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# Consensus phylogeny of *Dictyostelium*

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**Abstract.** The evolutionary relationship of *Dictyostelium discoideum* to the yeasts, fungi, plants, and animals is considered on the basis of physiological, morphological and molecular characteristics. Previous analyses of five proteins indicated that *Dictyostelium* diverged after the yeasts but before the metazoan radiation. However, analyses of the small ribosomal subunit RNA indicated divergence prior to the yeasts. We have extended the molecular phylogenetic analyses to six more proteins and find consistent evidence for a more recent common ancestor with metazoans than yeast. A consensus phylogeny generated from these new results by both distance matrix and parsimony analyses establishes *Dictyostelum*'s place in evolution between the yeasts *Saccharomyces cerevisiae* and *Schizzosaccharomyces pombe* and the worm *Caenorhabditis elegans*.
**Key words.** Distance matrices; parsimony; primary sequence comparisons; protist evolution.

## Introduction

Closely related organisms share molecular processes for growth and development to a greater extent than distantly related ones. Therefore, it is of more than taxonomic interest to determine when a given organism diverged from the line that leads to our own species, *Homo sapiens*. As this issue emphasizes, recent advances in molecular techniques have identified a wealth of new genes in *Dictyostelium discoideum* that are involved in cellular motility and multicellular cellular development, so that it has become a paradigm for understanding the role of the cytoskeleton, of surface receptors involved in intercellular communication, of adhesion and of the extracellular matrix. If *Dictyostelium* diverged from the line that gave rise to metazoa long ago, before yeast diverged[22], then the molecular components would have had more chance to drift and specialize than those in yeast or other protists. If, on the other hand, *Dictyostelium* diverged more recently[21], then physiological processes seen in *Dictyostelium* would have more bearing on similar functions in humans.

*Dictyostelium* is clearly not a true fungus since it lacks vegetative hyphae. Nevertheless, it has been classified in the Kingdom Mycetae[29] and is often referred to as a slime mold[4]. The characteristic fruiting bodies are only a few millimeters high and carry spores on a cellulose encased stalk. While spore formation is a fungal trait, it is also found in algae, moss, and even some trees. However, the first person to isolate and describe a dictyostelid, Oskar Brefeld, was a mycologist. While he was analyzing the spores found in horse dung, he noticed some that were much smaller than those of the prevalent fungus, *Mucor mucedo*, but considered them sufficiently related that he named the species *Dictyostelium mucoroides*[5]. He subsequently succeeded in growing the small spores on cooked horse dung and realized that they gave rise to free-living cells which grew independently and divided by binary fission. During the developmental stage of the life cycle, hundreds of thousands of cells aggregated to form a compact slug-shaped mass within which he assumed the cells fused to form a plasmodium[5]. For this reason he related *Dictyostelium* to the true slime molds, the Myxomycetes, which form syncytial plasmodia before fruiting. A few years later, van Tieghem[31] corrected this error by demonstrating that the cells of *Dictyostelium mucoroides* never fuse. Brefeld subsequently referred to the multicellular slug stage as a pseudoplasmodium[6], but by then the damage was done and texts still treat *Dictyostelium* and the true slime molds, such as *Didymium nigripes* and *Physarum polycephalum*, as if they were closely related. Further evidence that *Dictyostelium* shares little with the lifestyles of the fungi came from the studies of Potts[28] who showed that the amoebae are not saprophytes but feed upon bacteria which he thought were digested extracellularly. Shortly thereafter it became clear that *Dictyostelium* cells engulf bacteria and digest them in internal vesicles[27,32]. Based on this fact alone, the similarity of *Dictyosteluim* cells to mammalian macrophages appears stronger than to molds.

Modern microscopic techniques have revealed *Dictyostelum* cells to have all the expected eukaryotic organelles[20]. The plasma membrane is of typical dimensions and appearance, as is the nuclear membrane. The endoplasmic reticulum and Golgi apparatus can be visualized although they are not as regular or extensive as in cells of tissues specialized for massive secretion such as the mammalian pancreas. Neither flagellae nor cilia have ever been observed and so affinity to the ciliates is ungrounded. Microtubules are as prevalent as in many mammalian cells and consist of

polymerized tubulins closely related to metazoan tubulins (see below). Clathrin coated vesicles can be seen[25] as can the spongiform osmoregulatory network that drains into the highly active contractile vacuoles[15,24]. Mitochondria can be clearly recognized although the cristae are less regular than in mammalian cells[20]. Nevertheless, the 50 kb mitochondrial genome carries most if not all the same genes as are found in the mammalian mitochondrial genome[13].

Highly motile *Dictyostelium* cells continuously extend and retract filopods that expand into pseudopods when they form contacts with the substratum. Filopods are formed by protrusion of the plasma membrane as G-actin polymerizes to form microfilaments. A bed of myosin underlies the filopod and holds flanking membrane in place until signalled to allow expansion of the filopod into a pseudopod[14]. Both actin and myosin in *Dictyostelium* are more closely related to their homologs in metazoa than they are to those in yeast[21].

Comparison of the enzymes and pathways of central intermediary metabolism or macromolecular biosynthesis does not shed much light on phylogenetic relationships among eukaryotes since almost all the components have been highly conserved in this monophyletic group[19]. However, the structure of the chromosomes and their genes can be quite informative. The 40 Mb haploid genome of *Dictyostelium discoideum* is carried on six chromosomes. As is the case for the chromosomes of metazoa, each *Dictyostelium* chromosome has a single kinetocore to which microtubulues attach at mitosis[23]. Multiple short repeats of a G-rich sequence are found at the telomeres of *Dictyostelium* chromosomes[8]. Similar sequences cap all eukaryotic telomeres analyzed to date. The overall nucleotide composition of *Dictyostelium* is highly skewed towards adenine (A) and thymine (T) such that the A + T content is about 85%[12]. Within coding regions of genes the nucleotide composition is less skewed since it is constrained by codon usage. When averaged over 50 genes, the A + T content is found to be 62% (Loomis, unpublished results). These numbers suggest that less than 25% of the genome (=10Mb) encodes proteins and indicate that there are less than 7000 genes in the *Dictyostelium* genome, calculated on the basis of an average gene encoding a 40 kDa protein. In addition to the coding portions of genes, transcripts also carry introns that are spliced out during the processing that generates mature mRNA. Most *Dictyostelium* genes have been found to carry a small intron although genes have been encountered that have multiple introns and others have been found to be free of introns[17]. The invariant bases found at the ends of all spliceosomal introns are faithfully conserved in *Dictyostelium* introns. The short intron sequence which is recognized by U2 for lariat attachment in the yeast *Saccharomyces cerevisiae* but not in mammalian introns is not found in *Dictyostelium* introns. Sequence comparison of some of the small nucleolar RNAs in *Dictyostelium* has shown them to be more similar to those in mammals than to those in yeast[33,34]. Thus, both the chromosomes and the genes of *Dictyostelium* appear to be more closely related to those of metazoans than to those of yeast. However, detailed studies of primary sequences from a large number of varied organisms are needed to position the time of divergence accurately[16].

## Molecular phylogenetic analyses

Due to its relative ease of isolation, the gene which encodes the RNA of the small ribosomal subunit (18s rRNA) has been isolated and sequenced from the greatest representation of diverse organisms[30]. Multiple copies of this gene are present in every eukaryotic genome analyzed to date. However, the individual copies are almost identical to each other. In some organisms these multiple copies are found in tandem arrays along a chromosome while in others, including *Dictyostelium discoideum*, they are found on multiple copies of an extrachromosomal palindromic DNA element[6b]. Sequences of 18S rDNA from over 1000 organisms including bacteria, fungi, plants and animals of diverse phyla are available in the data bases. This sequence collection provides a robust data set for comparative phylogeny. When the 18s rDNA sequences are aligned and compared, the degree of divergence of the genes from most organisms is that expected from the relationships derived from diverse characteristics both molecular and morphological[22]. However, the degree of divergence of *Dictyostelium* rDNA from that of metazoan organisms is greater than that of the yeasts, *Saccharomyces cerevisiae* or *Schizzosaccharomyces pombe*, although anatomically the amoebae appear to be more closely related to metazoan cells than are yeast. While it is possible that the anatomical characteristics are misleading, it is also possible that the analysis of rDNA sequences is misleading due to species-specific parameters. For instance, the inheritance of multi-copy extrachromosomal rDNA may have resulted in more rapid divergence than chromosomally inherited rDNA genes due to 'bottle-necks' in the passage of copies to progeny during the less precise segregation of the plasmids. Population genetics has shown that when the number of genes (or individuals) in a population is drastically reduced and then re-amplified, the rate of genetic drift can increase significantly. Another species-specific drive found in *Dictyostelium* is towards high A + T. While rDNA is not as skewed towards high A + T as are non-coding portions of the *Dictyostelium* genome (57%), it is still at the high end when compared to rDNA sequence in other organisms. Changes from guanine (G) or cytosine (C) to adenine (A) or thymine (T) can be readily accommodated in rDNA since the RNA

product is not translated and so is not constrained by codon requirements. As long as G or C changes to A or T are compensated by complementary changes in other portions of the gene, the secondary structure of rRNA can be maintained by Watson-Crick bonding. In fact, the predicted secondary structure of *Dictyostelium* 18S RNA is almost identical to that in the amphibian *Xenopus laevis*[26]. However, the skewing towards high A + T content of rDNA results in a high degree of divergence of the primary sequence of the gene from those of metazoa. One of the ways to settle the apparent conflict is to expand the number of characteristics used in determining the time of divergence of *Dictyostelium*. Protein sequences provide a separate data set which do not suffer from the same drawbacks as untranslated DNA sequences or morphological properties.

Table. Similarity of *Dictyostelium* proteins.

| Protein (*Dictyostelium*) | Percent identity to mammalian homologs | Percent identity to *S. cerevisiae* homologs |
|---|---|---|
| Aspartate transcarbamylase | 59 | 54 |
| Dihydroorotase | 55 | 21 |
| UMP synthetase | 47 | 34 |
| Actin | 91 | 88 |
| Myosin heavy chain (head) | 51 | 44 |
| Calmodulin | 88 | 60 |
| Ras | 66 | 32 |
| Gα2 | 47 | 46 |
| Gβ | 67 | 40 |
| PKA catalytic subunit | 50 | 47 |
| PKA regulatory subunit | 52 | 41 |
| α tubulin | 67 | 62 |
| β tubulin | 74 | 68 |

## Phylogenetic divergence based on protein sequences: old trees

A few years ago the sequences of only a few proteins had been determined in *Dictyostelium discoideum* as well as in representative members of diverse phyla and so the database used to determine the relative degrees of relatedness among the organisms was somewhat restricted[21]. One of the genes, *pyr*5-6, encodes a protein able to catalyze the last two steps in pyrimidine biosynthesis. In both the eubacterium *Escherichia coli* and the yeast *S. cerevisiae* these steps are catalyzed by separate proteins encoded by independent genes, but in both *Dictyostelium* and mammals the genes encoding these proteins are fused. This shared characteristic is of significance by itself. The primary sequence of the *Dictyostelium* protein is 47% identical to that in mammalian homologs while only 34% identical to the *S. cerevisiae* homologs when the two yeast proteins are aligned along the single *Dictyostelium* protein[21]. In fact, all of the pyrimidine biosynthetic enzymes, as well as the other *Dictyostelium* proteins used in the analysis, are more similar to their mammalian homologs than they are to the yeast homologs (table).

When these sequences were used to generate trees by maximum parsimony, it was found in every case that the yeast *S. cerevisiae* diverged from the line leading to vertebrates earlier than *Dictyostelium*. These results were confirmed by analyses using distance matrices[21]. We have now extended this analysis by comparing six additional *Dictyostelium* proteins that have homologs in the data base from representative organisms.

## New trees

The sequences of two G-proteins, two cAMP-dependent protein kinase subunits, and two tubulins were analyzed by distance matrix to determine the relative branching of yeast and *Dictyostelium* from the line leading to humans. Sequences for these six proteins were available in the databases for our reference set of four metazoans (*Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Caenorhaditis elegans*) and two yeast (*Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*). Where more than one copy of the homologous gene was available for an organism, the one most closely related to the *Dictyostelium* gene was used for the comparison. In every case, the analysis indicated that *Dictyostelium* diverged after the yeasts but before the round worm *C. elegans* (fig. 1). The branching pattern indicated by parsimony analyses confirmed the branching pattern in every case but showed some uncertainty within the metazoans (data not shown).

Divergence of *S. pombe* in the distance matrices rooted on *S. cerevisiae* established a base line for estimating the time of divergence of the other eukaryotes (fig. 1). The branch lengths to the first two internodes are a significant proportion of the total distance, such that it can be taken with confidence that *Dictyostelium* diverged after the yeasts and before the radiation of metazoans. Likewise, the evolutionary distances to subsequent internodes show that *C. elegans* and *Drosophila* diverged somewhat later, as expected from the fossil record.

Analyses of the tubulins provides even more convincing evidence for the order of divergence since the databases include sequences for the fungus *Aspergillus* as well as the mustard plant *Arabidopsis* (fig. 1). The tree constructed for α tubulin suggests that *Aspergillus* shared a common ancestor with *S. pombe* following divergence, while the tree constructed for β tubulin suggests that both the yeasts diverged before *Aspergillus*. In both these trees *Dictyostelium* is grouped with the metazoans rather than with the yeasts or fungi. Although the data are too sparse to be fully confident of the exact time of divergence, these trees suggest that *Dictyostelium* diverged more recently than the yeasts and fungi about 1500 million years ago, but before the plant/animal split about 600 million years ago.

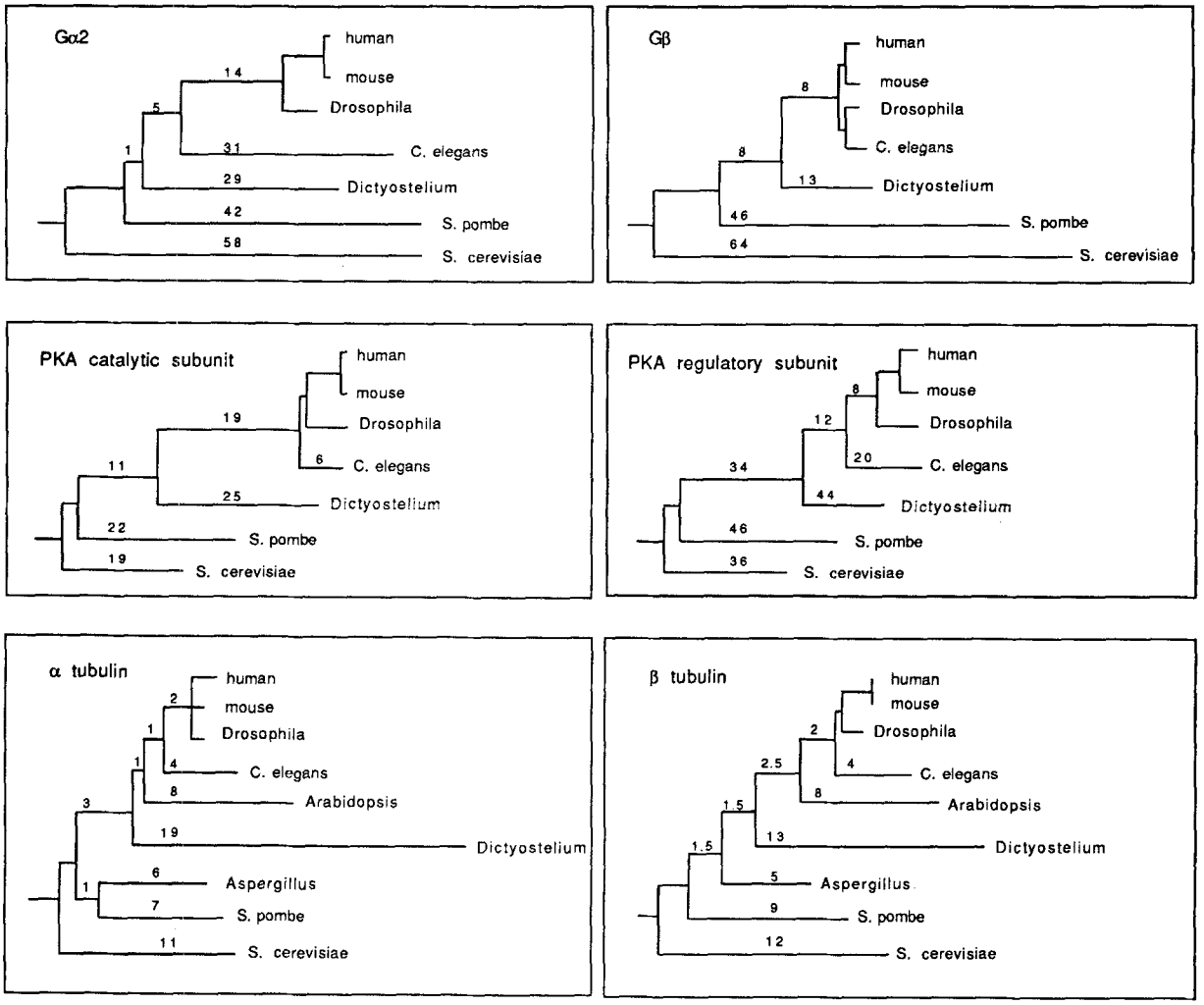


Figure 1. Phylogeneic trees based on protein sequences. Homologs of G proteins, cAMP-dependent protein kinase (PKA), and tubulins in seven different species were aligned. Trees were constructed by using the distance matrix approach rooted on *S. cerevisiae*. Branch lengths are shown on the branches which are drawn proportionally. The trees based on the tubulins include *Aspergillus* and *Arabidopsis* which bracket *Dictyostelium*.

## Conclusions

While molecular studies based on sequence comparisons of the small ribosomal subunit RNA suggest that *Dictyostelium* diverged before yeast[22], all of the studies based on protein sequences of this organism indicate that it diverged after the yeast (ref 21; this work). All 11 of the proteins used in these studies show greater identity to mammalian homologs than they do to yeast homologs (table). Likewise, the branching patterns generated either by distance matrix or parsimony analyses indicate that *Dictyostelium* diverged significantly later than the yeasts (ref 21; fig, 1). The score stands at 11 to 1 in favor of later divergence for *Dictyostelium*.

The structures of each of the six new trees generated from either distance matrix or parsimony data are sufficiently similar that we have been able to analyze all of the data together by either of two methods to give a consensus phylogeny (fig. 2). Since this 'tree of trees' approach is based on a larger number of comparisons, the branching pattern can be taken with greater confidence than those generated on the basis of single sets of homologs. Both distance matrix and parsimony analyses of the composite data showed that *Dictyostelium* diverged significantly later than either the budding yeast *S. cerevisiae* or the fission yeast *S. pombe* and that the metazoans diverged about an equal time later. Therefore, we can expect processes that function in *Dictyostelium* to be more closely related to mammalian processes than those of yeast.

Generating a consensus phylogeny based on protein sequences settles the previous conflict between the phylogeny based on morphological and biochemical characteristics and the phylogeny based on rDNA sequences. We can now be fairly confident that *Dictyostelium* diverged after the yeasts but before the plant/animal divergence. Further refinement of the time of divergence

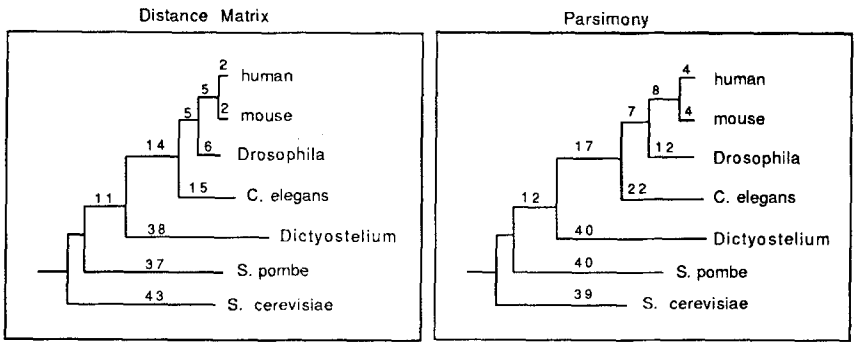


Figure 2. Consensus phylogeny. Averaged branch lengths were analyzed by distance matrix or parsimony to generate a 'tree of trees'. The lengths generated by the two different approaches are presented above each branch.

will require sequence comparisons with organisms more closely related to *Dictyostelium discoideum* such as *Dictyostelium mucroides* and *Polysphondylium pallidum* as well as such comparisons with additional fungal and plant sequences.

## Methods

### Sequences

Sequences were obtained from the SwissProt (SP) protein library[1], version 28.0, or from the National Biomedical Research Foundation (NBRF) protein libraries[2], version 40.0, or from the National Center for Biotechnological Information (NCBI) GenPept (GP) transaltion of the GenBank (GB) DNA libraries[3], version 83.0. For proteins with multiple functional copies per genome, the sequence most similar to that in *D. discoideum* was used.

All accession numbers are from SwissProt unless otherwise indicated; for Gα proteins: *Homo sapiens* (human), P29777; *Mus musculus* (mouse), P18873; *D. melanogaster*, P16377; *C. elegans*, P22454; *D. discoideum*, P16051; *S. pombe*, Q04665; and *S. cerevisiae*, P08539; for Gβ proteins: human, P04901; mouse, P29387; *D. melanogaster*, P26308; *C. elegans*, P17343; *D. discoideum*, NBRF A47370; *S. pombe*, GP L28061, and *S. cerevisiae*, P18851; for the PKA catalytic subunits: human, P17612; mouse, P05132; *D. melanogaster*, P12370; *C. elegans*, P21137; *D. discoideum*, P34099; *S. pombe*, GB D23667; and *S. cerevisiae*, P06245; for the PKA regulatory subunits: human, P10644; mouse, P12849; *D. melanogaster*, P16905; *C. elegans*, P30625; *D. discoideum*, P05987; *S. pombe*, P36600; and *S. cerevisiae*, P07278; for the α tubulins: human, P04687; mouse, P05214; *Xenopus laevis*, P08537; *D. melanogaster*, P06603; *C. elegans*, P34690; *Arabidopsis thaliana*, P11139; *D. discoideum*, P32255; *S. pombe*, P04688; *Aspergillus nidulans*; P24633; and *S. cerevisiae*, P09733; for the β tubulins: human, P07437; mouse, P05218; *X. laevis*, P30883; *D. melanogaster*, P08840; *C. elegans*, P12456; *A. thaliana*, P24636; *D. discoideum*, P 32256; *S. pombe*, P05219; *A. flavus*, P22012; and *S. cerevisiae*, P02557.

### Analyses

Full-length sequences were used except for PKA catalytic subunit sequences of *D. discoideum* and *S. pombe* in which the 360 amino acids of the C-terminal end were compared to full length sequences from the other organisms. Sequences of homologous proteins were aligned by using the multiple sequence alignment programs of Feng and Doolittle[10,11], as previously described[21]. The branch lengths of the resulting unrooted trees were determined by a least-squares method[18] from a standard difference score distance matrix analysis[11]. In addition, unrooted trees were determined from a column-by-column analysis of the aligned sequences by using the parsimony program PROTPARS available in the PHYLIP phylogeny package[9], as previously described[21]. For comparison, the PAPA3 (parsimony after pairwise alignment) program of Doolittle and Feng[7] was also used. In PAPA3, a four-taxon parsimony analysis using mutational probability parameters to calculate branch lengths is applied after sequences have been aligned using progressive alignment[10,11]. Only a single most-parsimonious tree was found for each set of homologous proteins except for the β tubulins, in which the phylogeny of *C. elegans* was variable. The length of each branch was determined using PAPA3 and roughly coincided with the branch lengths presented in figure 1 after rooting on *S. cerevisiae*.

Two approaches were used to construct the 'tree of trees'. In the first, the interspecies branch lengths in each of the six trees were used. For the α and β tubulins, lengths from analyses comparable to those of figure 1 but including only the seven basic species (human, mouse, *D. melanogaster*, *C. elegans*, *D. discoideum*, *S. pombe*, and *S. cerevisiae*) were used. These lengths were first normalized to a maximum of 100, to take into account different rates of evolution for each of the six proteins. The individual normalized lengths were then averaged, and the averaged lengths used to calculate

new branch lengths and trees. The trees shown in figure 2 result from such analysis using interspecies branch lengths determined by the distance matrix and parsimony approaches after pairwise alignment, as described above. Trees resulting from similar analyses but without normalization of the interspecies branch lengths were qualitatively identical to those shown in figure 2.

In the second approach, all six sequences used in this analysis were concatenated together for each species. The distance matrix and parsimony analyses described above were then performed on these concatenated sequences. The results were not significantly different from those obtained using the first approach.

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