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Multiple secondary origins of the anaerobic lifestyle in eukaryotes

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Classical ideas for early eukaryotic evolution often posited a period of anaerobic evolution producing a nucleated phagocytic cell to engulf the mitochondrial endosymbiont, whose presence allowed the host to colonize emerging aerobic environments. This idea was given credence by the existence of contemporary anaerobic eukaryotes that were thought to primitively lack mitochondria, thus providing examples of the type of host cell needed. However, the groups key to this hypothesis have now been shown to contain previously overlooked mitochondrial homologues called hydrogenosomes or mitosomes; organelles that share common ancestry with mitochondria but which do not carry out aerobic respiration. Mapping these data on the unfolding eukaryotic tree reveals that secondary adaptation to anaerobic habitats is a reoccurring theme among eukaryotes. The apparent ubiquity of mitochondrial homologues bears testament to the importance of the mitochondrial endosymbiosis, perhaps as a founding event, in eukaryotic evolution. Comparative study of different mitochondrial homologues is needed to determine their fundamental importance for contemporary eukaryotic cells.

Keywords: mitochondria; hydrogenosomes; mitosomes; anaerobic eukaryote evolution

1. INTRODUCTION

Many classical theories for eukaryotic origins suggested that eukaryotes originated under anaerobic conditions, and were primitively without mitochondria (e.g. Goksoyr 1967; Cavalier-Smith 1983*b*). A member of this anaerobic eukaryotic community is posited to have subsequently phagocytosed a eubacterium that could carry out aerobic respiration and which subsequently became an endosymbiont (John & Whatley 1975). The presence of this eubacterial endosymbiont allowed the host to colonize aerobic environments. A variety of mechanisms have been suggested as to why this should be so; the most popular, and one that features in a variety of different formulations for mitochondrial origins (Woese 1977; Martin & Müller 1998; Vellai *et al.* 1998; Kurland & Andersson 2000), being the removal of cytosolic oxygen by the endosymbionts' respiratory activity. The feasibility of this idea, at least as a mechanism, is supported by a modern analogue among contemporary ciliate protozoa. *Strombidium purpureum* lives in anaerobic marine sands and contains facultatively aerobic photosynthetic endosymbiotic bacteria. In the light, *Strombidium* avoids even traces of oxygen (less than 1% atmospheric saturation) and the endosymbionts probably use host substrates for anoxygenic photosynthesis. In the dark, the ciliates, protected against O_2-toxicity by the endosymbionts' aerobic respiration, accumulate in water with a partial pressure of O_2 of 1–4% atmospheric saturation (Fenchel & Bernard 1993*a*,*b*; Bernard & Fenchel 1994).

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One contribution of 14 to a Discussion Meeting Issue 'Major steps in cell evolution'.

Despite the plausibility of the above scenario we will probably never know why two disparate ancient cells came, and then stayed, together. Consequently, opinions are diverse concerning the ecological context and selective advantages for host and endosymbiont (e.g. John & Whatley 1975; Martin & Müller 1998; Cavalier-Smith 2002; Embley & Martin 2006). What does seem well founded is that the resulting host–endosymbiont consortium forged the common ancestor of all of the mitochondrion-containing eukaryotes that are seen today. Thus, a single common origin for all mitochondria is supported by phylogenetic analyses of the genes encoded by mitochondrial genomes (Yang *et al.* 1985; Gray *et al.* 2004). The anaerobic eukaryotes that did not participate in the mitochondrial endosymbiosis, have been posited either to have gone extinct, due to an inability to deal with rising oxygen levels or some other catastrophe (Philippe & Adoutte 1998; Vellai *et al.* 1998), or to have persisted in anaerobic habitats to the present day (Cavalier-Smith 1983*b*; Margulis *et al.* 2005).

The persistence hypothesis is plausible for several reasons. Anaerobic habitats have existed throughout Earth's history and they harbour anaerobic bacteria, providing food for phagotrophic eukaryotes. Extinction in the microbial world may also be rare because of large population sizes, ease of dispersal and the occurrence of resistant stages (Fenchel 1993). Even transient anaerobic habitats generally possess protist inhabitants. As ancient oxygen levels rose, the occupants of anaerobic habitats on the margins of the aerobic world would actually benefit from the reduced carbon derived from oxygenic photosynthesis. In modern situations, the activities of oxygenic phototrophs often provide the reducing power, in the form of organic material, to maintain anoxic environments;

contemporary anaerobic life is most rich in the vicinity of aerobic life (Fenchel & Finlay 1995). In conclusion, there are no compelling reasons drawn from contemporary ecosystems, to suppose that primitively amitochondriate eukaryotes, if they ever existed, would have gone extinct simply because they did not participate in the mitochondrial endosymbiosis.

The persistence hypothesis was supported by the discovery of contemporary eukaryotes that apparently lacked mitochondria. These eukaryotes, called Archezoa to indicate that the absence of mitochondria was a primitive state, not a derived state resulting from secondary loss, included *Entamoeba*, *Giardia*, *Trichomonas* and Microsporidia, and their various close relatives (Cavalier-Smith 1983*a*,*b*). As well as not having mitochondria, these species were thought to lack other features common to most eukaryotes, including Golgi dictyosomes and peroxisomes, supporting their early emerging status (Cavalier-Smith 1987*a*; Patterson & Sogin 1992). Archezoa or Hypochondria as they were also known (Patterson & Sogin 1992), thus became models for studying early eukaryotic evolution and the features of the first eukaryotic cells. Some suggested that the peculiar features of Archezoa could also be a derived state (Wolters 1991; Siddall *et al.* 1992), caused, or perhaps facilitated, by the adaptation of Archezoa to their parasitic or anaerobic lifestyles. But that debate was unresolved because there was no clear comparative evidence that convincingly linked the Archezoa to eukaryotic groups that possessed mitochondria or the other debated features.

The Archezoa hypothesis was founded in comparative cytology as a taxonomic hypothesis designed to draw attention to a group of eukaryotes which might provide a glimpse into a pre-mitochondrial phase of eukaryotic evolution (Cavalier-Smith 1983*a*,*b*). It was thus enormously useful as a guide to study. It gained considerable support when the first phylogenetic trees based upon molecular sequence data (Vossbrinck *et al.* 1987; Sogin *et al.* 1989; Leipe *et al.* 1993; Hashimoto *et al.* 1997) did indeed place key archezoans before other eukaryotes with mitochondria. Doubts were expressed about the veracity of these trees (e.g. Wolters 1991; Siddall *et al.* 1992), but the apparent agreement between molecules and morphology in establishing the timing of the mitochondrial endosymbiosis, one of the pivotal events in eukaryotic evolution, was compelling and generally seen as a major breakthrough.

2. THE DEMISE OF ARCHEZOA

In an area of research where speculation and conjecture abound, the Archezoa hypothesis for early eukaryotic evolution stood out as testable in obvious ways. It could be rejected for individual archezoans if they were shown to contain genes of mitochondrial ancestry, to branch among eukaryotes with mitochondria, i.e. the trees placing them deep were wrong, or to contain previously overlooked mitochondria. Using these criteria the Archezoa hypothesis has now been rejected for all of the best-studied archezoans. The data and arguments underpinning the conclusion that the Archezoa hypothesis can now be rejected, for the key species for which it was formulated, have been extensively reviewed (Embley *et al.* 2003*b*; van der Giezen *et al.* 2005; Embley & Martin 2006), so only a few points will be made here in relation to each type of evidence and to controversies, where they exist, surrounding the interpretation of these data.

All of the best-studied archezoans have now been shown to contain host–nuclear-encoded genes of putative mitochondrial ancestry (table 1). That is, they contain genes that encode proteins that in aerobes typically function in mitochondria and, when the proteins are subjected to phylogenetic analysis, they form a monophyletic group with the *alpha*-proteobacteria. This is the group of bacteria from which the mitochondrial endosymbiont is held to have originated based upon analyses of genes that are encoded by mitochondrial genomes (Yang *et al.* 1985; Gray *et al.* 2001). One criticism of using the genes in table 1 to reject the Archezoa hypothesis is that none of them have ever been found on a mitochondrial genome (Gray *et al.* 2004; Gray 2005). Such a location, which is found for some of the proteins involved in oxidative phosphorylation, the canonical function of aerobic mitochondria, would make a direct link between gene and organelle. To explain the localization of genes of mitochondrial ancestry on host nuclear genomes, it is necessary to posit that they were transferred from the endosymbiont genome to the host nuclear genome during the process of transforming endosymbiont into an organelle (John & Whatley 1975; Viale & Arakaki 1994). This process has been called endosymbiotic gene transfer and it is still ongoing (Timmis *et al.* 2004). For example, among plants there are examples where particular genes appear on the mitochondrial genome in some lineages, but in other lineages they are encoded by the host nuclear genome (Adams & Palmer 2003). Despite the need to invoke such gene transfer, the hypothesis that the genes in table 1 came from the mitochondrial endosymbiont is probably now the standard interpretation of these data (Boorstein *et al.* 1994; Viale & Arakaki 1994; Clark & Roger 1995).

It has also been suggested that archezoans could have acquired the genes in table 1 from a source other than the mitochondrial endosymbiont. Alternative sources that have been posited include *alpha*-proteobacterial food bacteria (Doolittle 1998) and bacteria related to, but distinct from, the mitochondrial endosymbiont (Sogin 1997). Although these ideas have been presented as credible alternatives to the standard interpretation discussed above (Dyall & Johnson 2000; Kurland & Andersson 2000; Knight 2004; Dyall *et al.* 2004*b*; Margulis *et al.* 2005), there is no unambiguous support from phylogenetic analyses for the separate origin of these Archezoan genes (Horner & Embley 2001; Williams *et al.* 2002; Hrdy *et al.* 2004).

The strongest evidence that archezoans are not primitively without mitochondria has come from discoveries that some of the bacterial proteins in table 1 are housed in double-membraned organelles. These organelles are called hydrogenosomes (Müller 1993; Bui *et al.* 1996), remnant mitochondria (Williams *et al.* 2002) or mitosomes (Tovar *et al.* 1999, 2003), with the latter two terms sometimes used

Table 1. Proteins of mitochondrial ancestry in former Archezoa.

species	protein	references for tree	location in cell	reference for location
Entamoeba histolytica	Mitochondrial 60-kDa chaperonin (Cpn60)	Clark & Roger 1995	Mitosome	Tovar *et al.* 1999
	Mitochondrial 70-kDa heat shock protein (Hsp70)	Bakatselou *et al.* 2000	Not determined (ND)	
Giardia intestinalis	Cpn60	Roger *et al.* 1998 Horner & Embley 2001	Mitosome	Regoes *et al.* 2005
	Hsp70	Morrison *et al.* 2001	Mitosome	Regoes *et al.* 2005
	Cysteine desulphurase (IscS)	Tachezy *et al.* 2001 Emelyanov 2003	Mitosome	Tovar *et al.* 2003
Trichomonas vaginalis	Cpn60	Horner *et al.* 1996 Bui *et al.* 1996 Roger *et al.* 1996	Hydrogenosome	Bui *et al.* 1996 Bozner 1997
	Hsp70	Bui *et al.* 1996 Germot *et al.* 1996	Hydrogenosome	Bui *et al.* 1996 Bozner 1997
	IscS	Tachezy *et al.* 2001 Emelyanov 2003	Hydrogenosome	Sutak *et al.* 2004
	Mitochondrial NADH-dehydrogenase	Hrdy *et al.* 2004 Dyall *et al.* 2004*a*,*b*	Hydrogenosome	Hrdy *et al.* 2004 Dyall *et al.* 2004*a*,*b*
Microsporidia:				
Nosema locustae	Hsp70	Germot *et al.* 1997	ND	
	Pyruvate dehydrogenase	Fast & Keeling 2001	ND	
Vairimorpha necatrix	Hsp70	Hirt *et al.* 1997	ND	
Encephatitozoon cuniculi	Hsp70	Peyretaillade *et al.* 1998	ND	
	IscS	Emelyanov 2003	ND	
Encephalitozoon hellem	Hsp 70	Peyretaillade *et al.* 1998	ND	
Trachipleistophora hominis	Hsp70	Williams *et al.* 2002	Mitosome	Williams *et al.* 2002

interchangeably for the same organelle. There are only two double-membraned organelles, mitochondria and primary plastids, of undisputed bacterial origin in eukaryotes. Thus the simplest, and hence preferable, hypothesis to explain the observations that proteins typically found in mitochondria are present in double-membrane-bounded Archezoan organelles is that all such organelles share common ancestry with mitochondria (Bui *et al.* 1996; Tovar *et al.* 1999, 2003; Williams *et al.* 2002). Mitochondria, mitosomes and hydrogenosomes are evolutionary homologues *sensu* Owen and Darwin (Owen 1843; Darwin 1859), and the differences between them are the products of descent with modification from a common ancestral organelle. The hypothesis that mitochondria, mitosomes and hydrogenosomes are homologues, predicts that, as the organelles are studied more deeply, additional similarities between them will emerge to corroborate the hypothesis. So far this is what has happened.

With one notable exception (Boxma *et al.* 2005), hydrogenosomes and mitosomes lack a genome, presumably because they no longer require the proteins it encodes for oxidative phosphorylation. Any proteins functioning within hydrogenosomes and mitosomes must therefore be synthesized in the cytosol, and then correctly targeted and imported into the organelle. Although the mitochondrion contains a genome, most of its proteins are encoded by the host nuclear genome, so there is a similar requirement for a protein import machinery. Mitochondria have evolved two main protein import pathways (Pfanner & Geissler 2001). Proteins that are destined for the inner mitochondrial membrane are guided to their destination by internal targeting signals. Others, destined for the mitochondrial matrix, are synthesized as pre-proteins carrying a targeting sequence at their amino-terminus that is cleaved during import into the organelle. The development of a system for protein import must have been an early and critical step in the evolution of the mitochondrial organelle (Cavalier-Smith 1987*b*). The resulting mitochondrial protein import machinery is sufficiently complicated, and fidelity of import sufficiently important, that the same pathways are very unlikely to have evolved in different organelles.

The hydrogenosomes of *Trichomonas vaginalis* import proteins using both mitochondrial pathways. Hydrogenosomal ferredoxin carries an N-terminal targeting sequence that guides it into isolated *Trichomonas* hydrogenosomes, and the same targeting sequence can also sort a marker protein into yeast mitochondria (Plumper *et al.* 1998). *Trichomonas* hydrogenosomes also use a member of the mitochondrial carrier family (MCF) to transport ADP and ATP (Tjaden *et al.* 2004). Members of the MCF are eukaryotic proteins that are inserted into the inner mitochondrial membrane by internal targeting signals, where they mediate the import and export of substrates, including ADP and ATP, required or produced by the mitochondrion (Kunji 2004). The insertion of an ADP/ATP carrier into the protomitochondrion membrane was a key step in its transition into an organelle, because it made symbiont-generated ATP available to the host cell (John & Whatley 1975).

The *Trichomonas* ADP and ATP carrier is correctly targeted to yeast mitochondria in heterologous transfection experiments, suggesting that targeting signals for import have been conserved between the *Trichomonas* hydrogenosome and yeast mitochondria (Dyall *et al.* 2000).

Trichomonas hydrogenosomes have other features in common with mitochondria. They catalyse the enzymatic assembly and insertion of Fe–S centres into apoproteins, using the same enzymes, inherited from the mitochondrial endosymbiont, as do yeast mitochondria (Sutak *et al.* 2004). *Trichomonas* hydrogenosomes also contain the NADH dehydrogenase module of complex I of the mitochondrial respiratory chain (Hrdy *et al.* 2004), although the reader should be aware that this last conclusion is controversial (see Gray 2005), because an alternative origin from another bacterium had previously been suggested for this module (Dyall *et al.* 2004*b*). While these data are clearly difficult to analyse using conventional phylogenetic methods, it is important to appreciate that they cannot reject a common origin of *Trichomonas* and mitochondrial proteins using standard tests (Hrdy *et al.* 2004). Moreover, when corrections are made to mitigate obvious problems with the data, such as amino acid differences between sequences, the trees do support a mitochondrial origin (Hrdy *et al.* 2004). The discovery of the NADH dehydrogenase activity solves the long-standing puzzle of how the *Trichomonas* hydrogenosome regenerates NAD^+ after malate oxidation (Müller 2003).

Entamoeba mitosomes (Tovar *et al.* 1999) also contain a mitochondrial carrier protein that transports ADP and ATP, and in heterologous transfection experiments it too is translocated to the inner mitochondrial membrane of yeast (Chan *et al.* 2005). Unlike typical mitochondrial ATP/ADP carriers (Klingenberg 1985), the *Entamoeba* carrier does not require a positive-outside membrane potential, to transport ADP and ATP (Chan *et al.* 2005). This can be rationalized by reference to biochemical data (Reeves 1984) and to the recently published *Entamoeba* genome (Loftus *et al.* 2005), which show that *Entamoeba* lacks the electron transport chain necessary to make this gradient.

Import pathways are also conserved between *Giardia* mitosomes and mitochondria (Dolezal *et al.* 2005; Regoes *et al.* 2005). For example, the N-terminal presequence from the *Giardia* mitosomal ferredoxin is both necessary and sufficient to guide green fluorescent protein into mammalian mitochondria (Regoes *et al.* 2005). Study of the microsporidian mitosome has lagged behind study of other mitochondrial homologues, because Microsporidia are obligate intracellular parasites, and there are no homologous transfection methods like those established for *Entamoeba*, *Giardia* and *Trichomonas*. Nevertheless the published genome of the microsporidian *Encephalitozoon cuniculi* (Katinka *et al.* 2001) already provides clues to putative shared functions, e.g. homologous mechanisms of Fe–S cluster assembly, between its mitosome and mitochondria (Vivares *et al.* 2002).

Phylogenetic analyses previously taken to support the deep branching positions of archezoans have also come under increasing scrutiny with the result that they are no longer viewed as reliable (Stiller & Hall 1999; Hirt *et al.* 1999; Philippe *et al.* 2000; Inagaki *et al.* 2004; Thomarat *et al.* 2004; Roger & Hug 2006). The molecular sequences of the former Archezoa often evolve differently to those of other eukaryotes to which they are being compared. However, most methods of phylogenetic analysis assume a homogeneous process, i.e. that all sequences evolve in the same way, so the aberrant behaviour of Archezoan sequences can make their phylogenetic position difficult to infer reliably (see Roger & Hug 2006). The difficulty experienced when trying to resolve the origins of the *Trichomonas* hydrogenosomal NADH dehydrogenase provides just one example of the kind of problems that can be encountered (Dyall *et al.* 2004*b*; Hrdy *et al.* 2004; Gray 2005). The case for a rethink is clear-cut for Microsporidia because most data and analyses now agree in placing them with fungi rather than as deep branching eukaryotes (Hirt *et al.* 1999; Keeling *et al.* 2000; Inagaki *et al.* 2004; Thomarat *et al.* 2004). The hypothesis that *Entamoeba* is related to *Dictyostelium*, an aerobic amoeba that contains mitochondria, is also now well supported by a number of proteins (Horner & Embley 2001; Bapteste *et al.* 2002). The position of *Giardia* and *Trichomonas* is uncertain, but there are sufficient rooted trees that argue otherwise, to be wary of making strong claims that they branch before other eukaryotes (Horner & Embley 2001; Emelyanov 2003; Arisue *et al.* 2005).

Freed from the prejudice that Archezoa must be 'early branching' or in some sense 'primitive', their mitochondrial homologues fit perfectly comfortably within the spectrum of 'non-Archezoan' eukaryote biology.

3. REMNANT MITOCHONDRIA IN CRYPTOSPORIDIUM

Cryptosporidium parvum is an apicomplexan parasite related to the malaria parasite *Plasmodium*, and together they are part of a broader predominantly aerobic group comprising apicomplexans, ciliates and dinoflagellates, called the alveolates (Cavalier-Smith 2002; Adl *et al.* 2005). *Cryptosporidium parvum* contains a double-membraned organelle that has been called a relict (Riordan *et al.* 2003) or degenerate mitochondrion (Abrahamsen *et al.* 2004), but from its known features it could just as easily be called a mitosome. The *Cryptosporidium* mitochondrion has lost its genome and the capacity for oxidative phosphorylation (Abrahamsen *et al.* 2004), but it still imports mitochondrial heat shock proteins (Riordan *et al.* 2003; Slapeta & Keithly 2004). There is also circumstantial evidence that it makes Fe–S clusters, but *in situ* localization of the enzymes has not been done (LaGier *et al.* 2003).

4. HYDROGENOSOMES: MITOCHONDRIA THAT MAKE HYDROGEN

Hydrogenosomes were originally discovered (Lindmark & Müller 1973) in the cattle parasite *Tritrichomonas foetus*, a close relative of *T. vaginalis*, but they are also found in anaerobic chytrid fungi and diverse lineages

of ciliate protozoa (Müller 1993). The evidence for common ancestry of chytrid and ciliate hydrogenosomes with mitochondria is now very strong (van der Giezen *et al.* 2002, 2003; Voncken *et al.* 2002*a*; Boxma *et al.* 2005). Among ciliates, mitochondria- and hydrogenosome-containing groups freely intermingle; there are at least four and probably more ciliate lineages that possess hydrogenosomes within this predominantly aerobic group (Fenchel & Finlay 1995; Embley *et al.* 1995). The transformation of mitochondrion to hydrogenosome can occur over relatively short genetic distances as illustrated by the genus *Cyclidium*, which contains *Cyclidium glaucoma* with mitochondria and *Cyclidium porcatum* with hydrogenosomes (Esteban *et al.* 1993; Embley *et al.* 1995). Given the ease by which ciliates have evolved hydrogenosomes it was perhaps inevitable (Embley *et al.* 1997) that the strongest evidence for the common ancestry of hydrogenosomes and mitochondria would eventually come from this group. The hydrogenosomes of the anaerobic ciliate *Nyctotherus ovalis* uniquely retain a mitochondrial genome (Boxma *et al.* 2005), providing the long-sought direct link between the two organelles (Martin 2005).

Like those of *Trichomonas* (Clemens & Johnson 2000), the hydrogenosomes of the anaerobic fungus *Neocallimastix* apparently lack a genome (van der Giezen *et al.* 1997). *Neocallimastix* also contains a member of the MCF on its genome (van der Giezen *et al.* 2002; Voncken *et al.* 2002*a*). This protein has been shown to import and export ADP and ATP *in vitro* and, crucially, it can restore the function of mutant yeast mitochondria that lack their own ADP/ATP carrier (van der Giezen *et al.* 2002). Thus, fungal hydrogenosomes and yeast mitochondria use the same pathway for ADP/ATP exchange and they import the relevant protein in the same way. *Neocallimastix* hydrogenosomes also contains mitochondrial Cpn60 and Hsp70 and these proteins contain targeting signals that are capable of sorting them, or a green fluorescent reporter protein, into mammalian mitochondria (van der Giezen *et al.* 2003).

Anaerobic eukaryotic diversity is poorly sampled for organelle function so it is unlikely that these groups will be the final additions to the list of eukaryotes shown to possess hydrogenosomes (Fenchel & Finlay 1995). Transforming mitochondria into hydrogenosomes appears to be something that diverse eukaryotes have accomplished, raising the question of how it has been done.

5. EVOLUTIONARY ORIGINS OF THE ENZYMES USED TO MAKE HYDROGEN

Hydrogenosomes are defined by their ability to make hydrogen so it is the source of the biochemistry to do this, i.e. key to the transformation of mitochondria into hydrogenosomes (Embley *et al.* 1997). In *Trichomonas* hydrogenosomes, which are the best studied for their biochemistry, pyruvate:ferredoxin oxidoreductase (PFO) carries out the metabolism of pyruvate, and the electrons generated are transferred via ferredoxin to [Fe]-hydrogenase, producing hydrogen as the reduced end product (Müller 1993; Hrdy & Müller 1995*a*,*b*). There are biochemical data consistent with the presence of both enzymes in the hydrogenosomes of anaerobic ciliates and fungi (Yarlett *et al.* 1981, 1984, 1986). By contrast, aerobic eukaryotes use the non-homologous pyruvate dehydrogenase, located in the mitochondrion, to decarboxylate pyruvate and the electrons flow via the electron transport chain to oxygen.

The presence in hydrogenosomes of PFO and [Fe]-hydrogenase, enzymes typical of anaerobic bacteria, prompted the early suggestion, recently restated in modified form, that *Trichomonas* hydrogenosomes were descended from an endosymbiotic anaerobic bacterium (Whatley *et al.* 1979; Dyall *et al.* 2004*a*). However, neither PFO nor [Fe]-hydrogenase is uniquely associated with hydrogenosomes, and thus they are not reliable indicators of a separate organelle origin. The mitochondria of *Euglena* contain pyruvate:NADP oxidoreductase, a fusion protein containing PFO domains linked to a C-terminal NADPH-cytochrome P450 reductase domain. The same fusion is found in *C. parvum*, but its localization is unknown (Rotte *et al.* 2001). Large fragments of PFO have also been found in *Saccharomyces cerevisiae* where they combine with other redox proteins to participate in methionine biosynthesis (Horner *et al.* 1999). PFO also occurs in *Entamoeba*, *Giardia* and *Spironucleus* (a close relative of *Giardia*), but the location of the protein in these species has not been unambiguously demonstrated (Reeves *et al.* 1977; Townson *et al.* 1996; Brown *et al.* 1998; Rodriguez *et al.* 1998).

Hydrogenase genes have now been cloned from *T. vaginalis* (Bui & Johnson 1996; Horner *et al.* 2000), the ciliates *N. ovalis* (Akhmanova *et al.* 1998) and *Trimyema* sp. (Embley *et al.* 2003*a*), and the chytrid fungi *Neocallimastix frontalis* L2 and *Piromyces* sp. E2 (Davidson *et al.* 2002; Voncken *et al.* 2002*b*). All of them encode iron-only [Fe]-hydrogenases of a type that is found in eubacteria, but not in archaebacteria. Green algae, such as *Chlorella fusca*, *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* also produce hydrogen, using [Fe]-hydrogenases located in their chloroplasts (Horner *et al.* 2002). The presence of a [Fe]-hydrogenase in green algal plastids is surprising, since cyanobacteria, the endosymbiotic progenitor of plastids, use the non-homologous [NiFe]-hydrogenase to make hydrogen. The original [NiFe]-hydrogenase brought by the endosymbiont appears to have been replaced by a host-nuclear encoded [Fe]-hydrogenase of non-cyanobacterial origin.

Genes encoding [Fe]-hydrogenases similar in structure to *Trichomonas* enzymes, were also recently discovered in *Giardia*, in its relative *Spironucleus*, and in *Entamoeba histolytica* (Horner *et al.* 2000; Nixon *et al.* 2003). The intracellular location of these enzymes is unknown at present. *Giardia* can actually make small amounts of hydrogen, the elimination of which may help to maintain redox balance in this species (Lloyd *et al.* 2002). Given that the structures of the *Entamoeba* and *Spironucleus* genes closely resemble the one in *Giardia*, it seems likely that these species can also make hydrogen.

Most surprisingly, genes encoding short proteins related to [Fe]-hydrogenases also occur in the human

genome (Barton & Worman 1999; Horner *et al.* 2002). These genes have been called NARF-like, after human nuclear prelamin A recognition factor (Barton & Worman 1999). In humans, the Narf protein is thought to be involved in the processing of lamin, a protein required for maintaining the structural integrity of the nucleus (Barton & Worman 1999). However, detailed studies on the yeast homologue Nar1p, suggest a rather different role for this protein. In yeast, which lacks lamin, Nar1p appears to be an essential protein located in the cytosol where it functions in the synthesis of extramitochondrial Fe–S proteins (Balk *et al.* 2004, 2005). Database searches have revealed that NARF-like genes are present in all of the available eukaryotic genomes (Horner *et al.* 2002; Balk *et al.* 2004), including the smallest eukaryote genome yet sequenced, from the intracellular microsporidian parasite *E. cuniculi* (Katinka *et al.* 2001). Once thought to be exotic exceptions, it seems entirely possible that [Fe]-hydrogenases, in the form of Narf-like proteins, have an important role to play in all eukaryotes.

It has been suggested that the genes for PFO and [Fe]-hydrogenase could have originated from the mitochondrial endosymbiont, as both genes are common among proteobacteria (Embley *et al.* 1997; Martin & Müller 1998). Phylogenetic analyses have not provided any support for this hypothesis. Like many eukaryotic metabolic enzymes (Ribeiro & Golding 1998; Rivera *et al.* 1998; Esser *et al.* 2004), eukaryotic PFOs are more similar in structure to eubacterial enzymes than to those from archaebacteria. Moreover, published analyses of PFO (Horner *et al.* 1999; Rotte *et al.* 2001; Embley *et al.* 2003*a*), recover eukaryotic sequences as a single cluster consistent with an ancient common origin for eukaryotic enzymes (figure 1). However, the eukaryotic PFO sequences are not the nearest neighbours of sequences from *alpha*-proteobacteria. Interestingly, the two *alpha*-proteobacterial sequences do not cluster together in the tree, suggesting that the evolution of eubacterial PFO genes is more complex than can be explained by vertical inheritance of a single copy gene (Horner *et al.* 1999).

The relationships between eukaryotic [Fe]-hydrogenase sequences are not well resolved, to which saturation of variable sites is probably a contributing factor (Horner *et al.* 2000). Thus, although they do not cluster together in the tree (figure 2), the data cannot decisively reject the hypothesis of a common and early origin for eukaryotic sequences using standard tests (Horner *et al.* 2000). There is strong support for the coherence of individual clusters of eukaryotic sequences, consistent with the hypothesis that the respective ancestors of the sampled green algae and chytrid fungi already contained a gene for [Fe]-hydrogenase. The observation (Embley *et al.* 2003*a*) that the enzymes from the two ciliates *Nyctotherus* and *Trimyema* cluster together is particularly interesting, since these two morphologically distinct species are separated by aerobic mitochondria-containing lineages in the ciliate tree (Embley *et al.* 1995; Akhmanova *et al.* 1998). This is consistent with the hypothesis that the common ancestor they shared with aerobic ciliates, already contained a [Fe]-hydrogenase (Embley *et al.* 2003*a*), thus helping to explain the apparent ease by which diverse ciliates have made hydrogenosomes (Embley *et al.* 1995). The tree provides no support for a close relationship between eukaryotic [Fe]-hydrogenases and the only sequence from an *alpha*-proteobacterium, that of *Rhodopseudomonas palustris* (Davidson *et al.* 2002). The *Rhodopseudomonas* sequence clusters strongly with one of two different [Fe]-hydrogenases from *Desulfovibrio vulgaris* (strain Hildenborough), a member of the *delta*-proteobacteria. As for the PFO tree, there is evidence that gene duplications and/or potential horizontal gene transfers have influenced the tree topology for prokaryotic sequences. For example, the [Fe]-hydrogenases from *Desulfovibrio* species do not cluster together (figure 2), as their common classification suggests they should.

6. CONCLUSIONS, SPECULATIONS AND OUTLOOK

The idea that primitively amitochondriate protists—Archezoa—were alive and well was an attractive one, not least because it lent credibility to theories for eukaryote origins that required a host of this kind to engulph the mitochondrial endosymbiont. Better trees, the discovery of genes of mitochondrial origin and, latterly, the discovery of mitochondrial homologues have allowed the rejection of the Archezoa hypothesis for the key species for which it was formulated. It thus seems possible that all eukaryotes might contain an organelle of mitochondrial ancestry, emphasizing the pivotal role that the mitochondrial endosymbiosis has played in eukaryotic evolution. Although we still need a reliable root for the eukaryotic tree (see Cavalier-Smith 2006; Roger & Hug 2006), it is evident that anaerobic eukaryotes have repeatedly arisen from within ancestrally aerobic groups with mitochondria. Anaerobic eukaryotes are neither rare nor primitive as once thought.

Most of what is known about mitochondrial function and the importance of mitochondria for the eukaryotic cell is drawn from the study of yeast, mammal and plant mitochondria. Apart from oxidative phosphorylation, other important reactions include the Krebs cycle, haem biosynthesis, *beta*-oxidation of fatty acids, amino acid biosynthesis and the formation and export of iron–sulphur (Fe/S) clusters (Reichert & Neupert 2004; Lill & Muhlenhoff 2005). It is already evident, from biochemical and genomic data, that hydrogenosomes and mitosomes can have retained only a limited subset of these reactions (Katinka *et al.* 2001; Müller 2003; Abrahamsen *et al.* 2004; Loftus *et al.* 2005). This raises important questions concerning the fundamental importance of this compartment of endosymbiotic ancestry for the eukaryotic cell, its biochemical flexibility and the limits of organelle reduction. For example, MCF protein diversity closely mirrors important facets of mitochondrial metabolic diversity; non-parasitic aerobic eukaryotes typically have between 30 and 60 MCF genes, to transport different substrates required or produced by the mitochondrion (Kunji 2004). By contrast, *E. histolytica* has lost all but a single member of the MCF which

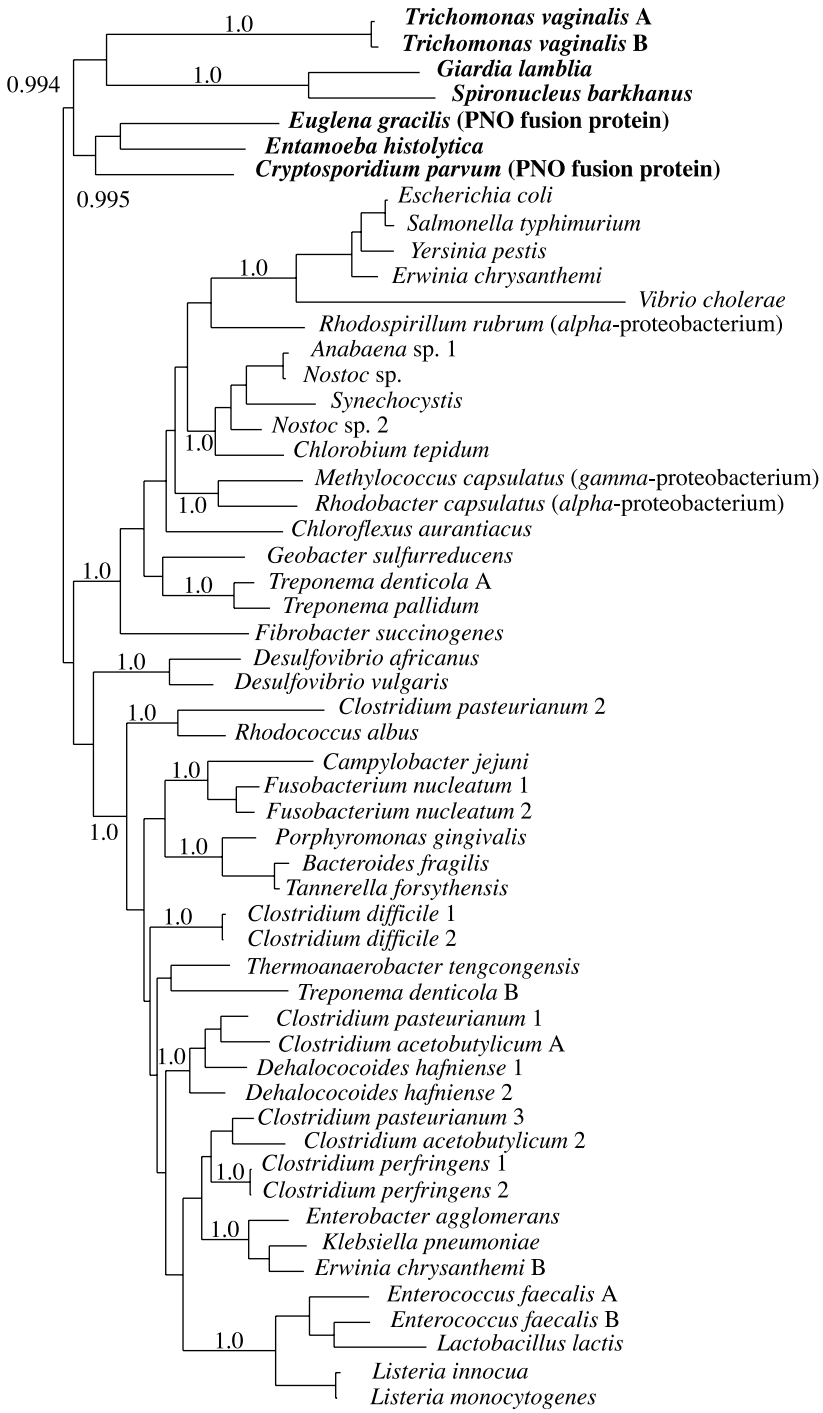

Figure 1. Phylogenetic tree showing the evolutionary relationships between pyruvate:ferredoxin oxidoreductase (PFO) sequences from eukaryotes and eubacteria. Eukaryotes are shown in bold. The tree is a consensus tree from a Bayesian analysis of aligned protein sequences using custom software (available from p.foster@nhm.ac.uk). Aligned positions (715 sites) were recoded into the six Dayhoff groups: C, STPAG, NDEQ, HRK, MILV and FYW to reduce the effects of mutational saturation (Hrdy *et al.* 2004). Posterior probabilities for some groups are shown, with a value of 1.0 representing maximum support. The strongly supported groups have also been recovered in previously published analyses using different methods, including maximum-likelihood, and different support measures including bootstrapping (Horner *et al.* 1999; Rotte *et al.* 2001).

functions in its mitosome to transport ATP and ADP (Chan *et al.* 2005; Loftus *et al.* 2005). The microsporidian *Encephalitozoon*, an obligate intracellular parasite, has gone even further, because its genome lacks any MCF genes (Katinka *et al.* 2001). This raises the intriguing question of how the *Encephalitozoon* mitosome acquires ATP.

The maturation of Fe/S clusters, for insertion into the Fe/S proteins that are crucial for all cellular life, is to date the only biosynthetic function for which mitochondria are essential to the yeast cell (Lill *et al.* 1999). Key proteins in this pathway; cysteine desulphurase (IscS) and scaffolding protein (IscU), appear to have originated from the mitochondrial endosymbiont (Tachezy *et al.* 2001; Emelyanov 2003; van der Giezen *et al.* 2004). The maturation and export of Fe/S clusters has been suggested as a possible common function of all mitochondrial homologues (Tachezy *et al.* 2001; Embley *et al.* 2003*a*). Key genes for this process are indeed present on all eukaryotic genomes

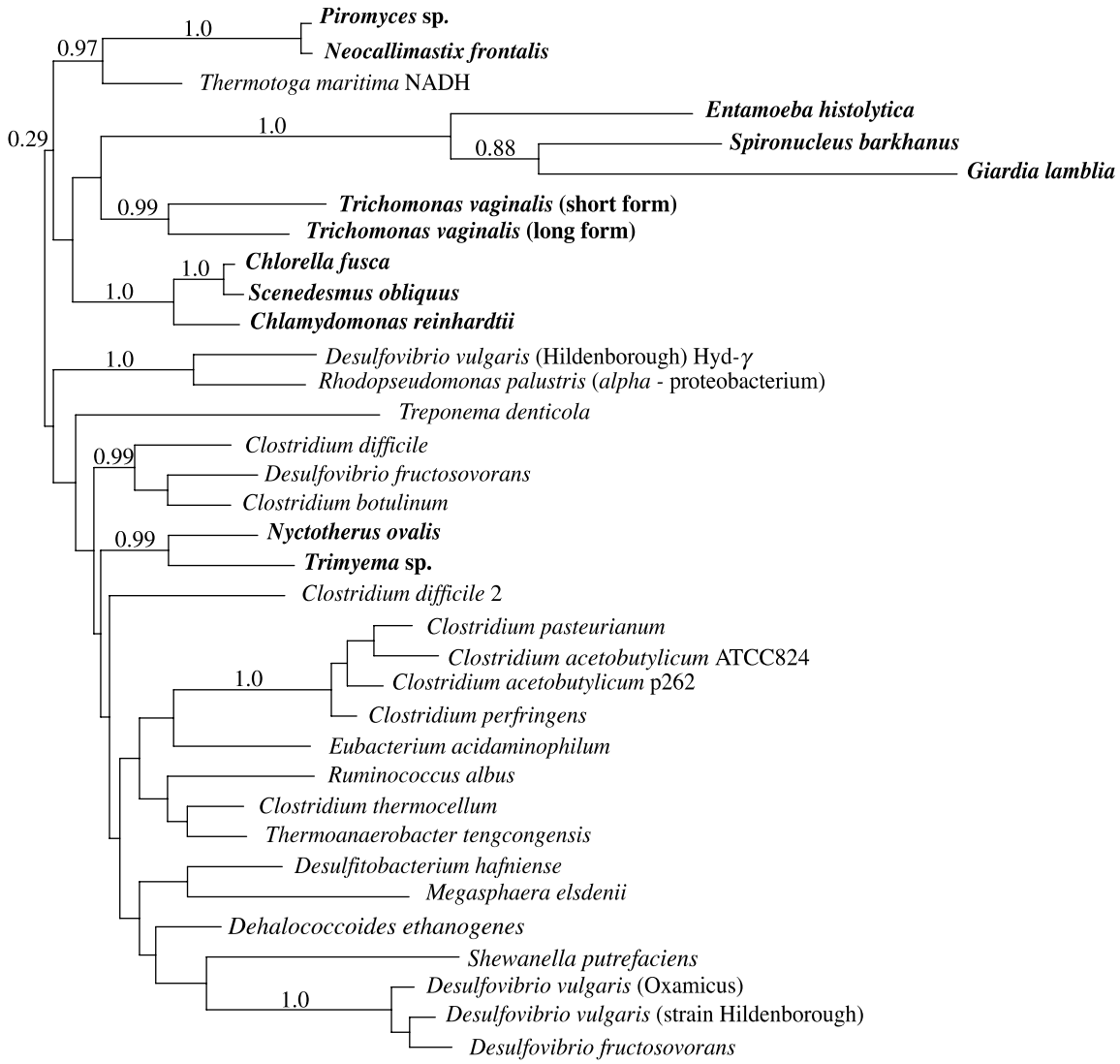

Figure 2. Phylogenetic tree showing the evolutionary relationships between [Fe]-hydrogenase sequences from eukaryotes and eubacteria. Eukaryotes are shown in bold. Details of the analysis on 196 aligned positions are given in the legend to figure 1. The strongly supported groups have also been recovered in previously published analyses using different methods, including maximum-likelihood, and different support measures including bootstrapping (Horner *et al.* 2000).

examined (Katinka *et al.* 2001; LaGier *et al.* 2003; Lill & Muhlenhoff 2005) but only the *Trichomonas* hydrogenosome (Sutak *et al.* 2004) and *Giardia* mitosome (Tovar *et al.* 2003) have actually been demonstrated to contain the pathway. *Entamoeba* is so far unique among eukaryotes in that it has lost the mitochondrial homologues of IscS and IscU (Loftus *et al.* 2005), and instead it possesses genes for the homologous proteins NifS and NifU, acquired through horizontal gene transfer from a bacterium related to *Campylobacter* (Ali *et al.* 2004; van der Giezen *et al.* 2004). The location of this pathway is central to testing the hypothesis that Fe/S cluster assembly is the common function of all mitochondrial homologues.

So far there is no evidence that the genes for [Fe]-hydrogenase and PFO, which mediate the conversion of mitochondria to hydrogenosomes, originated from the mitochondrial endosymbiont (Embley *et al.* 1997; Martin & Müller 1998). However, phylogenetic analyses do suggest that early eukaryotes likely contained both PFO and [Fe]-hydrogenase, and thus it is possible that they could make hydrogen. The two proteins, in various forms, have been found in diverse eukaryotes, where they are targeted to the cytosol, hydrogenosomes, mitochondria, nucleus and plastids. Although hydrogenase and PFO fail to display a unique affinity for the 'mitochondrial' compartment, their widespread retention among eukaryotes has undoubtedly contributed to the facility by which eukaryotes have repeatedly evolved hydrogenosomes (Embley *et al.* 1997). The origins of eukaryotic [Fe]-hydrogenase and PFO are also part of a bigger question concerning the origins of the eubacterial-like genes that encode much of eukaryote metabolism (Ribeiro & Golding 1998; Rivera *et al.* 1998; Esser *et al.* 2004). There are a number of imaginative hypotheses to explain these genes; as the product of multiple lateral transfers from different prokaryotes (Doolittle 1998), or the legacy of different eubacteria that participated in eukaryogenesis (Martin & Müller 1998; Moreira & LopezGarcia 1998; Rivera & Lake 2004; Cavalier-Smith 2006). Genomics coupled with more sophisticated phylogenetic analyses, should in principle be able to resolve gene origins, or at least reveal the limits of resolution that the data can provide (Penny *et al.* 2001; Ho & Jermiin 2004; Roger & Hug 2006). Published work on whole genomes has

so far failed to provide strong support for any of the hypotheses for eukaryogenesis in common currency (Rivera & Lake 2004; Cavalier-Smith 2006).

The discovery that all eukaryotes, or at least the ones that have been studied in any detail, contain a mitochondrial homologue, potentially places the mitochondrial endosymbiosis at the very dawn of eukaryotic evolution. The demise of Archezoa also means that we can no longer be confident that the host for the mitochondrial endosymbiont was already a eukaryote. Prokaryote-host models for the mitochondrial endosymbiont (e.g. Searcy 1992; Martin & Müller 1998; Vellai & Vida 1999), which the existence of Archezoa had seemed to exclude, can now be judged on their merits as predictive hypotheses that can be tested. Of course, many anaerobic habitats and the eukaryotes that populate them remain poorly characterized and a bona fide Archezoan might have evaded discovery. However, given what is known about the ubiquity of anaerobic habitats and their persistence over time, it is difficult to see why archezoans should have proved so elusive.

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