Phylogeny of the "Forgotten" Cellular Slime Mold, *Fonticula alba*, Reveals a Key Evolutionary Branch within Opisthokonta

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The shared ancestry between Fungi and animals has been unequivocally demonstrated by abundant molecular and morphological data for well over a decade. Along with the animals and Fungi, multiple protists have been placed in the supergroup Opisthokonta making it exceptionally diverse. In an effort to place the cellular slime mold *Fonticula alba*, an amoeboid protist with aggregative, multicellular fruiting, we sequenced five nuclear encoded genes; small subunit ribosomal RNA, actin, beta-tubulin, elongation factor 1-alpha, and the cytosolic isoform of heat shock protein 70 for phylogenetic analyses. Molecular trees demonstrate that *Fonticula* is an opisthokont that branches sister to filose amoebae in the genus *Nuclearia*. *Fonticula* plus *Nuclearia* are sister to Fungi. We propose a new name for this well-supported clade, Nucletmycea, incorporating *Nuclearia*, *Fonticula*, and Fungi. *Fonticula* represents the first example of a cellular slime mold morphology within Opisthokonta. Thus, there are four types of multicellularity in the supergroup—animal, fungal, colonial, and now aggregative. Our data indicate that multicellularity in *Fonticula* evolved independent of that found in the fungal and animal radiations. With the rapidly expanding sequence and genomic data becoming available from many opisthokont lineages, *Fonticula* may be fundamental to understanding opisthokont evolution as well as any possible commonalities involved with the evolution of multicellularity.

Introduction

Among eukaryotes, multicellularity and its associated cellular differentiation are manifested by a tremendous diversity of forms and functions. Multicellular organization of organisms has evolved numerous times in the evolutionary history of eukaryotes—for most people, vascular green plants, animals, and fungi come to mind first. However, almost all major eukaryotic lineages have multicellular representatives; such as kelps (stramenopiles), some ciliates (*Zoothamnium* and *Sorogena*), most red algae, social amoebae (e.g., *Dictyostelium*, Amoebozoa), and a variety of other lesser known and often forgotten cellular slime molds such as *Acrasis* and *Pocheina* (Heterolobosea) in which multicellularity arises from the aggregation of amoebae.

Two of the most prominent examples of complex multicellular eukaryotic lineages, animals (Metazoa) and Fungi, are part of a monophyletic supergroup along with several unicellular and colonial protist groups collectively known as Opisthokonta (Adl et al. 2005). The monophyly of Opisthokonta is supported by single-gene to multigene phylogenomic data (Amaral Zettler et al. 2001; Hertel et al. 2002; Lang et al. 2002; Cavalier-Smith and Chao 2003; Medina et al. 2003; Bullerwell and Lang 2005; Ruiz-Trillo et al. 2006, 2008; Steenkamp et al. 2006; Carr et al. 2008; Shalchian-Tabrizi et al. 2008) as well as morphological and molecular synapomorphies, such as a single posteriorly directed pushing flagellum in taxa with flagellated life stages, typically flat mitochondrial cristae, and the ubiquity of a ~12 amino acid insertion in the translation elongation factor 1-alpha (EF1α) protein in the taxa that still possess the gene (Baldauf and Palmer 1993; Steenkamp and Baldauf 2004; Ruiz-Trillo et al. 2006). Opisthokonts are split into two major evolutionary branches in multigene phylogenies (Cavalier-Smith and Chao 2003; Ruiz-Trillo et al. 2006, 2008; Shalchian-Tabrizi et al. 2008). One branch includes the Fungi and the other includes the Metazoa (Holozoa branch). The preponderance of unicellular taxa reside on the Holozoa side of the opisthokont tree, including the choanoflagellates that robustly branch as the unicellular sister group to Metazoa (Leadbeater and Kelly 2001; Cavalier-Smith and Chao 2003; King 2004; Steenkamp and Baldauf 2004; Carr et al. 2008; King et al. 2008; Ruiz-Trillo et al. 2008; Shalchian-Tabrizi et al. 2008). Recent molecular analyses of both large subunit and small subunit ribosomal RNA (LSU and SSU rRNA) genes and protein-coding genes have shown that *Nuclearia* is the only unicellular protist taxon to fall on the fungal side of the opisthokont tree (Bullerwell and Lang 2005; Ruiz-Trillo et al. 2006; Steenkamp et al. 2006).

Within the opisthokonts, there are currently three representative types of multicellular organization: animal, fungal, and to a degree colonial. Metazoan multicellularity is found only within the group and is characterized by embryogenesis, where a single cell (the zygote) develops into numerous differentiated cells and tissue types. Mycelia, interconnected, walled filamentous cells, which may exhibit varying degrees of multinuclearity, characterize fungal multicellularity. The few colonial examples are exemplified by some Choanoflagellata, which display very little to no cellular differentiation, as well as two known examples of "pseudomulticellular" colonial forms found in Ichthyosporea (Ruiz-Trillo et al. 2007; Marshall et al. 2008).

The transitional events from unicellular to multicellular lifestyles are considered among the most significant evolutionary adaptations in the eukaryotes. Recent reviews have begun to examine the genomic data that are rapidly accumulating from both multicellular taxa and their unicellular relatives to address possible molecular and developmental commonalities among all of the multicellular lineages (Grosberg and Strathmann 2007; Rokas 2009). The initiative spearheaded by Ruiz-Trillo et al. (2007) will generate a wealth of genomic data from a wide variety of unicellular opisthokonts that may provide the foundation for understanding the molecular machinery(ies) involved in the evolution of multicellularity within a supergroup that diversified from a single, still unknown, common unicellular ancestor.

Key words: *Fonticula alba*, Opisthokonta, multicellularity, aggregation, Nucletmycea, protist.

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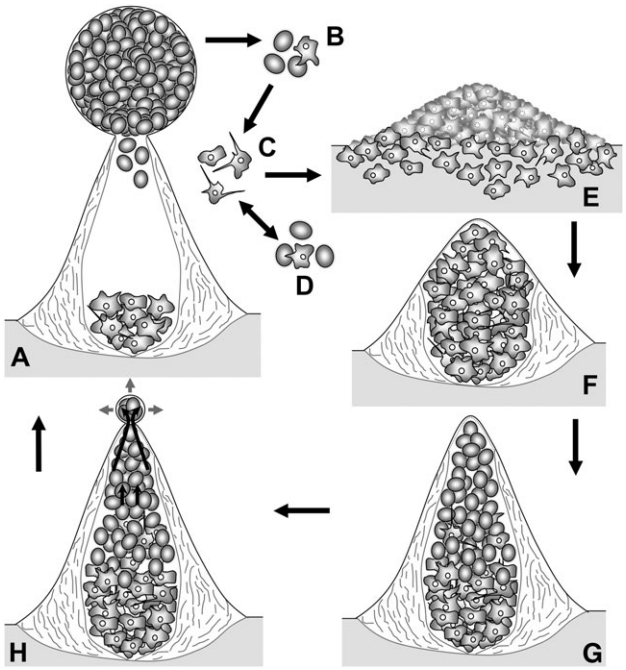

FIG. 1.—Life cycle of *Fonticula alba* based on (Worley et al. 1979; Deasey 1982). (*A*) Mature fruiting body (sorocarp) with a mucoid spore mass (sorus) atop the stalk made of extracellular matrix material. Not all spores get incorporated into the spore mass at maturity, note spores subtending the sorus. At the bottom of the sorocarp, some amoebae remain, which do not become spores. (*B*) Spores, which are surrounded by a mucus sheath, germinate as amoebae. (*C*) Trophic amoebae with filopodia. (*D*) Amoebae can encyst to form cysts morphologically identical to spores. Cysts germinate as amoebae. (*E*) Amoebae aggregate to form a mound. (*F*) The aggregate forms a common slime sheath, and sorogenic amoebae secrete an extracellular matrix of stalk material. (*G*) The upper two-thirds of amoebae within the stalk begin to encyst to form spores. (*H*) When the stalk reaches maturity, a bulge forms at the apex and spores are mechanically forced upward into the sorus, which expands as spores are forced upward.

Fonticula alba, the only species in its genus, is an enigmatic cellular slime mold. It is an amoeboid protist that forms a multicellular fruiting body through aggregation of individual amoebae (fig. 1) (Worley et al. 1979). The taxon is extremely rare—having been isolated only once to the authors' knowledge. Fortunately, the type culture is still available. *Fonticula*'s multicellular organization is unique among cellular slime molds (Olive 1975; Raper 1984). In *Fonticula*, the process of fruiting starts with aggregation of amoebae to form a mound of cells that secrete a Golgi-derived extracellular matrix that transforms the mound to a tapered, extracellular stalk containing sorogenic (spore forming) cells (fig. 1) (Deasey and Olive 1981; Deasey 1982). At stalk maturity, the fruiting body is volcano shaped with the tapering stalk widest at the base. Once mature, the stalk apex opens and sorogenic cells within the upper part differentiate into encysted spores that are forcibly expelled through the stalk apex by an unknown mechanism (Worley et al. 1979; Deasey 1982). Some amoebae located at the base of the stalk persist and continue to produce Golgi-derived stalk material even after the spore mass is removed (Deasey 1982). Spores germinate under appropriate conditions into amoebae that possess "true" filose pseudopodia that are not supported by microtubules (filopodia) (fig. 2*a–d*) and have discoid mitochondrial cristae (Deasey 1982). These morphological features are also present in *Nuclearia*, which prompted Cavalier-Smith (1993) to place these taxa together into the Rhizopoda, Class Cristidiscoidea. Later, Cavalier-Smith (1998) emended Cristidiscoidea by including *Ministeria*, a genus of filopodial amoebae with nearly discoid, flat mitochondrial cristae (Cavalier-Smith and Chao 2003) and suggested an affiliation with Choanozoa within Opisthokonta.

Because *Fonticula* is a protist that develops into a multicellular entity, its phylogenetic placement has significant implications on the evolution of multicellularity, no matter if it branches with *Nuclearia*, *Ministeria*, or even as an independent lineage within the opisthokonts, or elsewhere among the eukaryotes. Therefore, we have sequenced its SSU rRNA gene as well as four phylogenetically informative protein-coding genes. These multigene analyses demonstrate that *Fonticula* is an opisthokont that branches sister to *Nuclearia*. In addition, we provide basic morphological details of this organism that have not been sufficiently illustrated in the past literature.

Materials and Methods

Culturing

The type culture of *F. alba* (strain K2) was obtained from the American Type Culture Collection (ATCC accession 38817). This strain was received in frozen stasis from ATCC contaminated with an unidentified filamentous fungus. Through spore isolation using a flame sterilized minuten needle in accordance with the technique described in Brown et al. (2007), we successfully established a mono-eukaryotic culture consisting of *Fonticula* and unidentified bacteria, devoid of other eukaryotes. The culture has been maintained through monthly serial transfer onto nutrient agar plates (8.00 g Nutrient Broth, 15.00 g Difco Bacto Agar, 1.0 l deionized H_2O) inoculated with the Gram-negative bacterium *Klebsiella pneumoniae*. Fruiting diminished and then ceased when the culture was grown exclusively on bacteria.

Nucleic Acid Extraction, Polymerase Chain Reaction (PCR), Cloning, and Sequencing

Cultures were grown to adequate cell densities for nucleic acid extraction. Total genomic DNA was obtained through a modified Chelex Resin (BioRad, Hercules, CA) extraction method (Singer-Sam et al. 1989). Total RNA was isolated using TRI reagent (Sigma–Aldrich, St Louis, MO) following the manufacturer's recommended protocol. Purified polyA+ RNA was obtained using the Poly(A) Purist mRNA purification kit (Ambion, Austin, TX). First-strand cDNA was synthesized using a Smart cDNA synthesis kit using the SMART oligo-dT primer following manufacturer recommendations (Clontech, Mountain View, CA). Complete second-strand cDNA (ds cDNA) synthesis was performed using the Advantage 2 DNA polymerase (Clontech) following the manufacturer's suggested protocols.

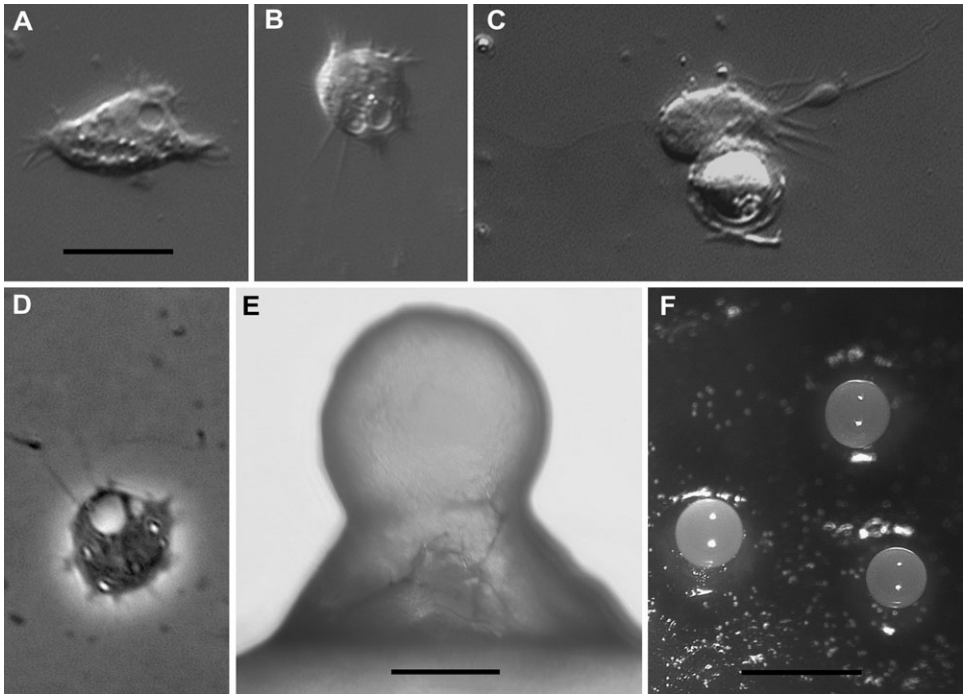

FIG. 2.—Light micrographs of *Fonticula alba*. (*A–C*) Typical trophic amoebae on an agar culture slides. Note filose pseudopodia (differential interference contrast). (*D*) An amoeba with filopodia on an agar culture slide (phase contrast). (*A–D*) Scale bar = 10 μm. (*E*) Multicellular fruiting body on a dry slide (brightfield). Scale bar = 100 μm. (*F*) Three fruiting bodies on a culture plate viewed from above under reflected light. Scale bar = 500 μm.

The SSU rRNA, β-tubulin, and actin genes were PCR amplified directly from genomic DNA using GoTaq DNA polymerase (Promega, Madison, WI). The SSU rRNA gene was amplified using universal eukaryotic specific primers (primers A and B from Medlin et al. 1988) and the cycling parameters described in Brown et al. (2007). The actin gene was amplified using ActN2F (Fahrni et al. 2003) and ACT-BXR (Brown et al. 2007). The β-tubulin gene was amplified using Btub20F (5′-CAR ATH GGN GCN AAR TTY TGG GA-3′ [peptide motif: QIGAKFW]) and Btub412R (5′-TCC ATN CCY TCI CCN GTR TAC CA-3′ [peptide motif: WYTGEGMD]). The EF1α and the cytosolic isoform of heat shock protein 70 (Hsp70c) genes were PCR amplified from ds cDNA. The EF1α gene was amplified in two overlapping fragments using primers 1F (Baldauf and Doolittle 1997) with EF4R (5′-CAT GTC ACG GAC GGC GAA ACG AC-3′ [peptide motif: GRFAVRD]) and FaEF1aF1_mb (5′-CGA CCG CCG CTC GGG TAA-3′ [*F. alba* gene-specific primer] with 3′ SMART cDNA primer (Clontech). The Hsp70c gene was amplified in two overlapping fragments using primers HSP70F61 (5′-GGI ATH GAY YTI GGN ACN ACN TA-3′ [peptide motif: GIDLGTTY]) with HSP701470R (5′-GCY TCR TCD GGG TTG ATR GA-3′ [peptide motif: SINPDE]) and FaHSP70F1_mb (5′-CTC TGC TCG GAC CTC TTC CGT GG-3′ [*F. alba* gene-specific primer]) with HSP70R_mb (5′-CGN CCY TTR TCR TTN GT-3′ [peptide motif: TNDKGR]). All protein-coding genes were amplified using the step-down cycling parameters employed in Brown et al. (2007). PCR products were cloned into TOPO-TA vector pCR4 (Invitrogen, Carlsbad, CA). The SSU rDNA, actin, and β-tubulin clones were sequenced in both orientations using vector and internal primers. The primary PCR products of the EF1α and Hsp70c genes, 1F with EF4R and HSP70F61 with HSP701470R, respectively, were TOPO-TA cloned and sequenced in both orientations with vector primers. The 3′ end of EF1α (PCR product generated with FaEF1aF1_mb with 3′SMART) and the 3′ end of Hsp70c (PCR product generated with FaHSP70F1_mb with HSP70R_mb) were sequenced with the 5′ (forward) PCR primers and internal primers in only the forward orientation from purified PCR products, each as two independent sequencing reads. These PCR products were purified with the QIAquick Gel Extraction kit (Qiagen, Valencia, CA) using the manufacturer's protocols. Sequence chromatograms were manually verified and contigs assembled in Sequencher 4.8 (GeneCodes, Ann Arbor, MI). For cloned genes, between one and four clones were sequenced. Sequences obtained from *Fonticula* that were used in this study have been deposited to GenBank under accession numbers FJ816014 (actin), FJ816015 (β-tubulin), FJ816016 (EF1α), FJ816017 (Hsp70c), and FJ816018 (SSU rRNA).

Phylogenetic Analyses

Multigene phylogenetic analyses were performed on data sets consisting of the concatenation of actin, β-tubulin, EF1α, and Hsp70c protein sequences, with (five-gene) and without nuclear encoded SSU rRNA (four-gene) from 42 eukaryotic taxa including *Fonticula*. Accession numbers of sequences used in our analyses can be found in supplementary table S1, Supplementary Material online. Initial protein alignments were created in ClustalW (Larkin et al. 2007) and subsequently refined by eye in MacClade

v4.08 (Sinauer Associates, Sunderland, MA). The *Fonticula* SSU sequence was laced into preexisting alignments. Only unambiguously aligned positions were utilized in phylogenetic analyses comprising a total of 2,802 aligned characters with 1,234-nt characters from SSU rDNA sequences, 288 amino acid characters from actin, 388 from β-tubulin, 411 from EF1α, and 481 amino acid characters from Hsp70c. The total amount of missing data across the entire five-gene and four-gene data sets was 10.01% and 17.47%, respectively.

The protein-coding genes used in our analyses were assessed for phylogenetic congruence in a likelihood framework by means of the program Concaterpillar v1.3 (Leigh et al. 2008). An amino acid alignment of each gene (same inclusion sets as above) was given as a separate input from which we removed the taxa that were missing that specific gene in our alignment. This input method allowed us to retain all taxa used in our analyses. As Concaterpillar was designed for patchy data sets, this method was appropriate. Using the default P value cutoff of 0.05, all genes and branch lengths were found to be congruent. Because of a current limitation of Concaterpillar, we were unable to analyze our full mixed data set (i.e., amino acid + nucleotide characters), but the optimum five-gene tree (fig. 3), the optimum four-gene tree inferred from the protein-coding genes alone (fig. 4), and the single-gene SSU rDNA tree (fig. 5) topologies are largely congruent.

Bayesian inference (BI) and maximum likelihood (ML) analyses were run under per gene partition models. The SSU rDNA partition was analyzed under the general time reversible model + gamma distribution + estimation of proportion of invariant sites (GTR + Γ + I) as suggested by the Akaike information criterion (AIC) in Modeltest v3.7 (Posada and Crandall 1998), with six discrete rate categories. All amino acid partitions were run under the RTREV amino acid model + gamma distribution + empirical base frequencies (RTREV + Γ + F) as suggested by AIC in ProtTest (Abascal et al. 2005) on the ProtTest server (http://darwin.uvigo.es/software/prottest_server.html). Bayesian analyses run in MrBayes v3.12 (Ronquist and Huelsenbeck 2003; Altekar et al. 2004) consisted of two independent Markov chain Monte Carlo runs of 2,000,000 generations printing trees every 1,000 generations with a burn-in of 346,000 generations for the five-gene analysis and 200,000 generations for the four-gene analysis, by which time all parameters converged as assessed by an average standard split deviation $\leq$ 0.01 and the potential scale reduction factor convergence diagnostic. All BI analyses were carried out on the University of Oslo's Bioportal (www.bioportal.uio.no). Topological support was assessed through 1,000 bootstrap replicates in RAxML v7.0.4 (Stamatakis et al. 2008) on the freely available CIPRES portal (www.phylo.org). Bootstrap values were drawn onto the best-scoring tree of 300 ML tree searches carried out in RAxML.

The SSU rDNA phylogenetic analyses consisted of 44 SSU rDNA sequences from various opisthokonts and other protist taxa that have been shown elsewhere to be closely related to the opisthokonts. Numerous *Nuclearia* SSU rRNA gene sequences were included to determine if *Fonticula* branched among these amoebae. Phylogenetic analyses were based on 1,234 unambiguously aligned nucleotide characters. Trees were built using the same methods and model as above. The burn-in for the BI analyses of the SSU rDNA data set was 200,000 generations, by which time all parameters converged (assessed as above). Topological support was assessed by 1,000 ML bootstrap replicates in both Garli v0.951 (Zwickl 2006) and RAxML.

Approximately Unbiased (AU) Topology Testing

The AU tests were preformed independently for the four-gene, five-gene, and SSU rDNA data sets. *Fonticula* alone, as well as the clade of *Fonticula* + *Nuclearia simplex* (in the four- and five-gene AU tests) were constrained to branch with various taxa followed by reoptimization of the unconstrained nodes by 10 ML tree searches using RAxML with the models mentioned above. The best-scoring ML tree from each constraint tree search was added to a text file in Newick format that also contained the best-scoring unconstrained ML tree and a set of 300 plausible trees (i.e., bootstrap trees). Site likelihoods were calculated in RAxML and the AU tests were performed with Consel v0.1i (Shimodaira and Hasegawa 2001). The set of trees with $P \geq 0.05$ should contain the true tree with a probability of 95%. Therefore, constrained trees not inside the set ($P < 0.05$) were ruled out for further consideration.

Results

Morphological Observations

The amoebae of *F. alba* are small and irregular in form, ranging in size from 7 to 13 μm with an average length–breadth ratio of ~1.4. Amoebae have a single indistinct nucleus (fig. 2*a–d*). Trophic amoebae can have long filopodia that may extend to several body lengths (4–15 μm) (fig. 2*c*). These observations are consistent with the excellent descriptions in earlier studies (Worley et al. 1979; Deasey 1982; Raper 1984). However, no illustrations have been published that actually show the filopodia that are described in these works; published micrographs of *Fonticula*, show the amoebae with retracted pseudopodia that appear more lobose with broad lobopodia (see fig. 7 of Worley et al. 1979). Figure 2*a–d*, therefore, are the first high-quality images of this character. When amoebae are kept "happy" at appropriate conditions, their filopodia are evident (fig. 2*a–d*). As a voucher for the organism from which our molecular data were obtained, we also present micrographs of fruiting bodies (fig. 2e and *f*).

Multigene Phylogenetic Analyses

Analyses of congruence among the protein-coding genes using Concaterpillar suggest that concatenation of these genes for phylogenetic analyses was acceptable ($P \geq 0.05$). This indicates that the genes were compatible both in phylogenetic signal and individual branch lengths. The tree topologies inferred using ML and BI of concatenated SSU rRNA, actin, β-tubulin, EF1α, and Hsp70c sequences (five-gene data set) and protein-only (four-gene

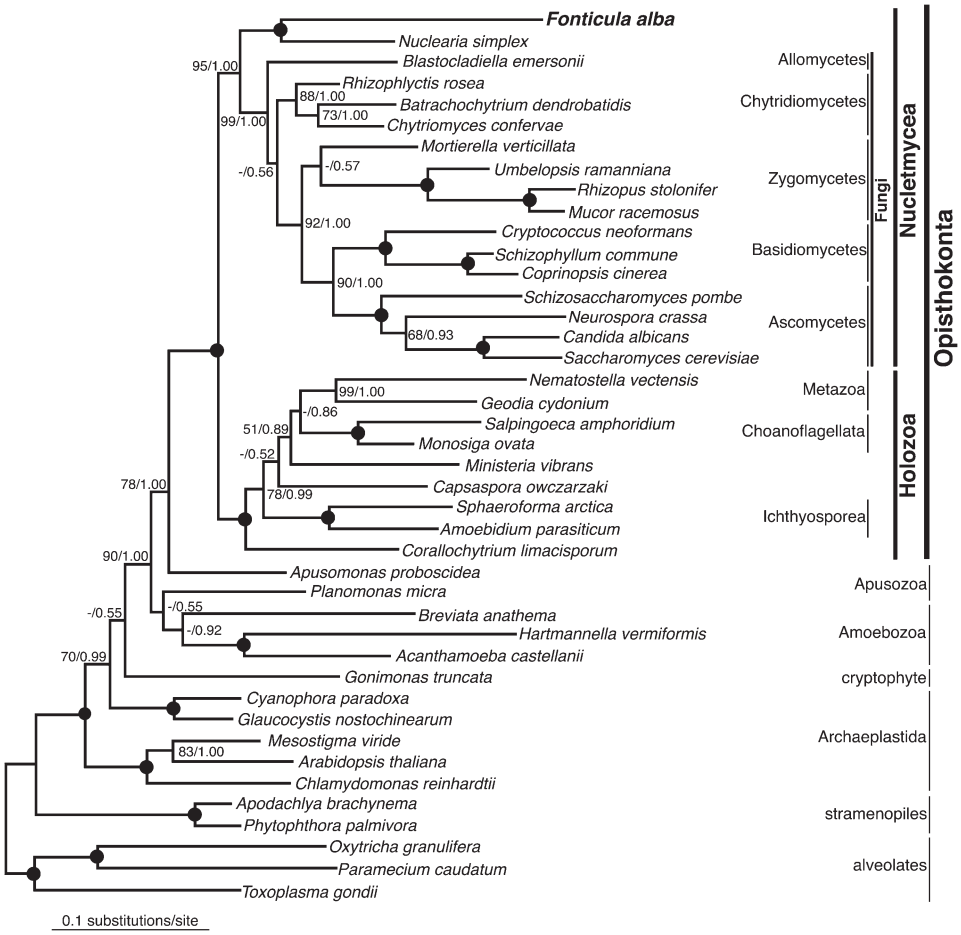

FIG. 3.—Bayesian tree constructed from five concatenated genes. Support values at each node are presented for RAxML/BI. ML bootstrap values and BI posterior probabilities equal to 100%/1.00, respectively, are represented by a black dot. Unrecovered topologies and support values under 50% or 0.50 for the respective method are represented by a -. The scale bar represents evolutionary distance in changes per site.

data set) were largely congruent with very similar support values for all nodes (figs. 3 and 4). For the five-gene data set (to which we will restrict our subsequent observations), ML and BI yielded similar tree topologies only differing in the placement of *Blastocladiella emersonii* within the Fungi and the placement of *Breviata anathema* in the Amoebozoa.

Fonticula alba is an opisthokont (fig. 3). Opisthokonta, including *Fonticula*, is monophyletic with high ML bootstrap and BI posterior probability support (100%/1.00, respectively). The 12 amino acid insertion in EF1α common to opisthokonts is found in *Fonticula* supporting this phylogenetic placement (see supplementary fig. S1, Supplementary Material online). *Fonticula* branches with high support as sister to the filose amoeba, *N. simplex* (100%/1.00), the only species of *Nuclearia* (to date) from which all the genes used in these analyses were publicly available. The *Nuclearia* + *Fonticula* clade (from here on referred to as nuclearioid amoebae) branches on the fungal side of the opisthokont dichotomy as sister to a monophyletic Fungi with high support (95%/1.00) (fig. 3). The fungal clade is recovered with high support (99%/1.00). Within the fungal lineage, *Blastocladiella* (Allomycetes) occupies the basal-most position followed by a monophyletic Chytridiomycetes clade that is sister to the Zygomycetes + dikaryomycetes (Ascomycetes + Basidiomycetes). A monophyletic Holozoa is recovered with high support (100%/1.00). *Corallochytrium* branches as an independent lineage basal to the rest of Holozoa (fig. 3). Ichthyosporea are monophyletic with high support (100%/1.00). Choanoflagellata is recovered as sister to the metazoans with very low support (38%/0.86). Outside of Opisthokonta, Apusozoa as represented by *Apusomonas proboscidea* and *Planomonas micra*, is paraphyletic. *Apusomonas proboscidea* is sister to Opisthokonta with moderate support (78%/1.00), whereas the newly described apusozoan, *P. micra* (Cavalier-Smith et al. 2008), previously known as *Ancyromonas sigmoides,* is sister to Amoebozoa but with little topological support (fig. 3). A monophyletic Amoebozoa received low ML bootstrap support but moderate BI support (0.92). We do not recover a "unikont" clade (Amoebozoa + Opisthokonta) to the exclusion of either of the apusozoans. The topology of outgroup taxa is consistent with other studies of similar content (Kim et al. 2006). We recover a monophyletic alveolate clade sister to a stramenopile clade with high support (100%/1.00). The supergroup Archaeplastida, represented by a highly supported clade of glaucophytes and a highly supported clade of green plants/algae, is paraphyletic in our five-gene analyses,

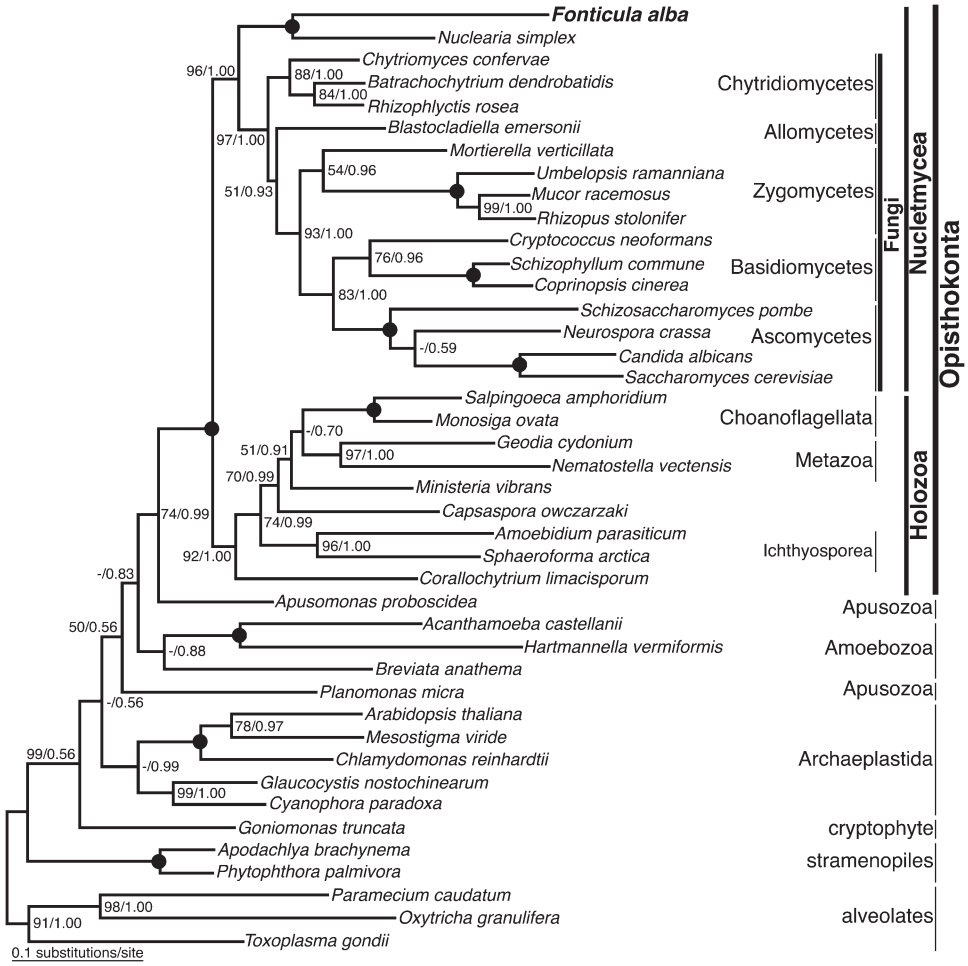

FIG. 4.—ML tree constructed from four concatenated protein-coding genes. Support values at each node are presented for RAxML/BI. ML bootstrap values and BI posterior probabilities equal to 100%/1.00, respectively, are represented by a black dot. Unrecovered topologies and support values under 50% or 0.50 for the respective method are represented by a -. The scale bar represents evolutionary distance in changes per site.

which is common in analyses of so few genes (Rodríguez-Ezpeleta et al. 2005; Kim and Graham 2008).

SSU rDNA Phylogenetic Analyses

A rooted, opisthokont-rich, SSU rRNA gene data set was analyzed to determine if *Fonticula* branched within the genus *Nucleariа* (fig. 5). *Fonticula* is recovered as sister to the genus in SSU rDNA analyses that includes all available *Nucleariа* sequences. The genus *Nucleariа* is a very highly supported clade to the exclusion of *Fonticula*. The support for the placement of *Fonticula* in relationship to the other well-supported opisthokont lineages is inconclusive because of low backbone support among deep nodes recovered in these analyses (fig. 5).

AU Testing

Several competing hypotheses relating to the evolutionary position of *Fonticula* within the eukaryotic Tree of Life were tested in a likelihood framework using the AU test. Table 1 lists the results of AU tests of the five-gene and four-gene analyses where *Fonticula* or the clade *Fonticula* + *Nucleariа* are constrained with various lineages. This latter constraint was performed because *Fonticula* and *Nucleariа* form a highly supported clade in optimum trees. The *P* value for most alternative hypotheses for the placement of *Fonticula* and *Fonticula* + *Nucleariа* was less than 0.05 and these trees were not considered further. Of note, all trees constraining nuclearioid amoebae within Holozoa or with any nonopisthokont outgroup fell outside the 95% confidence interval. Conversely, the AU test cannot reject the placement of *Fonticula* + *Nucleariа* as sister to the other opisthokonts or sister to Holozoa.

The phylogenetic affinity of *N. simplex* to Fungi is apparent because it remains sister to the Fungi when *Fonticula* was constrained to branch with most nodes of the five- and four-gene trees. In a few reoptimized constraints (five-gene: *Fal* + *Pmi*, *Fal* + *Gtr* and four-gene: *Fal* + *Pmi*, *Fal* + *Ban*, *Fal* + *Gtr*), *N. simplex* followed *Fonticula* and grouped as sister to the constraint (see supplementary table S3, Supplementary Material online), perhaps leading to the rejection of these hypotheses. Therefore, we also constrained *N. simplex* to Fungi in addition to the constraint of *Fonticula* to these taxa (table 1). These constraint topologies could be rejected.

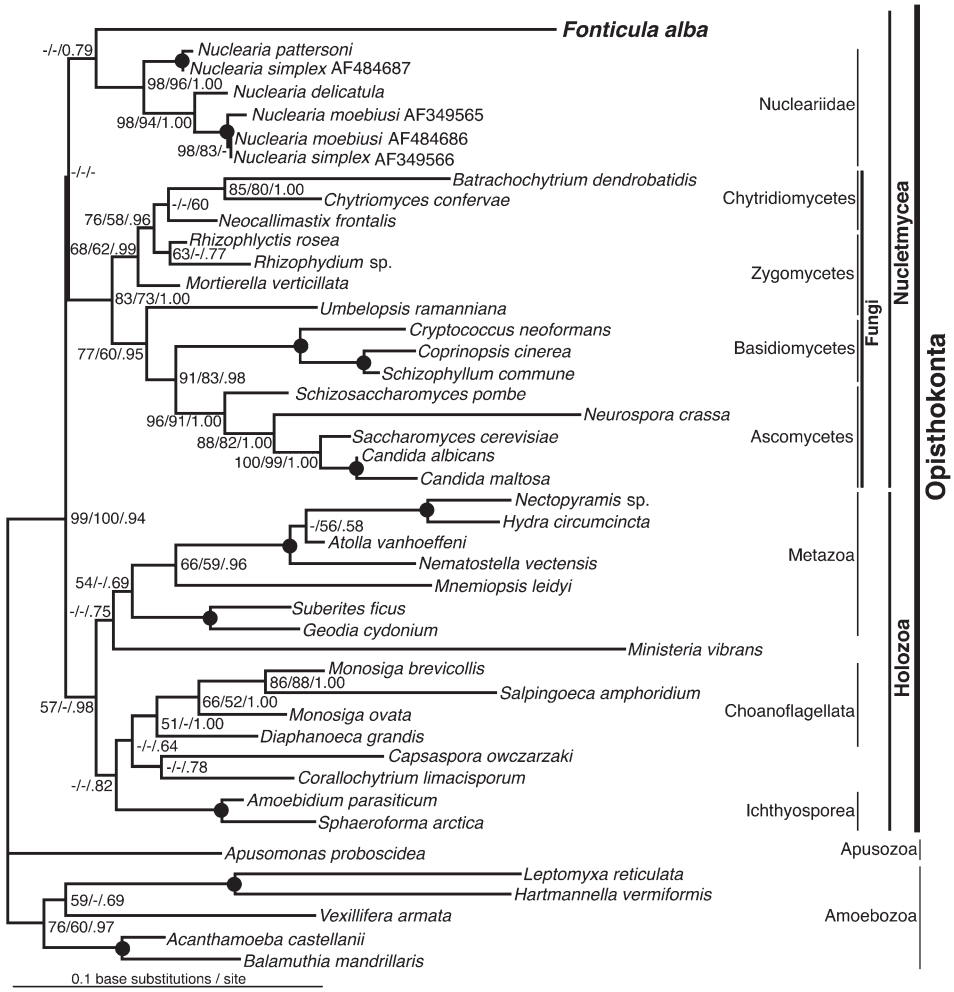

FIG. 5.—ML tree inferred from SSU rRNA gene sequences. Support values at each node are presented for RAxML/GarliML/BI. RAxML ML bootstrap values, Garli ML bootstrap values, and BI posterior probabilities equal to 100%/100%/1.00, respectively, are represented by a black dot. Unrecovered topologies and values under 50% or 0.50 for the respective method are represented by a -. The scale bar represents evolutionary distance in changes per site.

The AU testing of the multigene and SSU rDNA data yielded drastically differing results when similar hypotheses are tested (table 1, supplementary table S2, Supplementary Material online). In the single-gene SSU data set, most alternative branching positions for *Fonticula* cannot be rejected. This is likely due to the poorly resolved deep nodes in SSU rDNA analyses. The AU testing of the SSU rDNA data is somewhat useful to test the possibility that *Fonticula* may be a *Nuclearia* with the ability to fruit (supplementary table S2, Supplementary Material online). The placement of *Fonticula* with *N. simplex* (AF349566) + *Nuclearia moebiusi* was rejected ($P = 0.035$). However, the placement of *Fonticula* with *Nuclearia pattersoni* + *N. simplex* (AF484687) and *Fonticula* with *Nuclearia delicatula* cannot be rejected with P values of 0.344 and 0.162, respectively.

Discussion

Fonticula alba is an opisthokont protist (figs. 3–5). The optimum tree topologies of our multigene phylogenetic analyses recover Opisthokonta including *Fonticula* with high support. All trees within the 95% confidence interval established in the AU test results have *Fonticula* branching with Opisthokonta (table 1). In addition, *Fonticula* has the EF1α amino acid insertion that is unique to Opisthokonta (supplementary fig. S1, Supplementary Material online).

The molecular data show *Fonticula* as closely related to the genus *Nuclearia* (figs. 3–5). This is also supported by morphology (Cavalier-Smith 1993). In our *Nuclearia*-rich SSU rDNA phylogenetic analyses, *Fonticula* does not appear to be a *Nuclearia* with the ability to fruit but rather an independent lineage sister to *Nuclearia* (fig. 5). Nucleariod amoebae, *Fonticula* + *Nuclearia*, consistently group away from *Ministeria vibrans*, making Cristidiscoidea, at best, a paraphyletic assemblage (Cavalier-Smith and Chao 2003; Steenkamp et al. 2006). This is consistent with the most recent interpretation of Cristidiscoidea sensu Shalchian-Tabrizi et al. (2008), which excludes *M. vibrans*.

Our optimal trees recover the Holozoa and the Fungi clades each with high support and recover *Fonticula* + *Nuclearia* as sister to the Fungi with high support. Potential uncertainties of the exact placement of *Fonticula* within

Table 1
The AU Topological Test Constraints and *P* Values Obtained for Each

Tree	Description	AU (SABEH)	AU (ABEH)
1	Optimal Tree RAxML	0.853	0.913
2	*Fal*, *Nsi*, (opisthokont)	0.530	0.402
3	*Fal*, *Nsi*, *Bem*	0.209	0.402
4	*Fal*, *Nsi*, Holozoa	0.111	0.173
5	*Fal*, Fungi	0.052	0.043
6	*Fal*, *Nsi*, Chytridiomycetes	0.043	0.022
7	*Fal*, *Bem*	0.036	0.032
8	*Fal*, (Fungi, *Nsi*)	0.022	0.163
9	*Nsi*, Fungi	0.022	0.163
10	*Fal*, *Gtr*	0.022	0.006
11	*Fal*, *Nsi*, plant	0.021	3.00E−37
12	*Fal*, Metazoa	0.020	2.00E−125
13	(*Fal*, *Pmi*), (*Nsi*, Fungi)	0.018	1.00E−79
14	*Fal*, (opisthokont)	0.010	0.006
15	*Fal*, Basidiomycetes	0.007	0.026
16	(*Fal*, *Gtr*), (*Nsi*, Fungi)	0.007	0.003
17	*Fal*, *Nsi*, glaucophytes	0.006	0.008
18	*Fal*, *Nsi*, *Pmi*	0.005	0.003
19	*Fal*, *Ban*	0.004	2.00E−04
20	*Fal*, *Cow*	0.003	6.00E−06
21	*Fal*, Ichthyosporea	0.002	0.045
22	*Fal*, Ascomycetes	0.002	0.030
23	*Fal*, *Nsi*, Zygomycetes	0.002	0.028
24	(*Fal*, *Ban*), (*Nsi*, Fungi)	0.002	4.00E−04
25	*Fal*, *Nsi*, Amoebozoa	0.002	3.00E−05
26	*Fal*, *Nsi*, *Cow*	0.002	6.00E−54
27	*Fal*, Amoebozoa	0.002	8.00E−60
28	*Fal*, stramenopiles	0.001	8.00E−58
29	*Fal*, *Nsi*, *Ban*	4.00E−04	0.034
30	*Fal*, *Nsi*, Amoebozoa (- *Ban*)	3.00E−04	0.007
31	*Fal*, *Nsi*, *Gtr*	2.00E−04	0.011
32	*Fal*, *Nsi*, *Apr*	1.00E−04	0.011
33	*Fal*, *Nsi*, *Cli*	1.00E−04	0.001
34	*Fal*, Holozoa	6.00E−05	0.023
35	*Fal*, *Nsi*, Ascomycetes	5.00E−05	0.003
36	*Fal*, *Nsi*, Ichthyosporea	1.00E−05	0.018
37	*Fal*, plant	1.00E−05	0.003
38	*Fal*, Amoebozoa (- *Ban*)	1.00E−05	2.00E−57
39	*Fal*, *Cli*	7.00E−06	0.008
40	*Fal*, alveolates	1.00E−06	2.00E−44
41	*Fal*, *Mvi*	4.00E−08	1.00E−30
42	*Fal*, *Nsi*, Basidiomycetes	8.00E−12	0.001
43	*Fal*, Zygomycetes	1.00E−14	0.002
44	*Fal*, *Nsi*, alveolates	7.00E−35	2.00E−65
45	*Fal*, Choanoflagellata	5.00E−38	0.001
46	*Fal*, *Nsi*, Metazoa	5.00E−51	0.004
47	*Fal*, *Apr*	3.00E−60	0.003
48	*Fal*, *Nsi*, *Mvi*	5.00E−62	5.00E−31
49	*Fal*, *Nsi*, stramenopiles	4.00E−64	0.029
50	*Fal*, Chytridiomycetes	1.00E−66	1.00E−04
51	*Fal*, *Pmi*	3.00E−78	0.010
52	*Fal*, glaucophytes	1.00E−86	2.00E−31
53	*Fal*, *Nsi*, Choanoflagellata	4.00E−87	0.004

S = SSU rDNA, A = actin, B = β-tubulin, E = EF1α, and H = Hsp70c. Binomen abbreviations used for taxa as *Fonticula alba* = *Fal*. *Fal*, (opisthokont) = *Fonticula* constrained as the sister to all other opisthokonts. *Fa1*, Amoebozoa (- *Ban*) = Amoebozoa less *Breviata anathema*. plant = *Arabidopsis thaliana*, *Chlamydomonas reinhardtii*, and *Mesostigma viride*; glaucophytes = *Cyanophora paradoxa* and *Glaucocystis nostochinearum*. Tree numbers correspond to the tree numbers in supplementary table S3 (Supplementary Material online). The hypotheses within the 95% confidence interval that could not be rejected are where $P \geq 0.05$.

Opisthokonta are raised by the AU test results (table 1). Of note, *Fonticula* + *Nuclearia* could not be rejected as sister to either the rest of Opisthokonta or sister to Holozoa because these topologies are found within the 95% confidence interval of the AU test results. Morphology does not discount these alternative hypotheses. However, because no more than one tree within the 95% confidence interval could possibly be the correct tree, we contend that our optimum trees are more likely to be correct than these other alternatives. It is apparent that both *Fonticula* and *Nuclearia* have an affinity toward Fungi. 1) When either *Fonticula* or *Nuclearia* (data not shown) is constrained elsewhere within the tree, the other taxon usually remains as sister to the Fungi. 2) Bootstrap analyses robustly support the sister relationship of *Fonticula* + *Nuclearia* with Fungi in the optimum trees. Taken together, these observations support a higher level group with *Fonticula* + *Nuclearia* as sister to Fungi.

Nucleariod amoebae are not fungi, as opposed to what Bullerwell and Lang (2005) recently suggested. Although this interpretation is phylogenetically acceptable based on the molecular data presented here, a key synapamorphy of Fungi is not present in either *Fonticula* or *Nuclearia*, that is, presence of a chitinous cell wall covering assimilative cells that results in a loss of phagotrophy leading to a lysotrophic, absorptive heterotrophic, lifestyle (Cavalier-Smith 1987). The presence of the fungal cell wall throughout the assimilative phase of the life cycle is unique to Fungi among opisthokonts and is not known to be present in phagotrophic *Nuclearia* or *Fonticula*, which are only walled in the dormant cyst and, in *Fonticula,* spore stages. Given these walled, dormant states, we would not be surprised if chitin synthase were found in nucleariod amoebae, as it appears to be present in the last common ancestor of the opisthokonts (Cavalier-Smith and Chao 2003), if not of all eukaryotes (Mulisch 1993; Cavalier-Smith and Chao 2003). A character further corroborating the exclusion of *Fonticula* and *Nuclearia* from the Fungi are the discoid mitochondrial cristae found in the former, whereas Fungi usually have flat cristae, as do most other opisthokonts. The sister relationship of nucleariod amoebae and Fungi is noteworthy and has yet to be named. Here, we propose a higher order taxon name for this clade: Nucletmycea (figs. 3–5). The etymology of the name stems from nucleariod amoebae (Nucl) and (et) Fungi (myc). The phylogenetic dichotomy of Opisthokonta is represented by the higher order taxa Nucletmycea and Holozoa (figs. 3–5).

Multigene acquisition and analyses from additional species of *Nuclearia* and other taxa with suggested close affiliation to *Nuclearia* (i.e., *Pinaciophora*, *Pompholyxophrys*, *Rabdiophrys*, and *Vampyrellidium*) (Patterson et al. 2000) may prove invaluable in firming up the evolutionary history of *Fonticula* and its closest relatives.

Our trees reliably resolve commonly observed opisthokont lineages, specifically Holozoa, Metazoa, Ichthyosporea, Choanoflagellata, Fungi, and fungal specific lineages (figs. 3–5). The specific phylogenetic relationships among holozoan lineages are not well resolved and have low support in our analyses (figs. 3 and 4). This is a common occurrence in both single and few gene phylogenies (two to four genes) of opisthokonts (Amaral Zettler et al. 2001; Medina et al. 2003; Ruiz-Trillo et al. 2004, 2006; Steenkamp and Baldauf 2004; Steenkamp et al. 2006). Our results show *Corallochytrium*, *Capsaspora*, and *Ministeria* as independent unicellular opisthokont lineages and

Ichythosporea, Choanoflagellata, and Metazoa as monophyletic lineages branching within Holozoa (figs. 3 and 4). These results have been demonstrated in other studies of a similar nature (Ruiz-Trillo et al. 2006; Steenkamp et al. 2006).

Regardless of its final place within Opisthokonta, *Fonticula* represents a fourth and novel type of multicellularity in the supergroup, an aggregative fruiting form, that is, a life cycle in which a multicellular structure develops by the aggregation and subsequent differentiation of individual cells. The ability of cells to aggregate in opisthokonts is not novel. For example, in sponges, the basal-most animals (see Halanych 2004 for a review), aggregation has been described in the classical experiments of dissociated sponge cells (Wilson 1907; Curtis 1962). Likewise, mixed cell types from dissociated amphibian embryonic cells aggregate when mixed, followed by like-cell migration and reassociation (Townes and Holtfreter 1955). During embryogenesis in metazoans, cells migrate from their points of origin and, in effect, aggregate at their final places of development, for example, multipolar ingression and germ-line migration (Mergner 1957; Savage and Danilchik 1993). In certain fungi, hyphae grow chemotropically toward points where sex organs develop; an example is the growth of investing hyphae toward the ascogonia of many pezizomycetes (Bistis 1956; Butler 1966). Further, in yeasts (secondarily unicellular fungi), aggregates can also be formed (Morris 1966). However, the aggregative origin of a multicellular state, like that found in *Fonticula*, has never been reported in Opisthokonta, and our findings highlight a novel path leading toward organismal complexity in the supergroup.

Opisthokonts seem to have a great propensity toward multicellularity with at least three independent origins, less the colonial forms, that is, metazoan, fungal, and aggregative. The molecular underpinnings of multicellularity warrant further study as the type of multicellularity differs among the three. Therefore, Opisthokonta provides the perfect microcosm to examine hypotheses of the transitional events to a multicellular lifestyle from protist ancestors. The last common ancestor of Opisthokonta had to be sexual, must have had one stage with a posteriorly directed flagellum with an accessory nonflagellated basal body, was capable of amoeboid motion, and could probably encyst (form walled cells). Choanoflagellates are likely the protist group sister to the metazoans, suggesting that the last common ancestor of both groups was a protist with a choanoflagellate-like appearance (see King 2004 for a review). The origin of multicellularity in Fungi is much more contentious and less understood. For example, it is unclear if there is a single origin of multicellularity in Fungi with multiple losses or multiple independent origins among the group. The protist ancestor to the Fungi has not been identified. The first fungus may have been a unicellular, chytrid-like, flagellated organism (Cavalier-Smith 1987), but, given our recognition of Nucletmycea, its ancestor may have been an amoeboid, sexual organism with a flagellated state. The unicellular ancestor to *Fonticula* may have been a filose, nuclearioid amoeba that had lost a flagellated stage. Given the phylogenetic trees presented here and elsewhere, these are the most parsimonious explanations for the evolutionary history leading to the diversity of multicellular forms in Opisthokonta.

If further research in Opisthokonta does not yield homologous mechanisms for the underpinnings of at least some aspects of the three types of multicellular development, then attempting to equate commonalities among the breadth of multicellular eukaryotes is likely to be a futile endeavor. However, if a common toolkit for any aspect of multicellular development is uncovered within Opisthokonta, then systematic analyses of multicellular lineages and their respective unicellular ancestors throughout the eukaryote Tree of Life may possibly uncover common evolutionary stepping stones responsible for these major transitions.

As an enormous amount of genomic data encompassing the breadth of both unicellular and multicellular opisthokont lineages is rapidly becoming available (see Ruiz-Trillo et al. 2007), powerful comparative genomic analyses will soon be possible. As *Fonticula* is the only taxon with an aggregative fruiting type of multicellularity in Opisthokonta, examining its transcriptome through expressed sequence tags represents an exciting opportunity to explore the evolutionary similarities of multicellularity across the diversity of Opisthokonta (work in progress). Insights into ancestral and derived molecular innovations involved in becoming multicellular may be gained by comparative studies of gene families already identified as important to this process; for example, cell adhesion genes such as cadherins and integrins, and cell–cell communication genes such as receptor kinases, components of the cell signaling pathways (such as Wnt components), and transcription factors. Meaningful comparative analyses between early animal and fungal multicellularity may be remiss without the inclusion of *Fonticula*.

Supplementary Material

Supplementary tables S1–S3 and supplementary figure S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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