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Origin and evolution of the slime molds (Mycetozoa)

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ABSTRACT The Mycetozoa include the cellular (dictyostelid), acellular (myxogastrid), and protostelid slime molds. However, available molecular data are in disagreement on both the monophyly and phylogenetic position of the group. Ribosomal RNA trees show the myxogastrid and dictyostelid slime molds as unrelated early branching lineages, but actin and β -tubulin trees place them together as a single coherent (monophyletic) group, closely related to the animal–fungal clade. We have sequenced the elongation factor-1 α genes from one member of each division of the Mycetozoa, including *Dictyostelium discoideum*, for which cDNA sequences were previously available. Phylogenetic analyses of these sequences strongly support a monophyletic Mycetozoa, with the myxogastrid and dictyostelid slime molds most closely related to each other. All phylogenetic methods used also place this coherent Mycetozoan assemblage as emerging among the multicellular eukaryotes, tentatively supported as more closely related to animals + fungi than are green plants. With our data there are now three proteins that consistently support a monophyletic Mycetozoa and at least four that place these taxa within the “crown” of the eukaryote tree. We suggest that ribosomal RNA data should be more closely examined with regard to these questions, and we emphasize the importance of developing multiple sequence data sets.

Olive defines the Mycetozoa as consisting of three distinct groups (1). The true or plasmodial slime molds (Myxogastria—e.g., *Physarum polycephalum*) are amoeboid flagellates, most of which develop into large, reticulate plasmodia with $>10^4$ synchronously dividing nuclei. The cellular slime molds (Dictyostelia—e.g., *Dictyostelium discoideum*) are strictly amoeboid, and, under conditions of nutrient starvation, aggregate to form large, motile, multicellular slugs (1). The Protostelia, first described in the 1960s (2), are mostly microscopic but morphologically diverse organisms, with different taxa exhibiting various combinations of myxogastrid- and/or dictyostelid-like traits (1). All Mycetozoa share a structurally similar fruiting body consisting of a cellulosic stalk of one to many sterile cells supporting the spore-bearing sori (1). A fourth group of “slime molds,” the Acrasids, now appear to be entirely unrelated, on the basis of both ultrastructural (1) and molecular (3) data.

Since the slime molds were first described in the mid-1800s, opinions on the monophyly and phylogenetic affinity of these organisms have varied widely. The striking contrasts in the trophic stages of the myxogastrids and dictyostelids have often led to their being classified as entirely unrelated. Furthermore, the motile slug stage of the dictyostelids, the fungal-like plasmodia of the myxogastrids, and the plant-like fruiting bodies of both have led them, in whole or in part, to be classified as plants, animals, or fungi. In his original five-kingdom scheme of life, Whittaker placed the slime molds together at the base of the fungi (4), while admitting that they

stuck out of his mitten scheme “like a sore thumb” (5). Olive, however, argued that the slime molds have little in common with fungi and should be classified as protists (6).

Molecular phylogenies of rRNA genes show little or no support for a coherent Mycetozoa. In addition, these analyses usually show *Physarum* as arising early in the tree, among the first “mitochondriate” eukaryotes. These studies include analyses of the small subunit (SSU) or 16S-like rRNA using whole sequences (7) or universally alignable portions only (8), as well as analyses of the large subunit (23S-like) rRNA (9) and 5S rRNA (10). In contrast, actin and β -tubulin trees place *Physarum* and *Dictyostelium* together with generally high confidence (11–14). Furthermore, these trees, along with trees of α -tubulin (11, 14), RNA polymerase largest subunit (15), and glyceraldehyde-3-phosphate dehydrogenase (3), all place the represented mycetozoans among the multicellular eukaryotes, consistently closer to animals + fungi than are green plants in all but the polymerase trees. This position is also supported for *Dictyostelium* alone with a combined maximum likelihood analysis of 19 proteins (16).

The protein synthesis elongation factor-1 α (EF-1 α) appears to be well suited for deep-level phylogeny due to its slow rate of sequence evolution, its single or low copy number in all taxa examined to date, and the fact that the eukaryote EF-1 α tree can be rooted by using closely related archaeobacterial homologs (17). To evaluate the origin and possible phylogenetic coherence of the Mycetozoa, we have sequenced the EF-1 α -encoding (*tef*) genes from *Physarum polycephalum*, *Dictyostelium discoideum*, and an amoeboid flagellate protostelid, *Planoprotostelium aurantium*. Molecular phylogenetic analyses of these sequences strongly support the Mycetozoa as a monophyletic group. Furthermore, all methods of analysis place this group among the eukaryote “crown” taxa, possibly more closely related to the animal–fungal clade than are green plants.

METHODS

Cell Culture and DNA Extraction. *Planoprotostelium aurantium* was grown on the pink yeast, *Rhodotorula mucilaginosa*, on agar plates and in liquid media as described (18). DNA was extracted from 125-ml liquid cultures grown with gentle shaking at 25°C for 7–10 days. Cells were harvested by centrifugation at 500 \times g, leaving most yeast cells in suspension. The cell pellet was lysed in 0.1 M EDTA/0.25% SDS containing 50 mg/ml proteinase K for 1 hr at 37°C, extracted once each with equal volumes of chloroform and phenol/chloroform (1:1), and precipitated with ethanol. After resuspension in 10 mM Tris·NaOH, pH 8.0/1 mM EDTA, the DNA was purified once by extraction with glass beads. T. Burland (University of Wisconsin, Madison) provided *P. polycephalum* genomic

Abbreviations: EF-1 α , protein synthesis elongation factor-1 α ; SSU rRNA, small subunit ribosomal RNA.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF016239–43). A commentary on this article begins on page 11767.

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DNA, D. Pallotta and A. Laroche (Université Laval, Ste-Foy, PQ, Canada) provided *P. polycephalum* cDNA, and C. Singleton (Vanderbilt University, Nashville, TN) provided genomic DNA from *D. discoideum*.

PCR Amplification, Cloning, and DNA Sequencing. DNAs were amplified with various combinations of the primers described in Table 1. All amplifications used 40 cycles of 1 min each at 95°C, 50°C, and 72°C followed by a final, 10-min step at 72°C. Initial amplification products were electrophoresed through low-melting-point agarose gels, from which individual bands were excised and melted at 65°C, and 1–5 μ l was used in a second round of amplification in a total volume of 100 μ l. Secondary amplification products were also separated on low-melting-point agarose gels, and the appropriate bands were excised, extracted from the gel with glass beads, ligated into a T-tailed vector (InVitrogen), and used to transform the competent cells provided (INVaF').

Positive clones were initially identified by thermocycle screening of whole colonies using M13 primers (19). For each amplification product, a minimum of five clones were further screened by partial sequencing (20). Final sequencing was done on an Applied Biosystems and/or Licor automatic sequencer. Both DNA strands were sequenced in their entirety, and a minimum of one complete DNA strand was sequenced from at least two separate clones to control for *Taq* DNA polymerase errors. An error rate of approximately 1.2 errors per kilobase of sequence was found, and all discrepancies were resolved by partial sequencing of additional clones.

Phylogenetic Analyses. Because only four small, well defined areas of length variation are found in eukaryote EF-1 α (positions 1–7, 160–164, 217–228, and 450–end, Fig. 1), sequences were aligned by eye. Regions of length variation were omitted from analysis, as were the amino and carboxyl termini (positions 1–20 and 438–end; Fig. 1), which are missing from all PCR-generated sequences. The *Toxoplasma gondii* *tef*, encoding $\approx 75\%$ of the EF-1 α protein, was compiled from the EST (expressed sequence tag) database, using only those regions for which at least two ESTs were available. Preliminary distance trees (see below) of all available sequences were used to trim the initially large plant, animal, and fungal clades, with an emphasis on minimizing terminal branch lengths while retaining a broadly representative sampling of taxa. All non-constitutively expressed, developmental-stage-specific EF-1 α s were also omitted.

Sequences were analyzed at the amino acid level and at the nucleotide level, using first and second codon position nucleotides. Amino acid distance analyses utilized the PHYLIP 3.57c program PROTDIST with its Dayhoff weighting matrix (21) and a single outgroup (*Desulfurococcus*) as recommended (22). Trees were constructed separately by neighbor-joining and by the method of Fitch and Margoliash using 100 replicates of jumbled taxon addition order and global branch swapping (21). Distance analyses of nucleotides used the PHYLIP 3.57c program DNADIST with the Kimura two-parameter model and trees constructed by neighbor-joining (21). Distance bootstrap analyses consisted of 100 replicates with trees constructed by neighbor-joining.

Parsimony analyses of both amino acids and nucleotides utilized the program PAUP 3.1.1 (23). Shortest tree searches consisted of 100 rounds of random addition using TBR (tree

bisection-reconnection) branch-swapping and the steepest descent option (23). Parsimony bootstrap analyses consisted of 500 replicates of simple addition holding one tree at each step.

Maximum likelihood analyses of amino acids utilized the program fastPROTML (24). Bootstrap values were calculated by the REL method on the 1,000 best trees, using the weighting matrix of Jones *et al.* (25) normalized to the amino acid composition of the data set (-jf option) on a semiconstrained starting tree. To avoid prohibitively long PROTML analysis times, deeply branching, single-representative-clade taxa not directly related to the Mycetozoa, based on the results of parsimony and distance analyses (see *Results*), were omitted. These include *Trichomonas*, *Entamoeba*, and *Glugea* for all analyses and also *Blastocystis* and *Stylonychia* for analyses testing the monophyly of the Mycetozoa. On the basis of the strong results of the latter analyses, the Mycetozoa were constrained as monophyletic for PROTML analyses testing the phylogenetic position of the group as a whole. Maximum likelihood analysis of nucleotides utilized the PHYLIP 3.57c program DNAML (21) with empirical base frequencies, a transition-to-transversion ratio of 1.0, and 100 bootstrap replicates.

RESULTS

Mycetozoan *tef* Gene Sequences and Intron Positions. The 5' two-thirds of the *Physarum* *tef* gene was amplified from genomic DNA, while the 3' half of the gene was amplified from cDNA. The latter was necessary because all primer combinations for the 3' half of the gene preferentially amplified the retrotransposon *Tp1*, which constitutes 10–20% of the *Physarum* genome (26). All 12 1F-7R clones screened were identical to each other, as were the 4 2F-10R clones screened. The 3' and 5' clones were also identical to each other in their 260 nucleotides of overlap, suggesting the presence of a single, active *tef* locus in this genome. The *Physarum* *tef* gene contains a single 142-nucleotide intron, which lies at a position identical to that of an intron found in both vertebrates and invertebrates (Fig. 1).

Both *Dictyostelium* *tef* genes, for which cDNA sequences were previously determined (27), were amplified and sequenced in the region covered by primers 1F and 10R (Table 1). A single 147-nucleotide intron was found in the *tef2* gene at amino acid position 53. This intron position is clearly unrelated to that of the *Physarum* intron, although it is close to another intron position shared by vertebrates and invertebrates (Fig. 1). Otherwise, both *Dictyostelium* *tef* genomic sequences were identical to their cDNA sequences, which are also identical to each other at the amino acid level (27).

Initial amplification of the protostelid DNA revealed the presence of three *tef* sequences (Fig. 1). Two of these, designated *tef1* and *tef2*, are very similar to each other and were presumed to be from the protostelid. The third sequence appears to be a fungal *tef*, presumably from the protostelid food source (see below). The two presumed protostelid sequences are intronless and differ at 32 nucleotide positions, all of which are silent except for position 377, which gives a glutamate in *tef1* and a glycine in *tef2* (Fig. 1). The five Mycetozoan *tef* genes show strong codon bias: both the protostelid and *Physarum* sequences are 74–75% G+C at silent

Table 1. EF-1 α PCR primers

Forward primers:					Reverse primers:				
name	start	end	nucleotide sequence (5'→3')	amino acid sequence	name	start	end	nucleotide sequence (5'→3')	amino acid sequence
1F	37	60	GGATTTCGTTATTGGNCAYGTNGA	XXVIGHVD	2R ₃	402	378	CCNGTYTGNTCTCTTGTACGAGAG	GQTREHAX
2F	301	324	CTCGAGAARAAYATGATNACNGG	XXKNMITG	4R	762	738	GTCTRCANATGTTCTAGCCAGCC	QDVYKIGG
3F	477	499	CGGAAGCTTTTCAAYAARATGGA	XXXNKMND	7R	924	901	CCNCTRTTRCAACCTAGGTGCA	GDNVXXXX
4F ₁	804	827	GGAATCGATACAGTNCNGTNGG	GIGTVPVG	10R ₁	1311	1287	GTYTGNCAACGSCAGCCTTAAGAC	QTVVGXX
4F ₂	771	794	GTACGTGAGCTCCARGAYGTNTA	XXXXQDVY	10R ₂	1320	1297	CGNCANCTCATTACCTAGGTCT	AVGVIXXX

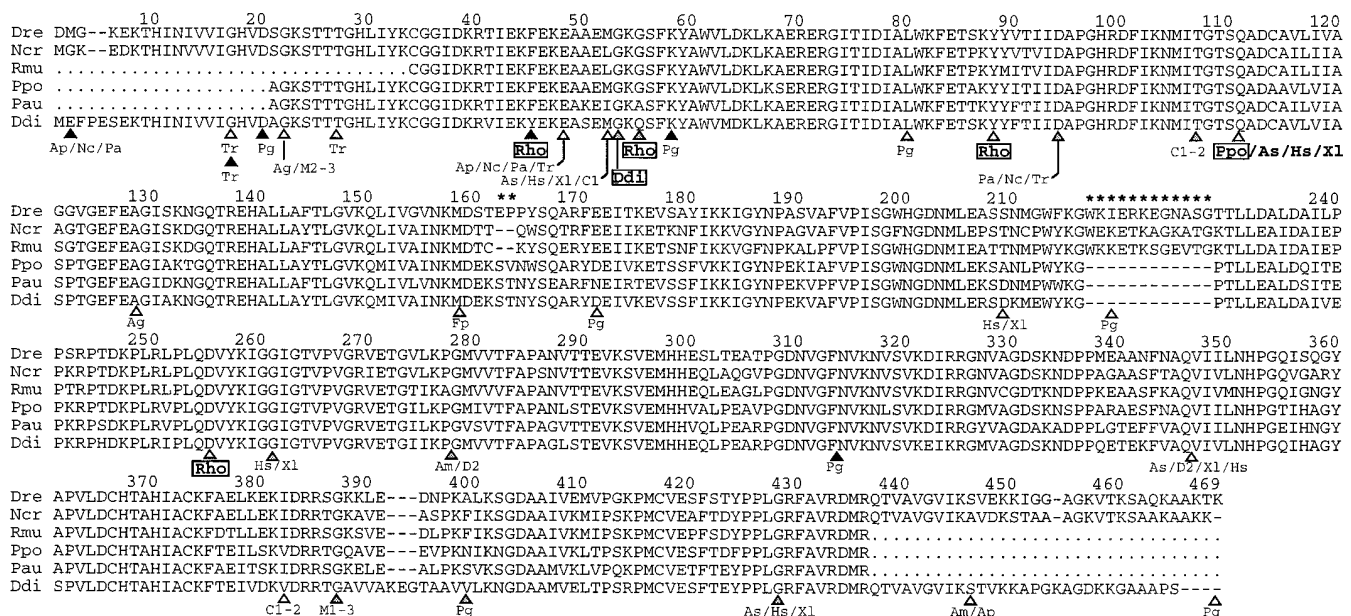


FIG. 1. EF-1 α sequence alignment and intron positions. The deduced amino acid sequences of the *Dictyostelium discoideum* (Ddi), *Physarum polycephalum* (Ppo), *Planorhynchus auranitum* (Pau), and *Rhodotorula mucilaginosa* (Rho) EF-1 α s are shown aligned with those of the fungus *Neurospora crassa* (Ncr) and the fish *Danio rerio* (Dre). Gaps in the alignment are indicated by hyphens and missing data by periods. An insertion and deletion, which are together diagnostic of fungi, are indicated above the alignment by asterisks (see text). Intron positions are shown below the alignment with open, shaded, or solid triangles to indicate phase 0, 1, or 2 introns, respectively; phase 0 introns are indicated below their 3' flanking amino acid. Organisms in which the indicated introns are found are indicated below the alignment and abbreviated as follows: Ag, *Absidia glauca*; Am, *Apis mellifera*; Ap, *Aureobasidium pullulans*; As, *Artemia salina*; C, *Caenorhabditis elegans* (two loci); D2, *Drosophila melanogaster* F2; Fp, flowering plants; Hs, *Homo sapiens*; M, *Mucor racemosus* (three loci); Nc, *Neurospora crassa*; Pa, *Podospora anserina*; Pg, *Puccinia graminis*; Tr, *Trichoderma reesei*; and Xl, *Xenopus laevis*. All sequences are available from the GenBank database.

codon positions, versus 32–34% G+C at silent positions for the *Dictyostelium* genes (27).

The third *tef* sequence amplified from the protostelid DNA preparation appears to belong to the protostelid food source, *Rhodotorula mucilaginosa*, as it encodes an insertion found exclusively in all animals and fungi (positions 217–228, Fig. 1; ref. 11) and a two amino acid gap found in all fungi† (positions 162–163, Fig. 1). The sequence contains four introns ranging in size from 67 to 156 nucleotides. None of these introns occurs at a previously described intron position (Fig. 1). A fungal origin of this sequence was also confirmed by phylogenetic analysis (see below). Some contamination of the protostelid DNA preparation with yeast DNA is expected due to incomplete separation of cells prior to extraction (see *Methods*) and the presence of undigested yeast cells in protostelid food vacuoles.

Molecular Phylogeny of EF-1 α Sequences Strongly Supports a Monophyletic Mycetozoa. A data set consisting of all known, constitutively expressed, protistan EF-1 α sequences and a representative set of animal, fungal, and angiosperm sequences was analyzed at the amino acid and nucleotide levels, using maximum parsimony and two distance-matrix methods, with more limited questions tested by maximum likelihood analysis (Fig. 2). All phylogenetic methods used place the Mycetozoa together as a monophyletic group, with the myxogastriid and dictyostelid sequences more closely related to each other than either is to the protostelid sequence (Fig. 2). Both the monophyly of the Mycetozoa and of the myxogastriid–dictyostelid clade are strongly supported by bootstrap analysis of amino acid sequences using all methods (81–100% and 89–97%, respectively, Fig. 2). Nucleotide analyses also support both a monophyletic Mycetozoa and a

myxogastriid–dictyostelid clade, although with consistently lower bootstrap values (56–84% and 77–92%, respectively).

Parsimony and distance analyses also place the putative *Rhodotorula mucilaginosa tef* gene together with that of *Puccinia graminis* (88–91% bootstrap, Fig. 2). This is consistent with the current classification of *Rhodotorula mucilaginosa* as a basidiomycete fungus (28) and confirms its identity. Our analyses also show a moderately strong affinity between the basidiomycete and zygomycete fungi (76–77% bootstrap, Fig. 2). This contradicts both SSU rRNA trees and traditional taxonomy (29) and warrants further study.

EF-1 α Phylogeny Tentatively Supports the Mycetozoa as a Sister Group to Animals + Fungi. All methods of analysis also place the Mycetozoa within the crown of the eukaryote tree, closer to the animal–fungal clade than are the green plants (Fig. 2). This topology is reconstructed in the optimal trees by all methods of analysis used with both amino acids and nucleotides (Fig. 2). However, no significant bootstrap support is found for this specific placement of the Mycetozoa or for any other higher order placement of these taxa by any of these methods.

Inspection of individual distance bootstrap trees shows that 71% of these actually reproduce an animal–fungal–Mycetozoa clade (Fig. 2), but in 36% of the trees this clade also includes, alone or in combination, *Porphyra*, *Stylonychia*, Euglenozoa, or *Blastocystis* (33%, 7%, 6%, and 3% of total, respectively). Only 14% of replicates place the Mycetozoa together with green plants, with or without other taxa, and only 10% place the Mycetozoa deep to animals + fungi + green plants. Otherwise, the *Porphyra* sequence is also found as the outgroup to an animal–fungal–mycetozoa clade with green plants, or with the Mycetozoa (19%, 27%, and 13% of trees, respectively), whereas the *Stylonychia* sequence is found most frequently with the Euglenozoa or near the other ciliates (45% and 34% of trees, respectively). Because such poorly resolved, unstable branches can obscure otherwise stable relationships among their neighboring branches within a tree (30), analyses

†This latter region is also variable in some protists and archaeobacteria. Therefore, it is only the combination of the insertion together with the deletion that defines this as a fungal EF-1 α (S.L.B., unpublished data).

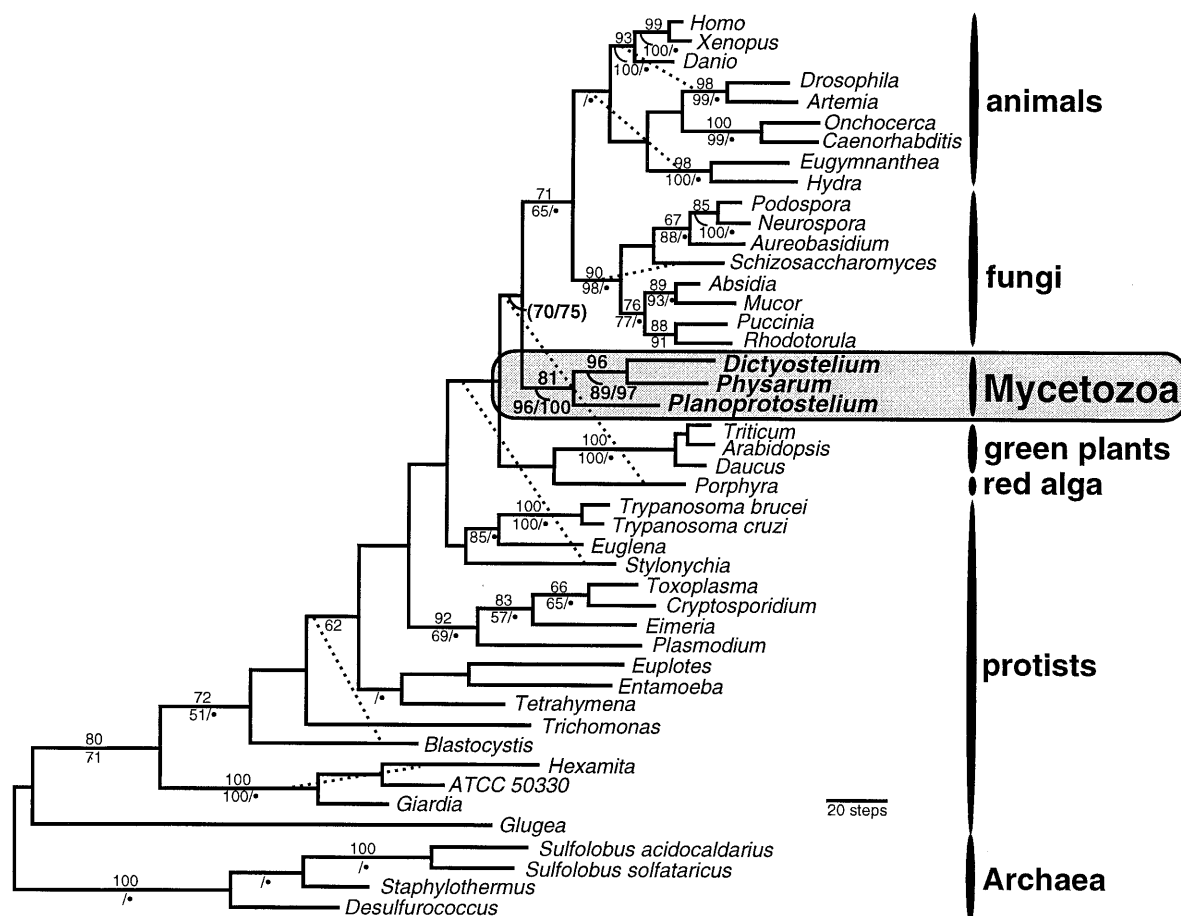


FIG. 2. Phylogenetic analyses of EF-1 α amino acid sequences show a monophyletic, late-branching Mycetozoa. The tree shown is one of two shortest trees found by parsimony analysis; the other tree at this length places *Schizosaccharomyces* at the base of the Zygomycete + Basidiomycete clade. The tree is 2,319 steps long, and branches are drawn to scale as indicated. Dotted lines indicate the differences in the tree topology reconstructed by distance analysis. Bootstrap values over 50% are indicated above the nodes for parsimony analysis and below the nodes and to the left of the "/" for distance analysis. Dots (·) below the nodes (to the right of a "/") indicate which nodes were constrained for maximum likelihood analysis. The results of bootstrap analyses with the *Porphyra* and *Stylonychia* sequences excluded are indicated in parentheses to the left and right of the "/" respectively, for distance and maximum likelihood analyses.

were repeated with the *Stylonychia* and *Porphyra* sequences deleted.

Distance analyses with the *Porphyra* and *Stylonychia* sequences deleted show 85% bootstrap support for the Mycetozoa as members of a larger crown group including green plants, animals, and fungi and 70% bootstrap support for the Mycetozoa as closer to the animal–fungal clade than are the green plants (Fig. 2). Likewise, protein maximum likelihood analysis without these sequences shows 82% bootstrap support for a crown placement of the Mycetozoa and 75% support for their sisterhood with animals + fungi (Fig. 2). However, parsimony analysis, albeit the most refractory to the correction of long-branch effects (31), still finds less than 50% bootstrap support for either relationship.

DISCUSSION

EF-1 α Phylogeny Strongly Supports a Monophyletic Mycetozoa. We have enzymatically amplified and sequenced the EF-1 α -encoding genes from representatives of each of the three recognized subclasses of Mycetozoa, the cellular (dictyostelid), acellular (myxogastrid), and protostelid slime molds (Fig. 1). Phylogenetic analyses of a broadly representative EF-1 α data set show strong support for the monophyly of the group by all methods of analysis used (86–100% bootstrap, Fig. 2). Strong support for a monophyletic Mycetozoa, represented by *Dictyostelium* and *Physarum*, is also found by analyses of

actin (72–95% bootstrap, refs. 12 and 13) and of β -tubulin (74–91% bootstrap, ref. 14).

The EF-1 α data further subdivide the Mycetozoa into a myxogastrid–dictyostelid clade strongly excluding the amoebflagellate protostelid, *Planoprotostelium aurantium* (89–97% bootstrap, Fig. 2). Thus, the myxogastrid–dictyostelid divergence does not appear to represent the deepest division within the Mycetozoa. This suggests that the differences between these taxa, such as an amoebflagellate versus strictly amoeboid condition and plasmodial versus aggregative development, may not be as profound as many have considered them to be. Both Olive (1) and Spiegel (18) have argued that a strictly amoeboid trophic stage, at least, has probably evolved multiple times among the protostelids.

The Mycetozoa as Members of a Eukaryote Crown Group. Phylogenetic analyses of EF-1 α sequences also place the Mycetozoa among the multicellular eukaryotes as the immediate outgroup to the animal–fungal clade (Fig. 2). This topology is favored by all analytical methods used (Fig. 2), although there is no immediate bootstrap support for this specific topology by any method. However, distance and maximum likelihood analyses of the EF-1 α data with the *Porphyra* and *Stylonychia* sequences deleted show greatly increased bootstrap support for both the placement of the Mycetozoa within the eukaryote crown and for these taxa as more closely related to the animal–fungal clade than are green plants (82–85% and 70–75%, respectively). Since bootstrap

values greater than 70% have been shown likely to correspond to confidence levels of 95%, except under conditions of extreme substitutional saturation or highly unequal rates (34), both methods seem to strongly suggest that the Mycetozoa are crown eukaryotes, probably more closely related to the animal-fungal clade than are green plants. Nonetheless, unweighted parsimony analysis, a method highly sensitive to long-branch effects (31), still shows no significant support for these or most other major clades in the EF-1 α tree (Fig. 2).

Thus the apparent lack of support for the higher order placement of the Mycetozoa with the full EF-1 α data set appears to be due, at least in part, to poor resolution of the branching positions of several taxa, most notably *Porphyra* and *Stylonychia* (Fig. 2). Inspection of individual bootstrap trees shows that these sequences are weakly supported at various positions in the tree, *Porphyra* being found mostly among the "crown" taxa, whereas *Stylonychia* ranges from among the relatively deeply branching ciliates to within fungi. Such unstable branches can decrease bootstrap values, apparently even for relatively distantly related nodes (11, 32). This appears to be due, at least in part, to a combination of the tendency of poorly resolved taxa to obscure underlying tree structure (30) with the requirement of bootstrap analysis, as currently implemented, for strictly monophyletic groups (33).

Although increased taxon sampling to break up long branches should help alleviate this problem with bootstrap analysis (22, 35)—with all methods except perhaps parsimony (31)—the gathering of protein sequence data to evaluate ancient divergences is still a relatively slow process. Nonetheless, it may still be possible, with caution, to answer more limited but still highly relevant questions (11, 33). In this case, we are asking only whether the Mycetozoa are early- or late-emerging eukaryotes, possibly more closely related to the animal-fungal clade than are green plants. It is important to note that we are in no way precluding the possibility that other taxa, most notably the red alga *Porphyra*, may be more closely related to the animal-fungal clade than are the Mycetozoa.

An origin of the Mycetozoa from within a eukaryote "crown" group—i.e., among animals, fungi, and green plants to the exclusion of most or all protistan lineages represented, is also supported by individual analyses of actin (12–13, 36), RNA polymerase largest subunit (15), glyceraldehyde-3-phosphate dehydrogenase (3), and most analyses of α - and β -tubulin (refs. 11 and 14, but see ref. 36) as well as a combined analysis of all relevant, currently available, protein data (16). Furthermore, both the actin and the combined protein analyses specifically support the Mycetozoa as more closely related to the animal-fungal clade than are green plants (56–60% and 83–86% bootstrap, respectively). This relationship is also suggested by analyses of both α - and β -tubulin (67% and 73% bootstrap, respectively, ref. 11), although the rooting of these trees is clearly problematic (14, 36).

Although nucleotide-level analyses of actin place the Mycetozoa closer to animals than fungi (64% bootstrap, ref. 12), this is not supported by amino acid-level analyses of the same data (11, 13, 36). Loomis and Smith (37) also noted strong similarity between animals and *Dictyostelium* based on six small protein data sets. However, because none of these data sets included an outgroup, these results cannot be meaningfully interpreted. A specific relationship between animals and *Dictyostelium* to the exclusion of fungi is further ruled out by its lack of an 11- to 13-amino acid insertion found exclusively in all animal and fungal EF-1 α s (Fig. 1, ref. 11).[‡]

Ribosomal RNA Phylogeny of Mycetozoa. Although three protein sequence data sets, actin, α -tubulin, and EF-1 α ,

strongly support a monophyletic Mycetozoa (refs. 12–14; Fig. 2) and at least four place these taxa in the eukaryote crown (refs. 3, 11–15, and 36; Fig. 2), rRNA trees consistently show the Mycetozoa to be polyphyletic as well as early branching (7–10). *Physarum*, especially, appears as one of the earliest branches of mitochondrial eukaryotes in nearly all rRNA trees (7–10). Although Cavalier-Smith finds very weak evidence for a monophyletic Mycetozoa with SSU rRNA (40), this clade still arises very deeply in the tree. Because a growing body of protein sequence data contradicts these results (3, 11–16, 36), including the data presented here (Fig. 2), it is necessary to consider the possibility that current rRNA trees may be misleading with respect to these questions.

If the current rRNA phylogenies are indeed incorrect with regard to the phylogeny of the Mycetozoa, it should be considered that increased taxon sampling has been shown to potentially overcome many sources of both random and systematic error in phylogenetic analyses (22, 35). Thus, inclusion of additional rRNA sequences from all three classes of Mycetozoa, especially protostelids, might help to resolve some of these questions. This is suggested by the results of Spiegel *et al.* (41), who analyzed the first protostelid molecular sequence, a 310-nucleotide portion of the SSU rRNA gene of *Protostelium mycophaga*. Analyses of this sequence with a limited set of taxa showed strong support for a monophyletic Mycetozoa, although the method of sequence alignment may have biased the results in this direction (41).

Accuracy of the Current EF-1 α Data Set for Deep-Level Phylogeny. Besides a relatively broad representation of the animals, fungi, Mycetozoa, and Apicomplexa (Fig. 2), most of the EF-1 α tree is still sparsely sampled. Thus most of the deeper branches are only tentatively resolved, and the placement of these taxa in the tree should be considered a general indication of their true phylogenetic position, at best. Perhaps most problematic is the fact that the ciliates do not form a clade in the EF-1 α tree, contradicting considerable morphological and molecular data (3, 8–14, 36, 42). The instability of the *Stylonychia* EF-1 α branch was noted above, and the grouping of *Entamoeba* with the ciliate *Euplotes* is almost certainly a spurious long-branch attraction as well (Fig. 2, ref. 31). Better resolution of the relationships among the various protistan taxa in the EF-1 α tree will almost certainly require both more thorough sampling of taxa and careful analysis of specific questions.

The *Glugea* EF-1 α is especially noteworthy in that it gives an extremely long branch and is more distant from the rest of the eukaryotes than even the archaebacterium *Desulfurococcus* (Fig. 2), more than twice as far in distance analyses (21)! The *Glugea* EF-1 α also contains many nonconservative amino acid substitutions at otherwise universally conserved positions, including active site residue changes unlikely to be compatible with enzymatic function (A. Roger and S.L.B., unpublished data). This sequence also appears to encode an insertion otherwise found only in animals and fungi (11). The latter is consistent with the placement of the Microsporidia with fungi in α - and β -tubulin phylogeny (14, 39). Thus the *Glugea* EF-1 α may be artefactually drawn toward the base of the EF-1 α tree due to an accelerated rate of evolution, as previously observed with the *Xenopus* EF-1 α -derived protein, thesaurin (43).

Implications of a Mycetozoan Sister Clade to Animals + Fungi. Placement of the Mycetozoa among the "crown" eukaryotes is consistent with a large body of data on their physiology, biochemistry, molecular biology, behavior, and development (1, 44–46). Perhaps most notable among these is the Mycetozoan fruiting body, which shows characteristics of true multicellularity by including functionally specialized, non-reproductive cells (1). This is especially striking in the dictyostelids, where the developmental fates of fruiting body cells are predetermined in the slug (1, 44, 45).

[‡]*Dugesia japonica* (38) EF-1 α has a 4-amino acid insertion within this larger insertion. Although Microsporidia may encode a form of the 11- to 13-amino acid insertion, this is consistent with other data suggesting that they may be fungi (refs. 14 and 39; see below).

Thus, a growing body of protein sequence data supports a monophyletic Mycetozoa (Fig. 2; refs. 12–14), and all currently available, broadly representative protein data sets support these taxa as late-emerging eukaryotes (Fig. 2; refs. 3, 11–16, and 36). This suggests that the rRNA data should be more closely examined with regard to these questions. In addition, the possibility that the Mycetozoa may be more closely related to the animal–fungal clade than are green plants clearly warrants further study. The results of our work and others (Fig. 2; refs. 3, 11–16, and 36) indicate that animals, fungi, and slime molds may still represent only a small corner of eukaryote diversity, and it should not be assumed that traits shared by these taxa are ancient or universal among eukaryotes. On the other hand, these results support the continued use of mycetozoan taxa as model systems for studying the origin, evolution, and function of a wide range of characteristics of “higher” eukaryotes (44–47).

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