Origins of microsporidia

Patrick J. Keeling and Geoff I. McFadden

Microsporidia are obligate intracellular parasites that infect a wide range of eukaryotes, causing severe diseases in immunocompromised humans and losses to apiaries, fisheries and silk farms. They have often been considered to be primitive eukaryotes; however, more recent evidence suggests they are more closely related to fungi.

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The microsporidia, or phylum Microspora, were first recognized in the last century when *Nosema bombycis* was found to cause the destructive pébrine disease in the commercially important silkworm, *Bombyx mori*. Since then, they have been shown to parasitize many other eukaryotes, and the list of harmful diseases attributed to them has grown apace[1,2]. Until the last decade, microsporidian parasites were not recognized to be of particular human medical importance, as they were mainly known for the diseases they cause in fish and in insects such as silkworms, bees and locusts. In 1985, however, *Enterocytozoon bieneusi* was discovered to cause chronic diarrhoea in HIV-infected patients[3] and, subsequently, the number of microsporidian pathogens described in humans has grown considerably. Such human infections are most prevalent and most dangerous in patients with compromised immune systems and are frequently, but not always, associated with AIDS (Refs 4,5).

In the last decade, the microsporidia have also attracted increasing attention from evolutionary biologists. These tiny parasites have several very strange characteristics, and it has been difficult to provide a satisfactory explanation as to how they evolved and how they are related to other eukaryotes[6]. Lately, however, we have gained new insights into microsporidian biology that change the way we regard their evolution and perhaps also how we regard them as parasites (for a complementary review of this subject, see Ref. 6).

Unusual parasites

Microsporidia are obligate intracellular parasites of eukaryotes: outside another cell they can only exist as infective spores that are protected by walls of protein and chitin. The primary distinguishing features of spores are their small size, thick walls and the presence of an organelle called the polar tube. This long, thin tube lies tightly coiled within the cytoplasm of the spore, contacting the thinnest point of the spore coat at its anterior end. When triggered, the polar tube can be rapidly everted. It breaks through the spore coat at the anterior and, if a host cell is nearby, the polar tube may penetrate its membrane, allowing the microsporidian cell to pass directly into the host cytoplasm (Fig. 1, stages b and c). Once inside the host, there is a great

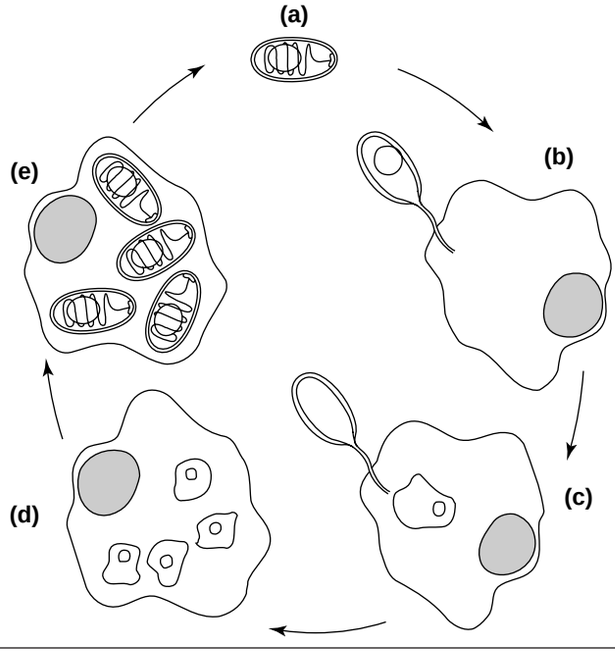

Fig. 1. Generalized microsporidian life cycle. The host nucleus is depicted in grey, and the microsporidia are not drawn to scale. **(a)** Infective spores in the environment showing the thick wall and the polar tube coiled about the nucleus and infective cytoplasm. A single nucleus is shown, but in many genera the spores are diplokaryotic. **(b)** Eversion of the polar tube to pierce the membrane of the host cell. This eversion typically takes place within a fraction of a second. **(c)** Invasion of the host cytoplasm. The infective cytoplasm of the parasite is squeezed through the polar tube (leaving a residue of cytoplasm in the spore coat) into the cytoplasm of the host. **(d)** Parasites grow and divide in the host cytoplasm. At this stage, there is considerable variation between genera in the behaviour of the parasites and the way they affect and interact with the host. **(e)** Differentiation of parasites into mature spores. This stage is also marked by considerable variation. Mature spores are eventually released from the host cell (either while it remains alive or when it dies) into the environment. For descriptions of the various cell cycles and diagrams, see Refs 1,2.

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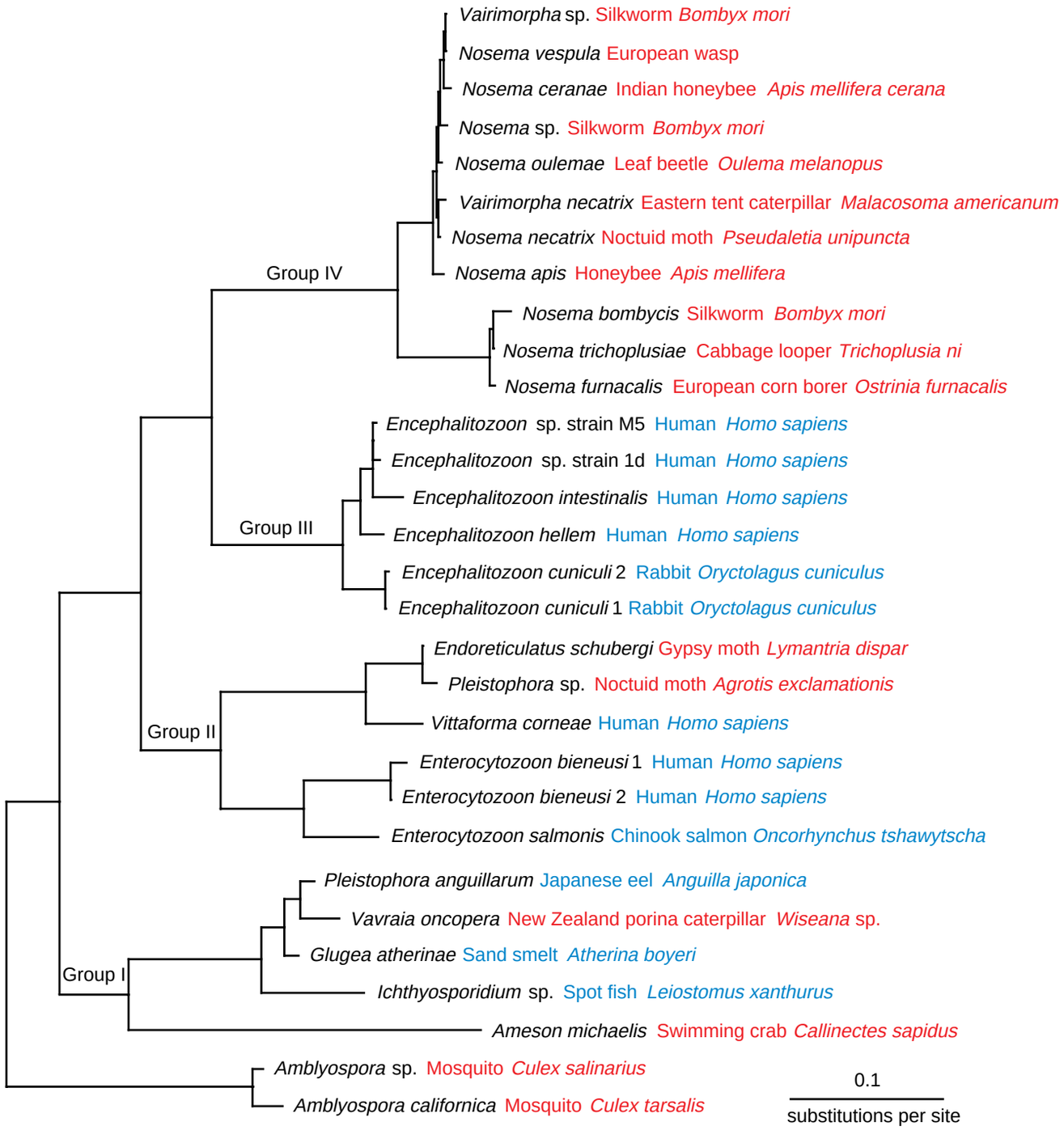

Fig. 2. Maximum likelihood phylogenetic tree of microsporidian small subunit rRNA sequences showing the distribution of host species. Vertebrate hosts are indicated in blue, while invertebrate hosts are in red. The root and groups I–IV are defined according to Refs 25,26. *Encephalitozoon cuniculi* is represented by the two highly similar sequences numbered 1 and 2 according to the Ribosomal Database Project[27]. Parasites of group III are only known to infect vertebrates, whereas those of group IV are only known to infect invertebrates. However, vertebrate and invertebrate species are hosts to group I and II parasites, showing that parasites have adapted to infecting distantly related animals in the past. Although a single species of host has been listed, many microsporidia are known to infect a range of hosts. The species listed is simply one common host or the host where the parasite was first described.

deal of diversity in how the parasites proliferate (Fig. 1, stages d and e). In some species, this intracellular phase is very simple: the parasites grow and divide individually, eventually differentiating into mature spores that are released from live host cells or when the host dies. However, the propagation of other species can be complex and variable. Parasites can contain a single isolated nucleus, can grow as multinucleate plasmodia, or can contain nuclei in one or more diplokaryotic arrangements. They can also divide in isolation or be held

together in membrane-bound clusters. In some species, the life cycle also includes meiosis, whereas in others there is no evidence of karyogamy. Descriptions of these life cycles and their accompanying terminology can be found in Refs 1,2.

Although microsporidian infections have been characterized in a few protists (ciliates and gregarine Apicomplexa), the vast majority have been described in animals. Within metazoa, microsporidian parasites are known to infect members of every phylum and to infect a broad range of diverse tissues. Any one species of microsporidia is often associated with a single host animal or a single tissue type, but some microsporidia are known to have both intermediate and definitive hosts[7], and others have a broad host range. Usually, the hosts of any one microsporidian are related[8,9]; however, some microsporidia have been found to infect both vertebrate and invertebrate hosts[10,11], and closely related microsporidia can also infect very distantly related animal hosts (Fig. 2).

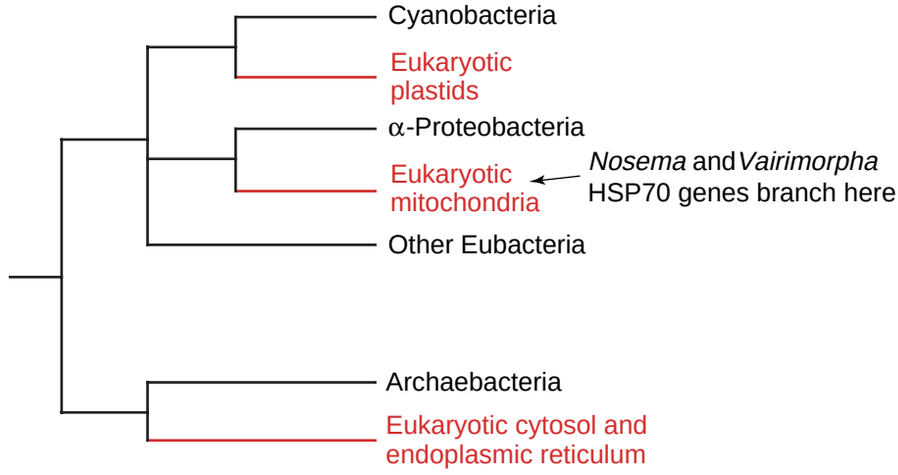

Fig. 3. Organellar genes encoded in the nucleus can still be recognized by their evolutionary origin in the Eubacteria. Genes encoded in the eukaryotic nucleus are shown as red branches, but mitochondrion- and plastid-targeted proteins branch with α-proteobacteria and cyanobacteria, respectively, and not with other eukaryotic genes. The recently discovered microsporidian HSP70 (heat-shock protein 70) genes are related to mitochondrial HSP70s in other eukaryotes, which in turn are related to α-proteobacterial genes[18,19]. The presence of these genes in microsporidia shows that their ancestors contained mitochondria.

Origins of microsporidia – ancient or recent?

Before the introduction of molecular phylogeny, microsporidia posed a difficult evolutionary problem because they lack several features that are considered to be universal to eukaryotes. These include mitochondria, peroxisomes, classical stacked Golgi membranes, 80S ribosomes (microsporidia have 70S ribosomes) and 9 + 2 microtubule structures such as cilia or flagella. On the basis of these observations, it was proposed that microsporidia and certain other amitochondrial protists are the descendants of primitive eukaryotes. These organisms were called the Archezoa and included the Microspora, Metamonadina (e.g. *Giardia*), Parabasalia (e.g. *Trichomonas*) and Archamoebae (e.g. *Entamoeba*)[12]. The theoretical reasoning behind the Archezoa was that they lack many 'eukaryotic' characteristics, particularly mitochondria, because their ancestors split from other eukaryotes before these characteristics evolved.

Phylogenetic studies based on molecular sequences initially supported an early origin of microsporidia. In trees inferred from both small and large subunit rRNA, elongation factors EF-1α and EF-2, and isoleucyl-tRNA synthetase, microsporidia branch very early among eukaryotes, often before any other eukaryotic lineage[13–15]. In addition, in microsporidian rRNAs, the sequence corresponding to 5.8S rRNA is fused to the large subunit rRNA as it is in prokaryotes[16]. This characteristic should be interpreted with caution, however, as this fused rRNA could arise *de novo* by the loss of a single processing site in the rRNA operon[17].

Initially, the combination of molecular phylogenetics and the dearth of eukaryotic characters in microsporidia provided a comfortable congruence of data supporting the concept that this group was among the most primitive of extant eukaryotes. But this comfort was short lived. New evidence favours a more recent origin of microsporidia, suggesting that they are actually part of the 'crown' of eukaryotes, a loosely defined group that includes animals, plants, fungi and certain recently evolved protist taxa.

One of the original reasons behind the proposal that the microsporidia are ancient eukaryotes is their lack of mitochondria. Although no one has yet identified an actual mitochondrion in microsporidia, it has now been found that they do contain molecular relics of one. Many of the genes for proteins essential for mitochondrial function are encoded by nuclear chromosomes. Originally, these genes were part of the genome of the mitochondrial symbiont but were transferred to the nucleus as the symbiont was transformed to an organelle. The mitochondrial provenance of these genes can still be recognized in two ways: their products are targeted to the organelle using a transit peptide, and they are most similar to homologues from α-proteobacteria (the type of bacterium from which the mitochondrion evolved by endosymbiosis). The presence of such mitochondrion-derived genes reveals that the ancestor of the lineage contained a mitochondrion, even though an organelle has not been identified in the lineage. Very recently, genes encoding HSP70 (a heat-shock protein or chaperonin) have been identified in the microsporidia *Nosema locustae* and *Vairimorpha necatrix*, and phylogenetic analyses have shown unequivocally that these genes are most closely related to HSP70 proteins from the mitochondria of other eukaryotes[18,19] (Fig. 3).

Interestingly, of all the mitochondrial HSP70s, these microsporidian genes prove to be most like those from fungi. This bolsters an already growing list of reasons to believe that microsporidia are closely related to fungi.

Box 1. Microsporidian genomes

Compared with other eukaryotes, microsporidia have extremely small genomes, often in the size range of bacterial genomes. The smallest to date is that of *Encephalitozoon cuniculi*, which was recently found to be only 2.9 Mbp (Ref. 28). To put this in perspective, the genome size of *Escherichia coli* is 4.6 Mbp and that of humans is 3200 Mbp. In light of the apparent relationship between microsporidia and crown eukaryotes, the small genomes of microsporidia are clearly a derived characteristic, perhaps related to their highly adapted parasitic lifestyle. So how does a eukaryote manage to reduce its genome size so drastically? Presumably, they have lost many of the genes essential in free-living organisms, packed the remaining genes very tightly on the chromosomes and reduced the number and size of elements such as introns and non-coding control regions. These are some of the characteristic features of other eukaryotes with apparently severe selection on genome size[29,30], but there will surely be some surprises in microsporidian genomes – means of conserving space that we have never seen or considered before. It will be interesting to learn what strategies microsporidian genomes use and how different organisms have found solutions to the same evolutionary pressures.

First, although microsporidia branch early in EF-1α phylogeny, the microsporidian gene contains an insertion that is otherwise unique to animals and fungi[15]. Similarly, in plants and other protists where it has been examined, thymidylate synthase and dyhydrofolate reductase are part of a single protein, whereas in animals, fungi and microsporidia they are encoded by two distinct genes[20]. These characteristics link microsporidia with the animal/fungal lineage, and other features hint specifically at a relationship with fungi. Like the mitochondrial HSP70, both the α- and β-tubulins of microsporidia show similarities to fungal tubulins and branch very securely within the fungal radiation in phylogenetic trees[21,22]. Other similarities that strengthen the relationship between microsporidia and fungi include the presence of chitin and trehalose and certain characteristics of the meiotic and mitotic cycles (reviewed in Refs 18,21).

Why do different microsporidian genes suggest contradictory phylogenies? The answer probably lies in the fact that sequences of microsporidian genes tend to be highly divergent, and this rapid rate of evolution makes it difficult to accurately infer the phylogenetic position of a gene. Rapidly evolving sequences result in long branches on phylogenetic trees, and these long branches tend to cluster with other long branches, even if they are not actually related[23]. In the case of microsporidia, their very long branches may result in an artifactual, but often statistically convincing, position at the root of eukaryotes, where there are several other long-branch eukaryotic taxa and the long branch to prokaryotes[18,21,24]. Why some genes might be less susceptible than others to this effect is not easily explained, but it does point to the dangers of placing too much confidence in the phylogeny of a single gene or even a few genes. Much of our evolutionary thinking is currently based in some way or another on the phylogeny of small subunit rRNA. This phylogeny has made an unparalleled contribution to our understanding of the origin and diversity of eukaryotes and prokaryotes alike, but, like any other single-gene phylogeny, it may be misleading in some respects while correct in others[24].

Questions for future research

- Do primitively amitochondrial eukaryotes still live today?
- Do microsporidia contain a mitochondrion and, if so, what does it do?
- Microsporidia have severely reduced genome sizes: what genes have they retained and how are they organized?
- What are the relationships among animals, fungi and microsporidia?
- If microsporidia are closely related to fungi, can this knowledge provide new and useful ideas for treatment?

Conclusions: implications of a late origin of microsporidia

It seems increasingly certain that the microsporidia are neither ancient nor primitive eukaryotes, but are instead a relatively recently evolved group of highly adapted parasites. The close relationship now apparent between microsporidia and fungi changes many of our beliefs about early eukaryotic evolution and also modifies how we interpret the biology of microsporidia (Box 1). This new perspective may also have important implications for how we might deal with them as pathogens.

It is now important to narrow the evolutionary position of microsporidia as accurately as possible to determine whether they have actually evolved from true fungi, whether they are sisters to the fungi or whether they are sisters to both animals and fungi. This may prove difficult with molecular tools, given the typically divergent nature of microsporidian gene sequences, but it may nevertheless be possible to identify informative insertions and deletions (such as the one in EF-1α) that link microsporidia specifically to their closest relatives. Also, although the presence of mitochondria in the ancestors of microsporidia has been established by the finding of mitochondrial HSP70 genes, the organelle itself (should it still exist) has yet to be identified. Chaperonins of this type are generally used for importing proteins to the mitochondrion, and both the *Nosema* and *Vairimorpha* HSP70 proteins have amino-terminal extensions that might serve to target these proteins to a specific subcellular compartment[18,19]. Both these facts point to the possible presence of an organelle homologous to the mitochondrion, but to be certain it must be identified and its function explored.

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The role of CpG dinucleotides in DNA vaccines

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DNA vaccines can induce potent humoral and cellular immune responses without any additional adjuvant. Recent studies indicate that unmethylated CpG dinucleotides within DNA vaccines are immune stimulatory and exert an essential endogenous adjuvant activity. These CpG motifs can be added deliberately to DNA or conventional protein vaccines to enhance the Th1 immune response.

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Unlike vertebrate DNA, bacterial DNA induces B-cell proliferation and immunoglobulin (Ig) and cytokine secretion[1]. The lymphocyte activation is caused by unmethylated CpG dinucleotides, which are present at the expected frequency of one per 16 dinucleotides in bacterial DNA, but are underrepresented (CpG suppression) and methylated in vertebrate DNA. B-cell activation can also be triggered by synthetic oligodeoxynucleotides (ODNs), which contain an unmethylated CpG dinucleotide in a particular sequence context.

CpG DNA activates all subsets of B cells and can drive more than 95% of them into the cell cycle[1] (Fig. 1). Thus, B-cell activation by CpG DNA is T-cell-independent and antigen nonspecific. Nevertheless, B-cell activation by low concentrations of CpG DNA *in vitro* synergizes strongly with signals delivered through the B cell antigen receptor for both B-cell proliferation and Ig secretion[1]. This strong synergy between the B-cell signaling pathways triggered through the B-cell antigen receptor and by CpG DNA promotes antigen-specific immune responses and suggests that CpG DNA may be of value as a vaccine adjuvant.

In addition to its direct effects on B cells, CpG DNA also directly activates monocytes, macrophages and dendritic cells *in vitro* to upregulate their expression of co-stimulatory molecules that drive immune responses and to secrete a variety of cytokines, including high levels of interleukin 12 (IL-12) (Fig. 1)[2–4]. These cytokines stimulate natural killer (NK) cells to secrete interferon γ (IFN-γ) and have increased lytic activity[2,5–7] (Fig. 1). Overall, CpG DNA induces a Th1-like pattern of cytokine production dominated by IL-12 and IFN-γ and with little secretion of Th2 cytokines[2]. It appears likely that the rapid immune activation in response to CpG DNA may have evolved as one component of the innate immune defense mechanisms that recognize structural patterns specific to microbial molecules.

Mechanisms of immune stimulation by CpG DNA

Although it has been suggested that activation of human B cells by CpG DNA is mediated via membrane receptors specific for CpG motifs[8], we have found that in

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