Evolution of a morphological novelty occurred before genome compaction in a lineage of extreme parasites

Karen L. Haag[a,b,1], Timothy Y. James[c], Jean-François Pombert[d], Ronny Larsson[e], Tobias M. M. Schaer[a], Dominik Refardt[a,f], and Dieter Ebert[a]

[a]Zoological Institute, Basel University, 4051 Basel, Switzerland; [b]Department of Genetics, Federal University of Rio Grande do Sul, Porto Alegre, 91501-970 RS, Brazil; [c]Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109; [d]Department of Biological and Chemical Sciences, Illinois Institute of Technology, Chicago, IL 60616; [e]Department of Biology, University of Lund, SE-223 62 Lund, Sweden; and [f]Institute of Natural Resource Sciences, Zurich University of Applied Sciences, 8820 Wädenswil, Switzerland

Edited by Patrick J. Keeling, University of British Columbia, Vancouver, BC, Canada, and accepted by the Editorial Board September 10, 2014 (received for review June 9, 2014)

Intracellular parasitism results in extreme adaptations, whose evolutionary history is difficult to understand, because the parasites and their known free-living relatives are so divergent from one another. Microsporidia are intracellular parasites of humans and other animals, which evolved highly specialized morphological structures, but also extreme physiologic and genomic simplification. They are suggested to be an early-diverging branch on the fungal tree, but comparisons to other species are difficult because their rates of molecular evolution are exceptionally high. Mitochondria in microsporidia have degenerated into organelles called mitosomes, which have lost a genome and the ability to produce ATP. Here we describe a gut parasite of the crustacean *Daphnia* that despite having remarkable morphological similarity to the microsporidia, has retained genomic features of its fungal ancestors. This parasite, which we name *Mitosporidium daphniae* gen. et sp. nov., possesses a mitochondrial genome including genes for oxidative phosphorylation, yet a spore stage with a highly specialized infection apparatus—the polar tube—uniquely known only from microsporidia. Phylogenomics places *M. daphniae* at the root of the microsporidia. A comparative genomic analysis suggests that the reduction in energy metabolism, a prominent feature of microsporidian evolution, was preceded by a reduction in the machinery controlling cell cycle, DNA recombination, repair, and gene expression. These data show that the morphological features unique to *M. daphniae* and other microsporidia were already present before the lineage evolved the extreme host metabolic dependence and loss of mitochondrial respiration for which microsporidia are well known.

microsporidia | genome evolution | phylogenomics | evolution of extreme parasitism | *Daphnia*

Microsporidia are intracellular parasites that represent the extreme of known genome simplification and size reduction among eukaryotes (1). There are more than 1,200 described microsporidia species (2), with several having an economic impact by causing disease in animals such as fish and honey bees, and are a problem to human health, in particular since the AIDS pandemic. Microsporidia are currently placed on an ancestral branch within the fungi, although this placement has only recently been worked out, due to the absence of clear morphological and physiological connections to other eukaryotes and the profound changes resulting from adaptations to an intracellular lifestyle (3–6). The evolutionary history of microsporidia is marked by the loss of several eukaryotic features, such as mitochondria (7), a typical Golgi apparatus (8), a flagellum (3), and the evolutionary innovation of an infection apparatus, the polar tube. Microsporidia evolved distinctive genetic features, such as massive loss of genes, leading to the smallest known eukaryotic genomes (1). Remnants of mitochondria appear in microsporidian cells as DNA-free organelles called mitosomes (9, 10) that perform simplified versions of the original mitochondrial functions, such as the assembly of iron-sulfur clusters (11), but no ATP production via citrate cycle and oxidative phosphorylation (12, 13). For example, the human parasite *Enterocytozoon bieneusi* seems to have no fully functional pathway to generate ATP from glucose (12), relying on transporters to import ATP from its host. These ATP transporters have been acquired by horizontal gene transfer (HGT) from intracellular parasitic bacteria (13). Despite major progress in the understanding of microsporidian biology, it is still unclear how this clade of extreme parasites evolved.

The key to understanding the sequence of events leading to extreme parasitism is a well-resolved phylogeny. Microsporidia pose a problem in this regard due to their phenomenal molecular rate acceleration, possibly related to loss of cell cycle control genes (14). A phylogenetic analysis of 53 conserved concatenated genes supports a topology in which microsporidia is the most basal branch in the fungal tree (5). Another phylogenetic analysis based on 200 genes included the endoparasite *Rozella allomycis*, a representative of the recently discovered basal fungal lineage Cryptomycota (15, 16), and placed microsporidia and Cryptomycota together on the most basal fungal branch (4). One of the shared genomic elements between microsporidia and *R. allomycis* is the nucleotide transporter that is used by microsporidia for stealing energy in the form of ATP from their hosts;

Significance

Intracellular obligate parasitism results in extreme adaptations, whose evolutionary history is difficult to understand, because intermediate forms are hardly ever found. Microsporidia are highly derived intracellular parasites that are related to fungi. We describe the evolutionary history of a new microsporidian parasite found in the hindgut epithelium of the crustacean *Daphnia* and conclude that the new species has retained ancestral features that were lost in other microsporidia, whose hallmarks are the evolution of a unique infection apparatus, extreme genome reduction, and loss of mitochondrial respiration. The first evolutionary steps leading to the extreme metabolic and genomic simplification of microsporidia involved the adoption of a parasitic lifestyle, the development of a specialized infection apparatus, and the loss of diverse regulatory proteins.

Author contributions: K.L.H. and D.E. designed research; K.L.H., T.Y.J., J.-F.P., R.L., T.M.M.S., and D.R. performed research; R.L. and D.E. contributed new reagents/analytic tools; K.L.H., T.Y.J., J.-F.P., R.L., and D.E. analyzed data; and K.L.H., T.Y.J., J.-F.P., R.L., and D.E. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. P.J.K. is a guest editor invited by the Editorial Board.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database for GenBank BioProject PRJNA243305 and BioSample SAMN02714227 (accession no. JMKJ00000000).

[1]To whom correspondence should be addressed. Email: karen.haag@ufrgs.br.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1410442111/-/DCSupplemental.

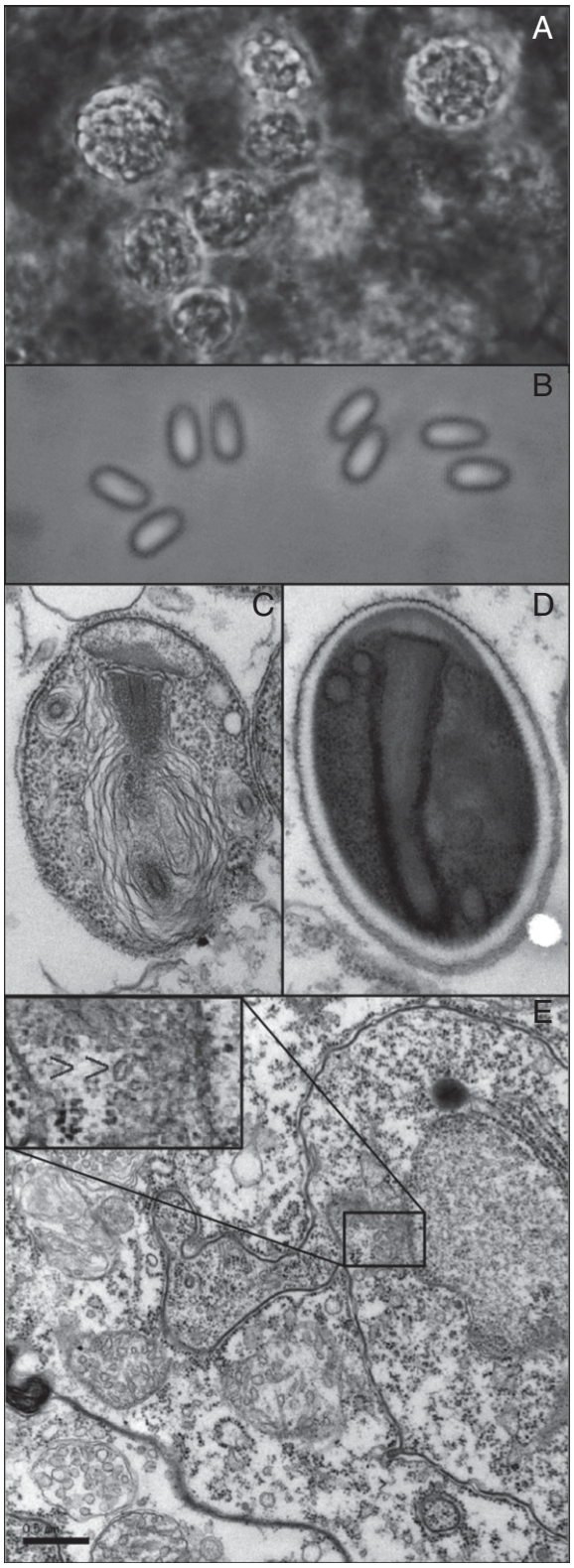

Fig. 1. Light (*A* and *B*) and transmission electron microscopic (*C*–*E*) pictures of *M. daphniae*. (*A*) Part of the posterior gut of *Daphnia magna* with vesicles of *M. daphniae*. (*B*) Individual mature spores of *M. daphniae*. (*C* and *D*) Longitudinal sections of immature (*C*) and mature (*D*) spores. A three-layered spore wall with exo-and endospore layers is present. A structure similar to the microsporidian infection apparatus with polar sac, polar filament, and polaroplast is clearly visible. (*E*) *Plasmodium* interdigitating with the host cell (not a microsporidian character). In the proximity of the nucleus a mitochondrium-like structure with a double membrane can be seen (enlarged in the *Inset* and marked with double arrowheads).

however, *Rozella* harbors a mitochondrion containing a genome, its proteome has not undergone major contraction, and it does not show the typical infection apparatus of microsporidia: the polar tube. However, the sequence of events that occurred at the root of microsporidian evolution is still unclear. Here we describe the evolutionary history of an unusual intracellular parasite of the hindgut of *D. magna* that morphologically resembles gut-inhabiting microsporidia (17, 18). Ultrastructural examination reveals profound morphological similarities to microsporidia, including a polar tube. However, the genome of the new species surprisingly differs from known microsporidia by the presence of a mitochondrial genome and genes coding for ATP production from glucose via citrate cycle and oxidative phosphorylation. Its genome sequence allows us to produce a well-resolved phylogeny, which shows that the new parasite, *Mitosporidium daphniae*, is the most early diverging microsporidian described to date. Here we provide a full morphological description of the new species, analyze its genome, and use the underlying data to make inferences about the order of events that occurred during the evolution of microsporidia.

Results and Discussion

Species Description. The new species, which we formally name *Mitosporidium daphniae* gen. et sp. nov., is known from northwestern Europe (Belgium, Germany, and United Kingdom) and has been used in earlier studies, without formal classification, where it was shown to negatively affect the fitness of the host (19). Most distinctly, this gut parasite has a three-layered spore wall with an endospore, exospore, and plasma membrane and contains a structure similar to the microsporidian infection apparatus with polar sac, polar filament, and polaroplast (Fig. 1). Briefly, the new genus and species are described as follows. *Mitosporidium* gen. nov.: all stages have isolated nuclei. A multinucleate sporogonial plasmodium generates a great and irregular number (>20) of ovoid or slightly bent, uninucleate spores in a sporophorus vesicle lacking prominent inclusions of the episporontal space. Spores have a thin, electron opaque exospore and a wide, electron lucent endospore, a uniform, weakly developed polaroplast of concentrically arranged lamellae, and an anisofilar polar filament with three regions of posteriad reduced width. Polar filament connects anteriorly to a curved polar sac. *M. daphniae* sp. nov.: with the basic character of the genus and the following additions: spore size range, 2.31–2.67 × 1.05–1.29 μm (live material, $n = 40$); polar filament arranged as two steeply tilted coils (coiling starts the posterior pole of the spore, angle of tilt about 35°), wide anterior part twice as wide as the narrowest coil; polaroplast reaches the equator of the spore. Type host: *Daphnia magna* (Crustacea, Cladocera). A full description is presented in *SI Text* and Figs. S1–S4.

Genome Sequencing, Annotation, and Phylogenetics. Using shotgun sequencing of DNA extracted from isolated parasite spores, we generated a draft genome of *M. daphniae* (*SI Materials and Methods*) assembling into 612 contigs totaling 5.64 Mbp with an N50 of 32.031 kbp (350× average coverage). A total of 3,300 proteins were predicted, for a coding density of 0.585 proteins/kbp, with an average of 1.27 introns per protein. Despite showing a twofold difference in size, the *M. daphniae* genome is only slightly more compact than that of the water mold parasite *R. allomycis* (11.86 Mbp; 0.535 proteins/kbp). The number of predicted proteins in *M. daphniae* is roughly half that of *R. allomycis* and on par with the 3,266 ORFs predicted in 8.5+ Mbp genome of the microsporidian human pathogen *Trachipleistophora hominis* (20). The relative levels of similarity observed for the *M. daphnia*

proteins suggest that it is closely related to both other microsporidian species and to *R. allomycis* (Table S1).

A phylogeny based on 53 conserved orthologous genes (5) gives strong support for placing *M. daphniae* as the most early diverging microsporidian and, together with the microsporidia, sister to the cryptomycete *R. allomycis* (Fig. 2). The statistical robustness of this phylogenetic placement was tested by rearranging the position of *M. daphniae* to every other possible position on the tree, and using this rearranged tree as a constraint for a maximum likelihood search in which the relationships between the other taxa were free to vary. We statistically rejected ($P \leq 0.001$) each of the 50 possible alternative placements of *M. daphniae* as a likely alternative placement using the approximately unbiased test (21). Because concatenation may produce incorrect phylogenetic relationships due to systematic biases in the data (22), we evaluated the phylogenetic relationships suggested for each protein partition. The basal position of *M. daphniae* among microsporidia was observed in 37% of the individual genes, similar to the support for Dikarya (40%) or Mucoromycotina + Mortierellomycotina (37%). Our tree, therefore, provides strong evidence that *M. daphniae* is the earliest-diverging microsporidian sequenced to date and suggests that microsporidia are derived from within or are sister to the Cryptomycota. A phylogenetic analysis using solely the fast-evolving ribosomal RNA genes recovers a phylogeny consistent with this finding (Fig. S5). Furthermore, the *M. daphniae* rRNA operons contain eukaryotic-sized rRNA subunits that lack the characteristic 5.8S/23S gene fusion observed in derived microsporidia (Fig. 3) (21, 23).

Comparative Genomics. We analyzed the *M. daphniae* genome to identify genes that it shares uniquely with microsporidia or other fungi. Foremost among microsporidia traits is the absence of a mitochondrial genome, yet the genome assembly of *M. daphniae* revealed a single contig of 14,043 bp with a sequencing coverage of 765× representing a mitochondrial genome (Fig. S6). This mitochondrial genome seems to be linear, because it has terminal inverted repeats of 477 bp. Linear mitochondrial chromosomes with terminal inverted repeats are known in fungi and supposedly represent an evolutionary transition between circular and "true" linear (with telomeric ends) mitochondrial genomes (24). The mt-genome of the cryptomycete *R. allomycis* is circular, but otherwise, the *M. daphniae* and *R. allomycis* mt-genomes have an nearly identical gene content, and a phylogenetic analysis of mt-genome encoded proteins shows that the two genomes are closely related (Fig. S7). The *M. daphniae* mitochondrial genome encodes 17 tRNAs, small and large ribosomal subunits, and six proteins belonging to complexes II, III, and IV of the electron transport chain (ETC). The mitochondrial genome is also apparently functional; intact reading frames for the mitochondrial RNA and DNA polymerases are found in the nuclear genome (Table S1). Genes encoding proteins involved in pathways related to energy metabolism are more numerous in *M. daphniae* compared with the well-studied microsporidium *Encephalitozoon cuniculi* (Fig. S8) or other microsporidian species. On the other hand, *M. daphniae* lacks all genes of respiratory chain complex I (NADH dehydrogenase), just as observed in *R. allomycis* (Fig. 3). However, the *M. daphniae* nuclear genome encodes two genes, internal NADH dehydrogenase and alternative oxidase, that are presumably capable of moving electrons through the ETC without pumping protons. *R. allomycis* differs from *M. daphniae* in having an additional dehydrogenase, external NADH dehydrogenase that presumably projects into the intermembrane space (4). This partial oxidative phosphorylation pathway combined with a complete citrate cycle suggest that *M. daphniae* mitochondria are capable of producing ATP, although without generating as much energy as a complete ETC (Fig. 3 and Fig. S9). Surprisingly, we did not find an ATP transporter gene that is common to all microsporidia and used to steal ATP from the host, which was also detected in the *Rozella* genome. This suggests that the ATP transporter gene has either been lost in *M. daphniae*, was horizontally transferred independently to microsporidia and *Rozella* from the same lineage of intracellular parasitic bacteria (*Chlamydia*), or is not present in the current assembly of the *M. daphniae* genome. Because our genome has about 350× sequencing coverage, failing to assemble the gene is unlikely. However, the ATP transporter gene might have been lost or diverged to become unrecognizable in the genome of *M. daphniae*. Additional cryptomycete and microsporidian genomes will be needed to work this out. The presence of an ATP transporter gene in the early stages of microsporidia evolution, when mitochondria were still functional, might have been transient.

Fungal genomes encode a diverse set of chitin synthase genes that are involved in producing the polymer that provides structure to the fungal cell wall. Among the known chitin synthase genes, one is restricted to fungi, known as the division II chitin synthases (25). Moreover, only fungi possess a chitin synthase gene with a myosin domain—the class V, division II chitin synthases (26)—with the exception of the choanozoan *Corallochytrium limacisporum* (27). The myosin motor domain of the chitin synthase is believed to function by depositing chitin at particular regions of the plasma membrane via the cytoskeletal highway and is associated with polarized secretion and apical growth in fungi (28). The genome of *M. daphniae* encodes four chitin synthases, all of division II; however, there is no class V chitin synthase containing a myosin domain (Fig. 3 and Table S1). Because microsporidia germinate via a highly specialized polar tube extrusion mechanism (29) and grow in vivo by schizogony, the myosin domain might no longer have been necessary as there is no polarized growth phase. If this hypothesis is correct, the related group aphelids, which like *Rozella* appear to grow into their hosts through the formation of a germ tube (30) with a cell wall, would be predicted to have a chitin synthase with a myosin domain. *M. daphniae* and other microsporidia presumably solely require chitin for the development of the cell wall of the resting spore. Resting spores disperse passively without motility, unlike *Rozella* and aphelids, which use a flagellum for dispersal. Indeed, none of the proteins that are found in all eukaryotes exhibiting flagellar movement (31) are found in the proteomes of *M. daphniae* and other microsporidia (Fig. 3 and Table S1).

The proteome of *M. daphniae*, albeit smaller, resembles to a large extent the fungi (Fig. S8*A*). We identified 3,330 proteins, of which 2,200 belong to 1,878 gene families, or orthologous groups (OGs). Thus, about 34% of the predicted proteome corresponds to proteins without detectable similarity to any other available sequences. We compared *M. daphniae* OGs to those of its most closely related nonmicrosporidian fungus (*R. allomycis*), as well as OGs from the well-studied fungus *Saccharomyces cerevisiae* and the microsporidium *Enc. cuniculi*. The largest fraction of all *M. daphniae* protein families (65%) is either shared with *R. allomycis, S. cerevisiae,* and *Enc. cuniculi* (619/1,878 OGs) or with only *R. allomycis* and *S. cerevisiae* (606/1,878). The majority of protein families involved in basic cellular processes or features, such as repair and recombination, transcription, ribosome biogenesis and function, and chromosome structure are shared with *R. allomycis, S. cerevisiae*, and *Enc. cuniculi*. Most OGs related to mitochondrial biogenesis, carbon metabolism, and cellular transport are not found in *Enc. cuniculi* (Fig. S8*B*). The *M. daphniae* proteome also contains most of the so-called microsporidian-specific domains, which appear in all microsporidia with a known genome and are shared with some other eukaryotes, but not with fungi other than *Rozella* (18) (Table S1). Thus, we conclude that the metabolic profile of *M. daphniae* is not as simplified as in other microsporidia, and yet already shows microsporidia-specific features.

Given that *M. daphniae* is the earliest diverging microsporidian parasite, we expected to find orthologs that had already

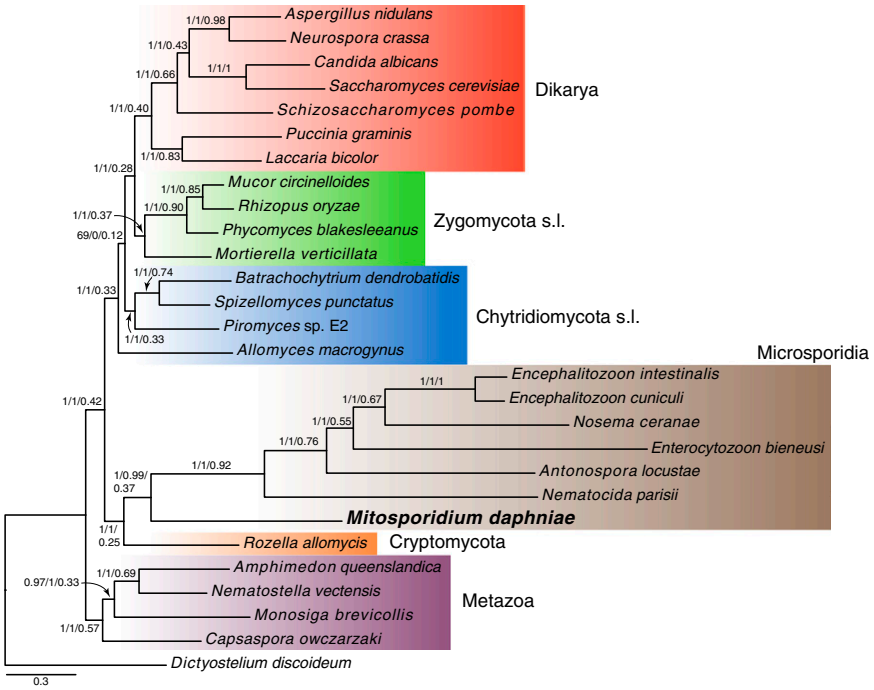

Fig. 2. *M. daphniae* is the most early diverging microsporidian genome sequenced to date. Phylogeny based on concatenated analysis of 53 conserved orthologs identified by Capella-Gutiérrez et al. (5). Tree shown is the maximum-likelihood tree found using RAxML 7.2.8a (47). Indicated at each node is the bootstrap percentage/Bayesian posterior probability/percentage of individual genes containing clade (see *SI Materials and Methods* for full details). Because of missing data, not every taxon is present in each protein alignment, and therefore the percentage of genes reflects only a proportion from which the relationship could be evaluated. The placement of *Allomyces* was different in the Bayesian consensus tree in which it formed the sister taxon to the Dikarya + Zygomycota sl.

evolved specifically in the microsporidian lineage that were coincident with the evolution of some of the morphological features, such as the polar filament. However, we found only four orthologs that were shared between *M. daphniae* and more derived microsporidia, but not with other fungi (Fig. 3 and Table S2); one (OG5_127467; PF01139) encodes a RtcB-like ligase involved in tRNA splicing and repair (32), which is ubiquitous in all kingdoms, except the fungi. Another ortholog (OG5_181516) contains a DNA-binding homeodomain of the Knotted (KN) family (PF05920) that is common in plants (33). The other two are microsporidian-specific orthologs (OG5_182073 and OG5_188308): a protein containing a mechanosensitive (MS) ion channel domain (PF00924) and another containing a Sec6 exocyst component domain (PF06046). MS ion channels have a variety of roles in the physiology of eukaryotic cells, such as osmotic gradients, cell swelling, gravitropism, and control of cellular turgor (34). We speculate that the microsporidian-specific MS channel could be used for spore germination, which involves increased intrasporal osmotic pressure (29). The exocyst complex, which is well known in yeast, functions by the interaction of four subunits—Sec6, Sec8, Sec10, and Exo70—delivering proteins that are essential for cell separation after division (35). Blast searches using the *Schizosaccharomyces pombe* Sec6 (NP_587736) as query identified a single protein in *M. daphniae* (OG5_188308; Fig. 3) and in all other microsporidian proteomes that we examined (Table S1). *S. pombe* has three paralogous genes encoding proteins with a Sec6 domain (PF06046)—OG5_128060, OG5_129076, and OG5_129499—but none of them is orthologous to the microsporidian Sec6-like proteins. No blast hits are found in the proteomes of any microsporidia using other *S. pombe* exocyst proteins as queries. Nonetheless, the genomes of *Enc. cuniculi*, *Enc. intestinalis*, and *Enterocytozoon bieneusi* contain one microsporidian-specific ortholog gene (OG5_196856) encoding a Sec8 (PF04048) domain-containing protein, but no homologous gene or domain was found in *M. daphniae*. These results illustrate the difficulties in performing microsporidian comparative genomics, because distinguishing gene loss from extreme molecular divergence is not straightforward. Indeed, about one third of the *M. daphniae* proteome could not be assigned to any OG. The involvement of Sec6 and Sec8 in microsporidian schizogony deserves more evaluation in the future.

Implications of the Discovery of *M. daphniae* in the Understanding of Microsporidia Evolution. The success of microsporidia as intracellular parasites had been linked to their ability to rapidly proliferate, as observed for the killer parasite *Nematocida parisii* (14). Our data suggest that the first steps that resulted in the extreme genome reduction of microsporidia might have involved changes in basic cellular processes such as cell cycle, meiosis, DNA repair, and gene expression (Table S1 and Fig. S8*C*). One hypothesis for a genetic feature linked to an accelerated microsporidian cell cycle is the lack of tumor suppressor RB that interacts with the E2F-DP transcription factor within a crucial pathway for cell cycle control (36), which is also absent in *M. daphniae,* but present in *R. allomycis* (18, 33) (Fig. 3 and Table S1). Genomic changes in microsporidia may have been further accelerated by the loss of several proteins involved in DNA repair and recombination. Overall, starting with the ancestors of *R. allomycis* and *M. daphniae*, transcription factor and DNA repair complexes seem to have degenerated (Table S1). Basal transcription factors TFIIF and TFIIH, which control both DNA repair and the initiation of transcription by recruiting specific chromatin components to particular sites of the genome, have lost subunits Tfb1 and Tfg1, respectively, in the *M. daphniae*–microsporidian lineage (Fig. 3 and Table S1). In parallel, microsporidia evolved peculiar gene promoters and mRNA processing mechanisms (37, 38). A similar phenomenon is observed for the protein repertoire involved in meiosis and recombination (Table S1). For example, Mcd1, a protein required for sister chromatid cohesion, is lacking in *M. daphniae* and all microsporidian proteomes (Figs. 2 and 3 and Table S1). Other proteins participating in the

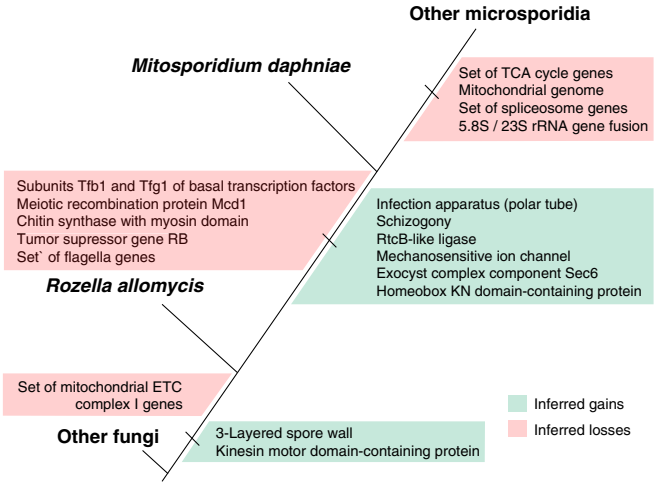

Fig. 3. Evolution of microsporidia-derived features. Inferred gains and losses of morphological characters, genomic features, and protein encoding genes in the evolutionary lineage leading to microsporidia are shown in green and red backgrounds, respectively. The following proteins are found in most other fungi but not predicted from microsporidia genomes and therefore inferred as gene losses (National Center for Biotechnology Information accessions in parenthesis): tumor supressor gene RB (NP_525036), Tfb1 (P41895), Tfg1 (P32776), Mcd1 (NP_010281), and chitin synthase with myosin domain (cd00124). Orthologous proteins containing domains found in other organisms but showing a microsporidian-specific distribution are inferred as gains (OrthoMCL database accessions in parenthesis): RtcB-like ligase (OG_12746), kinesin motor domain-containing protein (OG5_127467), homeobox KN domain-containing protein (OG5_181516), mechanosensitive ion channel (OG5_182073), and exocyst complex component Sec6 (OG_188308). For more information, see Tables S1 and S2.

synaptonemal complex and meiotic recombination, such as Hop1 and Hop2, are lacking in derived microsporidian such as *Enc. cuniculi*, *Edhazardia aedis, Nosema ceranae*, and *Nem. parisii*, but not in *M. daphniae*. It is known, however, that missing parts of the meiotic toolkit are not an impediment to perform meiosis (39). There is evidence for gametogenesis in a few microsporidian life cycles (40), but meiosis within the group has long been regarded as atypical, because chiasmata were never observed when chromosomes desynapsed in diplotene (41).

Our data provide, to our knowledge, the first picture of the sequence of events during the morphological and genomic transitions that occurred during the massive genome compaction that led to the evolution of the smallest eukaryotic genomes in the microsporidia. *M. daphniae* has similar morphological features to typical microsporidia, such as a polar filament, lack of a flagellum, three-layered spore wall, and sporogony in vesicles. However, many of its genomic features are more like the majority of fungi, including a mitochondrial genome. Thus, several of the characteristics of most microsporidia, such as the degeneration of the mitochondrion into a relictual mitosome, extremely compacted genomes, and reduced rRNA genes, seem to have occurred after the adoption of the microsporidian-like parasitic lifestyle (Figs. 2 and 3 and Table S1).

Microsporidia are characterized by strongly accelerated rates of molecular evolution (5). Using our new phylogeny, we tested where this acceleration is first detectable. Investigating nucleotide substitution rates using relative rate tests, our analysis reveals that the acceleration process began with the common ancestor of microsporidia, including *M. daphniae* (Fig. S10). Rates of evolution of orthologous genes are largely influenced by structural–functional constraints; that is, the robustness against protein misfolding is negatively correlated with molecular evolutionary rates (42). Protein sites or domains that interact with other molecules are under functional constraint or purifying selection. A reduced number of protein interactions (as observed in small microsporidia genomes) would increase the neutrality of existing gene networks; that is, the networks of sequences connected by single-step mutation distances, with similar effects on fitness (43). Thus, because *M. daphniae* and other microsporidia lack several subunits of basic cellular machineries (Table S1), we speculate that the increase in molecular evolutionary rates may involve a reduction in the number of protein interactions. Additionally, the loss of proteins responsible for DNA repair and recombination such as Tfb1, Tfg1, and Mcd1, as well as the shortening of generation times derived from the loss of tumor suppressor RB, may have led to a larger number of mutations being produced and accumulated in a shorter period.

Currently, the classification and nomenclature of microsporidia and Cryptomycota as true fungi is a subject of debate (30, 44). The finding that Cryptomycota are the sister clade or are even the paraphyletic ancestral clade from which microsporidia are derived has important implications for our understanding of the evolution of the microsporidial lifestyle, genome compaction, and the morphology and ecological function of the mysterious group Cryptomycota (15, 45). Recently, Corsaro et al. (44) described a parasite of amoebae (*Paramicrosporidium*) with overall similarity to *Mitosporidium*, having a microsporidian-like spore stage with a chitinous wall but with an amorphous, possibly nonfunctional polar filament, and unfused rRNA 5.8S/23S. In *Paramicrosporidium*, the authors were unable to detect a mitochondrion in micrographs of the parasite, and, indeed, even in *Mitosporidium*, the mitochondrion is difficult to indentify. *Mitosporidium* and *Paramicrosporidium* (KSL3 in Fig. S5) do not group together in the rRNA tree. In this tree, only *Mitosporidium* groups at the base of the microsporidia, although with weak statistical support. The overall morphologies of *Mitosporidium* and *Paramicrosporidium* are similar to an ancient group of microsporidia, the metchnikovellids, which parasitize gregarines that live in the gut of polychaetes and other marine invertebrates (46). However, the defining feature of the metchnikovellids is the presence of distinct uncoiled manubrium instead of the polar filament. The phylogenetic placement of metchnikovellids has yet to be determined. The results here and in Corsaro et al. (44) provoke speculation that many of the diverse environmental sequences attributed to Cryptomycota may ultimately be associated with microsporidian-like organims.

Conclusions

M. daphniae bears morphological features that are exclusively found in the microsporidia—schizogony and a polar filament that resembles the microsporidian infection apparatus—but retains a mitochondrial genome and the genes necessary for producing ATP from glucose. *M. daphniae* allows us to infer the sequence of some of the key events leading to the morphological, physiological, and genomic peculiarities of the microsporidia. We conclude that the morphological traits indicative of a parasitic lifestyle that characterize modern microsporidia evolved before the loss of mitochondrial function, reduced genome complexity, and high evolutionary rates. What physiological changes and adaptations triggered genome reduction and rapid molecular evolution in extant microsporidia remains still unresolved, but our findings demonstrate that extreme parasitism was achieved because a highly efficient apparatus for invasion that eliminates all extracellular growth and a peculiar mode of proliferation within host cells evolved first.

Materials and Methods

One *M. daphniae* isolate (UGP3) obtained in Kaimes (United Kingdom) was used for further laboratory procedures. Genomic DNA was extracted from spores purified by centrifugation in 60% Percoll (Sigma-Aldrich). The genome was sequenced using the Illumina shotgun approach and assembled with

Velvet (www.ebi.ac.uk/), Ray (http://denovoassembler.sourceforge.net/), and Geneious (www.geneious.com/). The mitochondrial genome was annotated with MFannot version 1.31 (http://megasun.bch.umontreal.ca/). Proteins were predicted with Maker (www.yandell-lab.org/), and their putative functions were assigned by homology searches against the UniProt-TrEMBL database. Spurious ORFs were deleted after manual curation and the remaining 3,300 proteins used to perform BLAST searches on 11 proteomes of reference (www.broadinstitute.org/) with an *E*-value cutoff of 1×10^{-5}. Proteins were classified according to the orthologous groups defined by OthoMCL version 5 (www.orthomcl.org/) and KEGG (www.genome.jp/) databases. Phylogenetic placement of *M. daphniae* was estimated using three gene sets: five mitochondrial proteins, a conserved set of 53 nucleus-encoded orthologs (5), and the 28S, 18S, and 5.8S subunits of the ribosomal RNA genes. The full materials and methods are found in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Jürgen Hottinger, Urs Stiefel, and Rita Wallén for technical assistance; Fasteris for genome sequencing; Loïc Baerlocher and Jean-Claude Walser for preliminary genome assembly; and Louis du Pasquier for fruitful discussions. This project was supported by grants from the Swiss National Science Foundation and the European Research Council.

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