Molecular Evolution of the Fungi: Relationship of the Basidiomycetes, Ascomycetes, and Chytridiomycetes [1]

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Establishing the phylogeny of fungi and protists often has proved difficult owing to the simple morphologies and convergent characters in these organisms. We used DNA sequences of nuclear small-subunit ribosomal RNA genes to determine phylogenetic relationships among three major classes of organisms considered to be fungi—Basidiomycetes, Ascomycetes and Chytridiomycetes—and to assess the taxonomic position of *Neocallimastix,* an economically important anaerobic rumen microorganism whose classification is controversial. The Basidiomycetes and Ascomycetes, two classes of nonflagellated fungi, are the most closely related taxa. Chytridiomycetes, though bearing flagella, group with these higher fungi rather than with the protists. *Neocallimastix,* a eukaryote lacking mitochondria and variously classified as a protist or as a fungus, shows closest molecular affinities with the Chytridiomycete fungi in the order Spizellomycetales.

Introduction

The fungi, historically considered to be plants because of their form and apparent lack of locomotion, have been recognized as a kingdom in their own right, a classification championed by Whittaker (1969). Two of the classes of fungi that lack flagella—Basidiomycetes (mushrooms, "shelf fungi," and their allies) and Ascomycetes (mostly molds and yeasts)—are universally allied by morphological characters (Tehler 1988). Also traditionally classified as fungi are three classes of flagellated organisms—Chytridiomycetes ("chytrids"), Oomycetes (water molds), and Hyphochytriomycetes. Of these, only the chytrids share with the nonflagellated classes their cell-wall polymers (Bartnicki-Garcia 1970) and lysine synthetic pathway (Vogel 1964).

Phylogenetic analysis based on the ~120-base 5S ribosomal RNA (rRNA) suggested that the nonflagellated fungi do not form a natural (monophyletic) group (Hori and Osawa 1987); indeed, in that analysis, green plants branched between the two fungal classes. Other researchers (Van de Peer et al. 1990) have found these nonflagellated classes to form a monophyletic group, also based on 5S rRNA. However, because of the small amount of information in the 5S molecule, neither of these results is likely to be strongly supported (Halanych 1991; Steele et al. 1991). Hendriks et al. (1991), using 18S rDNA, found that the nonflagellated classes were not monophyletic when a limited number of sequence positions were used in the analysis but that these classes became so when a less conservative subset was used. To determine whether

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these conflicting observations can be resolved with any confidence, we have used our sequence of nuclear small-subunit ribosomal DNA (18S rDNA) from the basidiomycete shelf fungus *Spongipelis unicolor,* comparing it to sequences already known from Ascomycetes and other organisms.

Phylogenetic placement of the class Chytridiomycetes is a matter of current debate. These organisms are claimed for the protists on the basis of possession of flagella (Margulis and Schwartz 1988, pp. 77, 140–141, and 153) and are claimed for the fungi on the basis of characters that they share with the nonflagellated classes. Förster et al. (1990), using 18S rDNA sequences, placed the chytrid *Blastocladiella emersonii* as a sister group to the ascomycetes, but the degree of support for this conclusion was not addressed. We present two new sequences for 18S rDNA from members of the Chytridiomycetes and determine with a high degree of confidence their relationship to the nonflagellated fungi.

Neocallimastix and related anaerobic gut microbes also have controversial classifications. Although these agriculturally important, cellulolytic microbes share characters with Chytridiomycetes, many of their characteristics are unique, i.e., multiple flagella (Braune 1913), genomic G+C content <20% (Brownlee 1989), lack of mitochondria (Heath et al. 1983), strict anaerobiosis (Orpin 1975), and hydrogen-generating hydrogenosomes (Yarlett et al. 1986). The zoospores of these gut microbes share ultrastructural features with one order of Chytridiomycetes (Spizellomycetales) (Barr 1980; Heath et al. 1983) and share developmental features with another (Blastocladiales) (Wubah et al. 1991); but intense interest in these microbes has resulted in many new descriptions, and their classification is anything but settled (Barr et al. 1989; Breton et al. 1990; Ho et al. 1990). To determine the phylogenetic placement of *Neocallimastix,* we sequenced its nuclear 18S rDNA, for comparison with our chytrid sequences and other known sequences.

We therefore address three questions that are well suited to molecular evolutionary investigation: (1) Are the Basidiomycetes, Ascomycetes, and Chytridiomycetes part of a monophyletic fungal lineage? (2) Are the anaerobic gut microorganisms members of the Chytridiomycetes? (3) Are gut chytrids most closely related to the order Spizellomycetales?

Material and Methods

Neocallimastix species, culture LM-2, was obtained from the Anaerobic Culture Collection, CSIRO, Prospect, New South Wales. *Spizellomyces acuminatus,* strain Barr 62A, was a gift from Donald Barr. *Chytridium confervae* 81-1 was obtained from the U.C. Microgarden (University of California, Berkeley). Total DNA isolated from *C. confervae* (Lee and Taylor 1990) and *Neocallimastix* (Brownlee 1988) and pSK2 plasmid DNA from *Spongipelis unicolor* (Kwok et al. 1986) was amplified using conditions which minimize the errors introduced by the *Taq* DNA polymerase (<1 error/5 kb after 35 cycles) (Gelfand and White 1990) with modified versions of primers NS1 and NS8 (White et al. 1990): SL21—CCGAATTCGTAGTCATATGCTTGTCT; SL27—CCAAGCTTAAACCTTGTTACGACTT. Amplified DNA was purified by precipitation, treated with Klenow fragment, purified by agarose electrophoresis, and blunt-end ligated into the *Hinc*II site of the pUC18 vector. Transformed *Escherichia coli* strain DH5 was screened with the probe NS2 (White et al. 1990), and clones were sequenced from both strands (Sequenase protocol; U.S. Biochemical). Sequences obtained from the cloned polymerase chain reaction (PCR) products of *Neocallimastix, C. confervae,* and *Spongipelis unicolor* were verified against partial sequences obtained

from direct sequencing of 800–1,100 bases each from single-stranded DNA, asymmetrically amplified (Kwok et al. 1986; Gyllensten and Erlich 1988) either from 1–10 ng of genomic DNA or from the cloned *Spongipelis unicolor* rDNA repeat.

PCR reactions on *Spizellomyces acuminatus* total DNA were performed using primers NS1 (White et al. 1990) and NS24 (AAACCTTGTTACGACTTTTA) (A. Gargas, personal communication), one of which was biotinylated in each of two reciprocal reactions. *Spizellomyces acuminatus* DNA was diluted 1:100 with autoclaved distilled water from the extracted stock DNA. Fifty microliters of diluted DNA was amplified in a 100-μl reaction containing 1 × GeneAmp Buffer (Perkin Elmer—Cetus); 62.5 μM each dGTP, dATP, dTTP, and dCTP; 5% glycerol (Smith et al. 1990); 50 pmol of each primer; and 2.5 units AmpliTaq (Perkin Elmer—Cetus). The reaction mixture was heated to 95°C for 5 min and then was subjected to 40 cycles, each of 40 s at 95°C, 25 s at 50°C, and 3 min at 72°C; these were followed by a single 10-min extension at 72°C. Products were stored at 4°C briefly before single-stranded-DNA preparation.

Spizellomyces acuminatus single-stranded 18S rDNA for sequencing was prepared using a streptavidin agarose (SA) technique based on that of Mitchell and Merrill (1989), with the variation that columns were eliminated, and the entire procedure was done in microcentrifuge tubes. All procedures were performed at room temperature. After each step, the mixture was spun briefly in a microcentrifuge to pellet the SA beads, and the supernatant was removed with an Eppendorf micropipetter. SA beads (Bethesda Research Laboratories 5942SA) were rinsed five times with storage buffer (20 mM Tris, 200 mM NaCl, 1 mM ethylenediaminetetraacetate, pH 7.6) before use. The slurry of SA and storage buffer [~1:1 (v:v)] was stored at 4°C. Approximately 90 μl of double-stranded, biotinylated PCR product was captured on 200-μl SA slurry in a 2-ml microcentrifuge tube (Sarstedt 72.689) by agitation on a Labquake rotator (Labindustries, Berkeley, Calif.) for 30–40 min. After being washed twice with 500 μl storage buffer, the double-stranded DNA (still attached to SA) was denatured by rinsing the beads twice, for 6 min each time, with 150 μl freshly prepared 0.2 M NaOH, and the nonbiotinylated single strand of DNA was recovered in the supernatants. The combined supernatant containing the ssDNA was neutralized with 200 μl of 5 M ammonium acetate, pH 6.8, and was desalted and concentrated to a volume of 35–60 μl by using three 2-ml washes with autoclaved distilled water on Centricon-100 (Amicon 4212). Of the resulting ~40 μl of single-stranded DNA solution, 7.5 μl was sequenced using standard Sequenase 2.0 protocol (U.S. Biochemical), with the exception that the labeling mix was diluted 1:20 before use. Bases at 1,417 positions were sequenced on both strands of the DNA. Single-stranded sequence was accepted when the data were clear, were read identically by two investigators who had no knowledge of the other's reading, and were alignable with published fungal sequences.

Results and Discussion

We obtained sequences for 18S rRNA genes, complete except for an estimated 38 bases at the 5′ end and 49 bases at the 3′ end, for *Neocallimastix* species, the two chytrids *Chytridium confervae* and *Spizellomyces acuminatus,* and the basidiomycete shelf fungus *Spongipelis unicolor* (fig. 1). These could be unambiguously aligned with published sequences from the chytrid *Blastocladiella emersonii* (Förster et al. 1990), the ascomycete bread mold *Neurospora crassa,* the soybean *Glycine max,* and the ciliate protist *Stylonychia pustulata,* at 1,368 positions (underlined in fig. 1); 308 of these positions showed at least one substitution event. The maximum number of

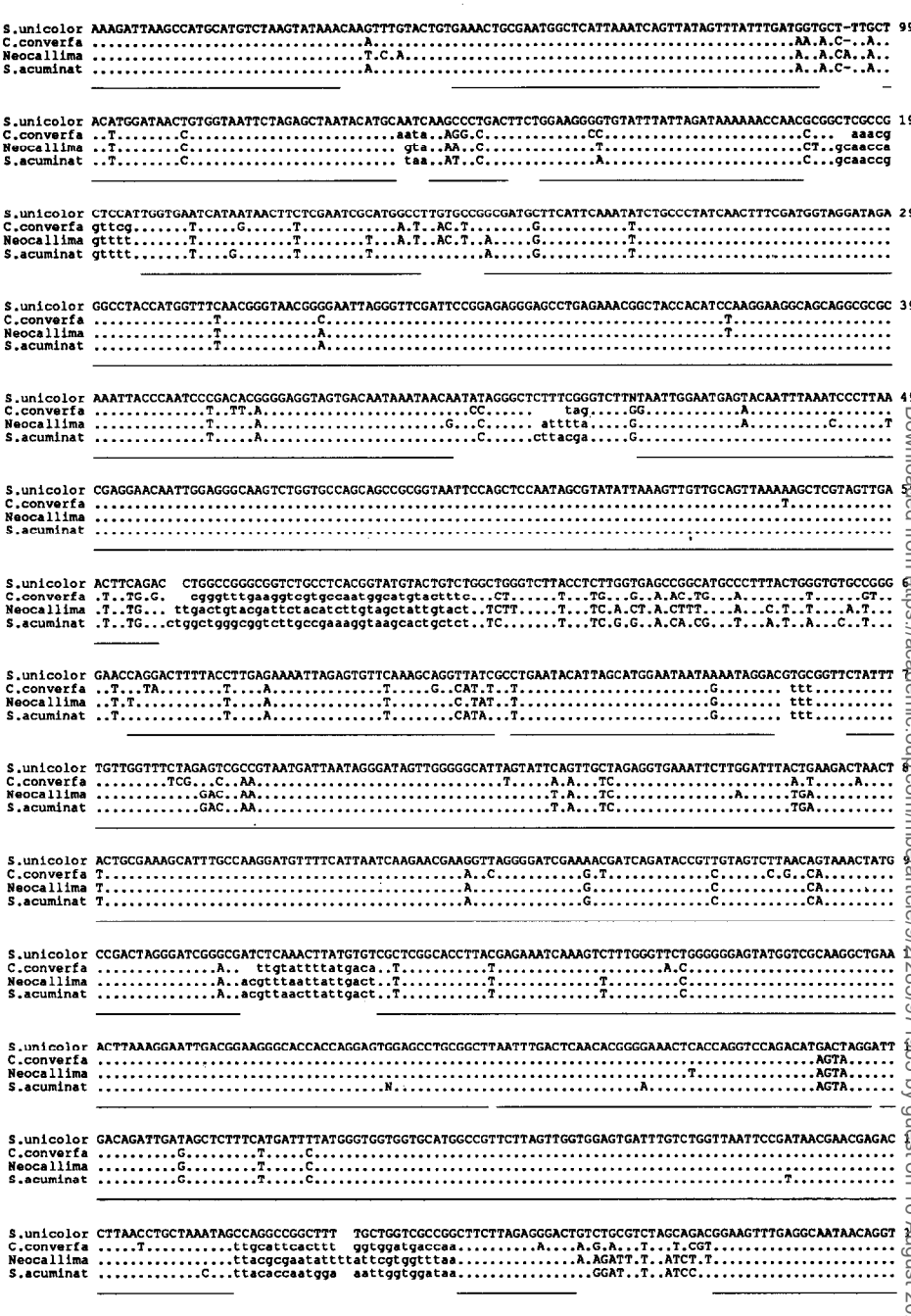

FIG. 1.—Aligned small subunit rRNA sequences from four species. Sequence data were obtained from the anaerobic rumen fungus *Neocallimastix* (1,717 bases; GenBank accession number M59761), the chytrids *Chytridium confervae* (1,707 bases; M59758), *Spizellomyces acuminatus* (1,718 bases; M59759), and the basidiomycete shelf fungus *Spongipelis unicolor* (1,718 bases; M59760), between PCR primers NS1 and NS24. These were aligned with published sequences of the ascomycete bread mold *Neurospora crassa* (GenBank NEURRNAS), the chytrid *Blastocladiella emersonii* (Förster et al. 1990), the ciliate protist *Stylonychia pustulata* (GenBank SLURGSS), and the soybean *Glycine max* (GenBank SOYRGE). (These four sequences are not shown, but the complete alignment will be supplied on request.) Sequences were aligned manually, using the Eyeball Sequence Editor ESEE version 1.09 (Cabot and Beckenbach 1989) for

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S.unicolor CTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGAGCCAGCGAGTTATTCACCGGCCTTGGAAGGTCTGGGTAATCTTGTGAAACT 1495
C.converfa ..........................................AT.G....A...attcaccttggcc ......C...........C......A
Neocallima ..........................................TGA..T..A.....ttcatccttgatc ........C..........T.......
S.acuminat ..........................................TGA..G....A...aattcaccttggcc..................C.......

S.unicolor CTGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAACGAGGAATTCCTAGTAAGCGTGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTTT 1595
C.converfa T..............T.TC.C.............GA.A.G.....................CA...........T.....................
Neocallima TCA..............TC.C.........T...GA...A.....................................A.....................
S.acuminat TCA.............T.TC..............GA.A.....................................................

S.unicolor GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGTCTTGGGATTGGCTTCGGGGAGCCGGCAACGGCACCCTGTTGCTGAGAACTTGAT 1695
C.converfa ..........................................gacctccggattgaaagctcttgggcagcaatgcccgatagctgttg....G...G.
Neocallima ..........................................atcttcggattggctatttttttctggcaacagaattaaactgcta .A..G...GG
S.acuminat ..........................................gcctccggattgggagcttggtaccggcaacggcaccgagctcactg....G...GG

S.unicolor CAAACTTGGTCATTTAGAGGAAG                                                                        1718
C.converfa ........................
Neocallima ........................
S.acuminat ........................
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the IBM PC. A dot indicates a base both homologous to and identical to the corresponding base in the *Spongipelis unicolor* (reference) sequence. A capital letter indicates a base that differs from the base in the reference sequence. A lowercase letter indicates a base at a position whose homology to positions in the reference sequence is undetermined. These include areas in which the sequences are sufficiently diverged that positional homology cannot be inferred directly from sequence similarity and in which, in addition, small or large length variations are evident. When short (<12 bases) areas of substantial sequence divergence were bounded by regions of certain homology and included no length variation, these were considered alignable (i.e., positional homology was inferred—e.g., see positions 743–747 in fig. 1). Unalignable areas were omitted from all phylogenetic analyses. In some regions, a subset of the sequences was alignable, and for those sequences these positions were included in parsimony analyses. Positions used in distance analyses (neighbor joining) are underlined and include only those positions alignable in all eight species (including *Neurospora crassa, B. emersonii, Stylonychia pustulata,* and *G. max*). The boundary of a sequence that was alignable despite high sequence variation was differentiated from an adjacent unalignable area if (1) three of the four bases at the margin of the aligned region were identical to those at the same positions in at least one other sequence of certain alignment, (2) these three matching bases included the position bounding the aligned area, and (3) no length variation was involved (e.g., see *C. confervae* at positions 605–608, which is identical both to the reference sequence at positions 606 and 608 and to the well-aligned *Neocallimastix* sequence at position 605).

pairwise substitutions in the aligned regions was 169 (12.4%) between the bread mold and the ciliate protist.

We used three different methods of phylogenetic analysis to determine evolutionary relationships and to evaluate the strength of the branching order: the neighbor-joining method (Saitou and Nei 1987), based on genetic distances; a bootstrap test of the neighbor-joining method (Whittam 1991); and the parsimony-based Winning Sites Test (Prager and Wilson 1988).

The neighbor-joining method (Saitou and Nei 1987) was used to construct, from the unambiguously aligned positions, the branching order shown in figure 2. This tree unites the two classes of nonflagellated fungi as closest relatives, in accordance with morphological and physiological criteria. The anaerobic rumen fungus *Neocallimastix* falls within a monophyletic group formed by the flagellated class Chytridiomycetes. This group is united with the nonflagellated fungal classes, to the exclusion of the protist.

Any phylogenetic tree must be considered a hypothesis; it is necessary to examine rigorously any specific inferences drawn from this tree, to see whether the data are sufficiently robust to support definitive conclusions. The neighbor-joining method usually returns the minimum-length tree (Saitou and Nei 1987; Saitou and Imanishi 1989), but this is not necessarily the correct tree. (Neighbor-joining is guaranteed to return the correct tree only if distance data satisfy the condition of being additive.) In order to evaluate the strengths of particular inferences derived from the neighbor-

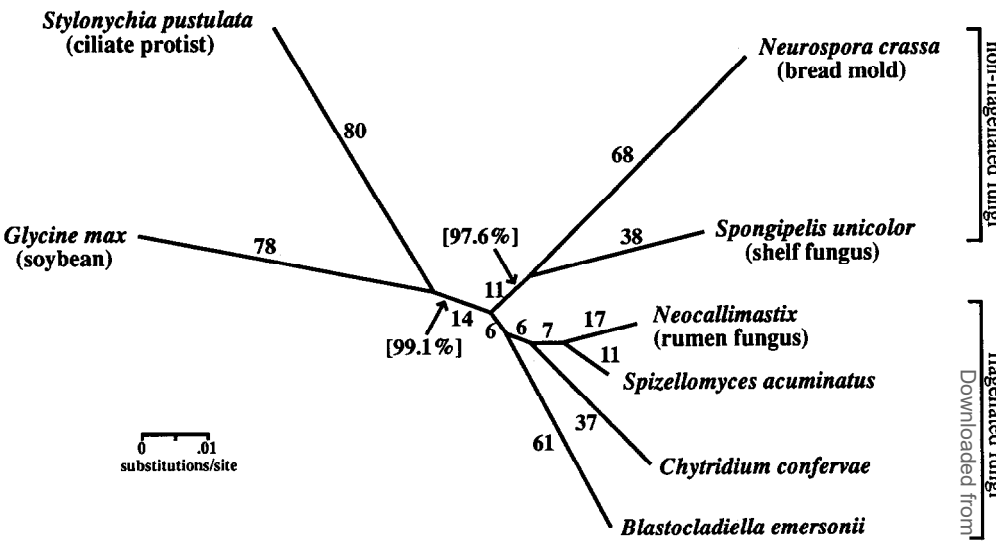

FIG. 2.—Neighbor-joining tree of eight species. The computer program NJTREE (Jin and Ferguson 1990) was used to estimate the branching order and branch lengths for the eight species. Of the 1,368 alignable sequence positions, a maximum of 169 (12.4%) differed in any pairwise comparison. Uncorrected differences were used to construct the tree. Branch lengths have been rounded to the nearest whole number of substitutions, and branches are drawn proportional to their lengths. A scale is provided to show the inferred number of substitutions per sequence position. In this tree, nonflagellated fungi form a monophyletic group. The anaerobic rumen flagellate *Neocallimastix* falls within the monophyletic group formed by the Chytridiomycetes (flagellated fungi). Chytrids and the nonflagellated fungi form a monophyletic group to the exclusion of the protist and green plant. The two significant bootstrap results (Whittam 1991), returned after 1,000 trials, are shown in brackets. Bootstrap support for other branches was not significant at the 95% level (Felsenstein 1985): there was 93.1% support for the branch uniting *Neocallimastix* with *Spizellomyces acuminatus*, 81.3% support for the branch uniting these two with *Chytridium confervae*, and 93.4% support for the monophyly of the chytrids (flagellated fungi).

joining tree, we used both a bootstrapping test based on the neighbor-joining algorithm (Whittam 1991) and the parsimony-based Winning Sites Test (Prager and Wilson 1988).

The Winning Sites Test determines how strongly the branching order is supported by the data, by contrasting the number of sequence positions that support alternative, unrooted tree topologies. For the simplest test, four taxa are arranged into the three possible topologically distinct, bifurcating networks (e.g., see fig. 3). Phylogenetically informative sequence positions for this test are those at which two species share one base while the two others share a different base. When a common base is shared by the two species on the same side of the network, the data can be explained by a single substitution event, occurring on the central branch between the nodes connecting the pairs of sequences. The other two topologies would require at least two events. The network requiring a single event is counted as "winning" at that sequence position. The total numbers of sequence positions favoring each of the three networks are compared, to see whether support for one topology significantly exceeds support for the alternatives (Li and Gouy 1991). Because the Winning Sites Test can be led astray by base-compositional bias among the sequences compared (Irwin and Wilson, accepted) the base compositions of the sequences analyzed were evaluated. *Neocallimastix* genomic DNA is known to have an unusually low percentage of G+C (18%; Brownlee

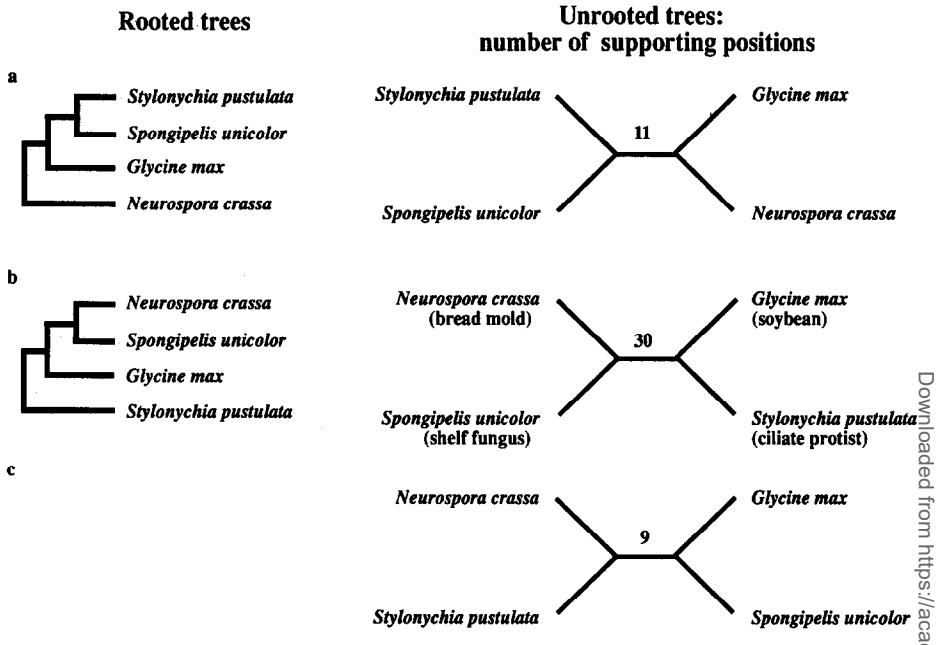

FIG. 3.—Evaluation of relationship of Ascomycetes and Basidiomycetes. The Winning Sites Test (Prager and Wilson 1988) was used to compare support for the branching order suggested by 5S rRNA sequence comparisons and for the morphologically based branching order, which is also that returned by the neighbor-joining method using 185 rDNA sequences. a, 5S-based rooted tree (*left*) and corresponding unrooted network. b, One possible rooted tree that unites nonflagellated fungi in monophyletic group. The unrooted network (*right*) represents any rooted tree uniting these two taxa. The third possible network is also shown. Fifty sequence positions are phylogenetically informative for these four taxa, with two taxa sharing one base and the other two sharing a different base. Of these, 11 support (require only one substitution in) network a, 30 support network b, and 9 support network c. When the four-taxon method of Li and Gouy (1991) is used, this result is significant at the 0.15% level.

1989); nevertheless, the percentage of G+C in *Neocallimastix* 18S rDNA is 41.9% and does not differ markedly from those of the other fungi evaluated, whose percentages of G+C are 44.3%–48.2%.

Using the Winning Sites Test, we first evaluated whether the nonflagellated fungal classes are closest relatives. In addition to these fungi, green plants and ciliate protists were represented both in the present study and in the 5S rRNA study by Hori and Osawa (1987) that found the nonflagellated fungi not to be monophyletic. The 5S rRNA tree grouped the Ascomycetes (represented here by the bread mold *Neurospora crassa*) with green plants (the soybean *G. max*) and grouped the Basidiomycetes (represented here by the shelf fungus *Spongipelis unicolor*) with ciliate protists (*Stylonychia pustulata*). (This branching order, obtained using a simplified unweighted pair-group method, was not strongly supported, having substantial error bars on the closely spaced branch points.) We compared the network representing the 5S tree (fig. 3a) both with that representing the 18S neighbor-joining tree (fig. 3b) and with the third alternative. Of 50 sequence positions phylogenetically informative for these four taxa, 11 supported the 5S branching order, 30 supported the 18S branching order, and 9 supported the third alternative. This support for the unity of the nonflagellated fungal classes is significant at the 0.15% level, by the four-taxon method of Li and Gouy (1991).

Because the protists are a paraphyletic group, their relationship to the fungal classes might not be accurately represented by a single phylum. We repeated this test, using the chrysophyte alga *Ochromonas danica* (GenBank OCHAB), whose divergence from other lineages was close in time to the divergence of plant and fungal lineages (Gunderson et al. 1987). Thus *Ochromonas* stands the best chance of disrupting the monophyletic grouping of the fungi. Of 43 phylogenetically informative positions, 26 grouped the two nonflagellated fungal lineages, while only 6 and 11 positions, respectively, supported the two alternatives (support again was significant at the 0.15% level) (Li and Gouy 1991). Thus the Winning Sites Test strongly favors the branching order suggested by morphological and physiological criteria.

In addition, a bootstrapping test using the neighbor-joining algorithm (Whittam 1991) supported the association of the nonflagellated fungal classes, in 976 out of 1,000 replicates (fig. 2). The greater quantity of information in the 18S molecule (relative to 5S) can indeed be used to resolve with a high degree of confidence the branching order of these four taxa, supporting the morphological classification uniting the two classes of nonflagellated fungi.

A second inference from the neighbor-joining tree is that the Chytridiomycetes are the closest relatives of the nonflagellated fungi. Chytrids traditionally have been classified as flagellated fungi, along with the Oomycetes (water molds) and Hyphochytriomycetes. Margulis and Schwartz (1988, p. 76) show a morphologically based tree in which chytrids are not closely related to the nonflagellated fungi and are classed as protists. Recent molecular studies (Kwok et al. 1986; Förster et al. 1990) have suggested instead both that chytrids are part of a monophyletic lineage that includes the nonflagellated fungal classes and that Oomycete water molds have their closest relatives among the protists.

We used the Winning Sites Test to determine whether the proposition that chytrids shared their most recent common ancestry with nonflagellated fungi, rather than with protists, could be supported with a high degree of confidence. For this analysis, we compared the soybean 18S rDNA sequence both with those of the ciliate protist *Stylonychia pustulata,* a species from the Chytridiomycete type genus *Chytridium,* and with the nonflagellated shelf fungus *Spongipelis unicolor.* Twenty-five sequence positions supported grouping *C. confervae* with the shelf fungus, while only 12 united it with the protist, and 7 favored the third alternative, supporting, at the 0.9% level, the association of the chytrids with the nonflagellated fungi (Li and Gouy 1991). Substitution of the bread mold for the shelf fungus gave similar results, as did using the chrysophyte *O. danica* in place of the ciliate. In addition, a bootstrapped neighbor-joining test (Whittam 1991) supported (in 991 of 1,000 replicates) the branch unifying the flagellated (Chytridiomycete) and nonflagellated fungal lineages, to the exclusion of the protist and green plant (fig. 2).

A third inference tested using the Winning Sites Test is that the anaerobic rumen fungus *Neocallimastix* is part of a monophyletic fungal lineage, rather than being a protist. Because of the paraphyly of the protists, testing the simple association of *Neocallimastix* with another fungus, to the exclusion of a representative protist, is insufficient. It is necessary to demonstrate that *Neocallimastix* branches within the fungi and is not an exterior branch.

Along with *Neocallimastix,* the ciliate protist *Stylonychia pustulata,* the bread mold *Neurospora crassa,* and the chytrid *Spizellomyces acuminatus* were used to test this inference (fig. 4). If the tree uniting *Neocallimastix* with the protist is favored (fig. 4a), then *Neocallimastix* might not be a fungus. If it unites with either of the

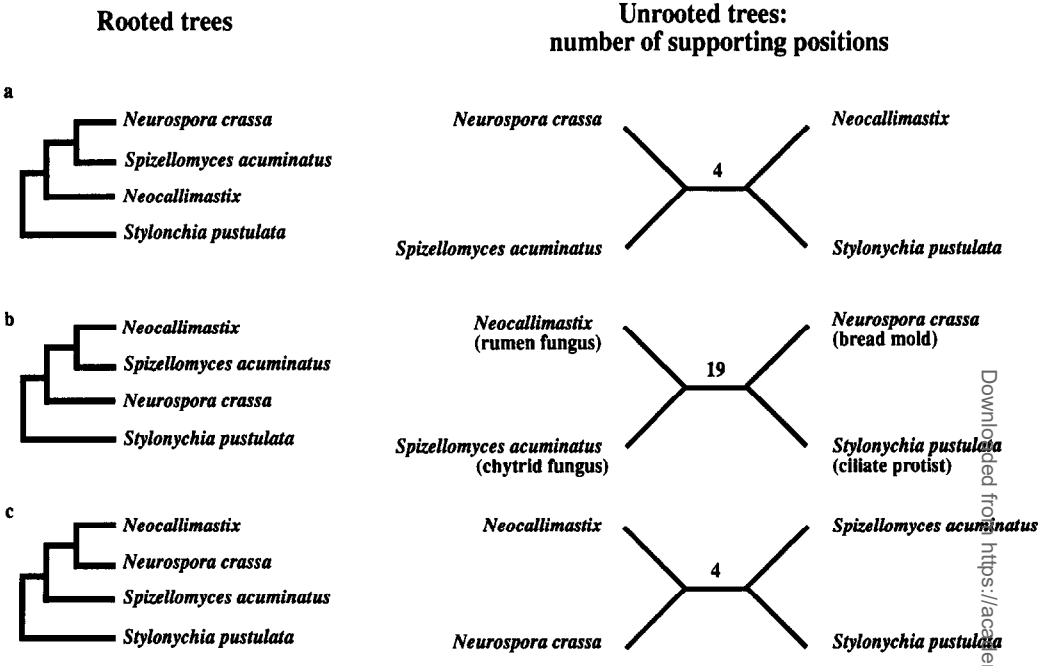

FIG. 4.—Evaluation of placement of anaerobic rumen flagellate *Neocallimastix* in Fungi kingdom. The Winning Sites Test (Prager and Wilson 1988) was used to evaluate the strength of the inference that *Neocallimastix* belongs in the Fungi kingdom and is not a protist. Support of network a (topologically equivalent to the rooted tree to its left) would allow the conclusion that *Neocallimastix* is a protist; support for either network b or network c would place *Neocallimastix* within the Fungi kingdom. Of the positions phylogenetically informative for these taxa, 19 favor placing *Neocallimastix* within the fungi and associated with the chytrid *Spizellomyces acuminatus,* and 4 positions favor each of networks a and c. When the four-taxon method of Li and Gouy (1991) is used, this result is significant at the 0.15% level.

other species, it can with confidence be called a fungus (fig. 4b and c). Of the 27 positions phylogenetically informative for these four taxa, 19 unite *Neocallimastix* with the Chytridiomycete fungus, while only 4 place it with the protist, and 4 associate it with the bread mold (this distribution is significant at the 0.15% level) (Li and Gouy 1991). This result supports the placement of *Neocallimastix* within the fungi. Specifically, *Neocallimastix* is allied with the chytrid branch, to the exclusion of the nonflagellated fungal lineage. The more conservative bootstrap test of neighbor-joining (Whittam 1991), however, provided nonsignificant support (e.g., uniting the four flagellated fungi as a monophyletic group in only 931 of 1,000 trials.)

To evaluate with which order *Neocallimastix* is allied within the class Chytridiomycetes, we compared its sequence with those of three other fungi: (1) *B. emersonii,* representing the order Blastocladiales, which shares some developmental features with *Neocallimastix* (Wubah et al. 1991); (2) *Spizellomyces acuminatus,* representing the order Spizellomycetales, into which Heath et al. (1983) proposed placing *Neocallimastix;* on the basis of zoospore ultrastructure; and (3) the shelf fungus as outgroup. The network uniting *Neocallimastix* with the order Spizellomycetales was overwhelmingly supported (22 sequence positions favoring *Spizellomyces acuminatus* vs. 4 and 3 favoring *B. emersonii* and the outgroup, respectively), verifying this aspect of the neighbor-joining tree (with support at the 0.15% level) (Li and Gouy 1991) and supporting Heath et al.'s (1983) classification based on zoospore ultrastructure. When

we substituted *C. confervae* (Order Chytridiales) for *B. emersonii* in the Winning Sites Test, the test could not exclude the possibility that *Neocallimastix* united with the order Chytridiales instead of with the Spizellomycetales. Bootstrap analysis (Whittam 1991) returned nonsignificant results, uniting *Neocallimastix* with Spizellomyces in 934 of 1,000 trials. Nevertheless, the phylogenetic position of *Neocallimastix* is as a late branch within the fungi, suggesting that its lack of mitochondria is due to secondary loss.

In summary, we have presented four new essentially full-length fungal 18S rRNA sequences from one basidiomycete and three chytrid fungi. Within the organisms represented by these sequences, our data support the association of the nonflagellated fungal classes Ascomycetes and Basidiomycetes, in keeping with morphological classification. We have further shown, with a high degree of confidence, that the flagellated class Chytridiomycetes and the nonflagellated fungal classes together form a monophyletic unit. Although the Chytridiomycetes were the first class to diverge at the base of the fungal branch, there is no strong reason to exclude them from the Fungi kingdom.

We demonstrate with the present study an instance in which molecular data have been used successfully to discern the phylogenetic status of an unusual organism whose unique biochemical properties made its classification by morphological and physiological criteria a matter of contention. The rumen flagellate *Neocallimastix* is certainly a Chytridiomycete fungus. It has been possible, with a high probability, to assign this genus to the order Spizellomycetales, to the certain exclusion of the order Blastocladiales.

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