A kingdom's progress: Archezoa and the origin of eukaryotes

Patrick J. Keeling*

Summary

The taxon Archezoa was proposed to unite a group of very odd eukaryotes that lack many of the characteristics classically associated with nucleated cells, in particular the mitochondrion. The hypothesis was that these cells diverged from other eukaryotes before these characters ever evolved, and therefore they represent ancient and primitive eukaryotic lineages. The kingdom comprised four groups: Metamonada, Microsporidia, Parabasalia, and Archamoebae. Until recently, molecular work supported their primitive status, as they consistently branched deeply in eukaryotic phylogenetic trees. However, evidence has now emerged that many Archezoa contain genes derived from the mitochondrial symbiont, revealing that they actually evolved after the mitochondrial symbiosis. In addition, some Archezoa have now been shown to have evolved more recently than previously believed, especially the Microsporidia for which considerable evidence now indicates a relationship with fungi. In summary, the mitochondrial symbiosis now appears to predate all Archezoa and perhaps all presently known eukaryotes. BioEssays 20:87–95, 1998.

INTRODUCTION

Prior to the popularization of the endosymbiotic theory, it was widely believed that the evolutionary link between prokaryotes and eukaryotes was the presence of photosynthesis in cyanobacteria and algae. The biochemistry of oxygenic photosynthesis was considered too complicated and too similar in detail to have arisen twice independently. Therefore, it was reasoned that all photosynthetic organisms were related, and by extension that cyanobacteria had evolved into photosynthetic eukaryotes. This ancestral eukaryote was thought to be like red algae because their pigments and light-harvesting antennae most closely resemble those of cyanobacteria and they also lack flagella and basal bodies (for discussion see Ref. 1). However, according to the endosymbiotic theory, the reason photosynthesis is so similar in cyanobacteria and photosynthetic eukaryotes is that the plastids of plant and algal cells are derived from a cyanobacterial symbiont. With increasing acceptance of the origin of plastids from cyanobacteria, links between cyanobacteria and the nucleus dissolved, and with it our explanation for the origin of eukaryotes.

An alternative to the cyanobacterial origin of eukaryotes arose from what seemed like an unlikely source when Carl Woese and his colleagues discovered an unexpected division in prokaryotes in 1977. Woese's group showed that prokaryotes are composed of two very distantly related groups,[2] which they named Eubacteria and Archaebacteria, now synonymous with Bacteria and Archaea. Archaebacteriologists soon began to find molecular links between archaebacteria and eukaryotes,[3] and these observations were brought into focus by the demonstration that archaebacteria and eukaryotes are one another's closest relatives in rooted universal trees.[4–6] This means that archaebacteria share a recent common ancestor with eukaryotes, so it makes

School of Botany, University of Melbourne, Parkville, Victoria, Australia.
Contract grant sponsor: Natural Sciences and Engineering Research Council of Canada Fellowship.
*Correspondence to: Dr. Patrick J. Keeling, Department of Biology, Indiana University, Bloomington, IN 47405. E-mail: pkeeling@sunflower.bio.indiana.edu

TABLE 1. Some Common Names and Characteristics of Archezoan Groups

Group	Some common genera	Mitochondria	Peroxisomes	Golgi	Flagella
Metamonads	*Giardia* *Hexamita* *Trepomonas* *Retortamonas* *Pyrsonympha*	Not recognized	Not recognized	Not recognized	Yes
Microsporidia	*Encephalitozoon* *Nosema* *Spraguea* *Vairimorpha*	Not recognized	Not recognized	Not recognized	No
Parabasalla	*Trichomonas* *Tritrichomonas* *Monocercomonas* *Trichonympha*	Hydrogenosome?	Not recognized	Yes	Yes
Archamoebae	*Entamoeba* *Pelomyxa* *Mastigamoeba* *Phreatamoeba*	Not recognized	Not recognized	In some	In some

perfect sense that many aspects of archaebacterial molecular biology also should be found to resemble their eukaryotic counterparts.[7] Each of these shared similarities clarifies the prokaryote-eukaryote transition in a small way by showing that molecular traits previously considered ‘‘eukaryotic’’ actually predate nucleated cells. However, these shared characters tie the archaebacteria to *all* eukaryotes. Unlike the cyanobacteria-to-alga hypothesis, no single group of eukaryotes specifically resembles the archaebacteria. So what was the nature of the first eukaryote?

ARCHEZOA: ARCHETYPICAL EUKARYOTES

When the endosymbiotic theory became fashionable and the photosynthetic origin of eukaryotes less so, the absence of mitochondria in certain eukaryotic cells started to attract attention. If the mitochondrion, like the plastid, originated by an endosymbiotic event, then it was also possible that some amitochondrial eukaryotes diverged prior to this event. This was first suggested for the hypermastigotes, a group of Parabasalia,[8] and was expanded and refined by Cavalier-Smith in 1983.[9] Cavalier-Smith proposed the Archezoa to contain the descendants of ancient premitochondrial eukaryotes: the Metamonads, Parabasalia, Microsporidia, and a new group, the Archamoebae. Each of these groups comprised anaerobic, amitochondrial cells that are morphologically very simple. Not only do they lack mitochondria but also peroxisomes or microbodies, in most cases Golgi dictyosomes, and in some also flagella (see Table 1). What’s more, the ribosomes of Metamonads, Parabasalia, and Microsporidia also were known to be about the same size as those of prokaryotes. Eukaryotic ribosomes are typically 80S in size, whereas those of archezoa and prokaryotes are 70S. Similarly, eukaryotic rRNA molecules are for the most part 18S and 28S in contrast to the smaller rRNAs found in prokaryotes and archezoa.

Almost immediately after the Archezoa was proposed, evidence from molecular phylogeny was produced to support its validity. The small subunit rRNA genes from a microsporidian, a diplomonad (the most studied subdivision of Metamonada), and a parabasalian were all published within a short space of time, and these three genes branched deeper in the rRNA tree than any previously known eukaryotic sequence.[10–12] Although the order of the three groups remains contentious in rRNA phylogeny, trees of other molecules involved in gene expression, such as EF-1a, EF-2, RNA polymerase subunits, isoleucyl-tRNA synthetase, and large subunit rRNA, corroborated the deep branching position of these organisms.[6,13–16]

This phylogenetic evidence fulfilled the most basic prediction of the Archezoa hypothesis for these groups: if they predate the origin of the mitochondrion, then they must branch earlier than mitochondrion-containing eukaryotes in phylogenetic trees (see Fig. 1). However, this was the high-water mark for the Archezoa. In recent years evidence has emerged that several of these organisms contain a genetic residue of the mitochondrion, which means that they could not have evolved before the endosymbiosis. The Archezoa is fast losing membership, and it now appears that the mitochondrial endosymbiosis may have taken place before the evolution of any of the presently known eukaryotic lineages.

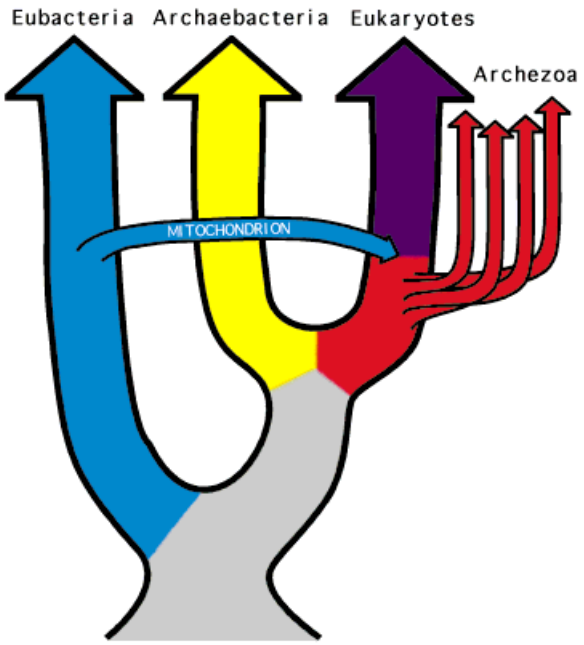

Figure 1. The Archezoa as originally conceived. Mitochondria containing eukaryotes are purple, Archezoa are red, Eubacteria are blue, and Archaebacteria are yellow. The symbiosis that led to the mitochondrion was proposed to have taken place after the divergence of at least four lineages from other eukaryotes, the Metamonads, Microsporidia, Parabasalia, and Archamoebae. The order that these four groups diverged was not clear, and all have since been argued to be the first.

ARCHAMOEBAE: FIRST TROUBLE FOR ARCHEZOA

Little can be said about the Archamoebae as a group because they share almost no unifying characters other than the fact that they are all amitochondriate amoebae. Otherwise, they are a very diverse group inhabiting a wide variety of environments and making a living in many diverse ways. The diversity of the Archamoebae has been reason to doubt the phylogenetic unity of the group,[17] but at the same time the unparalleled simplicity of the members also has led to the suggestion that of all Archezoa, Archamoebae are the most primitive[18] (Fig. 2).

The first hint that any Archamoeba may be secondarily amitochondrial came from the enteric pathogen, *Entamoeba histolytica*. When the small subunit rRNA gene from *Entamoeba* was characterized, it was found to branch later than some mitochondria-containing protists (herolobosea and euglenozoa), which suggested that the ancestor of *Entamoeba* had a mitochondrion.[12] Since then, the small subunit rRNA from *Phreatamoeba balamuthi* and large subunit rRNA from a species of *Pelomyxa* also have been sequenced. In phylogenetic trees these also branch within the mitochondria-containing eukaryotes,[19,20] so these too appear to have arisen from mitochondria-containing ancestors. Furthermore, in the small subunit rRNA tree, *Entamoeba* and *Phreatamoeba* sometimes branch together,[21] and sometimes do not,[19] raising doubts as to the validity of the Archamoebae as an evolutionary lineage, let alone a primitively amitochondrial one. These phylogenetic arguments are not beyond reasonable doubt, however, because the Archamoebae often branch very near the origin of mitochondria, and other molecular trees disagree with rRNA on the order of these deep branching taxa.[22]

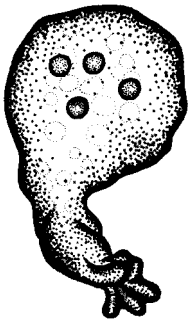

Figure 2. An archamoeba, *Pelomyxa.*

More substantial evidence came from a direct search of the *Entamoeba* genome for molecular relics of the mitochondrial symbiont. Most of the many hundreds of mitochondrial protein-coding genes are encoded in the nucleus and targeted to the organelle after they are translated in the cytoplasm. These genes were transferred from the symbiont genome to the nucleus but are recognizable today both because of this targeting and because the genes themselves closely resemble homologues from the type of bacteria from which the mitochondrion evolved, the alpha-proteobacteria.[23] Finding such genes in the nucleus of an organism shows that ancestors of that lineage contained a mitochondrion, even if the organelle cannot otherwise be recognized today. This concept proved to be a breakthrough for testing the Archezoa and was first applied to the nuclear genome of *Entamoeba histolytica*. Clark and Roger[24] found two genes of mitochondrial origin in *Entamoeba*, one for pyridine nucleotide transhydrogenase and another for a 60-kilodalton chaperonin (cpn60). In phylogenetic analyses of cpn60, the *Entamoeba* protein branched very strongly with homologues from the mitochondrion of other eukaryotes, which in turn were related to cpn60 proteins from alpha-proteobacteria.

These observations could be interpreted as evidence for a lateral transfer or symbiosis involving some other eubacterium[25,26] but to do so would require a number of lateral

transfers or symbioses involving alpha-proteobacteria that happened to be *very* closely related to the mitochondrial symbiont. The simplest explanation is that *Entamoeba* evolved from mitochondrial-containing ancestors,[24] and this may be said with some confidence because the *Entamoeba* chaperonin not only conforms to the phylogenetic expectations of a mitochondrial-derived protein but also is specifically related to eukaryotic homologues that are targeted to the mitochondrion.

PARABASALIA AND THE HYDROGENOSOME

Although they lack classical mitochondria, the Parabasalia do contain a double membrane-bound metabolic organelle called the hydrogenosome whose origin has been the source of some debate. Parabasalian hydrogenosomes do not resemble mitochondria in morphology, they do not appear to contain a genome, and unlike oxidative phosphorylation in the mitochondrion, energy is released in the hydrogenosome from the conversion of pyruvate or malate into acetate, carbon dioxide, and hydrogen gas (see Ref. 27 for review). However, despite these differences, there are reasons to suspect that parabasalian hydrogenosomes may share common ancestry with mitochondria. First, hydrogenosomes are not restricted to Parabasalia but are found in isolated members of several unrelated eukaryotic lineages (percolozoa, ciliates, and chytrid fungi), and these organisms invariably *lack* mitochondria. Moreover, some of these hydrogenosomes do resemble mitochondria morphologically.[28] The mutual exclusion of mitochondria and hydrogenosomes throughout eukaryotes led to the suggestion that mitochondria may have turned into hydrogenosomes in these organisms, and for this reason, the Parabasalia were removed from Archezoa.[29] However, in some of these organisms the hydrogenosome also has been argued to have evolved from peroxisomes,[30] and considering the differences between hydrogenosomes and mitochondria, their mutual exclusion is not in itself sufficient evidence that they are of common ancestry.[27] Without a direct link between the mitochondrion and hydrogenosome, the question remains open (Fig. 3).

For the Parabasalia, such a link has now been clearly established by the application of the same strategy that was so successful in *Entamoeba* to the parabasalian pathogen, *Trichomonas vaginalis*. Chaperonin genes, cpn10, cpn60, and cpn70, were sought and found in the genome of *Trichomonas*. In each case these genes were found to bespecifically related to mitochondrial homologues.[31–34] In addition, *T. vaginalis* cpn60 and cpn70 antibodies were shown to cross-react specifically with the purified hydrogenosomes,[32] and bacterial cpn60 antibodies have been localized specifically to the hydrogenosome by in situ immunoelectron microscopy.[35] These chaperonins are apparently localized in the hydrogenosome and derived from the same

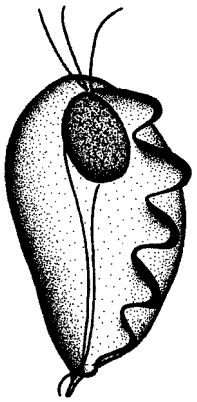

Figure 3. A parabasalian, *Tritrichomonas*.

endosymbiont as the mitochondrion, creating a compelling argument that the parabasalian hydrogenosome and the mitochondrion descended from a common ancestor.

How, then, are the hydrogenosome and mitochondrion related? In those ciliates and fungi where hydrogenosomes evolved from mitochondria, they must have evolved from a highly specialized organelle because mitochondria were well developed in the ancestors of these groups. But this is not necessarily the case in Parabasalia; mitochondria are not found in any lineage known to predate Parabasalia, so all that can be said is that their hydrogenosome evolved from the same symbiont. Whether that symbiont was anything like what we would call a mitochondrion is not certain. If the ancestral parabasalian had a ''proper'' mitochondrion, then the transformation to the hydrogenosome may have occurred as it did in ciliates and fungi. This process would entail the loss of the critical metabolic enzymes found in mitochondria and the conscription of the nonmitochondrial enzymes hydrogenase and pyruvate:ferredoxin oxidoreductase from some other source. The loss of the mitochondrial genome would be expected to occur when the enzymes that it encoded were no longer used in the organelle.[36] Alternatively, if the hydrogenosome evolved from a not-yet-specialized symbiont, then it merely followed a different evolutionary trajectory than did the mitochondrion, and this would reflect the likelihood that the symbiont had a greater metabolic diversity than contemporary mitochondria.[37] Indeed, this also would bring into question the widely held assumption that the original reason the symbiont was retained was for the reactions that the mitochondrion now performs.

MICROSPORIDIA: ARCHEZOA, PROTISTS, OR FUNGI?

Microsporidia are obligate intracellular parasites that share a complex and unique infection strategy. Outside their host

Figure 4. A microsporidian, *Nosema*.

cells, Microsporidia can only survive as spores with a tough double coat of chitin and protein. Inside the cytoplasm is a tightly wound projectile known as the polar tube. This organelle could be thought of as a hybrid between a harpoon and a hypodermic needle; when a spore encounters a susceptible host, it rapidly everts the polar tube, which then penetrates the host membrane. The infectious cytoplasm is squeezed through the polar tube (which is typically only approximately 0.1 μm in diameter) and is injected directly into the host cytoplasm where it lives as an amoeba, dividing and producing more spores (see Ref. 38 and references therein for details) (Fig. 4).

Microsporidia are obviously accomplished parasites, but they stand out among eukaryotes in others ways too. Microsporidia have the smallest known nuclear genomes, in some instances smaller even than many bacterial genomes.[39] Moreover, microsporidian ribosomes resemble prokaryotic ribosomes in that the sequence homologous to the 5.8S rRNA molecule is covalently linked with the 23S rRNA; in other eukaryotes this rRNA is a separate molecule.[40] Because the fused 5.8S-23S rRNA is unique to Microsporidia and prokaryotes, it has been cited to support the notion that Microsporidia are the most primitive of all eukaryotes.[41] However, the 5.8S gene is located immediately upstream of the large subunit gene in the rDNA operon of eukaryotes. When this operon is transcribed, these two species of rRNA are cleaved at specific processing sites. Microsporidian rRNA sequences are very odd in general and contain numerous deletions. If one of these deletions affected a single processing site in the ancestor of Microsporidia, it could easily have led to the reformation of fused 5.8S-23S rRNA.[42]

All of these characteristics can be interpreted as evidence that Microsporidia are descendants of ancient or primitive eukaryotes, but they also could be the result of the highly adapted, parasitic lifestyle characteristic of the group. This consideration has always figured in speculation of their evolutionary origin,[42] but even with such doubts, current arguments regarding the nature of their evolutionary history still come as a surprise. Evidence is now emerging that suggests that Microsporidia actually evolved recently from so-called "crown" eukaryotes (the twigs, such as animals, plants, and fungi) and may share a close ancestry with fungi. The strongest evidence supports a general relationship between Microsporidia and crown-taxa. First, EF-1a proteins of microsporidia, animals, and fungi all contain an insertion that is unique to these taxa.[15] Similarly, the dihydrofolate reductase and thymidylate synthase are two separate enzymes in microsporidia, animals, and fungi but are fused in plants and other protists.[43] A specific relationship with fungi was first proposed on the basis of parallels found between the unusual meiotic cycle of Microsporidia and that of certain fungi[44] and also has now been supported by the phylogeny of both alpha- and beta-tubulins, which place Microsporidia within the fungal radiation.[45,46]

The balance between evidence for the ancient or crown status of the Microsporidia was tipped in favor of the latter by the recent discovery of mitochondrial cpn70 genes in the genomes of *Nosema locustae*[47] and *Vairimorpha necatrix.*[48] In phylogenetic trees these sequences branch convincingly with mitochondrial homologues, undermining the argument that they are primitive descendants of amitochondrial eukaryotes. Interestingly, the microsporidian cpn70 genes also branch, albeit weakly, with mitochondrial genes from fungi.[47,48]

So why would the Microsporidia branch at the base of the eukaryotes in phylogenetic trees if they actually arose from the crown of the tree? Highly divergent sequences will often fall in the wrong place in phylogenetic trees, and the rate of substitution in microsporidian genes is exceptionally high.[10,15,45–48] Such genes will sometimes branch preferentially with other highly divergent sequences, and in the case of the eukaryotic tree, this means branching deeply. In fact, the divergence rate of most of the deep branching eukaryotes is relatively high, although to a much lesser extent than Microsporidia. It may be that some of these other taxa also are misplaced due to substitution rate, but there is no reason to dismiss the rRNA tree without evidence for some other relationship, such as we now have for Microsporidia.

MITOCHONDRIAL RELICS IN METAMONADS?

Most of what we know about the Metamonads is from one type, the diplomonads. This is perhaps unfortunate because diplomonads are probably the most highly derived Metamonads and, therefore, poorly represent the ancestral state of the group. Other Metamonads are flagellates with a single nucleus and four kinetosomes, one of which is recurrent and often has a flagellum associated with a feeding

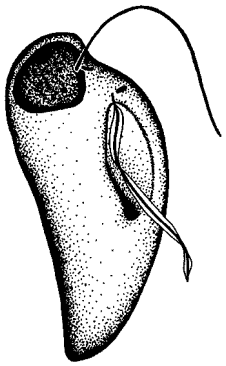

Figure 5. A metamonad, *Retortamonas*.

organelle or cytostome. Most diplomonads are essentially two such cells fused back-to-back in axial symmetry. There are two nuclei associated with four kinetosomes each, and in heterotrophic species there are also two symmetrical cytostomes (see Refs. 49,50 for review) (Fig. 5).

Historically, there has been little reason to suspect Metamonads of ever having harbored a mitochondrion. They contain no mysterious organelle comparable with the parabasalian hydrogenosome and, unlike the Microsporidia, their molecular sequences are not so unusual that they stand out as obvious candidates for misplacement in phylogenetic trees. Indeed, molecular data have provided little reason to doubt the early divergence of these organisms; genes for transcription and translation genes commonly used for phylogeny consistently place diplomonads at the very base of eukaryotes.[14,16,21]

So what reasons are there to doubt the primitively amitochondrial nature of the Metamonads? There is an intriguing report that the diplomonad *Giardia lamblia* contains a protein that cross-reacts with antibodies against mitochondrial cpn60.[51] However, in situ immunofluorescent labeling with this antibody is scattered throughout the cytoplasm and not concentrated in a particular part of the cell as one would expect of an organellar protein. One possibility is that this chaperonin is derived from the mitochondrion but has assumed a cytosolic role. This would contrast with other chaperonin proteins in amitochondrial eukaryotes: the *Trichomonas* chaperonins are apparently targeted to the hydrogenosome by using a transit peptide,[32] and both *Vairimorpha* and *Nosema* chaperonin genes also have amino-terminal peptides that probably direct the protein to some as yet unidentified compartment.[47,48]

The process by which a protein of organellar origin takes on a cytosolic role has been proposed to take place in other instances, however, and has been called endosymbiotic gene replacement.[52] These proteins have lost all functional links to the organelle, but their evolutionary history is revealed in their phylogenetic position among bacteria. There is compelling evidence that this is the source of 3-phosphoglycerate kinase in plants because the cytosolic protein is more closely related to homologues from plastids and bacteria than it is to other eukaryotic cytosolic genes.[52] There is also reason to suspect that certain glycolytic proteins in eukaryotes may be derived from the mitochondrial symbiont. These proteins are more related to homologues from proteobacteria (the closest relatives of the mitochondrion) than they are to homologues from archaebacteria (the closest relatives of the nuclear-cytosolic lineage). This relationship has been seen in both glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[53] and triosephosphate isomerase (TPI).[54] In both cases the genes from diplomonads are known, and they do not differ remarkably from those of other eukaryotes. If the source of either of these proteins is the mitochondrial symbiont, then it implies that diplomonads descend from a lineage that also contained the symbiont. Unfortunately, neither GAPDH nor TPI is totally unambiguous. The phylogeny of GAPDH is complex, with numerous paralogous gene families distributed in a pattern whose interpretation is not yet clear from the available data.[53,55] TPI, on the other hand, does not contain enough phylogenetically useful information to discriminate between a specifically alpha-proteobacterial origin of eukaryotic TPI or simply the proteobacteria in general.[54]

The idea that the mitochondrial symbiont could have contributed more to the nucleus and cytosol than just mitochondrially targeted genes is an interesting assertion and may partially explain a few nagging problems in the phylogeny of several other proteins. There are a number of eukaryotic proteins that appear to be closer to proteobacterial homologues than expected or at least closer to eubacteria than to archaebacteria. These have generally been interpreted as the result of lateral transfers or even an ancient cellular fusion event, or chimerism.[26,56] Alternatively, *some* of these incongruencies may be the result of genes derived from the mitochondrial symbiont acquiring a role in cytoplasmic metabolism (variations on this theme are discussed in Ref. 57). Unfortunately, for many of these genes there is either insufficient information in the sequences or inadequate taxon sampling to make any believable conclusions as to their origin. Moreover, because there are no mitochondrially targeted homologues of any of the genes for which this has been proposed (including TPI or GAPDH), their evolutionary origin in eukaryotes will never be as clear-cut as proteins, such as cpn60, which have a functional link to the organelle in mitochondrion-containing eukaryotes. This also means that, even if these genes are derived from the mitochondrial symbiont, their presence is not evidence for the presence of the organelle. With no functional link between the protein and the organelle, the organelle could be

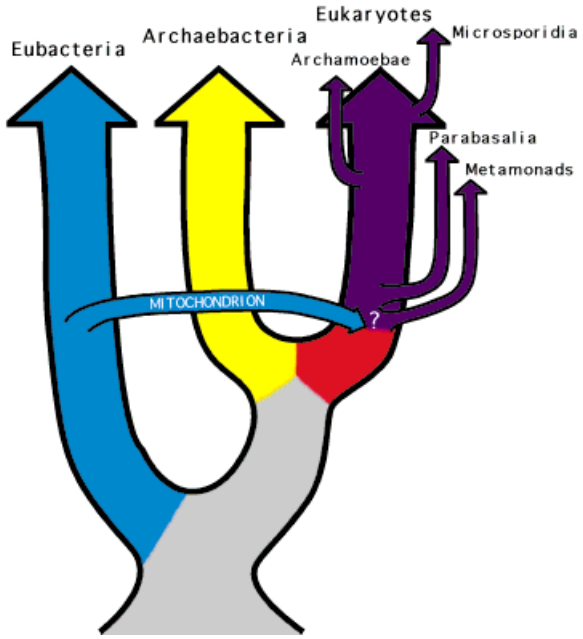

Figure 6. The Archezoa today. The color scheme is as in Figure 1. The Archamoebae and Microsporidia are shown branching much later than previously proposed, and the Parabasalia and Metamonads are shown still branching deeply among eukaryotes but after the mitochondrial symbiosis. This can be said with some confidence in the case of Parabasalia, but there is only preliminary evidence to support this conclusion for Metamonads (hence the lingering question mark).

lost without affecting the protein, and similarly, the protein could be the result of a transfer that took place when the symbiosis was still transient.[54]

CONCLUSIONS: THE RIGHT ANCESTORS FOR THE WRONG REASONS

The purpose for creating the Archezoa was that it united primitively amitochondrial eukaryotes. This is not fulfilled for Parabasalia, Microsporidia, or Archamoebae, and there are now growing doubts for the Metamonads as well.

However, the kingdom Archezoa also was proposed explicitly as a ''phylogenetic hypothesis''[9] intended to draw attention to these organisms as putative descendants of early eukaryotes. In this regard it has been an outstanding success because two of the original Archezoa, Parabasalia and Metamonads, probably really are descendants of early eukaryotes whether they had a mitochondrion or not (Fig. 6). The ancestor of extant eukaryotes will be clearer once we have a better and broader understanding of these groups. Retortamonads have strong morphological similarities to diplomonads,[49,58,59] and the hypermastigotes are likewise strongly allied with other Parabasalia, but these relationships have yet to be tested with molecular data. Moreover, some other relationships are not so obvious. Oxymonads, for instance, are only tenuously classified with Metamonads and have characteristics, such as meiosis and a sexual cycle[60] that make them stand out among Metamonads. It is distinctly possible that Oxymonads are not closely related to diplomonads or retortamonads at all. These ''details'' are central to our understanding of the nature of these groups, and the nature of the ancestral eukaryote.

Of course, there is also hope that new groups and deeper branches will be revealed to us in the future as organisms known only from morphology or some never seen before are characterized or identified. The molecular approach to biological diversity has greatly increased our understanding of diversity and the ancestral state of eubacteria and especially archaebacteria.[25,61] The same has yet to be applied to eukaryotes on as grand a scale, but when it is, it will hopefully yield as many surprises and confirmations as it has in the prokaryotes. Even if none of the eukaryotes we know today evolved before the acquisition of the mitochondrion, we might still find an archezoan somewhere in the long branch between archaebacteria and eukaryotes.

ACKNOWLEDGMENTS

I thank A.J. Roger, W.F. Doolittle, M. Müller, G.C. Clark, G. Brugerolle, and T. Cavalier-Smith for past discussions and lessons on this subject, and G.I. McFadden and N.M. Fast for critical reading of the manuscript. *Retortamonas* and *Tritrichomonas* line drawings were based on reconstructions and diagrams in References 58,59.

NOTE ADDED IN PROOF

The assertion that diplomonads once contained mitochondria has received considerable support with the recent finding of a mitochondrial-type *cpn*60 in *Giardia lamblia* (Roger, A.J., Svärd, S.G., Tovar, J., Clark, C.G., Smith, M.W., Gillin, F.D., and Sogin, M.L. (1998) A mitochondrial-like chaperonin 60 gene in *Giardia lamblia*: Evidence that diplomonads once harbored an endosymbiont related to the progenitor of mitochondria. *Proc Natl Acad Sci USA* 95, 229–234).

REFERENCES

1 Dougherty, E.C., and Allen, M.B. (1960). Is pigmentation a clue to protistan phylogeny? In M.B. Allen (ed): *Comparative Biochemistry of Photoreactive Systems.* New York: Academic Press, pp. 129–144.

2 Balch, W.E., Magrum, L.J., Fox, G.E., Wolfe, R.S., and Woese, C.R. (1977). An ancient divergence among the bacteria. *J Mol Evol* 9, 305–311.

3 Huet, J., Schnabel, R., Sentenac, A., and Zillig, W. (1983). Archaebacteria and eukaryotes posses DNA-dependent RNA polymerases of a common type. *EMBO J* 2, 1291–1294.

4 Iwabe, N., Kuma, K.-I., Hasegawa, M., Osawa, S., and Miyata, T. (1989). Evolutionary relationship of archaebacteria, eubacteria, and eukaryotes inferred from phylogenetic trees of duplicated genes. *Proc Natl Acad Sci USA* 86, 9355–9359.

5 Gogarten, J.P., Kiblak, H., Dittrich, P., Taiz, L., Bowman, E.J., Bowman, B.J., Manolson, N.F., Poole, R.J., Date, T., Oshima, T., Konishi, J., Denda, K., and Yoshida, M. (1989). Evolution of the vacuolar H^+-ATPase: Implications for the origin of eukaryotes. *Proc Natl Acad Sci USA* 86, 6661–6665.

6 Brown, J.R., and Doolittle, W.F. (1995). Root of the universal tree of life based on ancient aminoacyl-tRNA synthetase gene duplications. *Proc Natl Acad Sci USA* 92, 2441–2445.

7 Keeling, P.J., and Doolittle, W.F. (1995). Archaea: Narrowing the gap between prokaryotes and eukaryotes. *Proc Natl Acad Sci USA* 92, 5761–5764.

8 Stewart, K.D., and Mattox, K. (1980). Phylogeny of phytoflagellates. In E.R. Cox (ed): *Phytoflagellates, Development in Marine Biology.* Amsterdam: Elsevier/North Holland, pp. 433–462.

9 Cavalier-Smith, T. (1983). A 6-kingdom classification and a unified phylogeny. In H.E.A. Schenk and W.S. Schwemmler (eds): *Endocytobiology. II. Intracellular Space as Oligogenetic*. Berlin: Walter de Gruyter, pp. 1027–1034.

10 Vossbrinck, C.R., Maddox, J.V., Friedman, S., Debrunner-Vossbrinck, B.A., and Woese, C.R. (1987). Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. *Nature* 326, 411–414.

11 Sogin, M.L., Gunderson, J.H., Elwood, H.J., Alonso, R.A., and Peattie, D.A. (1989). Phylogenetic meaning of the kingdom concept: An unusual ribosomal RNA from *Giardia lamblia. Science* 243, 75–77.

12 Sogin, M.L. (1989). Evolution of eukaryotic microorganisms and their small subunit ribosomal RNAs. *Am. Zool.* 29, 487–499.

13 Viscogliosi, E., Philippe, H., Baroin, A., Perasso, R., and Brugerolle, G. (1993). Phylogeny of trichomonads based on partial sequence of large subunit rRNA and on cladistic analysis of morphological data. *J Eukaryot Micorbiol* 40, 411–421.

14 Klenk, H.-P., Zillig, W., Lanzendörfer, M., Grampp, B., and Palm, P. (1995). Location of protist lineages in a phylogenetic tree inferred from sequences of DNA-dependent RNA polymerases. *Arch Protistenkd* 145, 221–230.

15 Kamaishi, T., Hashimoto, T., Nakamura, Y., Nakamura, F., Murata, S., Okada, N., Okamoto, K.-I., Shimzu, M., and Hasegawa, M. (1996). Protein phylogeny of translation elongation factor EF-1a suggests Microsporidians are extremely ancient eukaryotes. *J Mol Evol* 42, 257–263.

16 Yamamoto, A., Hashimoto, T., Asaga, E., Hasegawa, M., and Goto, N. (1997). Phylogenetic position of the mitochondrion-lacking protozoan *Trichomonas tenax*, based on amino acid sequences of elongation factors 1alpha and 2. *J Mol Evol* 44, 98–105.

17 Cavalier-Smith, T. (1987). Eukaryotes with no mitochondria. *Nature* 326, 332–333.

18 Cavalier-Smith, T. (1991). Archamoebae: The ancestral eukaryotes? *Biosystems* 25, 25–38.

19 Hinkle, G., Leipe, D.D., Nerad, T.A., and Sogin, M.L. (1994). The unusually long small subunit ribosomal RNA of *Phreatamoeba balamuthi. Nucleic Acids Res* 22, 465–469.

20 Morin, L., and Mignot, J.-P. (1995) Are Archamoebae true Archezoa? The phylogenetic position of *Pelomyxa* sp., as inferred from large subunit ribosomal RNA sequencing. *Eur J Protistol* 31, 448.

21 Cavalier-Smith, T., and Chao, E.E. (1997). Molecular phylogeny of the free-living archezoan *Trepomonas agilis* and the nature of the first eukaryote. *J Mol Evol* 43, 551–562.

22 Hasegawa, M., Hashimoto, T., Adachi, J., Iwabe, N., and Miyata, T. (1993) Early branchings in the evolution of eukaryotes: Ancient divergence of *Entamoeba* that lacks mitochondria revealed by protein sequence data. *J Mol Evol* 36, 380–388.

23 Yang, D., Oyaizu, Y., Oyaizu, H., Olsen, G.J., and Woese, C.R. (1985). Mitochondrial origins. *Proc Natl Acad Sci USA* 82, 4443–4447.

24 Clark, C.G., and Roger, A.J. (1995). Direct evidence for secondary loss of mitochondria in *Entamoeba histolytica. Proc Natl Acad Sci USA* 92, 6518–6521.

25 Pace, N.R. (1997) A molecular view of microbial diversity and the biosphere. *Science* 276, 734–740.

26 Rosenthal, B., Mai, Z., Caplivski, D., Ghosh, S., de la Vega, H., Graf, T., and Samuelson, J. (1997) Evidence for the bacterial origin of genes encoding fermentation enzymes of the amitochondriate protozoan parasite *Entamoeba histolytica. J Bacteriol* 179, 3736–3745.

27 Müller, M. (1993) The hydrogenosome. *J Gen Microbiol* 139, 2879–2889.

28 Finlay, M., and Fenchel, T. (1989). Hydrogenosomes in some anaerobic protozoa resemble mitochondria. *FEMS Microbiol Lett* 65, 311–314.

29 Cavalier-Smith, T. (1987) The origin of cells: A symbiosis between genes, catalysts, and membranes. *Cold Spring Harb Symp Quant Biol* 52, 805–824.

30 Marvin-Sikkema, F.D., Kraak, M.N., Veenhuis, M., Gottschal, J.C., and Prins, R.A. (1993) The hydrogenosomal enzyme hydrogenase from the anaerobic fungus *Neocallimastix* sp. L2 is recognized by antibodies, directed against the C-terminal microbody protein targeting signal SKL. *Eur J Cell Biol* 61, 86–91.

31 Horner, D.S., Hirt, R.P., Kilvington, S., Lloyd, D., and Embley, T.M. (1996). Molecular data suggest an early acquisition of the mitochondrion endosymbiont. *Proc R Soc Lond [Biol]* 1263, 1053–1059.

32 Bui, E.T., Bradley, P.J., and Johnson, P.J. (1996). A common evolutionary origin for mitochondria and hydrogenosomes. *Proc Natl Acad Sci USA* 93, 9651–9656.

33 Germot, A., Philippe, H., and Le Guyader, H. (1996). Presence of a mitochondrial-type HSP70 in *Trichomonas* suggests a very early mitochondrial endosymbiosis in eukaryotes. *Proc Natl Acad Sci USA* 93, 14614–14617.

34 Roger, A.J., Clark, C.G., and Doolittle, W.F. (1996). A possible mitochondrial gene in the early-branching amitochondriate protist *Trichomonas vaginalis. Proc Natl Acad Sci USA* 93, 14618–14622.

35 Bozner, P. (1997). Immunological detection and subcellular localization of Hsp70 and Hsp60 homologs in *Trichomonas vaginalis. J Parasitol* 83, 224–229.

36 Palmer, J.D. (1997) Organelle genomes: Going, going, gone! *Science* 275, 790–791.

37 Müller, M. (1997) Evolutionary origin of trichomonad hydrogenosomes. *Parasitol Today* 13, 166–167.

38 Canning, E.U. (1990). Phylum Microsporidia. In L. Margulis, J.O. Corliss, M. Melkonian, and D.J. Chapman (eds): *Handbook of Protoctista.* Boston: Jones and Bartlett Publishers, pp. 53–72.

39 Biderre, C., Pages, M., Méténier, G., Canning, E.U., and Vivarès, C.P. (1995) Evidence for the smallest nuclear genome (2.9 Mb) in the microsporidium *Encephalitozoon cuniculi. Mol Biochem Parasitol* 74, 229–231.

40 Vossbrinck, C.R., and Woese, C.R. (1986). Eukaryotic ribosomes that lack a 5.8S RNA. *Nature* 320, 287–288.

41 Siddall, M.E., Hong, H., and Desser, S.S. (1993) Phylogenetic analysis of the Diplomonadida (Wenyon, 1926) Brugerolle, 1975: Evidence for heterochrony in protozoa and against *Giardia lamblia* as a "missing link". *J Protozool* 39, 361–367.

42 Cavalier-Smith, T. (1993). The kingdom Protozoa and its 18 phyla. *Microbiol Rev* 57, 953–994.

43 Vivarès, C., Biderre, C., Duffieux, F., Peyretaillade, E., Peyret, P., Méténier, G., and Pagès, M. (1996). Chromosomal localization of five genes in *Encephalitozoon cuniculi* (Microsporidia). *J Eukaryot Microbiol* 43, 97S.

44 Flegel, T.W., and Pasharawipas, T. (1995). A proposal for typical eukaryotic meiosis in microsporidians. *Can J Microbiol* 41, 1–11.

45 Keeling, P.J., and Doolittle, W.F. (1996). Alpha-tubulin from early-diverging eukaryotic lineages and the evolution of the tubulin family. *Mol Biol Evol* 13, 318–326.

46 Edlind, T.D., Li, J., Visvesvara, G.S., Vodkin, M.H., McLaughlin, G.L., and Katiyar, S.K. (1996). Phylogenetic analysis of β-tubulin sequences from amitochondrial protozoa. *Mol Phylogenet Evol* 5, 359–367.

47 Germot, A., Philippe, H., and Le Guyader, H. (1997). Evidence for loss of mitochondria in microsporidia from a mitochondrial-type HSP70 in *Nosema locustae. Mol Biochem Parasitol* 87, 159–168.

48 Hirt, R.P., Healey, B., Vossbrinck, C.R., Canning, E.U., and Embley, T.M. (1997) *Curr Biol* 7, 995–998.

49 Kulda, J., and Nohynková, E. (1978). Flagellates of the human intestine and of intestines of other species. In J.P. Kreier (ed): *Parasitic Protozoa*, vol. II. New York: Academic Press, pp. 1–138.

50 Vickerman, K. (1990). Phylum Zoomastigna class Diplomonadida. In L. Margulis, J.O. Corliss, M. Melkonian, and D.J. Chapman (eds): *Handbook of Protoctista*. Boston: Jones and Bartlett Publishers, pp. 200–210.

51 Soltys, B.J., and Gupta, R.S. (1994). Presence and cellular distribution of a 60-kDa protein related to mitochondrial Hsp60 in *Giardia lamblia. J Protistol* 80, 580–588.

52 Brinkmann, H., and Martin, W. (1996). Higher plant chloroplast and cytosolic 3-phosphoglycerate kinases: A case of endosymbiotic gene replacement. *Plant Mol Biol* 30, 65–75.

53 Henze, K., Badr, A., Wettern, M., Cerff, R., and Martin, W. (1995). A nuclear gene of eubacterial origin in *Euglena gracilis* reflects cryptic endosymbioses during protist evolution. *Proc Natl Acad Sci USA* 92, 9122–9126.

54 Keeling, P.J., and Doolittle, W.F. (1997). Evidence that eukaryotic triosephosphate isomerase is of alpha-proteobacterial origin. *Proc Natl Acad Sci USA* 94, 1270–1275.

55 Roger, A.J., Smith, M.W., Doolittle, R.F., and Doolittle, W.F. (1996). Evidence for the Heterolobosea from phylogenetic analysis of genes encoding glyceraldehyde-3-phosphate dehydrogenase. *J Eukaryot Microbiol* 43, 475–485.

56 Gupta, R.S., and Golding, G.B. (1996) The origin of the eukaryotic cell. *Trends Biochem Sci* 21, 166–171.

57 Martin, W., and Schnarrenberger, C. (1997) The evolution of the Calvin cycle from prokaryotic to eukaryotic chromosomes: A case study of functional redundancy in ancient pathways through endosymbiosis. *Curr Genet* 32, 1–18.

58 Brugerolle, G., and Taylor, F.J.R. (1977). Taxonomy, cytology and evolution of the Mastigophora. In S.H. Hutner (ed): *Proceedings of the Fifth International Congress of Protozoology*. New York: Pace University, pp. 14–28.

59 Brugerolle, G., and Mignot, J.P. (1990). Phylum Zoomastigna class Retortamonadida. In L. Margulis, J.O. Corliss, M. Melkonian, and D.J. Chapman (eds): *Handbook of Protoctista*. Boston: Jones and Bartlett Publishers, pp. 259–265.

60 Raikov, I.B. (1995). Meiosis in protists: Recent advances and persisting problems. *Eur J Protistol* 31, 1–7.

61 DeLong, E.F. (1992). Archaea in coastal marine environments. *Proc Natl Acad Sci USA* 89, 5685–5689.