Available online at www.sciencedirect.com
SCIENCE DIRECT
Gene 375 (2006) 103–109
GENE
SECTION
EVOLUTIONARY GENOMICS
www.elsevier.com/locate/gene

Assessing the microsporidia-fungi relationship: Combined phylogenetic analysis of eight genes

Erin E. Gill *, Naomi M. Fast

Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada

Received 24 November 2005; received in revised form 15 February 2006; accepted 27 February 2006
Available online 20 March 2006
Received by T. Gojobori

Abstract

Microsporidia are unicellular eukaryotes that are obligate parasites of a variety of animals. For many years, microsporidia were thought to be an early offshoot of the eukaryotic evolutionary tree, and early phylogenetic work supported this hypothesis. More recent analyses have consistently placed microsporidia far from the base of the eukaryotic tree and indicate a possible fungal relationship, but the nature of the microsporidian–fungal relationship has yet to be determined. The concatenated dataset employed in this analysis consists of eight genes and contains sequence data from representatives of four fungal phyla. A consistent branching pattern was recovered among four different phylogenetic methods. These trees place microsporidia as a sister to a combined ascomycete+basidiomycete clade. AU tests determined that this branching pattern is the most likely, but failed to reject two alternatives.

Keywords: Ascomycetes; Basidiomycetes; Zygomycetes; Chytrids; Concatenated phylogeny; Evolution

1. Introduction

Microsporidia are a fascinating group of organisms from both a medical and an evolutionary point of view. These unicellular eukaryotes infect at least 1200 species of animals from every major evolutionary lineage, from crustacean to mammal, with a large proportion infecting insects (Wittner and Weiss, 1999). Microsporidia first came to the attention of humans when a strange parasite decimated lucrative European silkworm populations in the 19th century, but the drive to investigate these organisms was drastically increased by the more recent discovery of microsporidian infections in immuno-compromised humans, such as AIDS, organ transplant and cancer patients in the 1980s and 1990s.

Microsporidia alternate between two life forms — the spore and the meront, but only the spores are viable outside an animal host. Although diverse in size and shape, microsporidian spores have a typical morphology that includes a thick, protective proteinaceous coat containing chitin, and the polar filament, which is specialized for host invasion. This filament, also called the polar tube, is perhaps the most recognized microsporidian feature. It is attached to a plate at one terminus of the spore, is wound around the spore contents, and eventually ends near the posterior vacuole at the other end of the spore. Both the polar tube and the posterior vacuole play integral roles in spore germination and host infection. For a comprehensive review of the microsporidian life-cycle, see Keeling and Fast (2002).

Although microsporidia contain unique infective organelles, they lack several structures that are usually considered hallmarks of eukaryotic life, such as typical mitochondria, peroxisomes and centrioles. They also possess several seemingly "prokaryotic" characteristics, such as 70S ribosomes, tiny genomes and a fused 5.8S and 28S rRNA. For these reasons, microsporidia were long thought to be a primitive or ancestral eukaryotic lineage, that

Abbreviations: AU, approximately unbiased; BLAST, basic local alignment search tool; Cpn60, chaperonin 60; EF1-α, elongation factor 1-α; EST, expressed sequence tag; Hsp70, heat-shock protein 70; ML, maximum likelihood; NCBI, National Center for Biotechnology Information; PDH, pyruvate dehydrogenase; RAD25, DNA repair helicase; RPB1, largest subunit of RNA polymerase II; TBP, TATA-box binding protein; UBC2, subunit of the E2 ubiquitin conjugating enzyme.

* Corresponding author. Tel.: +1 604 822 6817; fax: +1 604 822 6089.
E-mail address: egill@interchange.ubc.ca (E.E. Gill).

0378-1119/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2006.02.023

diverged from the universal eukaryotic ancestor before the gain of the α-proteobacterial endosymbiont, which eventually became the mitochondrion (Cavalier-Smith, 1991). This hypothesis placed microsporidia into Kingdom Archezoa, a group of organisms defined by their primitive lack of mitochondria (Cavalier-Smith, 1991).

Initially, molecular data seemed to support the inclusion of microsporidia in the Archezoa. Notably, analyses of ribosomal RNA (Vossbrinck et al., 1987), elongation factor 1-alpha (EF1-α) and elongation factor 2 (EF-2) sequences (Kamaishi et al., 1996a,b) placed microsporidia at the base of the eukaryotic tree. Later, α- and β-tubulin phylogenies (Edlind et al., 1996; Keeling and Doolittle, 1996; Keeling et al., 2000) indicated a close relationship to fungi, in stark contradiction to the data supporting microsporidia as members of the Archezoa. Additional analyses conducted on mitochondrial Hsp70 (Germot et al., 1997; Hirt et al., 1997; Peyretaillade et al., 1998), TATA-box binding protein (Fast et al., 1999), the largest subunit of RNA polymerase II (RPB1) (Hirt et al., 1999), and pyruvate dehydrogenase subunits E1α and β (Fast and Keeling, 2001) bolstered the proposed microsporidia–fungi relationship. In line with the discovery of mitochondrion-derived genes in microsporidian genomes and a fungal ancestry for microsporidia, Williams et al. identified a cryptic mitochondrion in the microsporidian *Trachipleistophora hominis*, by immunolocalization of Hsp70 (Williams et al., 2002).

In addition, re-analysis of certain molecules, such as EF-2 (Hirt et al., 1999; Van de Peer et al., 2000) and LSU rRNA (Fischer and Palmer, 2005), did not support a basal origin of microsporidia when using a larger taxon set. As phylogenetic methodology advanced, shortcomings were also found with other previous analyses that had originally supported a basal position for microsporidia. For instance, EF1-α is not a suitable molecule to use in many phylogenetic analyses due to its mutation saturation and covarion behaviour, particularly in microsporidian sequences (Hirt et al., 1999; Inagaki et al., 2004).

Microsporidia share physiological and biochemical characteristics with fungi as well. Both have a similar meiotic mechanism, utilizing a closed spindle formation (Flegel and Pasharawipas, 1995), and they also share a common mRNA capping mechanism (Hausmann et al., 2002). However, these characteristics are not exclusive to fungi and microsporidia. Taken together, the phylogenetic, cytological and biochemical evidence indicate that microsporidia do have some tie with fungi, but the exact nature of this relationship has remained elusive, partially due to inadequate fungal representatives in analyses.

Two more recent analyses attempted to remedy this situation and came to differing conclusions. Tanabe et al. conducted analyses of RPB1 and EF1-α (Tanabe et al., 2002). These analyses included a wider sampling of fungal sequences, including representatives from four fungal phyla (ascomycetes, basidiomycetes, zygomycetes and chytrids), and results did not indicate a strong relationship between microsporidia and fungi. Instead, the microsporidia were placed at the base of a combined fungal/animal clade. In contrast to the Tanabe analysis, a phylogenetic study performed by Keeling not only strongly supported a relationship between microsporidia and fungi, but also proposed that microsporidia evolved from a zygomycete ancestor (Keeling, 2003). This is an important issue to be addressed herein: If microsporidia are related to fungi, as the majority of data indicate, are microsporidia the descendants of an actual fungus, or did they share a common ancestor with extant fungi?

Unfortunately, the evolutionary history of any lineage is difficult, if not impossible, to ascertain from the analysis of any single gene. In general, support values for branching patterns in trees are low with any dataset of restricted length. This is also true for phylogenies including microsporidia. By increasing the length of the dataset and the number of fungal species, our aim is to generate a more robust tree. Here we attempt to resolve the nature of the microsporidia–fungi relationship using a concatenated alignment of eight genes, containing 1666 amino acid characters.

2. Materials and methods

2.1. Gene and taxon selection

In order to clarify the relationship between microsporidia and fungi, genes were chosen for analysis based on the recent work of Thomarat et al. (Thomarat et al., 2004). Thomarat's group analyzed the genome of *Encephalitozoon cuniculi*, and conducted four types of phylogenetic analysis on each of several dozen genes. Each gene was annotated as branching at a specific point between fungi, animals and plants. Genes that consistently branched with fungi were selected for this study and the final dataset includes 8 genes: α-tubulin, β-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 E1α and β subunits of pyruvate dehydrogenase. Each of these genes was compared to available public sequence data using the BLAST algorithm (Altschul et al., 1997). The majority of sequences were retrieved from the NCBI databases, with the following exceptions. Data for the zygomycete *Rhizopus oryzae* were collected from the *Rhizopus* sequencing project performed at the Broad Institute of MIT and Harvard (http://www.broad.mit.edu/annotation/fungi/fgi/). *Spizellomyces punctatus* sequences were downloaded from the Protist EST Project public database (http://amoebidia.bcm.umontreal.ca/public/pepdb/agrm.php).

Taxa were identified that possessed sequences for all eight of the protein-coding genes listed above. Two representative species for each major fungal lineage were sought, however sequence data is relatively scarce for zygomycetes and chytrids. Although it is not ideal, given previous hypotheses regarding a relationship between microsporidia and zygomycetes, the limited available data only allowed for a single representative taxon for both zygomycetes and chytrids. Two animal taxa, a plant and an amoebozoan are also included. As *Blastocladiella emersonii* sequences were retrieved from an EST survey, not all of the genes used in the analysis were present at full-length. Therefore, significant portions of β-tubulin, RAD25, RPB1 and UBC2 were not included in the analysis. This dataset, hereafter refered to as the complete dataset, has 1666 characters and contains 12 species, for which all eight of the above genes are included.

A second dataset containing an additional chytrid representative, *S. punctatus*, was assembled (for explanation, see Section 3.1). Due to sequence availability, not all eight protein-coding genes could be included. This dataset has 858 characters — approximately half the number included in the complete dataset. UBC2, TBP and RAD25 sequences were not included.

For each individual protein-coding gene, up to 40 additional sequences were obtained from eukaryotic and bacterial representatives. These sequences were analyzed along with those of the 12 taxa present in the complete dataset in single gene phylogenetic analyses.

2.2. Phylogenetic analyses

Alignments of each of the eight protein sequences were constructed using CLUSTALW version 1.83 (Thompson et al., 1994) and manually edited using MacClade software version 4.06 (Maddison and Maddison, 1989). For the concatenated analysis, all eight alignments were placed sequentially in the same MacClade file. All analyses were conducted using the JTT substitution matrix (Jones et al., 1992) with 6 Γ rate categories, plus one invariable. The fraction of invariable sites and α-parameter were determined from each dataset using TREE-PUZZLE version 5.2 (Schmidt et al., 2002). TREE-PUZZLE settings were adjusted to calculate pairwise distances only and to generate exact parameter estimates. Maximum likelihood (ML) analyses were performed using IQPNNI (Important Quartet Puzzling and Nearest Neighbor Interchange) version 2.6 (Vin le and Von Haeseler, 2004) and PROML version 3.6b (Felsenstein et al., 2000) (for the concatenated datasets). Default settings were used for IQPNNI, while PROML settings were altered to perform a slow analysis, global rearrangements and randomize input order, jumbling ten times. Maximum likelihood–distance (ML–distance) analyses were performed using FITCH version 3.6b (Felsenstein et al., 2000), and Bayesian analyses (for the concatenated dataset) were performed with MRBAYES version 3.0b4 (Huelsenbeck and Ronquist, 2001). FITCH settings were altered to allow global rearrangements and randomize input order, jumbling ten times. MRBAYES was set to perform one million generations, sampling every 1000th generation. Four chains were used in the analysis, and the final consensus tree was constructed with a burn-in of 10,000 generations. Bootstrapping was conducted using PUZZLEBOOT and FITCH for ML–distance, and PHYML (Guindon and Gascuel, 2003) for ML analyses. One hundred pseudo-datasets were used for bootstrapping purposes for both methods. The approximately unbiased (AU) test of tree selection (Shimodaira, 2002) was performed using CONSEL version 1.10 (Shimodaira and Hasegawa, 2001) with default settings. Parameters were unlinked between genes. The site likelihoods for this method were determined by TREE-PUZZLE and reformatted utilizing a script written and provided by J. Leigh (Dalhousie University, Halifax, Nova Scotia, Canada).

The α-parameter was estimated by TREE-PUZZLE to be 0.85 for the full-length dataset, and 0.88 for the shorter dataset. The fraction of invariable sites was 0.2 for both datasets.

3. Results and discussion

3.1. Tree topologies and fungal phyla

Until recently, the lack of available sequences from both fungi and microsporidia prevented a large-scale phylogenetic analysis. In general, single gene phylogenies addressing the microsporidia–fungi relationship were not robust. Although microsporidian sequences consistently branched with fungal sequences in these analyses, the lack of sampling or poor phylogenetic resolution prevented the exact nature of the microsporidia–fungi relationship from being pinpointed. In 2003, Keeling's combined analysis of α- and β-tubulin sequences was the first to utilize data from more than one gene, and although support for placing microsporidia within the fungal radiation was high, the overall branching patterns of the trees were only moderately supported (Keeling, 2003). In the current study, we analyze more than twice as many characters to test the microsporidian phylogenetic position predicted by the tubulin trees. To date, this is the largest dataset employed to examine the relationship of microsporidia and fungi.

Before conducting the combined analysis, each molecule was analyzed separately. Maximum likelihood analyses of α- and β-tubulin, RPB1, RAD25, UBC2 and PDH E1α placed microsporidia within the fungal clade, whereas the TBP and PDH E1β analyses placed them at the base of a combined animal/fungal group. These results are similar to those obtained in previous analyses of RPB1, α- and β-tubulin, TBP and PDH E1α and E1β (Edlind et al., 1996; Hirt et al., 1999; Fast et al., 1999; Fast and Keeling, 2001; Thomarat et al., 2004). In general, bootstrap support values were very low. As the analysis of each of these molecules demonstrated either a microsporidia–fungi relationship or a fungi/animal–microsporidia relationship, all were included in the concatenated dataset. (See supplementary data for individual phylogenetic trees).

All of the concatenated analyses (ML, ML–distance and Bayesian), performed on the complete dataset recovered identical trees that placed microsporidia as a sister to a combined ascomycete+basidiomycete clade with 81% and 73% support for ML and ML–distance bootstrapping methods, respectively. (See Fig. 1) Bootstrap values for both methods lie above 70% at all nodes, with the vast majority being greater than 90%. Bayesian posterior probabilities are 100 at all nodes. To test the strength of the position of the microsporidia, AU tests were carried out. AU tests were conducted on topologies constructed by moving the microsporidia (as a pair) to all alternative positions within the original tree. Tests found the ML/ML–distance/Bayesian tree (i.e. Fig. 1) to be the most likely, with an AU probability of 0.872. However, the next two most likely trees, those placing microsporidia at the base of the fungal clade, and as a sister to the basidiomycetes, respectively, cannot be rejected at a significance level of 5%. In fact, the optimal tree (with microsporidia branching as the sister to ascomycetes+basidiomycetes) possesses a high degree of dominance over the alternatives that possess AU probabilities of 0.369 (base of the fungi) and 0.183 (sister to basidiomycetes). (See closed and open circles in Fig. 1).

A.

Position of Microsporidia	AU Probability
Sister to Ascomycetes + Basidiomycetes	0.872
Base of Fungi	0.369
Sister to Basidiomycetes	0.183

B.

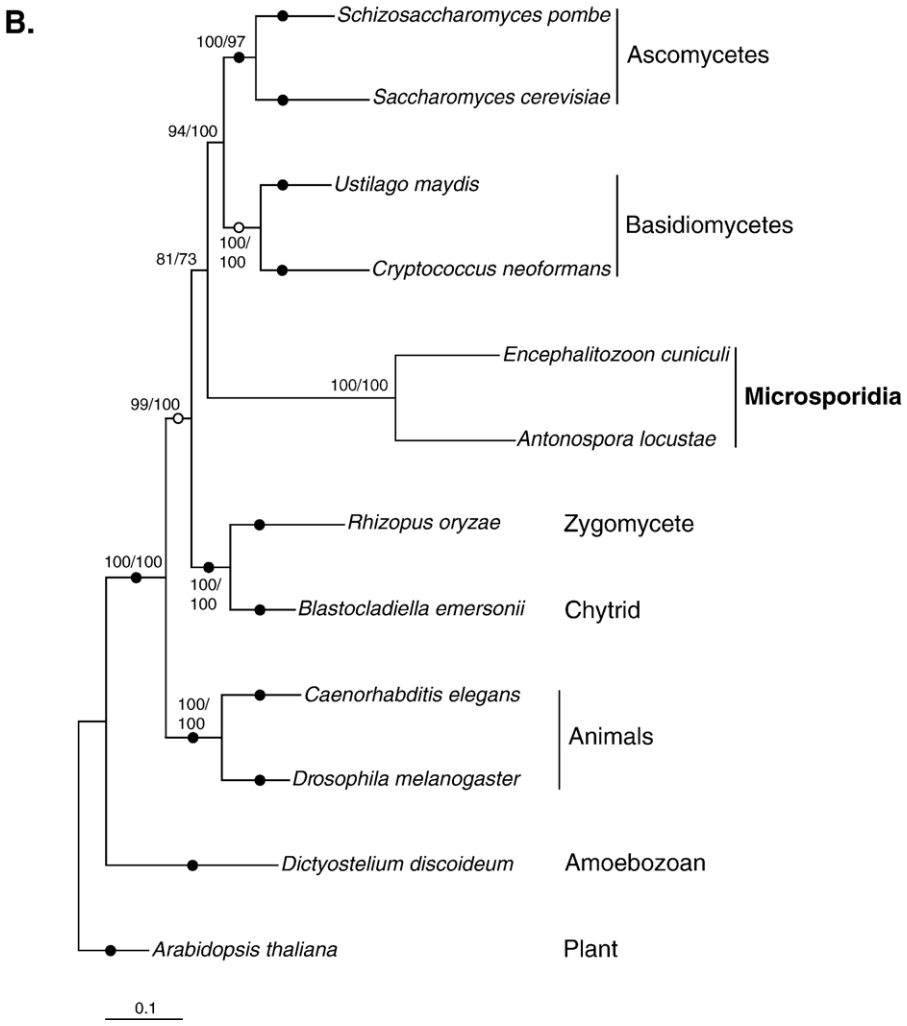

Fig. 1. A. AU probabilities for the three branching patterns not rejected at a 5% significance level. B. PROML tree generated from the complete concatenated dataset (1666 characters, 6Γ categories plus one invariable). Bootstrap values are indicated at nodes. The first and second numbers represent percentages from ML (PHYML) and ML–distance (FITCH) bootstrap methods, respectively. An open circle represents a position for microsporidia that was not rejected in AU tests at a significance level of 5%. A closed circle represents a position for microsporidia that was rejected in AU tests.

Strangely, *R. oryzae* and *B. emersonii*, a zygomycete and a chytrid, respectively, branched together with 100% bootstrap support in the recovered tree. This topology was somewhat unexpected, but probably reflects our current understanding of fungal systematics. A recent and comprehensive multi-locus analysis from the "Assembling the Fungal Tree of Life" (AFTOL) project indicates that the zygomycetes and chytrids are probably not monophyletic (Lutzoni et al., 2004). Indeed, the AFTOL analyses placed the blastocladialean chytrid representative, *Allomyces*, within one of several otherwise zygomycete clades. This result is not unique: an analysis conducted on ribosomal DNA sequences from dozens of chytrids placed the blastocladialean chytrid fungi within a combined zygomycete/blastocladialean clade (James et al., 2000). Ideally, several representatives of both the zygomycetes and the chytrids would have been included in this study, especially given the somewhat tenuous position of the Blastocladiales within the fungal tree. However, sequence sampling in these groups is slim. There is, however, a limited amount of sequence data from a different chytrid, *S. punctatus*, which is not a member of the Blastocladiales lineage. A second concatenated dataset was constructed including both chytrids, but it is much shorter, containing approximately half the characters of the complete dataset. (See Section 2.1).

Trees generated from the shorter dataset place *Blastocladiella* and *Spizellomyces* together as a sister clade to *Rhizopus*, again with 100% support. (See Fig. 2) The topology of this tree differs from that of the complete dataset in the placement of the

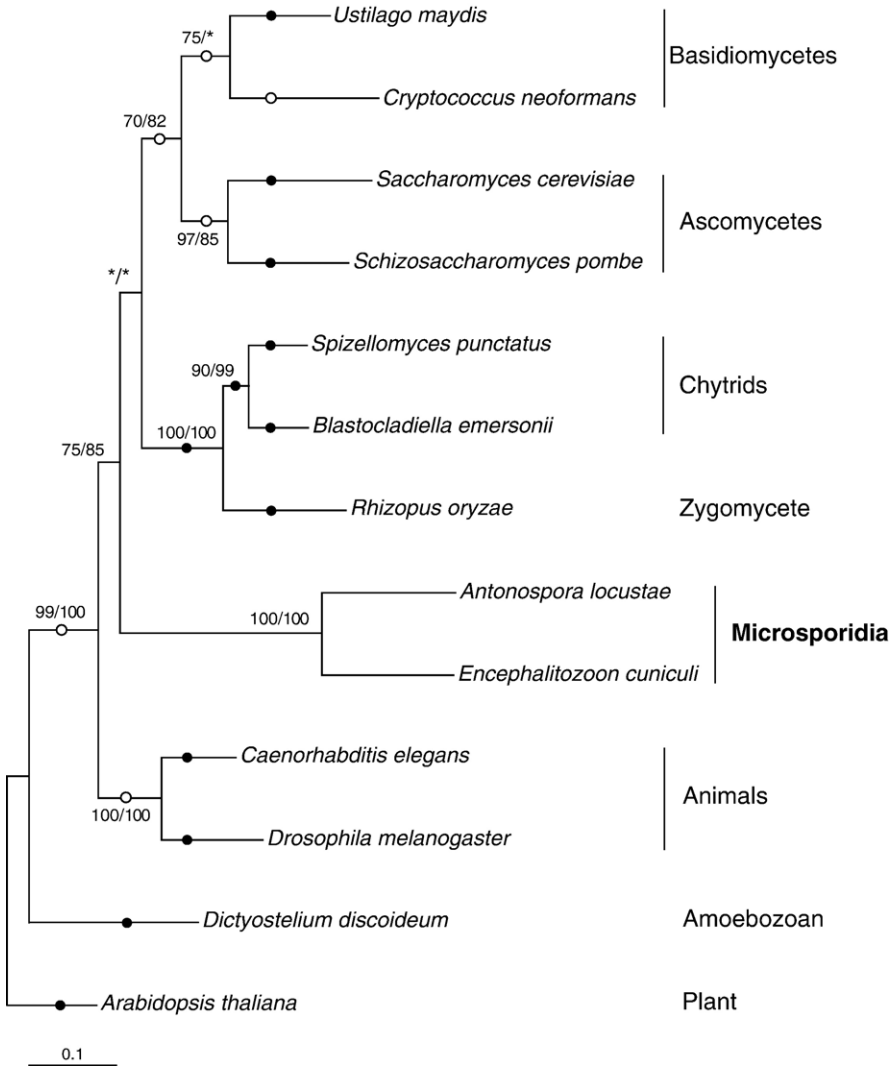

Fig. 2. PROML tree generated from the short concatenated dataset including *Spizellomyces punctatus* (858 characters, 6 Γ categories plus one invariable). Bootstrap values are indicated at nodes. The first and second numbers represent percentages from ML (PHYML) and ML–distance (FITCH) bootstrap methods, respectively. Open and closed circles are as described for Fig. 1. Asterixes indicate branching patterns that were not recovered by bootstrapping.

microsporidia. In this case, they occupy a position at the base of the fungi, one of the topologies not rejected by AU tests performed on the complete dataset. However, this position is not supported, as it was not recovered by either bootstrapping method. In general, this tree's topology is not nearly as well-supported as that of the complete dataset — likely because it is only based on approximately half as many characters. Bootstrap values range from 70% to 100%, with two nodes not recovered from the majority of bootstrapping datasets for one or both methods. This lack of support is also evident in AU tests, where the position of the microsporidian pair was tested as described above for the complete dataset. AU tests of 19 topologies fail to reject seven alternatives at a 5% significance level, including microsporidia as a sister to the animals, as a sister to the basidiomycetes, as a sister to the ascomycetes, as a sister to *Dictyostelium* and *Arabidopsis*, as a sister to *Cryptococcus* and at the base of the animal/fungal clade (see closed and open circles in Fig. 2). Perhaps it is notable that AU testing identified the most likely tree as that placing the microsporidian pair with the ascomycetes+basidiomycetes, the strongly supported topology recovered in the analysis of the complete dataset.

3.2. Considering the phylogenetic position of microsporidia

During the past 20 years, microsporidia have been placed at several different nodes on the eukaryotic tree. Initial molecular evidence seemed to indicate an ancient origin, while more recent single gene phylogenies consistently grouped microsporidia with fungi. However, the exact nature of the relationship between microsporidia and fungi has remained unclear. Tanabe and Keeling attempted to solve this problem by broadening the fungal representation in their analyses (Keeling, 2003; Tanabe et al., 2002). The results of Tanabe's EF1-α analysis were in concordance with early work, recovering microsporidia near the base of the eukaryotic tree, whereas his RPB1 analysis placed microsporidia at the base of an animal/fungal clade. Keeling's analyses placed microsporidia within the zygomycete clade. Trees generated from the multi-gene

analyses presented here recover another possibility: microsporidia as a sister to the ascomycetes and basidiomycetes. This topology is well supported statistically, however, other topologies – including microsporidia branching as a sister to the fungi – were not rejected by AU tests.

Although there are many ascomycetes and basidiomycetes that infect animals (for example, the basidiomycete *Cryptococcus*), these animal parasites tend to be found as terminal branches of the fungal tree, nested within free-living clades of ascomycetes and basidiomycetes (Berbee, 2001). This suggests that the ancestor of ascomycetes+basidiomycetes was free-living, and not an animal parasite. Although this seems at odds with microsporidia having a phylogenetic position as a sister to ascomycetes+basidiomycetes (as in Fig. 1), there remains the formal possibility that such a parasitic ancestor of ascomycetes+basidiomycetes did exist at some point but is now extinct. It is, perhaps, easier to reconcile microsporidian life style with a zygomycete heritage as suggested by the tubulins (Keeling, 2003). Zygomycetes and chytrids have many members (including putative basal lineages) that are animal parasites (with many infecting insects). However, tubulins are the only molecules to suggest this history, and one has to consider weaknesses inherent in using tubulins as phylogenetic markers. It has previously been noted that the loss of flagella and other 9+2 microtubule structures has led to accelerated rates of evolution in tubulin genes (Keeling et al., 2000; Keeling, 2003), and this trend is clearly evident in sequences from ascomycetes, basidiomycetes, zygomycetes and microsporidia. Chytrid sequences are much less divergent, as chytrids possess flagella for part of their lifecycle. This discrepancy in rates could compromise the phylogenetic usefulness of tubulins to resolve fungal and microsporidian relationships. In fact, long branch attraction has been raised as a reason to question the microsporidia–fungi relationship based on tubulin phylogenies. This may be a legitimate concern when resolving the position of microsporidia within the fungal radiation in tubulin trees. However, the suggestion that microsporidia and fungi only branch together in tubulin trees because of long branch attraction has been discredited by analyses showing a microsporidia–fungi relationship when the only fungal representatives in the tree were the short-branched chytrid sequences (Keeling et al., 2000). In the current combined analysis, the branch lengths are much more conservative and consistent across fungi, however, one could still argue that the microsporidia and the ascomycetes+basidiomycetes do possess the longer branches in the tree. Nevertheless, the bootstrap support for the sisterhood of microsporidia and ascomycetes+basidiomycetes is fairly strong.

Although the validity of EF-1α as a phylogenetic marker to assess microsporidian relationships has seriously been called into question, Tanabe et al. propose that indel information in EF-1α could shed light on the relationship between microsporidia and fungi (Tanabe et al., 2002). They identify a two amino acid deletion that is present in all examined fungi, but is absent in microsporidia. This character unites fungi to the exclusion of microsporidia, and implies that microsporidia and fungi could only be related as sisters. Although there are currently only three EF-1α sequences from microsporidia in the NCBI database (from *Antonospora locustae*, *E. cuniculi*, and *Glugea plecoglossi*), they do represent a large fraction of microsporidian diversity. On its own the indel data might not be too compelling, however it is consistent with results of this study where the sisterhood of microsporidia and fungi could not be statistically rejected by analysis of either dataset.

Although this study significantly increases the amount of data used to address the relationship between microsporidia and fungi, a single strongly supported phylogenetic position is not clearly evident. If microsporidia are sisters to ascomycetes+basidiomycetes – a provocative possibility that would have serious implications for the development of parasitism within the fungi – we expect that future phylogenetic analyses will recover similar results and bolster this position. However, we do not discount the possibility that microsporidia and fungi are sisters, based on the EF-1α indel and our own phylogenetic results that do not reject this possibility. When more EF-1α sequences become available, particularly from microsporidia considered to be basal, the usefulness of the indel as a phylogenetic marker can be more effectively evaluated. Until that time, the exact nature of the relationship between microsporidia and fungi remains unclear.

4. Conclusions

(1) A combined maximum likelihood analysis of 8 genes (1666 characters) placed microsporidia within the fungal clade, as a sister to the ascomycetes+basidiomycetes.
(2) This placement of microsporidia is supported by bootstrap values of 81% and 73% for maximum likelihood and maximum likelihood–distance methods, respectively.
(3) AU tests revealed that the ML/ML–distance/Bayesian tree is the most likely, however two alternative topologies could not be rejected at a significance level of 5%. These trees place the microsporidia as a sister to the basidiomycetes and at the base of the fungi. The latter possibility is in line with the distribution of an indel in EF-1α (Tanabe et al., 2002).

Acknowledgements

We would like to thank J. Leigh at Dalhousie University for her assistance with AU tests and provision of helpful programs, M. Berbee and M. Rogers for helpful discussions, and P. Keeling and R. Lee for providing comments on the manuscript. EEG's work is supported by a Canada Graduate Scholarship from NSERC and research in the Fast lab is funded by NSERC Discovery Grant No. 262988 to NMF.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2006.02.023.

References

Altschul, S.F., et al., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Berbee, M.L., 2001. The phylogeny of plant and animal pathogens in the Ascomycota. Physiol. Mol. Plant Pathol. 59, 165–187.

Cavalier-Smith, T., 1991. Archamoebae: the ancestral eukaryotes? Biosystems 25, 25–38.

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

Fast, N.M., Keeling, P.J., 2001. Alpha and beta subunits of pyruvate dehydrogenase E1 from the microsporidian *Nosema locustae*: mitochondrion-derived carbon metabolism in microsporidia. Mol. Biochem. Parasitol. 117, 201–209.

Fast, N.M., Logsdon, J.M., Doolittle, W.F., 1999. Phylogenetic analysis of the TATA box binding protein (TBP) gene from *Nosema locustae*: evidence for a microsporidia–fungi relationship and spliceosomal intron loss. Mol. Biol. Evol. 16, 1415–1419.

Felsenstein, J., et al., 2000. PHYLIP: Phylogeny Inference Package. http://evolution.genetics.washington.edu/phylip.html.

Fischer, W.M., Palmer, J.D., 2005. Evidence from small-subunit ribosomal RNA sequences for a fungal origin of microsporidia. Mol. Phylogenet. Evol. 36, 606–622.

Flegel, T.W., Pasharawipas, T., 1995. A proposal for typical meiosis in microsporidians. Can. J. Microbiol. 41, 1–11.

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

Guindon, S., Gascuel, O., 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

Hausmann, S., Vivares, C.P., Shuman, S., 2002. Characterization of the mRNA capping apparatus of the microsporidian parasite *Encephalitozoon cuniculi*. J. Biol. Chem. 277, 96–103.

Hirt, R.P., Healey, B., Vossbrinck, C.R., Canning, E.U., Embley, T.M., 1997. A mitochondrial Hsp70 orthologue in *Varimorpha necatrix*: molecular evidence that microsporidia once contained mitochondria. Curr. Biol. 7, 995–998.

Hirt, R.P., Logsdon, J.M., Healy, B., Dorey, M.W., Doolittle, W.F., Embley, T.M., 1999. Microsporidia are related to fungi: evidence from the largest subunit of RNA polymerase II and other proteins. Proc. Natl. Acad. Sci. U. S. A. 96, 580–585.

Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Inagaki, Y., Susko, E., Fast, N.M., Roger, A.J., 2004. Covarion shifts cause a long-branch attraction artifact that unites microsporidia and archaebacteria in EF-1 alpha phylogenies. Mol. Biol. Evol. 21, 1340–1349.

James, T.A., Porter, D., Leander, C.A., Vilgalys, R., Longcore, J.E., 2000. Molecular phylogenetics of the Chytridiomycota supports the utility of ultrastructural data in chytrid systematics. Can. J. Bot. 78, 336–350.

Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275–282.

Kamaishi, T., et al., 1996a. Protein phylogeny of translation elongation factor EF-1alpha suggests microsporidians are extremely ancient eukarotes. J. Mol. Evol. 42, 257–263.

Kamaishi, T., et al., 1996b. Complete nucleotide sequences of the genes encoding translation elongation factors 1 alpha and 2 from a microsporidian parasite, *Glugea plecoglossi*: implications for the deepest branching of eukaryotes. J. Biochem. (Tokyo) 120, 1095–1103.

Keeling, P.J., 2003. Congruent evidence from alpha-tubulin and beta-tubulin gene phylogenies for a zygomycete origin of microsporidia. Fungal Genet. Biol. 38, 298–309.

Keeling, P.J., Doolittle, W.F., 1996. Alpha-tubulin from early-diverging eukaryotic lineages and the evolution of the tubulin family. Mol. Biol. Evol. 13, 1297–1305.

Keeling, P.J., Fast, N.M., 2002. Microsporidia: biology and evolution of highly reduced intracellular parasites. Annu. Rev. Microbiol. 56, 93–116.

Keeling, P.J., Luker, M.A., Palmer, J.D., 2000. Evidence from beta-tubulin phylogeny that microsporidia evolved from within the fungi. Mol. Biol. Evol. 17, 23–31.

Lutzoni, F., et al., 2004. Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. Am. J. Bot. 91, 1446–1480.

Maddison, W.P., Maddison, D.R., 1989. Interactive analysis of phylogeny and character evolution using the computer program MacClade. Folia Primatol. 53, 190–202.

Peyretaillade, E., Broussolle, V., Peyret, P., Metenier, G., Gouy, M., Vivares, C.P., 1998. Microsporidia, amitochondrial protists, possess a 70-kDa heat shock protein gene of mitochondrial evolutionary origin. Mol. Biol. Evol. 15, 683–689.

Schmidt, H.A., Strimmer, K., Vingron, M., von Haeseler, A., 2002. TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics 18, 502–504.

Shimodaira, H., 2002. An approximately unbiased test of phylogenetic tree selection. Syst. Biol. 51, 492–508.

Shimodaira, H., Hasegawa, M., 2001. CONSEL for assessing the confidence of phylogenetic tree selection. Bioinformatics 17, 1246–1247.

Tanabe, Y., Watanabe, M.M., Sugiyama, J., 2002. Are microsporidia really related to fungi?: a reappraisal based on additional gene sequences from basal fungi. Mycol. Res. 106, 1380–1391.

Thomarat, F., Vivares, C.P., Gouy, M., 2004. Phylogenetic analysis of the complete genome sequence of *Encephalitozoon cuniculi* supports the fungal origin of microsporidia and reveals a high frequency of fast-evolving genes. J. Mol. Evol. 59, 780–791.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Van de Peer, Y., Ben Ali, A., Meyer, A., 2000. Microsporidia: accumulating molecular evidence that a group of amitochondriate and suspectedly primitive eukaryotes are just curious fungi. Gene 246, 1–8.

Vin le, S., Von Haeseler, A., 2004. IQPNNI: moving fast through tree space and stopping in time. Mol. Biol. Evol. 21, 1565–1571.

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

Williams, B.A.P., Hirt, R.P., Lucocq, J.M., Embley, T.M., 2002. A mitochondrial remnant in the microsporidian *Trachipleistophora hominis*. Nature 418, 865–869.

Wittner, M., Weiss, L.M., 1999. The Microsporidia and Microsporidosis. ASM Press, Washington, D.C.