zoospore of the Chytridiaceae primarily by the structure of the electron opaque plates adjacent to the kinetosome. Isolates with a Group II-type zoospore probably represent a separate family in the Chytridiales.

Clade 3. Blastocladiomycota.—Traditionally considered among phylum Chytridiomycota, the Blastocladiales diverges from the core chytrid clade and is sister of a clade including Zygomycota, the chytrid genus *Olpidium*, Glomeromycota and Dikarya (FIG. 2). They are saprotrophs as well as parasites of fungi, algae, plants and invertebrates, and may be facultatively anerobic. Two major subclades are resolved in molecular phylogenetic analyses (FIG. 2), one composed of the plant parasite *Physoderma* and the other containing the remaining blastocladialean genera. The morphology of thalli of Blastocladiales parallels forms found among the core chytrid clade, ranging from monocentric to polycentric and mycelial. However *Coelomomyces* produces a tubular unwalled thallus in its host, reminiscent of hyphal bodies in the Zygomycete group Entomophthorales. The zoospore is functionally similar to those found among core chytrids with a single posteriorly directed flagellum, stored lipid and glycogen reserves, a characteristic assemblage of lipids, microbodies, membrane cisterna called the side-body complex (=microbody-lipid globule complex) and a membrane-bounded ribosomal cap covering the anterior surface of a cone-shaped nucleus.

Major evolutionary changes have accompanied the divergence of the Blastocladiales from the core chytrids. For example the Blastocladiales have a life cycle with sporic meiosis whereas most core chytrids have zygotic meiosis, where known. The Blastocla-
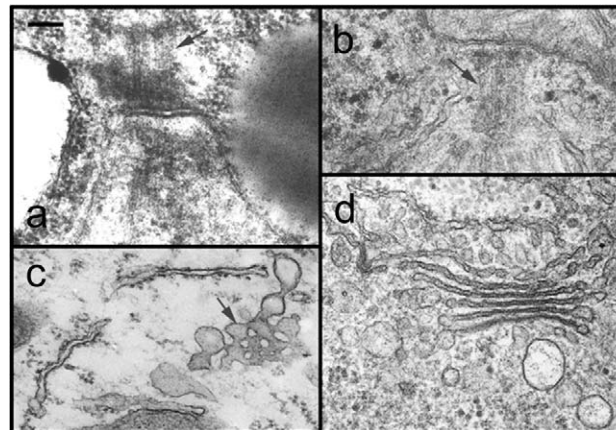

FIG. 3. Comparison of ultrastructural differences between the Blastocladiales and core chytrids. a. Longitudinal section through the closed mitotic pole of *Catenaria allomycis* with centriole (arrowed) separated from the internuclear spindle (Blastocladiales). b. Open mitotic pole of *Powellomyces variabilis* (*Spizellomyces* clade) with the centriole (arrowed) proximate to the mitotic spindle. c. Unstacked, single cisterna of a Golgi equivalent (arrowed) in *Allomyces arbusculus* (Blastocladiales) near rough endoplasmic reticulum. d. A Golgi apparatus with five stacked cisternae in *Powellomyces variabilis* (*Spizellomyces* clade). Notice smooth endoplasmic reticulum and small transport vesicles at the proximal (upper) side of the stacks and larger secretory vesicles at the distal (lower) side of the stack. Methods for sample preparation and microscopy are as previously published (Powell 1975). Bars: a, b = 0.25 μm; c, d = 0.125 μm.

diales exhibits several ultrastructural features more characteristic of higher filamentous fungi than of the core chytrids, including closed nuclear poles during mitosis (FIG. 3a) rather than open (fenestrate) poles (FIG. 3b) and Golgi equivalents (Bracker 1967, FIG. 3c) rather than stacked Golgi cisternae (FIG. 3d). A Golgi apparatus with stacked cisternae has been reported in *Physoderma* (Lange and Olson 1980), suggesting this subclade might have diverged before the loss of Golgi cisternal stacking in the Blastocladiales. These distinctive ultrastructural characteristics as well as the phylogenetic placement of the group warrant their elevation to a new phylum, the Blastocladiomycota.

Blastocladiomycota T. James, *phylum nov.*

Synonyms: Allomycota Cavalier-Smith, BioSystems 14:465, 1981 (not valid, no Latin diagnosis)

Archemycota Cavalier-Smith, Biol. Rev. 73:246, 1998 pro parta

Zoosporum cum flagellum unum, corpus-latus complexe, nucleus pileus ex ribosomata membrane confinium, nucleus cum conus forma terminari propinquus kinetosoma, microtubuli ex propinqui-

tate kinetosomatis per zoosporum radiant circum nucleus, flagellum obturamentum nullum. Generare nonsexus cum zoosporae, generare sexus ad conjiungare planogametum, orbis vita cum meiosis sporae.

Typus: Blastocladia Reinsch 1878

Zoospore with a single flagellum, side-body complex, nuclear cap of membrane-bounded ribosomes, cone-shaped nucleus that terminates near the kinetosome, microtubules radiate anteriorly from the proximal end of the kinetosome around the nucleus, zoospore flagellum lacks electron-opaque plug in transition zone. Asexual reproduction with zoospores, sexual reproduction through fusion of planogametes, life cycle with sporic meiosis.

Type: *Blastocladia* Reinsch 1878

Blastocladiomycetes cl. nov. T. James. Description as for Blastocladiomycota.

Reinsch described *Blastocladia* in 1878, and Petersen (1910) erected the family Blastocladiaceae to accommodate this single genus, classifying it in the class Oomycetes. Kanouse (1927) first used the order name, Blastocladiales (which she credited to Petersen), and included in it the single family with *Allomyces* and *Gonapodya* (now removed). Fitzpatrick (1930) placed the Blastocladiales in class Phycomycetes, a classification Sparrow (1960) followed. Alexopoulos (1966) separated fungi into eight classes, one of which was class Chytridiomycetes, including Blastocladiales. This placement for the Blastocladiales was retained as Chytridiomycetes was elevated to the phylum Chytridiomycota (not validly published, no Latin diagnosis; p 15 in von Arx 1967, Margulis et al 1989). Chytridiomycota will be validated when the phylogeny is more clearly resolved.

Although Tehler (1988) used Blastocladiomycota as a division (phylum) name, the taxon was not validly published because he did not provide or refer to a Latin description or diagnosis or explain the basis of or rationale for this name (Article 36.1, Greuter et al 2000). Consequently it is nomen nudum and furthermore the group that Tehler (1988) circumscribed with this name is not monophyletic. Thus we have used Blastocladiomycota as a phylum for a monophyletic clade (Clade 3), validated the phylum name with a Latin description and have designated a type. A potentially competing phylum name, Allomycota (Cavalier-Smith 1981), is invalid because Cavalier-Smith did not include a Latin diagnosis. We may select the phylum name Blastocladiomycota however because the principle of priority is not mandatory above the rank of family (Article 11.1, 11.9). Cavalier-Smith (1998) eventually validated class Allomycetes with Latin; however this validation is moot to our

selection because a name does not have priority outside its own rank (Article 11.2). As we have described the Blastocladiomycota, it is a natural group separate from the more heterogeneous phylum Archemycota (Cavalier-Smith 1998).

Clade 4. Olpidium brassicae.—*Olpidium brassicae* unexpectedly grouped among the polyphyletic Zygomycota and with the nonzoosporic fungi. *O. brassicae* currently is assigned to the Spizellomycetales (Barr 1980), although this placement was considered tenuous (Barr 2001). Few ecological and morphological features unite *O. brassicae* and Zygomycota; *O. brassicae* is a root pathogen of cucurbits, whereas most members of the Zygomycota II lineage (FIG. 2) are associated with animals. The zoospore of *O. brassicae* contains a unique combination of features, coneshaped striated rhizoplast, gamma-like particles and rough endoplasmic reticulum (Lange and Olson 1976). These ultrastructural features as well as holocarpic, endobiotic sporangia had suggested a relationship between *Olpidium* and *Rozella* (Held 1975), but *Olpidium* differs from *Rozella* in that sporangia of the former develop a cell wall inside the host cytoplasm while sporangia of *Rozella* do not and instead use the host's cell wall (Held 1981). Further data will be needed to determine the exact phylogenetic position of *O. brassicae* and whether it shares a common ancestor with the majority of terrestrial fungi. We predict that *Caulochytrium protostelioides* with a similar zoosporic ultrastructure and aerially produced sporangia (Powell 1981) also will be a member of this currently monotypic clade.

Toward a phylogenetic classification.—The paraphyly of the Chytridiomycota requires that its current circumscription be redefined to produce a phylogenetic classification system. Placing all flagellated true Fungi into one phylum does not reflect shared ancestry of these fungi because the flagellum was a character possessed by the common ancestor of all Fungi (i.e. it is a pleisiomorphic character. Furthermore the absence of a flagellated state (as in *Hyaloraphidium curvatum*) should not prevent the inclusion of nonzoosporic fungi among natural groups of Chytridiomycetes. The phylogeny (FIG. 2) suggests the existence of at least four major lineages of chytrids. The distinction of Blastocladiomycota from other chytrids is clear on the basis of life cycle (Sparrow 1960), ultrastructural characters (FIG. 3) and multiple molecular phylogenetic studies using rDNA (James et al 2000, this study), RPB1 (Tanabe et al 2004), RPB2 (T.Y. James unpublished) and whole mitochondrial genome sequencing (Seif et al 2005). The exact placement of the holocarpic parasites *Rozella* and *Olpidium*, formerly included in the

Spizellomycetales, is an open question because of limited sampling of taxa, a paucity of molecular data and phylogenetic uncertainty (in the case of *O. brassicae*).

Molecular studies have revealed that many currently defined genera in Chytridiales are polyphyletic (James et al 2000, Letcher and Powell 2005b, Letcher et al 2005). Our results show that neither developmental characters, such as polycentric growth, or sporangial characters, such as presence of an operculum, will be useful in defining families or even genera. These phylogenies demonstrate a repeated evolution of polycentric growth in nearly every well sampled clade, suggesting the evolution of this form of indeterminant growth does not require a complex genetic change. Some of the largest genera (e.g. *Rhizophydium, Entophlyctis* and *Chytriomyces*) are based on few distinctive characters and are dramatically in need of revision. Other genera are highly distinctive (e.g. *Loborhiza, Obelidium*) but are monotypic. Current efforts are focused on combining morphological, ultrastructural and molecular data to use a holistic approach for redefining genera of Chytridiales (e.g. Letcher and Powell 2005b).

Application of zoospore ultrastructural characters.—A resolved phylogeny of the chytrid fungi now permits the mapping of ultrastructural character states onto the gene tree to further our understanding of character evolution. For example the electron-dense plug in the base of the flagellum has been considered phylogenetically informative (Barr 2001). This structural character state is present in all members of the *Chytriomyces* (2K, Letcher et al 2005), *Cladochytrium* (2D) and *Polychytrium* (2C) clades examined thus far. It is absent in the *Rozella* (1), Neocallimastigales (2A), Blastocladiomycota (3) and *Olpidium* (4) clades (Barr 2001). When the presence or absence of this character is mapped onto the rDNA phylogeny, it appears that this character was present in the ancestor of the Chytridiales and Monoblepharidales (clades 2B–K) but has been lost repeatedly in independent lineages (FIG. 2). For example in the *Synchytrium* clade (2E) this structure may be either present (*S. endobioticum*, Lange and Olsen 1978) or absent (*S. macrosporum*, Montecillo et al 1980); in the Monoblepharidales (2B) it is either present (i.e. *Monoblepharis polymorpha*, Mollicone and Longcore 1994) or lost (*Gonapodya polymorpha*, Mollicone and Longcore 1999); it has been lost in the Spizellomycetales (2I and 2J, Barr 1980) and *Rhizophydium* clades (2G; Letcher et al 2004b, 2006). Thus an apparently identical character state, the absence of the flagellar plug, is a convergent character state if applied to

organisms from different lineages but it may be a valuable character for taxonomic purposes within a lineage. The heuristic exercise of mapping morphological characters on gene trees suggests that some character states have evolved repeatedly in distinct lineages, thus creating an opportunity for previous assumptions regarding character homology to be reassessed in a phylogenetic context.

Summary of current phylogenetic knowledge and remaining questions.—We have identified four major lineages of chytrid fungi, the largest of which can be subdivided into 11 additional clades. By increasing the sampling of both rRNA characters and species we have greatly expanded our knowledge of phylogenetic diversity and relationships among the groups of chytrids. Our results confirm previous phylogenetic studies and ultrastructural information that suggest the Blastocladiales is distinct from the remaining chytrids and possibly more closely related to the nonzoosporic fungi than the core chytrids. Thus we have elevated the order Blastocladiales to the level of phylum (Blastocladiomycota). Based on the present research, as well as the results of the Assembling the Fungal Tree of Life project (http://ocid.nacse.org/research/aftol/) and complete sequencing of fungal mitochondrial genomes (Seif et al 2005), the phylogeny of the core chytrids is beginning to be resolved with the Neocallimastigales as the earliest diverging lineage and the Monoblepharidales as sister taxon of a clade containing Chytridiales+Spizellomycetales. Phylogenetic investigations in the past three years suggest that much of the diversity in the chytrids is found in species that are not readily cultured (e.g. *Olpidium, Rozella, Synchytrium*). Remaining questions in the chytrid phylogeny are the exact placement of *Olpidium* and whether microsporidia are a part of the basal grade of chytrids. Further sampling of DNA sequences from species that are obligate biotrophic parasites with unique zoospore ultrastructure (e.g. *Caulochytrium* [Powell 1981], *Thalassochytrium* [Nyvall et al 1999] and *Zygorhizidium* [Beakes et al 1988]) might yet uncover additional clades. Furthermore, given that a number of chytrid species are facultative or obligate anaerobes, sampling of these environments also might uncover additional phylogenetic diversity.

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