Evidence from Beta-Tubulin Phylogeny that Microsporidia Evolved from Within the Fungi

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Microsporidia are obligate intracellular parasites that were thought to be an ancient eukaryotic lineage based on molecular phylogenies using ribosomal RNA and translation elongation factors. However, this ancient origin of microsporidia has been contested recently, as several other molecular phylogenies suggest that microsporidia are closely related to fungi. Most of the protein trees that place microsporidia with fungi are not well sampled, however, and it is impossible to resolve whether microsporidia evolved from a fungus or from a protistan relative of fungi. We have sequenced beta-tubulins from 3 microsporidia, 4 chytrid fungi, and 12 zygomycete fungi, expanding the representation of beta-tubulin to include all four fungal divisions and a wide diversity of microsporidia. In phylogenetic trees including these new sequences, the overall topology of the fungal beta-tubulins generally matched the expected relationships among the four fungal divisions, although the zygomycetes were polyphyletic in some analyses. The microsporidia consistently fell within this fungal diversification, and not as a sister group to fungi. Overall, beta-tubulin phylogeny suggests that microsporidia evolved from a fungus sometime after the divergence of chytrids. We also found that chytrid alpha- and beta-tubulins are much less divergent than are tubulins from other fungi or microsporidia. In trees in which the only fungal representatives were the chytrids, microsporidia still branched with fungi (i.e., with chytrids), suggesting that the affiliation between microsporidian and fungal tubulins is not an artifact of long-branch attraction.

Introduction

The evolutionary relationship between the microsporidia (Microspora) and other eukaryotes has been a long-standing and difficult issue to resolve. Based on structural characters, microsporidia have historically been classified with various combinations of other intracellular parasites such as myxosporidia, actinomyxidia, haplosporidia, and sporozoa (see, e.g., Lom and Vávra 1962; Kudo 1966; Desportes and Nashed 1983). However, many of the characters uniting these groups are associated with their highly specialized modes of parasitism or are present in many other groups of eukaryotes, and this raises the possibility of convergence. In addition, many of the other characters frequently used for eukaryotic classification (e.g., mitochondria, and 9 + 2 microtubule structures) are missing or unrecognizable in microsporidia, further obscuring their origins.

Eventually, this absence of characters, particularly of the mitochondrion, led to the inclusion of the microsporidia in the Archezoa, a group proposed to descend from eukaryotes that lived prior to the acquisition of the mitochondrial endosymbiont (Cavalier-Smith 1983). Initial molecular evidence provided support for this putatively ancient origin of microsporidia by showing that they were among the deepest eukaryotic branches in trees based on ribosomal RNA (Vossbrinck et al. 1987) and translation elongation factors (Kamaishi et al. 1996*a,* 1996*b*). However, these molecular data were not without complications. Like the cells in which they reside, microsporidian genes tend to be very odd—typically characterized by unique insertions or deletions, strong base-composition bias, and highly accelerated rates of substitution. Genes with accelerated rates are often erroneously placed in phylogenies because of attraction to other accelerated or divergent sequences (Felsenstein 1978; Philippe and Laurent 1998). Thus, even considering the apparent agreement between morphological and molecular data, the position of the microsporidia was never beyond skepticism (Cavalier-Smith 1993).

This caution proved to be well founded, as sampling of more microsporidian genes soon yielded a strong alternative to their ancient position, namely that microsporidia are somehow related to fungi. Similarities between microsporidian and fungal mitosis and meiosis have been noted over many years (Desportes and Théodoridès 1979; Desportes and Nashed 1983; Flegel and Pasharawipas 1995), but these similarities failed to generate much taxonomic enthusiasm because the shared characters were not unique to these two groups. The first molecular phylogenies to argue for a relationship between microsporidia and fungi were those of alpha- and beta-tubulins, both of which placed the microsporidia within the fungi (Edlind et al. 1996; Keeling and Doolittle 1996). Since then, only one additional gene (again involving the translation apparatus) has been found to support an ancient origin of microsporidia (Keeling, Fast, and McFadden 1998), whereas several more support a fungal relationship. These include genes encoding mitochondrial HSP70 (which also indicates that microsporidian ancestors did not lack mitochondria; Germot, Philippe, and Le Guyader 1997; Hirt et al. 1997; Peyretaillade et al. 1998*b*), the largest subunit of RNA polymerase II (Hirt et al. 1999), and TATA-box binding protein (Fast, Logsdon, and Doolittle 1999). A number of other molecular and morphological features have also been found or reinterpreted as supporting a relationship between microsporidia and fungi (reviewed in Keeling and McFadden 1998; Cavalier-Smith 1998; Hirt et al.

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1999). Furthermore, it has recently been shown that many of the genes that support an ancient origin of microsporidia are probably unreliable: there is now evidence that microsporidian EF-1 alpha is likely saturated and that EF-2 and large subunit rRNAs do not significantly exclude the possibility that microsporidia are related to fungi (Peyretaillade et al. 1998*a*; Hirt et al. 1999).

Altogether, there is a fairly strong body of evidence that microsporidia are related to fungi, but very little evidence that the microsporidia actually evolved from fungi, which is a very significant distinction. Molecular phylogenies that support the relationship between the two are generally only scantily sampled; almost all are restricted to sequences from only one or two microsporidia and a few ascomycete fungi (Germot, Philippe, and Le Guyader 1997; Hirt et al. 1997, 1999; Peyretaillade et al. 1998*b*; Fast, Logsdon, and Doolittle 1999). In these analyses, the microsporidia are a sister group to fungi but do not actually arise from within the group. Similarly, molecular features uniting microsporidia with fungi really unite them with both animals and fungi (Kamaishi et al. 1996*a,* 1996*b*; Vivarès et al. 1996). These phylogenies and features are not sufficiently resolved or sampled to address the issue of whether microsporidia are highly derived fungi or only close relatives of fungi.

In this respect, alpha- and beta-tubulin phylogenies appear to be exceptions: both include representatives from ascomycete and basidiomycete fungi, and in both, the microsporidia branch within the fungal clade, specifically associated with ascomycete sequences (Edlind et al. 1996; Keeling and Doolittle 1996; Keeling, Deane, and McFadden 1998). These results hint at the possibility that microsporidia may have evolved from a bona fide fungus; however, the sampling of fungal tubulins hardly approaches the known diversity of fungi. Ascomycetes and basidiomycetes are only two of the four fungal divisions and are generally thought to be the most closely related (Bruns et al. 1992; Berbee and Taylor 1993; Paquin et al. 1995). In addition, microsporidia branch within the fungi by only one or two nodes in these trees.

Here, we addressed the question of whether microsporidia evolved from a fungus or from a protozoan relative of the fungi by sampling beta-tubulin genes from a broad diversity of both microsporidia and fungi. We sequenced 29 beta-tubulins from microsporidia, zygomycetes, and chytrids, greatly increasing the diversity of microsporidian tubulins and expanding fungal tubulins to include representation from all four fungal divisions. The resulting beta-tubulin phylogeny is in general agreement with what is believed to be the organismal phylogeny of the two groups and shows that the microsporidian beta-tubulins emerge from within the fungal clade. These results provide the first clear demonstration that microsporidia evolved from a fungus.

Materials and Methods

Strains

DNA from the microsporidia *Glugea plecoglossi, Spraguea lophii,* and *Trachipleistophora hominis* were gifts from T. Hashimoto (Rockefeller University), Greg Hinkle (Cereon Genomics), and Sarah Cheney (Imperial College of Science, Technology and Medicine), respectively. DNA from the chytrids *Spizellomyces punctatus, Harpochytrium* sp. (strain 94), *Rhizophlyctis rosea,* and *Allomyces macrogynus* (strain ATCC 46923) and the zygomycetes *Mortierella verticillata* (strain NRRL 6337), *Linderina pennispora* (strain NRRL 3781), and *Rhizopus oligosporus* (strain NRRL 2710) were gifts from Franz Lang (Université de Montréal). DNA from the zygomycetes *Micromucor ramanniana* (strain NRRL 5844), *Smittium culisetae* (strain Col-18-3), *Furculomyces boomerangus* (strain AUS-42-7), *Spiromyces minutus* (strain NRRL 3067), *Syncephalis depressa* (strain NRRL 22627), *Basidiobolus ranarum* (strain NRRL 20525), *Conidiobolus coronatus* (strain NRRL 1912), *Entomophaga maimaiga* (strain ARSEF 1400), and *Capniomyces stellatus* (strain MIS-10-108) were gifts from Kerry O'Donnell (U.S. Department of Agriculture, National Center for Agricultural Utilization Research).

Amplification, Cloning, and Sequencing of Beta-Tubulins

Except where noted below, approximately 90% of the beta-tubulin-coding region was amplified using the primers GCCTGCAGGNCARTGYGGNAAYCA and GGCCTCAGTRAAYTCCATYTCRTCCAT. Similar-sized fragments of beta-tubulin were amplified from *Spizellomyces* with primers AACCAGATCGGCGCGAARTTYTGGGA and CTCGTCCATGCCYTCNCCNGTRTACCA. Smaller fragments of beta-tubulin were amplified from *Allomyces, Harpochytrium,* and *Mortierella* with primers GATACCGTNGTNGARCCNTAYAA and GGCCTCAGTRAAYTCCATYTCRTCCAT. Alpha-tubulin from *Spizellomyces* was amplified with primers GCGCGAATTCARGTNGGNAAYGCNTGYTGGGA and CGCGCCATNCCYTCNCCNACRTACCA. All amplifications were carried out with an annealing temperature of 45°C and an extension time of 1.5 min. PCR products were separated by agarose gel electrophoresis and cloned using either a TA or a TOPO TA cloning kit (Invitrogen). From each species, multiple clones were sequenced on both strands using ABI or LiCor chemistry. In most cases, all of the clones of one product proved to be identical, but in cases in which variation was observed, additional clones were sequenced and variants were treated as separate copies of the gene. The new sequences are deposited in GenBank under accession numbers AF162056–AF162085.

Phylogenetic Analyses

Conceptual translations of new tubulin genes were aligned with existing homologs from GenBank and an unpublished *Vittaforma corneae* sequence (generously provided by T. Edlind). Phylogenetic trees were inferred from an alignment of 428 amino acids using quartet puzzling, protein maximum likelihood (ML), and several distance methods. PUZZLE, version 4.0.1 (Strimmer and von Haeseler 1996), was used to calculate ML distances corrected by the JTT substitution frequency ma-

trix with amino acid usage estimated from the data, site-to-site rate variation modeled on a gamma distribution with six or eight rate categories plus invariant sites, and the shape parameter estimated from the data. (Typically six categories were used, but for the smaller data sets of 26–27 animal, fungal, and microsporidian genes, eight categories were used.) PROTDIST was also used to calculate distances corrected by the Dayhoff PAM 250 substitution frequency matrix. Trees were constructed from these distance calculations using the neighbor-joining and Fitch-Margoliash algorithms (with the BioNJ and FITCH programs, respectively: Gascuel 1997; Felsenstein 1993). Fitch-Margoliash trees were searched using the global rearrangement option and 10 random additions. Bootstrap data sets were created using SEQBOOT, and bootstrap distances were calculated with PUZZLE, version 4.0.1, using the settings described above (the alpha parameter was recalculated for each bootstrap in data sets with fewer than 30 taxa, and for others the alpha parameter from the original data was used) with the shell script puzzleboot (by M. Holder and A. Roger) and using PROTDIST. All trees shown are BioNJ trees of gamma-corrected ML distances; other distance analyses were very similar for strongly supported relationships and are only discussed if they differ from the trees shown.

Quartet puzzling trees were constructed using PUZZLE, version 4.0.1, with the settings described for calculating ML distances and 10,000 puzzling steps limited for the 26- and 27-sequence data sets and 5,000 for all others. Protein ML trees were inferred using PROTML, version 2.3 (Adachi and Hasegawa 1992), using a JTT substitution frequency matrix with amino acid usage estimated from the data. Trees were searched using the quick-add option for data sets with fewer than 30 taxa. Exhaustive searches of partially constrained trees were also performed as noted. Resampling estimated log likelihood bootstrap (RELL; Hasegawa and Kishino 1994) and relative likelihood support (RLS; Jermiin et al. 1997) values were determined from the best 1,000 trees with exhaustive and quick-add searches. RLS scores were determined with an alpha value of 0.01 and a class V (exponential) distribution. Kishino-Hasegawa tests (Kishino and Hasegawa 1989) were performed on partially unresolved trees using PROTML, version 2.3, or PUZZLE, version 4.0.1, with the parameters described above.

Results and Discussion

Sequencing of Microsporidian and Fungal Beta-Tubulin Genes

Tubulins are frequently found in multiple copies in a genome, and any two genes from a single organism can range from being identical to being highly divergent. Here, multiple beta-tubulin gene clones were sequenced from each organism, and these show similar ranges. In 10 cases, a single form of the gene was isolated. Among the nine taxa for which multiple distinct coding regions were isolated, the two genes isolated from *Glugea, Allomyces,* and *Conidiobolus* each varied at only a few synonymous positions, whereas the others contained more divergent paralogs. In the case of *Rhizopus,* three copies were found, with two being nearly identical and the third being more divergent. Sequence was obtained from only the 3′ ends of genes from *Allomyces* and *Mortierella* and one of two genes from *Harpochytrium,* as the whole gene could not be amplified. We attempted to amplify the remainder of these genes using various strategies, but in no case were we completely successful in doing so. (Additional sequence was obtained from one *Mortierella* gene, but it was still truncated within a large intron.) These genes are not included in the phylogenetic analyses shown below, since nearly half the region considered in the phylogeny is missing. Including these sequences has no effect on any of the strongly supported tree features: *Allomyces* branches with the main clade of chytrids with high support, *Harpochytrium* 2 branches with *Spizellomyces* 2, and *Mortierella* 1 and 2 branch with the other zygomycetes, or between the chytrids and zygomycetes (not shown).

Overall, the intron sizes and densities of the newly characterized genes were quite variable. Three groups of shared introns were found: four shared between *Rhizophlyctis* and *Harpochytrium* 1; one shared between *Conidiobolus, Smittium, Capniomyces,* and *Furculomyces*; and two shared between *Micromucor* and all three *Rhizopus* genes, with one of these also shared with *Entomophaga.* In addition, the *Micromucor* gene contained an additional intron bounded by noncanonical AT-AC splice sites. Such introns were only recently discovered and have been found to be rare but fairly widely distributed in eukaryotic genomes (Tarn and Steitz 1997; Burge, Padgett, and Sharp 1998).

Phylogeny of Fungal and Microsporidian Beta-Tubulins

The overall structure of beta-tubulin trees has been consistently found to include a moderately supported clade of microsporidia and fungi, with animals as their next closest relative and the rest of the eukaryotes more distant (Edlind et al. 1996; Keeling and Doolittle 1996; Keeling, Deane, and McFadden 1998). In trees constructed with a variety of eukaryotes plus the fungal and microsporidian sequences reported here, the same overall relationships are recovered with support values similar to or slightly lower than those reported in published analyses (fig. 1). The fungal sequences tend to be more divergent than most tubulins, and there is a considerable degree of heterogeneity in this divergence, especially in the chytrid and zygomycete sequences. Not surprisingly, in different analyses the branching positions of several of the more divergent fungal sequences are inconsistent, and many of these genes branch in unexpected phylogenetic positions (with little or no support). This is particularly evident among zygomycetes, which have the most pronounced rate heterogeneity and are multiply polyphyletic, and it is also seen in the comparatively divergent *Spizellomyces* 2, which branches outside the clade of otherwise highly conserved chytrid genes. Interestingly, trees constructed with diverse representation

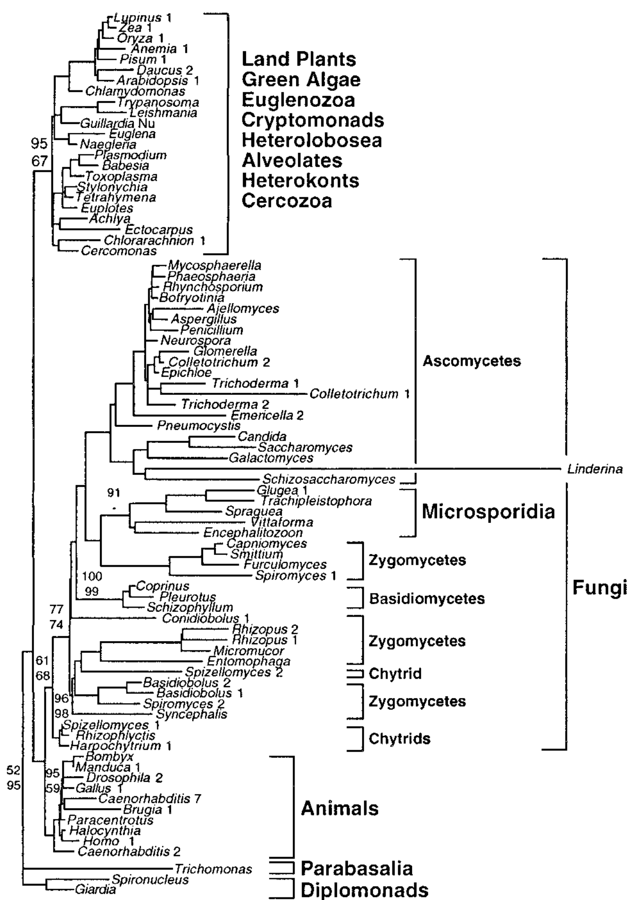

FIG. 1.—Phylogeny of diverse eukaryotes based on beta-tubulin; BioNJ tree of maximum-likelihood (ML) distances corrected for site-to-site rate variation. Numbers at selected nodes indicate support greater than 50% from neighbor-joining of gamma-corrected ML distance bootstraps (top) and percentage of occurrence in the quartet puzzling tree (bottom).

of eukaryotes, but only the most conserved representatives of each fungal division, are apparently much more robust. In these analyses (not shown), the fungal-microsporidian clade and the animal-fungal-microsporidian clade both gained much higher support from distance (81% and 87% bootstrap, respectively), and with ML, all nonfungal positions for microsporidia were rejected by Kishino-Hasegawa tests at confidence levels of >95%.

To allow more comprehensive analyses and to exclude the potential effects of distant outgroups, the branching order between fungi and microsporidia was further examined using only animals as an outgroup. Figure 2*A* shows a tree including the same selection of fungal tubulins with an animal outgroup. In contrast to figure 1, the four fungal divisions are monophyletic in this tree (with the single exception of the divergent *Spizellomyces* 2 gene), and their branching order reflects the expected pattern, with chytrids branching first, followed by zygomycetes and, finally, basidiomycetes and ascomycetes (Bruns et al. 1992; Berbee and Taylor 1993; Paquin et al. 1995). Nevertheless, the overall bootstrap and quartet puzzling support for this tree tends to be low, with one important exception being the relatively strong support for the divergence of chytrids before other fungi or microsporidia. As with the larger analysis shown in figure 1, many of the surprisingly poorly supported features were found to be affected by the more divergent sequences (especially *Linderina*), so once again, trees were inferred using only the most conserved representatives of each of the four fungal divisions (fig. 2*B* and *C*). In all such analyses, the chytrids were the deepest branch of fungi, followed by zygomycetes and then basidiomycetes and ascomycetes. The overall support for the branching order among the fungal divisions and microsporidia was generally much higher in these reduced data sets, and the basal divergence of the chytrids continued to be recovered with relatively high support. However, the position of the microsporidia within these trees varied depending on the presence or absence of a single zygomycete, *Conidiobolus.* When *Conidiobolus* was excluded, the microsporidia branched with the ascomycetes (fig. 2*B*), but when it was included, the microsporidia branched specifically with *Conidiobolus,* and zygomycetes were paraphyletic (fig. 2*C*). In summary, the phylogeny of fungal and microsporidian beta-tubulins strongly supports the inclusion of the microsporidia within the fungal clade by showing that they evolved sometime after the divergence of chytrids. The exact relationships between microsporidia and other fungal divisions is not clear, however, since microsporidia branch with either ascomycetes or zygomycetes, depending on the analysis.

Long-Branch Attraction and the Fungal-Microsporidian Relationship

The first molecular phylogenies to include microsporidia were rRNAs and elongation factors, and these invariably supported the ancient origin of the group by placing them deep among eukaryotes (Vossbrinck et al. 1987; Kamaishi et al. 1996*a,* 1996*b*). However, these microsporidian genes are all highly divergent compared with other eukaryotic homologs, and following the accumulation of evidence that microsporidia are actually related to fungi, it was suggested that these divergent microsporidian genes branched deeply due to spurious attraction to other long branches at the base of eukaryotes (Embley and Hirt 1998; Keeling 1998; Philippe and Adoutte 1998). Likewise, it is a serious concern that all previously characterized microsporidian and fungal tubulins are also quite divergent, as this might suggest that the position of microsporidia in tubulin phylogenies is also an artifact of long-branch attraction rather than a reflection of a close relationship between microsporidia and fungi (Keeling and Doolittle 1996).

The present finding that not all fungal tubulins are noticeably divergent, and specifically that most of the chytrid genes are quite highly conserved, leads to an important test of the relationship between microsporidian and fungal tubulins. If this relationship were due to long-branch attraction, then inferring the phylogeny of beta-tubulin with only the highly conserved chytrid sequences representing fungi should lead to the microsporidia branching with their true relatives or with some

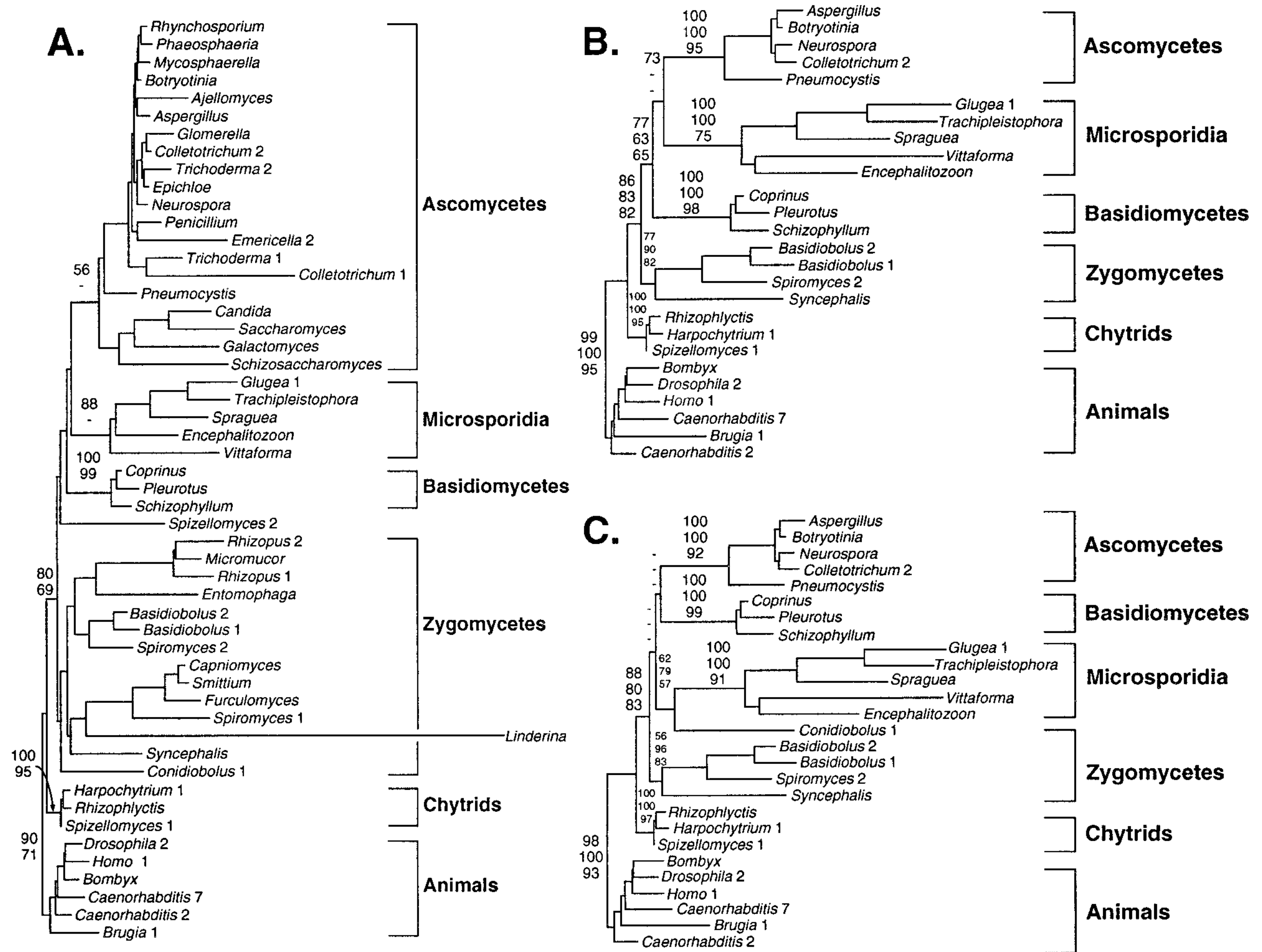

FIG. 2.—Phylogeny of microsporidian and fungal beta-tubulins with animals as the outgroup; BioNJ trees of maximum-likelihood (ML) distances corrected for site-to-site rate variation. *A,* Diverse fungi and microsporidia represented. Numbers at selected nodes refer to support greater than 50% from (top to bottom) neighbor-joining of gamma-corrected ML distance bootstraps, and percentage of occurrence in the quartet puzzling tree. *B* and *C,* Phylogeny of beta-tubulins from microsporidia and the most conserved representatives of the four fungal divisions, excluding *Conidiobolus* (*B*) and including *Conidiobolus* (*C*). Numbers at selected nodes indicate support greater than 50% from (top to bottom) neighbor-joining of gamma-corrected ML distance bootstraps, ML resampling estimated log likelihood (RELL) bootstraps from quick-add search, and percentage of occurrence in the quartet puzzling tree. Other distance bootstrapping scores were similar to those shown, and relative likelihood support scores were similar to RELL bootstraps, so for clarity, only representatives are shown.

other long branch. If, alternatively, the relationship between microsporidian and fungal tubulins is genuine, then the divergent microsporidian and conserved chytrid fungal tubulins should still branch together despite the high degree of rate heterogeneity between them.

In such an analysis of beta-tubulins from a wide variety of eukaryotes, the latter result is found (fig. 3*A*), suggesting that the relationship between microsporidian and fungal beta-tubulins is indeed genuine. Although the clade uniting the two is only weakly supported by bootstrap and quartet puzzling, it is found consistently with different methods, and the node uniting animals, fungi, and microsporidia is fairly well supported, excluding a relationship with any other eukaryote, including diplomonads and parabasalia. The position of the microsporidia with the chytrid fungi was also compared directly with six alternative positions by Kishino-Hasegawa tests (fig. 3*A*). Of these alternatives, positioning microsporidia with chytrids is favored, but not significantly so, over placing them with animals or animals plus chytrids. In contrast, all other positions, including those with diplomonads and parabasalia, are rejected at confidence levels between 90% and 95%.

That microsporidian beta-tubulins retain their phylogenetic affinity to even the highly conserved chytrid genes is compelling evidence that the beta-tubulin tree correctly reconstructs the relationship between microsporidia and fungi independently of divergence levels. To further test this hypothesis, we sequenced the alpha-tubulin gene from the chytrid *Spizellomyces,* expecting that the same would be true of alpha-tubulin phylogeny. As with beta-tubulin, the microsporidian and chytrid fungal alpha-tubulins retain a specific relationship in alpha-tubulin phylogeny despite an even greater disparity in evolutionary rates (fig. 3*B*). Similarly, the node uniting the microsporidia and *Spizellomyces* was not very

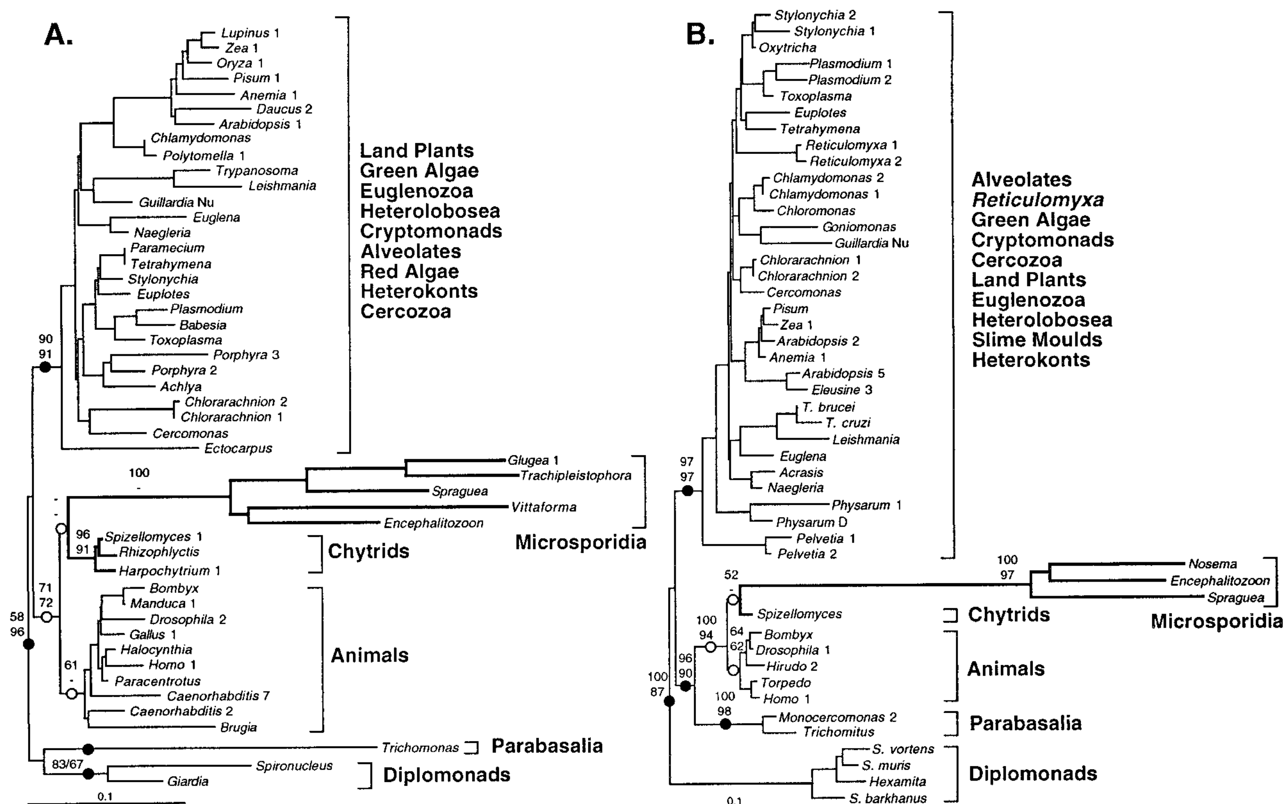

FIG. 3.—Phylogenetic relationships between (*A*) beta-tubulin and (*B*) alpha-tubulin from microsporidia and chytrid fungi, with diplomonads as the outgroup; BioNJ trees of maximum-likelihood (ML) distances corrected for site-to-site rate variation. Numbers at selected nodes indicate support greater than 50% from gamma-corrected neighbor-joining bootstraps (top) and percentage of occurrence in the quartet puzzling tree (bottom). Alternative positions for microsporidia were assessed with the Kishino-Hasegawa test at nodes with circles. In both trees, the fungal position was preferred, while nodes with open circles were not rejected at confidence levels of 90% and nodes with closed circles were rejected (at confidence levels over 90% for beta-tubulin and over 95% for alpha-tubulin).

strongly supported, but the node uniting animals, *Spizellomyces,* and microsporidia was quite strong. Kishino-Hasegawa tests also favored the grouping of microsporidian and chytrid alpha-tubulins, and, again, the alternative positions with animals or animals plus fungi were not excluded at a confidence of 95%, while all other alternatives were. In this case, the alternatives that placed microsporidia with the diplomonads and parabasalia were resoundingly rejected, with confidence levels over 99%.

It is interesting to note that alpha- and beta-tubulins from both of the other purportedly ancient protist lineages, diplomonads and parabasalia, are also relatively divergent. The fact that microsporidia never branch with these lineages in these analyses is a strong statement that microsporidia do not share a close phylogenetic relationship with these protists and that the position of the microsporidia in the tubulin trees is not dictated by their level of divergence.

While these analyses certainly argue that the overall relationship between microsporidia and fungi in tubulin phylogeny is not simply an artifact of long-branch attraction, the relative divergence of their tubulins is perhaps still relevant to the position of microsporidia within the fungi. On the one hand, it is possible that microsporidia actually arose from within one of the fungal divisions but are drawn outside of this clade by the long branch leading to that division. A potentially analogous situation is seen for ascomycetes, in which several divergent yeast beta-tubulins cluster together beneath the archeoascomycete, *Pneumocystis* (fig. 2*A*). On the other hand, we cannot rule out the possibility that the divergent zygomycete, basidiomycete, and ascomycete tubulins have artificially drawn microsporidia within the fungal clade, perhaps from a sister group relationship with fungi. Interestingly, organisms that have lost 9 + 2 structures in all stages of their life histories tend to have more divergent tubulins (Philippe and Adoutte 1998; Keeling, Deane, and McFadden 1998), and this is also true for the fungi: zygomycetes, basidiomycetes, ascomycetes, and microsporidia all lack 9 + 2 structures and have divergent tubulins, whereas (most) chytrids have flagella in sexual stages and have correspondingly conserved tubulins. Ironically, this shared divergence, which might spuriously group microsporidia within the fungi, is perhaps more likely a result of the shared absence of 9 + 2 microtubule structures, which is itself evidence that microsporidia evolved from within the fungi—after the evolution of chytrids and after the loss of flagella.

Conclusions—Reinterpreting Microsporidia

Ever since the first phylogenetic evidence from tubulins that microsporidia are related to fungi, a number of explanations have been proffered to account for the conflicting stories generated by different genes (e.g., Sogin 1997; Philippe and Adoutte 1998). Some of the criticisms aimed specifically at the position of microsporidia in tubulin trees are that it could be the result of long-branch attraction (Keeling and Doolittle 1996), that tubulins cannot be accurately rooted (Hirt et al. 1999), or that microsporidia acquired their tubulin genes from a fungal host (Sogin 1997). The new data from chytrid fungi argue against long-branch attraction being solely responsible for the placement of microsporidia with fungi (fig. 3). As for rooting tubulins, it has been shown that using one tubulin as an outgroup to another probably does generate an unreliable root of eukaryotes (Keeling and Doolittle 1996). However, this is probably true for all molecular phylogenies, regardless of whether eukaryotes are rooted using a paralog (as with tubulins) or a prokaryotic outgroup. More to the point, the position of the root is totally irrelevant to our interpretation of the relationship between microsporidia and fungi unless the root is actually within either of these groups. There is certainly no evidence to suggest this, and furthermore, it demands that both microsporidia and fungi are more ancient than all other eukaryotes. Lateral transfer of tubulins is also extremely unlikely, since no transfer of a tubulin has ever been observed, both the alpha- and beta-tubulins show the same relationship and hence both would have to have been transferred from similar donors, and no microsporidian has ever been reported to infect a fungus. Indeed, almost all described microsporidia infect animals, and the rest infect protists that are themselves animal parasites (gregarines, myxosporidia, and certain ciliates). This suggests that microsporidia originated as animal parasites and that other targets of microsporidian infection are probably later, opportunistic developments.

Rather than evoking a complicated scheme of evolutionary events to explain why various molecular phylogenies disagree, it seems simpler to conclude that phylogenetic reconstruction is breaking down with many microsporidian genes. Virtually all known microsporidian sequences show evidence of accelerated rates. Perhaps some genes, especially for the translation apparatus, have evolved so rapidly as to lose any evolutionary signal, while others have retained enough information to give a more or less accurate placement of the lineage. This kind of difficulty is acute for microsporidia, but is not unique to the group, and it is well known that the ‘‘deep’’ branches of a tree are generally the most challenging to accurately reconstruct and interpret (Philippe and Adoutte 1998; Philippe and Laurent 1998; Embley and Hirt 1998; Roger et al. 1999). In the case of microsporidia, the earlier evidence that placed them deep was widely distrusted only when an opposing camp of evidence was established from several different gene phylogenies that all converged on a single alternative—the fungal origin of microsporidia. Discerning whether microsporidia branch within fungi or as sisters to the fungi is a similar question on a different scale and will only be convincingly resolved with the convergence of several well-sampled gene trees on a single answer.

Morphological and life cycle data are consistent with microsporidia having originated from within fungi, but these data do not conclusively link microsporidia with a particular kind of fungus. What few characteristics microsporidia do share with fungi can be divided into two categories: some are stable absence characters that only separate microsporidia from most chytrids (lack of 9 + 2 microtubule structures and lack of stacked Golgi dictyostomes), while others are typically not very strong and are scattered among various zygomycetes, basidiomycetes, and ascomycetes, as well as nonfungi (the presence of centrosomal plaques, closed mitosis, and dikaryotic life stages). Such data have recently been summarized and used to argue that certain harpellalean zygomycetes may have evolved into microsporidia (Cavalier-Smith 1998). However, the primary structural characteristic uniting them is the possible homology of the harpellalean apical spore body and the microsporidian polar tube. This is reminiscent of previous classifications of microsporidia with actinomyxidia and myxosporidia based on apparent similarities between their extrusion apparatuses. In these cases, any similarities are now known to be superficial, so it is prudent to consider the relevance of the apical spore body only with the addition of more reliable evidence. Indeed, microsporidian polar tube eversion is also reminiscent of spore dispersion in *Conidiobolus* (Ingold 1971), and *Conidiobolus* branches with microsporidia in some analyses of beta-tubulin (fig. 2*C*). As a whole, the distribution of morphological characteristics that unite microsporidia and fungi is consistent with microsporidia originating from any one of a number of fungal lineages, but no single character is unique to microsporidia and one kind of fungus. Molecular data might well be the only evidence we ever have in cases such as this, in which microsporidia have undergone such extraordinarily divergent and reductive evolution, losing most of the morphological and molecular clues as to the exact nature of their fungal heritage.

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