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Animals and fungi are each other's closest relatives: Congruent evidence from multiple proteins

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ABSTRACT **Phylogenetic relationships among plants, animals, and fungi were examined by using sequences from 25 proteins. Four insertions/deletions were found that are shared by two of the three taxonomic groups in question, and all four are uniquely shared by animals and fungi relative to plants, protists, and bacteria. These include a 12-amino acid insertion in translation elongation factor 1α and three small gaps in enolase. Maximum-parsimony trees were constructed from published data for four of the most broadly sequenced of the 25 proteins, actin, α-tubulin, β-tubulin, and elongation factor 1α, with the latter supplemented by three new outgroup sequences. All four proteins place animals and fungi together as a monophyletic group to the exclusion of plants and a broad diversity of protists. In all cases, bootstrap analyses show no support for either an animal–plant or fungal–plant clade. This congruence among multiple lines of evidence strongly suggests, in contrast to traditional and current classification, that animals and fungi are sister groups while plants constitute an independent evolutionary lineage.**

The traditional classification of fungi as plants has been largely replaced over the past three decades by the five-kingdom classification of Whittaker (1, 2). In this scheme, the three major multicellular groups—animals, fungi, and green plants—are each given the status of kingdoms derived from different protistan lineages of uncertain affinities. More recently, however, based on a combination of ultrastructural and biochemical characters, Cavalier-Smith (3) proposed that animals and fungi are closely related and share a most recent common ancestor similar to present-day choanoflagellates. Molecular phylogenies of ribosomal RNA (rRNA) sequences have alternatively placed either plants or fungi as more closely related to animals with about equal frequency (4–9). However, the most recent and comprehensive rRNA analysis supported animals as the sister group to fungi (9), as did an analysis of a subset of elongation factor 1α (EF-1α) sequences (10).

The only analysis of this question using multiple lines of molecular evidence used rRNA, transfer RNA (tRNA), and protein sequences and supported plants as the sister group to the animals (11). However, this analysis was critically limited by the narrow representation of major taxonomic groups available at the time. We have reexamined this question by using several different proteins, each representing a wide diversity of eukaryotic phyla, as well as by surveying a large number of proteins for diagnostic insertions and deletions. From these, we find multiple lines of data all showing that animals and fungi are most closely related to each other to the exclusion of a broad diversity of eukaryotic phyla, including plants.‡

MATERIALS AND METHODS

An internal portion of the EF-1α gene was amplified from *Giardia lamblia*, *Trypanosoma brucei*, and *Staphylothermus marinus* by the polymerase chain reaction (PCR). DNA was amplified through 40 cycles of 1 min at 92°C, 1.5 min at 50°C or 55°C, and 2 min at 72°C with a set of 16- and 32-fold degenerate primers. Products were cloned, and sequence was determined on both strands for at least three independent clones. Clones were hybridized to Southern blots of at least two different restriction digests each of total DNA from *T. brucei*, *Paramecium aurelia*, and six different archaebacteria, and *G. lamblia* DNA from four independent sources. This confirmed the origin of each PCR product and also showed the gene to be single copy in all three taxa. The *S. marinus*, *T. brucei*, and *G. lamblia* sequences correspond to amino acids 20–304, 20–422, and 63–304 of animal EF-1α, respectively.

Sequences were aligned on a Sun workstation using the Wisconsin Genetics Computer Group program PILEUP (12) with default gap penalties. Alignments were then modified by eye to minimize insertion/deletion events. For phylogeny reconstruction, identical or nearly identical sequences were identified by pairwise comparisons, and a single representative was chosen for each set. For the four proteins analyzed, a total of five short amino- and carboxyl-terminal regions were omitted, as these were not alignable with confidence among all sequences; for EF-1α, a single region ambiguous among archaebacteria was included and scored as missing data for these taxa. All alignments and the coordinates of deleted regions are available from S.L.B. upon request.

Maximum-parsimony trees were derived from amino acid sequences by using PAUP 3.0r (13). Shortest tree, decay (14), and alternative topology searches used 50 replicates of random addition with branch swapping by tree bisection–reconnection (TBR). In all cases, all shortest trees were found within the first one to four random replicates, suggesting that this heuristic search strategy was effective in identifying all most-parsimonious trees (D. Swofford, personal communication). In addition, when trees were trimmed of closely related taxa, all shortest trees were found on the first replicate and discovered on all subsequent relicates, suggesting that any search islands (15) that might exist were restricted to terminal clades. Bootstrap analyses (16) used 100 replicates of simple addition, holding one tree at each step. Due to computer memory limitations, all data sets except EF-1α required trimming for either decay and/or bootstrap analyses as indicated in the figure legends, although this was still insufficient to allow decay analysis of actin. All trees are rooted with kinetoplastids except for the EF-1α tree, which is rooted with the archaebacteria. All data sets were also analyzed by distance with the PHYLIP 3.5C (17) programs PROTIST (weights based on Dayhoff PAM 001 and/or George–

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Abbreviation: EF-1α, elongation factor 1α.

‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L23957, L23984, and L25868).

Hunt–Barker categories) and FITCH. EF-1α and β-tubulin nucleotide sequences were analyzed—with third positions deleted—by both parsimony (13) and distance (DNADIST, all changes weighted equally; ref. 17).

RESULTS

Four Gaps Unite Animals and Fungi. In the course of our work (18) on EF-1α (also known as EF-Tu), we discovered a gap of 12 amino acids in an otherwise highly conserved region of the protein which appears to be an insertion (Fig. 1*A*). This sequence is found in all animal and fungal EF-1α proteins but not in those of any other organism examined, including plants, six diverse protists, and archaebacteria (Fig. 1*A*). Taken together, the conserved size, sequence, and placement of the insertion all make it unlikely that this sequence was either inserted twice independently, once in animals and once in fungi, or present earlier but deleted independently and precisely in one or more of the other lineages. The simplest interpretation of this gap is a single insertion event in a common ancestor shared solely by animals and fungi.

An extensive search of the nucleotide and amino acid sequence data bases identified 24 additional proteins for which plant, animal, fungal, and outgroup sequences were available and alignable with confidence. These are actin, α- and β-tubulins, alcohol dehydrogenase, calmodulin, catalase, citrate synthase, Cu-Zn and Fe-Mn superoxide dismutases, elongation factor G, enolase, fructose bisphosphatase, glyceraldehyde-3-phosphate dehydrogenase, hydroxymethylglutaryl-CoA reductase, malate dehydrogenase, phosphoglycerate kinase, phosphoglucose isomerase, pyruvate kinase, RNA polymerase II largest subunit, triosephosphate isomerase, vacuolar ATPase subunits 1 and 2, and the 60- and 70-kDa heat shock proteins.

Analysis of the 24 proteins revealed only three gaps, all in enolase, that united two of the three groups of interest. Like the EF-1α insertion, the two deletions and one insertion in enolase are all uniquely shared by animals and fungi (Fig. 1*B*). Parsimony analyses indicate a eukaryotic origin of the plant enolase (data not shown), ruling out a possible eubacterial/plastid ancestry for this protein (a cytoplasmic enzyme; ref. 20). Although the enolase gaps are small (one or two amino acids) and, in two cases, are in regions affected by additional insertions and deletions, the simplest interpretation in each case is a single event in an exclusive common ancestor of animals and fungi.

Molecular Phylogenetic Support for an Animal–Fungal Clade. Four criteria were set for selecting proteins for extensive phylogenetic analysis. Proteins were chosen that were (*i*) >300 amino acids long, (*ii*) available from multiple representatives each of animals, plants, and fungi; (*iii*) available from a range of protistan lineages; and (*iv*) free from problems of gene duplication, substitution, or horizontal evolution at relevant taxonomic levels. These proteins gave the most consistent results—i.e., branching patterns were least affected by inclusion or exclusion of individual taxa. Inclusion of multiple representatives of ingroup as well as outgroup lineages is especially important in phylogeny reconstruction in order to avoid systematic errors in the placement of long, sparsely sampled branches (21).

Of the 25 total proteins only 4 currently satisfy the above criteria: actin, α-tubulin, β-tubulin, and EF-1α. Although these four proteins include some duplication, preliminary results have shown that all duplications are restricted to individual terminal clades (see Fig. 2) and are therefore irrelevant to the branching order among major groups. For EF-1α, the least broadly represented of the four proteins, a wider diversity of outgroup taxa was provided by sequencing the corresponding gene from the early-branching protists *G. lamblia* and *T. brucei* and from the archaebacterium *S. marinus*.

Without exception, the maximum parsimony trees for all four proteins placed animals and fungi together as sister lineages to the exclusion of all other eukaryotes, including plants (Fig. 2). Bootstrap analyses showed varying levels of support for the animal–fungal clade (100% for α-tubulin, 85% for β-tubulin, 53% for EF-1α, and 28% for actin) but no support for either an animal–plant or fungal–plant clade (<1.0% for actin, 0.0% for all others). Trees uniting plants with either animals or fungi required an additional 32 or 34 steps, respectively, for the α-tubulin tree, an additional 13 or 14 steps for the β-tubulin tree, 8 or 10 steps for the EF-1α tree, and an additional 5 steps for either topology for actin. Although placement of the root in all trees except EF-1α is essentially arbitrary, it should be noted that there is no way to root any of these trees so that animals–fungi are not monophyletic, unless either animals or fungi are used as the actual root. Thus, parsimony analysis of all four proteins supports the same conclusion as structural (insertion/deletion) data, that animals and fungi are most closely related to each other.

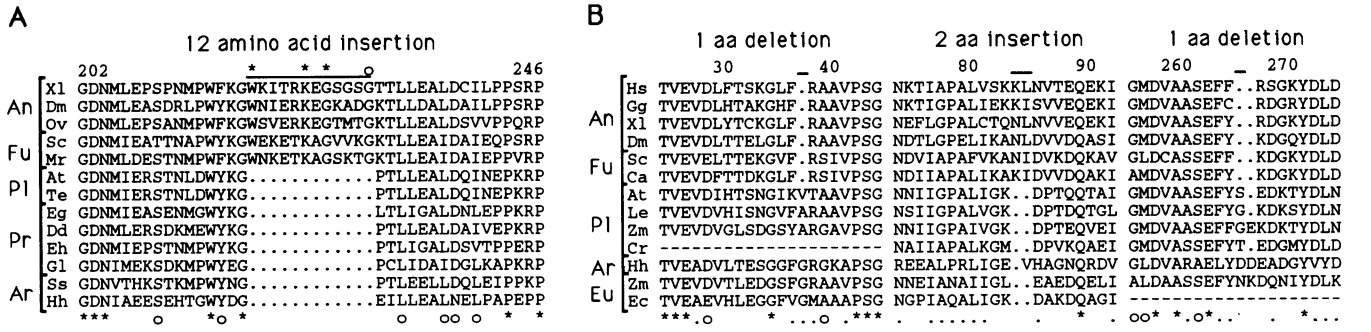

FIG. 1. A 12-amino acid insertion in EF-1α and three small gaps in enolase are all shared uniquely by animals and fungi. Portions of amino acid (aa) sequence alignments are shown for EF-1α (*A*) and enolase (*B*). Numbers above the sequence fragments indicate their position in the overall alignment. Within the alignment gaps are indicated by dots; missing data (incomplete sequences) are indicated by dashes. Universally conserved sites are denoted above and below the alignment by stars; sites with only conservative substitutions (19) are shown by open circles. Relatively conserved positions are defined as sites sustaining only one or two nonconservative substitutions and are denoted below the enolase alignment by dots. Letters to the left of the brackets indicate major taxonomic groups (Fu, fungi; An, animals; Pl, plants; Pr, protists; Ar, archaebacteria; Eu, eubacteria); letters to the right of the brackets indicate species (At, *Arabidopsis thaliana*; Ca, *Candida albicans*; Cr, *Chlamydomonas reinhardtii*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Ec, *Escherichia coli*; Eg, *Euglena gracilis*; Eh, *Entamoeba histolytica*; Gg, *Gallus gallus*; Gl, *Giardia lamblia*; Hh, *Halobacterium halobium*; Hs, *Homo sapiens*; Le, *Lycopersicon esculentum*; Ov, *Onchocerca volvulus*; Mr, *Mucor racemosus*; Sc, *Saccharomyces cerevisiae*; Ss, *Sulfolobus acidocaldarius*; Te, *Triticum aestivum*; Xl, *Xenopus laevis*; Zm, *Zea mays*; Zy, *Zymomonas mobilis*.

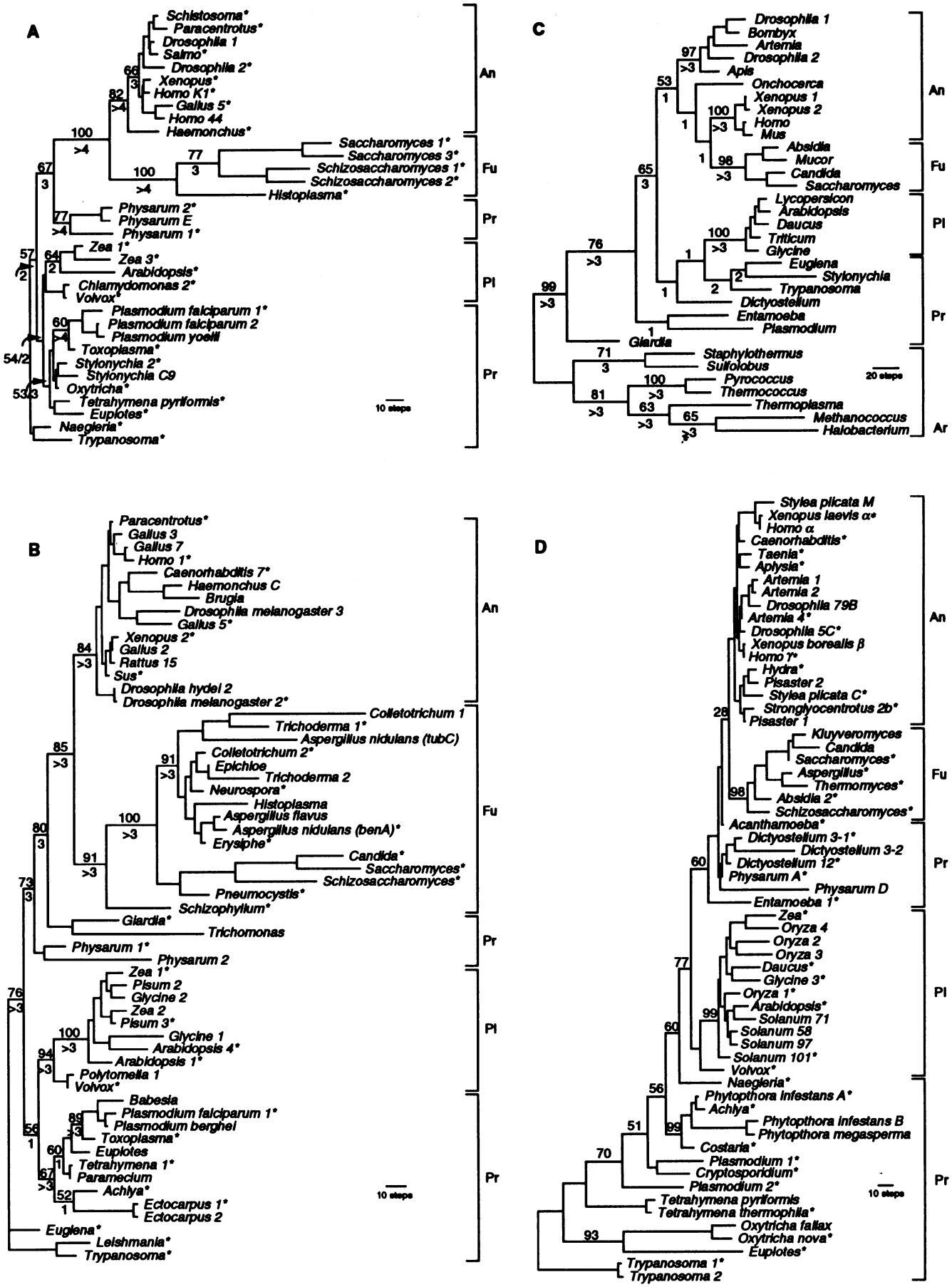

FIG. 2. (*Figure legend appears at the bottom of the opposite page.*)

Molecular phylogenetic support for an animal–fungal clade appears to be independent of the method of analysis used. Amino acid distance trees for all four proteins and nucleotide parsimony and distance trees for EF-1α and β-tubulin all placed animals and fungi together to the exclusion of all other taxa (data not shown). In fact, the overall branching pattern among major groups was nearly identical for all proteins by all methods used. Only EF-1α showed significant differences in topology depending on method; all distance-based analyses of EF-1α identified animals as a monophyletic group (vs. animals paraphyletic to fungi; Fig. 2*C*). In addition, all methods other than amino acid parsimony gave 99–100% bootstrap support for *Giardia* as the deepest branching eukaryote.

Interpretation of Bootstrap Values. Cursory examination of individual bootstrap replicates (17) showed that almost all replicates did in fact reproduce the animal–fungal clade but that this clade was often "contaminated" with various protists. This suggested that certain relatively derived protists might constitute unstable branches adversely affecting bootstrap support for nearby clades. Therefore, bootstrap analyses were repeated for actin and α- and β-tubulin with all sequences branching between the animal–fungal and plant clades deleted. With all relatively derived protists deleted, these data sets all showed >95% bootstrap support for animals–fungi (100% for α-tubulin without *Physarum*; 100% for β-tubulin without *Giardia* and *Trichomonas*; 97% for actin without *Acanthamoeba, Dictyostelium, Entamoeba,* and *Physarum*). For EF-1α, bootstrap values may also be affected by instability within the animal clade, as bootstrap support increased to 90–93% when animals were constrained to be a monophyletic group. Similarly, a maximum likelihood analysis using a subset of the EF-1α data and constraining animals as monophyletic found 93–96% bootstrap support for animals–fungi (10). In all cases, bootstrap support for a plant–animal or plant–fungal clade remained at 0.0%. These analyses show that all four proteins do, in fact, contain strong bootstrap support for the proposal that animals and fungi are more closely related to each other than either animals or fungi are to plants. It should be noted that these analyses no longer test the monophyly of animals–fungi *per se*, as they cannot rule out the possibility that the animal–fungal clade may also include one or more of the deleted protists. However, the instability of the various protistan clades, as well as their lack of congruence among our phylogenies, most likely reflects poor representation of these deep and diverse lineages. We suggest that more in-depth analyses of each individual protist group, similar to what we present here with plants/animal/fungi, will be necessary to accurately define their position in the eukaryote tree.

DISCUSSION

Animals and Fungi Are Sister Groups. Two kinds of molecular data, shared insertions/deletions and amino acid or nucleotide substitutions, provide strong corroborative support for fungi as the sister group to animals. These data are derived from five separate proteins of diverse function (EF-1α, protein synthesis; enolase, glycolysis; actin and tubulins, cytoskeleton). Although α- and β-tubulin are related by an early gene duplication, this duplication appears to predate the origin of all known eukaryotes (22) and to be unaffected by subsequent concerted evolution, making these proteins functionally independent data sets. Thus, five separate lines of evidence all place animals and fungi together to the exclusion of all other eukayotes examined. These results are also supported by small-subunit rRNA genes, which show 80–85% bootstrap support for an animal–fungal clade (9).

These results imply that animals and fungi should share a number of as yet undiscovered similarities at the molecular and cellular levels. Fungi and animals are unique in having a single, basal flagellum on motile cells (3). Both also contain mitochondria with a similarly reduced gene content and using UGA to encode tryptophan (23), which are clearly derived conditions but are also found in certain protists (23). Other features often cited as shared by animals and fungi, such as chitinous exoskeleta and the use of glycogen for carbohydrate storage, are also found in a variety of protistan lineages (24) and are therefore best interpreted as shared primitive characters probably lost in plants.

These results are of special relevance to the use of the yeast *Saccharomyces cerevisiae* as the model system of choice for eukaryotes. On the one hand, this yeast is now more clearly than ever the appropriate microbial model system for animals, including humans. On the other hand, the tendency to suppose that phenomena shared "from yeast to man" should also extend to other eukaryotes is even less appropriate than before and can now be viewed as anthropocentric in the broad sense. Since the best studied eukaryotes—i.e., yeast, human, fruit fly, and nematode—can now be seen to represent only a small corner of the eukaryotic kingdom, it becomes increasingly important that other eukaryotes, both the great diversity of protists as well as plants, be viewed as important objects of study in their own right.

If, as these results strongly suggest, animals and fungi are sister groups, then there may exist an as yet unsampled protistan lineage which is more closely related to both groups than are plants. One candidate is choanoflagellates, as suggested by Cavalier-Smith (3) and by rRNA analysis (9), or perhaps certain slime molds; *Physarum*, for example, branches closer to animals–fungi than to plants in all three protein phylogenies in which it is represented (Fig. 2 *A, B,* and *D*; but see, e.g., ref. 6). Choanoflagellates resemble both the probable earliest branch of animals, the sponges (25, 26), and the earliest branch of fungi, the chytrids (3). However, the similarity of fungi to the true slime molds has also been long recognized (27), and it has been noted that the latter share similarities with both animals (holotrophy, motility) and fungi (coenocytic condition, sessile fruiting body, meiospores) (31).

Phylogenetic Reconstruction: General Considerations. Molecular phylogenetic analyses of the four proteins that met our criteria showed universal support for animals as the sister group to fungi. Preliminary analyses of the other 21 data sets

FIG. 2. Four protein phylogenies support an animal–fungal clade. Phylogenetic trees derived by maximum parsimony analysis of amino acid sequences are shown for α-tubulin (*A*), β-tubulin (*B*), EF-1α (*C*), and actin (*D*). Bootstrap values above 50% are indicated above the nodes defining major groups only, and decay values (additional steps needed to collapse a node) below. Scale bars indicate numbers of inferred amino acid substitutions; brackets to the right indicate major taxonomic groups as in Fig. 1. Numbers or letters after species names indicate individual members of multigene families. Stars denote taxa used in bootstrap (α- and β-tubulin and actin) and decay (α- and β-tubulin) analyses performed with a subset of the taxa shown. (*A*) The α-tubulin tree shown is one of six shortest trees and has a total length of 749 steps, a consistency index (c.i.) of 0.65 (excluding uninformative characters), and a retention index (r.i.) of 0.80. Alternative shortest trees remove *Volvox* and *Chlamydomonas* from a sister-group relationship with angiosperms and place the ciliates as a monophyletic group. (*B*) The β-tubulin tree shown is one of eight shortest trees and has a length of 1295, c.i. = 0.49, r.i. = 0.73. The eight trees differ in the branching order within animals and within plants. (*C*) The EF-1α tree shown is the single shortest tree found and has a length of 1656, c.i. = 0.58, r.i. = 0.67. (*D*) The actin tree shown is one of two shortest trees, which differ in the branching order of *Naegleria* and the oomycete/Rhodophyte lineage. It has a length of 1268, c.i. = 0.56, r.i. = 0.70.

showed a roughly equal split between animals–fungi and animals–plants, with none supporting plants–fungi. However, most of these data sets are extremely small, often with only a single very distant outgroup and/or only a single plant and/or fungal sequence. Nonetheless, we can conclude that there is no support from this large body of data for the traditional classification of fungi as plants. The fact that all the data support only two of three possible alternatives suggests the presence of a specific artifact or artifacts which, considering the strength of our results and others (9), are either drawing plants and animals together or pushing animals and fungi apart. Possible sources of artifact include unequal rates of sequence evolution and paralogous or horizontal evolution. A striking example is glyceraldehyde-3-phosphate dehydrogenase; with yeasts alone representing fungi, animals and plants form a strong clade in the glyceraldehyde-3-phosphate dehydrogenase tree, but when a broad diversity of other fungi are included, these displace plants as the closest relative to animals (ref. 28; S.L.B. unpublished). Therefore, we emphasize again that proteins used in phylogeny reconstruction should include a broad representation of ingroup as well as outgroup lineages, both to help assess orthology and to minimize rate artifacts.

We find that all parsimony- and distance-based analyses of four large and diverse data sets support a sister-group relationship between animals and fungi. The varying levels of bootstrap support for animals–fungi are consistent with the tendency of bootstrap values to be inversely related to the size and complexity of the data sets analyzed (29). This is apparently due to the inability of the bootstrap, as currently implemented (16), to test other than strictly monophyletic groups (29). We chose to circumvent this problem by deleting an entire class of sequences of less immediate interest, the relatively-derived protists, in order to further examine relationships among our primary group of interest, plants/animals/fungi. The results of these analyses show that all our data sets do, in fact, strongly support animals–fungi over either alternative, consistent with the complete lack of bootstrap support for the latter, even with all taxa included. This use of the bootstrap to test alternative monophyletic groups is both an appropriate and highly informative application of the method (J. Felsenstein, personal communication). Recent findings also show that under a wide range of conditions, groups supported by bootstrap values above 70% have a >95% probability of representing true clades (30).

Despite the potential power of insertions and deletions as phylogenetic markers, our results suggest that they are probably too rare to be relied upon consistently for phylogeny reconstruction. Therefore, sequence-based phylogenies, of both proteins and rRNAs, will necessarily continue to be the focus of efforts to elucidate evolutionary relationships among eukaryotes. However, each phylogeny will have to be interpreted with caution, as no single gene or protein is likely to reproduce a completely accurate tree with all branches strongly supported. Rather the accumulation of congruent evidence from multiple independent sources, molecular and, inasmuch as possible, nonmolecular, will be required.

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