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Review

Microsporidia: a journey through radical taxonomical revisions

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ABSTRACT

Microsporidia are obligate intracellular parasites of medical and commercial importance, characterized by a severe reduction, or even absence, of cellular components typical of eukaryotes such as mitochondria, Golgi apparatus and flagella. This simplistic cellular organization has made it difficult to infer the evolutionary relationship of Microsporidia to other eukaryotes, because they lack many characters historically used to make such comparisons. Eventually, it was suggested that this simplicity might be due to Microsporidia representing a very early eukaryotic lineage that evolved prior to the origin of many typically eukaryotic features, in particular the mitochondrion. This hypothesis was supported by the first biochemical and molecular studies of the group. In the last decade, however, contrasting evidence has emerged, mostly from molecular sequences, that show Microsporidia are related to fungi, and it is now widely acknowledged that features previously recognized as primitive are instead highly derived adaptations to their obligate parasitic lifestyle. The various sharply differing views on microsporidian evolution resulted in several radical reappraisals of their taxonomy. Here we will chronologically review the causes and consequences for these taxonomic revisions, with a special emphasis on why the unique cellular and genomic features of Microsporidia lured scientists towards the wrong direction for so long.

1. Introduction

Our view of the tree of eukaryotic has undergone substantial revision in the past decade (Keeling *et al.*, 2005; Keeling and Palmer, 2008), but few lineages have seen their position within the tree as radically changed as have the Microsporidia. These organisms are tiny (from 2 to 40 μm in diameter) unicellular eukaryotes, all of which are opportunistic, obligate intracellular parasites of other eukaryotes (predominantly animals). Microsporidian spores are the only stage that lives outside their host, and spores are distinguished by a thick, rigid wall and a complex infection apparatus consisting of a long coiled polar filament, a posterior vacuole, and a system of stacked membranes called the polaroplast. When triggered to germinate, the spore starts to take up water through aquaporins, creating pressure and a swelling of the posterior vacuole. This pressure eventually ruptures the wall at the apex and forces the ejection of the polar filament, which everts to

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become a tube. The contents of the spore are forced through the tube and, if the tube has punctured another cell, the parasite is injected into the cytoplasm of that cell (Fig. 1).

To date, over 1300 species of Microsporidia (in 160 genera) have been formally described in the literature, based on their cellular structure, life cycle, and host specificity. Individual species typically infect a relatively narrow range of hosts, but overall the members of the group are found in a wide taxonomical range of hosts, including a few protists, many arthropods, and vertebrates, including humans (Becnel and Andreadis, 1999; Larsson, 1999; Vossbrinck and Debrunner-Vossbrinck, 2005; Weiss and Vossbrinck, 1998).

Aside from the elaborate infection mechanism in the spore, microsporidian cells are generally very simple, lacking peroxisomes and $9+2$ microtubular structures such as flagella, and also lacking conventional versions of other common eukaryotic organelles such as mitochondria and the Golgi apparatus. The coexistence of both extreme simplicity and complexity in Microsporidia has played a major role in the many and extensive revisions to our views about their evolutionary origins. On one hand they have lost many of the morphological features typically used to compare eukaryotes with one another, while on the other hand they are so highly adapted to intracellular parasitism that they have evolved many features that are unique to the group. As a result, they are difficult to compare with their relatives and over the last 150 y their placement in various taxonomic systems has been highly unstable. This has been particularly true in the last three decades, however, with the introduction of electron microscopy and then molecular data to address questions of microbial taxonomy, while at the same time interest in Microsporidia as pathogens began to grow with increasing reports of human infections, especially in patients under immunosuppressive treatment, and in individuals infected with HIV (Weiss, 2001).

While great progress has been made in elucidating the evolutionary origin of Microsporidia in recent years, primarily due to molecular data, a number of questions remain highly contentious and many aspects of the evolution of Microsporidia remain mysterious. In this review, we will discuss the causes and consequences that led to multiple and radical revisions of our taxonomic view of Microsporidia, how advances in our knowledge about their evolution have coincided with major technological innovations, and highlight the biological basis for those questions that remain problematic.

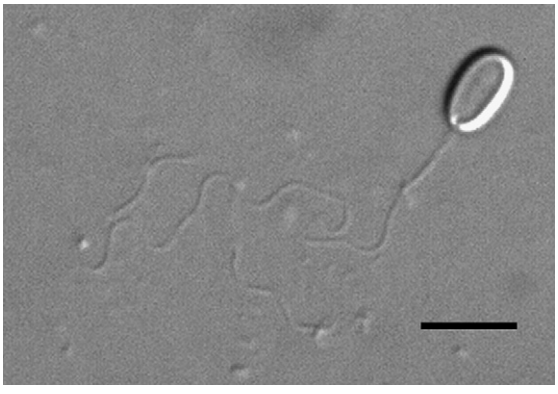

Fig. 1 – *Antonospora locustae* spore with ejected polar tube. Scale bar represents 5 μm.

2. The discovery and early views of microsporidian evolution

Under the microscope of a nineteenth century researcher, a microsporidian spore might not have been the most appealing organism to identify and to study: they are small, refractile, immotile, and lack most distinguishing features of a eukaryotic cell. Nevertheless, with their importance as disease-causing agents, the discovery of microsporidian parasites followed hard on the heels of an increased acceptance of the so called ''germ theory''. In the mid-1850s, the entire European silk industry was in decline due to a rapidly running and devastating disease affecting the silk-worm, the pébrine (or pepper-disease). Due to the economical importance of the silk production at that time, there was some pressure to search for a potential microbial agent, and an association between silk-worms affected with pébrine and the presence of characteristic globules was soon described. These globules were named *Nosema bombycis* by the Swiss microbiologist Karl Wilhelm von Nägeli in 1857, and the first Microsporidia was described in the literature (Nageli, 1857). The specific parasitic nature of the microsporidian spores was subsequently described in some detail by Louis Pasteur and his colleagues (Pasteur, 1870), eventually leading to a revival of the European silk industry.

Ironically, when Nägeli first identified *N. bombycis* (Nageli, 1857), he described it as a yeast-like fungus and included it in the Schizomycetes, which is superficially very close to our current concept of where Microsporidia fit into the tree of eukaryotes. Nägeli's prescience, to which we have (sort of) returned 150 y later, was unfortunately not the result of a particularly detailed insight, but rather derived from the then limited knowledge about microbial biodiversity and primitive microbial nomenclature. As a result, this early classification as a fungus was quickly revised following the growth of a more complex taxonomic system that took protists into full account. Indeed, the Schizomycetes as a group has been disbanded as it was in reality a collection of unrelated spore-forming eukaryotes and bacteria.

By 1882, Edouard-Gérard Balbiani had created a new group for *Nosema*, which he informally named the 'microsporidies' and included them in the protozoan group, Sporozoa (Balbiani, 1882). Sporozoa was a long-lived idea that united an assemblage of spore-forming parasites that are now known to be quite distantly related. It included several lineages now recognized as members of Apicomplexa (alveolates), the Haplosporidia (rhizarians), and a subgroup known as Cnidosporidia. Cnidosporidia comprised Myxosporidia (animals), Actinomyxidia (protists of unknown origin), Helicosporidia (green algae), and the Microsporidia (Kudo, 1947).

Despite their now-evident inaccuracy, these early classifications were nevertheless important for our understanding of the evolutionary origin of Microsporidia because they recognized it to be a natural group, composed of intracellular parasites and a unique host-infection mechanism. Most importantly, however, the inclusion of Microsporidia in the

Phylum Cnidosporidia had a significant impact on future thinking on the origins of the group. As advances in microscopy made the connection between the cnidosporidian subgroups increasingly less compelling, it became clear that the position of Microsporidia had to be revised (Lom and Vavra, 1961). It took some time, but eventually one influential hypothesis filled this void by providing a completely new and seemingly solid perspective about the evolutionary origin of Microsporidia.

3. The Archezoa hypothesis and the first microsporidian molecular data

The subjection of Microsporidia to analysis by electron microscopy (Krieg, 1955; Kudo and Daniels, 1963; Vavra, 1965; Weiser, 1959) not only led to the discovery of the most spectacular features of the spore, but also revealed the absences of a number of features common to more conventional eukaryotic cells, including mitochondria, Golgi bodies, peroxisomes, or flagella and other $9+2$ microtubule structures (Vávra and Larsson, 1999). At the same time, biochemical analysis of cell fractions revealed three distantly related Microsporidia harbored ribosomes that were not of the 80S class characteristic of other eukaryotes, but instead sedimented like prokaryotic 70S ribosomes (Curgy *et al.*, 1980; Ishihara and Hayashi, 1968). The absence of mitochondria was particularly influential, as this character was common to several other group of eukaryotes, and there was some speculation that these organisms might represent ancient, primitive eukaryotic lineages (Stewart and Mattox, 1980). This idea was eventually articulated in a formal way as the Archezoa hypothesis by Cavalier-Smith (1983), who postulated that the origin of the eukaryotic cell might have preceded the symbiotic origin of mitochondria, and that several extant eukaryotic lineages diverged between prior to this endosymbiosis. A total of four eukaryotic lineages were identified and collectively included in the new sub-kingdom Archezoa: Archamoebae, Metamonada, Parabasalia, and Microsporidia (Cavalier-Smith, 1983, 1987). While the relationships between Archezoan groups were never well defined, it was broadly assumed they were a paraphyletic group, and in some articulations of the hypothesis, the Microsporidia were argued to be the very deepest branch of eukaryotes on account of the fact that they alone also lacked $9+2$ microtubule structures (Patterson, 1994) (Fig. 2).

The formulation of the Archezoa hypothesis coincided with the first broad-based application of molecular tools to the problems of microbial phylogenetics and systematics. Soon after the Archezoa hypothesis was proposed, the first molecular data from Microsporidia were described, specifically the small and large subunits of the ribosomal RNA (SSU and LSU rRNA) from *Vairimorpha necatrix* (Vossbrinck *et al.*, 1987). Intriguingly, these data provided support for the Archezoa in two different ways. First, in phylogenetic analyses of SSU rRNA including members of several other eukaryotes lineages, *V. necatrix* was identified as the earliest branch of eukaryotes (Vossbrinck *et al.*, 1987), which was consistent with the prediction from the Archezoa hypothesis that Microsporidia were an ancient lineage (a requirement to being primitively amitochondriate). Secondly, the microsporidian 5.8S and LSU rRNAs was found to be fused as a single molecule (Vossbrinck *et al.*, 1987), a condition found in prokaryotes, but known in no other eukaryotic lineage, and thus interpreted as another 'primitive' characteristic of Microsporidia. For a time, each new genes sequenced and every new phylogeny inferred seemed to add more weight to the evidence for the early origin of Microsporidia, with analyses of isoleucyl aminoacyl-tRNA synthetase, elongation factor-1alpha, and elongation factor-2 all supporting a deep position

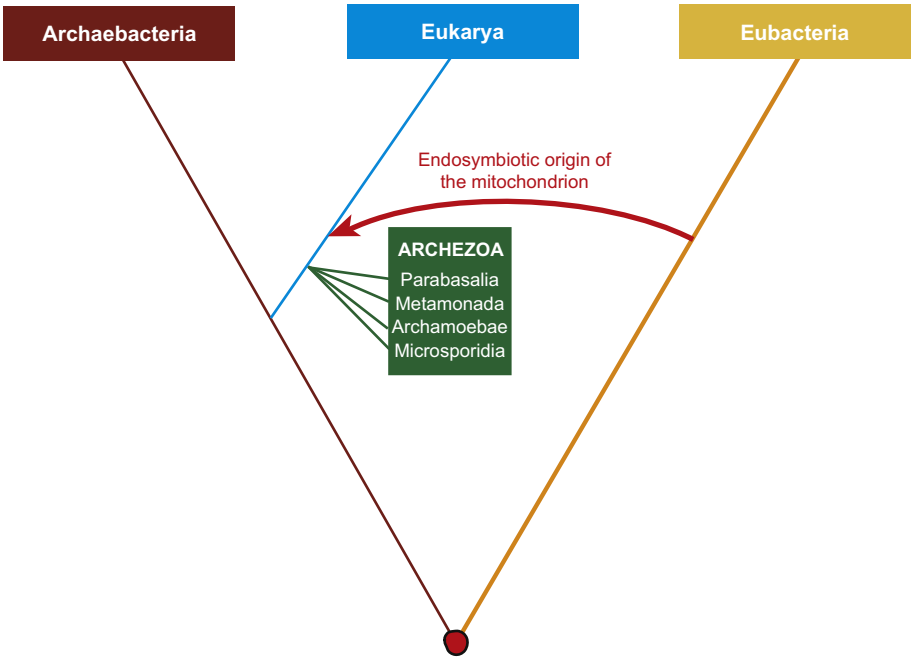

Fig. 2 – Archezoa hypothesis as originally conceived.

of the Microsporidia within the eukaryotes (Brown and Doolittle, 1995, 1999; Kamaishi *et al.*, 1996a,b). In parallel, similar evidence was accumulating for other members of the Archezoa, further bolstering confidence in the hypothesis (Cavalier-Smith, 1991). Indeed, at that time the evidence supporting the Archezoa seemed to be overwhelming, and the evolutionary origin of the Microsporidia accordingly appeared to be more or less resolved, with the major outstanding questions being their position among Archezoa and just how primitive they might be.

4. A fungal connection

Despite the sudden accumulation of phylogenetic evidence, the ancient origin of Microsporidia continued to be a source of doubt: the highly adapted nature of these parasites and the high divergence of their gene sequences were both troublesome characteristics with the potential to mislead. An obligate intracellular parasitic lifestyle could potentially lead to reduction or even loss of several organelles and cellular structures such as mitochondria, the peroxisomes, centrioles, or ribosomes, and the rRNAs contained many deletions so it was plausible that the 5.8S-LSU fusion was a reversion brought about by the loss of a processing sequence (Cavalier-Smith, 1993). At the same time, elevated rates of sequence divergence were well known to cause a phylogenetic artefact called ''long-branch attraction'', which could draw Microsporidia deeper to the base of the tree than they belong. Eventually, these doubts all proved to be well founded, but it took almost a decade before sufficient evidence against an ancient origin of Microsporidia could be gathered.

The first modern proposal that Microsporidia were similar to fungi came from a re-analysis of microsporidian meiosis, which suggested the process shared a number of similarities with certain groups of fungi (Flegel and Pasharawipas, 1995). Soon after the first phylogenetic evidence emerged from analyses of alpha- and beta-tubulins from a number of microsporidian species (Edlind *et al.*, 1994, 1996; Keeling and Doolittle, 1996). Within a short span of time various other fungal connections came to light: the identification of fungal-like chitinases (Hinkle *et al.*, 1997), phylogenetic analysis of TATA-box binding protein (Fast *et al.*, 1999), RNA polymerase II (Hirt *et al.*, 1999), HSP70 (Germot *et al.*, 1997; Hirt *et al.*, 1997; Peyretaillade *et al.*, 1998), glutamyl synthase (Brown and Doolittle, 1999), and both alpha and beta subunits of pyruvate dehydrogenase E1 (Fast and Keeling, 2001) all supported an association with fungi. Finally, the publication of the first microsporidian genome (Katinka *et al.*, 2001) also provided a wealth of data in favor of a fungal relationship. By the end of the 1990s, the possibility of fungal–Microsporidia connection found his place in the scientific community (Weiss *et al.*, 1999).

5. Undermining the ancient origin of Microsporidia

For a brief time, two incompatible views about the evolutionary origin of the Microsporidia were seemingly both well supported. But as data accumulated to support a connection between fungi and Microsporidia, the earlier data supporting an ancient origin of Microsporidia began to erode. In several cases, re-examination of the data that supported the Archezoa hypothesis using models that accounted for among-site rate variation, and by removing the most fast-evolving sites, led to radical changes in the topologies of the trees. Indeed, for some genes the change in topology was so radical that phylogenies that originally placed Microsporidia deep in the eukaryotic tree began supporting (albeit weakly) a Microsporidia–fungi relationship (Hirt *et al.*, 1999; Van de Peer *et al.*, 2000). Ultimately, analysis of the complete genome of *Encephalitozoon cuniculi* revealed a correlation between the divergence rate of a gene and the likelihood that it placed Microsporidia 'deep' in eukaryotes, while more conserved genes tended to place them with fungi (Thomarat *et al.*, 2004). This strongly reinforced the notion that early phylogenies were misleading due to long-branch attraction.

6. Undermining the absence of mitochondria in Microsporidia

The progress of molecular phylogeny clearly undermined the 'deep' position of Microsporidia, but the original reason for proposing the Archezoa hypothesis was not molecular trees, but the apparent absence of mitochondria. A close relationship with fungi would indicate that the *ancestor* of Microsporidia must have had mitochondria, but did not address whether cryptic mitochondria remained. This question was being asked for all the Archezoa by the mid-1990s because the basic premise of the hypothesis was at that time challenged by the identification of nuclear genes encoding mitochondrial proteins in the archamoeba, *Entamoeba histolytica* (Clark and Roger, 1995). This finding was the first direct assault on the idea that some archezoans might not have been ancestrally amitochondriate, and suggested they might even still have relict mitochondria. The same debate shortly began for Microsporidia as well, with the rapid-fire discovery of nuclear genes encoding mitochondrial HSP70 from *Antonospora (Nosema) locustae*, *V. necatrix*, and *E. cuniculi* (Germot *et al.*, 1997; Hirt *et al.*, 1997; Peyretaillade *et al.*, 1998). Subsequently the alpha and beta subunits of pyruvate dehydrogenase E1 were characterized in *A. locustae* (Fast and Keeling, 2001) and six other mitochondrial-associated genes were annotated in the genome of *E. cuniculi* (Katinka *et al.*, 2001). An additional ADP/ATP carrier protein and import processing peptidase were subsequently characterized in *A. locustae* (Williams and Keeling, 2005; Williams *et al.*, 2008b), revealing diversity in mitochondrial activities between species.

While these many genes each undermined the notion that Microsporidia are ancestrally amitochondriate, they still only suggested the organelle is still present, because predicting mitochondrial targeting peptides is far from unambiguous in the case of Microsporidia. To really determine if an organelle exists, it was necessary to physically locate it, and the first evidence of a mitochondrial relict was reported in *Trachipleistophora hominis*, by immuno-localising mitochondrial HSP70 (Williams *et al.*, 2002). The protein was found to be distributed in numerous, tiny, double membrane-bounded organelles throughout the *T. hominis* cytoplasm, dubbed mitosomes. Mitosomes have since also been found in *E. cuniculi*, where

they number only two or three per cell (Goldberg *et al.*, 2008; Williams *et al.*, 2008b), and are positioned such that they likely represent the 'polar vesicles' previously hypothesized to be relict mitochondria (Vávra *et al.*, 2005).

The function of these mitosomes is severely reduced compared with canonical mitochondria: They have no role in aerobic respiration or many other typically mitochondrial functions, and instead appear to primarily function in the assembly of iron–sulfur clusters (Katinka *et al.*, 2001). Interestingly, however, some of the proteins originally identified as being mitochondrion-derived have since been found to localize to the cytoplasm (Williams *et al.*, 2008a), suggesting mitochondrial degeneration in Microsporidia has taken place both by loss of function, and incorporating other functions into the cytosol.

Overall, the discovery that Microsporidia harbor mitochondrial genes and relic mitochondria put an end to their inclusion in the Archezoa, and undermined the original rationale for considering them to be an ancient, primitive lineage. Instead, they are now acknowledged to represent highly adapted parasites, which evolved long after the endosymbiotic origin of mitochondria, and harbor several cellular and molecular features that directly link them with the fungi. Current debates are now focusing on whether Microsporidia are sister group to the fungi, or whether they represent an early fungal lineage.

7. What kind of fungi are Microsporidia?

The phylogenetic analyses that first led to the suggestion that Microsporidia are related to fungi were all based on single genes, and typically included only a small fraction of the known diversity of both Microsporidia and fungi. For these reasons, none of these analyses could really distinguish between two very different possibilities: that Microsporidia are *related to* fungi, or that Microsporidia actually are fungi.

The first analyses to address this question were restricted to the few genes that reliably recovered some relationship to fungi, since other molecules were simply too divergent to be interpretable, which at the time meant tubulins (Keeling *et al.*, 2000). Tubulin trees had the strongest support for the Microsporidia–fungi relationship, but were not without problems since Microsporidia and most fungi share relatively high rates of substitution in their tubulins. Phylogenies of both alpha- and beta-tubulin, as well as concatenations of both genes, which contained a broad representation of both fungal and microsporidian diversity were consistent in placing the Microsporidia within the fungi, as opposed to sister to all fungi, and generally consistent in supporting a specific relationship with zygomycetes, specifically the entomophthorales (Keeling *et al.*, 2000; Keeling, 2003) (Fig. 3A). However, the chytrid tubulins proved to be substantially more conserved than those of other fungi, so any conclusions based on these trees needs to be taken with caution, since long-branch attraction could be drawing the Microsporidia *within* the fungi, just as it drew them deeper in the eukaryotes with other genes.

Ideally, a large multigene data set with broad taxonomic representation could be brought to bear on this problem, but it is questionable if such a data set exists. Microsporidia have relatively few genes (genomic evidence to date points towards a total number of about 2000), and most of these are too divergent at the sequence level to be of much use phylogenetically (Thomarat *et al.*, 2004). Nevertheless, a few multigene

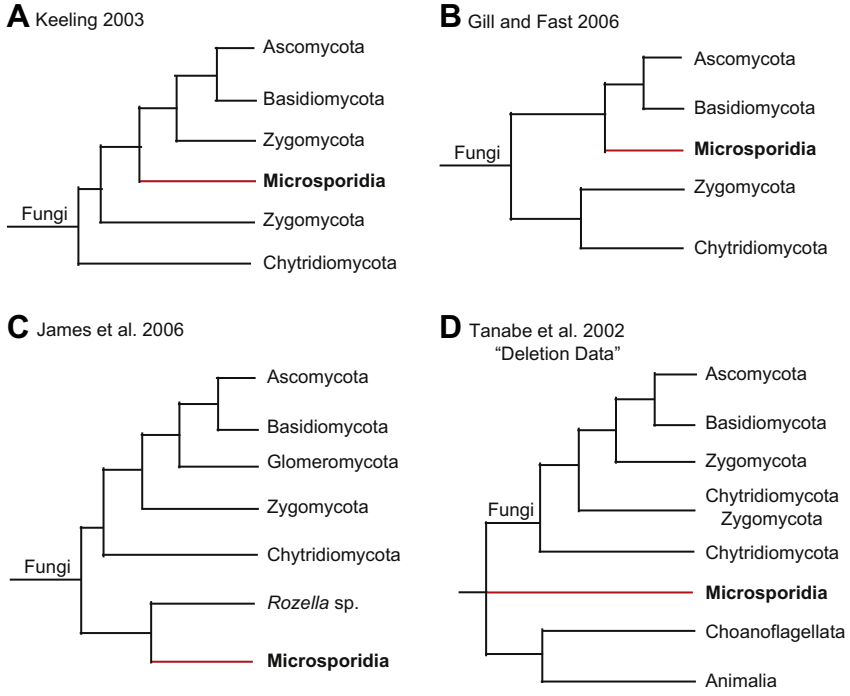

Fig. 3 – Schematic representation of the phylogenetic placement of the Microsporidia according to Keeling (2003) (A), Gill and Fast (2006) (B), James *et al.* (2006) (C), and Tanabe *et al.* (2002) (D).

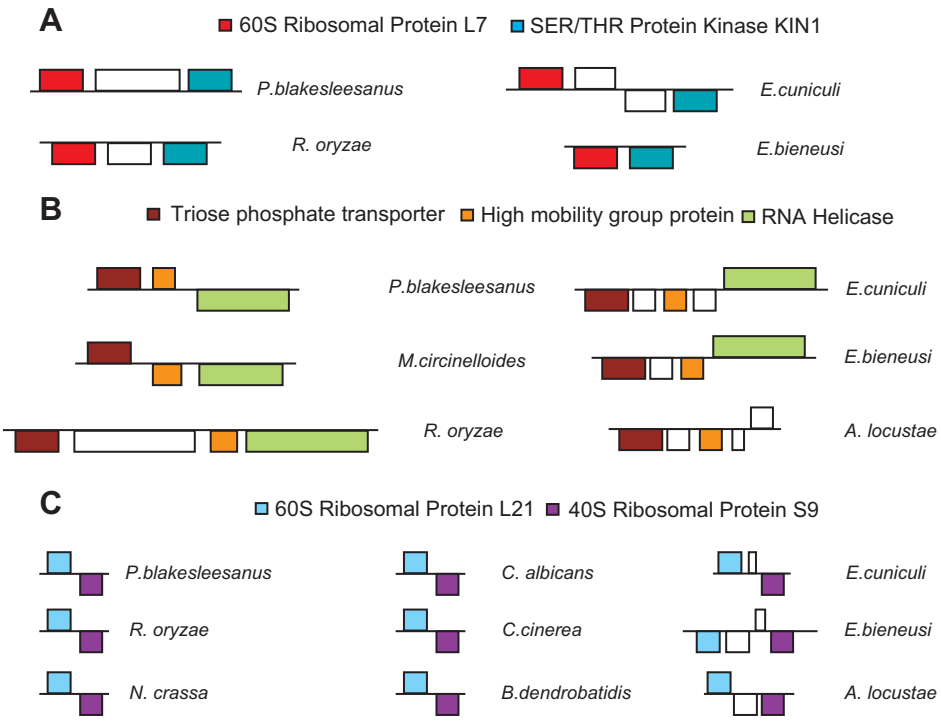

Fig. 4 – Examples of gene order conservation between Microsporidia and specific fungal groups according to Lee *et al.* (2008). (A) Example of gene order conservation between Microsporidia and zygomycetes. (B) Conservation of the zygomycete *MAT* locus in Microsporidia. (C) Example of gene order conservation exclusive to the fungi and Microsporidia.

analyses have been undertaken. A concatenation of 8 conserved protein encoding genes (alpha-tubulin, beta-tubulin, the largest subunit of RNA polymerase II (RPB1), the DNA repair helicase RAD25, TATA-box binding protein (TBP), a subunit of the E2 ubiquitin conjugating enzyme (UBC2), and the alpha and beta subunits of pyruvate dehydrogenase E1) also supported the Microsporidia branching within the fungi, and placed them as the sister group to ascomycetes and basidiomyces (Gill and Fast, 2006) (Fig. 3B). Using a different set of genes (rRNA, 28S rRNA, 5.8S rRNA, elongation factor-1 (EF-1), and two RNA polymerase II subunits (RPB1 and RPB2)), the Fungal Tree of Life project analysis proposed that the Microsporidia were closely related to *Rozella* (James *et al.*, 2006) (Fig. 3C), a putatively basal fungal lineage that is a parasite of chytrids. Another analysis of deletions in EF-1alpha suggested Microsporidia branched outside the fungi altogether, as their sister group (Tanabe *et al.*, 2002) (Fig. 3D). The difference between those conclusions is striking, and indeed, just about every possible relationship with major fungal divisions has now been proposed based on some kind of data (Fig. 3).

The combination of microsporidian genomes losing most of their genes, while those that have been retained have become highly divergent, stack the odds against the accurate reconstruction of their relationship to fungi. Evolutionary reconstructions based on gene sequences therefore might not be the best way to decipher the evolutionary origin of Microsporidia. However, genomes contain other kinds of information that have seldom been fully tapped. Rare structural genomic changes such as deletions and insertions in genes have been used in a number of cases to infer numerous phylogenetic relationships (Baldauf and Palmer, 1993). For instance, Microsporidia are known to harbor an eleven amino acid insertion in the EF-1alpha gene that is otherwise only found in fungi, animals, and close protist relatives (Kamaishi *et al.*, 1996a). Unfortunately, however, such characters are rare, and in reality are also very dependent on the conservation of gene sequences, since the homology of insertions and deletions can be difficult to interpret in highly divergent genes.

Recently, a different approach has been applied, based on the conservation of gene order (Lee *et al.*, 2008). It has been known for some time that, while microsporidian gene sequences are evolving very quickly, the order of genes within the genome is highly conserved (Corradi *et al.*, 2007; Slamovits *et al.*, 2004). This observation was recently extended by the demonstration that microsporidian genomes share higher frequency of gene order conservation with zygomycetes than they do with any other group of fungi for which genome data are available (Dyer, 2008; Lee *et al.*, 2008) (Fig. 4A). Interestingly, one of these regions is the zygomycete mating type locus (*MAT* locus), which also suggests a cryptic sexuality may exist in these Microsporidia (Fig. 4B). More broadly, this study also identified a conserved gene cluster of ribosomal proteins L21 and S9, which is not only shared by diverse Microsporidia and zygomycetes, but all other fungi as well (Fig. 4C), further supporting their overall relationship and suggesting some ancient selective pressure on these two genes.

8. Concluding remarks

150 y of research on microsporidian evolution has come full circle: they began as fungi for misguided reasons, and made their way through various groups of protozoa, to the Archezoa, and now have seemingly arrived once more as highly derived and adapted fungi. This has many implications for how we interpret the biology of these organisms and their evolutionary history, but also has unforeseen affects. For example, as fungi, the taxonomy of Microsporidia should be governed Botanical Code of Nomenclature, but Microsporidia have almost universally been named according to the Zoological Code. In theory many hundreds of microsporidian names would be invalidated by their relationship to fungi, but fortunately steps have now been taken to formally exclude them from the Botanical Code (Redhead *et al.*, 2009). What exactly is the relationship between Microsporidia and fungi is also not yet completely understood. There is evidence from both phylogeny and genome organization for a specific relationship to zygomycetes, and most molecular analysis with broad taxonomic representation agree they evolved somewhere within the fungi, so overall we conclude that a zygomycete origin of Microsporidia is currently the best working hypothesis.

The divergent nature of microsporidian genes remains a challenge, perhaps even affecting genome structure analysis, but there are a number of possible avenues to remedy this problem. Certainly a continued exploration of fungal diversity, particularly large scale genomic sequencing, may allow the identification of fungal lineages that share an especially close common ancestor with the Microsporidia. Other zygomycete lineages and *Rozella* are obviously candidates of interest. At the same time, it would be of great interest to increase the diversity of microsporidian genomic sequencing, in particular focusing on those lineages considered to be early-diverging Microsporidia, in the hopes that some of these larger genomes may contain more genes and, ideally, a much slower rate of sequence divergence. One group of possible interest are the metchnikovellids, hyperparasites that infect archaegregarine apicomplexa, themselves intracellular parasites of marine invertebrates. Metchnikovellids are poorly studied, but based on ultrastructure have been proposed to be a deep-branching member of the Microsporidia.

As a final note, the evidence for Microsporidia being at least related to the fungi seems now to be uncontestable, but it is worth remembering we have been in this situation before and were proved wrong. Though we cannot see how this could happen, it is always possible that future data will lead to a new, radical taxonomical twist for this challenging group of odd parasites. Whatever the future holds for this field, it is bound to be interesting, though perhaps not simple.

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