Emerging Genomic and Proteomic Evidence on Relationships Among the Animal, Plant and Fungal Kingdoms

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Sequence-based molecular phylogenies have provided new models of early eukaryotic evolution. This includes the widely accepted hypothesis that animals are related most closely to fungi, and that the two should be grouped together as the Opisthokonta. Although most published phylogenies have supported an opisthokont relationship, a number of genes contain a tree-building signal that clusters animal and green plant sequences, to the exclusion of fungi. The alternative tree-building signal is especially intriguing in light of emerging data from genomic and proteomic studies that indicate striking and potentially synapomorphic similarities between plants and animals. This paper reviews these new lines of evidence, which have yet to be incorporated into models of broad scale eukaryotic evolution.

Key words: genomics, proteomics, evolution, animals, plants, fungi

Introduction

The results of sequence-based, molecular phylogenetic analyses have reshaped current thinking about ancient evolutionary relationships, and have begun to establish a new framework for systematizing broad-scale eukaryotic diversity. Among the most widely accepted of the new evolutionary hypotheses is a proposed sister relationship between animals (+ choanoflagellates) and fungi (see ref. *1*, *2* for thorough reviews) and their combination into a new taxonomic kingdom, the Opisthokonta (*3*). Phylogenetic support for the Opisthokonta comes from analyses of several well-sampled individual genes (*4*–*6*) as well as combined analyses of these and other sequences in concatenated data sets (*7*). In addition, alignments of elongation factor 1α (EF-1α) sequences show that animal and fungal genes contain a unique and apparently conserved insertion that is not present in other eukaryotes (*5*). The cumulative evidence from these investigations is impressive and has convinced many evolutionary biologists of the validity of the Opisthokonta.

Support for a sister relationship between animals and fungi began to gain steam in the mid-1990s as molecular sequence data became available from a diverse array of eukaryotes. One of the enigmatic results of these early analyses (*5*, *6*) was the appearance of two different and conflicting phylogenetic signals; most available sequences supported an animals + fungi relationship, but a smaller subset of genes indicated a closer relationship between animals and plants. Curiously, there was little or no support for the third possible relationship (plants + fungi). This suggested that a persistent phylogenetic artifact, rather than noise, might be responsible for conflicts among gene phylogenies (*5*). Most of the genes examined in these early studies were too short in length and/or too poorly sampled to permit a more thorough investigation, and those that specifically supported an animal-plant relationship have not been the focus of subsequent phylogenetic investigations. Nevertheless, preliminary combined phylogenetic analyses of a number of these sequences, from nine completed genomes, confirm the presence of an additive tree-building signal that clusters animal and plant sequences (Figure 1), as do more widely sampled analyses of at least four of the genes individually (Figure 2).

The alternative tree-building signal present in these genes reinforces early cautions (*5*) that a pervasive and directional artifact is present in sequences used to resolve the animal-plant-fungus trichotomy. What phylogenetic analyses do not show, and what has not been investigated rigorously, is which of the signals is the artifact; that is, whether the pervasive artifact favors the Opisthokonta or whether it groups

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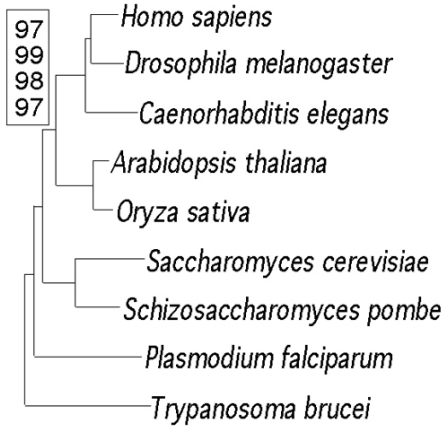

Fig. 1 Identical tree recovered from parsimony bootstrap (*33*), neighbor-joining bootstrap (*33*), quartet-puzzling maximum-likelihood (*34*) and Bayesian inference (*35*), with respective support values for the animal/plant grouping, on a combined alignment of 2,757 inferred amino acids from nine genes. These genes were chosen based on previously published analyses indicating that they contain a tree-building signal favoring animals + plants (*5*, *6*, *11*); to permit the most reliable estimate of orthologous relationships, only completed genomes were sampled for this analysis. The nine sequences are capping enzyme guanylyltransferase (*CEG*), casein kinase II alpha subunit (*CK-IIα*), citrate synthase (*CIT*), enolase, *F-ATPase β*, 70 kD heat shock protein (*HSP70*), 90 kD heat shock protein (*HSP90*), proliferating-cell nuclear antigen (*PCNA*) and triosphosphate isomerase (*TPI*).

animals with plants? Although further investigations certainly are needed to try to understand the underlying nature of this phylogenetic conflict, an ultimate determination of which tree-building signal is artifactual cannot be made directly from the phylogenetic analyses themselves.

As more sequences become available for global eukaryotic comparisons, a cumulative tree-building signal undoubtedly will emerge that favors one or the other hypothesis. There is no *a priori* basis, however, for assuming that historical signal will overwhelm a pervasive artifact as data sets grow larger. At deeper phylogenetic levels, artifacts can entirely dominate the tree topologies recovered (*8*). In analyzing the early evolution of metazoans, Rokas and colleagues (*9*) concluded that tree-building artifacts so pervade ancient phylogenetic reconstructions as to make them inherently unreliable. They suggest the use of genome-scale characters as an alternative. It seems increasingly clear that such alternative data are needed to test new hypotheses of ancient evolutionary relationships inferred from sequence-based phylogenies; such characters may be particularly useful in resolving the animal-plant-fungus trichotomy.

Alternative Data for Testing Sequence-based Trees

In addition to sequence-based phylogenetic analyses, one molecular character has been extremely influential in building a case for the Opisthokonta; that is, a unique shared insertion in the EF-1α genes of animals and fungi, which is absent from all other eukaryotes sampled (*1*, *5*). In its apparently ancestral state, this insertion encodes 12 residues with sequence similarity across animal and fungal genes. Because of its reasonable length (not easily explained by convergence), this insertion is interpreted most simply as a shared-derived character that was acquired in the common ancestor of the gene present in animals and fungi; its absence from plants and other eukaryotes suggests that no other major group shares a gene derived from that ancestor (*5*). Thus, the EF-1α insertion appears to offer compelling support for the Opisthokont hypothesis; it is an independent line of evidence that suggests sequence-based trees supporting an animal-plant relationship are due to phylogenetic artifacts.

Genomic-proteomic comparisons of molecular and cellular processes have begun to yield additional lines of evidence that are equally or perhaps more compelling. In a number of cases, however, these new data strongly support a sister relationship between plants and animals. Among of the most broadly investigated is the process of mRNA capping.

Evolution of Capping Enzymes

In eukaryotic mRNA processing, a 7-methylguanosine cap is linked to the 5′ end of pre-mRNA through a three-step pathway involving triphosphatase, guanylyltransferase and methyltransferase enzymes (*10*). In most eukaryotes, including fungi, the three enzymes are encoded by separate genes, and their structures and biochemical properties are conserved strongly (*11*). Animals and plants are exceptional; in both groups the triphosphatase and guanyltransferase genes are fused and encode a single, bi-functional polypeptide (*11*).

This fusion event, by itself, is not compelling evidence for an animal-plant relationship. Additional fusion events, some clearly convergent, have occurred

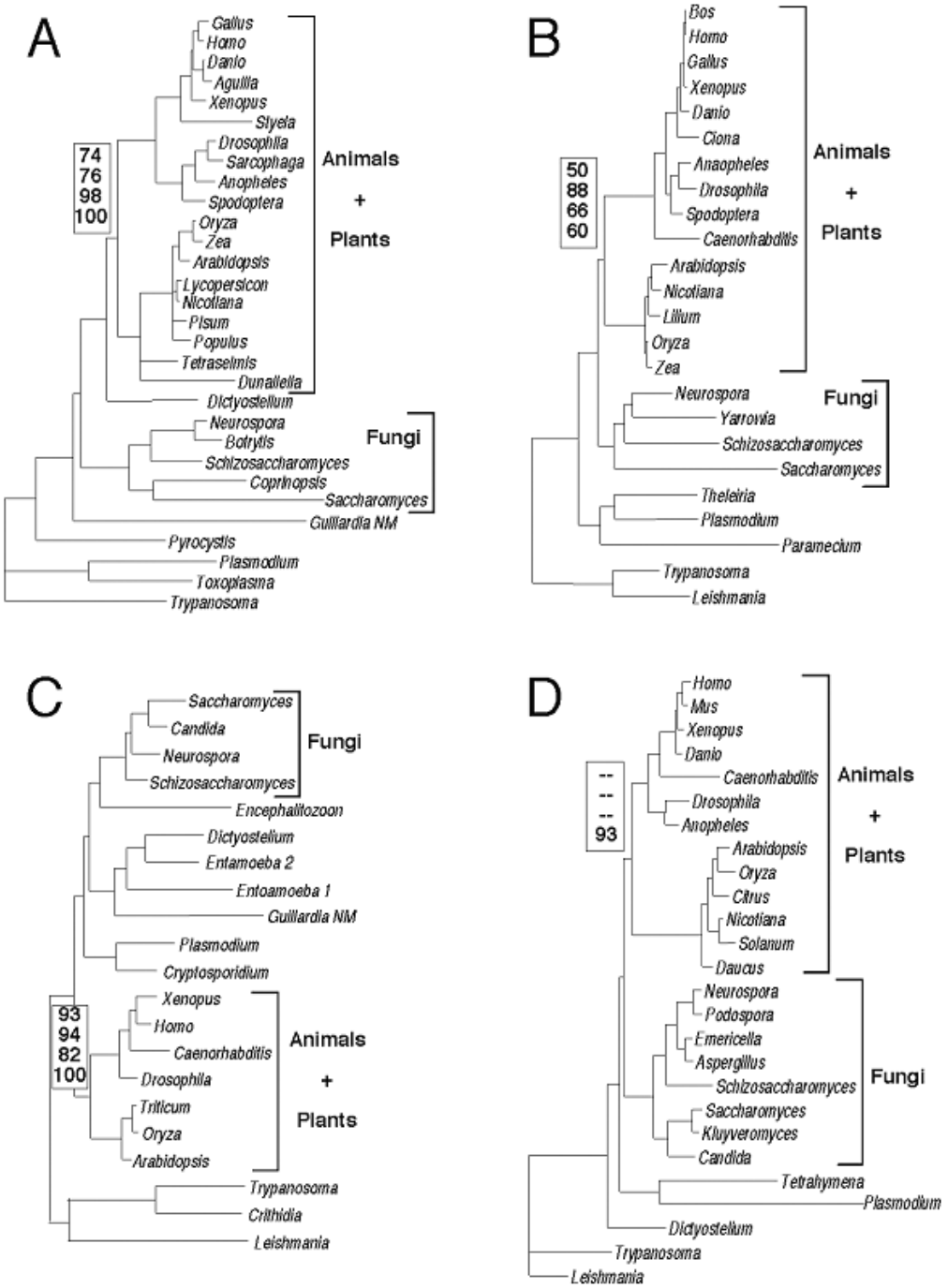

Fig. 2 Phylogenetic trees recovered from Bayesian inference of four examples of genes that provide support for an animal/plant grouping **(A)** *PCNA*, **(B)** *CKIIα,* **(C)** *CEG*, **(D)** *CIT*. Support values are, in descending order, from parsimony bootstrap, neighbor-joining bootstrap, quartet-puzzling maximum-likelihood and Bayesian inference. Values below 50% are indicated by (−−). In addition to sequences from the nine complete genomes used in Fig. 1, the following sequences were retrieved by reciprocal tBLASTn searches for each data set. **(A)** *Gallus gallus* AB053163, *Danio rerio* BC049535, *Aguilla japonica* AB025357, *Xenopus laevis* BC041549, *Styela clava* L42763, *Sacrophaga crassipalpis* AF020427, *Anopheles gambiae* XM319407, *Spodoptera frugiperda* AB069854, *Zea mays* AY110234, *Lycopersicon esculentum* AJ515474, *Nicotiana tobacum* AB025029, *Pisum sativum* Y16796, *Populus nigra* AB041506, *Tetraselmis chui* AF012212, *Dunaliella tertiolecta* AF034201, *Neurospora crassa* XM331630, *Botrytis cinerea* AL117064, *Coprinopis cinereus* AB056666, *Guillardia theta* NM AF083031, *Pyrocystis lunula* AF508260, *Toxoplasma gondii* AF242301; **(B)** *Bos Taurus* M93665, *G. gallus* M59456, *X. laevis* X62375, *D. rerio* BC044403, *Ciona intestinalis* AY092081, *A. gam-*

biae XM315576, *S. frugiperda* AF071210, *N. tobacum* AB077050, *Lilium davidii* AF517838, *Z. mays* AF239819, *N. crassa* AF494376, *Yarrowia lipolytica* Z83096, *Theileria parva* M92084, *Paramecium tetraurelia* AJ298914, *Leishmania major* AC103910; **(C)** *Candida albicans* D83180, *N. crassa* XM326114, *Crithidia fasciculate* AF059247, *Triticum aestivum* BT009633, *Cryptosporidium parvum* BX538351, **(D)** *Mus musculus* BC029754, *X. laevis* BC046571, *D. rerio* BC045362, *A. gambiae* XM320478, *Citrus maxima* U19481, *N. tobacum* X84226, *Solanum tuberosum* X75082, *N. crassa* XM328130, *Podospora anserine* AJ296102, *Emericella nidulans* AF468824, *Aspergillus niger* D63376, *Kluyveromyces lactis* AY145050, *C. albicans* AY126274, *Tetrahymena thermophila* D90117, *Dictyostelium discoideum* AC116305. Additional sequences (without listed accessions) were retrieved by tBLASTn searches from their respective genome databases. Links to completed genomes (*Encephlalitozoon cuniculi, Guillardia theta* nucleomorph) can be found at http://www.ncbi.nlm.nih.gov:80/genomes/static/euk_g.html; links to additional genomes in progress (*Dictyostelium discoideum, Entamoeba histolytica, Leishmania major*) at http://www.ncbi.nlm.nih.gov:80/genomes/static/EG_T.html.

among the three capping enzymes over the broad course of their evolution (*11*). Moreover, other gene fusions have been shown to support contradictory sets of relationships (*12*). It is reasonable that, although undoubtedly rare, fusions between proteins of related function would be favored by natural selection; thus, convergent events may turn out to be relatively common on the evolutionary time scale in question. What is highly significant about the capping enzyme, however, is the biochemical nature of the triphosphatase domain of the plant–animal fusion protein.

Animal and plant triphosphatases are members of the cysteine phosphatase super family and contain a conserved cys residue at the active site. This cys attacks the terminal phosphate of pre-mRNA to produce a covalently bonded intermediate and release the diphosphate mRNA product. This reaction does not require a metal ion co-factor; rather, it is inhibited in the presence of divalent cations (*11*). This contrasts sharply with the triphosphatase in fungi, which belongs to a different family of metal-dependent phosphohydrolases that specifically require divalent cations. Both the overall structure and catalytic properties of this enzyme clearly are distinct from the animal and plant triphosphatase (*11*). All other eukaryotes examined to date, including kinetoplastids, alveolates, microsporidians, *Dictytostelium*, *Entamoeba*, *Giardia*, the oomycete (stramenopile) *Phytophthora*, and the red alga *Cyanidioschyzon*, contain the type of metal-dependent triphosphatase that is present in fungi (*13*). Thus the switch to a new kind of triphosphatase and its fusion to the guanylyltransferase enzyme are best interpreted as shared-derived events that occurred in the common ancestor of plants and animals, after it diverged from other eukaryotes (*11*, *13*).

Although the EF-1α insertion cannot be dismissed lightly, it is not apparent that a shared 12-amino-acid insertion should be given greater phylogenetic weight than the shared origin of a new triphosphatase enzyme and its subsequent fusion to gualylyltransferase. Additional evidence from proteomic and genomic features will help to determine which of these characters reflects the evolutionary history of the organisms involved.

Comparisons of Genomic and Proteomic Networks

Comparative genomics is a potentially powerful tool for understanding eukaryotic evolutionary relationships. Orthologous gene families that are common to only a subset of eukaryotic taxa can represent shared-derived functions that evolved in the unique common ancestor of those taxa. Likewise, complex molecular or biochemical processes that are unlikely to be products of convergent evolution can offer compelling evidence for polarizing ancient relationships. Initial genome-wide comparisons have uncovered some remarkable similarities between plants and animals that are not shared by fungi or, at least thus far, by other eukaryotic groups.

Core functions of RNA metabolism are highly conserved across the breadth of evolutionary diversity. Nevertheless, major innovations and the origins of new processes have marked key evolutionary transitions, both from prokaryotic to eukaryotic cells and from simple to complex eukaryotes. An overall inventory of orthologous protein families from 31 completed genomes, including six from plants, animals and fungi, does not agree with the evolutionary concept of opisthokonts (*14*). Animals and plants share 41 orthologous groups, exclusive of fungi, whereas fungi and animals share only 15 groups exclusive of plants. The raw proportion of shared groups among the three lineages is complicated by the possibility of more extensive gene loss in the few complete fungal genomes examined thus far. However, in several cases

animal and plant orthologs share unique domain architectures that are not present in fungi or more distant outgroups. No such unique-shared architectures were reported that link animals with fungi to the exclusion of plants (see ref. *14* for full discussion).

There are additional intriguing examples of gene families and domain structures shared exclusively by animals and plants (*15–17*), but more comprehensive genome-scale comparisons, in particular sampling of more diverse fungal genomes, are required to determine whether overall inventories of other complicated metabolic machinery also favor an animal-plant relationship. Such inventories will provide powerful alternative sources of data for testing sequence-based phylogenetic hypotheses.

Control of Cellular Differentiation

Both animals and plants have the capacity to differentiate cell types into complex tissues that partition physiological functions. Remarkable similarities have emerged in how plants and animals regulate that differentiation. A key master control over animal development is exerted by retinoblastoma protein (Rb), which is important for integrating both cell division and differentiation (*18*). An Rb homologue in plants (*19*, *20*) has been shown to play comparable roles in regulation of the cell cycle (*21*). Even more remarkable has been the discovery that the Rb protein in plants is at the center of a complex array of interactions that occur during the G_1 phase of cell division and involve E2F transcription regulators (*22*) and D-type cyclin kinases (*21*). Animals and plants also have the same master controls that prevent cellular differentiation, thereby permitting the maintenance of totipotent stem/meristematic cell lines (*23*, *24*). All of these proteins and pathways appear to be homologous in plants and animals, performing the same roles in controlling cell division, coordinating growth and in the differentiation of tissue-specific cell type (*24*, *25*).

Previous genetic analyses, and a comprehensive search of available sequence databases (author's unpublished results), reveal that Rb protein is present across the broad diversity of green plants and animals, from nematodes to human and chlorophycean algae to angiosperms (*26*, *27*). In contrast, when queried with Rb genes from both human and *Arabidopisis*, tBLASTn (*28*) searches of all completed and partial fungal genomes, and of all other eukaryotic groups available through NCBI-linked BLAST resources (http://www.ncbi.nlm.nih.gov:80/BLAST/), return no significant hits from organisms outside the green plant and animal lineages (author's unpublished results). A protein similar to Rb was reported in the cellular slime mold *Physarum polycephalum* (*29*), although this sequence is not recovered in database searches and its subsequent functional characterization has not been reported.

It remains possible that Rb protein plays a role in cell division in eukaryotes other than plants and metazoans; however, given evidence that slime molds branch within the animal-plant-fungus trichotomy (*30*), even a true Rb orthologue in *Physarum* could be interpreted as a synapomorphy linking slime molds with an animal-plant grouping. In any case, clear Rb homologues and, most significantly, the suite of G_1 interactions that are central to controlling developmental and tissue specific cell differentiation, have been found only in green plants and animals (*21*). It has been hypothesized that the invention of the G_1 pathway was a defining moment in the evolution of eukaryotes, one that permitted the elaboration of complex organisms with multicellular tissues (*25*).

It remains possible that the G_1 pathway is more ancient and has been retained only in animals and plants, the two major groups that went on to develop true tissue differentiation. Under this scenario, the pathway would have been lost in all more developmentally simple eukaryotic lineages, including fungi; however, cell cycle regulation by Rb protein is conserved in the unicellular green alga *Chlamydomonas* (*27*). This suggests that, once fully integrated into the cell cycle, the G_1 pathway was not easily lost in groups that failed to attain multicellularity or more complex patterns of ontological development. Thus, the fact that fungi do not differentiate cells into true tissues may reflect their more ancient divergence from the ancestral lineage that led to plants and animals. Should additional sampling continue to support the current phylogenetic distribution of Rb protein and the G_1 pathway, it will offer strong support for the hypothesis that plants and animals share mechanisms for cell cycle control that originated and were canalized in their unique, common ancestor.

Conclusion

Analyses of multi-gene concatenated alignments generally introduce an implicit assumption: that is, as sequences are combined into ever larger data sets, the dominant tree-building signal that will emerge

from any given set of genomes comes from their historical pattern of relationships. Conflicting signals found among individual genes are then assumed to result from random noise or directional artifacts associated with smaller data sets. The actual source of the dominant signal, however, could be consistent biases that can come to dominate tree-building algorithms as data sets increase in size (*31*, *32*). Under these circumstances, only a minority of genes might retain enough historical signal to out-compete the pervasive artifact and recover the true evolutionary history.

Whatever relationship among plants, animals and fungi ends up favored by the global tree-building signal in eukaryotic molecular sequence data, it will be essential to determine whether that dominant signal is phylogenetic or artifactual in nature. That, in turn, requires alternative approaches for analyzing genome-level data. Initial investigations of proteomic networks and complex cellular processes have yielded intriguing insights into the workings of model plant, animal and fungal systems. Specifically a number of studies have suggested a greater similarity between animals and plants than between animals and fungi. It is too soon to know whether the totality of these data will support such a relationship. Certainly some shared molecular features, even those representing large and co-adapted processes, could have evolutionary histories complicated by parallel gains and/or losses. Caution is in order, especially when evolutionary hypotheses are based on features known from relatively few eukaryotic taxa; however, when broader taxonomic sampling continues to support the observed pattern of distribution, as has been true for the novel capping enzyme shared by animals and plants, the hypothesis of a unique, shared-derived character is strengthened.

As has been true for both morphological and primary sequence data, conflicts undoubtedly will arise in genomic and proteomic characters that need to be reconciled with any given phylogenetic hypothesis. Nevertheless, in building a strong consensus view on ancient eukaryotic relationships, evolutionary researchers must begin to take these alternative lines of evidence into account. As of submission of this review, the functional and genome-level data highlighted here have yet to be cited in any phylogenetic investigation of broad scale eukaryotic relationships. The goal of this contribution is to promote a broader appreciation of emerging bioinformatic, proteomic, and genomic data and their great potential for helping to resolve ancient evolutionary relationships.

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