# The Protistan Origins of Animals and Fungi

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Recent molecular studies suggest that Opisthokonta, the eukaryotic supergroup including animals and fungi, should be expanded to include a diverse collection of primitively single-celled eukaryotes previously classified as Protozoa. These taxa include corallochytreans, nucleariids, ministeriids, choanoflagellates, and ichthyosporeans. Assignment of many of these taxa to Opisthokonta remains uncorroborated as it is based solely on small subunit ribosomal RNA trees lacking resolution and significant bootstrap support for critical nodes. Therefore, important details of the phylogenetic relationships of these putative opisthokonts with each other and with animals and fungi remain unclear. We have sequenced elongation factor 1-alpha (EF-1α), actin, β-tubulin, and HSP70, and/or α-tubulin from representatives of each of the proposed protistan opisthokont lineages, constituting the first protein-coding gene data for some of them. Our results show that members of all opisthokont protist groups encode a ~12-amino acid insertion in EF-1α, previously found exclusively in animals and fungi. Phylogenetic analyses of combined multigene data sets including a diverse set of opisthokont and nonopisthokont taxa place all of the proposed opisthokont protists unequivocally in an exclusive clade with animals and fungi. Within this clade, the nucleariid appears as the closest sister taxon to fungi, while the corallochytrean and ichthyosporean form a group which, together with the ministeriid and choanoflagellates, form two to three separate sister lineages to animals. These results further establish Opisthokonta as a bona fide taxonomic group and suggest that any further testing of the legitimacy of this taxon should, at the least, include data from opisthokont protists. Our results also underline the critical position of these "animal-fungal allies" with respect to the origin and early evolution of animals and fungi.

## Introduction

An exclusive grouping of animals and fungi was first noted in evolutionary trees of small subunit ribosomal RNA (SSU rRNA; Wainright et al. 1993) and several proteins (Baldauf and Palmer 1993). This grouping was subsequently designated as "Opisthokonta" (Cavalier-Smith and Chao 1995) and is now strongly supported by phylogenetic analyses of all taxonomically well-represented and well-characterized molecular data sets. These include concatenated multigene analyses (e.g., Baldauf et al. 2000; Bapteste et al. 2001; Lang et al. 2002) as well as many single-gene trees (e.g., Baldauf 1999; Inagaki and Doolittle 2000; Van de Peer et al. 2000). Recent studies have suggested that the Opisthokonta may also include a diverse collection of poorly characterized single-celled "protists" (reviewed by Steenkamp and Baldauf 2004). To recognize the possible position of these taxa vis-à-vis the origins of animals and fungi, Cavalier-Smith has placed them in the new protozoan phylum, Choanozoa (Cavalier-Smith 1998*a*, 2002). These taxa include choanoflagellates (Cavalier-Smith and Chao 1995), ichthyosporeans (Ragan et al. 1996), corallochytreans (Cavalier-Smith and Allsopp 1996), nucleariids (Amaral-Zettler et al. 2001), and ministeriids (Cavalier-Smith and Chao 2003). Recent multigene phylogenies using mitochondrial proteins firmly place one ichthyosporean *Amoebidium parasiticum* and the choanoflagellate *Monosiga brevicollis* as progressively closer sister taxa to animals in a newly designated "Holozoa" clade (Lang et al. 2002). However, little is known about other members of these lineages or of the other proposed opisthokont protists.

Besides molecular phylogenetic data, few traits unite animals and fungi and their proposed protistan allies. Animals and fungi both possess uniflagellated reproductive stages (the male reproductive cells of animals and the zoospores of chytrid fungi) but are otherwise morphologically diverse, largely multicellular heterotrophs. Among the proposed protistan allies, choanoflagellates are aquatic uniflagellates (Sleigh 1989; Buck 1990; Hausmann and Hülsmann 1996); nucleariids are strictly amoebae with filose pseudopodia (Schuster 1990; Cavalier-Smith 1993; Patterson 1999); ministeriids are amoebae with symmetrically distributed, stiff, radiating pseudopodia (Patterson 1999); corallochytreans are simple free-living, nonflagellated saprotrophs (Cavalier-Smith and Allsopp 1996; Mendoza, Taylor, and Ajello 2002); and ichthyosporeans (also known as mesomycetozoea or DRIPs [{*Dermocystidium*, Rosette agent of fish, *Ichthyophonus*, and *Psorospermium*} Ragan et al. 1996; Herr et al. 1999; Mendoza, Taylor, and Ajello 2002]) are animal parasites with amoeboid (e.g., *Ichthyophonus* and *Amoebidium*) and/or uniflagellated stages (e.g., *Dermocystidium*) (reviewed by Mendoza, Taylor, and Ajello 2002).

The only obvious characters that Choanozoa share with animals and fungi are the presence of well-developed, platelike or flattened mitochondrial cristae (Patterson 1999; Cavalier-Smith and Chao 2003; Dykhová et al. 2003) and a single basal flagellum in motile reproductive stages, where present. In addition, certain ichthyosporeans (e.g., *Amoebidium*) display fungal-like traits such as hyphae and sporangia, but unlike true fungi (Kirk et al. 2001), they also display typical amoeboid cells similar to those of animals (Mendoza, Taylor, and Ajello 2002). The universal presence of the single flagellum in choanoflagellates (Buck 1990; Hausmann and Hülsmann 1996) is the main reason for the key position these taxa hold in some contemporary theories on the origins of animals and fungi. Most of these theories suggest that colonial naked choanoflagellate-like protists (similar to species in the family Codosigidae) gave rise to the first animals (i.e., sponges), while chitinous thecate choanoflagellate-like protists (similar to species in

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the family Salpingoecidae) gave rise to the first fungi (Cavalier-Smith 1987; Buck 1990; Hausmann and Hülsmann 1996; Cavalier-Smith 1998*a*).

However, none of the above described ultrastructural characters are unique or consistent synapomorphies for animals and fungi, much less their proposed protistan allies. The single posterior flagellum has been lost from most fungi and has not been detected in corallochytreans, ministeriids, and nucleariids (Patterson 1999; Cavalier-Smith and Chao 2003). Flattened mitochondrial cristae are also not found in all potential members of the Opisthokonta (e.g., the cristae of certain ichthyosporeans and nucleariids are discoidal or tubular [Ragan et al. 1996; Amaral-Zettler et al. 2001]). Furthermore, these ultrastructural characters are not necessarily limited to opisthokonts because the uniflagellate condition and flattened mitochondrial cristae have been detected in nonopisthokonts (e.g., some heterokonts and cryptophyte algae, respectively; Sleigh 1989). Also, a recently reported molecular character unique to opisthokonts, the unusual multimeric form of the β-thymosin gene, has only been detected in a single fungus (Telford 2004). Thus, the only character that so far appears to be universal and derived among all examined animals, fungi, and their potential protistan allies is the presence of a ~12-amino acid (aa) insertion in protein synthesis elongation factor 1-alpha (EF-1α) (Baldauf and Palmer 1993).

A few previous studies have attempted to resolve the exact phylogenetic branching order among some of the proposed opisthokont protistan taxa using phylogenetic analyses of single genes, specifically, SSU and EF-1α (e.g., Cavalier-Smith and Chao 2003; Hertel, Bayne, and Loker 2003; Ragan, Murphy, and Rand 2003), with limited success. This common limitation of single-gene phylogenies to resolve deep branches is well documented, and recent studies have shown that analyses of combined sequence data can provide resolution where single-gene phylogenies fail (e.g., Baldauf et al. 2000; Fast et al. 2002; Lang et al. 2002; Yoon et al. 2002). We have examined the deep phylogeny of opisthokonts by determining sequences for four to five slowly evolving nuclear protein–encoding genes from representatives of all potential protistan opisthokont lineages. Phylogenetic analyses of these data plus SSU sequences support a number of recently proposed theories on early opisthokont evolution and reject others. These data also allow us to examine whether the ~12-aa insertion in EF-1α is indeed a true synapomorphy for opisthokonts.

## Materials and Methods

### Cultures, Tissues, and Nucleic Acids

Cultures of various protists and fungi were obtained from culture collections and as gifts from individual researchers (table 1). DNA was isolated from these cultures using basic hexadecyltrimethylammonium bromide (CTAB) extractions (Steenkamp et al. 1999). For *Nuclearia simplex*, *Diaphanoeca grandis*, *Salpingoeca amphoridium*, and *Ministeria vibrans*, cDNA was synthesized using mRNA extracted with TRIZOL reagent (Invitrogen, Paisley, UK) followed by first-strand cDNA synthesis with SuperScript™ II RNAse H⁻ Reverse Transcriptase (Invitrogen) and Oligo(dT)$_{12–18}$ primer (Invitrogen). In some cases, DNA and/or cDNA were received as gifts from various sources (table 1).

### Polymerase Chain Reaction Amplification and Sequencing

Four nuclear protein–encoding genes (EF-1α, HSP70, actin and β-tubulin [EHAβ]) were selected for analysis based on their availability for a diverse set of eukaryotes and previously demonstrated phylogenetic utility. Except where indicated, all sequences were amplified with degenerate polymerase chain reaction (PCR) primers. EF-1α was amplified in two overlapping fragments with the primer sets 1F + 4R and 3F + 10R (Baldauf and Doolittle 1997), except for *Mi. vibrans*, where primer 1F was substituted with 13F (5′-gtgaattcaartaygcntgggt-3′). Primers 1F or 13F and 4R were also used for specifically assaying the ~12-aa EF-1α insertion. For amplification of EF-1α from *Sa. amphoridium*, three specific reverse primers were also used (EFSAM1R—5′-gtgcatctccacagacttgacttc-3′; EFSAM2R—5′-cacacactgacacacaacaaacgc-3′; and EFSAM3R—5′-gttgaacttctcagtggccttg-3′). Amplification of EF-1α from *Amoebidium* also required specific forward primers (EFAPA1F—5′-acatcaagaagatcggctacaacc-3′; and EFAPA2F—5′-ggcttcaacggtgacaacatgg-3′) and reverse primers (EFAPA1R—5′-agccgatcttcttgatgtagttgg-3′; and EFAPA2R—5′-cttgtaccagggcatgttgtcc-3′).

The three remaining genes were amplified as follows: actin—primers Act2F + ActR (5′-tatgtcactaaytgggaygayatg-3′ and 5′-gtacaccgaaaatgcttcgaggcc-3′, respectively; Keeling 2001); β-tubulin—primers btF2 + btRb (5′-gataccgttgttgagcctgataa-3′ and 5′-atggacgaaatggaattcactgaggc-3′, respectively; Keeling, Luker, and Palmer 2000); and HSP70—primers 2F + 4R (5′-gagactatagccaaygaycargg-3′ and 5′-tgntadtgnttgctattcgtggcg-3′, respectively). In some cases (see table 1), a fifth gene, α-tubulin, was amplified with the primers atF2 + atR2 (5′-cggcagctcttycayccngar-3′ and 5′-gcgcataacctcnccnacrtacca-3′, respectively). For *Amoebidium* HSP70, primer 4R was substituted with primer OR (5′-tagtcwacytcytckatrgt-3′; Borchiellini et al. 1998). In an attempt to amplify β-tubulin from this species, we also used various additional primers reported elsewhere (Keeling et al. 1999; Keeling, Luker, and Palmer 2000; Keeling 2003).

PCRs were performed with Invitrogen's *Taq* polymerase and 0.5 μM of each primer, using either DNA or cDNA as template. PCR cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 50°C, and 1 min elongation at 72°C, and followed by a final elongation at 72°C for 10 min. For tubulin and actin, an annealing temperature of 40°C was used.

All PCR products were electrophoresed on 1% agarose gels (Sambrook, Fritsch, and Maniatis 1989). Fragments with appropriate sizes were excised from the gels, purified with the QIAquick gel extraction kit (Qiagen, Crawley, UK), and cloned with the Promega pGEM-T Easy Vector System. Positive clones were screened for the presence of appropriate-sized inserts by PCR amplifications as described above but using the vector-specific primer pair T7 + SP6 (5′-taatacgactcactataggg-3′ [Dunn and Studier

**Table 1**
**List of Species, DNA and/or Isolate Source, and GenBank Accession Numbers for Sequences Generated in This Study**

| Species[a] | Isolate/DNA Source[b] | GenBank Accession Numbers[c] |
|---|---|---|
| *Chytridium confervae* (675.73) | CBS | AY582823(E), AY582833(H), AY582841(A), AY582848(β), AY582856(α) |
| *Rhizophlyctis rosea* (576.84) | CBS | AY582832(H), AY582857(α) |
| *Blastocladiella emersonii* (561.70) | CBS | AU582842(A), AY582849(β), AY582857(α) |
| *Smittium simulii* (CAL-8-1) | M. Cafaro | AY582822(E), AY852840(A), AY582855(α) |
| *Corallochytrium limacisporum* (DNA) | T. Cavalier-Smith | AY582826(E), AY582834(H), AY582844(A), AY582850(β), AY582859(α) |
| *Nuclearia simplex* (1552/4) | CCAP | AY582827(E), AY582835(H), AY582845(A), AY582851(β), AY582858(α) |
| *Nuclearia moebiusi* (1552/3) | CCAP | AY582852(β) |
| Stramenopile contaminant of *Nuclearia delicatula* (1552/1) | CCAP | AY582830(E), AY582838(H), AY582847(A), AY582854(β) |
| *Ministeria vibrans* (50519) | ATCC | AY582825(E), AY582836(H), AY582846(A), AY582851(β) |
| *Amoebidium parasiticum* (DNA) | R. Lichtwardt | AY582828(E), AY582831(H), AY582843(A) |
| *Monosiga ovata* (cDNA) | P. Holland | AY582824(E) |
| *Monosiga brevicollis* (cDNA) | N. King | — |
| *Salpingoeca amphoridium* | B. Leadbeater | DQ059027(E), DQ059029(A), DQ059030(β), DQ059032(S) |
| *Diaphanoeca grandis* | B. Leadbeater | DQ059028(A), DQ059031(β), DQ059033(S) |
| *Acanthamoeba culbertsoni* (DNA) | R. Gast | AY582829(E), AY582837(H), AY582853(β) |
| *Discodermia* sp. (DNA) | NHML | AY582839(A) |
| *Hartmannella abertawensis* (1534/9) | CCAP | — |
| *Ancyromonas sigmoides* (50267) | ATCC | — |
| *Apusomonas proboscidae* (1905/1) | CCAP | — |
| *Phalansterium solitarium* (50327) | ATCC and T. Cavalier-Smith | — |

[a] Isolate numbers are indicated in parentheses, but where nucleic acids were obtained as gifts, ''DNA'' or ''cDNA'' are indicated in parentheses.

[b] CCAP: Culture Collection of Algae and Protozoa, Natural Environment Research Council, Freshwater Biological Association, The Ferry House, Ambleside, Cumbria, LA22 OLP, United Kingdom; ATCC: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, United States; CBS: Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands; NHML: Department of Zoology, Natural History Museum London, Cromwell Road, London SW7 5BD, United Kingdom.

[c] E, A, H, S, β, and α, in parentheses refer to EF-1α, actin, HSP70, SSU rRNA, β-tubulin, and α-tubulin sequences, respectively. No sequences (indicated with ''—'') were deposited for *Mo. brevicollis*, nor for *H. abertawensis*, *An. sigmoides*, *Ap. proboscidae*, and *P. solitarium* (where only EF-1α fragment harboring the opisthokont-specific insertion was sequenced).

1983], and 5′-tatttaggtgacactatag-3′ [Butler and Chamberlin 1982]). DNA for sequencing was prepared by PCR amplification using the same vector-specific primers followed by precipitation of the resulting PCR products with equal volumes of a polyethylene glycol-NaCl solution (20% [w/v] polyethylene glycol and 2.5 M sodium chloride). Precipitates were washed with 70% (v/v) ethanol, resuspended in sterile water, and then subjected to automated sequencing using Applied Biosystems' (Foster City, Calif.) ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Kit on a 3730 DNA Analyzer at the Oxford Sequencing Facility (http://polaris.bioch.ox.ac.uk/dnaseq/).

To screen for multigene families, 15–80 clones per sample were assayed by restriction digests with 4 base pair (bp)–targeted restriction enzymes, and at least one example of each unique pattern was sequenced. Even where no differences in the restriction profiles could be detected, a minimum of three clones per PCR product were sequenced (in both directions) to control for PCR or sequencing errors. Sequences were initially analyzed using Chromas version 2.23 (Technelysium Pty Ltd, Tewantin, Australia) and then assembled into contigs and, where necessary, translated to aa, using BioEdit version 5.0.9 (Hall 1999) and DAMBE version 4.2.13 (Xia and Xie 2001). All sequences have been deposited in GenBank, and accession numbers are listed in table 1.

## Sequence Alignment and Taxon Selection

For the protein-coding genes, nucleotide (nt), and deduced aa sequences were aligned together with publicly available sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov) or individual genome sequencing projects (http://www.genomesonline.org/). All alignments were constructed first at the aa level using ClustalX version 1.83 (Thompson et al. 1997) and then corrected by eye as needed to minimize hypothesized insertion/deletion events using BioEdit. SSU rRNA sequences were downloaded from GenBank and those for some of the choanofla were obtained from B. Leadbeater (table 1). These sequences were aligned to the Ribosomal Database Project II (RDP II, http://rdp8.cme.msu.edu/html/) structure–based alignment using the RDP II Sequence Aligner invoking the "preserve common gaps" option. All alignments are available from S.L.B. upon request.

Where multiple copies of any gene were detected in a single taxon, only one of the copies was used for phylogenetic analysis being selected as follows. Initial alignments were compiled with all available homologues and subjected to neighbor-joining (NJ) distance analyses with 1,000 bootstrap replicates corrected for multiple substitutions using ClustalX. Assuming all sequences from any given taxon grouped first with each other, that is, represented taxon-specific duplications, the single sequence with the shortest terminal branch was selected for use in all further analyses. These NJ trees were also used to identify sequences with disproportionately long terminal branches, which were then excluded from subsequent analyses whenever possible.

## Phylogenetic Analyses

All phylogenetic analyses utilized unambiguously aligned positions only, and all nt-level analyses of

protein-coding sequences were conducted with third-codon positions deleted. Analyses were conducted at the nt and aa levels using Bayesian inference (BInt and BIaa), maximum parsimony (MPnt and MPaa), NJ distances (NJnt and NJaa), and maximum likelihood (MLnt and MLaa). The program Modeltest version 3.06 (Posada and Crandall 1998) indicated that the General Time Reversible (GTR) model (Rodríguez et al. 1990) with a designated proportion of invariant sites (I) and gamma correction (G) for rate variation among sites (GTR + I + G) gave the best fit to the data.

Bayesian analyses utilized the Metropolis-coupled Markov chain Monte Carlo search algorithm as implemented in the program MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). All Bayesian analyses consisted of 1,000,000 generations running one cold and three heated chains, with Bayesian inference posterior probabilities (biPP) calculated after discarding a burnin corresponding to roughly 50,000 generations past stationarity. BInt analyses utilized the GTR + I + G substitution model with separate parameters for each gene (partition) and an eight-category gamma model. BIaa analyses utilized a mixed aa model and four-category gamma distribution (Huelsenbeck and Ronquist 2001).

Maximum parsimony analyses (MPnt and MPaa) utilized the program PAUP* (Swofford 1998). Shortest tree searches consisted of 1,000 rounds of random sequence addition with all changes weighted equally and bootstrap analyses consisting of 1,000 replicates of one round of random addition each. NJnt analyses were also conducted with PAUP* (Swofford 1998) utilizing the GTR + I + G model with parameters estimated by the program and 10,000 bootstrap replicates. NJaa analyses utilized the programs PROTDIST and NEIGHBOR from the PHYLIP 3.6b package (Felsenstein 2002), while MLaa utilized the PROML program in this package. NJaa and MLaa both employed the Jones, Taylor, and Thornton substitution matrix (Jones, Taylor, and Thornton 1992) and a gamma rate correction of 0.56 as indicated by the results of the BIaa analyses (see above). NJaa and MLaa bootstrap analyses consisted of 1,000 replicates of the same. MLnt analyses were performed with the program Phyml version 2.4.3 (Guindon and Gascuel 2003), again using the GTR + I + G model. The likelihoods of alternative tree topologies were evaluated by the Approximately Unbiased (AU) and Shimodaira-Hasegawa (SH) tests (Shimodaira 2002; Shimodaira and Hasegawa 1999) as implemented in CONSEL version v0.1h (Shimodaira and Hasegawa 2001) and PHYLIP 3.6b (Felsenstein 2002).

## Results

### Sequences and Alignments

Sequences for EF-1α, actin, β-tubulin, and HSP70 were determined for at least one representative of each proposed protistan opisthokont lineage. The only exceptions were β-tubulin, which could not be amplified from *Amoebidium* despite numerous attempts with various primers (see above), and EF-1α from *Mo. brevicollis*, *D. grandis*, and *Blastocladiella emersonii*, all of which harbor an alternative form of the gene (Keeling and Inagaki 2004; Ribichich et al. 2005; unpublished data). In addition, α-tubulin sequences were determined for *Nuclearia*, *Smittium*, *Chytridium*, *Rhizophlyctis*, and *Corallochytrium*. All genes appear to be single copy with the exception of β-tubulin for which multiple copies were found in some of the opisthokont protists. In all cases, duplications were determined to be lineage specific by phylogenetic analysis; that is, each other's closest relatives with strong bootstrap support (data not shown).

Multigene data sets were assembled from these sequences together with a broad taxonomic sampling of animals and fungi from public domain data. Data sets for analysis were restricted to universally alignable and PCR-amplifiable portions of EHAβ for 360, 466, 310, and 230 aa positions, respectively. For specific analysis of the deeper branches in the animal subtree, SSU rRNA (1,453 universal positions) and α-tubulin (327 aa positions) were used instead of HSP70, which is multicopy in many animals. Due to the inclusion of SSU rRNA sequences, this data set was analyzed at the nt level with all third-codon positions deleted (see Supplementary Material online for nt substitution saturation plots). All protein-coding gene sequences displayed more than 65% identity at the aa level among all taxa, including the outgroups. Before combining, genes were examined individually and in various combinations by 1,000 parsimony bootstrap replicates. These analyses showed that there were no significant phylogenetic conflicts among the genes; that is, no major alternative topologies were found with >50% bootstrap percentage (BP) support (table 2). Taxa whose sequences tended routinely to form disproportionately long branches with all phylogenetic methods, such as nematodes and microsporidia (Keeling and Fast 2002), were excluded from these analyses to avoid potential long-branch attraction problems (Philippe and Germot 2000).

### Phylogeny of Opisthokonta

Phylogenetic analyses of a combined data set of EHAβ aa sequences including a broad taxonomic representation of animals, fungi, and other eukaryotes strongly support an exclusive grouping of animals and fungi together with all proposed opisthokont protists (84%–96% BP, 1.0 biPP, fig. 1). In addition to animals, fungi, and opisthokonts, these data also strongly recover all the other major eukaryotic groups represented, namely, Amoebozoa, Alveolata, Heterokonta, Viridiplantae, and Euglenozoa. The tree shows further strong support for the superassemblages Opisthokonta + Amoebozoa (71%–100% BP, 1.0 biPP, fig. 1) and Alveolata + Heterokonta (chromalveolates [Harper, Waanders, and Keeling 2005]; <50%–85% BP, 1.0 biPP, fig. 1), depending, as always, on the position of the root, which is currently debated (Stechmann and Cavalier-Smith 2003; Arisue, Hasegawa, and Hashimoto 2005).

Furthermore, these data place all the examined opisthokont protists in an exclusive clade with either animals or fungi. The choanoflagellates *Ministeria*, *Amoebidium*, and *Corallochytrium* are specifically associated with animals (84%–95% BP, 1.0 biPP, fig. 1), together representing the Holozoa (Lang et al. 2002; see *Discussion*). Within the

**Table 2**
**Analysis of Phylogenetic Compatibility Among Partitions (Genes)**

| | Partition Combinations[b] | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Nucleotides | | | | | | | | | | | | Amino Acids | | | | | | | | |
| Major Clades[a] | S | E | A | H | α | β | EAβ | EAH | SEAβ | SEAH | SEAαβ | SEAHβ | E | A | H | α | β | EAβ | EAH | EAαβ | EAHβ |
| Overall | | | | | | | | | | | | | | | | | | | | | |
| Opisthokonta | | ○ | | | | ○ | ● | ○ | - | ● | ● | ● | | | | ● | ● | ● | ● | ● | ● |
| Fungi + *Nulcearia* | | ○ | | | | | ● | ● | - | ● | ○ | ● | | ● | ○ | | | ● | ● | ● | ● |
| Animals + (*Min*, *Mon*, *Amo*, *Cor*) | | | | | | ○ | ● | ○ | - | ● | ○ | ● | | | | | ○ | ○ | ○ | ○ | ● |
| Opisthokonta + Amoebozoa | | | | | | | ● | | - | ● | | | ○ | ○ | | | | ● | ● | | ● |
| Animals | | | | | | | | | | | | | | | | | | | | | |
| Higher Animals | ● | | | | - | | | | ● | ● | - | ● | | | | - | | | | - | ● |
| Higher + Lower Animals | | | | | - | | | | ● | | - | ● | | | | - | | | | - | ○ |
| Animals + (*Min*, *Mon*) | | | | | - | | ○ | | ● | | - | ○ | | | | - | | | | - | ○ |
| Holozoa | ● | ● | | ○ | - | ● | ● | ● | ● | ● | - | ● | ● | ● | ● | - | ● | ● | ● | - | ● |
| Fungi | | | | | | | | | | | | | | | | | | | | | |
| Higher Fungi | ○ | | | - | | | ○ | ● | ● | - | ● | ● | | | - | ○ | | | ● | ● | ● |
| Higher Fungi + Zygomycetes | | | | - | | | | | | - | | | | | - | | | ○ | ○ | ● | |
| Fungi | | | | - | | | | | | - | | | | ● | - | | | ● | ● | ● | ● |
| Fungi + *Nuclearia* | | ○ | ○ | - | ● | ○ | ● | ● | ● | - | ● | ● | ● | ● | - | ○ | ○ | ● | ● | ● | ● |

[a] Major clades correspond to those recovered from the various overall eukaryotic, animal, and fungal sequence data sets. *Min*, *Mon*, *Amo*, and *Cor* refer to *Ministeria*, *Monosiga*, *Amoebidium*, and *Corallochytrium*, respectively.

[b] For these analyses, the data sets were subjected to parsimony analyses using different partitions and partition combinations. Solid circles (●) indicate bootstrap support of more than 70%, open circles (○) indicate bootstrap support of 50%–70%, and blank spaces indicate bootstrap support values below 50%. Hyphens indicate partition combinations that were not analyzed.

Holozoa, *Corallochytrium* is placed together with the ichthyosporean, *Amoebidium* (86%–100% BP, 1.0 biPP, fig. 1). The fifth protist, *Nuclearia*, is grouped together with the fungi (95%–100% BP, 1.0 biPP, fig. 1). Similar results were obtained with all examined subsets of the data (table 2). The only ambiguity in this phylogeny is the exact position of *Ministeria*, which is found inconsistently supported as the immediate sister group to animals (<50%–55% BP, 1.0 biPP, fig. 1, see below).

## Phylogeny of Holozoa

Having identified specific sister groups for animals and for fungi (fig. 1), we used these taxa as outgroups to examine separately the deepest branches within animals and within fungi. This use of closely related outgroup taxa should help increase the accuracy of reconstruction of deep ingroup branches over the traditionally used, much more distantly related nonopisthokont outgroups (Philippe and Germot 2000). A combined data set of SSU rRNA, EF-1α, actin, and β-tubulin (SEAβ) nt sequences was used to examine relationships among the holozoan lineages (animals and their protistan allies), with *Nuclearia* and chytrid fungi as outgroups (fig. 2). Although HSP70 sequences were also available for the majority of these taxa, they were excluded from these analyses, as the encoding gene appears to have undergone a number of duplications early in animal evolution (unpublished data).

As expected, phylogenetic analyses of holozoan sequences support the monophyly of true animals (i.e., Animalia or Metazoa; 86%–95% BP, 1.0 biPP, fig. 2, table 2). This confirms that choanoflagellates (*Monosiga ovata*, *Mo. brevicollis*, *D. grandis* and *Sa. amphoridium*), *Ministeria*, *Amoebidium*, and *Corallochytrium* are all sister taxa to the animals, as seen also in figure 1. Also similar to figure 1, these analyses consistently reject the monophyly of the protistan animal allies. Most strikingly, *Ministeria* appears as the closest sister taxon to the animals (86%–96% BP, 1.0 biPP, fig. 2), which is in agreement with the concatenated aa data (fig. 1). However, this position is not consistently supported with various subsets of the data, and this taxon may also be found weakly grouped with choanoflagellates depending on the combination of taxa and genes used to construct the trees (results not shown). A monophyletic choanoflagellate clade (81%–83% BP, 1.0 biPP, fig. 2) appears as the sister taxon to the *Ministeria* + Animals clade, albeit with less support than in figure 1 (fig. 2: 50%–52% BP, 0.94 biPP; fig. 1: 61%–94% BP, 0.1 biPP). Finally, a strongly supported group consisting of the ichthyosporean *Amoebidium* and *Corallochytrium* (74%–91% BP, 1.0 biPP, fig. 2) appears as the first branch of Holozoa (96%–100% BP, 1.0 biPP, fig. 2).

Within the animal clade, a grouping of the so-called "higher" animals (i.e., protostomes and deuterostomes, the "Bilateria") is strongly supported (100% BP, 1.0 biPP, fig. 2), while "lower" animals (Cnidaria, Ctenophora, and Porifera) consistently arise basal to this bilaterian clade. However, resolution within Bilateria is consistently problematic (results not shown). Most notably, the protostomes are weakly supported as arising from within deuterostomes, that is, more closely related to chordates (represented here by human, chicken, and zebra fish; <50%–60% BP, 1.0 biPP, fig. 2) than the latter are to urochordates and sea

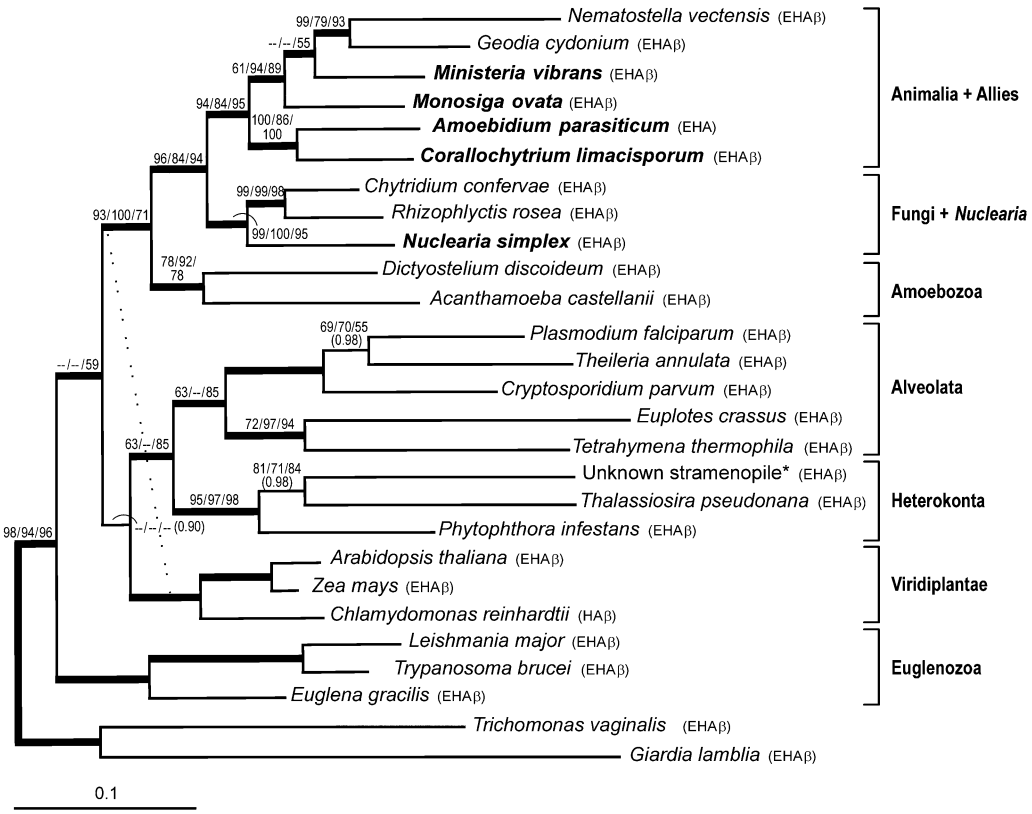


FIG. 1.—Monophyly of animals, fungi and their protistan allies based on concatenated EAHβ protein sequences. The tree shown was derived by Bayesian analysis (BIaa, 1347 universally aligned aa positions) and is rooted with *Trichomonas vaginalis* and *Giardia lamblia*. Identical trees were found by maximum likelihood analysis (MLaa) and parsimony analysis (MPaa), while the NJ distance tree (NJaa) differed only in placing Viridiplantae as sister to the Opisthokonta + Amoebozoa clade (85% BP, indicated with dashed line). Note that, in addition to its opisthokont-specific sequences, the *Nuclearia delicatula* culture harbored an unknown stramenopile (heterokont) contaminant (indicated with an asterisk). Thick lines separate clades with Bayesian posterior probabilities of 1.0. MPaa, NJaa, and MLaa BPs and biPP values <1.0 are given in that order and separated by forward slashes (i.e., MPaa/NJaa/MLaa [biPP]), except where all bootstrap values were ≥99%. Where sequences were not available for some taxa, sequences from different but closely related taxa were substituted as follows. For *Geodia cydonium*, the actin sequence of *Discodermia* sp. was used; for *Rhizophlyctis rosea*, the EF-1α of *Rhizophlyctis harderi* was used; and for *Acanthamoeba castellanii*, *Acanthamoeba culbertsoni*'s HSP70 and actin sequences were used. Genes for which sequences were available are indicated in parentheses after the taxon names by E, A, H, and β, for EF-1α, actin, HSP70 and β-tubulin (EAHβ), respectively.

urchin (*Ciona intestinalis*, *Halocynthia roretzi*, and *Strongylocentrotus purpuratus*). Similarly, none of the branches separating the lower animals receives significant statistical support. Problems associated with accurate reconstruction of evolutionary relationships within the animal clade have been repeatedly noted (e.g., Van de Peer and De Wachter 1997; Adoutte et al. 2000; Collins and Valentine 2001; Rokas et al. 2003). Our results suggest that the use of closely related outgroup taxa alone may not be enough to overcome these problems, although our data still include few taxa and only four genes.

## Phylogeny of Fungi + *Nuclearia*

The "fungal" half of the opisthokont clade was examined in more detail using the animal allies as outgroups and the EAαβ data set (concatenated EF-1α, actin, β-tubulin, and α-tubulin aa sequences), for which the broadest taxonomic sampling of fungal sequences was available. These analyses strongly place *Nuclearia* as the sister group to a monophyletic Fungi (84%–85% BP, 1.0 biPP, fig. 3, table 2). Similar results were obtained when the tubulins were replaced with HSP70 sequences (EAH data set) with the EAHβ data set with a broad taxonomic sampling of eukaryotes (table 2). The EAH data have the advantage of avoiding possible long-branch attraction among the highly divergent tubulins of the nonflagellated fungi (i.e., all fungi except chytrids [Tanabe et al. 2003; Keeling 2003]), but the disadvantage of currently having only one zygomycete (*Rhizopus microsporus*) represented.

As in most analyses of fungal phylogeny (e.g., Berbee and Taylor 2001), a strongly supported grouping of "higher fungi," containing typical ascomycete and basidiomycete clusters, is reconstructed by these data (fig. 3). In contrast, even with the limited number of lower fungi included here, the zygomycetes and chytrids both appear as paraphyletic (fig. 3). In the case of the zygomycetes, *Mortierella* appears as a separate, earlier branch with moderate to strong support (50%–95% BP, 1.0 biPP, fig. 3). In the case of the chytrids, *Blastocladiella* branches separate from a moderately supported *Chytridium* + *Rhizophylctis* clade (<50%–99% BP, 0.57 biPP, fig. 3). These results are consistent with previous, albeit inconclusive single-gene studies (e.g., Jensen

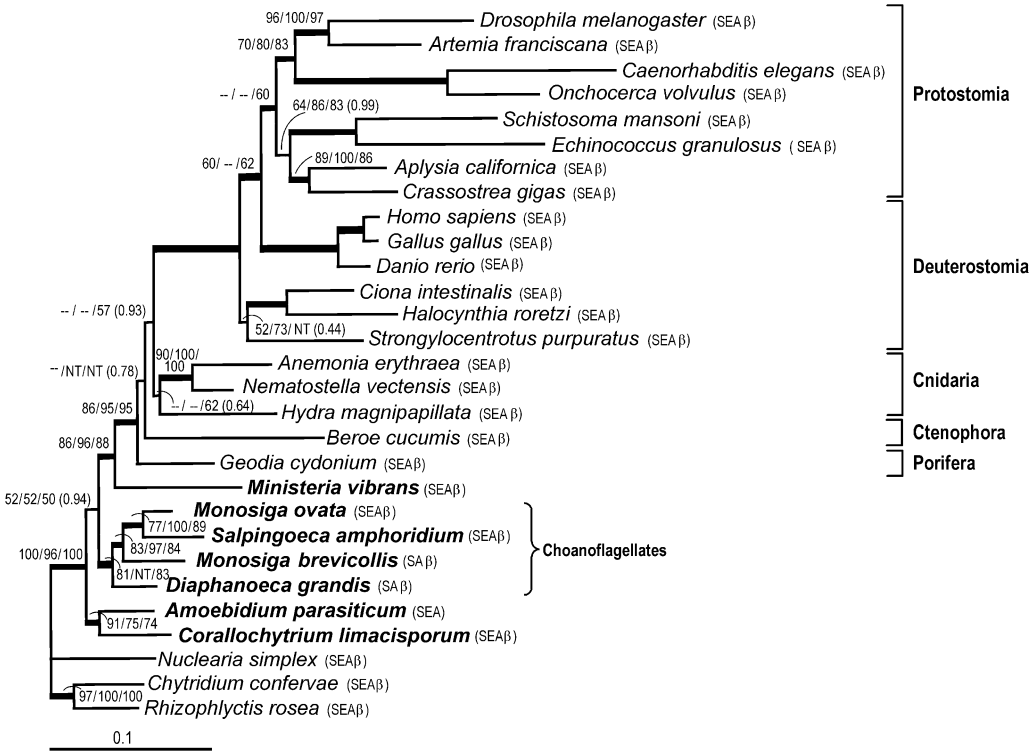


FIG. 2.—Phylogeny of animals and their protistan allies based on the combined SEAβ nt sequences. The tree shown was derived by Bayesian analysis of 2,809 universally aligned positions, with third-codon positions deleted for protein-coding genes and is rooted with *Chytridium confervae* and *Rhizophlyctis rosea*. Similar trees were generated using maximum parsimony (MPnt), maximum likelihood (MLnt), and distance (NJnt) methods, although only taxa for which all four sequences were available were used in the latter two analyses (see below). Where possible, missing sequences were substituted from closely related taxa as follows. For *Aplysia californica*, *Batillus cornutus*'s EF-1α was used; for *Artemia franciscana*, *Homarus americanus*'s β-tubulin was used; for *Geodia cydonium*, *Discodermia* sp.'s actin and SSU sequences were used; for *Crassostrea gigas*, *Crassostrea virginica*'s SSU sequence was used; for *Anemonia erythraea*, *Galaxea fascicularis*'s actin and *Montastraea faveolata*'s β-tubulin were used; for *Beroe cucumis*, *Mnemiopsis leidyi*'s β-tubulin was used; and for *Rhizophlyctis rosea*, *Rhizophlyctis harderi*'s EF-1α was used. Bayesian posterior probabilities are indicated as in figure 1. Bootstrap support values are listed at the corresponding nodes and separated by forward slashes in the order MPnt/NJnt/MLnt. Clade support not estimated in the distance or maximum likelihood analyses due to missing data are indicated with "NT." Branches not reconstructed or supported by a specific inference method are indicated with "–." Genes for which sequences were available are indicated in parentheses after the taxon names by E, A, β, and S for EF-1α, actin, β-tubulin, and SSU, respectively.

et al. 1998; James et al. 2000; Berbee and Taylor 2001; Schüßler, Scwarzott, and Walker 2001; Tanabe et al. 2003), where the base of the fungal tree usually appears as an unresolved mixture of various chytrid and zygomycete taxa. Our results are also consistent with recent combined SSU and large subunit rRNA (LSU) data (Lutzoni et al. 2004) and multiple mitochondrial protein phylogenies (Seif et al. 2005), both of which place the zygomycetes as "intermediate" lineages between higher fungi and the independent lineages of the uniquely flagellated chytrid fungi (fig. 3).

### An Insertion in EF-1α Is a Synapomorphy for Opisthokonta

To investigate the distribution of the ~12-aa EF-1α insertion among eukaryotes, we compared the corresponding portion of the gene in representative fungal and animal opisthokonts, the opisthokont protists, as well as the proposed closest relatives of opisthokonts (i.e., Apusozoa—represented by *Ancyromonas* and *Apusomonas* [Cavalier-Smith and Chao 1995; Atkins, McArthur, and Teske 2000] and Amoebozoa—represented by *Acanthamoeba*, *Phalansterium*, *Entamoeba*, *Dictyostelium*, and *Hartmannella* [Baldauf et al. 2000]). The resulting alignment also includes representatives for the major nonopisthokont eukaryote lineages (plants, heterokonts, alveolates, euglenozoa, heterolobosea, parabasalids, diplomonads, and oxymonads), as well as the archaebacteria. Our results revealed the presence of ~12-aa insertion in all protistan opisthokont lineages, and its absence from even the most closely related outgroup taxa (fig. 4, data not shown). The insertion is especially strikingly conserved among the fungi and *Nucleariа*, where it shows little variation in length or even sequence (fig. 4). In the holozoan lineage, on the other hand, the insertion has sustained a number of additional, internal insertions and deletions with the result that it varies in length from 9 aa in *Corallochytrium* to 17 aa in *Dugesia* (fig. 4).

## Discussion

Analyses of four protein sequences, together (fig. 1) and in various combinations (table 2), all support a monophyletic Opisthokonta consisting of animals, fungi, and four protistan lineages: *Ministeria*, Choanoflagellates, Ichthysporeans + *Corallochytrium*, and *Nucleariа* (figs. 1 and 2, table 2).

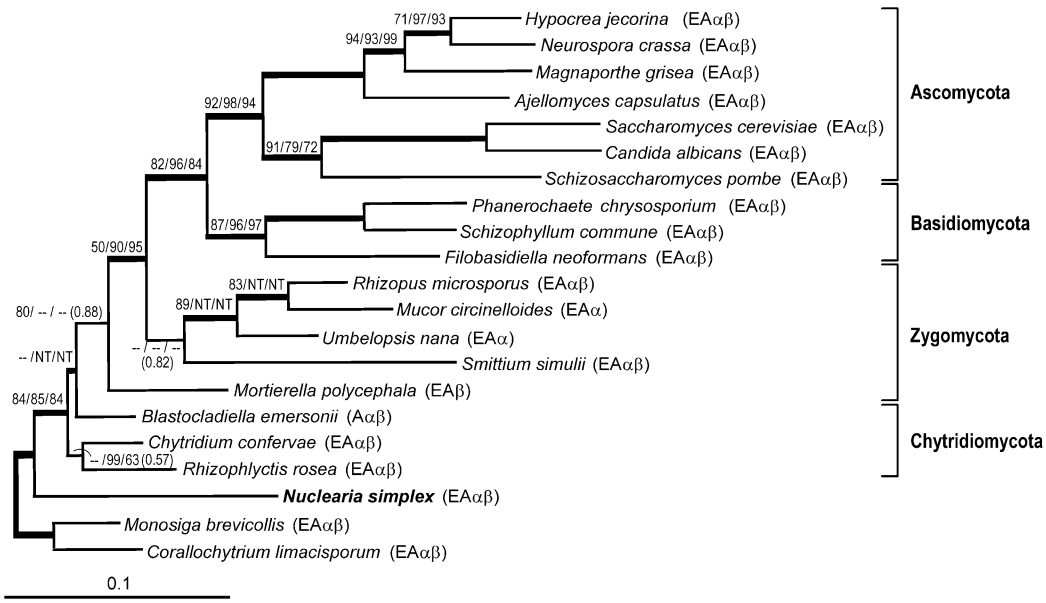


FIG. 3.—Phylogeny of fungi and their protistan allies based on combined EF-1α, actin, β-tubulin, and α-tubulin (EAαβ) protein sequences. The tree shown was derived by Bayesian analysis of 1,298 universally aligned aa positions and is rooted with *Corallochytrium limacisporum* and *Monosiga brevicollis*. Similar trees were generated using maximum parsimony (MPaa), maximum likelihood (MLaa), and distance (NJaa) methods, although only taxa for which all four sequences were available were included in the latter two analyses. Where possible, missing sequences were substituted from closely related taxa as follows. For *Umbelopsis nana*, *Umbelopsis ramanniana*'s α-tubulin sequence was used; for *Smittium simulli*, *Smittium culisetae*'s β-tubulin sequence was used; and for *Rhizophlyctis rosea*, *Rhizophlyctis harderi*'s EF-1α was used. Bootstrap support values and Bayesian posterior probabilities are indicated as in figure 2. Genes for which sequences were available are indicated in parentheses after the taxon names by E, A, β, and α for EF-1α, actin, β-tubulin, and α-tubulin, respectively.

In addition, one of these lineages, *Nuclearia*, is specifically assigned as the sister taxon to fungi (figs. 1 and 3), while the remaining three represent separate sister lineages to animals (figs. 1 and 2). Our data also identify for the first time the taxonomic position of the enigmatic protist *Corallochytrium*, grouping it strongly with the ichthyosporean (figs. 1 and 2). With only the opisthokont protists as outgroup, a consistent phylogeny of fungi is recovered which suggests that both Chytridiomycota and Zygomycota may be paraphyletic with respect to higher fungi (Ascomycota and Basidiomycota, fig. 3). However, even with four gene sequences combined and opisthokont protists as outgroups, the deep phylogeny of animals is still seriously problematic (fig. 2).

The supergroup Opisthokonta is an important taxon that includes both multicellular organisms and their extant single-celled relatives. This grouping is strongly supported by all concatenated nuclear and mitochondrial phylogenies (e.g., Baldauf et al. 2000; Bapteste et al. 2001; Lang et al. 2002). Recently proposed alternative groupings of Plants + Fungi (Philip, Creevey, and McInerney 2005) and Animals + Plants (Löytynoja and Milinkovitch 2001) are strongly rejected by our data (fig. 1 and table 3). Neither of these previous studies included representatives of the opisthokont protists or indeed most other protists nor did they include representatives of lower animals or fungi. Our results suggest that further tests on the legitimacy Opisthokonta would benefit substantially from inclusion of representatives of the opisthokont protists. These taxa should also prove useful in comparative studies of fundamental aspects of animal and fungal biology and help resolve important evolutionary questions in these two major groups, as these protists represent their closest sister taxa.

The specific association of certain protists and animals has been shown previously with phylogenetic analyses of concatenated mitochondrial genes (Lang et al. 2002). These data place the choanoflagellate *Monosiga* and the ichthyosporean *Amoebidium* as specific sister taxa to animals. Lang et al. (2002) have named this grouping of Animals + *Monosiga* + *Amoebidium*, Holozoa. Consistent with this grouping, we report here an *Amoebidium* + *Corallochytrium* clade sister to a Animals + Choanoflagellates + *Ministeria* clade (fig. 1, table 2). We therefore propose that the Holozoa should be extended to include corallochytreans and ministeriids.

One of the most surprising results from these data is the position of *Ministeria* as the closest sister taxon to animals (figs. 1 and 2), which is found to be particularly strongly supported by the SEAβ gene combination (86%–96% BP, 1.0 biPP, fig. 2). However, it is difficult to have complete confidence in this grouping because it is rejected by many combinations of genes and taxa, which tend instead to place *Ministeria* weakly with the choanoflagellates (results not shown). This and other groupings were also not rejected by the AU and SH maximum likelihood tests of alternative topologies (table 3). Additionally, the *Ministeria* sequences appear to have accelerated evolutionary rates relative to some of the holozoa, especially the holozoan protists, and thus tend to form long branches in phylogenetic trees. This suggests that the placement of *Ministeria* in these trees should be viewed with caution. Thus, these data do not allow us to confidently rule out the possibility that choanoflagellates are in fact the closest yet known sister group to animals or that *Ministeria* might be a highly derived choanoflagellate.

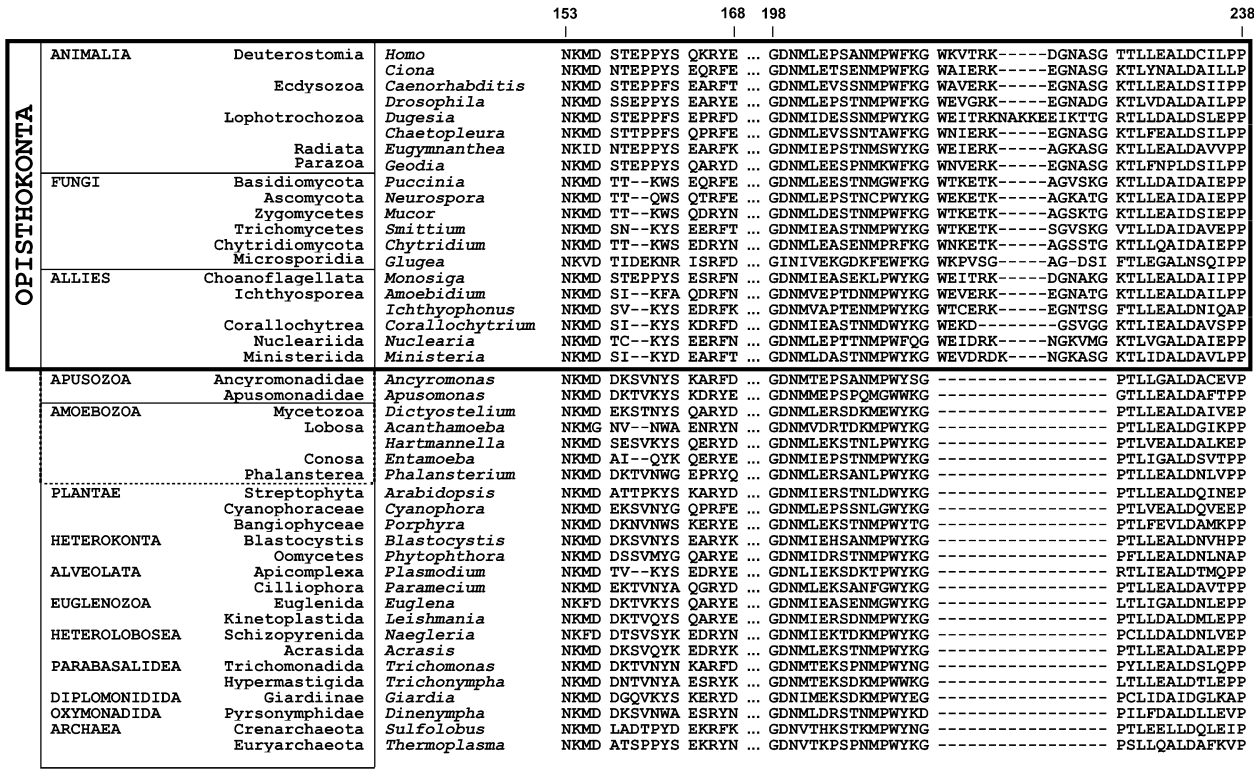


FIG. 4.—The ~12-aa insertion in EF-1α is found in all lineages of Opisthokonta and absent from all nonopisthokonts. The alignment shown includes representatives of all the major eukaryote and archaeal groups and corresponds to positions 153–238 of the *Homo sapiens* EF-1α sequence. Alignment gaps are indicated with hyphens. A conserved motif shared by animals and choanoflagellates is located between positions 157 and 163. Close relatives of opisthokonts are indicated in the dashed box and major higher order taxonomic designations are indicated in the column on the left. The database accession numbers of these sequences are as follows: *Homo*, X03558; *Ciona*, DGI-LQW213151.y1; *Caenorhabditis*, AAA81688; *Chaetopleura*, AAC03161; *Drosophila*, M11744; *Dugesia*, D49924; *Eugymnanthea*, BIAA08665; *Geodia*, CAA70221, *Puccinia*, CAA51932; *Neurospora*, BIAA08274; *Mucor*, AAG29019; *Smittium*, this study; *Chytridium*, this study; *Glugea*, BIAA12288; *Amoebidium*, this study; *Ichthyophonus*, AAL87078; *Corallochytrium*, this study; *Nuclearia*, this study; *Monosiga*, this study; *Ministeria*, this study; *Ancyromonas*, this study; *Apusomonas*, this study; *Dictyostelium*, CAA39442; *Acanthamoeba*, this study; *Hartmannella*, this study; *Entamoeba*, AAA29096; *Phalansterium*, this study; *Arabidopsis*, AAM91202; *Cyanophora*, AAD03711; *Porphyra*, AAA61790; *Blastocystis*, BIAA10962; *Phytophthora*, CAB65347; *Plasmodium*, CAA43018; *Paramecium*, AAD50290; *Euglena*, CAA34769; *Leishmania*, AAL08019; *Naegleria*, AAD15802; *Acrasis*, AAG48934; *Trichomonas*, AAD15799; *Trichonympha*, AAF62512; *Giardia*, BIAA03276; *Dinenympha*, BIAA22607; *Sulfolobus*, CAA36608; *Thermoplasma*, CAA37860. All numbers refer to GenBank accession numbers, except for those obtained from the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/, accessions prefixed with DGI).

In terms of morphology, the choanoflagellates are much more similar to animals than *Ministeria* in that they have uniflagellated motile stages and characteristic collars of tentacles that strongly resemble the choanocytes of sponges (Cavalier-Smith 1987). Our data also show that, among the opisthokonts, only the choanoflagellates share a conserved EF-1α motif with animals (the "STEPPYS" signature, fig. 4). *Ministeria*, on the other hand, has no obvious molecular characters or morphological features (based on the limited data available) that link it to any specific taxon, animals included. However, *Ministeria* and choanoflagellates appear to share certain traits. For example, *Ministeria* apparently uses its pseudopodia in the same way that choanoflagellates use their tentacles, that is, to trap bacteria for phagocytosis (Cavalier-Smith and Chao 2003). Thus, ministeriids could represent derived choanoflagellates that have lost their flagella or for which the flagellate stage has not yet been found. Resolving these questions will require additional sequences and the inclusion of closely related taxa to "break up" any potential long branches at this point in the tree. However, this could be difficult for ministeriids because the only other known species (*Mi. marisola*) is rarely found and is no longer in culture (Patterson 1999).

The results presented here clearly show the monophyly of choanoflagellates (fig. 2). Therefore, consistent with previous reports (e.g., Medina et al. 2003), our results contradict the idea of a choanoflagellate origin for both animals and fungi (Cavalier-Smith 1987). For this hypothesis to be valid, the choanoflagellates would have to be paraphyletic with the salpingoecid (*Sa. amphoridium*) appearing at the base of the fungal clade and the codosigids (*Mo. ovata* and *Mo. brevicollis*) at the base of animals. Instead, these taxa form a monophyletic clade together with the acanthoecid (*D. grandis*), which together form a sister group rather than the ancestral lineage of animals. Nevertheless, the last common ancestor of animals and choanoflagellates may have been a choanoflagellate-like organism somewhat resembling the acanthoecid *D. grandis* (shown here as the most basal choanoflagellate, fig. 2). The ability of

**Table 3**
**Comparison of Alternative Trees Using the SH Test[a]**

| Tree Topology[b] | Tree[c] | ln L | Δln L | *P* Value | Significantly Worse?[d] |
|---|---|---|---|---|---|
| Eukaryotes | | | | | |
| (((((H,(F,N),Am),((Al,S),P)),E),outgroup) | Tree in figure 1 | −21254.9 | 0.0 | | Best |
| (((((H,F),(Am,N),((Al,S),P)),E),outgroup) | *Nucl.* + Amoe. | −21379.9 | −125.0 | 0.001 | Yes |
| (((((H,N),F),Am),((Al,S),P)),E),outgroup) | *Nucl.* basal to Holo. | −21292.3 | −37.4 | 0.267 | No |
| ((((((H,F),Am),N),(Al,S),P)),E),outgroup) | *Nucl.* basal to Opis + Amoe. | −21379.8 | −124.9 | 0.001 | Yes |
| ((((((H,F),N),Am),(Al,S),P)),E),outgroup) | *Nucl.* basal to Opis. | −21291.5 | −36.7 | 0.265 | No |
| (((((((F,N),P),H),Am),(Al,S)),E),outgroup) | Plant basal to Fungi + *Nucl.* | −21388.0 | −133.1 | 0.000 | Yes |
| (((((((F,P),H),N),Am),(As,S)),E),outgroup) | *Nucl.* basal to Plant + Opis. | −21450.3 | −195.4 | 0.000 | Yes |
| ((((((H,P),(F,N)),Am),(As,S)),E),outgroup) | Plant basal to Holo. | −21394.6 | −139.7 | 0.000 | Yes |
| Holozoa | | | | | |
| (((((((B,H),C),P),M),Cf),AC),outgroup) | Tree in figure 2(a) | −28452.4 | −1.7 | 0.961 | No |
| ((((((B,H),C),P),(M,Cf)),AC),outgroup) | *Min.* basal to Choa. | −28477.3 | −26.6 | 0.307 | No* |
| (((((((B,H),C),P),Cf),M)AC),outgroup) | *Min.* basal to Anim. + Choa. | −28468.6 | −17.9 | 0.483 | No |
| (((((((B,H),C),P),Cf),(M,AC)),outgroup) | *Min.* basal to *Cor.* + *Amo.* | −28481.5 | −30.8 | 0.230 | No |
| (((((((B,H),P),C),M),Cf),AC),outgroup) | Cnid. basal to Bilat. | −28451.6 | −0.9 | 0.868 | No |
| (((((((B,C),H),P),M),Cf),AC),outgroup) | Ctenoph. basal to Bilat. | −28466.3 | −15.6 | 0.570 | No |
| ((((((((Pr,De),H),C),P),M),Cf),AC),outgroup) | Deuteros. monophyletic | −28476.0 | −25.3 | 0.338 | No |

[a] The SH test was performed with the PROML and DNAML programs of the PHYLIP package.

[b] The user-defined tree topologies (Newick format) tested in this study. For the eukaryote analyses, the EAHβ aa data set was used and for the holozoa analyses the SEAβ nt data set was used. Abbreviations are as follows for Eukaryotes: H = Holozoa; F = Fungi; N = *Nuclearia*; Am = Amoebozoa; Al = Alveolata; S = Stramenopile or Heterokonta; P = Plants; E = Euglenozoa. Abbreviations are as follows for Holozoa: B = Bilataria; H = Cnidaria; C = Ctenophora; P = Porifera; M = *Ministeria*; Cf = Choanoflagellata; AC = *Amoebidium* plus *Corallochytrium*; Pr = Protostomia; De = Deuterostomia. In the case of the eukaryotic analyses, *Trichomonas* and *Giardia* were used as ''outgroup,'' while chytrids and *Nuclearia* were used as outgroup in the holozoan analyses.

[c] The major feature of the various alternative trees tested. Abbreviations are as follows: *Nucl.* = *Nuclearia*; Holo. = Holozoa; Amoe. = Amoebozoa; Opis. = Opisthokonta; *Min.* = Ministeria; Choa. = Choanoflagellata; Anim = Animalia; *Cor.* = Corallochytrium; *Amo.* = Amoebidium; Cnid. = Cnidaria; Ctenoph. = Ctenophora; Bilat. = Bilataria; Deuteros. = Deuterostomia.

[d] Best = the best phylogenetic hypothesis to fit the respective aa and nt sequence data; No = tree not significantly worse ($P > 0.05$) than other trees; Yes = tree significantly worse ($P < 0.05$) than trees in figures 1 and 2. With the exception of the hypothesized basal position of *Ministeria* to choanoflagellates (indicated with an asterisk), similar results were obtained using the AU test implemented in CONSEL.

*D. grandis* to metabolize silica is also shared by some demosponges and the glass sponges, which together are thought to represent the most basal animal lineages (reviewed by Steenkamp and Baldauf 2004).

Our data resolve the phylogenetic affinity of *Corallochytrium* for the first time, by placing it with the ichthyosporean *Amoebidium* (figs. 1 and 2). This is an unexpected result as this relationship is not seen in SSU rRNA trees, the only previous molecular data for *Corallochytrium* (Cavalier-Smith and Allsopp 1996; Mendoza, Taylor, and Ajello 2002). The only morphological characters apparently linking the relatively simple *Corallochytrium* with the ichthyosporeans are the presence of an amoeboid-like stage, lack of flagellated stages, and presence of well-developed cell walls (Mendoza, Taylor, and Ajello 2002; Cavalier-Smith and Chao 2003), none of which are particularly unique features among eukaryotes. Our data further place this *Amoebidium* + *Corallochytrium* clade as the deepest branch in the holozoa, albeit strongly supported only by Bayesian inference (0.94–1.0 biPP, figs. 1 and 2). However, this placement of *Amoebidium* as sister to an Animals + Choanoflagellates clade is also strongly supported by mitochondrial protein trees (Lang et al. 2002). Together, these results should help to resolve the ongoing debate on the nature of ichthyosporeans (e.g., Ragan et al. 1996; Cavalier-Smith 1998*a*, 2000; Benny and O'Donnell 2000; Ragan, Murphy, and Rand 2003), which are a group of relatively well-studied and important pathogens of diverse animals, including humans, that have variously been suggested to be a sister group of animals, fungi, Animals + Choanoflagellates, and the entire Opisthokonta (Ragan, Murphy, and Rand 1996; Herr et al. 1999; Philippe and Germot 2000; Mendoza, Taylor, and Ajello 2002).

In terms of morphology, the appearance of *Nuclearia* at the base of the fungal clade is unexpected (figs. 1 and 3). Among the protistan opisthokonts, fungi share more morphological features with *Amoebidium*, which was previously classified as a trichomycete fungus (Benny and O'Donnell 2000), than with nucleariids, with which they share no apparent common features. Nucleariids are uni- or multinucleate heterotrophic amoebae with fine pseudopodia that phagocytose algae and bacteria for food (Patterson 1999; Amaral-Zettler et al. 2001). Fungi, on the other hand, are absorptive heterotrophs that usually produce networks of apically extending branched multinucleate tubes (hyphae) in which they live (Hawksworth et al. 1995; Cavalier-Smith 1998*b*, 2001).

The placement of *Nuclearia* in our trees contradicts various SSU-based phylogenies including those of Dykhová et al. (2003) who reported a *Nuclearia* + Ichthyosporeans clade at the base of an Animals + Choanoflagellates clade; Medina et al. (2003) who reported a *Nuclearia* clade basal to animals; and Amaral-Zettler et al. (2001) who found *Nuclearia* to be basal to holozoa. Although the possibility of such alternative groupings is not unequivocally rejected by our data (table 3), the placement of *Nuclearia* at the base of fungi is the only topology reconstructed regardless of the subset of taxa used, and it received strong statistical support from all combined aa data sets (EAHβ: 99%–100% BP, 1.0 biPP, fig. 1; EAαβ: 99%–100% BP,

1.0 biPP, fig. 3; and EAH: 72% BP, table 2) and all but one combined nt data set (table 2). This position for *Nuclearia* is also consistent with those from previous LSU + SSU (Medina et al. 2003) and actin + SSU (Ruiz-Trillo et al. 2004) trees.

Our results, together with the moderate length of the nucleariid branch in phylogenetic trees (figs. 1 and 3), suggest that *N. simplex* and its relatives *N. delicatula*, *N. moebiusi*, and *N. pattersoni* should be excellent outgroups for testing the deep phylogeny of fungi. Closer examination of nucleariid characteristics, such as modes of reproduction, cell wall chemistry, and ultrastructure, will most probably reveal traits that unite them with fungi. Moreover, the common occurrence of a single flagellum in chytrids, some ichthyosporeans, animals, and choanoflagellates suggests that the *Nuclearia* + Fungi ancestor also possessed a flagellum, which was subsequently lost in all fungi except chytrids. The alternative possibility, that chytrids secondarily acquired their flagellum, seems unlikely, but identification and analyses of additional protist taxa that are specifically related to the fungi should resolve this issue.

The exact phylogenetic relationship of the *Nuclearia*-like opisthokont protist, *Capsaspora owczarzaki* (Ruiz-Trillo et al. 2004), with the rest of the opisthokonts was not addressed in our study and remains unclear. At the time we conducted the study, this protist was classified in the genus *Nuclearia* and has only recently been shown to be distinct from the monophyletic and true nucleariids (*N. simplex* and its relatives) (Dykhová et al. 2003; Ruiz-Trillo et al. 2004). *Ca. owczarzaki* has been suggested to be closely related to the ichthyosporeans (Cavalier-Smith and Chao 2003; Hertel, Bayne, and Loker 2003). Other reports suggest that it represents an independent branch within Opisthokonta (Medina et al. 2003; Ruiz-Trillo et al. 2004). Phylogenetic analyses of a multigene data set representing animals, fungi, ministeriids, choanoflagellates, nucleariids, corallochytreans, and ichthyosporeans will undoubtedly clarify whether *Ca. owczarzaki* indeed forms part of the ichthyosporeans, whether it forms part of the Holozoa, and whether it represents a sixth lineage of protistan opisthokonts.

Taken together, our results indicate that the protistan phylum Choanozoa (Cavalier-Smith 1998*a*; Cavalier-Smith and Chao 2003) is deeply paraphyletic. Thus, instead of representing a coherent taxonomic group with a unique common ancestor, this taxon represents an evolutionary grade of premulticellular opisthokonts. Even within Holozoa, the protistan allies of animals are nonmonophyletic. We, therefore, recommend that until solid taxonomic characters are identified, clade names (e.g., Holozoa) rather than organismal names (e.g., "Choanozoa") should be emphasized. In this way, the fundamental relationships among the nonmonophyletic opisthokont protists would be recognized.

Besides molecular phylogenetic data, few traits unite animals and fungi with each other, much less with their protistan allies. The three known synapomorphies (plate-like mitochondrial cristae, uniflagellated motile stages, and the multimeric form of the β-thymosin gene) are not present in all, nor limited to, opisthokonts. Only one character, the ~12-aa insertion in the ancient housekeeping protein EF-1α, has been identified as potentially universal and unique to animals and fungi (Baldauf and Palmer 1993; Baldauf 1999). Alignment of our new EF-1α sequences from the protistan opisthokonts and closely related outgroup taxa, together with representatives of all major divisions of animals and fungi, confirms the presence of this insertion throughout the multicellular opisthokonts and, most importantly, demonstrates its presence in all the examined opisthokont protists where this gene was detected (fig. 4). The derived nature of the EF-1α insertion is confirmed by its absence from representatives of all the major groups of nonopisthokont eukaryotes (fig. 4, data not shown), including the closest proposed relatives of opisthokonts (Apusozoa and Amoebozoa), and by its absence from archaeal EF-1α (Baldauf and Palmer, 1993). This insertion is therefore the only known true synapomorphy that unites the "holo"-Opisthokonta and is a phylogenetic tree–independent marker that distinguishes these taxa from all other known eukaryotes.

The situation is somewhat complicated by the fact that some choanoflagellates and chytrids lack a canonical EF-1α altogether, which appears to have been replaced by an EF-related protein (EF1L; Keeling and Inagaki 2004). However, this replacement seems to be a derived condition within choanoflagellates and fungi (unpublished data). Thus, the EF-1α insertion appears to be a true synapomorphy of the holo-Opisthokonta. For diagnostic purposes, our universal (among eukaryotes) EF-1α primers 13F and 4R specifically target this insertion, while at the same time avoiding possible bacterial contaminants or the alternative forms of the EF-1α gene.

Opisthokonta is an ancient lineage, estimated at over 1 billion years old (Knoll 1992), possibly even representing one of the earliest divergences in the evolution of extant eukaryotes (Stechmann and Cavalier-Smith 2003; Arisue, Hasegawa, and Hashimoto 2005). It includes a group of morphologically highly diverse organisms in which multicellularity evolved at least twice independently (once in fungi and once in animals). Thus, it is not surprising that only a few synapomorphies have been tentatively identified. However, closer examination of representatives for all the opisthokont lineages might reveal additional molecular markers and/or biochemical characters that unite fungi, animals, and their protistan allies.

## Supplementary Materials

Nucleotide substitution saturation plots are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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