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Monophyletic Origins of the Metazoa: An Evolutionary Link with Fungi

Patricia O. Wainright, Gregory Hinkle, Mitchell L. Sogin,* Shawn K. Stickel

A phylogenetic framework inferred from comparisons of small subunit ribosomal RNA sequences describes the evolutionary origin and early branching patterns of the kingdom Animalia. Maximum likelihood analyses show the animal lineage is monophyletic and includes choanoflagellates. Within the metazoan assemblage, the divergence of sponges is followed by the Ctenophora, the Cnidaria plus the placozoan *Trichoplax adhaerens*, and finally by an unresolved polychotomy of bilateral animal phyla. From these data, it was inferred that animals and fungi share a unique evolutionary history and that their last common ancestor was a flagellated protist similar to extant choanoflagellates.

Haeckel (*1*) first recognized that metazoans must share a common ancestry with a unicellular protist. In spite of numerous comparative studies and theories that link metazoans to flagellates (*1*) and ciliates (*2*), the origin of multicellular animals remains an open question. Morphology, ultrastructure, and complex embryological data do not provide support for any one hypothesis. The fossil record documents the Cambrian explosion of multicellular organisms (*3*) but provides few clues about protists ancestral to contemporary metazoa.

Comparisons of ribosomal RNA (rRNA) sequences have successfully established phylogenetic relations within the prokaryotic (*4*) and protist worlds (*5*), but similar procedures have given contradictory topologies for early diverging metazoan lineages (*6*). Distance and parsimony analyses of rRNA data portray protists as a series of independent branches that precede the nearly simultaneous separation of plants, animals, fungi, stramenopiles (chromophyte algae and heterokont protists), alveolates (ciliates, dinoflagellates, and apicomplexans) (*7*), and other independent protist lineages. Collectively, these abruptly diverging lineages define the crown (*8*) of the eukaryotic tree. Because nodes for each of the crown groups are separated by fewer than five nucleotide changes per thousand positions, their relative branching order has proven difficult to establish. The absence of early branching lines within each of the crown groups and inadequate representation of eukaryotic microbial diversity have compromised the utility of molecular data in resolving the origins of animals (*6*). In some studies, the reconstruction of organismal phylogeny is further exacerbated by incomplete sequence information (*9*) and inadequate methods for inferring molecular frameworks that include both slowly and rapidly evolving lineages (*6*).

We have reexamined relations between the animal kingdom and other major eukaryotic groups with the use of maximum likelihood methods (*10*) to analyze an expanded small subunit (16*S*-like) rRNA sequence database. Complete 16*S*-like rRNA sequences were determined for the choanoflagellates *Diaphanoeca grandis* Ellis, 1930 and *Acanthocoepsis unguiculata* Thomsen, 1973 and several lower metazoans, including the ctenophore *Mnemiopsis leidyi* A. Agassiz, 1865; the cnidarian *Tripedalia cystophora* Conant, 1898; two porifera, *Microciona prolifera* (Ellis and Solander, 1786) and *Scypha lingua* Haeckel, 1872; and the placozoan *Trichoplax adhaerens* Schulze, 1883 (*11*).

Figure 1 is a maximum likelihood analysis (*12*) of 16*S*-like rRNA sequences from the groups that diverged at the crown of the eukaryotic tree. The fraction of 110 maximum likelihood bootstrap resamplings that support topological elements are shown above the branches (each resampling required 48 hours to compute). Values below the branches represent the fraction of 200 bootstrap neighbor-joining replicates (*13*) that support the evolutionary hypothesis in Fig. 1. In this phylogenetic framework, the metazoa comprise a monophyletic group that shares a most recent common ancestry with choanoflagellates. The animal and fungal lineages share a more recent common ancestor than either does with the plant, alveolate, or stramenopile lineages. Within the metazoan subtree, the sponge and ctenophore lineages diverge first, followed by placozoa and cnidarians, and then by a poorly resolved polychotomy leading to triploblast animal phyla. Although the

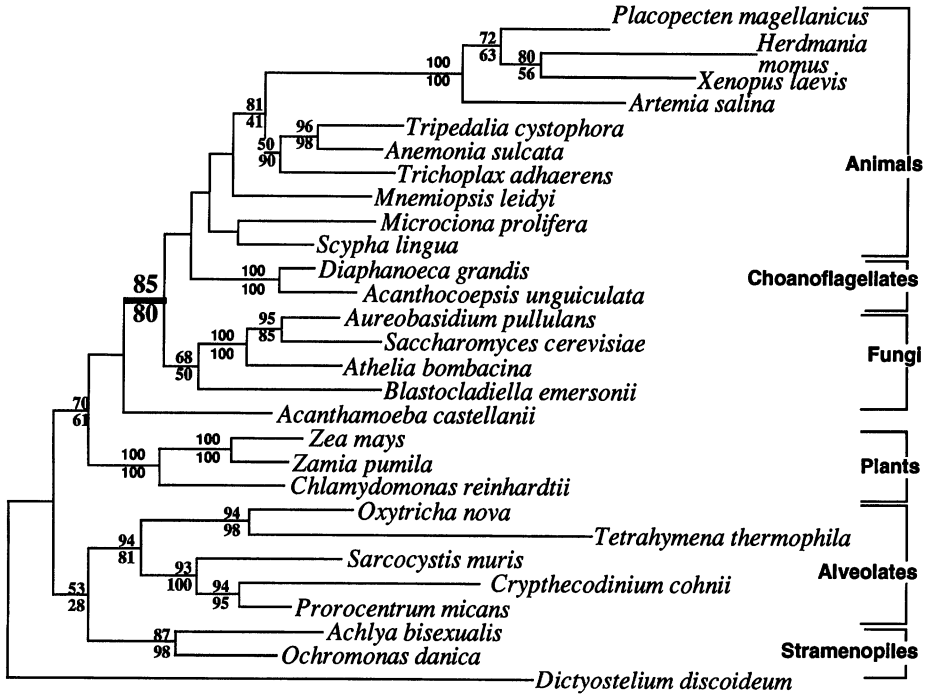

Fig. 1. Relation between animals, fungi, plants, and other eukaryotic groups inferred from complete 16*S*-like rRNAs. A computer-assisted method was used to align the 16*S*-like rRNA sequences from animals, plants, fungi, stramenopiles, alveolates, and other protist groups that diverged at the crown of the eukaryotic tree (*8*). Maximum likelihood methods (*10, 12*) were used to infer a molecular phylogeny with the use of sites that could be unambiguously aligned. The percentage of 110 bootstrap resamplings that support topological elements in maximum likelihood inferences is shown above the branches. The percentage of 200 neighbor-joining bootstrap replicates that corroborate topological elements in the figure is shown below the branches. The root of the tree is placed within the *Dictyostelium discoideum* lineage (*4, 5*). Confidence levels below 50% are not indicated.

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bootstrap values do not substantiate any single branching order for choanoflagellates, sponges, and ctenophores (*14*), the topology in Fig. 1 is consistent when alternative protist, fungal, and lower metazoan taxa are included in maximum likelihood analyses (*15*).

The early metazoan branching pattern confirms hypotheses about the relation of choanoflagellates and sponges to more complex animals (*16*). Haeckel (*1*) proposed that metazoans evolved from a colonial flagellate perhaps most similar to extant choanoflagellates. The cellular similarities between choanoflagellates and choanocytes of sponges are well known (*17*). Sponges can be loosely characterized as multicellular, and on a grade of relative complexity or organization lie between protists and metazoans (*18*). Multicellularity in the earliest diverging metazoans requires an extracellular matrix. In more complex animals, basement membranes provide structural support between epithelial and connective tissue (*19*). A central jellied matrix is found in the colonial choanoflagellate *Proterospongia haeckeli* (*20*), whereas in sponges more complex matrix components exist in the mesohyl (intercellular matrix) (*21*). Furthermore, basement membrane–like structures have been observed in sponges (*22*).

On the basis of gross morphology, ctenophores and cnidarians were at one time grouped in the phylum Coelenterata (*23*). Their deep divergence in our rRNA phylogeny is more consistent with the placement of ctenophores and cnidarians in separate phyla in accordance with differences in adult morphology and patterns of development (*24*). Our maximum likelihood analysis shows moderate bootstrap support for an earlier divergence of ctenophores relative to the separation of triploblastic animals from cnidarians and placozoans. Nonmolecular evidence in support of this branching pattern is equivocal. Ctenophores have mesenchymal muscle cells, gonoducts, and determinate embryological cleavage patterns (*25*), all characteristic of triploblastic animals. However, cell junctions that facilitate intercellular support and communication (*26*) are similar in sponges and ctenophores. Homologous structures are more highly developed in cnidarians (*27*). Finally, if the branching pattern in Fig. 1 accurately represents the evolutionary history of early diverging metazoan lineages, a loss of specialized intercellular structures in placozoans must have occurred (*28*).

The most important conclusion from our maximum likelihood analysis is the specific phylogenetic relation between animals and fungi. The fungi and animals represented in our data set are also united in maximum parsimony, least squares distance matrix, and neighbor-joining analyses (*29*). Independent confirmation of a conjoint evolutionary history for animals and fungi is provided by maximum likelihood analyses of amino acid sequences for elongation factors (*30*). At different times, fungi have been considered to be plants, members of other protist groups, or, in more contemporary schemes, worthy of kingdom level status (*31*). Molecular phylogenies based on 16*S*-like rRNA sequence comparisons (*32*) describe the higher fungi as a monophyletic group with flagellated chytrids representing the earliest diverging lineage. Most conventional phylogenies place the origin of Metazoa among protists, but Towe (*33*) and more recently Cavalier-Smith (*34*) argue that similarities in complex biosynthetic pathways, including syntheses of hydroxyproline, chitin, cellulose, and even ferritin, suggest a specific evolutionary relation between fungi and animals. Because chytrids and choanoflagellates both have flattened mitochondrial cristae and a single posterior flagellum, the hypothetical protist that represents the most recent common ancestor to the animals and fungi in the rRNA phylogeny very likely was a unicellular protist with these ultrastructural characteristics. This new rRNA phylogeny that recognizes the origins of animals and their relation to fungi provides a framework and a rational basis through which the origins and diversifications of metazoan phenotypes may be explored.

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9. Most analyses of animal origins have been based on partial sequences and hence lack a statistically significant number of independently variable sites. Furthermore, these sequences may be subject to systematic errors because of analyses of single-stranded rRNA sequencing templates. Sequence errors of 1% or more can lead to major rearrangements of deep interior nodes or terminal taxa separated by short branches [A. G. Clark and T. S. Whittam, *Mol. Biol. Evol.* **9**, 744 (1992)].
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12. The fastDNAml program written by G. Olsen (G. Olsen, M. Matsuda, R. Hagstrom, R. Overbeek, in preparation) is derived from J. Felsenstein's version 3.4 DNAML (part of his PHYLIP package) and uses the generalized two-parameter model of evolution [H. Kishino and M. Hasegawa, *J. Mol. Evol.* **29**, 170 (1989)]. Maximum likelihood trees were inferred from unambiguously aligned positions in the compared sequences. Using jumbled orders for taxa addition and allowing global swapping to cross three branches, we repeated the search for an optimal tree until the best log likelihood score (ln likelihood = −16987.92530 for the tree in Fig. 1) was reached in at least three independent searches. The average computation time for 28 taxa with a SUN SPARC2 workstation was 48 hours.
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Identification of a Mobile Endogenous Transposon in *Arabidopsis thaliana*

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A mobile endogenous transposable element, *Tag1*, has been identified in the plant *Arabidopsis thaliana*. *Tag1* was found in the nitrate transporter gene, *CHL1*, of a chlorate-resistant mutant present in a population of plants containing an active maize *Ac* transposon. *Tag1* excises from the *chl1* gene producing chlorate-sensitive revertants with *Tag1* or *Tag1*-related elements at different loci. *Tag1* and related elements are present in the Landsberg but not Columbia or Wassilewskija ecotypes of *Arabidopsis*. Thus, *Tag1* provides a tool for the insertional mutagenesis of plant genes essential for biological processes of agronomic importance.

Transposable elements have been invaluable for the identification and isolation of genes, as insertion of a transposon both disrupts and tags a gene with a known sequence (*1*). *Arabidopsis thaliana*, with its exceptionally small genome (100,000 kb), would be especially useful for the tagging of plant genes with transposons (*2*). *Arabidopsis* genes have been tagged by transferred DNA (T-DNA) from the soil bacterium *Agrobacterium tumefacians* (*3*) or isolated with the use of a map-based strategy (*4*). Endogenous transposons of *Arabidopsis* include *Ta1-10* (*5*) and a transposon-like element *Tat1* (*6*), but these are not mobile; that is, they do not transpose during development or transmission from one generation to the next. The maize *Ac* element, however, is mobile in *Arabidopsis* (*7*).

In an effort to exploit *Ac* as an insertional mutagen, we used several transgenic *Arabidopsis* lines carrying active *Ac* elements to search for mutants defective in the assimilation of nitrate. Such mutants can be selected with the herbicide chlorate. Chlorate is taken up by plants then reduced by nitrate reductase to chlorite, which is toxic (*8*). Mutants that are resistant to chlorate treatment are usually defective in chlorate (and nitrate) reduction (*9*). One exception is the *chl1* mutant of *Arabidopsis*, which is defective in chlorate and nitrate uptake (*10*). When we applied chlorate to the *Ac*-carrying *Arabidopsis* lines, we found a chlorate-resistant mutant with an endogenous transposable element integrated into the *CHL1* gene.

Arabidopsis seed (ecotype Landsberg) used for the chlorate selection originated from three independent transgenic plants containing an *Ac* element cloned into the 5′ untranslated leader region of a streptomycin-resistance gene (*11*). Progeny fully resistant to streptomycin (64 plants) were selected. These 64 plants were the product of an excision event of *Ac*, which restored the functional streptomycin resistance gene. Progeny (20 to 50 seeds from each of the 64 plants) were planted and self-fertilized, seed was harvested, and 100 to 200 seeds from each lineage were then germinated and treated with chlorate. Three chlorate-resistant mutants appeared in one family. A backcross to a *chl1* mutant (*chl1-1*) (*10*) indicated that the mutations were alleles of *chl1*. One of the three mutants, *chl1-6*, was characterized further.

We cloned the *CHL1* gene from a *chl1* mutant tagged with T-DNA (*12*). A *CHL1* cDNA clone (*12*) was used to analyze the

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