Molecular phylogenetics of the Chytridiomycota supports the utility of ultrastructural data in chytrid systematics

Timothy Y. James, David Porter, Celeste A. Leander, Rytas Vilgalys, and Joyce E. Longcore

Abstract: The chytrids (Chytridiomycota) are morphologically simple aquatic fungi that are unified by their possession of zoospores that typically have a single, posteriorly directed flagellum. This study addresses the systematics of the chytrids by generating a phylogeny of ribosomal DNA sequences coding for the small subunit gene of 54 chytrids, with emphasis on sampling the largest order, the Chytridiales. Selected chytrid sequences were also compared with sequences from Zygomycota, Ascomycota, and Basidiomycota to derive an overall fungal phylogeny. These analyses show that the Chytridiomycota is probably not a monophyletic group; the Blastocladiales cluster with the Zygomycota. Analyses did not resolve relationships among chytrid orders, or among clades within the Chytridiales, which suggests that the divergence times of these groups may be ancient. Four clades were well supported within the Chytridiales, and each of these clades was coincident with a group previously identified by possession of a common subtype of zoospore ultrastructure. In contrast, the analyses revealed homoplasy in several developmental and zoosporangial characters.

Key words: zoospore ultrastructure, Chytridiales, molecular phylogeny, Chytridiomycota, operculum.

Résumé : Les chytrides (Chytridiomycota) sont des champignons aquatiques morphologiquement simples qui se caractérisent par la présence de zoospores typiquement munies d'un unique flagelle dirigé vers l'arrière. Cette étude porte sur la systématique des chytrides en présentant une phylogénie basée sur les séquences de l'ADN ribosomale du gène codant pour la petite sous-unité, chez 54 chytrides, en mettant l'accent sur un échantillonage de l'ordre le plus important, les Chytridiales. Les auteurs ont également comparé des séquences sélectionnées de chytridiales avec des séquences provenant de Zygomycota, d'Ascomycota, et de Basidiomycota afin d'obtenir une phylogénie générale. Ces analyses montrent que le Chytridiomycota n'est probablement pas un groupe monophylétique; les Blastocladiales se regroupent avec le Zygomycota. L'analyse ne définit pas les relations entre les ordres de chytrides, ou entre les clades parmi les Chytridiales, ce qui suggère que les moments de divergences entre ces groupes pourraient être anciens. Quatre clades sont bien définis au sein des Chytridiales, et chacun de ces clades coïncide avec un groupe précédemment identifié par la possession d'un sous-type commun d'ultrastructures zoosporales. A l'opposé, les analyses révèlent la présence d'homoplasie chez plusieurs caractères au niveau du développement et des zoosporanges.

Mots clés : ultrastructure des zoospores, Chytridiales, phylogénie moléculaire, Chytridiomycota, opercule.

[Traduit par la Rédaction]

Introduction

The phylum Chytridiomycota is composed of five orders (Barr 1990; Alexopoulos et al. 1996) of fungi that are characterized by the production of motile reproductive cells that typically have a single, posteriorly directed flagellum. The chytrids, herein referring to the phylum Chytridiomycota, are among organisms sometimes known as "lower fungi" but have also been classified outside the fungi with various groups of protists (Barr 1990; Powell 1993). Recent molecular phylogenetic studies have confirmed their placement within the fungal kingdom (Förster et al. 1990; Bowman et al. 1992). Because the unwalled, flagellated zoospores of chytrids require water for dispersal, all members of this group are considered aquatic. Soils, when wet, also provide an aquatic habitat, and representatives of the Chytridiomycota are ubiquitous in soils as well as fresh water. Almost all chytrids are microscopic, and few species are of obvious economic importance (Powell 1993), which may be the reason they have received little attention by researchers in recent years. More attention has been focused on the Chytridiomycota currently, however, because *Batrachochytrium dendrobatidis* (Longcore et al. 1999), which lives in the keratinized epidermal cells of amphibians, is pathogenic and

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may be one of the causes of amphibian declines (Berger et al. 1998).

Among different chytrid species, the form of the thallus ranges from simple saccate to mycelial, but the thalli of most species possess few morphological characters with which to build a phylogenetically informative taxonomy. Similar thallus forms are common in different genera, orders, and even in other phyla. Consequently, it was not unexpected that many of the characters upon which the classical taxonomy (e.g., Sparrow 1960; Karling 1977) was built have been shown to be phylogenetically uninformative or misleading. During the 1970's and 1980's reports on the ultrastructure of chytrid zoospores were published (e.g., Barr 1978, 1981; Powell 1978, 1981; Lange and Olson 1978; Lucarotti 1981; Beakes et al. 1988). Out of these studies grew a consensus that certain zoosporic, ultrastructural characters were more conserved and thus phylogenetically more informative than morphological, thallic characters.

Orders of chytrids are currently recognized on the basis of ultrastructural characters of the zoospore (Barr 1980, 1990). For example, the analysis of zoospore characters resulted in the segregation of a new order, the Spizellomycetales, from the Chytridiales (Barr 1980) and later in the elevation of a family of rumen-inhabiting chytrids in the Spizellomycetales to ordinal status (Neocallimastigales; Li et al. 1993). Most chytrid orders are small, and the current taxonomy within them presents few problems. Within the Chytridiales, however, the lack of a phylogenetically based system of genera and families is a critical impediment to a new monograph, needed to update that of Sparrow (1960). This order contains nearly 80 genera and over 500 species. Classical taxonomic systems (e.g., Sparrow 1943, 1960, 1973; Karling 1977) emphasized the presence or absence of an operculum, the lid-like portion of the sporangium covering the site of zoospore release, and the type of development of the thallus. A comparison of ultrastructural characters of zoospores led to the conclusion that many families and genera are polyphyletic (e.g., Beakes et al. 1988; Longcore 1992*b*, 1995).

The current classification of the Chytridiales relies heavily on sporangial characters that have been shown to be phenotypically variable within a species (Booth 1971; Roane and Patterson 1974; Miller 1976; Powell and Koch 1977*a*, 1977*b*). Such plastic characters are likely to be phylogenetically labile, and could also affect classification above the species level. Unlike characters of the mature sporangium, patterns of development seem to be less variable within species. These patterns are descriptions of development from zoospore to mature sporangium, and Whiffen (1944), Roane and Patterson (1974), and Barr (1978) have proposed that such developmental characters could be used as the basis for a revised classification. When the Spizellomycetales were removed from the Chytridiales, Barr (1980) presented guidelines for a reorganization of the remaining chytridialean taxa into families based primarily on sporangial development and into subfamilial groups based on zoospore ultrastructural data. Since then, however, no further work has been done towards a revision of the taxonomy of the Chytridiales, although Longcore (1996) furnished a bibliography of taxonomic additions and changes since Sparrow's 1960 monograph.

Molecular approaches have revolutionized how the systematic relationships of many groups are inferred by generating data that are essentially independent of the morphological characters upon which classification typically rests. Molecular phylogenetic methods have never been employed to specifically elucidate the systematic relationships among orders of chytrids or within the Chytridiales; however, molecular phylogenies of the fungal kingdom agree in the basal placement of the chytrids within the fungi (Bruns et al. 1992; Bowman et al. 1992; Paquin et al. 1997). Sequencing of entire mitochondrial genomes has shown that the phylum Chytridiomycota is genetically diverse (Paquin et al. 1997) and likely to be polyphyletic; a hypothesis also suggested by other molecular phylogenetic studies (Bruns et al. 1992; Nagahama et al. 1995; Jensen et al. 1998).

We herein report new sequences for the small subunit ribsomal DNA gene (ssu rDNA) of 54 chytrids. Our principal objective was to determine the phylogenetic relationships among the orders of the Chytridiomycota as well as within orders, with emphasis on the Chytridiales. In addition we readdressed the position of the chytrids within a phylogeny of the fungal kingdom with published gene sequences. A molecular phylogeny allows the reassessment of historically important characters that may be useful in constructing a revised classification for the Chytridiales. These characters include type of zoospore discharge (Sparrow 1943, 1960, 1973; Dogma 1973), type of thallus development (Whiffen 1944, Roane and Patterson 1974; Karling 1977), as well as ultrastructural features of the zoospore (Barr 1980, 1990; Powell 1978; Powell and Roychoudhury 1992).

Materials and methods

Taxonomic sampling and sample preparation

The taxa used in these phylogenetic analyses are listed in Table 1. Sequences that are not newly reported in this study are from GENBANK or the DNA Database of Japan. Most species for which new data were generated are members of the Chytridiales (n = 38). All species represent axenic cultures from the chytridiomycete collection at the University of Maine or the culture collections at the University of California at Berkeley, the University of Georgia, and Duke University. Material for DNA isolation was obtained from fungal cultures by removing thalli from the surface of nutrient agar media (PmTG nutrient agar; Barr 1986) or from harvesting thalli from liquid media (PmTG broth) with suction filtration. Thalli were dehydrated with a Speed-vac concentrator (Savant Instr. Inc., Farmingdale, N.Y.), and DNA was isolated following standard protocols employing CTAB buffer (Zolan and Pukkila 1986).

DNA amplification and sequencing techniques

Polymerase chain reaction (PCR) amplifications followed the method of Vilgalys and Hester (1990) with the primer combinations NS1/NS4 and NS3/ITS2 (White et al. 1990). The primers NS1 and NS4 plus four additional primers were used for sequencing: NS2 (White et al. 1990), BMB-BR (Lane et al. 1985), SR6 (5′TGTTACGACTTTTACTT-3′), and SR1.5 (5′-AAGGCAGCAGGCGCGCAAATTAC-3′). Amplification products were purified with ULTRAfree-MC centrifugal columns (Millipore Corp., Bedford, Mass.). Sequences were produced with the use of automated sequencers (models ABI373 or ABI377, Perkin-Elmer Corp., Norwalk, Conn.) and dye terminator sequencing chemistries following the manufacturer's instructions. Sequence chromatograms were compiled with Sequencher software version 2.0 (Gene Codes Corp., Ann Arbor, Mich.).

Table 1. List of species used for phylogenetic analyses in this paper.

Species	Strain identification	Order	Sequence accession No.
Allomyces macrogynus (Emerson) Emerson & Wilson	GENBANK	Blastocladiales	U23936
Blastocladiella emersonii Cantino and Hyatt	GENBANK	Blastocladiales	X54264
Catenaria anguillulae Sorokin	JEL 194	Blastocladiales	AF164338-9
Gonapodya sp.	JEL 183	Monoblepharidales	AF164329-30
Harpochytrium sp.*	JEL 94	Monoblepharidales	AF164331-2
Monoblepharella elongata Springer	BK CR91	Monoblepharidales	AF164335
Monoblepharella sp.	BK 74-9	Monoblepharidales	AF164336
Monoblepharella mexicana Shanor	BK 78-1	Monoblepharidales	AF16433
Monoblepharis hypogyna Perrott	DDBJ	Monoblepharidales	ABO16019
Monoblepharis insignis Thaxter	BK 59-7	Monoblepharidales	AF164333
Gaertneriomyces semiglobiferus (Uebelmesser) D.J.S. Barr	BK 91-10	Spizellomycetales	AF164247-8
Powellomyces hirtus Longcore et al.	UGA-F18	Spizellomycetales	AF164239-40
Powellomyces sp.	JEL 95	Spizellomycetales	AF164245-6
Powellomyces variabilis Powell & Koch ex Longcore et al.	BK 85-1	Spizellomycetales	AF164243-4
Powellomyces variabilis	BK 91-11	Spizellomycetales	AF164241-2
Rhizophlyctis rosea (deBary & Woronin) Fischer	BK 57-5	Spizellomycetales	AF164249-50
Rhizophlyctis rosea	BK 47-07	Spizellomycetales	AF164251-2
Spizellomyces acuminatus (D.J.S. Barr) D.J.S. Barr	GENBANK	Spizellomycetales	M59759
Spizellomyces kniepii Gaertner ex D.J.S. Barr	UGA-F22	Spizellomycetales	AF164237-8
Caecomyces (*Sphaeromonas*) *communis* Gold et al.	GENBANK	Neocallimastigales	M62707
Neocallimastix frontalis (Braune) Vavra & Joyon ex Heath	GENBANK	Neocallimastigales	M62704
Orpinomyces joyonii (Breton et al.) Li et al. formerly *Neocallimastix joyonii* (Breton et al.)	GENBANK	Neocallimastigales	M62705
Piromyces (*Piromonas*) *communis* Gold et al.	GENBANK	Neocallimastigales	M62706
Allochytridium expandens Salkin	BK 69-3	Chytridiales	AF164291-2
Asterophlyctis sarcoptoides H.E. Petersen	JEL 186	Chytridiales	AF164317-8
Batrachochytrium dendrobatidis Longcore et al.	JEL 197	Chytridiales	AF164301-2
Catenochytridium sp.	JEL 145	Chytridiales	AF164289-90
Chytridium confervae (Wille) Minden	BK M62706	Chytridiales	M59758
Chytridium sp.	DU-DC2	Chytridiales	AF164321-2
Chytriomyces angularis Longcore	JEL 45	Chytridiales	AF164253-4
Chytriomyces annulatus Dogma	JEL 114	Chytridiales	AF164303-4
Chytriomyces spinosus Fay	JEL 59	Chytridiales	AF164323-4
Diplochytridium (*Chytridium*) *lagenarium* (Schenk) Karling (not same as in Barr and Hartmann 1976)	JEL 72	Chytridiales	AF164285-6
Cladochytrium replicatum Karling	JEL 38	Chytridiales	AF164297-8
Endochytrium sp.	JEL 49	Chytridiales	AF164293-4
Entophlyctis luteolus Longcore	JEL 129	Chytridiales	AF164325-6
Entophlyctis sp.	DU-DC1	Chytridiales	AF164255-6
Karlingiomyces sp.	JEL 93	Chytridiales	AF164278-80
Lacustromyces hiemalis Longcore	JEL 31	Chytridiales	AF164274-5
Nephrochytrium sp.	JEL 125	Chytridiales	AF164295-6
Nowakowskiella elegans (Nowak.) Schroeter	BK 50-1	Chytridiales	AF164281-2
Nowakowskiella hemisphaerospora Shanor	BK 85-6	Chytridiales	AF164283-4
Obelidium mucronatum Nowakowski	JEL 57	Chytridiales	AF164309-10
Phlyctorhiza endogena Hanson	JEL 80	Chytridiales	AF164313-4
Physocladia obscura Sparrow	JEL 137	Chytridiales	AF164327-8
Podochytrium dentatum Longcore	JEL 30	Chytridiales	AF164307-8
Podochytrium sp.	JEL 161	Chytridiales	AF164305-6
Polychytrium aggregatum Ajello	JEL 190	Chytridiales	AF164276-7
Rhizoclosmatium sp.	JEL 06	Chytridiales	AF164311-2
Rhizophlyctis harderi Uebelmesser	JEL 171	Chytridiales	AF164272-3
Rhizophydium chaetiferum Sparrow	JEL 39	Chytridiales	AF164263
Rhizophydium sp.	JEL 151	Chytridiales	AF164270-1
Rhizophydium sp.	UGA-F16	Chytridiales	AF164264-5
Rhizophydium sp.	JEL 136	Chytridiales	AF164268-9

Table 1 (*concluded*).

Species	Strain identification	Order	Sequence accession No.
Rhizophydium sp.	UGA-F15	Chytridiales	AF164319-20
Rhizophydium sp.	JEL 138	Chytridiales	AF164266-7
Rhizophydium sphaerotheca sensu Booth (not same as neotype in Barr 1969 and Barr and Hadland-Hartmann 1978*b*)	JEL 08	Chytridiales	AF164259-60
Septochytrium variabile Berdan	JEL 191	Chytridiales	AF164287-8
122 *Entophlyctis* development	JEL 122	Chytridiales	AF164257-8
142 multiple axes	JEL 142	Chytridiales	AF164299-300
155 cellophane chytrid	JEL 155	Chytridiales	AF164315-6
207 multiple rhizoids	JEL 207	Chytridiales	AF164261-2
Coemansia braziliensis Thaxter	GENBANK	Kickxellales	AF007532
Kickxella alabastrina Coemans	GENBANK	Kickxellales	AF007537
Spirodactylon aureum R.K. Benj.	GENBANK	Kickxellales	AF007541
Spiromyces aspiralis Benny & R.K. Benj.	GENBANK	Kickxellales	AF007543
Basidiobolus ranarum Eidam	GENBANK	Entomophthorales	D29946
Conidiobolus coronatus (Constantin) Batko	GENBANK	Entomophthorales	D29947
Entomophthora muscae (Cohn) Fresenius	GENBANK	Entomophthorales	D29948
Macrobiotophthora vermicola (McColloch) B. Tucker	GENBANK	Entomophthorales	AF052400
Strongwellsea castrans Batko & Weiser	GENBANK	Entomophthorales	AF052406
Kuzuhaea moniliformis R. K. Benjamin	DDBJ	Zoopagales	ABO16010
Piptocephalis corymbifer Vuillemin	DDBJ	Zoopagales	ABO16023
Genistelloides hibernus Peterson et al.	GENBANK	Harpellales	AF007536
Smittium culisetae Lichtwardt	GENBANK	Harpellales	D29950
Cunninghamella elegans Lender	Car. Biol. Supp.	Mucorales	AF164340-1
Micromucor ramannianus Möller	GENBANK	Mucorales	X89435
Mucor mucedo (Micheli) Fresenius	GENBANK	Mucorales	X89434
Mucor racemosus Fresenius	GENBANK	Mucorales	X54863
Pilobolus longipes van Tieghem	DU-DC7	Mucorales	AH006442
Syncephalastrum racemosum (Cohn) Schroeter	GENBANK	Mucorales	X89437
Entrophospora columbiana Spain & Schenck	GENBANK	Glomales	Z14006
Geosiphon pyriforme (Kutzing) F. v. Wettstein	GENBANK	Glomales	X86686
Gigaspora gigantea Gerde. & Trappe	GENBANK	Glomales	Z14010
Glomus etunicatus Becker & Gerd.	GENBANK	Glomales	Z14008
Glomus versiforme (Karsten) Berch	GENBANK	Glomales	X86687
Neolecta vitellina Korf & Rogers	GENBANK	Neolectales	Z27393
Neurospora crassa Shear & Dodge	GENBANK	Sordariales	X04971
Ophiostoma ulmi (Buism.) Nannf.	GENBANK	Ophiostomatales	M83261
Penicillium notatum Westling	GENBANK	Eurotiales	M55628
Pleospora rudis Berl.	GENBANK	Pleosporales	U00975
Saccharomyces cerevisiae Hansen	GENBANK	Saccharomycetales	Z75578
Schizosaccharomyces pombe Linder	GENBANK	Schizosaccharomycetales	X58056
Filobasidiella neoformans Kwon-Chung	GENBANK	Tremellales	D12804
Geastrum saccatum (Fr.) E. Fischer	GENBANK	Lycoperdales	AF026620
Rhodosporidium toruloides Banno	GENBANK	Sporidiales	D12806
Stereum hirsutum (Willd. ex Fr.) S. F. Gray	GENBANK	Aphyllophorales	U59095
Ustilago maydis (DC.) Corda	GENBANK	Ustilaginales	X62396
Acanthoecopsis unguiculata Thomsen	GENBANK	Choanoflagellida	L10823
Diaphanoeca grandis Ellis	GENBANK	Choanoflagellida	L10824
Emiliana huxleyi (Lohm.) Hay & Mohler	GENBANK	Haptophyta	M87327
Reticulosphaera socialis Grell	GENBANK	Haptophyta	X90992

Note: Species whose source is not listed as GENBANK or DNA Database of Japan (DDBJ) were generated by the authors during the present study. BK, Berkeley culture collection; UGA, University of Georgia; DU, Duke University; JEL, University of Maine, Longcore culture collection. More than one accession number is given for the taxa sequenced for the present study as these sequences appear as segmented sequences in the databases (see Materials).

*Although Barr (1990) placed *Harpochytrium* and the Harpochytriaceae in the Chytridiales, comparison of the kinetosome associated ultrastructure of zoospores and unpublished analyses of total mitochondrial DNA by Lang (Paquin et al. 1997; http://megasun.bch.umontreal.ca/People/lang/FMGP/phylogeny.html) clearly place this genus and its family in the Monoblepharidales.

Approximately 1600 bp coding for ssu rDNA were obtained for each taxon. Overlapping sequence data near the annealing site of the primer NS4 were not obtained; however, this region of the ssu rDNA is highly conserved in all organisms, and we were able to unambiguously align sequences on either side of this region. Difficulty was encountered in generating complete sequences for two taxa because of the presence of introns in the ssu rDNA of the chytrids *Karlingiomyces* sp. (JEL 93) and *Spizellomyces kniepii* Gaertner ex D.J.S. Barr (UGA F22). Two large introns were also detected in *Phlyctochytrium planicorne* Atkinson (unpublished sequence). Group I introns are found in the ssu rDNA of many fungi and other organisms (Gargas et al. 1995; Hibbett 1996) and may not be phylogenetically informative. Because of the large size of some of these introns (>400 bp), these regions were not sequenced, and thus contiguous sequences were not developed for the aforementioned species.

Data analysis

Three sequence alignments were generated for phylogenetic analyses. All sequences were hand aligned with the GeneDoc software package (Nicholas et al. 1997). The first alignment was for 61 chytrid taxa and a choanoflagellate outgroup. After the introduction of gaps, an alignment of 2064 nucleotides was produced (after subtracting 750 bp of intron sequences). After the exclusion of areas of ambiguous alignment, the remaining 1345 bp were used for data analyses. The second alignment was of 39 taxa in the Chytridiales plus *Monoblepharella elongata* as an outgroup. This alignment was 1804 nucleotides long (442 bp of intron sequence were excluded), and 1361 characters from this second alignment remained after excluding the areas of ambiguity in the alignment. The last alignment was of 69 taxa from the fungi, with a large representation of Chytridiomycota and Zygomycota. This last alignment was 2214 nucleotides long (469 bp of intron sequence were excluded), and 1303 characters from this second alignment remained after excluding the areas of ambiguity in the alignment. Gaps that remained in the alignments were scored as a fifth character state.

All three alignments were analyzed by maximum parsimony. Analyses were performed with PAUP* (Swofford 1998) running on a UNIX Sun Sparc 20 Station. Maximum parsimony was employed with the default PAUP settings, i.e., MULPARS = on and steepest decent not in effect. The most parsimonious trees were found by searching with tree-bisection–reconnection (TBR) branch swapping, and the starting trees were found by random sequence addition. One-hundred heuristic searches were performed, and the shortest trees over all replicates were kept and assumed to be the most parsimonious reconstructions. Support for each branch was estimated using 100 bootstrap replicates. Each replicate was a heuristic search using PAUP* with TBR branch swapping and MAXTREES set to 2000.

Topological constraints were employed on the larger (all fungi) data set to investigate alternative topologies that supported the monophyly of traditional groups such as the Chytridiomycota and Zygomycota. These constraints corresponded with searching for the most parsimonious trees that retained a monophyletic arrangement of a given taxonomic grouping. These trees were typically longer, i.e., less parsimonious, than the trees found without constraints. To test whether these constraints created significantly worse phylogenies, we compared the alternative topologies to the unconstrained trees with the Kishino–Hasegawa test (Kishino and Hasegawa 1989).

For the chytrid data set only, we explored the maximum likelihood (ML) alternative to phylogenetic reconstruction. The nature of the variation observed in the ssu rDNA region (Woese et al. 1983; Van de Peer et al. 1997) suggests that a complex model of sequence evolution that accounts for unequal mutation rates along the molecule may be appropriate for this gene. To determine the appropriate ML model for the chytrid data set, we evaluated the fit of various models to a best estimate of the phylogeny in question with a procedure similar to that described by Cunningham et al. (1998). For this data set, the best estimates of the true phylogeny were the most parsimonious (MP) trees. We chose a single most parsimonious tree from all of the MP trees by calculating the likelihood of each MP tree with a simple model of sequence evolution, the Hasegawa–Kishino–Yano model (HKY model, Hasegawa et al. 1985). The tree maximizing the likelihood under this model (the MLMP tree) was retained and then more complex models of evolution were used to calculate the likelihood of this single tree. The best-fitting model is one in which the addition of more parameters, i.e., degrees of freedom, does not significantly improve the likelihood score of a given topology, evaluated with the log-likelihood ratio test (Goldman 1993; Cunningham et al. 1998).

The best fitting maximum likelihood model of sequence evolution for the chytrid data set was a rather complex one. The estimated sequence composition showed a slight AT bias (about 56%). Nucleotide changes fit into three time-reversible substitution classes: (1) all transversions in one category, (2) A↔G transitions in a class about 3.0 times as fast as the first class, and (3) C↔T transitions about 4.5 times as fast as the first class. The model accounted for among-site rate variation with a gamma-shape parameter of 0.399 approximated by seven discrete classes, and the proportion of invariable sites was estimated to be 0.351. After model estimation, the MLMP tree was then used as the starting tree for TBR searching using maximum likelihood with the parameters of the model fixed to the estimates derived from model fitting. Swapping was performed for 500 h, and the most likely trees were retained. One hundred bootstrap replicates were used to evaluate the confidence for each node. For each replicate, trees were built with random sequence addition, but no swapping was performed.

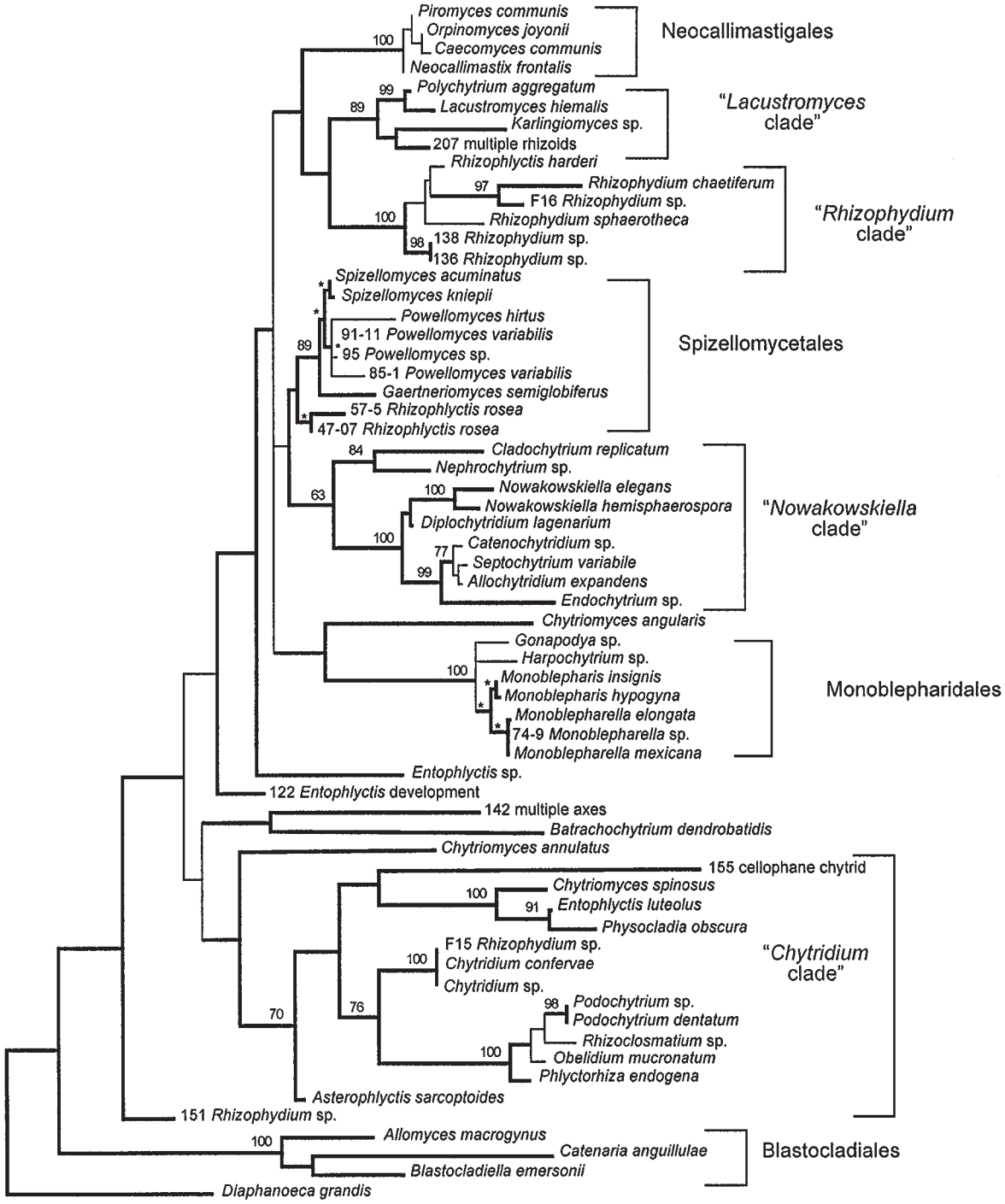
Results

Phylogeny of the Chytridiomycota

The alignment of sequences from 61 chytrids yielded 305 parsimony informative sites. The MLMP tree shown in Fig. 1 is the most likely of the 1080 most parsimonious reconstructions. Overall bootstrap support was 100% for the monophyly of each of the orders Neocallimastigales, Monoblepharidales, and Blastocladiales. Within the Chytridiales and Spizellomycetales, several groupings demonstrated high bootstrap support. The general topology of the tree, however, suggests that the Chytridiales either are not a monophyletic group or that the divergence of the clades within this order is so great that these molecular data do not resolve the relationships. The consistency index (CI) for the most parsimonious trees is 0.4740, suggesting that the molecular characters are subject to high levels of homoplasy.

The phylogenies of the chytridiomycetes found from searching with the maximum likelihood model (described in Materials) were significantly more likely than the most parsimonious trees ($P < 0.05$; Fig. 2). However, these maximum likelihood trees were 14 steps longer than the MLMP tree when evaluated with parsimony. Visual inspection shows that the maximum likelihood tree differs little from the parsimony tree. Differences are seen primarily in the arrangement among the well-supported clades and in the position of clades subtended by short branches. Trees from both analyses are consistent in containing monophyletic clades within the Chytridiales: one of *Chytridium*-like organisms, the "*Chytridium* clade"; a second of *Rhizophydium*-like organ-

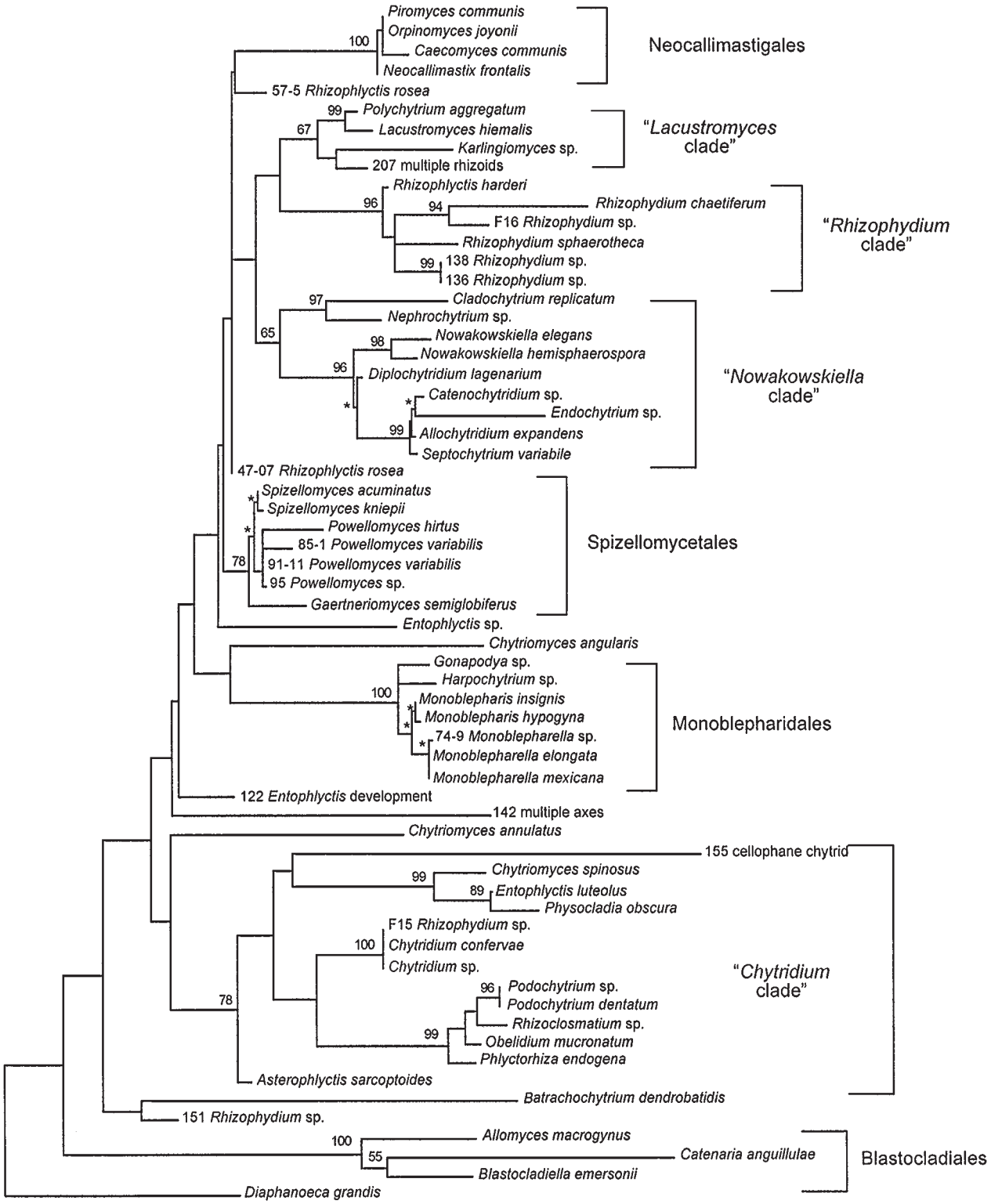
Fig. 1. Evolutionary hypothesis for the Chytridiomycota. Tree shown (MLMP tree) has the highest maximum likelihood (HKY model) of 1080 most parsimonious trees. Bootstrap values over 60% are shown. Asterisks indicate short internal nodes with greater than 60% bootstrap support. Branches in bold are present in the strict consensus of the 1080 trees.



isms, the "*Rhizophydium* clade"; a third "*Nowakowskiella* clade"; and a fourth "*Lacustromyces* clade" (Figs. 1 and 2). Further, MP and ML trees are also consistent in grouping the "*Lacustromyces* clade" with the "*Rhizophydium* clade" into a monophyletic lineage. In the ML tree (Fig. 2), the "*Nowakowskiella* clade" joins with the "*Lacustromyces*" and "*Rhizophydium*" clades to form a single lineage. In both ML and MP analyses, the "*Chytridium* clade" appears basal within a clade containing most of the Chytridiomycota except the Blastocladiales.

The same four chytridialean clades were recovered when the sequences for the Chytridiales were analyzed separately with maximum parsimony (Fig. 3). The Chytridiales tree again demonstrates the uncertainty about the relationships among the four major lineages, because their arrangement in the three trees (Figs. 1–3) are not in agreement. This

Fig. 2. Phylogeny of the Chytridiomycota reconstructed with maximum likelihood methods. Bootstrap values were calculated from 100 replicates without branch swapping; only values over 60% are shown. Asterisks indicate short internal nodes with greater than 60% bootstrap support. Tree shown is one of 15 trees of equal likelihood retained after swapping for over 500 h using the tree in Fig. 1 as the starting tree.



chytridialean tree was generated primarily to investigate the evolution of morphological characters important in chytridialean taxonomy, the implications of which are discussed below.

The Chytridiomycota within a global fungal phylogeny

The alignment of the entire fungal data set produced 490 parsimony informative characters of the 1303 aligned characters retained. The phylogenetic trees from this data set

showed well-supported groups above the ordinal level for nonchytrid lineages (Fig. 4), however, relationships among the chytrid orders were again not well supported by bootstrap analysis. Four major clades were detected within the fungi. First, a monophyletic clade that included all basidomycetes and ascomycetes was well supported. Further, this ascomycete–basidiomycete clade was part of a larger clade that included the endomycorrhizal zygomycetes in the Glomales. The second clade was comprised of the chytrids in the Blastocladiales and the zygomycetes, exclusive of the Glomales, and was basal to the lineages of higher fungi. The chytrids in the Monoblepharidales seem to be in a unique lineage within the fungi and comprised the third clade. The final and most basal clade was comprised entirely of chytrids plus the zygomycete *Basidiobolus ranarum*.

We evaluated whether topologies that supported classical taxonomic groupings created significantly less parsimonious trees by using constrained searches (Table 2). Our most parsimonius trees suggest that the Chytridiomycota is not a monophyletic group, but these trees were not significantly ($P = 0.1689$) more likely than trees in which the chytrids were constrained to be monophyletic. Thus, rejection of the monophyly of the chytrids was not possible with this data set, probably because of the high error in the phylogenetic estimation procedures. Constrained searches were also used to test the monophyly of the Chytridiales sensu Barr (1980). Contrary to the analysis of the data set that contained only representatives of the Chytridiomycota (Figs. 1 and 2), the most parsimonious trees of the fungal kingdom grouped the Chytridiales into a nearly monophyletic lineage (Fig. 4), and the constrained searches yielded trees that were the same length as the unconstrained trees. Therefore, the monophyly of the Chytridiales also could not be rejected.

Zygomycetes were highly polyphyletic in the parsimony analysis (Fig. 4) and constrained searches for trees that grouped all zygomycetes together yielded trees that were significantly longer ($P < 0.05$; Table 2). Although the paraphyletic position of the Glomales to the rest of the Zygomycota is an obvious departure from nonmonophyly of the zygomycetes, forcing this group to be monophyletic with the rest of the zygomycetes (excluding *B. ranarum*) recovers a phylogeny that is not significantly worse than the unconstrained phylogeny. It is primarily the position of *B. ranarum* that causes a monophyletic Zygomycota to be rejected from being a parsimonious explanation for the ssu rDNA data set. Searches that force this taxon to belong within the zygomycete clade result in a suboptimal phylogeny ($P < 0.05$).

Discussion

Relationship of the Chytridiomycota to other fungi

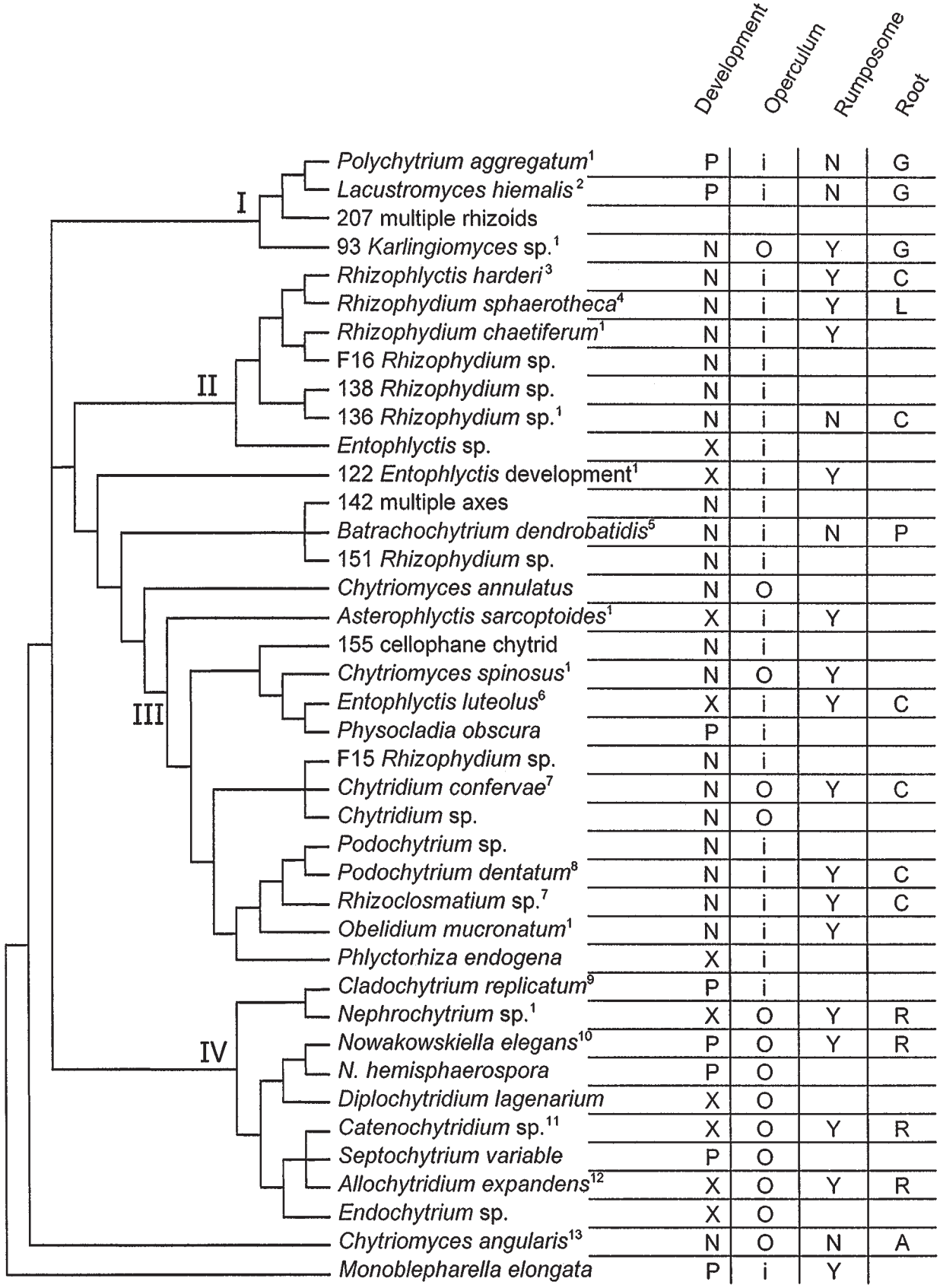
Because of the paraphyletic arrangement of the Blastocladiales and Monoblepharidales to the other chytrids, the Chytridiomycota do not appear monophyletic (Fig. 4). This result agrees with previous molecular phylogenies of the fungi (Bruns et al. 1992; Paquin et al. 1997). Although the placement of the Monoblepharidales within the fungal phylogeny is not supported by bootstrap analysis (Fig. 4), the most parsimonious phylogeny groups the Monoblepharidales closer to the "core chytrid clade" than the Blastocladiales, in agreement with both ultrastructural data (Barr 1981; Mollicone and Longcore 1994) and the phylogenies of Paquin et al. (1997). The monophyly of the chytridomycetes has previously been rejected by Jensen et al. (1998), however very few chytrids were included in their data set. All molecular analyses to date agree that the Blastocladiales are an independently evolving lineage of zoosporic fungi, and each study seems to support the monophyly of a "core chytrid clade" to the exclusion of the Blastocladiales.

Basidiobolus ranarum, a zygomycete in the order Entomophthorales, has attracted attention because of its possible phylogenetic affinity with the chytrid fungi (Nagahama et al. 1995; Jensen et al. 1998). One feature that suggests an alliance between *Basidiobolus* and the chytridiomycetes is the presence of a microtubule containing, nucleus-associated organelle (NAO), known from two *Basidiobolus* species (McKerracher and Heath 1985). *Basidiobolus* is currently the only genus of nonzoospore forming fungi known to have an NAO that contains microtubules. Although the *Basidiobolus* NAO contains a cylinder of 11 to 12 singlet microtubules rather than the 9 triplets found in centrioles of the Chytridiomycota, the presence of microtubules in the structure could suggest a homology with centrioles and a relationship with chytrids.

The unusual relationship between *B. ranarum* and the chytrids has suggested that the potential polyphyly of the zygomycetes with the chytrids should be explored. Analysis of the ssu rDNA sequences rejects the monophyly of the Zygomycota, but this seems to be only because of the association of *B. ranarum* with the "core chytrid clade" (Table 2). The ssu rRNA gene recently has been sequenced for representatives of poorly understood zygomycete groups (O'Donnell et al. 1998; Jensen et al. 1998). Our analysis of the new chytrid data combined with the recently published zygomycete data yielded no conclusive evidence of further zygomycete–chytridiomycete polyphyly. Of note, many zygomycetes are on very long branches (Fig. 4), suggesting an accelerated rate of sequence evolution of the ssu rDNA gene in these fungi. This rate heterogeneity creates an uncertainty as to whether convergent evolution has caused the positioning of *B. ranarum* within the chytridiomycetes in molecular-based trees. Additional evidence against the relationship of *B. ranarum* with the chytrids comes from molecular evidence using beta-tubulin gene phylogenies in which *B. ranarum* groups with other zygomycetes rather than chytrids (Keeling et al. 2000).

Phylogeny of the Chytridiomycota

Despite the lack of resolution among basal branching points within the chytridiomycetes, the molecular phylogenies were able to group chytrid taxa into well-supported clades (Fig. 1). Phylogenetic analyses of the chytridiomycetes strongly support the monophyly of the orders Blastocladiales, Monoblepharidales, and Neocallimastigales, and do not reject the monophyly of the Chytridiales and the Spizellomycetales. In addition, sub-ordinal clades within the Chytridiales were also well supported. Although analysis of ssu rDNA sequences identified super-ordinal clades within the zygomycetes, ascomycetes, and basidiomycetes, relationships among clades at the ordinal level in the chytridiomycetes were unresolved. This lack of resolution could be explained either by a rapid diversification in the past, thus



creating a "star-like" phylogeny, or by large levels of homoplasy in the ssu rDNA of chytridiomycetes because of the group's ancient origin (Berbee and Taylor 1993). Alternatively, the lack of resolution at the base of the "core chytrid clade" could be due to a lack of phylogenetic information in the ssu rDNA for level of phylogenetic branching.

Fig. 3. Strict consensus of six most parsimonious trees of members of the Chytridiales with *Monoblepharella elongata* (Monoblepharidales) as an outgroup. Clades observed in the phylogenies of the Chytridiomycota presented in Figs. 1 and 2 are marked at the node subtending the clade (I, "*Lacustromyces* clade"; II, "*Rhizophydium* clade"; III, "*Chytridium* clade"; IV, "*Nowakowskiella* clade"). Mapped onto the tree are two representative thallus characters: type of development (N, endogenous; X, exogenous; P, polycentric) and operculation (*i*, inoperculate; O, operculate); and two representative ultrastructural characters: rumposome (Y, present, N, absent) and type of microtubule root. More than one root occurs in some species and "root" here refers to the primary microtubule root, which in the Chytridiales, usually arises near triplets 9–2 of the kinetosome; G, irregular group of microtubules arising between two triplets that have projections; L, 1–7 microtubules in a single row, one above the other and separate from each other; P, group of separate microtubules extending parallel with the side of the kinetosome; C, a cord (usually 6–8) of microtubules with no space between microtubules; R, rope of microtubules (10 to more than 20), not in direct contact with each other, but connected by fibers; A, primary microtubule root absent. Information about the thallus is from species descriptions or observation of undescribed species. Information about ultrastructure of some taxa is from published descriptions of isolates identified as the same species as those in the molecular analysis, or as a morphologically similar species in the genus. Information about presence or absence of rumposome of some species is from unpublished photographs. 1, Longcore unpublished; 2, Longcore 1993; 3, Roychoudhury and Powell 1992; 4, Barr and Hadland-Hartmann 1978*b*; *Rhizophydium sphaerotheca* sensu Booth and most other species of *Rhizophydium* studied by Barr and Hadland-Hartmann have a root consisting of several microtubules, one above the other with space between; 5, Longcore et al. 1999; 6, Longcore 1995; 7, Barr and Hartmann 1976; 8, Longcore 1992*a*; 9, ultrastructual characters are not included for *Cladochytrium replicatum* because the morphology of our isolate differs from that used by Lucarotti (1981); 10, Lucarotti 1981; 11, Barr et al. 1987; 12, Barr 1986; 13, Longcore 1992*b*.

The current ordinal classification within the chytridiomycetes relies heavily on zoospore ultrastructure (Barr 1990). Consequently, the confirmation of the monophyly of these orders strongly supports the utility of zoospore characters in classification. Barr (1980, 1990) suggested that the Chytridiales could be classified by grouping taxa that possess the same subtype of zoospore. The four chytridialean clades that are well supported by analysis of the molecular data (Figs. 1 and 2) are coincident with taxa that have been grouped because they possess similar zoosporic ultrastructure. Chytrids with a *Chytridium* or group I (Barr 1980) zoospore subtype are all in the "*Chytridium* clade" in the molecular tree, those with a *Rhizophydium* or group III (Barr 1980) subtype of zoospore are in the "*Rhizophydium* clade," and those with a *Nowakowskiella* subtype of zoospore (Lucarotti 1981; Barr 1986; Barr et al. 1987) are in the "*Nowakowskiella* clade." *Lacustromyces hiemalis* is the only member of the "*Lacustromyces* clade" for which zoospore ultrastructure has been published (Longcore 1993), but our unpublished observations of the zoospores of *Polychytrium aggregatum* and *Karlingiomyces* sp. indicated that they have the same zoospore subtype.

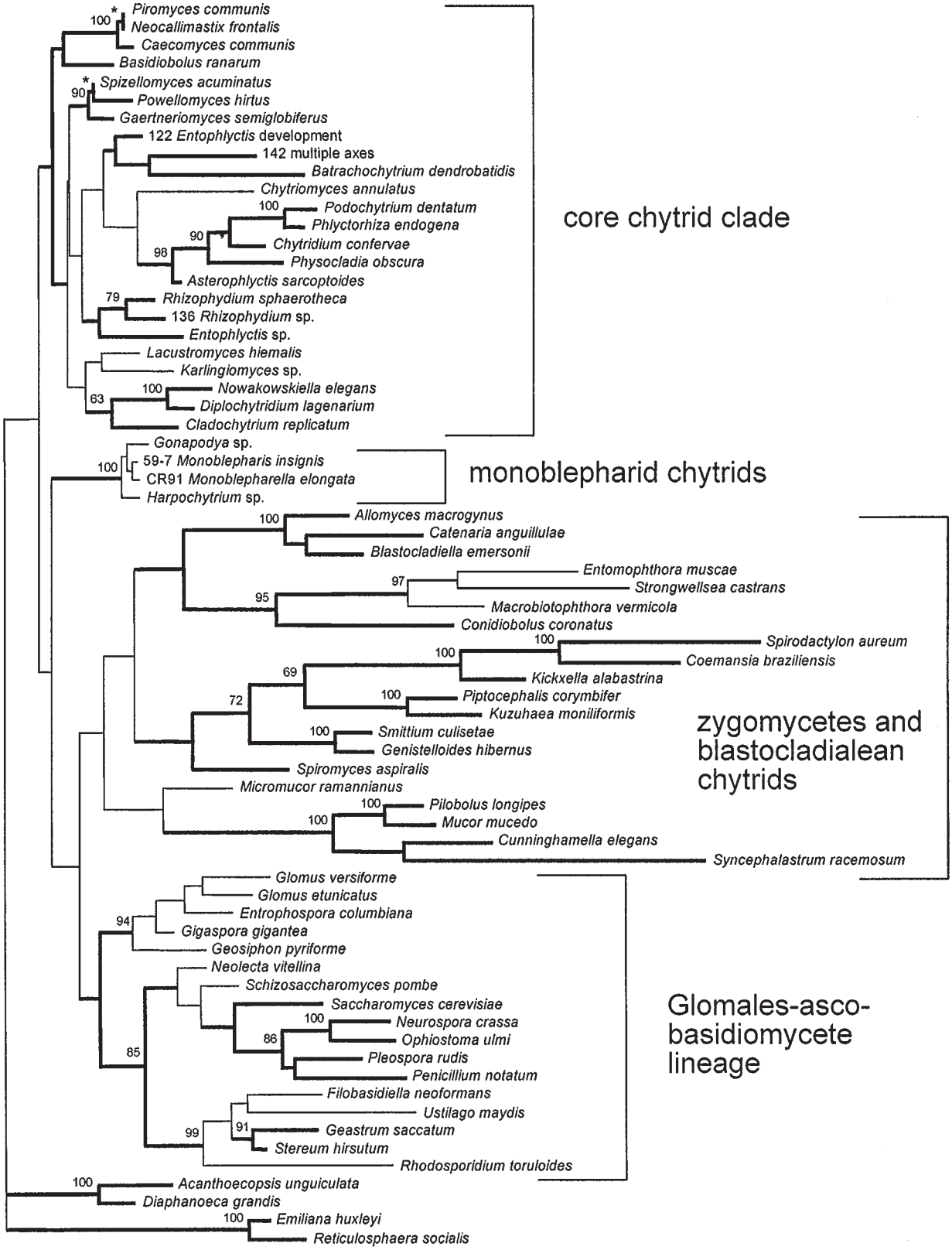
The ultrastructural features of *Chytriomyces angularis* (Longcore 1992*b*) and *Batrachochytrium dendrobatidis* (Longcore et al. 1999) zoospores are unique because they contain character states and combinations that do not occur in described zoospore subtypes. Their singular ultrastructural features are reflected in the isolated positions of these species in our phylogenetic trees. Other species with unique chytridialean zoospores that were not sampled in this molecular study have been reported. These include *Chytridium lagenarium* (Barr and Hartman 1976), *Synchytrium endobioticum* (Lange and Olson 1978), *S. macrosporum* (Montecillo et al. 1980), *Polyphagus euglenae* (Powell 1981), *Zygorhizidum* spp. (Beakes et al. 1988), and *Rhizophydium planktonicum* (Beakes et al. 1993). Addition of sequences from these taxa to the molecular analyses will likely add more chytridialean clades. Some chytrids in isolated positions in our phylogenies are isolates for which ultrastructural data are not yet available (e.g., *Entophlyctis* sp., 142 multiple axes, and 151 *Rhizophydium* sp.). These taxa show little relationship with the other chytrids in the data set, and their position may indicate the existence of additional zoospore subtypes within the Chytridiales. It is uncertain from our analyses whether the Chytridiales sensu Barr needs to be split into several orders to achieve monophyly.

Although the monophyly of the Chytridiales has seldom been disputed, monophyly of the Spizellomycetales has been challenged. Li et al. (1993) pointed out the differences between the zoosporic ultrastructure of *Karlingia* (*Rhizophlyctis*) and that of the core members of the Spizellomycetales (Barr 1980), as did Barr and Désaulniers (1986) when they reported on the types of zoospores found in *Rhizophlyctis*. Our maximum parsimony analysis (Fig. 1) suggests that *Rhizophlyctis* is likely to be a member of the Spizellomycetales; however, the two isolates of *Rhizophlyctis rosea* do not cluster together or with the Spizellomycetales in the maximum likelihood tree (Fig. 2). Taxonomic placement of the rumen chytrids (Neocallimastigales) has also been controversial (Heath et al. 1983; Barr 1988; Li et al. 1993). Heath et al. (1983) had previously placed *Neocallimastix* into a new family within the Spizellomycetales, citing synapomorphies between the zoospore ultrastructures of *Neocallimastix* and *Spizellomyces*. When Li et al. (1993) created a new order for the rumen chytrids, this evidence was based on a reanalysis of the ultrastructural characters of the rumen chytrids and from evidence derived from ssu rDNA sequencing (Li and Heath 1992). Although the close relationship of the rumen fungi to the Spizellomycetes has been argued (Barr 1988), our phylogenies suggest that this may not be the case, or that the relationship is not as close as previously indicated.

Interpretation of zoospore ultrastructure

The zoospores of most chytridiomycetes have several synapomorphies such as the presence of a concentric fiber (Barr 1992) in the transition zone between the kinetosome and flagellum and the presence of props between the kinetosome and the zoospore membrane (Barr and Hadland-Hartmann 1978*a*). The constancy of these ultrastructural characters throughout such a diverse group as the chytridiomycetes suggests that certain fine details of the zoospore

Fig. 4. Phylogenetic hypothesis for the fungal kingdom using maximum parsimony. Tree shown has the highest maximum likelihood (HKY model) of 41 most parsimonious trees. Bootstrap values over 60% are shown. Asterisks indicate short internal nodes with greater than 60% bootstrap support. Branches in bold are present in the strict consensus of 41 trees.



may be slow to change, and thus ultrastructural characters should be good indicators of phylogeny. Studies of the ultrastructure of chytrid zoospores typically uncover a wealth of information; however, the interpretation of this information can be difficult, because different researchers have emphasized different characters and the homology of organelles

Table 2. Evaluation of alternative, constrained topologies with the maximum likelihood approach of Kishino and Hasegawa (1989).

Constraint	Length	–ln (likelihood)	Difference in –ln (likelihood)	SD of difference	*T* value	Probability
None	2968	17 131.93	—	—	—	—
Chytridiomycota monophyletic	2981	17 190.96	59.03	42.88	1.377	0.1689
Chytridiales monophyletic	2968	17 147.42	15.49	37.83	0.409	0.6824
Zygomycota monophyletic	2984	17 218.12	86.19	34.02	2.533	0.0114*
Zygomycota excluding Glomales monophyletic	2978	17 194.50	62.56	30.99	2.019	0.0437*
Zygomycota excluding *Basidiobolus* monophyletic	2976	17 167.48	35.55	25.00	1.422	0.1552
Zygomycota excluding *Basidiobolus* and Glomales monophyletic	2972	17 151.60	19.67	20.25	0.971	0.3316

Note: One hundred heuristic searches were performed to find the shortest trees meeting the constraint criterion. The tree with the highest likelihood of the most parsimonious trees from a given set of topological constraints was compared with the unconstrained tree to determine if the constraint required a phylogeny that was significantly less likely. All maximum likelihood models corresponded with the HKY model of substitution with a transition/transversion ratio of two and observed base frequencies. No topologies were significantly different in length when evaluated with the parsimony based Templeton (Wilcoxon signed ranks) test.

*Significant at $P < 0.05$

found in different species is often uncertain (Powell and Blackwell 1995).

The most typical approach to interpreting zoospore ultrastructure has been to categorize chytrid zoospores into groups based on the configuration of internal contents (i.e., Barr and Hadland-Hartman 1978*a*; Powell 1978). Powell categorized zoospores by their organization of organelles in the microbody – lipid globule complex (MLC), which is "an assemblage of organelles, which always includes a microbody closely appressed to a lipid globule and often includes mitochondria and membrane cisternae or a tubular elaboration of membranes" (Powell 1978: 168). Five types of MLC's are now described (Powell and Roychoudhury 1992), with subtypes within these five. Four of these types of MLC coincide with the blastocladialean, chytridialean, monoblepharidalean, and spizellomycetalean clades that are supported by our molecular analyses. The Neocallimastigales are anaerobic and lack MLC's. The remaining type of MLC is specific for *Harpochytrium*, which in our analysis, is in the Monoblepharidales. The subtypes of MLC within the Chytridiales (type 5) do not correspond to clades defined by analysis of sequences from ribosomal DNA. As demonstration, the "*Rhizophydium* clade" contains three MLC subtypes: 5A, 5B, and 5D; in addition, subtype 5B is found in three of the four clades defined by the molecular phylogenies. The rumposome (Fuller and Reichle 1968), which is a fenestrated cisternum that is a part of the MLC, is a taxonomic feature that has been considered a hallmark of the Chytridiales and the Monoblepharidales. We have mapped the presence or absence of the rumposome onto the molecular phylogeny of the Chytridiales in Fig. 3. The rumposome is present in most zoospores of chytridialean taxa, however its absence in certain taxa suggests that this feature has been lost several times.

Grouping of species by possession of a common zoospore type and subtype as described by Barr (1980, 1990) yielded the same clades as our molecular phylogenetic analysis. Barr's zoospore types and subtypes include MLC and ribosomal characters, but emphasize features associated with the kinetosome. The kinetosomal root is a compound structure composed of organized microtubules or microfibrils that begin near the kinetosome and possibly function in anchoring the flagellum. As a representative kinetosomal character, we have mapped "type of microtubule root" onto the maximum parsimony tree of the Chytridiales (Fig. 3; see figure caption for a description of roots). Features of the primary kinetosomal root are specific to most clades of the Chytridiales as well as to the orders of the chytridiomycetes.

Evolution of the chytridialean thallus

Characters associated with sexual reproduction are important in the systematics of most fungal groups; however, sexual reproduction either does not occur or has not been observed under controlled conditions for most species of chytridialean fungi. Consequently these potentially important characters have not been available to help clarify the systematics of the Chytridiales. In the past, therefore, the search for taxonomic characters centered on characters of the asexual sporangia, such as the presence of an operculum, type of development, and resting spore morphology. Presence or absence of an operculum (a lid or flap opening of the zoosporangium) and type of development were the two most important characters in classical taxonomy (Sparrow 1943, 1960, 1973; Karling 1977) and are mapped onto Fig. 3. The presence of an operculum is not synapomorphic for any of the clades in the ssu rRNA phylogeny, although a high proportion of taxa in the "*Nowakowskiella* clade" produce opercula. Thus, operculation is not a phylogenetically informative character at the series level, as used by Sparrow (1943, 1960).

Type of development has been used in family and genus descriptions in the classical taxonomy, and Barr (1980) used this character to describe families of the Chytridiales and Spizellomycetales. Three common types of development (see Barr 1990 for a full description) are endogenous, exogenous monocentric, and exogenous polycentric. These descriptive terms relate to the behavior of the nucleus during development; whether it stays within the zoospore cyst (endogenous) or goes out of the zoospore cyst (exogenous). In endogenous development, the zoospore cyst enlarges to form a reproductive structure, which is usually a zoosporangium but may be a resting spore. In exogenous monocentric devel-

opment, the nucleus migrates out of the zoospore cyst and into a swelling in the germ tube. A single reproductive structure is then formed from the swelling. This type of development allows a zoospore to encyst on the outside of the substrate and the nucleus to migrate into the substrate through a germ tube to form an endobiotic zoosporangium. In exogenous polycentric development, the nucleus leaves the zoospore cyst and undergoes mitotic divisions throughout the developing branched mycelium. Many zoosporangia or resting spores are formed on each polycentric thallus. Inspection of these three types of development mapped onto the Chytridiales tree (Fig. 3) shows that, with the exception of the *Rhizophydium* clade, which is characterized by endogenous development, particular types of development are not specific for particular clades. We conclude that type of development is not a suitable major character for basing phylogenetically informative families.

Barr hypothesized a single line of evolution that led from simple thalli to polycentric thalli within the Chytridiales (Barr 1978), but later suggested that the evolutionary series may have taken place many times (Barr 1990). Our phylogeny (Fig. 3) shows that three of the major clades in the Chytridiales have polycentric representatives: *Cladochytrium replicatum*, *Nowakowskiella* spp., and *Septochytrium variable* in the "*Nowakowskiella* clade"; *Lacustromyces* and *Polychytrium* in the "*Lacustromyces* clade"; and *Physocladia obscura* in the "*Chytridium* clade." Sparrow (1960) described *Physocladia obscura* as being very similar to *Nowakowskiella*, and Karling (1977) thought that it was probably a species of *Cladochytrium*. The similarity of the polycentric mycelium of *Physocladia* to that of genera in distantly related clades emphasizes the problems with homoplasy of thallus characters.

The redefinition of families based on more evolutionarily stable characters is a priority of chytrid systematics. Increased taxon representation is needed before families and genera can be defined, but the trees presented here reveal well-supported clades in the Chytridiales that can serve as foci of additional research. By employing systematic data independent of morphology, thallus characters can be reevaluated and possibly defined more accurately so as to become more informative. As can be seen by the presence of a "*Rhizophydium*" in the *Chytridium* clade and a "*Diplochytridium* (≡ *Chytridium*)" in the *Nowakowskiella* clade (Figs. 1–3), it is currently difficult to identify genera with certainty based on their morphology alone. Barr (1980) proposed the use of zoosporic ultrastructural characters to describe genera in the Chytridiales. Whether enough diversity of zoospore characters within the Chytridiales exists to identify genera is not yet certain. Data from both ultrastructural and DNA studies will probably be needed to adequately determine genera. DNA sequencing is less time consuming and less material is needed than for ultrastructural studies; consequently new isolates can be categorized molecularly, and the most informative ones can be studied with electron microscopy.

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