

Early branching eukaryotes?

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Recent phylogenetic analyses suggest that *Giardia*, *Trichomonas* and Microsporidia contain genes of mitochondrial origin and are thus unlikely to be primitively amitochondriate as previously thought. Furthermore, phylogenetic analyses of multiple data sets suggest that Microsporidia are related to Fungi rather than being deep branching as depicted in trees based upon SSUrRNA analyses. There is also room for doubt, on the basis of a lack of consistent support from analyses of other genes, whether *Giardia* or *Trichomonas* branch before other eukaryotes. So, at present, we cannot be sure which eukaryotes are descendants of the earliest-branching organisms in the eukaryote tree. Future resolution of the order of emergence of eukaryotes will depend upon a more critical phylogenetic analysis of new and existing data than hitherto. Hypotheses of branching order should preferably be based upon congruence between independent data sets, rather than on single gene trees.

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Introduction

Establishing a phylogeny for eukaryotes is central to our attempts to understand contemporary eukaryote diversity: for example, patterns of character change can be mapped over a phylogeny to fuel hypotheses of common ancestral states prior to lineage splitting. Hypotheses of character evolution can be made without recourse to phylogenetic analysis but phylogenies help to identify independent evolutionary events and to distinguish cause from effect in comparative analysis [1]. If, through phylogenetic analysis, we were able to identify descendants of the first eukaryotic branches, it might even be possible to infer something about the early stages of eukaryote evolution. As such, there has been considerable effort spent in trying to identify early branching eukaryotes and the purpose of this review is to discuss prevailing ideas pertaining to this topic.

The Archezoa hypothesis for early branching eukaryotes

In the pre-molecular era, the absence of functional mitochondria in protists such as *Giardia*, *Trichomonas* and Microsporidia was interpreted [2] as resulting from their early separation from other eukaryotes (i.e. prior to the mitochondrion symbiosis) which is thought to have occurred once [3]. The hypothesis that *Giardia*, *Trichomonas* and Microsporidia were relicts of a pre-mitochondrial phase of eukaryote evolution was formalised by calling them 'Archezoa' to denote a

Table 1

List of nuclear encoded genes found in *Giardia*, *Trichomonas* and Microsporidia that probably originated from the endosymbiont that gave rise to mitochondria.

	<i>Trichomonas vaginalis</i>	<i>Giardia lamblia</i>	Microsporidia
Hsp10	+ [9]	?	?
cpn 60	+ [8–10]	+ [17**]	?
mHsp70	+ [9,11]	?	+ [14**–16**]
Valyl-tRNA synthetase	+ [18**]	+ [18**]	?
Triose-phosphate isomerase	?	P [13]	?
Adenylate kinase	+ [12]	– [12]	?

+, mitochondrial homologue detected; –, no mitochondrial homologue detected; P, likely α -proteobacterial origin (i.e. consistent with a mitochondrial origin); ?, no published data. mHsp70, mitochondrial Hsp 70.

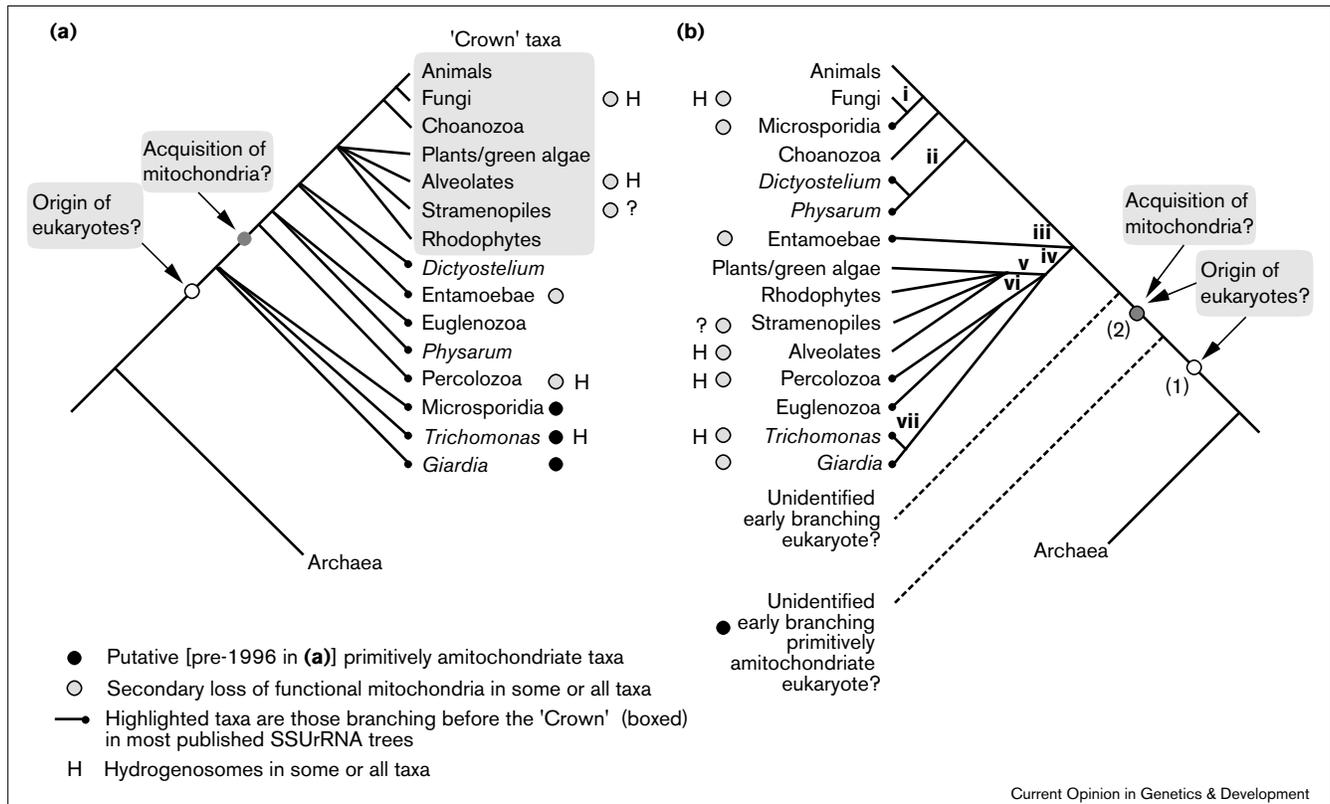
primitively amitochondriate condition [2,4*]. The Archezoa hypothesis was apparently supported for these taxa at least, when phylogenetic trees based upon small subunit ribosomal RNA [5] and translation elongation factors EF-1 α and EF-2 [6], showed *Giardia*, *Trichomonas* and Microsporidia branching before eukaryotes which contain mitochondria (see Figure 1a). The Archezoa hypothesis stimulated widespread interest in *Giardia*, *Trichomonas* and Microsporidia because their biology was expected to reveal stages in the acquisition of eukaryote cellular features; however, recent data now suggest that *Giardia*, Microsporidia and *Trichomonas* contain genes which are most likely of mitochondrial origin.

Mitochondrial genes in early branching eukaryotes

It is accepted that mitochondrial genes were transferred to the host nucleus during the evolution of the symbiosis because phylogenetic analysis of host nuclear genes betrays their mitochondrial origin (e.g. [7]). Genes for which phylogenetic analysis indicates (or is consistent with) a mitochondrial origin have now been reported in *Giardia*, *Trichomonas* and Microsporidia (Table 1). They include adenylate kinase, 10, 60 and 70 kDa heat shock proteins (Hsp10, cpn60, Hsp70), triose phosphate isomerase and valyl tRNA-synthetase [8–13,14**–18**].

Alternative explanations, other than one-time possession of mitochondria, have been proposed to account for the presence of these genes in *Giardia*, *Trichomonas* or Microsporidia [19*,20*] but these seem unnecessary to explain the strongly supported positions of cpn60s and/or Hsp70s within clades otherwise defined by mitochondrial homologues and

Figure 1



Alternative phylogenetic hypotheses for eukaryotes. **(a)** Relationships among eukaryotes as interpreted on a schematic SSUrDNA tree suggest the acquisition of mitochondria after the divergence of *Giardia*, *Trichomonas* and Microsporidia (all Archezoa *sensu* [2]) whose relative order of branching at the base of eukaryotes is uncertain. The placement of Archaea as the sister-group to eukaryotes is discussed in the text. **(b)** Composite phylogeny (i.e. no single gene tree supports all branches) depicting some alternative hypotheses for eukaryote relationships. The diagram was constructed by considering published trees from different protein datasets and some of these relationships are more speculative than others. All relationships should be viewed simply as more or less well-founded hypotheses which can be supported or refuted through more data and further analyses. The data considered to support particular hypotheses are: branch i, (see Table 2); ii, EF-1 α [50], actin [5] and β -tubulin (e.g. [35*]); iii–vii, actin and β -tubulin (e.g. [5,35*]) and valyl-tRNA synthetase [18*] (and see Table 2). Branch lengths are meaningless in both diagrams and polytomies indicate lack of resolution not support for explosive

radiation. Alternative hypotheses for the origin of the eukaryotic cell relative to the acquisition of mitochondria are indicated in (b): (1) The eukaryotic cell originated before the acquisition of the mitochondrial endosymbiont, thus there may be primitively amitochondriate eukaryotes remaining to be discovered. (2) The Hydrogen hypothesis [43**] posits that eukaryotes originated through symbiotic association between an Archaea (nominally the 'host') and the proteobacterium (the 'symbiont') which subsequently gave rise to mitochondria and hydrogenosomes. In this sense the pre-'mitochondrial' branches in 'host' trees will be Archaea. The documented distribution of hydrogenosomes among eukaryotes [51*] is shown on both trees, as are hypotheses of primitive or secondary absence of functional mitochondria. The possibility that Stramenopiles may lack functional mitochondria while retaining the organelle is discussed in [52]. Note that the perceived distribution of hydrogenosomes may well be conservative as anaerobic habitats are in general poorly described for the eukaryotes they contain or for the organelles that such eukaryotes may possess [38].

they are not founded upon phylogenetic analyses which support alternative origins for these or other genes (Table 1). We interpret the presence of genes in Archezoa which cluster with mitochondrial orthologues, as support for the hypothesis that *Giardia* and Microsporidia once had mitochondria, and for the hypothesis that *Trichomonas* has converted its mitochondria to hydrogenosomes [21,22].

Phylogenetic analyses of hydrogenosome-containing ciliates [23] and hydrogenosome-containing fungi [24] suggest that they are derived from aerobic ancestors with mitochondria. For ciliates, fungi and *Trichomonas*, there is also other data consistent with a mitochondrial origin for the hydrogenosome organelle (reviewed in [25]). The

hypothesis that a eukaryote may lose its mitochondria but still retain mitochondrial genes is now supported by recent phylogenetic analyses indicating a relationship between Microsporidia and Fungi (see below).

Alternative phylogenetic relationships for early branching eukaryotes

The finding of mitochondrial genes in *Giardia*, *Trichomonas* and Microsporidia does not preclude these taxa from branching early in eukaryote evolution but there are alternative, and in some cases now better supported, hypotheses of relationships for these taxa. The deep position of Microsporidia was first challenged by tubulin gene trees which depicted a relationship between Microsporidia and

Table 2

Alternative hypotheses of relationship for *Giardia*, *Trichomonas* and Microsporidia from two or more proteins.

	α -tubulin	β -tubulin	RPB1	EF-1 α	EF-2	mHsp70	cpn60	Valyl-tRNA synthetase	AK	GDH
Microsporidia + fungi	+ [27]	+ [26]	+ [29**]	-	-/+ [29**]	-/+ [14**, 15**]	?	?	?	?
<i>Giardia</i> + <i>Trichomonas</i>	-	+ [35*]	-	+ [6]	-/+ [29**]	?	+ [17**]	+ [18**]	-	?
<i>Giardia</i> +/- <i>Trichomonas</i> + plants	-	-	-	-	-	-	-	+ [18**]	+ [12]	+ [36]

+, support for relationship; -/+, support for relationship from some analyses; ?, data unavailable. -, no apparent support for this relationship. RPB1, largest subunit of RNA polymerase II; mHsp70, mitochondrial Hsp 70; AK, adenylate kinase; GDH, glutamate dehydrogenase.

Fungi [26,27]. This phylogenetic position for Microsporidia, surrounded by aerobic mitochondrial taxa, consistent with a mitochondria-loss hypothesis for Microsporidia discussed above. Judged against the apparently consistent story from SSUrRNA, EF-2 and EF-1 α , that Microsporidia branch deep, however, the tubulin trees were treated with scepticism. Methodological artefact caused by a high substitution rate for fungal tubulins [28] or lateral transfer of tubulin genes from Fungi to Microsporidia [19*] being proposed as explanations of the aberrant tubulin relationships.

Recent analyses of sequences from the largest subunit of RNA polymerase II (RPB1) also provide strong support for a relationship between Microsporidia and Fungi [29**] and analyses of mitochondrial Hsp70 support it weakly [14**, 15**]. Furthermore, re-analysis of the EF-1 α and EF-2 datasets show that they do not convincingly support the hypothesis that Microsporidia branch before other eukaryotes. When known sources of artefact affecting phylogenetic inference were accounted for, EF-2 was shown to support Microsporidia plus Fungi [29**]. The gene for EF-1 α in the microsporidian *Glugea plecoglossi* is potentially saturated [29**], making it unreliable for tree construction. It does, however, contain an insertion encoding 11 amino-acids which appears otherwise diagnostic for the EF-1 α genes of Fungi and Metazoa [30], which form a clade on the basis of phylogenetic analyses of different molecular datasets [5,30]. Thus, only SSUrRNA analyses appear to provide strong support for the deep divergence of Microsporidia [5] but even here there is some dissent; Kumar and Rzhetsky [31] concluded that the position of Microsporidia was difficult to resolve based on SSUrRNA. Furthermore, a recent analysis of large subunit rRNA, although not supporting a specific relationship with fungi, does not support an early divergence for the microsporidian *Encephalitozoon* [32*].

Are *Giardia* and *Trichomonas* really early branching?

The history of systematics suggests that it is unreasonable to expect any single gene (such as SSUrRNA) or another small sample of characters, to resolve all relationships equally and

some strongly supported patterns depicted in published gene trees may not reflect phylogeny [33,34]. It has been suggested already (e.g. [35*]) that the deep positions of *Giardia* and *Trichomonas* (and other protists) in SSUrRNA trees may represent examples of long branch attraction, whereby long branches in trees cluster together irrespective of phylogenetic relationships. Although we agree that this is plausible, the patterns in the data supporting this potential criticism of the SSUrRNA topology have not yet been identified analytically. Interestingly, neither RPB1 or reanalysis of EF-2 [29**] and EF-1 α [6,29**] provide compelling support for *Giardia* or *Trichomonas* diverging before other eukaryotes as is depicted in SSUrRNA trees. There are also alternative hypotheses of relationships for *Giardia* or *Trichomonas* (Figure 1b; Table 2) and for the protist lineages which branch before the 'crown' in SSUrRNA trees (Figure 1a). Some of these hypotheses are perhaps not as initially compelling as the signal from tubulin for Microsporidia plus Fungi but they cannot be dismissed on present analyses. For example, there is some support (Table 2, Figure 1b) for a sister group relationship between *Giardia* and *Trichomonas*. Much more speculatively — because limited data, uneven and limited taxon sampling, hinder tree comparison — there is a hint of a relationship to plants (Table 2) but not necessarily as a sister group.

A relationship between *Giardia* and *Trichomonas* would support, if the *Trichomonas* hydrogenosome is indeed a modified mitochondrion, the hypothesis of secondary loss of mitochondria by *Giardia*. A relationship to plants might explain, through hypothesised plastid loss in *Giardia*, the relationship between *Giardia* adenylate kinase and glutamate dehydrogenase and the same proteins located, respectively, in maize or *Chlorella* plastids [12,36] (but see also [37] for a discussion of when organelle localisation might not reflect symbiotic origins).

Where now in the search for the first branches in the eukaryote tree?

If we believe that an ancestral anaerobic or microaerophilic eukaryote gained mitochondria by engulfment of an

α -proteobacterium, this event need not necessarily exclude the descendants of other eukaryotes (which did not participate in this symbiosis) from persisting in anaerobic or microaerophilic habitats (branch [1] in Figure 1b). Anaerobic or microaerophilic habitats often support large communities of microbial eukaryotes but relatively few of these been studied in any detail [38]. As well as trying to isolate the eukaryotes in such samples, it might be of value to undertake a 'fishing expedition' for eukaryotic genes from anaerobic or microaerophilic samples, using the environmental gene library approach used to discover novel Archaea [39]. Which gene(s) might be most informative in such a fishing trip, which aims to discover early branching eukaryotes, is difficult to predict without doing the experiments but it would be sensible to use more than just SSUrRNA. Furthermore, even if *Giardia*, Microsporidia and *Trichomonas* are probably not primitively amitochondriate, it does not mean that all Archezoa which lack mitochondria once contained the organelle. For example, the phylogenetic positions of the amitochondriate oxymonads and retortomonads are still uncertain [40,41]. Lastly, we should not forget that the trees upon which existing phylogenetic hypotheses are based are poorly sampled and only a small number of anaerobic or aerobic protist lineages have been explored in any detail, or for more than a single gene; for example, Patterson recently listed his 'residua' — several hundred genera of unknown phylogenetic or ultrastructural affinity [42].

Interestingly, the 'hydrogen hypothesis' [43**] posits that eukaryotes originated through symbiotic association between an Archaea (nominally the 'host') and the proteobacterium (the 'symbiont') which subsequently gave rise to mitochondria and hydrogenosomes (branch [2] in Figure 1b). In this sense the pre-'mitochondrial' branches in 'host' trees will be Archaea. Interestingly, this prediction is consistent (ignoring contemporary phenotypes — see [43**]) with phylogenetic analyses of translation elongation factors [44–46] which suggest that some Archaea may be more closely related to eukaryotes than others.

Was there a 'big bang' in eukaryote evolution?

Most of the preceding discussion assumes that phylogenetic analyses will ultimately permit us to identify the early branches of the eukaryote tree, given more critical attention to methods and more data. It has been suggested recently, however, that the major eukaryote groups might have diversified so quickly that resolution of their order of emergence, early or late, may be either difficult or impossible. This hypothesis of a rapid cladogenesis or explosive radiation giving rise to most or all major groups of eukaryotes has been called the 'big bang' hypothesis [35*].

At present, this interesting idea has not been formulated sufficiently precisely for us to see what data actually supports it. For example, if it is based upon rates of lineage splitting as revealed by rigorously evaluated branching diagrams for different genes, then this data has not been

published. If it depends on the rapid emergence of the morphological or cellular features which are used to define taxonomic groups, then the characters and taxa supporting the hypothesis have not been described. And if the hypothesis uses both types of data, it has not been demonstrated that each can be analysed separately to provide independent support for the hypothesis. Without this information it is impossible to see what relationships or character distributions might critically test the big bang hypothesis. If, for example, tubulin trees (or Figure 1b) were to subsequently prove accurate for some of the relationships they display, would these relationships and character distributions either support or refute the big bang hypothesis?

Our suggestion that the big bang hypothesis needs to be stated unambiguously is motivated by its potential implications for our ability to resolve branching order, but also by a desire to avoid the confusion which has surrounded other explosion hypotheses including the most famous, the 'Cambrian explosion'. Here, the fossil data have been interpreted to support the hypothesis that the major metazoan phyla appeared during less than 20 million years in the early Cambrian [35*]. In contrast, Gould has written that "The Cambrian explosion embodies a claim for a rapid spurt of anatomical innovation within the animal kingdom, not a statement about times of genealogical divergence" [47]. Interestingly, a cladistic analysis of morphology [48], new fossils [47], and, most recently, new methods for analysing molecular data [49], are more consistent with a gradual pre-Cambrian diversification of metazoan phyla rather than an explosive appearance of new clades.

Conclusions

The results of phylogenetic analyses to date, have demonstrated that organelle absence, perceived cytological simplicity, or particular phenotypes are uncertain guides for identifying early branching eukaryotes prior to phylogenetic analysis. Thus *Giardia* and *Trichomonas* are probably not primitively amitochondriate and the strong support for them being deep-branching appears to come mainly from the SSUrRNA dataset. Microsporidia are related to Fungi on the basis of several datasets. Indeed, if early branching eukaryotes can be identified through new data, or more rigorous analysis can resolve the order of emergence supported by multiple data sets, their features may yet surprise us.

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Three papers which demonstrate through phylogenetic analysis the occurrence of mitochondrial Hsp70s in three different Microsporidia. In two [14**,15**] there is also evidence from some analyses for a relationship between Microsporidia and Fungi. Microsporidia are reported to lack mitochondria and none of the Hsp70s carry a convincing mitochondrial targeting signal; however, the Hsp70s from *Vairimorpha* and *Nosema* carry versions of a consensus PTS1 signal (peroxisomal type 1) which is absent from the third Hsp70 from *Encephalitozoon*. Microsporidia are reported to lack peroxisomes, therefore the functions of the proteins are cryptic at present.

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The valyl-tRNA synthetases presented in this paper are the latest examples of examples of most-likely genes occurring in *Giardia* mitochondrial genes most likely to occur in *Giardia* and *Trichomonas*. As well as indicating a mitochondrial origin, phylogenetic analysis indicates that *Giardia* and *Trichomonas* may be related to each other and, more surprisingly, that both may be related to plants. More data and analyses are needed to investigate further these potentially very exciting relationships, which are currently based upon very few sequences and limited taxa sampling.

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