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Louis M. Weiss
Aaron W. Reinke *Editors*

Microsporidia

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Microsporidia

Current Advances in Biology

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There have been many key research groups and individual researchers who have contributed to the development of the critical knowledge base required for progress on these enigmatic pathogens. This book is a testament to these researchers.

We would like to dedicate this book to Jiří Vávra PhD who was a long-standing faculty member of Charles University in Prague and the Institute of Parasitology of the Czech Academy of Sciences in České Budějovic. Jiří's intellectual rigor and deep thinking has had a significant influence on

current researchers on the Microsporidia, and we are all indebted to his generosity of spirit and profound insights into these pathogens.

Louis M. Weiss
Aaron W. Reinke

Jiří Vávra PhD (1933–2018). Photograph courtesy of Professor Lukeš Julius, Institute of Parasitology, České Budějovice

Preface

Microsporidia are a large group of medically and agriculturally important parasites. These enigmatic pathogens have fascinated biologists for over 160 years due to their ability to infect and cause disease in a large number of diverse animals, including humans, and also for their novel biology, such as their unique invasion apparatus, the polar tube. Indeed, microsporidia have been used to study host–pathogen interactions and immune responses in a variety of different hosts. Microsporidia have the smallest known eukaryotic genomes, and phylogenetic data suggest that microsporidia are related to fungi, with their closest relatives being the Cryptomycota. These features have made microsporidia a model for understanding parasite evolution though comparisons between canonical and early diverging microsporidia species.

This book describes recent advances in microsporidia research that have occurred since the publication of Weiss LM and Becnel JJ (Eds) *Microsporidia: Pathogens of Opportunity* in 2014. In Chaps. 1–4, authors cover the genomic reduction of microsporidia, what has been learned about microsporidia evolution through microsporidia genomics and comparisons to early diverging relatives of microsporidia, and the mechanisms controlling infection and host specificity. Chapters 5–7 discuss recent studies of microsporidia in the invertebrate species: *C. elegans*, silkworms, and honeybees. These animal models have helped to reveal mechanisms of microsporidia infection, shed light on host immune responses, and begin to develop techniques for genetic manipulation. Chapters 8 and 9 describe the structure and function of the polar tube as well as the physics and dynamics of spore firing. Chapters 10 and 11 review studies of the large and diverse group of aquatic microsporidia found in both invertebrates and fish. Finally, Chaps. 12–14 cover mammalian-infecting microsporidia, addressing the chronic nature of infections, host immunity, and disease epidemiology. Together, these chapters provide new insights into microsporidia biology and advance our understanding of these fascinating parasites.

Bronx, USA — Louis M. Weiss
Toronto, Canada — Aaron W. Reinke
2021

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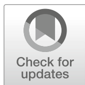
Chapter 1
Impact of Genome Reduction in Microsporidia



Nathan Jespersen, Leonardo Monrroy, and Jonas Barandun

Abstract Microsporidia represent an evolutionary outlier in the tree of life and occupy the extreme edge of the eukaryotic domain with some of their biological features. Many of these unicellular fungi-like organisms have reduced their genomic content to potentially the lowest limit. With some of the most compacted eukaryotic genomes, microsporidia are excellent model organisms to study reductive evolution and its functional consequences. While the growing number of sequenced microsporidian genomes have elucidated genome composition and organization, a recent increase in complementary post-genomic studies has started to shed light on the impacts of genome reduction in these unique pathogens. This chapter will discuss the biological framework enabling genome minimization and will use one of the most ancient and essential macromolecular complexes, the ribosome, to illustrate the effects of extreme genome reduction on a structural, molecular, and cellular level. We outline how reductive evolution in microsporidia has shaped DNA organization, the composition and function of the ribosome, and the complexity of the ribosome biogenesis process. Studying compacted mechanisms, processes, or macromolecular machines in microsporidia illuminates their unique lifestyle and provides valuable insights for comparative eukaryotic structural biology.

Keywords Microsporidia · Genome reduction · Comparative evolutionary structural biology · Ribosome structure and function · Ribosome biogenesis · Reductive evolution

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1.1 Causes and Effects of Reductive Evolution

There is a common bias to describe evolution as a march toward decreased entropy and increased complexity. After all, the regular ordering of atoms to create larger and more complex systems is an intrinsic feature of life on earth. While this suggests that a gradual increase in organism complexity is inevitable, complexity is balanced in many environmental niches by selective pressures that favor rapid and efficient reproduction, leading to the elimination of superfluous traits. This process of ablation is known as "reductive evolution" and can result in simpler organisms deriving from more complex ancestors.

Reductive evolution typically results from efficient nutrient usage. Macroscopic examples of this include the loss or atrophy of eyes in a wide variety of cave fish or the somewhat ironic loss of a digestive tract in tapeworms (Castro 1996; Morris et al. 2012). At the microscopic level, reductive evolution usually involves eliminating extraneous metabolic processes. Many freshwater chrysophytes, for example, have switched from autotrophy/mixotrophy to heterotrophy in order to combat limited carbon availability and have lost photosynthetic pathways and large swaths of their genomes along the way (Olefeld et al. 2018; Majda et al. 2021).

Gene loss is the most common example of reductive evolution and is often made feasible by co-occurring organisms. One interesting case of this is the loss of the catalase-peroxidase protein, KatG, in the marine cyanobacteria *Prochlorococcus* spp. (Scanlan et al. 2009). KatG serves an important role in protecting many cyanobacteria from hydrogen peroxide (Perelman et al. 2003), which builds up in oceans due to photooxidation of dissolved organic carbon (Cooper et al. 1988). In a few hours of direct sunlight, enough hydrogen peroxide can be produced to kill off *Prochlorococcus* cultures (Morris et al. 2011), indicating that they have a very high sensitivity for the molecule. It is surprising then that *Prochlorococcus* spp. would have lost *katG*. Instead, co-occurring cyanobacteria have retained *katG* and scavenge surrounding hydrogen peroxide from marine environments (Petasne and Zika 1997). The loss of *katG* is therefore only possible because organisms in the natural community provide a protective function.

Interactions between *Prochlorococcus* spp. and other marine cyanobacteria inspired the "Black Queen Hypothesis," which posits that natural selection for genomic streamlining breeds dependencies on co-occurring organisms (Morris et al. 2012). This contrasts with the "Red Queen Hypothesis," inspired by Lewis Carroll's *Through the Looking-Glass*, which postulates that competition breeds coevolution (Van Valen 1973). As a corollary to the Black Queen Hypothesis, the more dependent an organism is on other organisms, the more thoroughly a genome will be streamlined. We might therefore hypothesize that genome reduction scales with metabolic dependence on other organisms, i.e., the average genome size of evolutionarily related phototrophs > heterotrophs, and facultative parasites > obligate parasites, which seems to be the case (de Castro et al. 2009; Merhej et al. 2009; Clark et al. 2010; Majda et al. 2021) (Fig. 1.1).

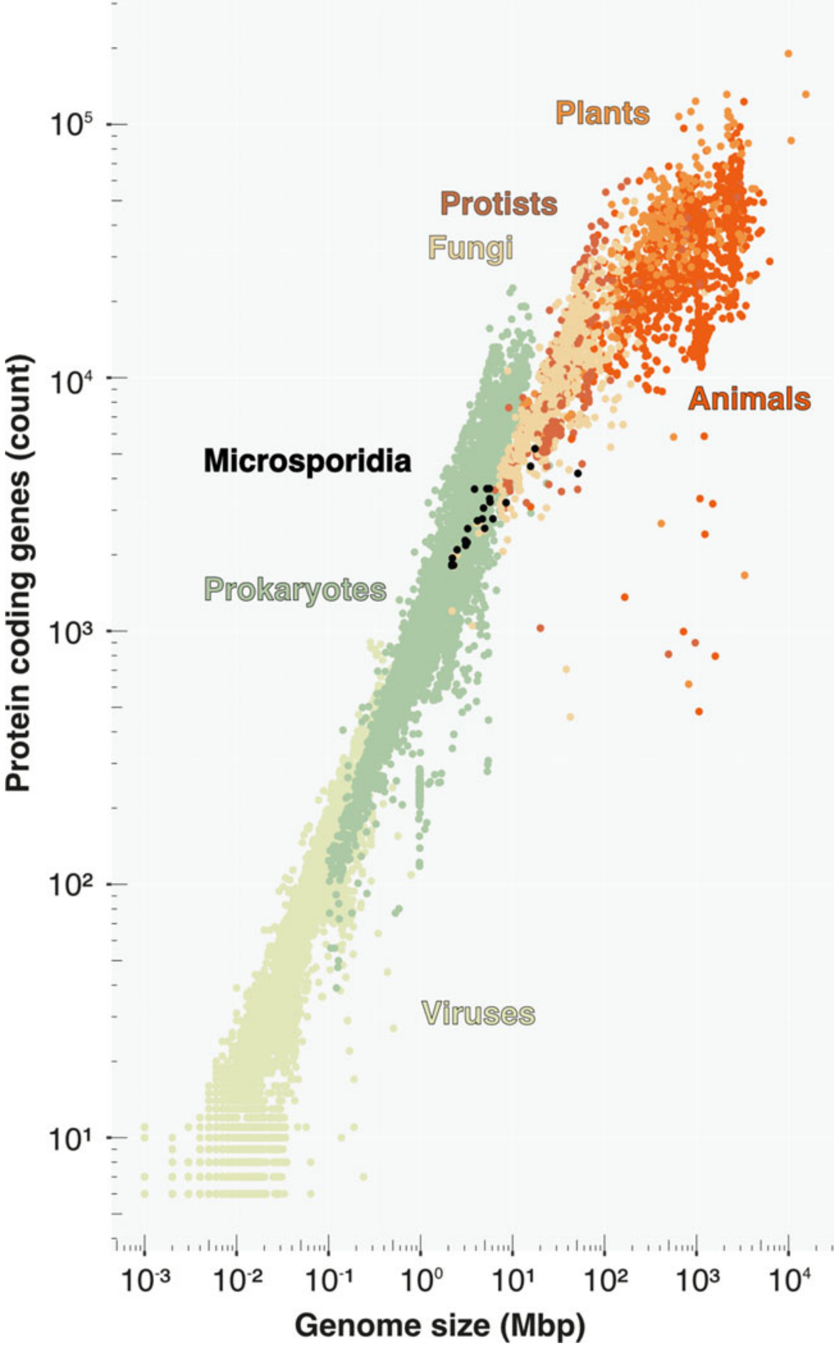

Fig. 1.1 Microsporidia have the smallest known eukaryotic genomes. Logarithmic plot of the number of annotated protein coding genes as a function of the respective organism's genome size. All entries present in NCBI (https://www.ncbi.nlm.nih.gov/) were included, but the data were broadly filtered to remove untenable outliers, partial sequences, and nucleomorphs. Because of

Parasites are some of the greatest beneficiaries of reductive evolution, and nowhere is this more conspicuous than in microsporidia. As obligate intracellular parasites, microsporidia have dramatically reduced many elements of their genomes. In the sections below, we describe the various factors facilitating genome reduction and outline elements of the genome that are absent in microsporidia. We then delve more deeply into the effects of genome ablation at the protein and RNA level by comparing aspects of ribosome structure, function, and maturation in microsporidia to other eukaryotes.

1.2 The Price of a Large Genome

The cost of genome replication is threefold and requires payment in time, nutrients, and space. All three costs increase with genome size, although there is some variation between prokaryotes and eukaryotes. In this section, we discuss the impact of each of these factors on genome replication and describe how they contribute to reduction in microsporidian genome sizes.

1.2.1 Time

Time is required both to collect materials for DNA synthesis and to physically duplicate the genome. The amount of time required for genome replication depends largely on the catalytic rate of the DNA polymerase. In *E. coli*, DNA polymerase III copies around 1000 nucleotides/second (Kelman and O'Donnell 1995; Naufer et al. 2017). On the other hand, the equivalent yeast polymerase, Pol ϵ, has a maximal catalytic rate of only 350 nt/s (Ganai et al. 2015). Yeast replication is further decreased to 50 nt/s by proofreading, lagging strand synthesis, etc. Fortunately, eukaryotes are able to offset slower catalytic rates and considerably larger genomes by segregating genetic material into different chromosomes and amplifying from multiple origins of replication. Consequently, yeast and *E. coli* grown in ideal conditions have replication times commensurate to their genome sizes: 90–120 min for 12 Mbp in yeast (Salari and Salari 2017), versus 40 min for 4.6 Mbp in *E. coli* (Fossum et al. 2007). Interestingly, replication rates for many cancerous human cells are on the order of only 20 h (Pereira et al. 2017), despite having genomes 250 times larger than yeast. This shows that various factors contribute to dramatically decrease the necessary time for eukaryotic replication,

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Fig. 1.1 (continued) the broad filtering, some partially sequenced or annotated entries are still present. The plot was generated using source code from https://github.com/smsaladi/genome_size_vs_protein_count. Eukaryotes are colored in different shades of red, with microsporidia in black. Prokaryotes and viruses are represented in shades of green

but that total replication time typically increases with increasing genome sizes. Thus, it is beneficial for intracellular parasites like microsporidia to reduce their genome size in order to decrease doubling times. Unfortunately, very little is currently known about microsporidian polymerases, or even whether their chromosomes harbor multiple origins of replication. One study on the microsporidia *Nematocida parisii* determined that their population doubles in around 140 min (Balla et al. 2016). Although there are a variety of confounding factors, such as growth occurring in infected nematodes rather than in an optimized broth, the replication rate of the 4.3 Mbp *N. parisii* genome is considerably slower than in yeast (about 1/4 the rate). This suggests that the catalytic rate of the polymerase is slower and/or that *N. parisii* has fewer chromosomes and origins of replication per Mbp than yeast.

1.2.2 Nutrients

Nitrogen and phosphorous are key elements in DNA and are considered the limiting nutrients for growth in most ecosystems (Ågren et al. 2012; Elser 2012). The biosynthesis of DNA is thus an extremely resource-intensive investment. In fact, comprehensive estimates for the ATP requirements for DNA replication suggest that it costs as much as 500 high-energy bonds/bp in diploid eukaryotes (Lynch and Marinov 2015). While this estimate includes indirect costs such as the production of nucleosomes to stabilize the DNA, most expenses scale linearly with genome size. The larger the genome, the more NTPs are required, and the less high-energy bonds are available for alternative functions like protein production or cell defense. Many organisms therefore pass through a cell-cycle checkpoint, called START, which acts as a nutrient-sensing step to assess available resources prior to replication (Foster et al. 2010). Cells lacking requisite nutrients enter a quiescent state until conditions are more favorable for DNA biosynthesis.

Nutrient limitations are even more restrictive for obligate intracellular parasites. Indeed, microsporidia are almost completely reliant on their hosts and are metabolically inactive in nutrient-poor, extracellular environments (Weiss and Becnel 2014). The hijacking of host systems allows them to bypass much of the innate cost of DNA replication, and simply importing nucleotides instead of synthesizing their own reduces the ATP requirements per base pair by nearly 50% (Lynch and Marinov 2015). Intriguingly, microsporidia have opted to eliminate the majority of enzymes required for nucleotide biosynthesis (Dean et al. 2016) and have instead expanded families dedicated to nucleotide import (Cuomo et al. 2012). This indicates that microsporidia have increased import proteins but greatly decreased biosynthetic pathways, facilitating a net decrease in overall genome size (Dean et al. 2016). Similar trends are identifiable in microsporidia for many other central eukaryotic pathways, such as glycolysis or fatty acid metabolism (Wiredu Boakye et al. 2017).

1.2.3 Space

Although space is perhaps the least conspicuous cost for DNA, many studies have noted and discussed the intricate relationship between genome size and cell size in eukaryotes (Gregory 2001; Cavalier-Smith 2005). The crux of this argument lies in the relatively invariant karyoplasmic ratio, i.e., the ratio of the nuclear volume to cytoplasm is important for cell function, and is generally conserved (Huxley 1925; Trombetta 1942; Cavalier-Smith 2005). The nuclear size is in turn proportional to the total volume of the chromatin (Cavalier-Smith 2005). Although the underlying causes of this effect are still being determined (Cantwell and Nurse 2019; Blommaert 2020), a decrease in genome size will generally lead to a decreased nuclear size, catalyzing a decrease in cell size. The reverse also holds true, where a decrease in cell size will herald a decrease in genome size. This relationship was cleanly demonstrated in a eukaryotic phytoplankton by Malerba et al. (2020). In this study, 72 different *Dunaliella tertiolecta* lineages with cell volumes spanning two orders of magnitude were placed under selective pressures favoring smaller cells. After 100 generations, lineages that were initially much larger displayed an up to 11% decrease in genome size, while smaller lineages were unaffected. This suggests that (1) selective pressures favoring smaller cells indirectly select for smaller genomes and (2) lineages with larger genomes contain a set of superfluous genes that can be lost, while smaller lineages are already operating at closer to the minimal genome (Malerba et al. 2020).

For intracellular parasites like microsporidia, the space available within their hosts directly restricts the number of spores produced. Cells infected with microsporidia are often saturated with spores (Weiss and Becnel 2014; Grigsby et al. 2020), suggesting the host cell walls limit the number of spores created per infection. In fact, the spatial costs of DNA are twofold, as not only does DNA indirectly determine the size of the spores or meronts, but it also takes up valuable real estate within the cell. It is therefore extremely beneficial for microsporidia to minimize genome size, and it is unsurprising that they are some of the physically smallest eukaryotes. As a consequence of cell-wall limitations to genome size, microsporidian species that exit via exocytosis may have less stringent spatial costs than lytic species. Mature spores are constantly being shed in exocytosed species, increasing the effective available space compared to lytic species. Currently, only one pair of species can be used as an example: *Nematocida displodere* is primarily released via cell lysis, while *N. parisii* can be exocytosed in vesicles (Luallen et al. 2016). Although the genome of *N. displodere* is, in fact, smaller than the genome of *N. parisii* (Luallen et al. 2016), more data are required to determine whether the "spore release method" contributes to genome size variation between related microsporidians.

1.3 Paths of Reductive Evolution in Microsporidia

Microsporidia are characterized by many unique and interesting features, such as a lack of innate mobility (Weiss and Becnel 2014) and a fishing-line like infection apparatus (Han et al. 2017). Despite their innovations, microsporidia are perhaps most frequently referenced for their exquisitely small genomes (Keeling and Slamovits 2004; Corradi et al. 2010; Corradi and Slamovits 2011) and minimized macromolecular complexes (Melnikov et al. 2018a; Barandun et al. 2019; Ehrenbolger et al. 2020). Microsporidian genomes are indeed very small and have the honors of claiming both the smallest known eukaryotic genome (Corradi et al. 2010) and one of the highest known eukaryotic gene densities (Fig. 1.2) (Keeling and Slamovits 2004; Keeling 2007). The genome of *Encephalitozoon intestinalis*, for example, is only 2.3 Mbp (Corradi et al. 2010). That is only half the size of the *E. coli* genome (4.6 Mbp) and 1/65,000 the size of *Paris japonica* (150 Gbp), a flowering perennial with the largest confirmed eukaryotic genome (Pellicer et al. 2010).

Early studies on microsporidia noted the absence or modification of several cellular structures characteristic of eukaryotes. For example, microsporidia lack peroxisomes, have unstacked Golgi bodies, and have highly reduced mitochondria called mitosomes (Corradi and Keeling 2009; Vávra and Ronny Larsson 2014). These observations led to speculation that microsporidia represent an ancient and

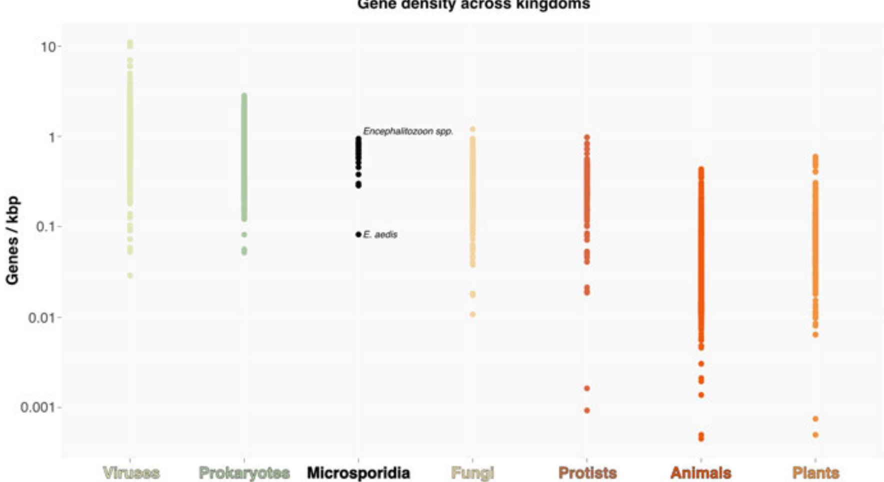

Fig. 1.2 Microsporidia have one of the most gene-dense eukaryotic genomes. Gene density across different kingdoms was calculated by dividing the number of annotated protein coding genes by the genome size of the respective organism in kilobase pairs. All entries present in NCBI (https://www.ncbi.nlm.nih.gov/) were included, but the data were broadly filtered to remove untenable outliers, partial sequences, and nucleomorphs. Because of the broad filtering, some partially sequenced or annotated entries are still present. Eukaryotes are colored in different shades of red, with microsporidia in black. Prokaryotes and viruses are represented in shades of green

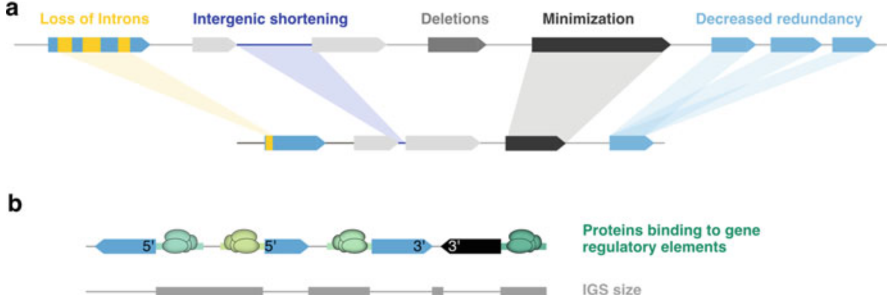

Fig. 1.3 Mechanisms of genome compaction in microsporidia. (**a**) Schematic representation of a relatively expanded (top) and compacted genome (bottom). Different genomic elements are colored, and the processes leading to their compaction (lower panel) are labeled on top. (**b**) The size of intergenic sequence (IGS) regions correlates with the directionality of adjacent genes, likely due to the presence of transcriptional control elements upstream of the transcriptional start site (e.g., enhancers, promotors). Gene directionality is indicated with arrows and 5′ or 3′ labels. Transcriptional control elements and their binding partners (e.g., transcription factors, RNA polymerases etc.) are shown as symbolic cartoons and colored in shades of green

unsophisticated eukaryotic lineage. They were therefore classified as Archezoa, with the prevailing hypothesis stating that they diverged prior to endosymbiosis of the mitochondrial ancestor (Cavalier-Smith 1983). This theory was disproven when further genetic analyses demonstrated that a subset of genes found in eukaryotic mitochondria have been transferred to microsporidian chromosomes (Germot et al. 1996; Katinka et al. 2001), indicating that microsporidia diverged after endosymbiosis and are therefore simplified organisms derived from more complex ancestors. Likewise, the small genomes of microsporidia are not a representation of a primitive ancestral state but are instead the result of minimization of multifarious genomic features. In this section, we describe several features affecting genome size, such as gene loss, intron minimization/removal, reductions in gene length, deletions of redundant genes, and the shortening of intergenic regions (IGRs) (Fig. 1.3a).

1.3.1 Non-coding Regions

Microsporidian genomes are similar to those of other eukaryotes in structure and organization. Multiple linear chromosomes can be segregated into telomeres, subtelomeres containing ribosomal DNA (rDNA) and repetitive elements, and gene-rich cores (Dia et al. 2016). Variation is more localized to individual elements of the genome, like coding sequences and intergenic regions. The regions between genes are essential for efficient transcription and contain binding sites for various promoters and enhancers, which are often thousands of nucleotides away from the gene they enhance. It is intriguing then that many microsporidia have tiny IGRs, with *E. intestinalis* averaging only 115 bp between genes (Corradi et al. 2010). The

genes themselves are an average of 1.04 kbp (Corradi et al. 2010). By taking into account the gene density (1.16 kbp/gene) (Corradi et al. 2010), we can determine that coding regions account for as much as 90% of the *E. intestinalis* genome. To put this in perspective, around 70% of the yeast genome codes for proteins (Dujon 1996) and only 2% of the human genome is protein coding (Piovesan et al. 2019). The low ratio of non-coding to coding sequences suggests that microsporidia have extremely streamlined IGRs. In fact, contrary to most eukaryotes, non-coding regions in microsporidia have higher sequence conservation than coding regions (Corradi et al. 2010; Corradi and Slamovits 2011; Whelan et al. 2019), indicating that the remaining bases form important molecular recognition motifs.

Most regulatory elements are found upstream of the 5′ end of a gene. Tellingly, the length of microsporidian IGRs appears to correlate with the directionality of adjacent genes (Fig. 1.3b) (Keeling and Slamovits 2004). For *Encephalitozoon cuniculi*, regions wedged between the termini of two genes (the 3′ ends) are about 20% shorter than regions between parallel genes (one 3′, and one 5′ end), while regions abutting divergent 5′ ends are a further 20% longer on average. This pattern is indicative of severe reductive selection operating on IGRs (Keeling and Slamovits 2004), as zero, one, or two sets of upstream transcription factors need to bind between convergent, parallel, and divergent genes, respectively.

Several other factors suggest that *Encephalitozoon* spp. are operating at the limit of IGR reduction. Firstly, the length of IGRs sometimes dips into negative values, i.e., genes overlap one another (Katinka et al. 2001; Akiyoshi et al. 2009; Corradi et al. 2010). Secondly, multiple studies have noted that transcripts initiate in upstream genes and read through into downstream genes (Williams et al. 2005; Corradi et al. 2008; Gill et al. 2010), suggesting that transcriptional start sites and termination sequences are often located within adjacent genes. Finally, microsporidia produce many multigene transcripts, which surprisingly encode both sense and antisense genes (Peyretaillade et al. 2009; Corradi and Slamovits 2011; Watson et al. 2015). These transcripts, known as "noncontiguous operons," are thought to regulate protein expression levels and result from evolutionary pressure to minimize genome size (Sáenz-Lahoya et al. 2019). These three examples provide evidence that microsporidia trim and eliminate IGRs wherever possible and have adapted more spatially efficient mechanisms to regulate protein expression levels.

Microsporidian parsimony is not only directed toward IGRs but also impacts other non-coding regions like introns. Splicing machinery and introns appear to have been convergently eliminated in at least three microsporidian genera: *Edhazardia*, *Nematocida*, and *Enterocytozoon* (Keeling et al. 2010; Desjardins et al. 2015). Even when introns are retained, they are reduced in both number and length (Lee et al. 2010; Campbell et al. 2013). In *E. cuniculi*, for example, a total of 36 introns have been identified, ranging from only 23 to 76 bases in length (Lee et al. 2010). The splicing efficiency for many of these introns is very low, often around 10–25%, and many putative introns display no active splicing (Grisdale et al. 2013; Campbell et al. 2013; Desjardins et al. 2015). For comparison, the yeast genome contains at least 300 introns ranging from around 100 to 1000 bases (Spingola et al. 1999; Xia 2020), which are frequently spliced with 100% efficiency (Xia 2020). The convergent loss

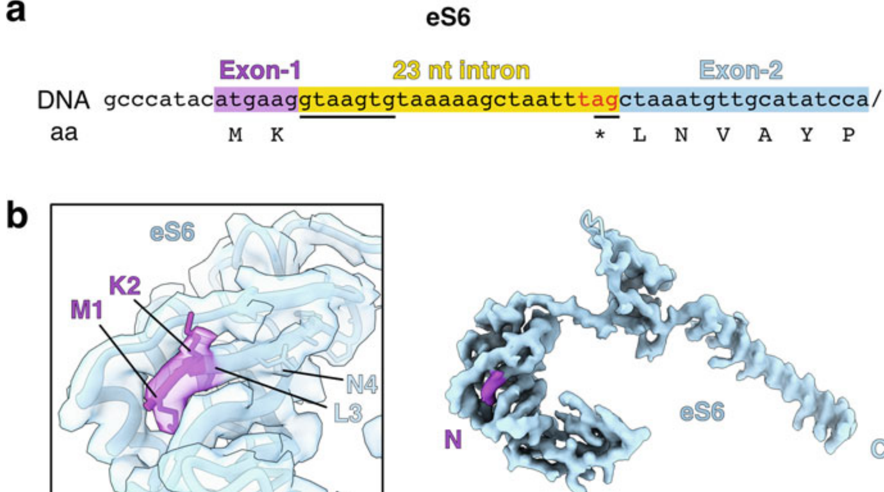

Fig. 1.4 Visualizing the protein product of eS6 mRNA splicing in *Paranosema locustae*. (**a**) Schematic representation of the 5′ end of the gene encoding eS6 in *P. locustae*, with DNA and corresponding amino acid sequence indicated. The splice sites are underlined. An in-frame stop codon in the intron is shown in red. (**b**) The corresponding cryo-electron microscopy density of eS6 is shown in isolation. The right side displays the entire ribosomal protein density in light blue with its N-terminus indicated in purple. The left side zoom in depicts the superposition of the model (PDB 6ZU5) and cryo-EM density (EMDB 11437) for the protein eS6 (Ehrenbolger et al. 2020). Density and cartoon model are colored according to their coding exons, analogous to (**a**) (Exon 1, purple; Exon 2, light-blue)

of introns and splicing machinery in several microsporidian clades implies there is little functional utility for most introns. Microsporidian species retaining spliceosomal machinery may therefore represent an earlier evolutionary snapshot in the decay of intron usage.

Interestingly, a large proportion of the retained introns are found in ribosomal proteins (Lee et al. 2010). Splice sites for ribosomal proteins are heavily biased toward the 5′ end of the coding sequence, and Exon-1 often encodes only a few amino acids (Fig. 1.4a). Ribosome structures are available for *Vairimorpha necatrix* and *Paranosema locustae*, two microsporidia that retain functional splicing machinery. The protein product of a spliced mRNA can be visualized by inspecting the cryo-EM density for proteins encoded by genes with introns. For example, the N-terminal region of eS6 in *P. locustae* displays apparent density for Met1-Lys2 amino acids (Exon-1), followed by amino acids from Exon-2, providing visual evidence for the successful splicing of a 23-nucleotide intron (Fig. 1.4b).

1.3.2 Gene Deletion and Minimization

Constant selective pressure favoring reductive evolution has led to widespread gene deletions, resulting in a core of only 800 conserved microsporidian proteins (Nakjang et al. 2013). These proteins are generally involved in essential processes, such as replication, DNA repair, and protein synthesis or recycling (Galindo et al. 2018). Categories of deleted proteins span the gamut; however, metabolic and regulatory pathways are particularly depleted (Nakjang et al. 2013; Dean et al. 2016; Wiredu Boakye et al. 2017; Galindo et al. 2018). *Encephalitozoon* spp. have lost almost all proteins involved in glycolysis, oxidative phosphorylation, fatty acid metabolism, and amino acid/nucleotide biosynthesis (Dean et al. 2016; Wiredu Boakye et al. 2017). Recent work identified one mechanism by which microsporidia survive without these important anabolic and catabolic pathways (Kurze et al. 2016; Luo et al. 2021). During infections, microsporidia secrete enzymatic proteins like hexokinase and trehalase into host cells (Senderskiy et al. 2014). These secreted proteins are incorporated into host metabolic pathways, leading to the upregulation of genes important for the biosynthesis of amino acids, nucleotides, and fatty acids (Kurze et al. 2016; Luo et al. 2021). Interestingly, transporters are one of the few classes of proteins that have experienced expansion rather than reduction in microsporidia (Nakjang et al. 2013; Dean et al. 2016). The slight radiation in transport genes has facilitated a much more substantial elimination of metabolic genes, allowing for a large net decrease in genome size.

Not only have many proteins been lost in microsporidia, the remaining proteins are also shorter. In fact, *E. cuniculi* proteins are on average 15% shorter than the yeast orthologs (Katinka et al. 2001). The impetus for this gene shortening remains to be proven, but Katinka et al. (2001) speculate that the loss of proteins led to more simplified interaction networks, which has facilitated the removal of protein-protein interaction domains from remaining proteins. One potential example of this can be seen in Taf5, a subunit of the transcription initiation factor TFIID. In most eukaryotes, Taf5 contains a conserved, N-terminal Lis1 Homology motif (LisH) (Romier et al. 2007; Wang et al. 2020). LisH domains are short, ~33 amino acid motifs that assist in protein dimerization and subcellular targeting (Gerlitz et al. 2005). In Taf5, the LisH domain is known to both facilitate dimerization (Bhattacharya et al. 2007) and help mediate interactions between Taf5 and the Spt20 subunit of the SAGA (Spt-Ada-Gcn5 acetyltransferase) complex (Wang et al. 2020). Interestingly, the LisH domain is absent in microsporidia (Romier et al. 2007). Although the absence of spt20 is yet to be verified, a BLAST (Altschul et al. 1990) search against all available microsporidian genomes produced no reliable hits. Additionally, other core components of the SAGA complex are absent in microsporidia (Miranda-Saavedra et al. 2007). As the LisH domain is primarily a structural domain that promotes protein-protein interactions, the loss of its binding partner would render its function moot. These data therefore support the hypothesis that simplified protein-protein interaction networks lead to the ablation of superfluous domains and the minimization of microsporidian gene length.

Although we have thus far described the loss of a gene as the loss of a function, functional redundancy is common in most eukaryotes (Dean et al. 2008). Organisms operating under strong reductive selection frequently minimize genomes by eliminating redundancies (Luo et al. 2011). In highly reduced picoplanktonic eukaryotic organisms, for example, the total number of gene families is conserved despite extensive gene loss (Derilus et al. 2020). Instead, the average size of each gene family is decreased as a result of deletions of paralogous genes. A similar trend can be identified in the most minimal microsporidia, like *Encephalitozoon* spp., which are largely devoid of duplications and repetitive elements (Katinka et al. 2001; Keeling and Slamovits 2004; Cormier et al. 2021). These findings only hold true, however, for the most reduced microsporidia. Other species, like *Nematocida* spp., have markedly expanded a small number of gene families (Reinke et al. 2017), while the comparatively large *Edhazardia aedis* and *Hamiltosporidium tvaerminnensis* are quite rich in duplications and repetitive elements (Williams et al. 2008; Cormier et al. 2021).

1.3.3 Gene Retention and Expansion

Gene families that are retained or expanded despite reductive selection provide an abundance of valuable information. By considering which proteins are retained, it is possible to identify biologically essential systems. As described above, gene families retained in microsporidia are often involved in core cellular processes, and their removal is typically lethal (Nakjang et al. 2013). Additionally, yeast orthologs of these conserved proteins are significantly more likely to be highly expressed and have a large number of interaction partners. These traits persist in microsporidia (Nakjang et al. 2013), revealing the importance of connectivity and expression levels in gene retention. Unexpectedly, conserved core proteins only account for around 800 of the 1750 (*Enterocytozoon bieneusi*) to 4500 (*Nosema bombycis*) predicted genes (Peyretaillade et al. 2009; Nakjang et al. 2013; Pan et al. 2013). The remaining genes serve species-specific functions and are often members of novel expanded gene families (Reinke et al. 2017).

One group that has undergone expansion is the Small Conductance Mechanosensitive Ion Channel (MscS) family. These membrane proteins are found in both prokaryotes and eukaryotes and are involved in the regulation of intracellular pressure in response to extracellular stimuli. Most frequently, this stimulus takes the form of mechanical stress on membranes resulting from hypo- or hyperosmotic conditions (Kung et al. 2010). These proteins function by forming a channel, which allows for the influx or efflux of water and small molecules to relieve the stress by reducing pressure. Microsporidia encode at least five copies of MscS proteins, derived from a combination of horizontal gene transfers and lineage-specific expansions (Nakjang et al. 2013). Based on other MscS functions, it has been proposed that they play a role in the regulation of osmotic stress during microsporidian germination (Nakjang et al. 2013). Previous studies have

demonstrated that the rapid degradation of metabolites like trehalose, followed by a subsequent increase in turgor pressure, provides the impetus for release of the microsporidian polar tube (Undeen and Vander Meer 1999). Therefore, it is unsurprising that proteins involved in the regulation of turgor pressure would be both enriched and conserved in microsporidian species.

The most expanded microsporidian gene families are novel and have no known function (Heinz et al. 2012; Peyretaillade et al. 2012). Examination of these proteins in *Nematocida* spp. demonstrated that they are recently generated and rapidly evolving, as many members are either species- or clade-specific (Reinke et al. 2017). Tellingly, the genes are typically located within the subtelomeric regions of the chromosomes, an area often associated with rapid evolution and immune evasion (Fischer et al. 2003; Brown et al. 2010; Pombert et al. 2012; Reinke et al. 2017). As such, it is likely that these families are involved in direct interactions with hosts and are expanding and evolving in a species-specific way in response to preferred hosts. In support of this idea, Reinke et al. (2017) identified host-exposed proteins in *N. parisii* using spatially restricted enzymatic tagging and found that 49% of the experimentally identified proteins belonged to a large gene family and that 88% of all host-exposed proteins lacked orthologs outside of closely related *Nematocida* spp. Although further work is required to understand the role these families play in host-parasite interactions, their expansion against a background of reductive evolution suggests a unique and important function.

1.3.4 Variation Between Species

The genome sizes of microsporidia differ considerably between species, from 2.3 Mbp (*E. intestinalis*) to 51.3 Mbp (*E. aedis*). This variation is not mirrored in the number of protein coding genes, which fluctuates within a much narrower window (1750–4500) (Peyretaillade et al. 2009; Pan et al. 2013). The larger genome size variation instead reflects the accumulation of non-coding regions in larger microsporidia. In fact, non-coding regions sometimes accrete to such a degree that the microsporidian clade contains both one of the most gene dense and one of the least gene dense fungal species (Fig. 1.2) (Muszewska et al. 2019). This surprising finding is evidence that not all microsporidia are undergoing aggressive reductive evolution.

Repetitive sequences make up a large proportion of the non-coding regions of gene-sparse species (Parisot et al. 2014). These repetitions, largely transposable elements, are associated with mildly deleterious effects in eukaryotes (Hua-Van et al. 2011). This begs the questions, what differs between gene-dense and gene-sparse species, and why are gene-sparse species experiencing less stringent reductive selection? To address these questions, recent work compared and contrasted the life cycles of various microsporidia and discovered a correlation between genome size and mode of transmission. Microsporidia that are transmitted through purely horizontal means have small and compact genomes, while microsporidia with

mixed-mode (vertical and horizontal) transmission have larger genomes with a higher concentration of transposable elements (Haag et al. 2020; De Albuquerque et al. 2020). These studies suggest that population bottlenecks resulting from vertical transmission lead to reduced selective pressure and facilitate the expansion of repetitive sequences. Although more work is required, it is clear that the mode of transmission contributes to the significant variation in microsporidian genome sizes.

1.4 The Ribosome as a Molecular Fossil Record

As seen above, reductive evolution operates on all facets of the microsporidian genome. Although we have thus far focused on the factors leading to genome reduction, it is just as important to understand the structural and functional adaptations resulting from that reduction, i.e., which pathways are lost or minimized, and how does the loss or minimization of these pathways fit in with what we know about the microsporidian life cycle? For the remainder of this chapter, we will describe the results of genome compaction on cellular systems, using ribosome structure, function, and biosynthesis as our case study. There are a variety of reasons to choose the ribosome for this analysis. Firstly, microsporidia suffer from a dearth of structural data. There are only 43 microsporidian structures available on the PDB (using keyword "microsporidia," www.rcsb.org; June 2021), as opposed to over 5000 for *Saccharomyces cerevisiae*. Two of the microsporidian structures encompass the whole ribosome, making it one of the most studied microsporidian structures (Barandun et al. 2019; Ehrenbolger et al. 2020). Secondly, the ribosome is an essential macromolecular complex responsible for protein synthesis in all known "living" organisms. Thirdly, despite a relatively conserved core, ribosomes differ significantly between clades (Fig. 1.5). They are dramatically expanded in most eukaryotes compared to prokaryotes, but highly reduced in intracellular parasites like microsporidia or apicomplexans. Finally, because of both its variation and ubiquity, the ribosome has long been used to build gene-based phylogenetic trees, promoting a function for ribosomes as evolutionary timekeepers.

In 1977 Carl Woese and George Fox recognized the ribosomal genes' potential to serve as a molecular fossil record of life and revolutionized biology by establishing ribosomal RNA (rRNA) sequencing as a tool in molecular phylogenetics (Woese and Fox 1977). Their work led to the discovery of the *Archaea* and a fundamental re-drawing of the tree of life. When analyzing the ribosomal RNA of the microsporidia *V. necatrix* in 1987, Vossbrinck and Woese found a highly reduced ribosomal RNA sequence, more akin to a prokaryote than a eukaryote. It was concluded with the data available at the time, that microsporidia might be early branching, primitive eukaryotes (Vossbrinck et al. 1987). However, with the advent of genome sequencing and the increasing availability of protein and ribosomal RNA sequences from different species in the decades since, mounting evidence has shown that microsporidia are closely related to fungi (James et al. 2006; Haag et al. 2014), rather than being primitive eukaryotes. Hence, the "prokaryote-like" ribosomal gene

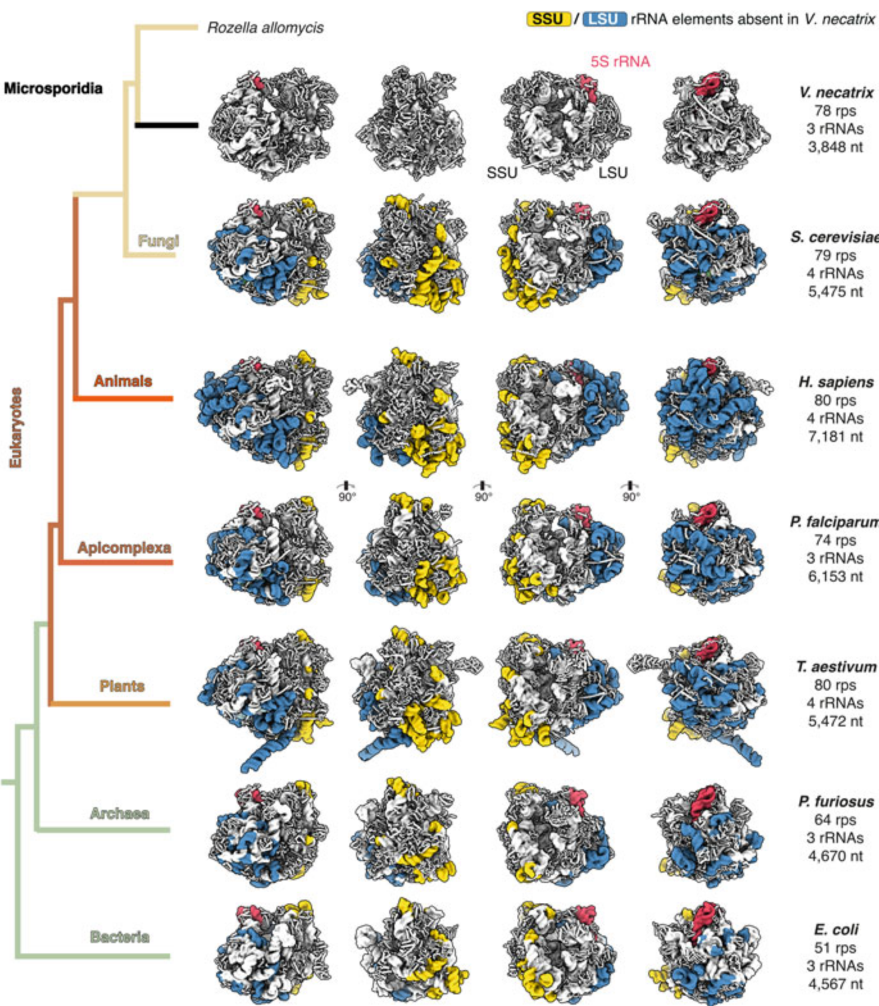

Fig. 1.5 The evolution of the microsporidian ribosome is shaped by an unusual reversal of eukaryotic expansions. A simplified schematic phylogenetic tree (James et al. 2006, 2013; Haag et al. 2014), depicting the expansive evolution of the ribosome from prokaryotes to eukaryotes and the reduction in microsporidia. Ribosomes are displayed for representative organisms in four views, related by 90-degree rotations [bacteria, *E. coli* PDB 4YBB (Noeske et al. 2015); archaea, *Pyrococcus furiosus* PDB 4V6U (Armache et al. 2013); plants, *Triticum aestivum* PDB 4V7E (Gogala et al. 2014); animals, *Homo sapiens* PDB 6EK0 (Natchiar et al. 2017); fungi, *S. cerevisiae* PDB 4 V88 (Ben-Shem et al. 2011); and microsporidia, *V. necatrix* PDB 6RM3 (Barandun et al. 2019)]. The shared rRNA core and all ribosomal proteins are shown in white, 5S rRNAs are in red, and rRNA elements absent in microsporidia are colored in blue (LSU rRNA) or gold (SSU rRNA). The organism's name, number and size of rRNAs, and number of ribosomal proteins (rps) are indicated on the right side

arrangement, reduced size of the ribosomal RNA, and minimized proteins in microsporidia are the result of genome compaction and represent an unusual reversal of the drastic expansion that occurred in eukaryotes (Fig. 1.5).

1.5 The Microsporidian Ribosome: An Outlier in Ribosome Evolution

The ribosome is a complex macromolecular machine responsible for the production of all proteins. To perform this vital function, many ribosomes are produced, accounting for nearly 30% of the dry mass of a rapidly dividing bacterial cell (Bremer and Dennis 2008; Piir et al. 2011). Ribosomes are composed of both proteins and RNA (i.e., they are ribonucleoproteins) and are typically segregated into two sections: the small subunit (SSU, 40S in eukaryotes) and the large subunit (LSU, 60S in eukaryotes). As a result of the ribosome's large size and essential role, as much as 80% of a cell's resources are dedicated to its biosynthesis and functional upkeep in nutrient-rich conditions (Tempest and Neijssel 1984; Maitra and Dill 2015). Although all known living organisms produce ribosomes, the ribosome composition varies significantly between clades (Fig. 1.5).

Most prokaryotes have simple genomes smaller than 10 Mbp (Fig. 1.1) and are tightly packed with protein-coding genes (Fig. 1.2). In extreme cases, genomes can be as small as 0.112 Mbp (*Nasuia deltocephalinicola*) (Bennett and Moran 2013), or as large as 13 Mbp (*Sorangium cellulosum)* (Han et al. 2013). Despite the tiny genome of *N. deltocephalinicola* containing only 137 coding genes, it still manages to produce ribosomes, which are composed of 3 rRNAs (4445 nucleotides total) and ~ 50 proteins (Moran and Bennett 2014). In more typical bacteria like *Escherichia coli*, ribosomes still consist of 3 ribosomal RNAs (rRNAs) with a combined size of 4567 nt. The surface of the rRNA is coated with 51 ribosomal proteins (Fig. 1.5), and the biogenesis process requires dozens of additional proteins (Shajani et al. 2011). The eukaryotic ribosome, on the other hand, is significantly expanded in size and number of components. For example, the cytoplasmic ribosome from *S. cerevisiae* contains 4 rRNAs of a combined 5475 nt, and a total of 79 proteins. The eukaryotic assembly process also differs drastically from prokaryotic ribosome assembly and utilizes over 300 trans-acting factors for ribosomal maturation (Woolford and Baserga 2013; Klinge and Woolford 2019).

While translational machinery is expanded in most eukaryotes, it has been significantly affected by genomic erosion in microsporidia. Recently, the ribosome structures have been solved for two microsporidian species: *V. necatrix* and *P. locustae* (Figs. 1.5 and 1.6) (Barandun et al. 2019; Ehrenbolger et al. 2020). These structures provide one of the first glimpses of the effects of reductive evolution on macromolecular complexes in microsporidia. Interestingly, microsporidian ribosomes have been reduced to such an extent that their rRNAs are smaller than many bacterial ribosomes (3 rRNAs totaling ~3850 nt), including those from

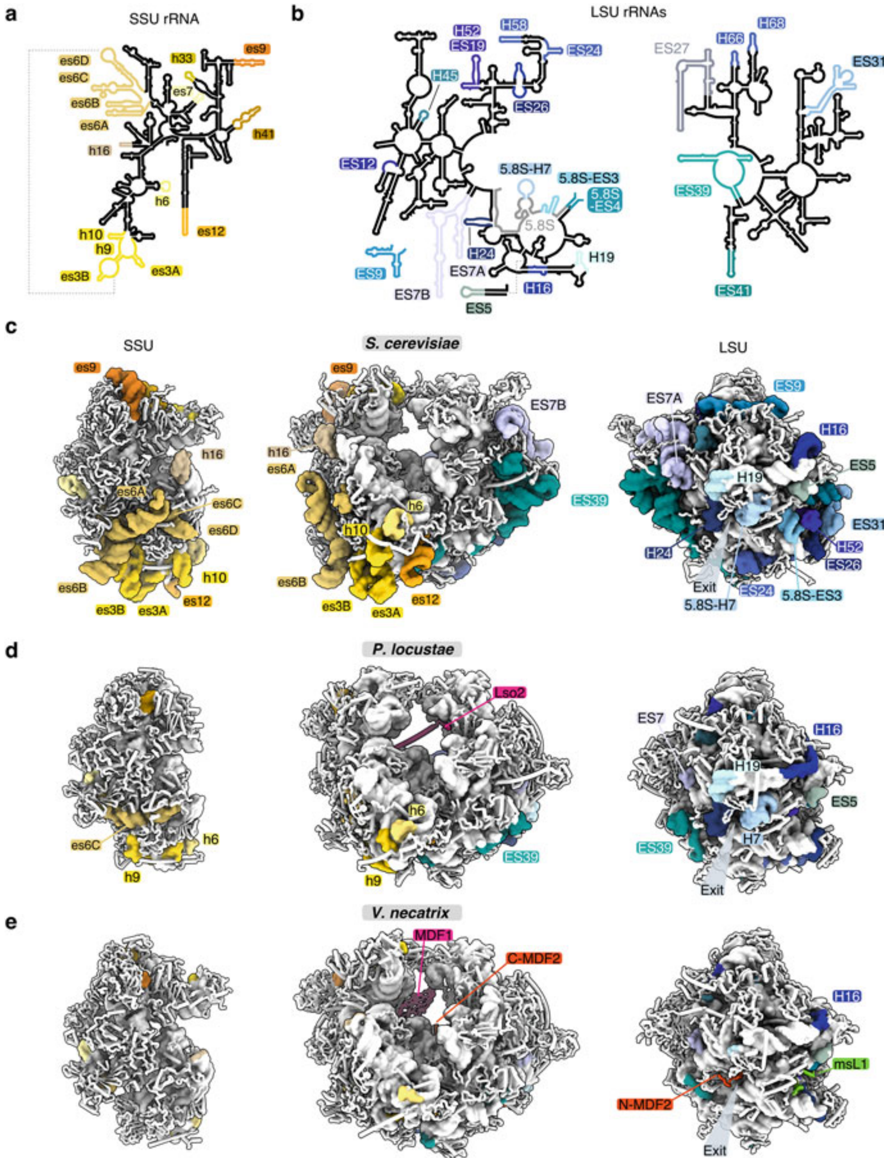

Fig. 1.6 Extensive rRNA expansion segment loss in microsporidia. (**a**, **b**) Schematic secondary structure diagram of the small (**a**) and the large subunit ribosomal rRNA (**b**), based on the *S. cerevisiae* structure, with expansion segments that have been lost in *V. necatrix* colored (SSU, shades of orange and yellow; LSU, shades of blue and green). (**c–e**) Related views of the ribosome from *S. cerevisiae* PDB 4 V88 (**c**), *P. locustae* PDB 6ZU5 (d), and *V. necatrix* PDB 6RM3 (**e**). The middle section displays the full ribosome, while two 90-degree-related views of the SSU and the LSU solvent-exposed sides are shown in isolation on the left and right. Ribosomes are colored in white, while locations of expansion segments or other elements that have been lost in *V. necatrix* are colored as in (**a**, **b**). The microsporidian ribosomal protein (msL1) is shown in light-green, while MDF1 and MDF2 are colored purple and red

N. deltocephalinicola, and are significantly smaller than the yeast ribosome. Many parts of the ribosome have been lost or reduced in microsporidia; in this section, we will describe some regions that have been altered, analyze the functional significance of those regions, and postulate on the implications of those changes for microsporidia.

1.5.1 Minimization of Expansion Segments

Eukaryotic ribosomes are characterized by approximately 30 additional eukaryote-specific proteins and 50 additional rRNA elements known as expansion segments (ES). These segments are aptly named, as they are regions of the rRNA that have been expanded in eukaryotes compared to prokaryotes. The functions of most ESs have not been thoroughly studied; however, many ESs seem to stabilize the additional layer of proteins present in eukaryotic ribosomes (Ben-Shem et al. 2011). Others aid in recruiting and organizing components of the much more complex eukaryotic ribosome biogenesis process (see Sect. 1.6) (Ramesh and Woolford 2016). More targeted studies have suggested that specific expansion segments are involved in recruiting regulatory factors (Fujii et al. 2018), or serving auxiliary roles by engaging and stabilizing mRNA during translation (Parker et al. 2018). Regardless of function, ESs are a hallmark of typical eukaryotic ribosomes.

Microsporidia have reversed the evolutionary trend to expand rRNA elements and have removed the vast majority of eukaryotic ESs (Fig. 1.6). Those that do remain are significantly reduced in size. A comparison of the SSU rRNAs indicates that microsporidia are indeed evolving toward the loss of ESs (Fig. 1.7), rather than simply not expanding in the first place. Step-wise deletions lead to early branching species like *Rozella allomycis* and *Mitosporidium daphinae* encoding partial versions of ESs, while more recently diverged species have removed many ESs altogether (see, e.g., es9 or es3; Fig. 1.7). If ESs were instead convergently evolving from a minimal core in a last common ancestor, we would not see ES sequence homology between early diverging microsporidia and other eukaryotes. Although most ESs do not have defined roles or specific known interaction partners, several segments have been studied in more detail, allowing us to draw conclusions on the causes and effects of ES loss in microsporidia.

N-terminal acetylation of proteins is extremely common, with 60% of the yeast proteome and 85% of the human proteome containing this modification (Arnesen et al. 2009). Acetylation plays a role in protein half-life, most commonly by protecting proteins from ubiquitination of N-terminal residues, thereby preventing their proteasomal degradation (Ree et al. 2018). Nearly 40% of all acetylation in humans happens co-translationally and is mediated by the NatA acetylation complex (Ree et al. 2018). Co-translational acetylation is achieved via direct interactions between NatA and multiple ribosome ESs, including H24, ES7, ES27, and ES39 (Knorr et al. 2019). In microsporidia, these ESs are extremely reduced (as in the case of *P. locustae*; Figs. 1.6 and 1.7), or completely absent (*V. necatrix*), indicating

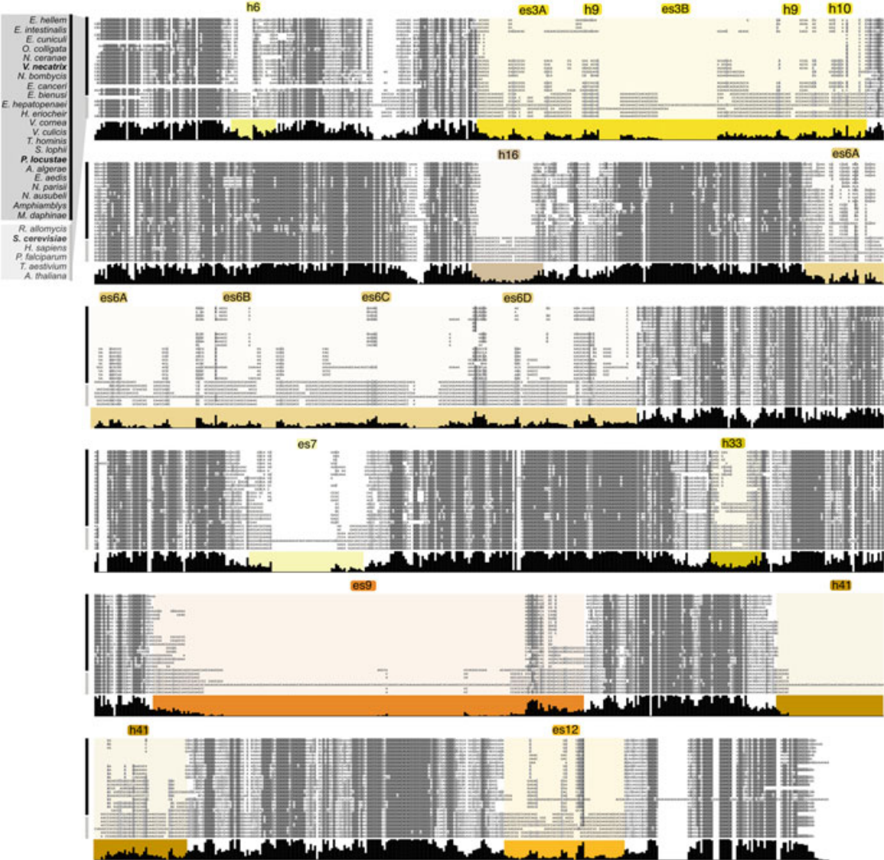

Fig. 1.7 Expansion segment loss and sequence divergence in the microsporidian ribosomal RNA. Small subunit rRNA sequence alignment of selected microsporidian and eukaryotic organisms, created with Clustal Omega using RNA settings (Sievers et al. 2011). A structurally impossible sequence insertion and potential sequencing issue in the *N. ceraneae* rRNA was manually removed (nt 700–728). Not included are sequences from *E. romaleae*, *P. neurophilia*, and *N. displodere*, which are only partially available. Organisms are labeled on the top left with microsporidia in the dark box and other eukaryotes in light-gray. Conservation is indicated with shades of gray, from white (variable) to dark gray (conserved). Elements which are not present in the *V. necatrix* rRNA are indicated with colored boxes and labeled on top using the same coloring scheme as in Fig. 1.6. The SILVA ribosomal RNA gene database (Quast et al. 2013) was used to obtain sequences

N-terminal acetylation is either not performed co-translationally or is mediated by a different complex. Consistently, an analysis of microsporidian proteomes demonstrated that, while some subunits of the NatA complex are present, there is a significant depletion in NatA substrate motifs in microsporidia (Rathore et al. 2016), suggesting a greatly diminished role for the acetylation complex. Intriguingly, N-terminal acetylation has also been associated with cellular targeting, where cytoplasmic proteins are enriched and secreted proteins are depleted in the

modification (Forte et al. 2011). As intracellular parasites, microsporidia interact extensively with hosts and are known to have a large number of secreted proteins (Reinke et al. 2017). It is therefore possible that the loss of NatA-interacting ESs serves to both conserve nutrients by minimizing the ribosome and facilitate protein secretion into host cells.

In addition to recruiting the acetylation complex, ES27 has a purported role in translational fidelity (Fujii et al. 2018). The deletion of segments of ES27 in yeast leads to the misincorporation of amino acids during translation. Microsporidia lack ES27 (Barandun et al. 2019; Ehrenbolger et al. 2020), indicating that they ensure translational fidelity via alternative mechanisms or have higher error rates. A recent study on the microsporidia *Vavraia culicis* supports the latter possibility and shows that nearly 6% of leucine residues are erroneously translated (Melnikov et al. 2018b). For comparison, *E. coli* has a mistranslation rate of only 0.2% (Zaher and Green 2009). A low translational fidelity is utilized by many organisms as an adaptive strategy, facilitating immune evasion by increasing proteomic diversity (Miranda et al. 2013; Ling et al. 2015). For organisms like microsporidia that have both high numbers of host-exposed proteins and extremely restricted proteomes, the additional flexibility garnered by mistranslation may be particularly beneficial. Therefore, the loss of ES27, and the potential decrease in translational fidelity, would be both economically and functionally advantageous.

The *P. locustae* ribosome structure provides an extremely economical example of ES ablation. In most eukaryotic ribosomes, ES39 contains a highly conserved nucleotide that appears to stabilize the interface between two ribosomal proteins (Ehrenbolger et al. 2020). The microsporidian ribosome has eliminated the vast majority of ES39; however, extra density consistent with a single nucleotide is present at the same location. These data indicate that the free nucleotide is a relic of ES39 and serves an important role as an architectural cofactor to stabilize the protein-protein interface. The near-complete reduction of ES39 to a single nucleotide is an exceedingly economical solution and lends credence to the idea that microsporidia are under high levels of reductive selection.

The minimization of this ES to a single-nucleotide relic, in conjunction with the previous examples of ES loss, demonstrates that ES deletion is a common mechanism by which microsporidia reduce genome size and cut nutrient costs in the biosynthesis of ribosomes. Interestingly, the localization of rRNA elements within the subtelomeric regions of the genome may facilitate rRNA minimization. Subtelomeres are often repetitive, associated with higher evolutionary rates, and have increased frequencies of double-strand breaks (Brown et al. 2010; Muraki and Murnane 2017). The high repetition may accelerate the deletion of ESs during double-strand break repair. Regardless of the underlying mechanism, it is clear that microsporidia have removed the vast majority of ESs, resulting in the smallest known cytoplasmic ribosome in eukaryotes.

1.5.2 Changes to the Proteinaceous Composition of Microsporidian Ribosomes

Ribosomal proteins are some of the most widely conserved across the tree of life. Approximately half of the protein subunits are present in both prokaryotic and eukaryotic ribosomes and are thus called the "universal" or "u" ribosomal proteins (Ban et al. 2014). However, as seen with ESs, eukaryotic ribosomes have greatly expanded their proteinaceous repertoire, developing the "eukaryotic" or "e" proteins of the ribosome. It is somewhat surprising then that the drastic microsporidian reduction in ESs is not accompanied by a concomitant loss in the number of ribosomal proteins (Fig. 1.5).

To better understand the proteinaceous changes to microsporidian ribosomes, we have collected sequences from genomes available on MicrosporidiaDB (Aurrecoechea et al. 2011) and compared their conservation in Fig. 1.8. Caution is advised while drawing conclusion from these data, as many microsporidian genomes are derived from incomplete assemblies and microsporidian proteins are rapidly evolving. It is therefore very likely that some of the proteins marked as absent were simply not identified via our methods. That said, microsporidia have retained most of the ribosomal proteins found in yeast, and only a few of the 80 yeast proteins are potentially absent in many microsporidian species. Remaining proteins have a 38% average sequence identity to yeast homologs and are often considerably shorter (Fig. 1.8a and b). Some proteins have lost loops or linkers, while others have been truncated at the N- or C-terminus. Additionally, low levels of sequence identity can be used to demarcate proteins that have structurally diverged from yeast (Fig. 1.8c).

Genome-wide knockout screens have been performed in yeast, which allows us to identify essential ribosomal proteins (Giaever et al. 2002; Gao et al. 2015) (Fig. 1.8a). These studies further noted knockouts that led to slow-growth defects. It is important to mention, however, that yeast have duplicated the majority of ribosomal genes. Some deleterious effects may have therefore been ameliorated by the presence of paralogs during single-gene deletion studies. Nevertheless, comparisons between gene conservation and essentiality reveal several interesting results. Firstly, as might be expected, many of the essential genes in yeast were not duplicated. Secondly, essential genes are still extant in almost all microsporidia. Instances of their loss, such as uL16 in *Enterocytozoon hepatopenaei*, are more likely a result of incomplete genome assemblies or low sequence conservation. This is evinced by the isolation of purported losses. Only in the case of uL23 are essential genes unidentifiable in a related cluster of microsporidia (*Trachipleistophora hominis* and *Pseudoloma neurophilia*). Numerous studies have demonstrated the essentiality of uL23 for the formation of the polypeptide exit tunnel (Kaur and Stuart 2011; Polymenis 2020). We therefore find it more probable that its absence is a matter of incomplete genome assemblies; however, a genuine absence would undoubtedly provide useful insights into evolutionary strategies developed by microsporidia to minimize the ribosome exit tunnel. Thirdly, all of the yeast proteins unidentifiable in most microsporidia are nonessential (see eL28, eL38, eL41, P1, and

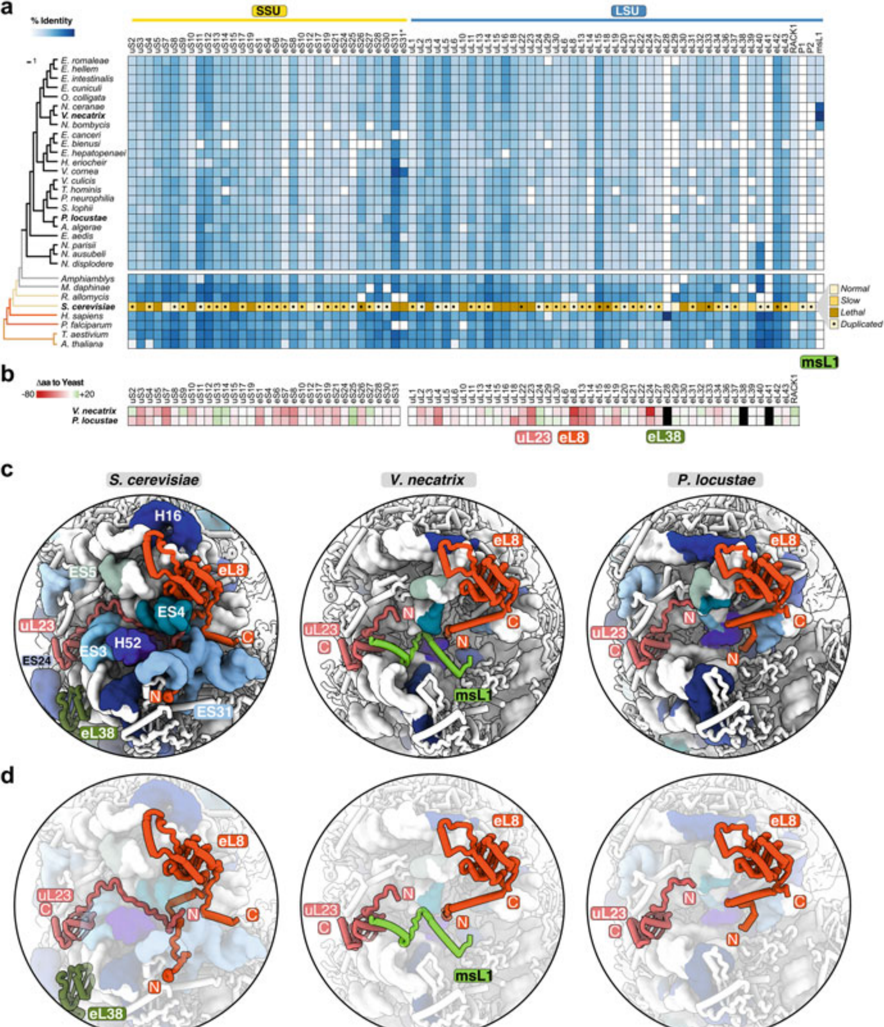

Fig. 1.8 Microsporidian ribosomal protein phylogeny, identity, and structure relative to their yeast homologs. (**a**) Ribosomal protein phylogeny generated by using protein sequences conserved in all listed microsporidian species. Connected to the microsporidian phylogeny (black) is a simplified tree for other non-microsporidian species, based on (James et al. 2006, 2013; Haag et al. 2014). For the microsporidian phylogenetic tree, the protein sequences were obtained by performing translated nucleotide blast (tblastn) searches with an E-value cutoff of 0.05, using the *S. cerevisiae* sequences or verified microsporidian hits as query and MicrosporidiaDB (Aurrecoechea et al. 2011) as database. For *P. locustae* and *V. necatrix*, protein sequences were obtained from (Barandun et al. 2019; Ehrenbolger et al. 2020) or local genome databases. For the non-microsporidian species, sequences were obtained from https://www.ncbi.nlm.nih.gov/. Proteins were aligned using MUSCLE 3.8.31 (Edgar 2004) and trimmed using trimAl (Capella-Gutiérrez et al. 2009) with the –gappyout option. The trimmed alignments were then concatenated using FASconcat 1.11 (Kück and Meusemann 2010). The phylogenetic tree was constructed with RAxML 8.2.12 using the model PROTGAMMAILGF, determined with ProtTest 3.4.2, and 1000 bootstrap replicates. The sequence identity heatmap was constructed using MUSCLE 3.8.31 (Edgar 2004) and Clustal-Omega (Sievers

P2), as are some of the frequently missing proteins (eS12, eS25, and eL29). The nonessential eL38 is present in all earlier branching eukaryotes and is absent in all but two microsporidian species (*M. daphinae*, *Amphiamblys* sp.), suggesting a relatively recent loss of this ribosomal protein (Barandun et al. 2019). These findings demonstrate that microsporidia have typically retained essential proteins and eliminated nonessential ones.

The nonessential protein eL41 is the only yeast subunit absent in all sequenced microsporidia (Fig. 1.8a) (Barandun et al. 2019; Ehrenbolger et al. 2020). It is remarkably short in other eukaryotes, only ~25 amino acids, and forms a small bridge between the LSU and the SSU (Tamm et al. 2019). Deletions of eL41 are easily tolerated, with knockout yeast strains displaying growth rates similar to wild-type strains (Giaever et al. 2002). More in-depth analyses have revealed that eL41 plays a role in translational efficiency (Dresios et al. 2003; Meskauskas et al. 2003). Ribosomes lacking eL41 had both lower translational fidelity and slower rates of peptidyltransferase activity. This suggests that the removal of eL41 in microsporidia may be another factor contributing to their markedly high rate of missense mutations (Melnikov et al. 2018b). The deletion of eL41 may also result in a slower translation rate, although no information is currently available on the kinetics of microsporidian ribosomes.

The ribosomal stalk proteins, which also have a purported role in translational efficiency (Wawiórka et al. 2017), are reduced in most microsporidia. A typical eukaryotic ribosomal stalk is composed of uL10, two subunits of P1, and two subunits of P2. All five protomers contain a highly conserved, C-terminal SDDDMGFGLFD motif, preceded by a long and flexible linker (Choi et al. 2015). This organization and motif is found in organisms as diverged as humans and the archaeon *Pyrococcus horikoshii* (Ito et al. 2014). During active translation, the C-termini of the pentamer bind to and recruit the essential elongation factor EF1α, which delivers charged aminoacyl-tRNA to the ribosome. It is proposed that the five redundant motifs aid in the rapid and efficient recruitment of the correct aminoacyl-tRNA, by greatly increasing the local concentrations of EF1α (Wawiórka et al.

Fig. 1.8 (continued) et al. 2011). The *S. cerevisiae* sequences were set as reference, except for eL28 and msL1, where *H. sapiens* and *V. necatrix* were used. The different shades of blue describe the percentage identity of the protein sequence compared to the reference. The row for *S. cerevisiae* contains viability data, color coded for lethal (dark yellow), slow-growing (yellow), and normal-growing (cream) ribosomal gene knockouts (Giaever et al. 2002; Gao et al. 2015). A black dot is used to mark genes that are duplicated in the yeast genome. Only single gene knockouts were performed in the referenced study. The sequence of eS31* was modified by removing the ubiquitin moiety to create the mature protein. (**b**) Difference in length between the *V. necatrix* or *P. locustae* and *S. cerevisiae* ribosomal proteins. (**c**) Comparison of the region around ES4 between the *S. cerevisiae* (left, PDB 4 V88), *V. necatrix* (middle, PDB 6RM3), and *P. locusate* ribosome (PDB 6ZU5). Selected ribosomal proteins are colored and labeled with name and N- and C-termini in shades of red. The lost eL38 and the gained msL1 are shown in shades of green. (**d**) The same view is shown as in (**c**) with selected proteins colored solid and the ribosome structure transparent

2017). Additionally, this kinetic model of decoding suggests that ribosomal pausing leads to the acceptance of near-cognate anticodons, resulting in missense mutations. It is therefore interesting that the majority of microsporidia do not to encode P1, and some may have lost P2 (Fig. 1.8), implying a single EF1α-binding motif is present. Previous work has demonstrated that P1 and P2 are nonessential in eukaryotes only because uL10 retains an EF1α binding domain (Santos and Ballesta 1995; Remacha et al. 1995). On the other hand, the prokaryotic equivalents to P1/P2 are required for translation (Huang et al. 2010), as prokaryotic L10 lacks the binding motif. Remarkably, the uL10 homologs for microsporidian clades have lost the linker and the SDDDMGFGLFD motif (data not shown). Some microsporidia therefore have no identified proteins that can recruit EF1α to ribosomes. This finding may indicate that the translation rate and fidelity are much lower in microsporidia. Alternatively, microsporidia might have developed novel proteins or binding motifs to recruit EF1α. This possibility is of particular interest, as the C-terminal motif utilized by eukaryotes and archaea is a common target for potent toxins like ricin (Choi et al. 2015; Fan et al. 2016). A unique motif would represent an attractive target for therapeutics or pesticides.

1.5.3 Retained and Gained Ribosomal Proteins

Most microsporidian proteins have relatively low sequence identity to yeast proteins (Fig. 1.8). This is not entirely unexpected, as even proteins from two closely related *Nematocida* species share only ~70% of their amino acid sequence (Balla and Troemel 2013). A noticeable outlier in this divergence is eS31, an essential protein located in the beak of the SSU. Interestingly, eS31 is always produced as a fusion with a ubiquitin moiety. The ubiquitin acts as a chaperone protein to assist in the production and folding of eS31 and is cleaved off before eS31 is incorporated into ribosomes (Martín-Villanueva et al. 2019). The high sequence identity for eS31 derives from this ubiquitin moiety, as a realignment without ubiquitin results in much lower values (see eS31 vs eS31* in Fig. 1.8). Another highly conserved protein is eL15, which is present in all sequenced microsporidia. Little is known about eL15's function other than that it is essential; however, it is a structural protein that is mostly buried and is therefore likely to have many conserved intermolecular interactions. Additionally, eL15 seems to mediate concentrations of other core ribosomal proteins, and its dysregulation leads to various cancers and diseases (Wlodarski et al. 2018; Ebright et al. 2020). Despite the lack of focused studies, the high conservation of eL15 in microsporidia evinces a high level of functional significance, which is not amenable to mutations in sequence or structure.

In addition to retaining most ribosomal proteins, microsporidia have also gained at least one novel subunit. The microsporidia-specific ribosomal protein (msL1) binds to *V. necatrix* ribosomes in a gap left by the loss of four ESs (Fig. 1.5) (Barandun et al. 2019). Although the specific role of this protein is unknown, it may be required to stabilize the ribosome in the absence of ESs. Genomic erosion in

organelles, such as mitochondria, has resulted in a similarly minimized rRNA. In response, many mitochondria have acquired unique proteins used to patch unstable ribosomes (Petrov et al. 2019). It is likely that msL1 serves a similar patching function in microsporidia where rRNA reduction led to structural instability.

1.5.4 Conserving Energy by Utilizing Ribosome Hibernation Factors

Translational costs are high, and an estimated 30 ATPs are required for the biosynthesis and attachment of each amino acid (Wagner 2007). Such costs are unsustainable in nutrient-poor conditions. Organisms therefore express proteins known as hibernation factors, which bind to and inhibit ribosomes when nutrients are scarce (Prossliner et al. 2018). These factors allow cells to sequester intact ribosomes instead of degrading them (Brown et al. 2018; Trösch and Willmund 2019). The ability to inactivate ribosomes and recover them post-quiescence is of vital importance to microsporidia, as they spend a significant portion of their lifecycle as metabolically inactive spores (Weiss and Becnel 2014).

Microsporidia encode multiple hibernation factors, including the late-annotated short open reading frame 2 (Lso2), and microsporidian dormancy factors (MDF) 1 and 2 (Barandun et al. 2019; Ehrenbolger et al. 2020). All three of these proteins block active sites of the ribosome (Fig. 1.6) and are incompatible with active translation. In yeast, Lso2 is important for recovery of ribosomes post-starvation (Wang et al. 2018), and roughly 10% of ribosomes isolated from starved yeast are bound by Lso2 (Wells et al. 2020). Microsporidian ribosomes isolated from spores, on the other hand, displayed an approximately 92% occupancy rate, indicating that the vast majority of ribosomes in spores are in an inactivated state (Ehrenbolger et al. 2020). MDF1 and MDF2 have not been biochemically characterized; however, their high occupancy in spores and mechanisms of binding indicate that they are likely hibernation factors (Barandun et al. 2019). While MDF1 is broadly conserved in eukaryotes, MDF2 may be species-specific. Orthologs have thus far only been identified in *V. necatrix*, *Nosema ceranae*, and *Nosema apis*. The high occupancy of these factors bound to spore-stage ribosomes, and the fact that microsporidia have potentially evolved species-specific hibernation factors, demonstrates that sequestration of ribosomes during the spore stage is crucial. Although hibernation factors are not specifically associated with reductive evolution, they provide an additional example of the mechanisms by which microsporidia conserve energy.

1.6 Microsporidian Ribosome Assembly

In eukaryotes, ribosome biogenesis is a multidimensional process requiring the action of all three RNA polymerases (Pol) and a complex repertoire of over 300 assembly factors and snoRNAs (Woolford and Baserga 2013; Ebersberger et al. 2014; Klinge and Woolford 2019). The pathway starts in the nucleolus, a subcompartment of the nucleus, where the transcription of a precursor ribosomal RNA (pre-rRNA) initiates a co-transcriptional maturation pathway. In yeast, the precursor contains the rRNAs of both the small subunit (18S) and the large subunit (5.8S, 25S). These rRNAs are flanked by four transcribed spacer regions, two external and two internal (ETS, ITS; Fig. 1.9a). The third rRNA of the large subunit (5S) is transcribed from a different locus and is not part of this long precursor RNA. Assembly factors associate in a co-transcriptional manner with the rRNA precursor, including the transcribed spacers, to assist in the folding and enzymatic processing of the pre-rRNA and to incorporate ribosomal proteins. Several co-transcriptional endonucleolytic cleavage events are required to process the spacers and release the partially matured pre-ribosomal particles. Maturation then continues in the nucleus, where the pre-mature rRNA ends (e.g., 5′ ETS or ITS2) are further processed and degraded. After a controlled export through the nuclear pore complex, the last ribosomal maturation steps and quality control events occur in the cytoplasm.

The transcribed spacers are not present in the mature ribosome, but are essential elements required to recruit ribosome assembly factors. The level of spacer processing is also used to demarcate the maturation stage of this complex particle (Klinge and Woolford 2019). In addition, eukaryotic ribosomal expansion segments, which are part of the mature ribosome, are also involved in recruiting specific assembly factors. Genome compaction in microsporidia has not only removed rRNA elements, such as eukaryotic ESs, but also drastically affected the transcribed

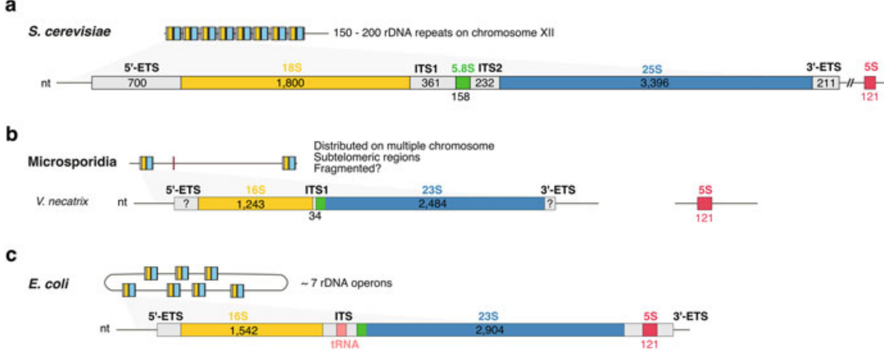

Fig. 1.9 Compaction of the microsporidian rDNA locus to a prokaryotic-like organization. Schematic representation of a single rDNA locus, below a diagram indicating the genomic distribution of all rDNA loci, from (**a**) *S. cerevisiae*, (**b**) microsporidia (nucleotide sizes from *V. necatrix*), and (**c**) *E. coli*. The genes and known spacer sizes are indicated and drawn to scale for comparative purposes

spacers of the ribosomal precursor (e.g., removal of ITS2; Fig. 1.9b). While the pre-ribosomal and ribosomal RNA have been minimized, the number of ribosomal proteins associated with the mature microsporidian ribosome has been less affected (see Sect. 1.5.2) (Barandun et al. 2019; Ehrenbolger et al. 2020). This raises the question of whether ribosome assembly factors and the maturation pathway have been similarly reduced overall, or if specific assembly factor categories have been more impacted by genome reduction than others. Have microsporidia lost ribosome assembly factors with a role in maturing eukaryotic-specific RNA or protein elements? The following section discusses the impact of reductive evolution on the organization of rDNA loci and the maturation of pre-rRNA in microsporidia.

1.6.1 Impact of Genome Compaction on Number and Localization of the rDNA Loci

In most organisms, the ribosomal RNAs are transcribed from one or more polycistronic ribosomal DNA loci. The number of rDNA loci increases considerably from prokaryotes to eukaryotes: from a single rDNA locus in slow-growing bacteria (e.g., *Mycobacterium tuberculosis*) to ~150–200 copies in yeast (Petes 1979) to more than 10,000 in some plants (Kobayashi 2014). In *S. cerevisiae*, the primary model organism to study eukaryotic ribosome assembly, all rDNA loci are clustered head to tail on a single chromosome (Petes 1979) (Fig. 1.9a). Within eukaryotes, the size of one pre-rRNA coding locus varies substantially. These size variations are mostly due to differences in the lengths of external and internal spacer elements or eukaryotic-specific ribosomal expansion segments. Eukaryotic rDNA sizes range from the minimal microsporidian version with approximately 4.5 kbp (calculated from the *V. necatrix* sequences), which has lost many regulatory spacers and ESs, to ~9.1 kbp in yeast or ~ 43 kbp in humans, which contain long ETSs and extensive intergenic spacer regions.

In microsporidia, the rDNA organization and localization within the genome differ between species. While other eukaryotes contain large numbers of clustered rDNA repeats, microsporidia are left with fewer and often not clustered rDNA genes. Twenty-two rDNA copies have been reported for *E. cuniculi*, located on both telomeric ends of its 11 chromosomes (Brugère et al. 2000; Katinka et al. 2001; Dia et al. 2016). Forty-six partial and polymorph rDNA loci have been found in *N. ceraneae* (Cornman et al. 2009), and similar to the rDNA loci in *N. bombycis*, they appear to be distributed over all chromosomes (Liu et al. 2008). While the individual loci are scattered throughout different chromosomes in many microsporidian species, in *N. apis*, the rDNA genes cluster as repeats head to tail (Gatehouse and Malone 1998), which is more similar to the classical arrangement observed in other eukaryotic organisms.

In most eukaryotes, the 5S encoding gene is dispersed throughout the genome and is not adjacent to the other three rRNAs. One exception to this observation is

S. cerevisiae, where the 5S rRNA gene clusters in the intergenic spaces between rDNA repeats (Fig. 1.9a). Both arrangements have been observed in microsporidia. Similar to yeast, in *N. bombycis*, the 5S gene is located next to the rDNA locus (Huang et al. 2004). Other species, such as *E. cuniculi* and *E. intestinalis*, have dispersed the 5S throughout the genome. In these two microsporidia, three copies for the 5S have been detected (Katinka et al. 2001; Corradi et al. 2010), in contrast to the 22 rDNA loci. While the rDNA locus is transcribed by RNA Pol I, the 5S rRNA is transcribed by RNA Pol III (Ciganda and Williams 2011). The microsporidian transcription machinery includes elements for RNA pol I, II, and III (Katinka et al. 2001), indicating that the use of separate polymerases for 5S and rDNA transcription may be retained in microsporidia.

The comparatively small number of rDNA repeats in microsporidia may be a result of their diminutive cell size, simple genomes, and low proteomic complexity. Fewer and shorter genes might require a reduced number of ribosomes, which in turn can be synthesized from fewer rDNA repeats. Indeed, a strong positive correlation between genome size and the number of rDNA repeats in eukaryotes has been noted (Prokopowich et al. 2003). Although this correlation exists, in general, only a fraction of all rDNA repeats are transcriptionally active. The actual rRNA synthesis rate is more so determined by the rate of RNA polymerase recruitment. A yeast strain with only 42 rDNA repeats, compared to the original 142 repeats, grows as well as wild type because two times more RNA polymerases are recruited to the rDNA locus (French et al. 2003). In addition to a potentially reduced need for ribosomes and increased RNA polymerase recruitment to a single locus, the simplified microsporidian rDNA gene organization might allow for a more streamlined ribosome maturation. Fewer pre-rRNA processing steps might be required than in other eukaryotes, due to missing pre-rRNA elements such as internal transcribed spacer 2 (ITS2).

1.6.2 Loss and Minimization of Transcribed Spacers

In many microsporidian species, genome compaction and gene fusion led to a reduction in the total number of ribosomal RNAs from four to three, which represents a reversal of the evolutionary trend seen in eukaryotic ribosomes. The eukaryotic 5.8S rRNA sequence and the 5′ end of the prokaryotic large subunit gene are homologous (Jacq 1981). In typical eukaryotes, ITS2 separates the 5.8S from the remainder of the large subunit rRNA gene (Fig. 1.9b). Early branching microsporidia like *M. daphnia* and *Chytridiopsis typographi* still contain highly reduced versions of ITS2 and thereby preserve the traditional eukaryote-specific separation of the 5.8S from the LSU gene (Corsaro et al. 2019). In all later-branching microsporidia, ITS2 has been removed (Vossbrinck and Woese 1986). The reductive evolution in these organisms led to a complete loss of ITS2 and fusion of the 5.8S rRNA with the LSU rRNA (23S), which has created a unique eukaryotic rDNA locus (Fig. 1.9b) with prokaryotic features (Fig. 1.9c). The remaining ITS has been

reduced to a surprisingly short sequence in some microsporidia. While *N. bombycis* (Huang et al. 2004) contains an ITS of ~179 nt, other microsporidians, such as *V. necatrix* or *N. apis* (Gatehouse and Malone 1998), compacted this element to only ~33/34 nt. The intergenic spacer regions are important signal sequences for co-transcriptional endonucleolytic processing of the pre-rRNA fragment. Together with an apparent reduction of the 5′ and 3′ ETS regions and the removal of ITS2, the shortening of the ITS has significant implications for the ribosome maturation process, which is tightly controlled by ribosome assembly factors binding to these regions.

1.6.3 Impact of rDNA Compaction on Ribosome Biogenesis Factors

In 2014, Ebersberger et al. performed an evolutionary analysis of 255 yeast protein factors involved in ribosome biogenesis and included four microsporidian species in their analyses (Ebersberger et al. 2014). From these initial factors, 244 were proposed to be present in the last common ancestor shared with the microsporidia. Remarkably, only about half of them could be identified in microsporidia, which was highlighted as "the most remarkable gene loss" observed among the eukaryotic supertaxa (Ebersberger et al. 2014). Although extensive lists of factors involved in yeast ribosome biogenesis existed at the time, the precise functions or binding sites of most of these factors were unknown due to a lack of structural and biochemical data. During the decade since, our knowledge of fungal ribosome biogenesis has advanced to a detailed structural and functional description of the individual factors. This is mainly due to the technical progress made in cryo-EM, which provided high-resolution information and enabled the study of previously inaccessible pre-ribosomal particles from the fungi *S. cerevisiae* or *Chaetomium thermophilum*. These structures now provide an updated and comprehensive picture of fungal ribosome maturation and depict the intricate interaction network of assembly factors and ribosomal proteins bound to pre-ribosomal rRNA elements (Barandun et al. 2018; Klinge and Woolford 2019). They show how ribosome maturation proceeds in a hierarchical manner through several different conformational states to produce the final mature eukaryotic ribosome (Klinge and Woolford 2019). The emerging structural data on the fungal biogenesis process, together with recent studies on the microsporidian ribosomes (Barandun et al. 2019; Ehrenbolger et al. 2020), allows us to give a few selected examples of why expansion segment and transcribed spacer removal or shortening might have enabled assembly factor loss (or vice versa).

In yeast, the 5′ ETS is 700 nt long and is involved in the co-transcriptional recruitment of up to 27 ribosome biogenesis factors and the formation of an assembly platform for the SSU. In microsporidia, the exact size and structure of the 5′ ETS pre-rRNA fragment are not known. However, several factors that

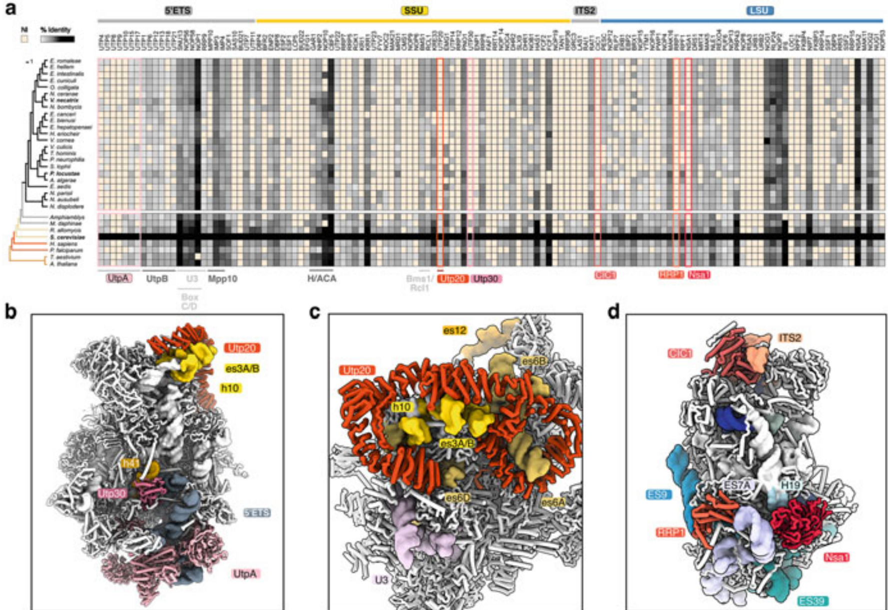

Fig. 1.10 A reduced set of ribosome biogenesis factors and selected examples of assembly factor and expansion segment loss in microsporidia. (**a**) Presence and conservation of ribosome assembly factors in selected eukaryotes and microsporidia. The protein sequences were obtained by performing translated nucleotide blast (tblastn) or protein blast (blastp) searches with an E-value cutoff of 0.05, using the *S. cerevisiae* sequences and MicrosporidiaDB (Aurrecoechea et al. 2011) as database. For *P. locustae* and *V. necatrix*, protein sequences were obtained from local genome databases. For the non-microsporidian species, sequences were obtained from https://www.ncbi.nlm.nih.gov/. For phylogenetic tree calculation, see legend of Fig. 1.8. Many biogenesis factors display significant sequence similarity (e.g., WD40 domain proteins). It was therefore common for the same open reading frame to be identified as homologous to multiple different biogenesis factors. In such cases, we selected hits with the lowest E-value. It should be noted that the figure thus serves as only a guide to general trends for absent and present proteins, since annotations may be inaccurate. Proteins that were not identifiable (NI) are shown in cream. Biogenesis factors are clustered based on known or predicted binding regions within the 5' ETS, SSU, ITS2, or LSU of the pre-rRNA. Conservation correlates between members of the same complex. Example complexes are labelled below (**a**) in shades of gray. (**b-d**) Structures of *S. cerevisiae* pre-ribosomal particles denoting selected maturation factors that are often absent in microsporidia, as highlighted in (**a**). Pre-SSU structures from PDB 5WLC (Barandun et al. 2017) (**b**), PDB 7AJU (Lau et al. 2021) (**c**), and a pre-LSU-structure from PDB 6C0F (Sanghai et al. 2018) (**d**) are displayed with expansion segments missing in *V. necatrix*, colored in shades of orange and yellow (SSU) or shades of blue and green (LSU), and selected biogenesis factors colored as in (**a**). These examples demonstrate a correlation between ES reduction and the loss of biogenesis factors that typically bind to those ESs

typically bind to this region have not been identified in microsporidia (Fig. 1.10). One of the first and largest multi-subunit complexes bound to the newly synthesized 5′ ETS is UtpA (Fig. 1.10b) (Hunziker et al. 2016). UtpA is a 7-subunit complex in yeast but appears to be absent or drastically reduced in microsporidia. The UtpA

binding site on the 5′ ETS is shared with Utp18, a subunit of another early binding biogenesis complex, UtpB. The potential absence of Utp18 and the entire UtpA complex (Fig. 1.10a) suggests microsporidia may contain a shorter 5′ ETS sequence, which recruits a minimal small subunit assembly platform. Alternatively, assembly factors may be too divergent to be identified.

During pre-rRNA maturation, several eukaryotic expansion segments of the ribosomal RNA are bound and remodeled by assembly factors. In general, the absence of an assembly factor correlates with removal of its binding site in other organisms (Fig. 1.10). One striking example includes the SSU segments es3 and es6, which are bound and stabilized by the large HEAT repeat protein Utp20 (Fig. 1.10c). Es3 and es6 are the two largest small subunit expansion segments and have been completely lost or strongly reduced in microsporidia (Barandun et al. 2019; Ehrenbolger et al. 2020). Similarly, Utp20 appears to be absent in all microsporidian species. This suggests the primary role of Utp20 in chaperoning the maturation of these two expansion segments is no longer required in microsporidia. Similarly, the loss of h41 correlates with the loss of Utp30, an assembly factor binding to this rRNA element in pre-ribosomal particles (Fig. 1.10b). In the large subunit, ES7 is bound by two assembly factors, Rrp1 and Nsa1. Again, both the ES7 and the two assembly factors seem to be eliminated from microsporidian genomes.

A key step in large subunit maturation in *S. cerevisiae* involves processing of the ITS2 prior to nuclear export. Absence of ITS2, the spacer separating the two LSU rRNAs, explains the absence of many ribosome assembly factors binding this RNA region, such as Cic1, Rlp7, or the Las1 complex (Woolford and Baserga 2013). ITS1 processing in yeast is catalyzed by the essential ribozyme-protein complex RNAse MRP. While microsporidia still contain a highly reduced version of RNAse MRP (Zhu et al. 2006), ITS1 has been ablated to only 33 nt. It is unclear if this short ITS region can fold into a structure recognized by the minimized RNAse MRP, or if a simpler mechanism is used.

Apart from the mature ribosome structure, genomic data, and bioinformatics, very little is known about ribosome assembly in microsporidia. By studying the process in these minimal organisms, we can learn more about the still relatively unknown role of expansion segments during the assembly process in other eukaryotic organisms. The compaction of rRNAs together with the removal of transcribed spacer regions appears to have significantly affected the assembly process in microsporidia. A more thorough analysis of how expansion segment removal correlates with assembly factor loss will be required to understand the process in microsporidia and relate loss and compaction to a potential functional role in other eukaryotes.

1.7 Conclusion and Future Perspectives

Genome reduction and size appear to correlate with the degree of metabolic dependence on other organisms. Consequently, an obligate intracellular lifestyle provides a plausible explanation for the loss of redundant metabolic pathways and the

invention of novel and more energetically efficient mechanisms of host exploitation. The drastic impact of genome compaction in microsporidia, however, has not only reduced the complexity of metabolic pathways but also affected intergenic regions, minimized gene sizes, and removed regulatory elements and features considered to be essential in eukaryotic organisms.

Genome erosion has significantly altered the microsporidian ribosomal DNA locus. By removing eukaryote-specific elements, such as ITS2 and nearly all expansion segments, the rDNA gene arrangement regressed to a prokaryote-like organization. The recent structural characterization of the microsporidian ribosome has illustrated the impact of genome reduction on the composition and assembly of this essential and ancient particle. It provided the surprising information that despite the loss of their rRNA binding site, almost all eukaryote-specific ribosomal proteins, albeit shortened, are still retained in the structure. Could limited access to primary metabolites precipitate a more compact ribosome? Are nucleotides more "rare" than amino acids, and could this be one reason why the rRNA is much more compacted than ribosomal proteins? Does the extensive rRNA loss affect the fidelity of the ribosome? Further studies are required to delineate the functional implications of ribosome compaction on protein synthesis and to reveal the suitability of ribosome-targeting antibiotics as translation inhibitors in microsporidia.

Microsporidia are of great interest in the fields of infection biology and comparative structural biology. They act as a reservoir for many unique and peculiar structures and have developed the most minimized versions of eukaryotic macromolecular complexes. Additional biochemical and structural studies in microsporidia not only will illuminate their own lifecycle but will also shed light on optional elements in many highly conserved cellular processes.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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Ethical Approval The chapter is a review of previously published accounts. As such, no animal or human studies were performed.

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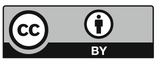
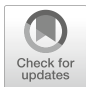


Chapter 2
Comparative Genomics of Microsporidia



Bryony A. P. Williams, Tom A. Williams, and Jahcub Trew

Abstract The microsporidia are a phylum of intracellular parasites that represent the eukaryotic cell in a state of extreme reduction, with genomes and metabolic capabilities embodying eukaryotic cells in arguably their most streamlined state. Over the past 20 years, microsporidian genomics has become a rapidly expanding field starting with sequencing of the genome of *Encephalitozoon cuniculi*, one of the first ever sequenced eukaryotes, to the current situation where we have access to the data from over 30 genomes across 20+ genera. Reaching back further in evolutionary history, to the point where microsporidia diverged from other eukaryotic lineages, we now also have genomic data for some of the closest known relatives of the microsporidia such as *Rozella allomycis*, *Metchnikovella* spp. and *Amphiamblys* sp. Data for these organisms allow us to better understand the genomic processes that shaped the emergence of the microsporidia as a group. These intensive genomic efforts have revealed some of the processes that have shaped microsporidian cells and genomes including patterns of genome expansions and contractions through gene gain and loss, whole genome duplication, differential patterns of invasion and purging of transposable elements. All these processes have been shown to occur across short and longer time scales to give rise to a phylum of parasites with dynamic genomes with a diversity of sizes and organisations.

Keywords Genome compaction · Microsporidia · Genome evolution · Gene loss · Transposable elements

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2.1 Why Sequences Genomes and Where Are We Now?

Over the past decades, genomic data have accumulated at a phenomenal rate with over 50 microsporidian genomes now available (Fig. 2.1). This abundance of sequence data, curated at www.MicrosporidiaDB.org (Aurrecoechea et al. 2011), has provided a springboard for a multitude of cell and molecular biological work characterising the microsporidian cell structure and its interactions with hosts. In addition, these genomes represent an important data set for understanding the evolution of small eukaryotic genomes and parasite genomes more generally. An increasingly large amount of data in this single phylum allows us to get a better handle on the dynamics of reductive genome evolution in eukaryotes, understanding changes over smaller and larger evolutionary timescales in a group with large-scale variation in genome architecture and in which genome size varies over an order of magnitude.

2.2 The First Sequenced Microsporidian Genome: *Encephalitozoon cuniculi*

The first genome of a microsporidian to be sequenced was that of *Encephalitozoon cuniculi* GB-M1 in 2001 (Katinka et al. 2001). Species within the clade *Encephalitozoon* emerged as opportunistically infecting organisms in AIDS patients in the 1980s (Terada et al. 1987). Prior to the full sequencing of this organism's genome, full sequences of chromosomes had been decoded revealing the compact nature of microsporidian genomes (Duffieux et al. 1998; Peyret et al. 2001). Through preceding cell and molecular experiments, it was also clear that microsporidia were unusually reduced eukaryotes that lacked the separate 5.8S rRNA gene distinctive of

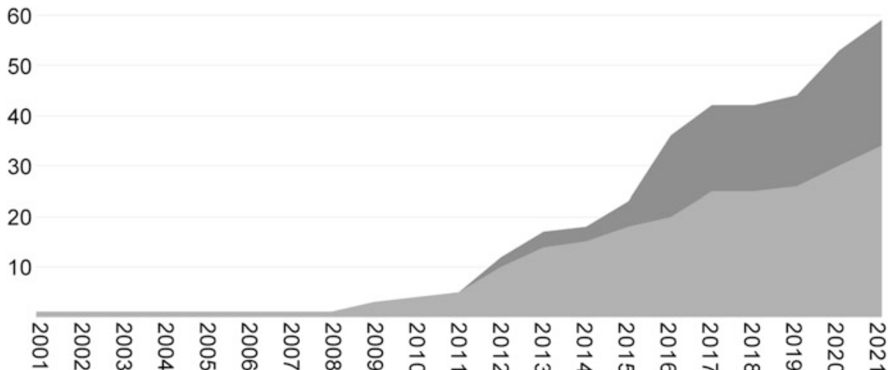

Fig. 2.1 Accumulation of microsporidian genomes assemblies over time in NCBI. The top line shows accumulation of numbers of assemblies, whilst the lower line indicates the number of new unique species assemblies. X axis shows years and Y axis, numbers of available genomes

eukaryotes and had ribosomes that settled at a sedimentation coefficient equivalent to those extracted from bacteria (Ishihara and Hayashi 1968) and seemed to lack characteristic organelles such as the mitochondrion or a typical Golgi (Cavalier-Smith 1983). Whilst for some time leading up to the sequencing of the *Encephalitozoon cuniculi* genome, these characteristics were viewed as primitive, there was growing evidence that microsporidia were related to fungi and that these reduced characteristics actually represented a secondary reduction (Cavalier-Smith 1983; Hirt et al. 1997, 1999; Roger 1999). However, it was with the full sequencing and annotation of the *Encephalitozoon cuniculi* genome that the extent and nature of reductive evolution within these organisms became clear (Katinka et al. 2001). At the time, the genome was completed by Sanger sequencing with the chromosome ends completely sequenced and annotated at a later date (Dia et al. 2016). The genome of *Encephalitozoon cuniculi* GB-M1 remains arguably the best annotated microsporidian genome and a key reference for all other microsporidia genomics studies.

Within the broader scope of microsporidian genome sizes, the *Encephalitozoon cuniculi* genome is small at 2.9 Mb, with few species having a smaller genome size. However, one characteristic of this genome which is common to all microsporidia is the small size of the predicted proteome; this currently stands at 2041 predicted protein coding genes (The UniProt Consortium 2021). The majority of these are single copy genes, and the predicted proteins are short (359 amino acid mean length) relative to the average across eukaryotes (472 aa), but still longer than those in bacterial or archaeal genomes (320 aa and 283 aa, respectively) (Katinka et al. 2001; Tiessen et al. 2012), which fits with the general trend of shorter proteins in genomes with small proteomes (Tiessen et al. 2012). These proteins have a simplified domain organisation with fewer domains on average than other eukaryotes (Koonin et al. 2004). It has been suggested that this results from a need for fewer interaction domains when there are fewer proteins to interact with (Zhang 2000; Koonin 2004). The genome is gene dense with a coding sequence for every 1.025 kb in the 'chromosomal core', just 38 introns and little repetitive DNA (Katinka et al. 2001; Pombert et al. 2013). The genome is organised as 11 linear chromosomes with a fairly homogeneous length ranging from 217 and 315 kb and is likely diploid (Brugère et al. 2000). Whilst remnant mitochondria named mitosomes have been uncovered in microsporidia (Williams et al. 2002), there is no mitochondrial genome; thus, all the genomic material is contained within these linear chromosomes. The ends of the chromosomes are characterised by a SSU-LSU rDNA 'transcription unit' and areas of segmental repeats between 3.5 and 23.8 kbp. These segmental repeats can contain genes in multiple copies and also encode four different gene families (*interAE*, *interB*, *interC* and *interD*), hypothesised to mediate host-cell interaction and/or immune escape (Dia et al. 2007, 2016). Variation in the size of these telomeric areas is thought to be responsible for variation in size of chromosomes within the species (Biderre et al. 1999).

2.3 How Do Other Microsporidian Genomes Differ from This Model?

The *Encephalitozoon cuniculi* genome size, content and organisation reflect three key processes that have shaped the generally streamlined genomes of microsporidia. These are (1) loss of genes, (2) shortening of genes and (3) loss of non-coding material including introns, intergenic regions repetitive DNA and transposable elements. Whilst there are mechanisms driving down genome size, within specific lineages or microsporidia, other mechanisms drive genome size in the opposite direction. These are the acquisition of new genes through gene and whole genome duplication, de novo gene origination, horizontal gene transfer, invasion and proliferation of transposable elements and re-expansion of intergenic regions. This interplay of mechanisms driving reduction and those driving genome expansion have led to a diversity of different genome sizes and organisations across the phylum where genome size varies by over 20-fold from 2.3 Mb to 53.1 Mb, whilst the number of predicted protein-coding genes varies over a much smaller range from approximately 1820 to 6442 (Corradi et al. 2010; Cuomo et al. 2012; Pombert et al. 2015; Cormier et al. 2021). Over the next paragraphs, we describe examples of how these different mechanisms have driven change in genome size, organisation and composition at different phylogenetic depths across the phylum (Fig. 2.2).

2.4 Massive Gene Loss at the Emergence of the Microsporidia

Comparisons of gene family evolution in microsporidians and their fungal relatives have identified core gene families conserved across microsporidia as well as major changes in gene content that occurred early in the evolution of microsporidia, during adaptation to a host-associated lifestyle (Keeling et al. 2010; Cuomo et al. 2012; Heinz et al. 2012; Campbell et al. 2013; Nakjang et al. 2013; Desjardins et al. 2015; Wiredu Boakye et al. 2017; Galindo et al. 2018; Cormier et al. 2021). These studies identify a microsporidian ‘core’ set of protein families that are usually conserved across microsporidia. Some of these are ancestor-derived conserved gene families including genes associated with basic cellular functions such as transcription, translation and DNA replication and repair, cell cycle control and the key enzymes for glycolysis, the pentose phosphate pathway, trehalose metabolism, chitin biosynthesis and those associated with the biosynthesis of structural components of the fungal cell membrane and spore wall (Nakjang et al. 2013). Proteins encoding a Fe/S cluster assembly pathway are also conserved across all sequenced microsporidian genomes (Freibert et al. 2017; Galindo et al. 2018). This pathway is localised to the mitosome and is likely essential for the maturation of certain nuclear and cytosolic Fe/S proteins (Freibert et al. 2017). The essentiality of this pathway provides a selection pressure for the retention of the mitochondrion in this highly minimal form across microsporidia.

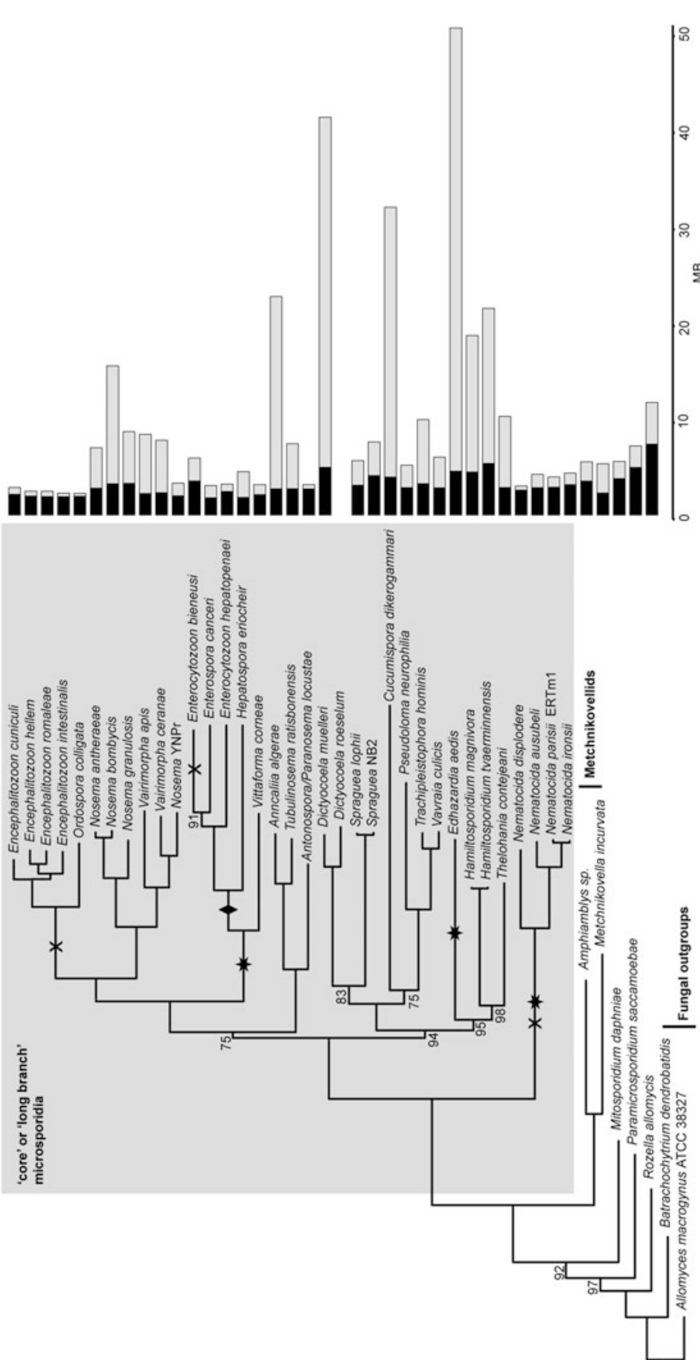

Fig. 2.2 Phylogenetic relationships between microsporidian species with sequenced genomes with to the right-hand side indication of genome/assembly size with proportion taken up by coding sequences in black (Note that for some species, the value for some figures was taken from published estimates which may not include gene duplicates). ◆ indicates putative loss of glycolysis, ✶ indicates putative loss of introns and a functional spliceosome and ✖ indicates putative losses of loss of the RNA interference pathway (available proteomes for sequenced genomes at NCBI were downloaded on the 18th of May 2021). For *Antonospora/Paranosema locustae* and *Metchnikovella incurvata* and *Spraguea* NB2, the nucleotide assembly was downloaded and EMBOSS getorf (min 100) used to predict proteins. OrthoFinder (default settings) was used to create orthogroups (Emms and Kelly 2019). Any orthogroups with representation for at least 20 taxa

In contrast some conserved microsporidia protein families are microsporidia-specific and are likely associated with and essential to the 'microsporidian lifestyle'. These include polar tube and spore wall protein families but also protein families without any characterised members or identifiable domains (Nakjang et al. 2013).

These studies also identified a massive loss of genes in the common ancestor of microsporidia involving the predicted loss of 1590 protein families leaving a common ancestor of the 'core' microsporidia with an estimated 1121 protein families (Nakjang et al. 2013). This early gene bottleneck left microsporidia bereft of pathways associated with energy metabolism including oxidative phosphorylation and the tricarboxylic acid (TCA), likely allowing the degeneration of mitochondria. In addition to loss of energy metabolism, there is a severely limited repertoire for the biosynthesis of amino acids and no genes involved in the de novo synthesis of nucleotides. There is also generally thought to be a streamlining of the protein network of most core pathways and cellular components. Other canonical eukaryotic pathways are differentially lost, and these include the RNA interference pathway, the spliceosome, fatty acid biosynthesis, the pentose phosphate pathway and glycolysis (Fig. 2.2) (Akiyoshi et al. 2009; Keeling et al. 2010; Cuomo et al. 2012; Wiredu Boakye et al. 2017; de Albuquerque et al. 2020).

Most recent phylogenetic analysis suggest that microsporidia are closely related to fungi (James et al. 2013), but for a long time, there was little genomic data for organisms that represent intermediate lineages between the microsporidia and other eukaryotes. However, there is now accumulating genomic data for many fascinating lineages that represent part of a sister group to the microsporidia or basal microsporidian lineages. These include *Rozella allomycis*, *Amphiamblys*, *Metchnikovella*, *Paramicrosporidium* and *Mitosporidium* (James et al. 2013; Mikhailov et al. 2017; Quandt et al. 2017; Galindo et al. 2018; Nassonova et al. 2021). *Rozella allomycis* is a cryptomycotan that is an obligate parasite of the water fungus *Allomyces* and morphologically quite distinct from the microsporidia, in particular in that it has a flagellated stage, whilst microsporidia have lost the flagellum (James et al. 2006). *Amphiamblys*, *Metchnikovella*, *Paramicrosporidium* and *Mitosporidium* are morphologically more similar to the microsporidia, and all possess a form of the polar filament or a manubrium analogous to the polar filament which is characteristic of microsporidia (Nassonova et al. 2021). The relationship between these groups is unclear, but phylogenetic analysis suggests that these organisms alongside microsporidia form a clade and that the microsporidia evolved from within a group of organisms called the *Cryptomycota* or *Rozellomycota* or *Rozellida* (Bass et al. 2018; Corsaro et al. 2019).

Fig. 2.2 (continued) were selected and aligned using MAFFT and trimmed using trimAl (Katoh et al. 2002; Capella-Gutiérrez et al. 2009). Initial trees (IQ-TREE) were inspected, and close protein orthologs that were the result of duplication within a single species were removed to leave a single copy (Nguyen et al. 2015). Alignments were concatenated, and IQ-TREE was run on the partitioned file with best model indicated for each aligned protein and 1000 UltraFast bootstrap replicates. Values for bootstraps were 100% unless indicated)

Extensive phylogenomic analyses have now shown how these lineages compare in gene content and organisation to those that have now been dubbed the 'canonical', 'long branch', or 'core' microsporidia giving some insight into events that shaped early microsporidia evolution (Bass et al. 2018; Timofeev et al. 2020; Nassonova et al. 2021). These studies have shown that the functional gene content of both *Amphiamblys* and *Metchnikovella* genomes is more similar to that of the 'core' microsporidia, whilst that of *Paramicrosporidium* and *Mitosporidium* is more similar to *Rozella allomycis* and other eukaryotes, suggesting that much of the genome remodelling in the microsporidian lineage occurred after the divergence of these latter taxa (Fig. 2.2). The genomes of *Paramicrosporidium* and *Mitosporidium* are relatively small and compact (7.28 Mb and 5.64 Mb, respectively) (Haag et al. 2014; Mikhailov et al. 2017), but they nonetheless possess some metabolic characteristics that clearly set them apart from the microsporidia, such as mitochondrial genomes (Haag et al. 2014; Quandt et al. 2017). In contrast *Amphiamblys* and *Metchnikovella* are more metabolically reduced, and like in 'core' microsporidia, mitochondrial pathways associated with the synthesis of nucleotides and amino acids are absent, and those associated with fatty acid metabolism are reduced (Galindo et al. 2018).

These data indicate that whilst genome compaction occurred sometime between the divergence of *the Fungi* and *the Cryptomycota*, there were different rounds of gene loss that marked the transition from free-living to parasitic organisms with the most dramatic loss in metabolic pathways likely occurring sometime before the divergence of *Amphiamblys* and *Metchnikovella* but after the divergence of the less metabolically simplified *Mi. daphniae*, *Pa. saccamoebae* and *R. allomycis* (Galindo et al. 2018).

After these periods of massive gene loss, there is evidence of substantial gene family expansions in different microsporidian lineages, giving rise to microsporidia specific gene families (Nakjang et al. 2013). These extensive expansions have in particular affected transporter proteins, many of which are predicted to target the parasite cell membrane. The hypothesis is that these transporters allow import of metabolites from hosts filling the gaps created by the past large-scale loss of biosynthetic capacity (Nakjang et al. 2013). Two key examples of microsporidian and microspordian-lineage-specific expansion of transporters include the nucleotide transporters NTT proteins that transport ATP and GTP and NAD+ from the host and the Major Facilitator Superfamily proteins (MFS) that transport ATP and GTP from the host. Both of these protein families are universally present across microsporidia, one having been acquired by horizontal gene transfer in the ancestor of the *Cryptomycota* + Microsporidia (see below) and the other being present in other eukaryotes with a canonical vertical inheritance from ancestors (Tsaousis et al. 2008; Cuomo et al. 2012; Major et al. 2019). Both have undergone multiple independent duplications within the phylum microsporidian lineages giving rise to different ortholog numbers in different lineages (Cuomo et al. 2012; Dean et al. 2018; Major et al. 2019).

In summary, the ancestor of 'core or 'long branch' microsporidia was already a parasite that likely had a relatively compact genome, and lacked a mitochondrial genome, the genes for the TCA cycle and oxidative phosphorylation and de novo nucleotide and amino acid biosynthesis. However, it encoded proteins that allowed

ATP uptake from the host, glycolysis, the pentose phosphate pathway, trehalose metabolism, RNAi and splicing.

2.5 Genomes Changes over Smaller Time Scales: Small Largely Static Genomes in the Genus *Encephalitozoon*

The genus *Encephalitozoon* harbours species with the smallest microsporidian genomes among this phylum of already reduced species. Genomes from multiple species and multiple isolates of the same species have now been sequenced from within *Encephalitozoon* providing a glimpse into how these compacted genomes are evolving at the level of the genus (Corradi et al. 2010; Pombert et al. 2012, 2013; Selman et al. 2013; Pelin et al. 2016). Multiple different strains of *Encephalitozoon cuniculi* named genotypes EC I-IV have been identified and are distinguished on the basis of the number of GTTT repeats within the internal transcribed spacer locus in the rRNA (Xiao et al. 2001). Representatives of genotypes ECI, ECII and ECIII have been fully sequenced and compared to with the originally sequenced *Encephalitozoon cuniculi* GB-M1 (ECI) strain to unveil patterns of change over short evolutionary time scales (Pombert et al. 2012, 2013; Selman et al. 2013; Pelin et al. 2016). This revealed genomes with near identical gene contents and gene arrangements; however, there was no evidence of recombination indicating that whist they are very closely related, they represent distinct strains with no exchange of material. In spite of their close relationship and highly similar genomes, this comparative study showed that the level of genetic diversity between strains was relatively high among ECI, ECII and ECIII (4.2 SNPs/kb) relative to SNPs numbers among strains of other species of single-celled pathogenic eukaryotes. This indicates a set of genomes that are largely frozen in content and organisation but fast evolving at the level of the nucleotide and therefore individual genes (Pombert et al. 2013).

Whilst nucleotides are changing at a relatively fast rate, differences in the coding content or significant changes to coding sequences were very few: One gene encoding a protein with a homeobox domain present in ECI is absent from ECII/ECIII genomes, there is a gene fission in ECI relative to other strains, and the ECI strain encodes 3 similar paralogs of one protein whilst other strains have a single paralog of the same protein (Pombert et al. 2013). However, the genome sequence of a wild isolate of genotype III from a steppe lemming did reveal a small-scale change with a large potential impact on phenotype: A frameshift mutation in a key meiosis gene Spo1 which likely renders it incapable of meiosis and sexual reproduction (Pelin et al. 2016). This demonstrates that, despite very high levels of similarity in the genome, there do exist small differences between very closely related strains that may result in fundamental differences in biology.

Within the same genus, further *Encephalitozoon* species have been sequenced (Corradi et al. 2010; Pombert et al. 2012). Whilst the genome of *Encephalitozoon cuniculi* is already highly compact, *Encephalitozoon intestinalis* takes that reduction

a step further: Its genome is just 2.3 Mb and thus 20% smaller than that of *E. cuniculi* (Corradi et al. 2010). Its genome is even more gene dense, its genes are even shorter and it has fewer hypothetical proteins and gene duplicates (Corradi et al. 2010). The two genomes share the same introns and are almost colinear in their gene order across their chromosomal cores, emphasising very few differences between the two genomes, but large blocks of the subtelomeric regions present in *Encephalitozoon cuniculi* are absent in *Encephalitozoon intestinalis*. This results either from a process of expansion in *Encephalitozoon cuniculi* or further contraction in *Encephalitozoon intestinalis* relative to their common ancestor (Corradi et al. 2010).

The genomes of *Encephalitozoon romaleae*, a grasshopper pathogen, and *Encephalitozoon hellem*, another human pathogen, have also been sequenced (Pombert et al. 2012). Again, these show very high levels of genome reduction and an extremely similar gene content to *Encephalitozoon cuniculi* with the same structure of 11 linear chromosomes (Pombert et al. 2012). One striking observation, however, was the presence of a particular set of genes encoding pathways for folate salvage, de novo folate biosynthesis and purine metabolism. Components of these pathways are represented by genes across four chromosomal regions and are absent from the other sequenced *Encephalitozoon* genomes (Pombert et al. 2012). These are pathways that are not typically complete in other sequenced microsporidia species, and phylogenetic analysis suggests that these genes were acquired by horizontal (or lateral) gene transfer, but interestingly not from a single donor organism source. Rather this pathway has been cobbled together by an ancestor of *Encephalitozoon hellem* and *Encephalitozoon romaleae* using genes from different sources, apparently including several prokaryotes and, for one gene, an animal or fungal source (Pombert et al. 2012). The genes exist in the subtelomeric regions of these genomes which, as seen in *Encephalitozoon cuniculi*, are the sites of rapid change and recombination that are home to multicopy protein families and pseudogenes, and insertion of a horizontally acquired gene here is far less likely to cause a disruption than in the extremely dense 'chromosomal cores'. Curiously, whilst the pathways appear intact in *Encephalitozoon hellem*, they look to be in the process of degeneration in *Encephalitozoon romaleae* (Pombert et al. 2012).

Looking just outside the genus, the genome of close relative *Ordospora colligata*, a pathogen of *Daphnia*, has also been sequenced. This shows a very similar genome content and organisation with a small compact genome (1820 predicted coding sequences within a 2.3 Mb assembly), with little repetitive DNA, a handful of introns, but organised into 10 chromosomes, rather than the 11 seen in *Encephalitozoon* spp.). Like the genomes of *Encephalitozoon hellem* and *Encephalitozoon romaleae*, this genome has been shaped by horizontal gene transfer. In this case, the *Daphnia* host is putatively the source for a Septin gene within the *Ordospora colligata* genome. This Septin shares structural features with Septin 7 which in the pathogen *Candida albicans* is an effector that induces the uptake of the pathogen by the host. Septin 7 is speculated to have an analogous function in *Ordospora colligata*, allowing an alternative means of entry into the host cell than via the polar tube (Pombert et al. 2015). These examples of pathway acquisition through horizontal gene transfer (and others below) demonstrate a mechanism of

expansion of the metabolic repertoire after the initial large-scale gene and pathway loss in the ancestor of the microsporidia.

2.6 Horizontal Gene Transfer Driving Innovation and Change in Microsporidian Genomes

The extent to which eukaryotic genomes are shaped by horizontal gene transfer is a subject of active debate (Ku and Martin 2016; Martin 2017; Leger et al. 2018; Van Etten and Bhattacharya 2020). Within the microsporidia, horizontal gene transfer has unquestionably been important in driving at least one evolutionary transition within the phylum. This key horizontal gene transfer was the acquisition of nucleotide transport proteins (NTT). This acquisition likely occurred from bacteria into the common ancestor of Microsporidia and *Rozella* spp. (Dean et al. 2018). This was followed by multiple duplication events within the microsporidian lineage giving rise to multiple copies (often 4) of this protein within microsporidia genomes (Dean et al. 2018). As proteins that allow the acquisition of host ATP, GTP and NAD+, this horizontal gene transfer event led to the evolutionary transformation of the microsporidian lineage into energy parasites and likely allowed the loss of energy metabolism pathways and the degeneration of the mitochondrion. More broadly it appears that horizontal gene transfer has been an important force in shaping microsporidia genome content, with apparently up to 2.2% of microsporidian genes derived from horizontal gene transfer (Alexander et al. 2016). It is likely the intracellular lifestyle and the intimate association between parasite and host that have facilitated the transfer of DNA and genes between animals and microsporidia perhaps through integration of reverse transcribed host mRNA into the microsporidian genome (Alexander et al. 2016). Examples of host to microsporidia transfer include multiple transposable elements (see below) and the septin and a purine nucleotide phosphorylase (PNP) mentioned above (Selman et al. 2011; Pan et al. 2013; Parisot et al. 2014; Pombert et al. 2015). However, intracellular life is also hypothesised to drive horizontal gene transfer from prokaryotes into microsporidia (Campbell et al. 2013; Alexander et al. 2016). One way in which this may occur is through contact with bacterial DNA within host phagocytic vesicles (Alexander et al. 2016). Coinfection of a host cell alongside intracellular bacterial pathogens such as *Chlamydia* spp. has also been suggested to facilitate prokaryote to microsporidian horizontal gene transfer (Lee and Heitman 2017). This scenario might have allowed the acquisition of the NTT transporters by microsporidia from *Chlamydia* spp., which are also energy parasites and one of the bacterial lineages whose genomes encode homologs of these NTT proteins (Major et al. 2017).

2.7 Small Genomes with no Introns: The Genus *Nematocida*

Among the highly derived, "long branch" or "core" microsporidians, the deepest phylogenetic split appears to lie between the genus *Nematocida* and the rest of "long branch"-microsporidian diversity (Fig. 2.2). Multiple *Nematocida* species' genomes have now been sequenced, and comparisons between these and other microsporidians can therefore provide insight into the early evolution of the group following the transition to an obligate intracellular parasitic lifestyle. Species within this genus, as the name implies, infect nematodes, and despite the large phylogenetic distance between this genus and *Encephalitozoon*, the two clades share some general genome characteristics. Within this genus, we observe another example of very small and compacted genomes ranging in assembly size from 3 to 4.4 Mb. For example, the *Nematocida parisii* ERTm1 genome is ~4.1 Mb in size and predicted to be comprised of 72.8% coding sequence with a mean distance of 418 bp between genes (Cuomo et al. 2012). *Nematocida* genomes also show the same level of reduced metabolic capacity as species within the genus *Encephalitozoon*, except for presence of a CTP synthase which would allow *Nematocida* spp. to synthesise CTP from UTP (Cuomo et al. 2012). Within the genus, there exists some size variation in genomes over a relatively short evolutionary timescale. The earliest diverging known *Nematocida* species *Nematocida displodere* has a more reduced genome, similar to species of *Encephalitozoon* in genome size with a higher proportion of coding material (85.8% compared to 69.2% for *Nematocida parisii* and 63.7% for *Nematocida ausubeli*) and fewer proteins (2278 compared to 2661 in *Nematocida parisii*) (Cuomo et al. 2012; Luallen et al. 2016). However, interestingly, *Nematocida displodere* has a considerable proportion of its genome occupied by members of a single large, expanded gene family. Whilst all sequenced *Nematocida* genomes house expanded gene families (Reinke et al. 2017), the *Nematocida* large gene family 2 has 235 members in *Ne. displodere*, and contributes over 10% of its predicted proteome (Luallen et al. 2016*)*. Many of the protein products (152/235) have predicted signal sequences and/or a RING domain (113/235), and like the *interAE*, *interB*, *interC* and *interD* gene families in *Encephalitozoon*, these genes are thought to encode proteins that mediate interactions with hosts (Dia et al. 2007; Reinke et al. 2017). Similar features are found in gene families across many other lineages of microsporidia (see below) and expanded gene families within the genus *Nematocida* where they represent a substantial proportion of identified 'host exposed proteins' (Reinke et al. 2017). These 'host exposed proteins' are parasite proteins that have been identified within the host cell cytoplasm or nucleus using a method called spatially restricted enzymatic tagging (Reinke et al. 2017).

Whilst *Nematocida* species share the same reduced metabolic capability as *Encephalitozoon*, one key difference between *Nematocida* and *Encephalitozoon* genomes is that in *Nematocida* spp., no introns were detected in gene sequences, there was no evidence of spliced transcripts and in addition, many components of the spliceosomal machinery are lost (Cuomo et al. 2012).

2.8 Independent Losses of Splicing and Introns

Although introns are sparse within the microsporidia, a small number have been found in most microsporidian genomes (Whelan et al. 2019). These introns appear to fall into two broad categories. The first includes short introns of around 25 bp which typically occur in ribosomal proteins right at the start of the coding region (typically after the first ATG). The second category includes two specific introns found in the same two genes across different species of microsporidia (Whelan et al. 2019). The longer introns are almost always found to have been removed by splicing (in transcriptomes 80% of the time), whereas the short introns are rarely removed (splicing rates are as low at 20%) (Whelan et al. 2019).

On at least three independent occasions, introns and the splicing machinery have been entirely lost during microsporidian evolution: Once in the *Nematocida* clade, once in the *Vittaforma*/Enterocytozoonidae clade and once in *Edhazardia aedis* (Fig. 2.2) (Akiyoshi et al. 2009; Cuomo et al. 2012; Wiredu Boakye et al. 2017). This is supported by both an absence of observable introns and a loss of many genes encoding components of the spliceosome from these genomes (Akiyoshi et al. 2009; Cuomo et al. 2012; Wiredu Boakye et al. 2017; Whelan et al. 2019). This pattern of retention and loss of introns and splicing is hard to explain. Introns in microsporidia typically fall in ribosomal proteins which are obviously fundamental to cell function. It has been suggested that there may be a role for these short introns in regulating their own expression and therefore ribosome synthesis as is seen in *S. cerevisiae* (Roy et al. 2020), yet why they are retained in some species, but not others, remains unresolved.

2.9 Larger Genomes with Polyploidisation and Transposable Elements: *Nosema/Vairimorpha* spp.

The closest relatives to the *Ordospora/Encephalitozoon* clade with sequenced genomes are those in the *Nosema/Vairimorpha* group. Here, again, there exists extensive genome data both within genera and within a single species, and this shows an upward shift in genome size relative to *Ordospora/Encephalitozoon* and the *Nematocida* genomes. The genus *Nosema* has been somewhat problematic in phylogenetic placement in the past as it has included multiple species that have since been transferred to different groups within the phylogenetic tree of microsporidia (Sokolova et al. 2005; Lord et al. 2010). In addition, *Nosema* and *Vairimorpha* species did not separate into clades and were intermixed in molecular phylogeny. This group contains species isolated from arthropods, mainly insects and particularly Lepidoptera and Hymenoptera (Tokarev et al. 2020). Recently, however, these clades have been redefined on the basis of phylogenetic position with the key bee pathogens *Nosema apis* and *ceranae* being redefined as *Vairimorpha* (Tokarev et al. 2020). Nevertheless, these two genera together form a monophyletic clade of closely

related pathogens that infect arthropods. As a pathogen that has been implicated in poor honeybee health, *Vairimorpha ceranae* has been of intense interest to the insect pathology community, and genomes of multiple isolates of *Vairimorpha ceranae* have now been sequenced (Cornman et al. 2009; Pelin et al. 2015; Peters et al. 2019; Huang et al. 2021).

The first genome sequence of *Vairimorpha ceranae* revealed an estimated genome size of 7.86 Mbp and a total of 2641 putative protein-coding genes, and whilst larger than those of *Encephalitozoon* species, it is less gene dense, with 0.60 genes/kb (64.8% coding sequence), and contains more repetitive DNA, particularly transposable elements (Cornman et al. 2009). These transposable elements are diverse including members of the gypsy-type LTR retrotransposons and DNA transposons such as Merlin, Helitron, piggyBac and MULE and occupy over 20% of the genome (Cornman et al. 2009; de Albuquerque et al. 2020). *Vairimorpha ceranae* infects *Apis mellifera* worldwide but was originally described in the Asiatic honeybee (*Apis cerana*), and extensive genome sequencing has been used to better understand the origins of this pathogen and its global spread within bee populations (Pelin et al. 2015; Peters et al. 2019). SNP phylogenies inferred from globally distributed isolates demonstrated a lack of geographic structure, suggesting a recent spread of the pathogen among hives, perhaps as a result of human activity (Pelin et al. 2015).

This study also revealed a surprising level of within-individual genetic variation in global *V. ceranae* isolates that might be explained by a polyploid (at least tetraploid) genome in comparison to the ancestral diploid population in Asia (Pelin et al. 2015; Peters et al. 2019). Within the genomes, both of the Asian and global populations, there are high of levels of linkage disequilibrium pointing to a likely clonal life style and a lack of recombination (Pelin et al. 2015; Peters et al. 2019).

Vairimorpha ceranae forms a clade with two other species that have sequenced genomes, one also infecting honeybees, *Vairimorpha apis*, and one infecting lepidopterans, *Nosema* YnPr (Fig. 2.2 and Tokarev et al. 2020), although the evolution of host preference within the clade remains unclear. The genome of *Nosema* YnPr is smaller at 3.4 Mb and is more compact with shorter genes, whilst that of *Vairimorpha apis* is larger at 8.5 Mb (Chen et al. 2013; Xu et al. 2016). A major contributor to this difference in size is a difference in the number and diversity of transposable elements in the two genomes, with the larger genome having a greater proportion of transposable elements, suggesting that these can invade and/or expand within genomes rapidly over relatively short evolutionary time periods within the microsporidia.

Transposable element expansion is perhaps more obvious in the true *Nosema* clade in which the silkworm pathogens *Nosema antheraeae*, *Nosema bombycis* and the gammarid pathogen *Nosema granulosis* have genome assembly sizes of 6.6 Mb, 15.7 Mb and of 8.8 Mb, respectively (Pan et al. 2013; Cormier et al. 2021). In fact, *Nosema bombycis* is reported to have the largest proportion of transposable element content of any microsporidia genome sequenced so far (Pan et al. 2013; de Albuquerque et al. 2020). This has likely resulted from the expansion of transposable elements common across microsporidia such as Ty3/Gypsy LTR retrotransposons

and also through acquisition of new elements from insect hosts, particularly the relatively recent acquisition of multiple *Piggybac* elements from lepidopteran hosts possibly in the ancestor of *Nosema antheraeae* and *Nosema bombycis*, followed by more extensive proliferation in the genome of *Nosema bombycis* (Pan et al. 2013).

2.10 Enterocytozoonidae: Metabolic Reductionism to the Extreme

The Enterocytozoonidae clade is home to some of the economically most important microsporidia species, for example, *Enterocytozoon bieneusi*, the microsporidian species most commonly found in humans (Didier and Weiss 2006), and *Enterocytozoon hepatopenaei* (dubbed EHP), a microsporidian that is currently causing extensive damage to the shrimp industry (Thitamadee et al. 2016). Several genomes within this family have been sequenced, and whilst they are not the smallest from the perspective of a number of nucleotides (3.1–6 Mb), they are unique with respect to the extent of loss of metabolic pathways (Akiyoshi et al. 2009; Keeling et al. 2010; Wiredu Boakye et al. 2017). The loss of the ability to generate ATP in mitochondria is a key characteristic of the microsporidia, and whilst the intracellular milieu allows access to ATP via transporters, it is thought that glycolysis is crucial in the extracellular spore stage when host resources are inaccessible (Dolgikh et al. 2011; Heinz et al. 2012; Timofeev et al. 2020). In the spore stage, the glucose to generate ATP through glycolysis likely comes from the breakdown of trehalose (Timofeev et al. 2020). Thus, the enzymes of the glycolytic pathway and trehalose metabolism are considered core to microsporidian energy metabolism (Nakjang et al. 2013).

However, the genomes sequenced from the Enterocytozoonidae and that of the closely related *Hepatospora eriocheir* do not encode full glycolytic pathways, but partial pathways with several components lost which likely render these organisms incapable of glycolysis (Akiyoshi et al. 2009; Keeling et al. 2010; Wiredu Boakye et al. 2017). Additionally, these species have lost the trehalase enzyme that could potentially break down trehalose into glucose in the spore and key components of the pentose phosphate pathway such as transketolase (Keeling et al. 2010; Wiredu Boakye et al. 2017). These losses seem to leave these species apparently entirely dependent on their hosts for ATP. Whilst there has been little research on the physiological state of microsporidian spores, it has been suggested that the process of rapid polar tube extrusion during spore germination must be ATP dependent (Timofeev et al. 2020). However, the absence of ATP generating pathways in these taxa suggests that either it is not or that these particular species enter the host by another mechanism, potentially phagocytosis (Wiredu Boakye et al. 2017).

In addition to the loss of these core energy metabolic pathways, these species have also far fewer proteins associated with fatty acid biosynthesis in comparison to other microsporidia. Microsporidia are generally limited in their repertoire of fatty

acid biosynthesis enzymes, and elegant experiments to knock down host lipid metabolic processes in *Tubulinosema ratisbonensis*-infected *Drosophila* have started to unpick the dependency of microsporidia on host lipids (Franchet et al. 2019). However, within the Enterocytozoonidae, there are further losses of enzymes associated with glycerophospholipid metabolism and specifically the generation of phosphatidylethanolamine and phosphatidylcholine (Keeling et al. 2010; Wiredu Boakye et al. 2017). These enzymes are otherwise considered broadly conserved within the microsporidia, and their loss in the Enterocytozoonidae suggests a potential dependence of the host for these key components of biological membranes (Nakjang et al. 2013; Wiredu Boakye et al. 2017). In addition to these losses of key metabolic pathways, there are no introns in these genomes, nor do they encode all the genes necessary to form the spliceosome (Akiyoshi et al. 2009; Keeling et al. 2010; Wiredu Boakye et al. 2017).

2.11 Species with Larger Proteomes: What Accounts for the Differences?

With the publication of the *Trachipleistophora hominis*, *Nosema ceranae*, *Hamiltosporidium tvaerminnensis* and *Enterocytozoon bieneusi* genomes, it became clear that some species of microsporidia had larger proteomes than others (Corradi et al. 2009; Akiyoshi et al. 2009; Cornman et al. 2009; Heinz et al. 2012). *Trachipleistophora hominis*, for example, with a genome size of ~11.6 Mb has a predicted proteome that is approximately 30% larger than those of small-genome microsporidia (Heinz et al. 2012; Watson et al. 2015). More recently, the genome sequence of the *Gammarus*-infecting species *Dictyocoela muelleri* was published with 6442 predicted genes (Cormier et al. 2021). Whilst there are some differences in the complement of metabolic pathways between microsporidia (as seen above), larger proteome size is not associated with a greater metabolic capacity. In those microsporidia that have larger proteomes, the vast majority of extra proteins have no annotated function, and many are a result of expansion of microsporidian or species-specific gene families through gene duplication (Heinz et al. 2012; Nakjang et al. 2013; Pan et al. 2013; Cormier et al. 2021). For example, 2591 of 6442 genes predicted in the *Dictyocoela muelleri* genome can be clustered in just 233 orthogroups (Cormier et al. 2021). The genome of *Nosema bombycis*, the pathogen of the domesticated silkworm, encodes 4458 predicted proteins relative to 3413 in the genome of the close relative *Nosema antheraeae* which infects the Tussar silk moth (Pan et al. 2013), and many of the extra genes in *Nosema bombycis* have arisen as tandem repeats since the divergence of *Nosema bombycis* and *Nosema antheraeae* (Pan et al. 2013). As seen in *Nematocida*, one or a handful of gene families can account for a considerable number of predicted proteins, and this is also the case in many other microsporidian genomes (Reinke et al. 2017). These extreme gene family expansion events can substantially add to the predicted proteome and

Fig. 2.3 Putative de novo gene area in *Spraguea* spp.: Shown is the same area of the genome of two populations of the same pathogen. In the East Atlantic genome, it is possible to see an area with an open reading frame that is transcribed and putatively translated into a population specific protein. In the West Atlantic genome, the same area does not contain an open reading frame. Differences at the DNA level between these two populations are indicated by black boxes

has led to both species-specific expansions such as the '*Nematocida* expanded gene family 1' proteins or the more widespread InterB proteins found in *Encephalitozoon*, *Vittaforma* and *Anncaliia* (Dia et al. 2007). These families can have over 100 members and are often enriched with motifs that mediate protein-protein interactions, and many of them are predicted to have secretion signals that may direct them out of, or to the surface of the microsporidian cell (Heinz et al. 2012; Campbell et al. 2013; Reinke et al. 2017). The functional importance of these expanded protein families is not fully understood, but they are strong candidates for characterisation as potential secreted parasite effector proteins.

In other instances, proteome increase has come about through whole genome duplication. Within the genus *Spraguea*, there is evidence of a recent whole genome duplication in the ancestor of the North American population of *Spraguea* inevitably leading a duplicate copy of each gene (Williams et al. 2016). Many of the resultant duplicates are pseudogenes but some are retained as intact open reading frames. As a result, the lineage that has experienced this whole genome duplication has a higher predicted protein number than close relatives (Williams et al. 2016). Novel lineage specific genes can also arise de novo from previously non-coding material rather than via gene duplication or rearrangement of exons/introns or other genomic elements. These de novo genes can be identified as open reading frames that are unique to one species and are actively transcribed, but where there is clear sequence similarity at the DNA level to non-coding regions from close relatives (Fig. 2.3) (Nakjang et al. 2013; Williams et al. 2016).

2.12 Genome Inflation Through Transposable Element Bursts and Intergenic Region Expansion: *Edhazardia aedis* and *Anncaliia algerae*

Differences in genome organisation and content, such as smaller or larger gaps between genes or the presence or absence of certain genes or gene families, can account for some of the difference between genome sizes, but large genome differences over short times scale are sometimes driven by bursts of transposable elements (Naito et al. 2006). There is a general correlation between genome size and transposable element content in eukaryotes broadly (Elliott and Gregory 2015), and this same trend applies broadly to the microsporidia: The smallest genomes have no or very few transposable elements, whilst the largest harbour large and diverse transposable element populations (Parisot et al. 2014).

Genome size expansion through the acquisition and spread of transposable elements has occurred multiple times within the microsporidia. One example seen above is in the genome of *Nosema bombycis* compared to the genomes of its close relatives (Pan et al. 2013). However, transposable elements are found in genomes across the diversity of microsporidia and have been shown to include both non-LTR retrotransposons and LTR retrotransposons and DNA transposons (including *Helitron*, *Mariner*, *Tc1*, *Merlin* and *piggyBac* groups). One genome that has become particularly expanded by a transposable element burst is that of the species *Anncaliia algerae* (Parisot et al. 2014). This pathogen primarily infects insects but is also a serious opportunistic pathogen of humans (Coyle et al. 2004). With a genome size of 23 Mb, it has one of the larger microsporidian genomes, and in this species, there is a large diversity of transposable elements with 97 LTR retrotransposons, four non-LTR retrotransposons and 139 DNA transposons identified within a single *Anncaliia algerae* genome (Parisot et al. 2014). Many of the transposable elements seen in microsporidia likely originate through horizontal gene transfer from hosts, permitted by the intimate association between microsporidian and their host organism. Interestingly, recent work has shown that there are substantial differences in transposable element content between different strains of *Anncaliia algerae*, indicative of a recent change in of transposable element content driven primarily by the spread of DNA transposons (de Albuquerque et al. 2020).

It has been suggested that the RNAi pathway in microsporidia has a role in transposable element defence and the retention or loss of the pathway is driven by the presence or absence of transposable elements within microsporidian genomes (Nakjang et al. 2013), and whilst there is an association between the retention of Dicer and Argonaute and the accumulation of transposable elements in microsporidian genomes, there is not a complete match, and other forces such as drift may also play a role in dictating when and where they are retained across the phylum (de Albuquerque et al. 2020).

Whilst larger microsporidian genomes have often become swollen through acquisition of transposable elements, one genome that bucks this trend is that of the mosquito parasite *Edhazardia aedis* which has the largest genome of any sequenced

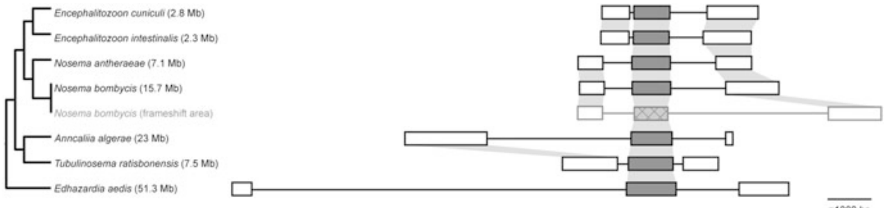

Fig. 2.4 Polar tube 2 region of the various genomes to illustrate variation in intergenic spacing between species. Shown is the PTP2 gene shaded in grey which is chosen as a reference point. Surrounding genes are shown in white. Homologous genes are indicated by light grey shading linking genes between species. Relationships between species shown to the left hand side are extracted from the phylogeny in Fig. 2.2. The hatched box in the second genomic area for *Nosema bombycis* indicates a possible pseudogene. It is also possible to see the synteny within *Encephalitozoon* and *Nosema* species, but this synteny breaks down with increasing phylogenetic distance which may reflect increased time since divergence but also a higher chance of fixation of recombinations as genome density decreases and the chance of disrupting an existing open reading frame decreases

microsporidian to date at 51.3 Mb (Desjardins et al. 2015). This genome, however, is not characterised by more transposable elements: Only 5.5% of the *Edhazardia aedis* genome could be classified as repetitive, and – in common with some smaller microsporidian genomes – it entirely lacks introns (Desjardins et al. 2015). *Edhazardia aedis* encodes 4190 protein-coding genes, a relatively large complement for a microsporidian but not making up the difference in genome size, with only 9% of the genome coding for protein. Intergenic distance tends to increase with increased genome size in the microsporidia (Fig. 2.4), but this is particularly apparent in the genome of *Edhazardia aedis* which has become bloated to a large size primarily through the accumulation of expanded AT-rich intergenic regions (Desjardins et al. 2015). These can be tens of thousands of bases in length (Desjardins et al. 2015) and are speculated to allow additional regulation of gene expression (Troemel and Becnel 2015). Phylogenetically, *Edhazardia aedis* emerges from within a clade of small-genome microsporidians, suggesting that its larger genome is the result of secondary re-expansion rather than the retention of the larger intergenic regions more typical of other eukaryotes.

2.13 What Drives the Evolution of Microsporidian Genome Size?

Adaptive or functional explanations for diminutive microsporidian genome sizes have been invoked in the past (Cavalier-Smith 2005). Three factors have been implicated in driving down genome size. The first is metabolic reduction, explained by loss of selective pressure to retain certain pathways after the adoption of an intracellular lifestyle as the host allows access to these resources. The second is

economy of resources required to replicate DNA in the nucleus as a force driving miniaturisation. The third is spatial reduction with the small cell size driving decreased genome size to maintain a 'karyoplasmic ratio" (Cavalier-Smith 2005). In contrast it has also been proposed that larger genomes with more genes are the result of selection for a large gene repertoire in species that have more than one host type (Peyretaillade et al. 2012; Pan et al. 2013).

However, these functional explanations, as in other eukaryotes, do not provide a complete account of differences in genome sizes and organisations over short time scales, or vast differences in genome sizes between organisms with very similar hosts and fundamentally the same intracellular niche. In addition to selection acting on genome size changes, genome size variation can also be driven by neutral processes. Whether genomic changes such as point mutations; gene duplications, transfers or losses; or acquisition of transposable elements will become fixed within a lineage depends largely on the fitness cost or benefit of that change. In populations with large effective population sizes, natural selection will allow changes with a small benefit to be fixed and mutations with small costs to fitness will be purged (Ohta 1992; Lynch and Conery 2003). However, in organisms with small effective population sizes, natural selection is less effective, and slightly deleterious mutations such as the addition of a new non-coding genomic element may become fixed through drift (Lynch and Conery 2003).

Non-adaptive processes in shaping microsporidian genomes have been recently investigated using genomic data (de Albuquerque et al. 2020; Haag et al. 2020). Firstly, in order to investigate how genome architecture has evolved in distantly related microsporidian species with similar hosts but different life histories, Haag et al. sequenced seven new microsporidian genomes infecting *Daphnia magna*: *Hamiltosporidium tvaerminnensis* (× 2 strains) and *Hamiltosporidium magnivora (× 2 strains)* and *Ordospora colligata* (× 4 strains) (Haag et al. 2020). These organisms were chosen because they inhabit the same host, but they display a mix of sexual and asexual lifestyles and patterns of horizontal transmission or vertical transmission (or even a mix of both within the same species) allowing the impact of these differences to be evaluated. These species also differ vastly in genome size and organisation. *Ordospora* is strictly horizontally transmitted and has a compact genome of 2.3 Mb in comparison to the genomes of *Hamiltosporidium* spp. which range from 17.2 Mb to 25.2 Mb and are a mixture of vertically and horizontally transmitted (Haag et al. 2020). The *Hamiltosporidium* genomes, like many expanded microsporidian genomes, are characterised by longer intergenic regions, segmental duplications and a greater proportion of transposable elements. One explanation for the differences in architecture between these groups is that vertical transmission creates a population bottleneck decreasing effective population size and thus decreasing the power of selection to remove slightly deleterious mutations (Haag et al. 2020). This is borne out by the observation that those species with larger genomes and vertical transmission as a part of their life stage also have an excess of nonsynonymous substitutions in single-copy orthologous genes relative to horizontally transmitted species (Haag et al. 2020).

Building on this hypothesis, Alberquerque et al. looked at the distribution of transposable elements across the phylum studying the pattern of where transposable elements occur and where they have expanded within genomes (de Albuquerque et al. 2020). Comparing 47 microsporidian genomes confirms the positive relationship between genome size and transposable element content that exists in eukaryotes generally (Elliott and Gregory 2015). It also showed statistically significant differences in the percentage of the genome taken up by transposable elements and genome size generally in those species with vertical transmission versus those that only transmit horizontally where species with vertical transmission have a higher proportion of transposable elements and larger genomes (de Albuquerque et al. 2020). This reinforces the idea that vertical transmission is associated with genome expansions and the spread of transposable elements in microsporidia, and this difference is the result of genetic drift in populations with a small effective size rather than selection (de Albuquerque et al. 2020).

2.14 The Power, Limitations and Future of Genomic Studies in Microsporidia

Microsporidian genomes are small, and these organisms evolve under very different selective constraints compared to free-living eukaryotes. Nonetheless, genomic studies of microsporidia have demonstrated that, particularly in regard to evolutionary dynamics of genome size change, the group represents a microcosm of evolutionary processes occurring within eukaryotes more generally.

Now comparative genomic studies are not only describing the differences in organisation and content and the mechanisms that led to these changes but also beginning to provide some answers as to which evolutionary pressures drive the differences in microsporidian genome size.

Genomic studies have given real insight into the nature of reductionism and patterns of differential reduction of metabolic and cellular processes during the evolution of the phylum. This has revealed clear patterns of pathways that are universally lost across the phylum, for example, the electron transport chain and pathways that are universally conserved across the microsporidia (e.g., mitochondrial iron sulphur cluster assembly proteins in the mitosome). In addition, genomic studies have highlighted pathways and complexes that have degenerated at certain points during the evolution of the phylum (RNAi, glycolysis and the spliceosome) and the general paring down of eukaryotic complexes to something far more minimal than seen in 'typical' eukaryotes.

In terms of understanding cellular processes, genome data can suggest hypotheses that can be tested by experiment. For example, comparative genomic studies can generate lists of candidate ORFs associated within particular unusual lifestyles, for example, feminisation of hosts or occupation of an intranuclear niche (Wiredu Boakye et al. 2017; Cormier et al. 2021). However, the lack of a genetic system

for microsporidia continues to impede progress in understanding the biological significance of these ORFs. Nonetheless, innovative experiments informed by genomic observations continue to provide new insights into microsporidian biology and the biology of parasitism, as illustrated, for example, by recent work on the evolution of the microsporidian ribosome (Barandun et al. 2019; Ehrenbolger et al. 2020). The ribosome was one of the earliest noted examples of reduction in the microsporidia, and when the *Encephalitozoon cuniculi* genome was first sequenced, 70 different ribosomal proteins were identified in contrast to the ~80 typically found in other eukaryotes, suggesting the loss of several proteins (Katinka et al. 2001). In addition, the ribosomal RNA genes in microsporidia are extensively shortened so that the eukaryote-specific expansion segments that mediate many interactions with proteins are lost, and it was therefore suggested that several ribosomal proteins are no longer part of the ribosome but have extra-ribosomal functions (Melnikov et al. 2018). However, recent generation of cryo-EM structures and proteomic characterisation of the *Vairimorpha necatrix* and *Paranosema locustae* ribosomes give an in-depth insight into the nature of ribosomal reduction (Barandun et al. 2019; Ehrenbolger et al. 2020). These studies demonstrated that, in fact, some microsporidia have retained all the ribosomal proteins typically present in fungi apart from eL38 and eL41. Whilst expansion segments are lost from rRNA, this loss is compensated for proteins being held in place by protein-protein interactions. This illustrates that whilst reduction of the ribosome has occurred through shortening of genes and proteins, proteins are retained, and the core structure of the eukaryotic ribosome is preserved. This study highlights just how challenging it is to make predictions about the biology of microsporidia on the basis of bioinformatic analysis, and whilst this is true of studies in all organisms, it is particularly pertinent in the microsporidia. This is partly because the very high rates of evolutionary change in protein sequences mean that it is not always easy to identify homologs of short or particularly divergent proteins, and because the process of reduction in microsporidia has resulted led to unique solutions, and highly derived states, which cannot be predicted fully without functional data.

However, the available genomes represent an essential resource to support in-depth studies like those of the ribosome, and as new emerging technologies are applied to this fascinating group of organisms, it will undoubtedly reveal novel ways in which microsporidia have adapted eukaryotic complexes in a way that cannot be understood with genomic data and bioinformatics alone.

Compliance with Ethical Standards

Conflict of interest: The authors declare that there is no conflict of interest.

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Chapter 3
Insights into Microsporidia Evolution from Early Diverging Microsporidia

Daniele Corsaro

Abstract Microsporidia have drastically modified genomes and cytology resulting from their high level of adaptation to intracytoplasmic parasitism. Their origins, which had long remained enigmatic, were placed within the line of *Rozella*, a primitive endoparasitic chytrid. These origins became more and more refined with the discovery of various parasites morphologically similar to the primitive lines of microsporidia (Metchnikovellids and Chytridiopsids) but which possess fungal-like genomes and functional mitochondria. These various parasites turn out to be distinct missing links between a large assemblage of chytrid-like rozellids and the true microsporidians, which are actually a very evolved branch of the rozellids themselves. The question of how to consider the historically known Microsporidia and the various microsporidia-like organisms within paraphyletic rozellids is discussed.

Keywords Early microsporidians · Rozellomycota · *Mitosporidium* · *Morellospora* · *Nucleophaga* · *Paramicrosporidium* · Chytridiopsids · Metchnikovellids

3.1 Introduction

Tracing the evolutionary history of Microsporidia and elucidating their enigmatic origin has, until recently, been problematic due to the extreme cellular and genetic modifications resulting from their adaptation to obligate intracellular parasitism. The most distinctive characteristic of microsporidians, the polar tube injection apparatus, with a unique structure and function, allowed them to be recognized as a homogeneous group while hampering reliable inference of their relationship to other eukaryotic lineages (Vávra and Lukeš 2013; Han and Weiss 2017). On the other hand, the congruence of various biomolecular analyses has led Microsporidia to be considered as highly derived parasitic fungi, with the parasitic chytrid *Rozella* ultimately being the closest relative (James et al. 2013). Ironically, it is the fungal nature of *Rozella*

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that has been questioned because, unlike fungi, it is phagotrophic and naked (lacking a cell wall) (Powell 1984; Powell et al. 2017). Some authors therefore placed *Rozella* and Microsporidia as the sister group of *Fungi*, alongside another group of phagotrophic parasites, the aphelids (Karpov et al. 2014). Others accepted them as early fungi (Naranjo-Ortiz and Gabaldón 2019; James et al. 2020), since osmotrophy through a chitinous wall (Cavalier-Smith 1987, 2013) might not yet have developed in the very first lines (Corsaro et al. 2014a) and would therefore no longer be a sufficient criterion to define the kingdom. At the same time, the *Rozella* line turned out to be richer, mostly comprising uncultivated clones (Lara et al. 2010), some of which resemble chytrids (Jones et al. 2011). Microsporidia-like parasites of amoebae are positioned within the rozellids, thus filling the deep morphological gap between *Rozella* and Microsporidia (Corsaro et al. 2014b), and providing new insights on their emergence (Corsaro et al. 2016).

How these various organisms were discovered, what evolutionary relationships they have with the historical microsporidians, and how they should be considered and classified are the main points covered in this review.

3.2 *Rozella* and Allied Taxa: From the *Olpidiaceae* to Rozellomycota

For their historical and evolutionary importance, a brief reminder on *Rozella* and other related organisms is presented. Sparrow (1960), in his authoritative work on the *Phycomycetes* (primitive fungi whose artificial grouping was by him already recognized), placed *Rozella* in the family *Olpidiaceae* (order *Chytridiales*) which includes only intracellular parasites (endobiotic) with a simple vegetative stage (unicellular thallus) which completely transforms into a sporangium (holocarpic) (Table 3.1). The most specious and perhaps the best-known types at the time, *Rozella* and *Olpidium*, proved difficult to classify, resulting in a multigene phylogeny placed at opposite ends of chytrid radiation, i.e., *Rozella* as the very early lineage and *Olpidium* as the closest to zygomycotans (James et al. 2006; Chang et al. 2021). This approach also revealed a link between Microsporidia and *Rozella*, thus reviving interest in this organism which already intrigued researchers for its atypical features. Indeed, *Rozella* develops inside its hosts as a naked multinucleated plasmodium capable of phagocytosis, using the wall of the host cell as its own sporangial wall, and producing thick-walled resting spores whose immature stages have chitin (Held 1981; Powell 1984; Powell et al. 2017). Like canonical chytrids, *Rozella* forms naked zoospores with a single posterior flagellum, which, when attached to the host, encyst and produce a penetration tube to pierce the host cell wall through which the parasite protoplast enters. Other former *Olpidiaceae* show overall similarity with *Rozella*, namely *Plasmophagus*, whose thallus is initially a naked plasmodium and becomes walled during its development (De Wildeman 1895; Sparrow 1960), and *Dictyomorpha* (Mullins 1961), which additionally forms spiny resting spores like

Table 3.1 Endobiotic chytrid-like parasites

Taxon	Main host	Zoospore	Current affiliation
Olpidiaceae sensu Sparrow			
Olpidium (Braun) Rabenhorst 1868 (*Pleotrachelus* Zopf 1884 pro parte)	Algae and plants	Posteriorly uniflagellate; encysts to form penetration tube	Zygomycota *Olpidiomycetes*
Rozella Cornu 1872	Chytrids, oomycetes, and some algae	Posteriorly uniflagellate; encysts to form penetration tube. Spiny resting spore	Rozellomycota Rozellales
Nucleophaga Dangeard 1895	Amoebae (intranuclear)	Non-flagellate, walled; preformed polar filament	Rozellomycota Nucleophagales
Sphaerita Dangeard 1886	*Euglena* (intracytoplasmic)	Anteriorly uniflagellate	Inc. sed. possibly Pseudofungi (Rozellopsidales)
Morellospora Corsaro et al. 2020 (*Sphaerita* pro parte)	Amoebae (intracytoplasmic)	Non-flagellate, walled; preformed polar filament. Spiny resting spore	Rozellomycota Morellosporales
Olpidiomorpha Scherffel 1926	Zoocyst of *Barbetia* (not *Pseudospora*) (Cercozoa) living in ochrophyte and volvocine algae	Posteriorly uniflagellate; encysts to form penetration tube. No resting spore	Inc. sed. possibly Rozellomycota
Dictyomorpha Mullins 1961 (*Pringsheimiella* Couch 1939)	*Oomycetes*	Posteriorly uniflagellate; encysts to form penetration tube. Spiny resting spore	Inc. sed. possibly Rozellomycota
Plasmophagus De Wildeman 1895	Filamentous algae	Posteriorly uniflagellate; encysts to form penetration tube. No resting spore	Inc. sed. possibly Rozellomycota
Blastulidium Pérez 1903	Freshwater crustaceans (eggs and embryos)	Anteriorly biflagellate	Pseudofungi Leptomitales
Chytridhaema Moniez 1887	Freshwater crustaceans (body cavity)	Flagellate	Inc. sed.
Not included in Sparrow's list			
Cibdelia infestans Juel 1925	*Pseudonectria buxi* (Ascomycota)		Inc. sed.
Sagittospora Lubinsky 1955	Rumen ciliates (intracytoplasmic)	Non-flagellate, walled	Inc. sed. possibly Rozellomycota

(continued)

Table 3.1 (continued)

Taxon	Main host	Zoospore	Current affiliation
Paramicrosporidium Corsaro et al. 2014	Amoebae (intranuclear)	Non-flagellate, walled; preformed polar filament	Rozellomycota Paramicrosporidiales
Mitosporidium Haag et al. 2014	Freshwater crustaceans (intracytoplasmic)	Non-flagellate, walled; preformed polar filament	Rozellomycota Morellosporales

those of *Rozella* and also present in the spore-forming *Sphaerita*, now *Morellospora* (Corsaro et al. 2020). Recent reviews of the literature consider it plausible that *Plasmophagus* and *Dictyomorpha* are affiliated with *Rozella* (Blackwell et al. 2016, 2017). New research on these organisms would therefore be very important to obtain molecular data and thus clarify their position. On the other hand, the anteriorly uni-/biflagellate zoospores of *Sphaerita* and *Pseudosphaerita* (Dangeard 1933) would suggest their affinity with the *Olpidiopsis*/*Rozellospis* group, belonging to the unrelated line of *Oomycetes* (Chromista; Pseudofungi). Interestingly, the oomycete grouping of *Blastulidium*, also suggested by its biflagellate spores (Manier 1976), has recently been confirmed by molecular studies (Duffy et al. 2015). A naked plasmodium also characterizes the intranuclear (*Nucleophaga*, *Paramicrosporidium*) and intracytoplasmic (*Mitosporidium*, *Morellospora*) parasites, which were confirmed to be related to *Rozella* by molecular phylogeny (Corsaro et al. 2014a, 2014b, 2016, 2020). However, there is no evidence that these parasites are capable of phagocytosis. Finger-like protrusions, similar to those allowing *Rozella* to engulf the host protoplasm, are present in both *Mitosporidium* (Haag et al. 2014) and *Nucleophaga* (Michel et al. 2009a), suggesting a possible residual phagocytic capacity (Corsaro et al. 2014a) that was however not found in successive observations. Such protrusions likely serve to increase the exchange surface with the host. The claim that *Nucleophaga ranarum* engulfed the nucleolus and parts of the nucleus of *Entamoeba* (Blackwell et al. 2019) is due to a poor translation of the original French text (Lavier 1935). Unlike *Rozella*, these parasites do not have a flagellate stage but form walled spores similar to those of microsporidians, containing a preformed polar filament (and other associated structures) (Corsaro et al. 2014b, 2016, 2020; Haag et al. 2014). In microsporidians, the polar filament is extruded and acts like a syringe to inject the sporoplasm through the plasma membrane of the host cell, but the polar tube in these parasites is not involved in invasion as the spores are engulfed by phagocytosis. Its activation could occur once the spore is in phagocytic vacuoles, similar to microsporidian spores which can enter by a similar route (Franzen 2005). Note that, following early descriptions of Dangeard on *Sphaerita* and *Nucleophaga* in euglenae and amoebae (Dangeard 1886, 1895), similar parasites have been reported in a wide variety of microbial eukaryotes (Kirby 1941), likely forming an assemblage of unrelated species. Among them, one probably affiliated with Rozellomycota is *Sagittospora* (Lubinsky 1955), a cytoplasmic parasite of rumen ciliates, superficially reminiscent

of microsporidians, which has a naked plasmodium and produces spores similar to those of some *Sphaerita*-like species found in parabasalid flagellates.

There is striking evidence that these parasites have developed two different ways to infect their hosts: one by swimming like a flagellated zoospore toward a walled host (e.g., algae and water molds) on which to encyst and penetrate and in another by forming an immobile but walled spore that waits in the environment to be swallowed by a phagocytic host (i.e., most protozoans). Swimming is probably the ancestral strategy, as it is most common among parasitic chytrids which almost all attack walled hosts. This strategy also occurs in aphelids, a group of algal parasites, which are placed at the base of the fungal tree. The development of a walled spore to access a new type of host, i.e., phagocytic, could therefore have taken place several times separately. Current molecular phylogenetic data seem to support this scenario.

3.3 From Environmental Clones to Early Microsporidians

Much of the diversity of rozellids consists of environmental rDNA sequences from uncultivated organisms. The first clones were obtained from a study on microbial diversity in a continuous-flow system on algal detritus with water from Lake Ketelmeer, The Netherlands, and form the LKM11 clade with fungi as their closest relatives (van Hannen et al. 1999) (Fig. 3.1a). As these clones have only a weak similarity (86%), a greater diversity was expected, and indeed subsequent environmental investigations attributed an increasing number of sequences to the LKM11 clade. The group was generally considered to be constituted of small (<5 μm) saprotrophic or parasitic eukaryotes (e.g., Lefèvre et al. 2007; Lepère et al. 2010). The phylogenetic inclusion of *Rozella* (Lara et al. 2010) and the demonstration of zoospores in at least two subgroups, LKM11 and LKM46, which parasitized diatom algae (Jones et al. 2011), indicate a chytrid-like appearance. The entire group was renamed Rozellida or Cryptomycota, to highlight their possible non-fungal nature or their hidden diversity, respectively (Fig. 3.1b). Whole genome sequencing confirmed the relationship between *Rozella* and Microsporidia (James et al. 2013), and further molecular analyses revealed a link with another line of endoparasites, the Aphelids, partly already included in both the Rozellida and Cryptomycota, suggesting an Aphelids/Rozella/Microsporidia (ARM) clade (Karpov et al. 2013; Letcher et al. 2013). The three lineages were thus united in a new superphylum called Opisthosporidia, claimed to be essentially phagotrophic, and a sister of true *Fungi* (Karpov et al. 2014) (Fig. 3.1c). The discovery of *Paramicrosporidium*, an intranuclear parasite of amoebae which ultrastructurally resembles microsporidia (Michel et al. 2000, 2009b) but which was clearly positioned within rozellids in rDNA phylogeny (Corsaro et al. 2014b), revealed the missing link with Microsporidia. Thus, the paraphyletic status of a newly defined phylum Rozellomycota which, in contrast to Rozellida/Cryptomycota, excluded aphelids. The contemporary recovery of *Mitosporidium*, a putative very ancestral microsporidium still possessing mitochondria (Haag et al. 2014), appeared

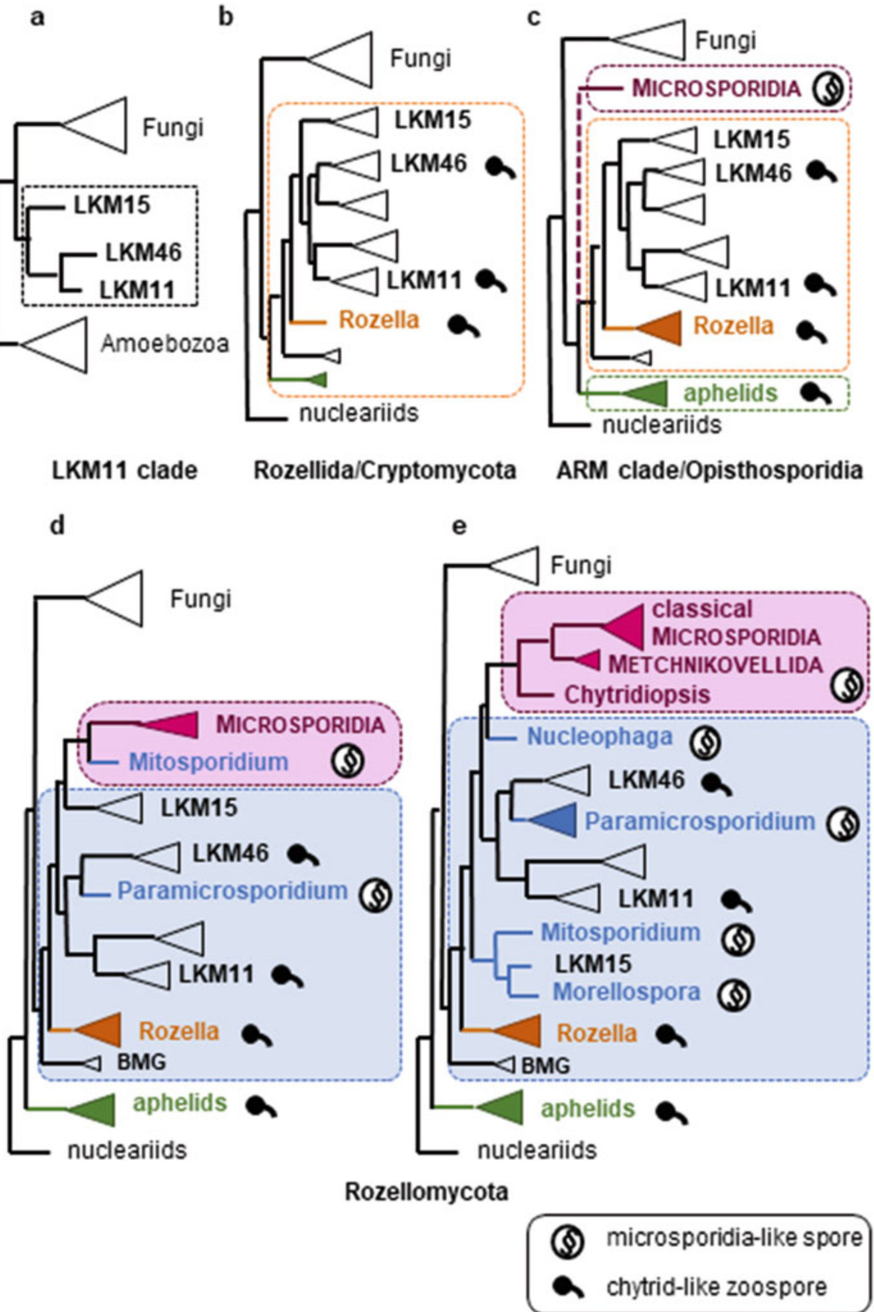

Fig. 3.1 Historical summary. During a mesocosm study, a clade of eukaryotes close to fungi was discovered and named LKM11 based on one of its sequences (**a**). Successive environmental surveys revealed that the clade is widespread and diverse and also includes the chytrid-like *Rozella* and other chytrid-like parasites of algae (**b**), as well as the aphelids, and it could be related to microsporidians (**c**). The recovery of an increasing number of parasites resembling microsporidia and the genetic

consistent with such a scenario (Fig. 3.1d). A set of marine sequences, labeled NCLC1 (Novel Chytrid-Like-Clade 1) for their phylogenetic position close to chytrids, have also been shown to be intracellular parasites of diatom algae. Inappropriately, these sequences have been reported as a new line, sister of "Opisthosporidia" (Chambouvet et al. 2019), despite the fact that they are always found as the most basal branch of the Rozellomycota, and therefore called Basal Marine Group A (BMG), in studies involving more and longer sequences (Fig. 3.1d). Relationships have continued to be reshaped with the characterization of new isolates, particularly other microsporidia-like organisms known for more than a century, like *Nucleophaga* (Michel et al. 2009a; Corsaro et al. 2014a, 2016) and *Morellospora* (Corsaro et al. 2020), and by the first molecular data from primitive microsporidians, metchkinovellids (Mikhailov et al. 2017; Galindo et al. 2018) and chytridiopsids (Corsaro et al. 2019) (Fig. 3.1e). Finally, microsporidia-like organisms have apparently functional mitochondria and fungal-like genomes (Haag et al. 2014; Quandt et al. 2017) but appear to have arisen separately during the rozellid evolution among chytrid-like lines, while true microsporidians form a monophyletic line which turns out to be the most derived branch of Rozellomycota, deserving of a high-rank distinction.

3.4 Morphological Similarities

The diagnostic characteristic of microsporidia, until now considered to be synapomorphic of the group, is the injection apparatus contained within the spore (Vávra and Lukeš 2013; Vávra and Larsson 2014). Classical microsporidia eject the filament in a unique way, but this does not seem to happen in primitive microsporidia (Larsson 2014), which were recognized as such only after electron microscopy studies (Vivier 1965; Manier and Ormieres 1968). Metchnikovellids and chytridiopsids have simpler polar filaments, consisting of only a manubrium, and a short coiled polar filament, respectively. The size of the polar tubes, along with the absence or reduction of the polaroplast and posterior vacuole, suggests that a lower pressure is likely required for extrusion. The spores are dispersed throughout the environment inside elongated or spherical cyst-like sacs that are ingested with food and are thus delivered close to host cells, i.e., gregarines infecting the gut of marine annelids (metchnikovellids) or almost always gut epithelium of terrestrial or freshwater arthropods (chytridiopsids). Once in the gut, the spores are released from the cyst and can more easily reach host cell areas allowing sporoplasm injection through their reduced filaments (Sokolova et al. 2014). Interestingly, *Mitosporidium* (Haag et al. 2014) and *Morellospora* (Corsaro et al. 2020) both have a polar filament very

Fig. 3.1 (continued) data of primitive microsporidia (**d**, **e**) have made it possible to refine the relationships between the lines which are however susceptible to other changes

similar to that of chytridiopsids, crowned by a curved polar sac devoid of an anchoring disk (Larsson 2014). For *Paramicrosporidium* and *Nucleophaga*, the structure of spores is less clear as attention has focused on their molecular phylogeny. Vacuolated structures of different electron densities that were initially interpreted as an irregularly coiled filament or as a prominent anchoring disk (Michel et al. 2009b, 2012) might in fact correspond to manubrial cisterns and a manubrium-like filament as in metchnikovellids. Further investigations are obviously necessary. In any case, *Paramicrosporidium* and *Nucleophaga* could use their devices once the spores are inside the amoeba, e.g., to invade the nucleus. For them as for *Morellospora*, the amoeba phagosome would be equivalent to the intestinal lumen of invertebrates, while *Mitosporidium* reaches the intestinal epithelium of *Daphnia* by the same route as chytridiopsids. Furthermore, metchnikovellids, chytridiopsids and the various microsporidia-like parasites also share the particular formation of spores by endogenous vacuolation of the sporogonial plasmodium (enveloped sporogony in sporont-derived sac). The vacuoles will form the plasma membranes of the sporoblasts which will mature into spores, while the plasmodium membrane remains as a sac forming the inner layer of the cyst. In metchnikovellids and chytridiopsids, dissemination to new hosts requires sac-bound spores, a constraint that the higher microsporidians overcame by developing a more complex and efficient injection apparatus.

It seems obvious that the various morphological traits that the microsporidia-like organisms share with the primitive microsporidians reflect a shared ancestry. However, they do not form a unique clade in molecular phylogeny, and these features might also represent a similar way of adapting to the transition from walled to phagotrophic preys (i.e., algae to amoebae), probably occurring more than once.

3.5 The rDNA Unit and the Loss of ITS2

In eukaryotes, ribosomal RNA genes are transcribed by RNA polymerase I into a single precursor (pre-rRNA) containing the small (18S), 5.8S and large (23S/28S) rRNAs separated by two internal transcribed spacers (ITS1 and ITS2). Mature rRNAs are released after complex processing involving nucleases and other factors, the initial steps of which are cleavages in ITS1 and ITS2 (Tomecki et al. 2017). Microsporidians are an exception as they have rRNA genes of prokaryote-like size and structure. Here, the small subunit (SSU) is greatly reduced (16S-like), and they lack ITS2 and have 5.8S fused with large subunit (LSU), as it occurs in prokaryotes where the first 150 nt of LSU are homologous to eukaryotic 5.8S. While these features were initially interpreted as further evidence of their primitive nature (Vossbrinck and Woese 1986; Vossbrinck et al. 1987), it is now evident that they are the result of reductive evolution. In fact, recent work has shown that more complex 18S-like SSU and ITS2 are present in primitive microsporidians (Corsaro et al. 2019) as well as in the various microsporidia-like organisms putatively ancestral to true microsporidians (Corsaro and Venditti 2020; Corsaro et al. 2014b,

2020). These early analyses used a 5.8S/LSU interaction model (Vaughn et al. 1984) that large, extensive studies have shown to be inaccurate (Schnare et al. 1996), and new models according to the currently accepted secondary structure (Wuyts et al. 2001) have been proposed (Mikhailov et al. 2021). The entire 5.8S/LSU interaction of *Paramicrosporidium* and the ITS2 structures of some key genera are reproduced with few modifications in Figs. 3.2 and 3.3, respectively. Interestingly, the new models better confirm the presence of ITS2 in both *Paramicrosporidium* and *Chytridiopsis*, but also in metchnikovellids, although in the latter, the portion probably remains as an uncleaved expansion (Fig. 3.3). The Las1/Grc3 tetrameric complex is responsible for releasing the precursors of 5.8S and LSU rRNAs, ensuring cleavage and phosphorylation in the process that begins at the C2 site in ITS2 (Frazier et al. 2021). Both enzymes are absent in higher microsporidians which have lost ITS2, and are not detectable in the available genomes of *Metchnikovella* and *Amphiamblys*, whereas they are present in those of *Paramicrosporidium* and *Mitosporidium*. In addition, Las1 recognizes C2 in a terminal bulge of the long stem III of ITS2 (Burlacu et al. 2017). A similar bulge is formed by the corresponding stems of the various microsporidia-like organisms including *Chytridiopsis*, but not by those of the Metchnikovellidae (Fig. 3.3). This would confirm that ITS2 is still removed in chytridiopsids but remains as a vestigial residue, consistent with the scenario of reductive evolution. Overall, the structure of *Chytridiopsis* is more similar to that of *Paramicrosporidium* than to that of metchnikovellids, while new results confirm that *Nucleophaga* has a classical four-stem conformation, indicating incompleteness of previous data. *Morellospora* has the most complex ITS2 structure (Corsaro et al. 2020), while the related *Mitosporidium* has a curious, very long sequence that might contain a group I intron (Corsaro and Venditti 2020).

3.6 Molecular Phylogeny and Systematics

A relationship between metchnikovellids and chytridiopsids has already been suggested based on their similar morphology and development, placing them as primitive lines compared to all other microsporidia possessing the typical characteristics of the group (Larsson 2014; Weiser 1977). SSU rDNA and multigene molecular phylogeny recovered metchnikovellids as a sister group to typical microsporidians (Frolova et al. 2021; Galindo et al. 2018; Mikhailov et al. 2017; Nassonova et al. 2021), both lines being highly supported holophyletic clades. Molecular data for metchnikovellids are consistent with their classification into a separate class, Rudimicrosporea, further suggesting the possible presence of additional lineages at the genus and family levels (Frolova et al. 2021; Mikhailov et al. 2021) (Fig. 3.4). Typical microsporidians are highly diversified, initially separated by SSU rDNA phylogeny into five main lineages which have been further refined in recent works (Park and Poulin 2021; Dubuffet et al. 2021). They deserve to be placed in the same class, Eumicrosporea cl. n. (eu-, Gr. for true, noble, higher), defined by their more complex life cycle including merogony and by a fully

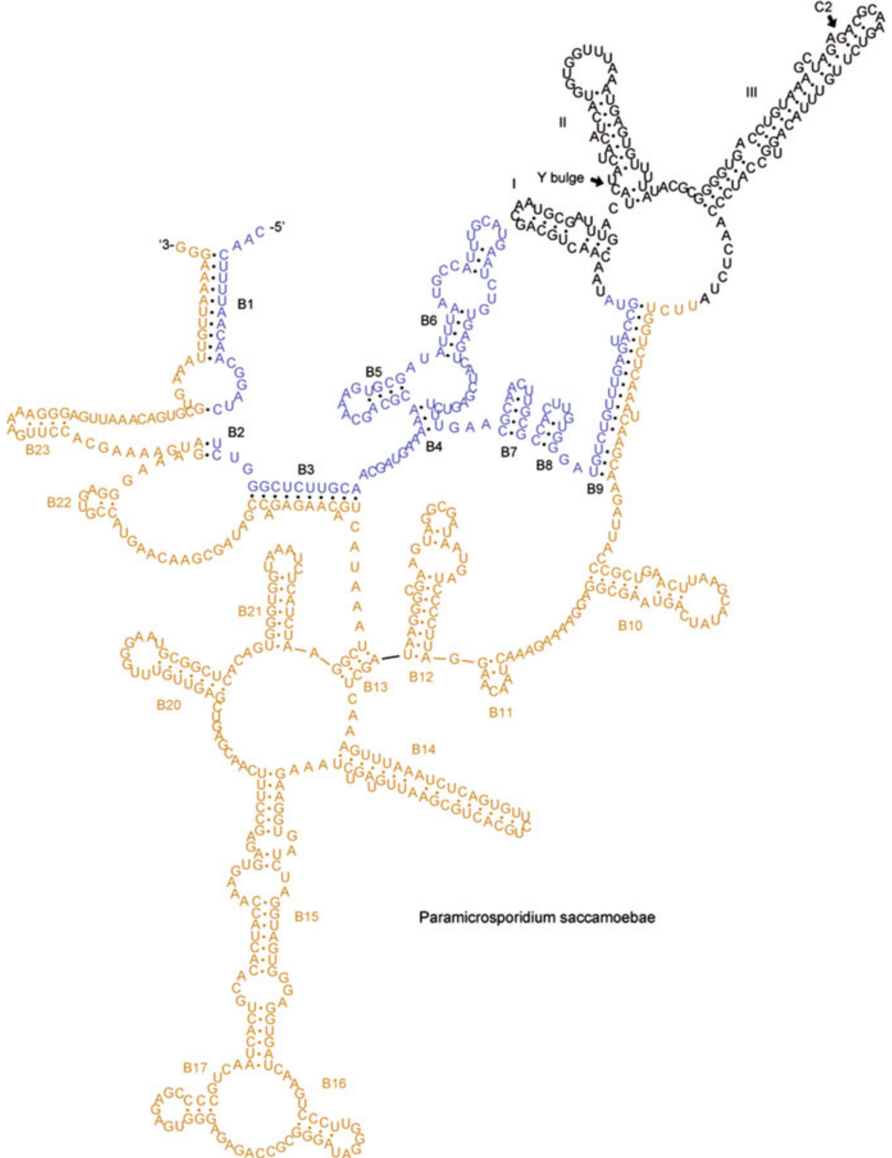

Fig. 3.2 *Paramicrosporidium* 5.8S/LSU interaction. Secondary structure of the ITS2 and 5.8S/LSU interaction of *Paramicrosporidium*, according to the current model (Wuyts et al. 2001)

developed injection apparatus, the latter being the characteristic feature of their advanced state. In this way, Eumicrosporea and Rudimicrosporea form two well-balanced groups strongly supported by morphological and molecular evidence. Likewise, it seems justified to place the different members traditionally assigned to

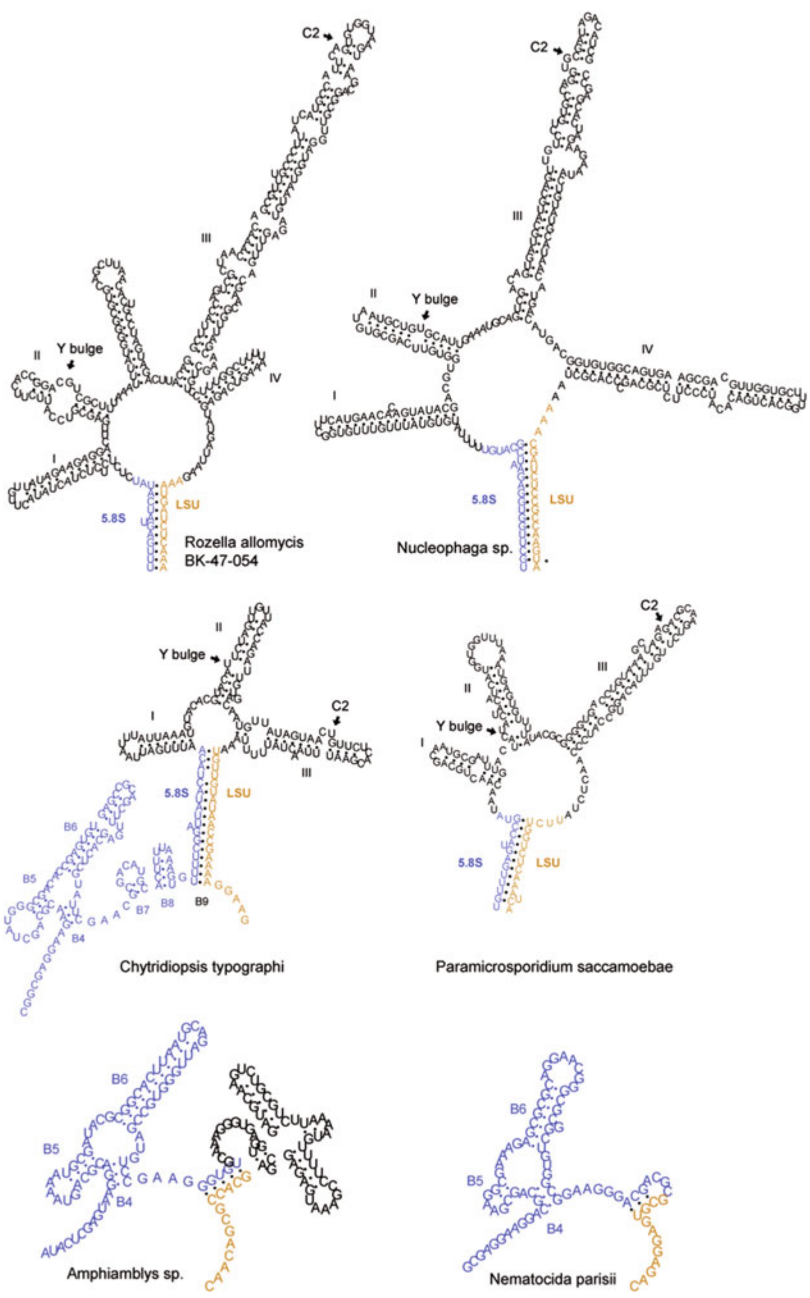

Fig. 3.3 ITS2. The secondary structures of ITS2 according to the revised model are presented for the main genera. For other species, see Mikhailov et al. (2021). Note the presence of a possible C2 site at the end of the long stem III of ITS2 in all except in metchnikovellids

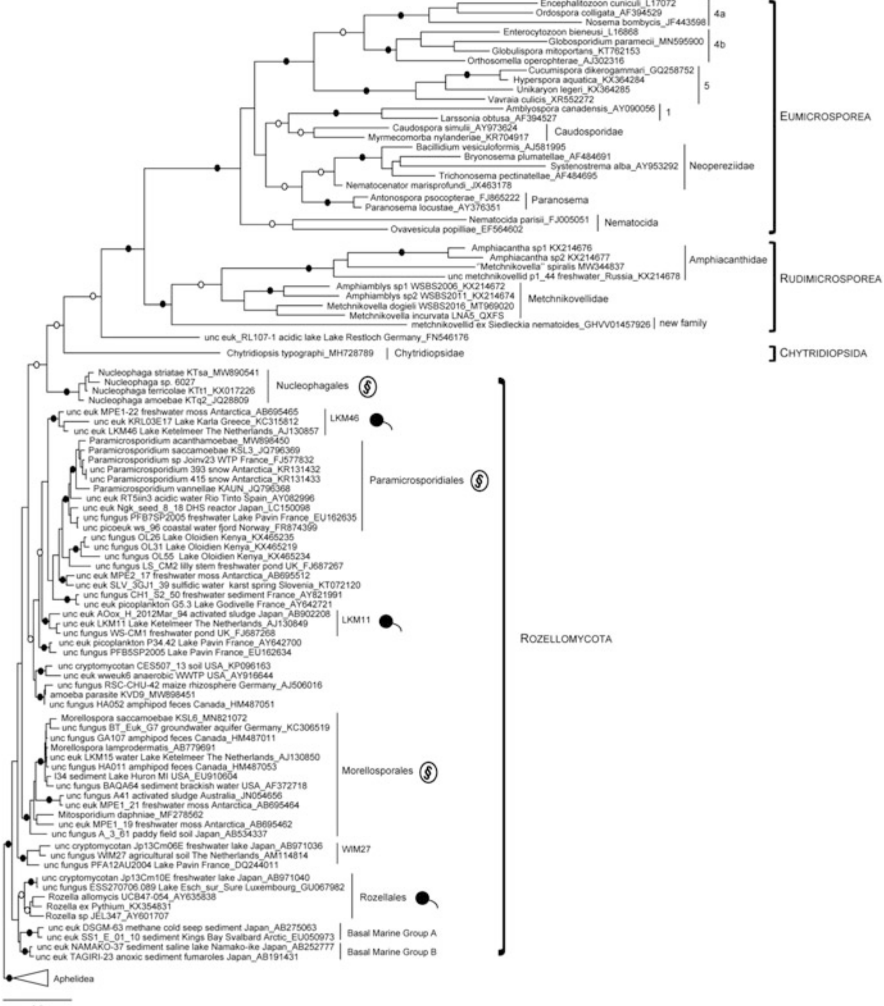

Fig. 3.4 18S rDNA phylogeny. Molecular phylogeny was carried out as described previously (Corsaro et al. 2019, 2020) on SSU rRNA gene sequences aligned with MAFFT and manually refined with BioEdit. Partial sequences representing less than 70% of the gene length were excluded from the final tree. Support values for Bayesian posterior probability and maximum likelihood bootstraps are shown at nodes by filled (1/100%) or open (0.9/95%) circles. Icons as in Fig. 3.1

chytridiopsids (Table 3.2) in their own class, Chytridiopsidea (Issi 1986) st. n., because overall their morphologies and developmental cycles are similar and clearly distinguishable from those of both the other classes (Larsson 2014). Molecular phylogeny, although based on a single species, *Chytridiopsis typographi*, supports the position of chytridiopsids as a distinct third branch (Corsaro et al. 2019). Obviously, this must be consolidated by characterizing other representatives of the

Table 3.2 Taxonomy of early diverging microsporidians and relatives

Phylum Rozellomycota Corsaro and Michel 2014	
Order 1. Rozellales Ord. n.	
Family 1. Rozellaceae Doweld 2013 st. n.	*Rozella*
Order 2. Paramicrosporidiales Ord. n.	
Family 1. Paramicrosporidiaceae fam. n.	*Paramicrosporidium*
Order 3. Morellosporales Ord. n.	
Family 1. Morellosporaceae fam. n.	*Morellospora*
Family 2. Mitosporidiaceae fam. n.	*Mitosporidium*
Order 4. Nucleophagales Ord. n.	
Family 1. Nucleophagaceae fam. n.	*Nucleophaga*
Phylum Microsporidia Balbiani 1882	
Class 1. Chytridiopsidea Issi 1986 st. n.	
Order 1. Chytridiopsida Weiser 1977	
Family 1. Chytridiopsidae Sprague et al. 1972	*Acarispora*
	Chytridiopsis
	Intexta
	Nolleria
	Sheriffia
Family 2. Burkeidae Sprague 1977	*Burkea*
Family 3. Buxtehudeidae Larsson 1980	*Buxtehudea*
	Jiroveciana
Order 2. Hesseida Weiser 1977	
Family 1. Hesseidae Ormières and Sprague 1973	*Hessea*
Class 2. Rudimicrosporea Sprague 1977	
Order 1. Metchnikovellida Vivier 1975	
Family 1. Metchnikovellidae Caullery and Mesnil 1914	*Amphiamblys*
	Metchnikovella
Family 2. Amphiacanthidae Larsson 2000	*Amphiacantha*
Class 3. Eumicrosporea cl. n.	

group. However, until new information, especially molecular data, become available to demonstrate the misplacement of either taxon, there is no reason to change the current composition. A complete SSU rDNA, intermediate in size and structure between that of *Chytridiopsis* and eumicrosporidians, was recovered from an acidic mining lake in Germany. This clone, RL107-1, is positioned between *Chytridiopsis* and the other microsporidians (Fig. 3.4), in place of the BAQA065 clone shown previously, which is now discarded because it is strongly suspected to be a chimeric sequence of gregarine giving a long branch attraction effect (Mikhailov et al. 2021).

Microsporidia-like organisms display various morphological characteristics in common with either Rudimicrosporea (*Paramicrosporidium*) or Chytridiopsidea (*Mitosporidium*, *Morellospora*), but all have apparently still functional mitochondria (Haag et al. 2014; Quandt et al. 2017). In all phylogenetic analyses, they are not directly related to microsporidians, nor do they cluster in a single group, but they

form strongly supported lineages emerging in distinct branches scattered among lineages containing chytrid-like flagellate forms (Fig. 3.4). Some of these organisms (*Morellospora*, *Nucleophaga*) were already affiliated with *Rozella* (Table 3.1), clearly supporting the paraphyletic status of Rozellomycota. To regard this large assemblage of microsporidia-like and chytrid-like organisms as "short-branched microsporidia," as in some recent publications, means to ignore the synapomorphies of Microsporidia.

Besides *Rozella*, only microsporidia-like organisms are currently well characterized, deserving to be recognized at a high taxonomic level (family, order), to obviate the recent oddities in the classification of these lineages. The proposed classification (Table 3.2) takes into account the morphological characteristics and the robust rDNA phylogeny, excluding poorly defined sequences. The choice of "botanical" endings is to emphasize that they do not belong to the Microsporidia.

Paramicrosporidium and *Nucleophaga* form two separate monotypic families (Paramicrosporidiaceae fam. n.; Nucleophagaceae fam. n.) and orders (Paramicrosporidiales ord. n; Nucleophagales ord. n.) comprising several species (Fig. 3.4). Both are endonuclear parasites of protozoa, especially amoebae, producing spores containing what appears to be a metchnikovellid-like injection apparatus. *Paramicrosporidium* infects several types of free-living amoebae (Corsaro et al. 2014b; Hoffmann et al. 1998; Michel et al. 2000, 2009b), and a new strain found in *Acanthamoeba* is named *P. acanthamoebae* (species name, L. gen. of *Acanthamoeba*). *Nucleophaga* has been found in *Thecamoeba* spp. (Corsaro et al. 2014a, 2016; Michel et al. 2009a, 2012, 2021), but it has already been reported in several types of protozoa (Kirby 1941). *Morellospora* (Corsaro et al. 2020) and *Mitosporidium* (Haag et al. 2014) are intracytoplasmic parasites of amoebae (*Saccamoeba*, *Lamproderma*) or freshwater invertebrates (*Daphnia*), respectively, and both have spores with chytridiopsid-like characteristics. They emerge as two separate branches, Morellosporaceae fam. n., and Mitosporidiaceae fam. n., within Morellosporales ord. n. (formerly LKM15 group). Functional mitochondria are present in members of all families.

The morphology and ecology of most of the remaining lines are unknown, as only the rDNA sequences are available. As chytrid-like forms that infect algae have been found in some groups, it would seem that the entire lineage contains phytoplankton parasites. However, as previously assumed (Corsaro et al. 2014b), it is very likely that a significant portion of rozellids do not interact with algae but with amoebae and other protozoa. Amoebae as hosts have surely been overlooked, and further support for this comes from the recovery of an additional amoeba parasite, KVD9. Although the morphology of this organism is still undetermined, it is placed in a lineage distinct from other known parasites.

Concerning *Rozella*, this endoparasite forms a basal lineage, comprising several species, and possibly more than one genus, as suggested by the low rDNA similarity values, linked to the clade C (Ishida et al. 2015) in the same order Rozellales ord. n. As stated earlier (Corsaro et al. 2014b), the characteristics listed by Doweld

2013 do not describe the phylum, but are suitable for a family; Rozellaceae stat. n.

Aphelids are not considered here. They emerge as the most basal lineage, after nuclearids, in rDNA trees, but in a multigene study, they are placed as the sister group of *Chytridiomycota* and classical fungi (Torruella et al. 2018). In any case, the "opisthosporidia clade" is never recovered, as they do not group with Rozellomycota and Microsporidia.

3.7 Conclusions

Cytology and type of development support an evolutionary link between Rudimicrosporea and Chytridiopsidea while recognizing the distinction of the two groups and their basal position relative to Eumicrosporea (Larsson 2014). Molecular analyses have largely confirmed this scenario, though some points require clarification. The Chytridiopsidea indeed seem to be the first line of Microsporidia, suggesting that the more simplified cytology of Rudimicrospores could be the result of a subsequent reduction due to hyperparasitism (Corsaro et al. 2019). This result, however, is based only on rDNA analysis including a single chytridiopsid, and more taxa or multigene analyses could paint a different picture. On the other hand, those morphologically more similar to Rudimicrosporea (Paramicrosporidiales) and Chytridiopsidea (Morellosporales) are clearly in separate unrelated groups, while the rDNA phylogeny suggests that *Nucleophaga* is the closest relative of Microsporidia. All of this raises doubts about a single origin of the injection apparatus that defines the microsporidians and leads us to wonder where and when the mitochondria-to-mitosome transition occurred, and if this should still be considered as typical of the group. Current data would allow speculation that either *Nucleophaga* could in fact be an early microsporidium or that *Chytridiopsis* should be placed among microsporidia-like organisms. Several of these points may be clarified when the genomes of other strains, especially *Nucleophaga* and *Chytridiopsis*, become available.

Other organisms requiring further investigation are those corresponding to the various chytrid-like flagellated lineages. Their isolation in culture could improve their morphological characterization and refine the genetic analysis, elucidating puzzling points currently present in the rozellid phylogeny.

Compliance with Ethical Standards

Conflict of Interest The author declares that there is no conflict of interest.

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Chapter 4
Factors That Determine Microsporidia Infection and Host Specificity

Alexandra R. Willis and Aaron W. Reinke

Abstract Microsporidia are a large phylum of obligate intracellular parasites that infect an extremely diverse range of animals and protists. In this chapter, we review what is currently known about microsporidia host specificity and what factors influence microsporidia infection. Extensive sampling in nature from related hosts has provided insight into the host range of many microsporidia species. These field studies have been supported by experiments conducted in controlled laboratory environments which have helped to demonstrate host specificity. Together, these approaches have revealed that, while examples of generalist species exist, microsporidia specificity is often narrow, and species typically infect one or several closely related hosts. For microsporidia to successfully infect and complete their life cycle within a compatible host, several steps must occur, including spore germination, host cell invasion, and proliferation of the parasite within the host tissue. Many factors influence infection, including temperature, seasonality, nutrient availability, and the presence or absence of microbes, as well as the developmental stage, sex, and genetics of the host. Several studies have identified host genomic regions that influence resistance to microsporidia, and future work is likely to uncover molecular mechanisms of microsporidia host specificity in more detail.

Keywords Microsporidia · Host specificity · Host range · Tissue specificity · Genetic resistance

4.1 Introduction

Microsporidia are a large phylum of obligate intracellular parasites. They belong to the earliest diverging fungal lineages but have undergone extreme genomic reduction and possess among the smallest eukaryotic genomes (Wadi and Reinke 2020). A defining feature of microsporidia is their ability to infect most types of animals.

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About half of all animal phyla have been reported to be infected with microsporidia (Murareanu et al. 2021). Though 1400 species of microsporidia have been identified, the true number is likely much higher, with some estimating over 100 million species (Larsen et al. 2017). As emerging pathogens, microsporidia are also expanding their host range, with several infections in shrimp and honey bees only recognized in the last few decades (Tang et al. 2016; Martín-Hernández et al. 2018). The ability of microsporidia to infect a diverse range of animals has also been exploited, with several being explored as potential biocontrol agents, and one species has already been approved for use for controlling grasshopper populations (Bjørnson and Oi 2014).

Microsporidia infection begins when spores come into contact with a host species. The environmentally resistant spores are typically ingested and initiate infection in the intestinal tract (El Jarkass and Reinke 2020). In the presence of a poorly defined host signal, these spores germinate, or "fire," rapidly releasing a unique infectious apparatus known as the polar tube (Jaroenlak et al. 2020). The polar tube is then used to inject the cellular contents of microsporidia (the sporoplasm), which includes the parasite's genetic material, into a host cell. Intracellularly, sporoplasms develop to form meronts, which in turn differentiate into spores, and these can exit the host to initiate new infections. Microsporidia life cycles can also be more complex and may involve generating multiple classes of spores, the need to pass through multiple hosts, and vertical transmission through the germline (Vávra and Lukeš 2013).

In this chapter, we describe the extensive field and laboratory studies that have helped to define the host range of many microsporidia species. We also discuss the host and environmental factors known to influence microsporidia infection. These insights suggest potential mechanisms controlling host specificity in microsporidia.

4.2 Determining the Host Range of Microsporidia Species

The host range and specificity of microsporidia species is a topic that has fascinated scientists for many decades (Steinhaus and Hughes 1949; Weiser and Coluzzi 1972). Two key approaches have been used to determine the host range of microsporidia: field studies and controlled laboratory infection experiments. Field studies are a necessary starting point to identify any microsporidia species. However, determining the full range of host species in nature is challenging as it requires extensive sampling of many potential hosts in multiple geographic areas. Additionally, field studies with low sampling might identify rare infections and assume they are common or misinterpret animals that have been exposed to spores, but are not infected (Quiles et al. 2019; Gisder et al. 2020). As a complementary approach, microsporidia isolated in nature can be used to infect animals reared under laboratory conditions. This approach involves examining if a defined set of potential host species can be infected. Importantly, however, infections often occur more easily under controlled conditions, and this should be considered when scrutinizing results

(Solter and Maddox 1998; Solter et al. 2005). Below, we highlight both field and laboratory studies that have informed our understanding of host specificity in microsporidia (Table 4.1).

4.2.1 Studies on the Natural Host Range of Microsporidia

Field studies can help provide a broad overview of microsporidia host relationships. For example, extensive sampling over a 3-year period in Siberia identified 21 species of microsporidia which were found to infect nine species of mosquitos. Analysis of this host-range data revealed that closely related microsporidia often infect hosts from the same genera. Mosquitoes of the genus *Culex* were only observed to be infected by one species of microsporidia each, while *Aedes* and *Ochlerotatus* mosquitoes were host to multiple species, including microsporidia from different genera (Andreadis et al. 2012).

Field studies can also reveal variability in host infections that occur as a result of geographical and ecological differences. Sampling from ~100 field sites identified 18 different microsporidia infecting the gammarid *Gammarus roeselii*. While vertically transmitted microsporidia species were found to infect this host over most of its range, other microsporidia species were more geographically localized. Many of the microsporidia only infected gammarids in areas that were recently colonized by this invasive host, suggesting that the infections were a result of host shifts from other gammarids (Quiles et al. 2019). Although many of the microsporidia infect multiple species of gammarids, there is evidence of specificity at the strain level, as strains are specific to a particular host (Quiles et al. 2020). Interestingly, although gammarids are infected by many genera of microsporidia, the species *Gammarus lacustris* is infected primarily by microsporidia from the genus *Dictyocoela*, demonstrating specificity for this host (Drozdova et al. 2020).

Another example of ecological constraints to microsporidia infection comes from a study monitoring three species of the water flea *Daphnia* in three ponds in southern England over a 1-year period. While the microsporidia species G*urleya vavrai* showed host specificity and mostly only infected *D. pulex*, the parasite *Pleistophora intestinalis* infected all three species of *Daphnia* hosts. Interestingly, this microsporidia showed local host specificity, infecting *D. longispina* in just two of the three ponds, even though the host was present in all three ponds (Stirnadel and Ebert 1997). If microsporidia are introduced into a susceptible population however, these ecological barriers can be overcome. Following introduction of the grasshopper-infecting *Paranosema locustae* from North America to Argentina, this parasite has now become endemic to many species of Argentinian grasshoppers (Lange 2010; Lange et al. 2020).

Field approaches have been especially useful for hosts such as humans and other vertebrates for whom careful laboratory experiments are not feasible. *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, and *Encephalitozoon intestinalis* infect a wide variety of mammals and birds, but there do appear to be some differences in host specificity. While *E. cuniculi* has a very broad host range,

Table 4.1 Examples of laboratory and field studies used to determine microsporidia host range

Field studies			
Host species	Microsporidia species	Summary of results	Refs.
Mosquitos	Several genera including *Amblyospora*, *Parathelohania*, *Trichoctosporea*, *Novothelohaniaovalae*	Nine mosquito species in Siberia were found to be infected with 21 species of microsporidia. *Aedes* and *Ochlerotatus* are host to multiple microsporidian species, whereas *Culex* hosts are only infected by a single microsporidia species each	Andreadis et al. (2012)
Amphipods	Several genera including *Nosema*, *Cucumispora*, *Dictyocoela*	These crustaceans are commonly infected in nature by a diverse set of microsporidia species	Wattier et al. (2007), Ironside et al. (2008), Krebes et al. (2010), Grabner et al. (2015), Bacela-Spychalska et al. (2018), Quiles et al. (2019, 2020, 2021), Drozdova et al. (2020)
Mammals and birds	*Encephalitozoon cuniculi*, *Encephalitozoon hellem*, *Encephalitozoon intestinalis*,	These species can infect a wide range of hosts, but there are differences in specificity. *E. hellem* is found most commonly in birds. *E. cuniculi* is found in diverse mammals. *E. intestinalis* is found in humans and domestic animals.	Hinney et al. (2016)
	Enterocytozoon bieneusi	*E. bieneusi* is a generalist that infects a wide range of hosts, including humans, other mammals, and birds. Some genotypes appear to be host specific	Li et al. (2019)
Laboratory studies			
Host species	Microsporidia species	Summary of results	Refs.
Fish	*Pseudoloma neurophilia*	*P. neurophilia* was first discovered in a pet zebrafish, but its natural host is unknown. *P. neurophilia* infected all eight host fish species tested	Sanders et al. (2016)
Mosquitos	*Edhazardia aedis* *Amblyospora connecticus*	These microsporidia were tested against many mosquito species. Infection of some mosquitoes, typically in the same genus as the natural host was observed.	Andreadis (1989, 1994), Becnel (1992), Becnel and Johnson (1993)

(continued)

Table 4.1 (continued)

Field studies			
Host species	Microsporidia species	Summary of results	Refs.
		Infection of the ovaries (needed to complete life cycle) was only observed in the natural host	
Laboratory and field studies			
Host species	Microsporidia species	Summary of results	Refs.
Moths and butterflies	Many species that either infect the gypsy moth (*Lymantria dispar*) or other insects	Microsporidia that infected gypsy moths were used to infect other insects, and microsporidia that infected other insects were used to infect gypsy moths. In both experiments, some non-natural infections occurred, but they appeared atypical and mostly could not be horizontal transmitted. Field studies show that microsporidia species that infect gypsy moths are highly specific	Solter et al. (1997, 2000, 2010); Solter and Maddox (1998)
Terrestrial nematodes	Several genera including *Nematocida*, *Enteropsectra*, *Pancytospora*	Many species of microsporidia infect terrestrial nematodes, with examples of both narrow and broad host ranges	Zhang et al. (2016)
Fire ants	*Vairimorpha invictae*	In laboratory tests with several ants, only the natural host *Solenopsis invicta* was infected. Hundreds of insects were tested in areas where *V. invictae* was found, and none were infected	Porter et al. (2007), Oi et al. (2010)
Fish	*Loma salmonae*	In laboratory tests of 12 species, only salmonids were infected. Three species (pacific salmon, rainbow trout, brook trout) were also infected in nature	Shaw et al. (2000), Brown et al. (2010)
Grasshoppers	*Paranosema locustae*	Over 100 species in the order *Orthoptera* can be infected by *Paranosema locustae*, as determined by a mixture of laboratory and field studies	Lange (2005)

(continued)

Table 4.1 (continued)

Field studies			
Host species	Microsporidia species	Summary of results	Refs.
Daphnia	*Hamiltosporidium tvaerminnensis* *Gurleya vavrai* *Pleistophora intestinalis*	Specificity between different host species and between different strains of the same host was observed	Stirnadel and Ebert (1997), Lange et al. (2015)
Brine shrimp	*Anostracospora rigaudi* *Enterocytospora artemiae*	Each microsporidian species could infect each host, but had a preferred host in both the lab and nature	Rode et al. (2013), Lievens et al. (2018, 2019)

E. hellem is mostly found in birds, and *E. intestinalis* is the most likely to infect humans and is often found in domestic animals (Hinney et al. 2016).

One challenge of field studies is that different strains of a single microsporidia species can be adapted to different host species. An example of this is the human-infecting microsporidia *Enterocytozoon bieneusi.* This species can infect many diverse hosts in nature, including humans, dogs, cats, livestock, wild mammals, and birds (Li et al. 2019). Over 500 *E. bieneusi* genotypes have been determined. Although many closely related *E. bieneusi* sequences have been isolated from diverse hosts, some groups of genotypes show host specificity, such as those that only infect either deer or horses (Li et al. 2019). Testing host specificity experimentally has not been possible for this species, so further sequencing of isolates will be needed to determine the extent of host adaptation.

4.2.2 Experimental Infection Studies

Laboratory experiments allow carefully controlled infection studies of potential hosts, but even though infection can occur, the parasite cannot always complete its full life cycle. For example, *Edhazardia aedis* (which naturally infects the mosquito *Aedes aegypti*) was found to infect seven of 12 mosquito species tested, including the natural host (Becnel and Johnson 1993). However, infection of the ovaries was only observed in the natural host *A. aegypti*. Infection of the ovaries is needed for the parasite to complete its life cycle and for long-term persistence within a population. A second study replicated these findings and showed that although five of the 12 mosquito species tested could be infected by *E. aedis* (Andreadis 1994), some species had lower prevalence of infections and less severe infections compared to the natural host. Critically, the microsporidia could not be vertically transmitted in these alternative species and was thus unable to complete its life cycle. A further study of a wide range of animals including insect, crustacean, planarian, and annelid representatives showed that none of these animals could be infected by *E. aedis*, again demonstrating the specificity of this species for its natural host (Becnel 1992).

Laboratory experiments have demonstrated that both horizontal and vertical infection can be restricted to natural hosts and can be used to define microsporidia host specificity. Using corn borers as a test system, Solter and colleagues (Solter et al. 2005) tested seven species of microsporidia (one known to infect corn borers, six that infect other hosts) and found that all seven species could infect the corn borer. By comparing each microsporidia species to its natural host, it was observed that only one species infected corn borers more than the microsporidia's natural host. Additionally, horizontal transfer in the corn borer was only observed for one non-natural microsporidia species. Only microsporidia that naturally infects corn borers could be transmitted both horizontally and vertically.

For microsporidia infection to be successful, all steps of the life cycle must be completed; however, the life cycle can be blocked at multiple stages. The microsporidia species *Amblyospora connecticus* has a complex life cycle of a copepod intermediate host and undergoes vertical transmission in mosquitoes. In one study, Andreadis (Andreadis 1989) tested *A. connecticus* against 20 mosquito species and found that four alternative hosts were infected, and these were all in the same genera as the natural host, *Aedes cantator*. This study revealed host specificity working on three levels: firstly, infection of the larvae, as only four alternative hosts became infected; secondly, sporulation, as this was only seen in one of the alternative hosts; and thirdly, the requirement of spores to germinate to infect the ovaries, which was not observed in alternative hosts. Thus, several steps must occur for the full life cycle to be completed.

Although infections can occur under laboratory conditions, they do not always lead to long-term prevalence within a host population. This was demonstrated using *Hamiltosporidium tvaerminnensis* infection of *Daphnia magna* (Lange et al. 2015). Here, 43 strains of *Daphnia* were infected for either 5 or 25 weeks. Though 31 strains displayed infection at 5 weeks, only 20 strains were infected at 25 weeks. The infected *D. magna* strains correlated with geographic distance and habitat, and the 25-week infections were better at predicting the geographic range than the 5-week experiments. These experiments also showed that host genetic factors can dictate geographical range of parasite infections.

4.2.3 Comparisons Between Laboratory and Field Studies

The complementary approaches of field and laboratory studies together have been used to understand microsporidia host specificity. One interesting example comes from a pair of brine shrimp species, *Artemia franciscana* and *Artemia parthenogenetica.* In nature, both hosts are infected by *Anostracospora rigaudi* and *Enterocytospora artemiae*; however, *A. rigaudi* is four times more prevalent in *A. parthenogenetica*, and *E. artemiae* is three times more prevalent in *A. franciscana* (Rode et al. 2013). This difference in prevalence can be recapitulated in the laboratory, as both microsporidia can complete their life cycle in each host, but they prefer the host they more commonly infect in nature (Lievens et al. 2018).

Although *A. rigaudi* can infect *A. franciscana*, its ability to infect appears to be dependent on the presence of *A. parthenogenetica.* Thus, host specificity can be facilitated by one species acting as a reservoir and allowing spillover into a different host species (Lievens et al. 2019).

Although a microsporidia species may display specificity in the laboratory, extensive field testing is necessary to truly confirm that no other hosts are infected in nature. One great example comes from assaying *Vairimorpha invictae* infection in *Solenopsis invicta* fire ants. In laboratory testing, *V. invictae* infected only *S. invicta*, and not the three other ant species tested (Oi et al. 2010). To more extensively test host specificity in nature, five field sites in Argentina, with *S. invicta* colonies that were 28–83% infected with *V. invictae*, were tested. Several hundred insects, including many other ant species, were collected, and none showed infection by *V. invictae*, demonstrating this parasites narrow host range (Porter et al. 2007).

Although microsporidia can often infect alternative hosts, infections do not always progress the same as in their natural hosts. One example of this comes from the gypsy moth which has been an important system for understanding host specificity in microsporidia. Using five different microsporidian pathogens that naturally infect European populations of gypsy moth, 49 other butterflies and moths were challenged with infection. Between 54 and 84% of alternative hosts were infected, depending on the microsporidia species. Although some infections looked similar to infections in the gypsy moth, many of the infections in alternative hosts looked atypical and did not produce the final stage of environmentally resistant spores used for horizontal transmission. This suggests that these atypical infections are unlikely to be transmitted to other species (Solter et al. 1997). In a related set of experiments, 21 microsporidia isolates that infected other insects were fed to gypsy moths. Though 18 of these microsporidia could infect the gypsy moth, many resulted in atypical infections compared to how the infection developed in the natural host. To test whether these atypical infections could result in horizontal transmission, transfer between individuals was tested. Here, only three of the nine species that reached the environmental spore stage could be horizontally transmitted, and even in these cases, only low levels of transmission were observed (Solter and Maddox 1998).

To confirm if laboratory observations of gypsy moth host specificity were consistent with infections in nature, several field studies were performed. Four sites in Bulgaria were monitored over a multiyear period. Interestingly, three sites each contained gypsy moths infected with a single species of microsporidia, while no gypsy moth infections were observed at the fourth site. Eleven microsporidia species were isolated from other insects that cohabitated these collection sites. None of the microsporidia infecting the gypsy moths were found in any of the other insects (Solter et al. 2000). To further test the specificity of gypsy moth infections in the environment, controlled introductions were performed. Two microsporidia species that infect gypsy moths were sprayed in field sites in Slovakia, and a very low number of individuals from other insect species were found to be infected. In 2 subsequent years, no other insects were infected. Interestingly, gypsy moths were also not found to be infected in subsequent years (Solter et al. 2010). Together,

these results suggest that laboratory infections can be much more permissive than the selectivity of infections found in nature and that environmental factors can play a large role in determining microsporidia infection success.

4.3 Mechanisms of Host Specificity in Microsporidia

4.3.1 Factors That Restrict Microsporidia Infection

For microsporidia to invade host tissues, spores must enter and remain in the host intestine for long enough to germinate. *E. aedis* spores have been shown to enter the gut and fire in a wide range of hosts from different phyla that could not be infected by this species (Becnel 1992; Becnel and Johnson 1993). In a key study, Undeen challenged five species of mosquitoes with *Anncaliia (Nosema) algerae* and measured the percentage of spores that had fired in the midgut of each animal. Although the rate of germination ranged from 25 to 81%, there was only partial correlation with how infected each host species was, and the host with the highest rate of germination had very little infection compared to other species. A known trigger for inducing spore germination in vitro is pH (Han et al. 2020). Measurement of the pH in the mosquito's midgut revealed only a partial correlation with spore firing frequency, indicating that other factors besides pH are likely to contribute to spore germination (Undeen 1976). It was reported that bumblebees could not be infected by *Nosema ceranae*; interestingly, the amount of time that the spores were in the bumblebee intestine was less than in infected honey bees. However, it is not known how rapidly the spores fire in the bumblebee intestine and if they germinate at the same rates as in honey bees (Gisder et al. 2020). In mosquitoes, it was determined that the amount of time it takes to passage particles through the gut is likely sufficient for germination (Undeen 1976).

The invasion, survival, and development of sporoplasms are critical steps in the microsporidian life cycle. A seminal study by Malone looked at *Vairimorpha plodiae* infection of three insect species. Although only two of these hosts were susceptible, similar levels of spore firing occurred in all three species. Though sporoplasms were observed in the tissues of the non-susceptible host, these sporoplasms either didn't develop or were destroyed by host cells. Interestingly, when spores were injected into the hemocoel, infection occurred in the tissues of the non-susceptible host that were not accessible during feeding (Malone 1984). In a different study, Undeen and Maddox fed *Anncaliia (Nosema) algerae* spores (which infect mosquitos in nature) to 12 non-mosquito arthropods and four non-arthropod hosts. Only a corn earworm was infected via feeding. In contrast, when spores were injected into animals, all arthropods became infected, but none of the non-arthropods. Spores that developed in all of the infected hosts could be used to infect a mosquito, indicating that infective spores were produced (Undeen and Maddox 1973). Several studies have proposed that lack of infection in some species is caused by the polar filament or sporoplasm not being able to pass into host cells

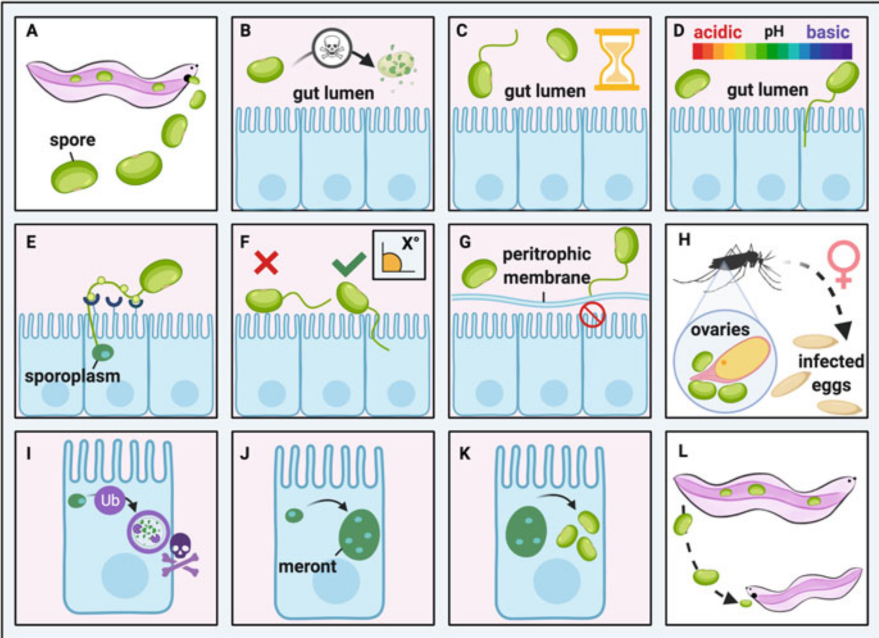

Fig. 4.1 Potential mechanisms that restrict host specificity in microsporidia. (**a**) Ingestion of spores; (**b**) survival of spores in the intestinal lumen; (**c**) retention of spores in the gut for sufficient time to infect host cells; (**d**) conditions which trigger firing of spores, e.g., the intestinal pH; (**e**) adhesion of spores and/or polar tube to target cells; (**f**) orientation of spores with respect to target cell; (**g**) physical barriers which obstruct host target cells, e.g., the intestinal peritrophic membrane; (**h**) infection of the female germline (e.g., ovaries in mosquitos) for vertical transmission; (**i**) survival of the sporoplasm in the host cell; (**j**) proliferation of the sporoplasm to form meronts; (**k**) formation of mature spores; (**l**) transfer of spores to a new host for sustained infection in a population

(Undeen and Maddox 1973; Undeen 1976). Support for this idea comes from experiments showing that the polar tube of *E. aedis* could only cross the peritrophic membrane in mosquito species that were infected (Becnel and Johnson 1993). This specificity may reflect unique protein-protein interactions between polar tube proteins or spore wall proteins and host cell receptors.

Collectively, studies on microsporidia host specificity suggest several barriers that can prevent infection and ensure host specificity: (1) the entry and occupancy of spores into the host intestine; (2) the germination of spores in the presence of a host signal; (3) the passage of the polar tube across the host cell membrane and the deposition of a sporoplasm into a host cell; (4) the survival and proliferation of sporoplasms within host cells; and (5) completion of the microsporidia's full life cycle, including the generation of environmentally resistant spores and/or infection of the host germline. These barriers to host infection are illustrated in Fig. 4.1.

4.3.2 Specialist and Generalist Microsporidia

Although microsporidia often only infect several hosts, some species have much broader host ranges. Recent quantification of data from 1435 species showed that while ~80% of species are known to infect only a single host, 2.2% of species can infect five or more hosts (Murareanu et al. 2021). For microsporidia that infect more than a single host, one of the biggest determinants of microsporidia host range is how closely related hosts are to one another. Experiments with many different types of hosts, including fish, nematodes, and mosquitoes, have demonstrated that microsporidia are more likely to infect hosts of the same genus as the natural host (Andreadis 1989, 1994; Shaw et al. 2000; Zhang et al. 2016). There are also a number of examples of microsporidia that have the ability to infect a much broader set of hosts. *P. locustae* can infect over 100 grasshopper species (Lange 2005). *Pseudoloma neurophilia* was first discovered in zebrafish, but later shown to infect at least five families of fish (Sanders et al. 2016). In a large-scale survey of microsporidia infecting nematodes, all microsporidia were restricted to infecting a single genera except for *Nematocida homosporus*, which could infect two genera in nature and a third in laboratory experiments (Zhang et al. 2016). *Encephalitozoon* species and *Enterocytozoon bieneusi* can infect a wide range of mammals and birds (Hinney et al. 2016; Li et al. 2019). Two species that infect bees, *Nosema apis* and *Nosema bombi*, have each been reported to infect bees in two genera, whereas *N. ceranae* can infect at least 15 genera (Ii and Quandt 2020). These observations suggest that although most microsporidia are specialized for an individual host, there are many examples of generalist species.

4.3.3 Tissue Specificity

In addition to possessing specificity for a particular host species, microsporidia also display specificity within a host by infecting a particular set of host tissues. Several studies have shown that the same species of microsporidia can have dramatically different somatic tissue distributions depending on the host species infected (Hazard and Lofgren 1971; Andreadis 1989, 1994). This suggests that tissue specificity isn't just an innate ability of the microsporidia species but depends on the host as well. One factor in determining tissue specificity is access to host tissues. In several cases, although microsporidia spores fed to alternative hosts are unable to infect, spores can successfully infect a number of different tissues when injected into the animal (Undeen and Maddox 1973; Malone 1984). The length of the polar tube has been proposed to impact tissue specificity. Interestingly, in microsporidia species that infect tissues besides the intestine, the polar tube is ~threefold longer, which could allow access to a broader set of tissues (Luallen et al. 2016; Murareanu et al. 2021).

4.3.4 Evolution of Host Specificity

Most closely related microsporidia species infect similar hosts, but there are some examples of dramatic shifts in host specificity. Comparing the sequence identity and host specificity of 270 microsporidia species revealed that closely related species are much more likely to infect the same host family (Murareanu et al. 2021). Interestingly, however, there are several examples of closely related species that can infect different host phyla (Stentiford et al. 2017; Yakovleva et al. 2020). Microsporidia from different clades can also evolve to infect the same hosts, demonstrating convergent evolution (Murareanu et al. 2021).

4.4 Factors That Influence Microsporidia Infection

Many different factors can influence microsporidia infection. These include the genetics, sex, and stage of the host and environmental factors such as microbes, nutrients, temperature, and seasonality. These factors are illustrated in Fig. 4.2 and described in the sections below.

4.4.1 Host Genetics

Susceptibility to microsporidia infection depends on host strain, as individuals with a population can have differing levels of resistance to infection. For example, studies

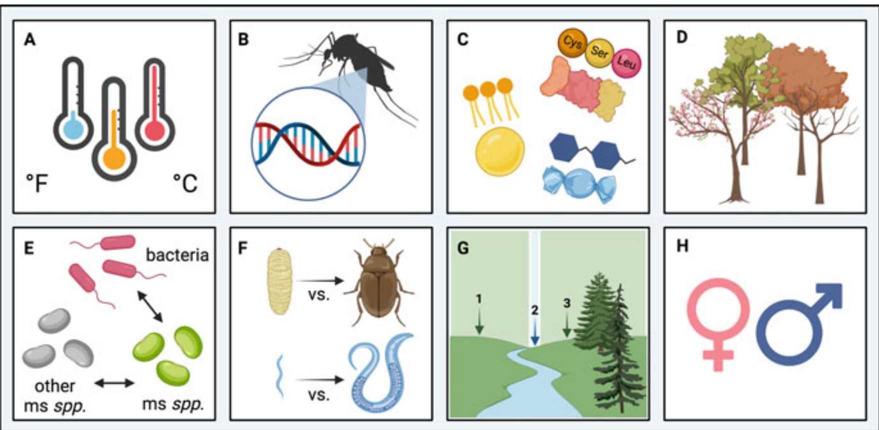

Fig. 4.2 Factors that influence microsporidia infection: (**a**) temperature, (**b**) host genetics, (**c**) host nutrition, (**d**) seasonality, (**e**) interactions with other pathogens or microbes, (**f**) developmental stage of the host, and (**g**) sex of the host

in which *Daphnia* were infected with several microsporidia species either in laboratory conditions or outdoor containers showed that the frequency of different strains in the population changed upon infection with microsporidia. Interestingly, different species of microsporidia selected for different host strains, suggesting that resistance can be specific to individual microsporidia species (Capaul and Ebert 2003; Haag and Ebert 2004). Differing levels of susceptibility and resistance to microsporidia infection have also been reported for strains of *Caenorhabditis elegans* (Balla et al. 2015).

Several studies have used quantitative genetic approaches to identify genomic loci responsible for resistance to microsporidia infection. A panel of 195 clonal F2 lines of *D. magna* were infected with *H. tvaerminnensis*, revealing five quantitative trait loci (QTL) that were responsible for horizontal transmission, including one loci that was also responsible for vertical transmission (Routtu and Ebert 2015). Another study examined long-term prevalence in this experiment by looking at clones of infected *D. magna* at 30 weeks, instead of the original study's 40 days. This experiment identified two QTL for long-term persistence including one which overlapped with vertical transmission from the previous experiment (Krebs et al. 2017). Another experiment employed a similar panel of *D. magna* clones but infected them with the microsporidia *Ordospora colligata*. This experiment revealed three QTL that impacted infectivity and one QTL impacting pathogen burden. Two of the QTL identified overlapped with regions identified for *H. tvaerminnensis* (Keller et al. 2019). In a study of *N. ceranae* infection in honey bees, researchers performed crosses between one susceptible strain and one bred for resistance and identified four QTL associated with reduced spore levels (Huang et al. 2014). Additionally, a study in *C. elegans* tested recombinants resulting from crosses of two worm strains infected with *N. ironsii* and identified four QTL associated with microsporidia resistance (Balla et al. 2015). Taken together, these results suggest that multiple genomic loci influence different traits related to microsporidia infection.

Several mechanisms have been suggested to explain how individuals within a population can be resistant to microsporidia infection. Quantitative genetic experiments with *Daphnia* revealed different genetic regions were involved in infection prevalence and infection burden, suggesting that these two forms of resistance are independent from one another (Keller et al. 2019). In *C. elegans*, the strain CB4856 is resistant to *N. ironsii* infection as a result of both reduced parasite invasion and enhanced clearing of the invaded pathogen. This clearance correlates with localization of host ubiquitin and the autophagy protein LGG-2 to the pathogen. Though LGG-2 is not required for pathogen clearance, it is needed to reduce invasion in this strain (Balla et al. 2019). A candidate for resistance within a QTL in honey bees is the argonaute protein Aubergine, a gene that is also upregulated in response to *N. ceranae* infection (Huang et al. 2014). Although there is no evidence for their existence in natural populations, several mutations can cause *C. elegans* to become resistant to infection. Deletion of either *pals-22* or *lin-35*, which are negative regulators of the *C. elegans* immune response to microsporidia infection, results in resistance to *Nematocida parisii* infection, although these mutations also cause

delayed animal development (Reddy et al. 2019; Willis et al. 2021). Mutation of the gene *aaim-1*, which is needed for microsporidia to efficiently invade *C. elegans*, also increases resistance to infection (Tamim El Jarkass et al. 2022).

4.4.2 Host Developmental Stage and Sex

Microsporidia infection can occur throughout host development or can be restricted to particular life stages. For example, infection of red flour beetles by *Nosema whitei* is greatest at the youngest larval stages, and animals become resistant as they progress through development (Blaser and Schmid-Hempel 2005). Similarly, only the early larval stages of fruit flies are susceptible to *Tubulinosema kingi* (Vijendravarma et al. 2008). In contrast, while a strain of *C. elegans* is resistant to infection at the earliest larval stage of development, these worms can be efficiently infected at later developmental stages. Additionally, the inherited immunity of progeny passed on from infected *C. elegans* parents is also strongest at the L1 stage and diminishes over development (Balla et al. 2015; Willis et al. 2021).

The sex of the host can also affect microsporidia infection. Several species of *Aedes* mosquitoes infected with *Amblyospora connecticus* under experimental conditions showed moderate but significant differences in infection prevalence that were dependent upon sex. Here, two mosquito species showed a higher prevalence of infection in males, and another showed a higher prevalence in females (Andreadis 1989). In some species of amphipods, females are infected at a high prevalence, but males are not infected (Gismondi et al. 2012). Higher prevalence of infected females is common and attributed to vertically transmitted microsporidia that can feminize their hosts, leading to distorted sex ratios in host populations (Terry et al. 2004). Infections can also impact hosts differently depending on sex. For example, some species that cause lethal infections in males are mostly benign in females (Dunn and Smith 2001).

4.4.3 Impact of Nutrients and Microbes

Nutrients and chemicals in the environment can influence microsporidia infections. Food quality has been shown to influence both the prevalence and intensity of microsporidia infection in *Daphnia* (Aalto et al. 2014; Decaestecker et al. 2015; Narr et al. 2019). One suggested explanation for this effect is that elevated nutrients increase feeding rates and cause higher levels of spores to be ingested (Narr et al. 2019). Feeding honey bees pollen, which is a rich source of protein, increased intensity of *N. ceranae* infection, but not prevalence (Porrini et al. 2011; Jack et al. 2016). Similarly, fruit flies fed a nutrient-rich diet of yeast displayed increased pathogen load. Injecting flies with phosphatidic acid, a potential precursor for membrane biosynthesis, also increased infection intensity (Franchet et al. 2019).

Chemicals released by hosts may also impact infection. For example, *Daphnia* became less infected with *Glugoides intestinalis* when treated with medium that had contained a crowded population of *Daphnia* (Pulkkinen 2007).

Microsporidia infections can be affected by other microsporidia and bacteria. For example, host infection by microsporidia can prevent infection by other microsporidia species. When gypsy moths are infected with *Nosema lymantriae* and *Vairimorpha disparis* at the same time, the levels of each parasite are similar. However, when infections are carried out 7 days apart, the species that infects first excludes the second. Interestingly, another microsporidia species, *Endoreticulatus schubergi*, does not alter the infection by the other two species (Pilarska et al. 2006). A similar situation was seen in honey bees, whereby animals infected with either *N. ceranae* or *N. apis* were much less susceptible to subsequent infection with the other species (Natsopoulou et al. 2015). However, when animals were infected with both species at the same time, there was no advantage for either species (Forsgren and Fries 2010; Milbrath et al. 2015). Microsporidia infection can also influence infections across generations. For example, *C. elegans* parents infected by *N. parisii* produce progeny that are resistant to subsequent infection (Willis et al. 2021). Other parasites can also alter microsporidia infection as infection of *Daphnia* by the pathogenic bacteria *Pasteuria ramosa* prevents microsporidia infection by *Octosporea bayeri* (Ben-Ami et al. 2011). Non-pathogenic bacteria can also affect microsporidia infection. *N. ceranae* infection in honey bees can be modulated with probiotic bacteria, causing a modest decrease in spore load and a reduction in host mortality (Borges et al. 2021).

4.4.4 Temperature

Because microsporidia grow exclusively inside of their hosts, the temperature of the parasite is dictated by the host, and the environmental temperature can have large effects on microsporidia growth. For example, lady beetles infected with *Nosema adaliae* and reared at 25 °C produce ~30-fold more spores than infected animals raised at 30 °C (Steele et al. 2020). Similarly, mammalian cells infected with *Anncaliia algerae* formed more spores at 33 °C than at 37 °C (Leitch and Ceballos 2008). There can even be differences between closely related species. While *N. ceranae* and *N. apis* produce a similar number of spores in honey bees maintained at 33 °C, *N. ceranae* forms more spores than *N. apis* when kept at 37 °C (Martín-Hernández Raquel et al. 2009). Temperature-dependent differences can also occur between strains of the same species. *A. algerae* isolated from humans can infect mammalian cells better at 37 °C compared to strains isolated from insects (Kucerova et al. 2004). In addition to upper limits for growth, minimum temperatures have been described. Infection of *D. magna* by *O. colligata* occurs between 11.8 and 29.7 °C, but 2 to 3 degrees lower or higher yields no observable infection (Kirk et al. 2018). The range of temperatures needed to support microsporidia infection can be quite narrow, as the development of spore-filled xenomas in rainbow trout infected with

Loma salmonae occurred only between 9 and 20 °C. Because microsporidia are so dependent on host temperature, the use of environmental temperature to treat microsporidia infections has been proposed. For example, the ayu *Plecoglossus altivelis* can be cured of *Glugea plecoglossi* by incubating fish at an elevated temperature (Takahashi and Ogawa 1997). How temperature directly affects microsporidia infection is not well-known, but germination can be impacted at lower temperatures, and parasite clearance has been observed at temperatures both below and above the species growth limit (Sanchez et al. 2000; Leitch and Ceballos 2008).

4.4.5 Seasonality

In nature, microsporidia infections often display strong seasonal dependencies (Andreadis 1984; Araújo-Coutinho et al. 2004; Lass and Ebert 2006; Martín-Hernández Raquel et al. 2007; Valles et al. 2010; Traver et al. 2012; Copley et al. 2012; Chen et al. 2012; Preston et al. 2020). One interesting example is that of *N. ceranae* infections in honey bees. Monitoring of apiaries in Taiwan revealed the highest frequency of infections occurred during the winter in December and January, and the lowest levels occurred in July through September (Chen et al. 2012). Seasonality was also observed for *N. ceranae* infections in Virginia, where infections peaked in the months of April–June (Traver et al. 2012). In contrast, monitoring of honey bees in Spain showed no evidence of seasonality, and a study in Quebec, Canada, showed variation in seasonality from year to year (Martín-Hernández Raquel et al. 2007; Copley et al. 2012). The related species *N. apis* shows a different pattern, with infection peaking in the spring and fall (Copley et al. 2012; Chen et al. 2012). Taken together, these studies show that seasonality in microsporidia infections often exist, but can be specific to individual locations and species.

There are several reasons why seasonal effects may have such a strong impact on microsporidia infections. Honey bees infected by *N. ceranae* show a negative correlation between temperature and infection intensity. However, no relationship with humidity is observed, suggesting that temperature is the major factor driving seasonal patterns for this species (Chen et al. 2012). Although temperature is a major factor in seasonality of microsporidia infections, the effect is not always directly on the parasite. In the case of two microsporidia that infect brine shrimp, the prevalence of *A. rigaudi* is strongly dependent upon the season, whereas *E. artemiae* is not. Though both microsporidia species grow at similar temperatures, the abundance of the primary host of *A. rigaudi* displays strong seasonal patterns, while the host of *E. artemiae* does not (Lievens et al. 2019). The abundance of other host populations can also be seasonal, and in several cases, host numbers were seen to decrease as infections became more prevalent (Andreadis 1984; Araújo-Coutinho et al. 2004). Seasonality can also have an impact on hosts beyond just affecting their abundance. *O. bayeri* infections of *D. magna* increase in the summer and decrease in the winter,

and this effect appears to be driven by the life cycle of the host, and not by the direct effect of temperature on parasite growth (Lass and Ebert 2006).

Infection patterns can also change over time. Infection of English sole by the microsporidian parasite *Glugea stephani* was monitored in 1971 and again in 1997 through 2000. An increase in infection prevalence during the later years was suggested to be due to increased temperatures caused by El Niño (Olson et al. 2004). Prevalence of microsporidia over time can also be affected by other species. At the beginning of a 3-year study, *N. apis* could be found infecting hives on its own, but by the end, only mixed infections with *N. ceranae* were observed, suggesting that *N. ceranae* may be displacing *N. apis* (Copley et al. 2012).

4.5 Conclusions and Future Perspectives

4.5.1 Considerations for Testing Host Specificity in Microsporidia

From the many studies on microsporidia host specificity, a number of principles emerge that should be taken into consideration when designing laboratory experiments to test microsporidia interactions in other hosts (Solter 2006). The conditions that the infection will take place under can dictate whether infection occurs. These conditions include the temperature that hosts are reared under, the developmental stage and gender of the hosts, and the presence of any other beneficial or pathogenic microbes. As different strains of hosts can be infected to different extents, testing several strains of each host is beneficial (Capaul and Ebert 2003; Haag and Ebert 2004; Balla et al. 2015; Zhang et al. 2016; Orlansky and Ben-Ami 2019). Choosing how to quantify infection is important, and quantifying the presence of the infective spores is likely to be more meaningful than measuring intermediate stages of infection. Challenging animals with multiple concentrations of microsporidia reveals whether alternative hosts can be infected to the same extent as the natural host, when using the same dose (Lievens et al. 2018). Although it is more laborious, testing transmission and demonstrating long-term persistence within a population provides further assurances that alternative hosts can be successfully infected (Solter 2006; Lange et al. 2015).

4.5.2 Future Directions

Despite decades of research, there is still much to learn about what determines microsporidia host specificity. One major challenge that remains is identifying molecular mechanisms that determine host range. In the last two decades, there has been a rapid increase in our understanding of how microsporidia invasion,

proliferation, and exit occur, including the identification of both microsporidia and host factors involved in these processes (El Jarkass and Reinke 2020). By taking a similar molecular approach, the determinants of microsporidia host specificity can be identified. There are reports of microsporidia strains isolated from nature that have different infection characteristics (Kucerova et al. 2004; Li et al. 2019; Orlansky and Ben-Ami 2019). Experimental evolution has been used to select for strains with specialist or generalist properties (Legros and Koella 2010; Lievens et al. 2020). Using whole genome sequencing, candidate genetic changes could be identified from these different microsporidia strains. Although the ability to genetically modify microsporidia doesn't currently exist, the development of such technology would allow the reintroduction and testing of candidate changes (Reinke and Troemel 2015). Although no microsporidia proteins are known to determine host specificity, a large number of microsporidia proteins predicted to come in contact with the host are some possible candidates (Reinke et al. 2017). Genetic approaches have identified several regions of host genomes that are associated with resistance to microsporidia infection (Huang et al. 2014; Balla et al. 2015; Routtu and Ebert 2015; Krebs et al. 2017; Keller et al. 2019). Further work identifying and characterizing the causative genes in these regions will likely reveal the different mechanisms that hosts can employ to prevent microsporidia infection. Through these molecular approaches, both microsporidia and host factors involved in microsporidia host specificity can be determined.

Taxonomic Note

Nosema apis and *N. ceranae* have recently been redefined as *Vairimorpha apis* and *V. ceranae* based on a recent molecular phylogenetics analysis of the *Nosema* and *Vairimorpha clades* (Tokarev et al. 2020). For the purposes of this review the Nosema Genus will still be used.

Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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Chapter 5
Insights from *C. elegans* into Microsporidia Biology and Host-Pathogen Relationships

Eillen Tecle and Emily R. Troemel

Abstract Microsporidia are poorly understood, ubiquitous eukaryotic parasites that are completely dependent on their hosts for replication. With the discovery of microsporidia species naturally infecting the genetically tractable transparent nematode *C. elegans*, this host has been used to explore multiple areas of microsporidia biology. Here we review results about microsporidia infections in *C. elegans*, which began with the discovery of the intestinal-infecting species *Nematocida parisii*. Recent findings include new species identification in the *Nematocida* genus, with more intestinal-infecting species, and also a species with broader tissue tropism, the epidermal and muscle-infecting species *Nematocida displodere*. This species has a longer polar tube infection apparatus, which may enable its wider tissue range. After invasion, multiple *Nematocida* species appear to fuse host cells, which likely promotes their dissemination within host organs. Localized proteomics identified *Nematocida* proteins that have direct contact with the *C. elegans* intestinal cytosol and nucleus, and many of these host-exposed proteins belong to expanded, species-specific gene families. On the host side, forward genetic screens have identified regulators of the Intracellular Pathogen Response (IPR), which is a transcriptional response induced by both microsporidia and the *Orsay virus*, which is also a natural, obligate intracellular pathogen of the *C. elegans* intestine. The IPR constitutes a novel immune/stress response that promotes resistance against microsporidia, virus, and heat shock. Overall, the *Nematocida*/*C. elegans* system has provided insights about strategies for microsporidia pathogenesis, as well as innate defense pathways against these parasites.

Keywords *C. elegans* · Microsporidia · Nematocida · Tissue tropism · Syncytium · Intracellular Pathogen Response · Host-exposed proteins

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5.1 Introduction

Study of the genetically tractable nematode *Caenorhabditis elegans* has resulted in seminal discoveries ranging from RNA interference (RNAi) to apoptotic signaling pathways (Singh 2021). *C. elegans* has several advantages for research in the lab including reproduction by selfing of hermaphrodites, which allows for propagation without crossing, a 3-day reproductive life cycle, and a 1-mm-long transparent body plan that facilitates imaging. Over 20 years ago, *C. elegans* was established as a powerful system to study innate immune responses in a whole animal model, using bacterial and fungal pathogens that cause lethal infections in this host (Darby et al. 1999; Mahajan-Miklos et al. 1999; Pujol et al. 2001). The majority of infection studies in *C. elegans* have been performed with clinically relevant bacterial pathogens, but these studies have now broadened to include natural pathogens, including microsporidia infections as described below.

The Microsporidia phylum contains over 1400 species of obligate intracellular pathogens related to fungi, and they can infect a wide range of hosts, from single-celled protists to humans (Han and Weiss 2017). Historical observations of microsporidia species that infect non-*C. elegans* nematodes date back to the early 1900s, but it was not until 2008 that a microsporidia species was identified that infects *C. elegans*. This pathogen was found in a wild *C. elegans* from a compost pit outside Paris, France, and causes a lethal intestinal infection. Based on ribosomal sequence, it was given a new genus and species name *Nematocida parisii* (in Latin *Nematocida* means nematode killer and *parisii* means from Paris) in the Microsporidia phylum (Troemel et al. 2008). Since that time, the *Nematocida* genus has grown to include eight species, which have been identified from wild *Caenorhabditis* and related nematodes across the globe (Zhang et al. 2016). Based on these studies, microsporidia are a common cause of infection for *C. elegans* in the wild, and *N. parisii* infection is the most common microsporidia species to infect *C. elegans*. This chapter will describe the isolation and characterization of these microsporidia species, their impact on host physiology, and the *C. elegans* innate immune response to these naturally occurring intracellular infections.

5.2 Identification of Microsporidia That Infect *C. elegans*

Species in the *Caenorhabditis* genus are free-living, bacterivorous nematodes that belong to the family Rhabditidae. Among the fifty-plus species in the *Caenorhabditis* genus, *C. elegans* and *C. briggsae* are the best studied (Stevens et al. 2019). The standard laboratory strains of each of these species are "domesticated" in that they have been grown for decades in a controlled laboratory environment. Driven by the interest to describe the natural ecology and biology of these species, researchers increased efforts to sample wild worms from across the world in the early 2000s, and there are now hundreds of wild-caught isolates of these two

species, as well as several other *Caenorhabditis* species (Kiontke et al. 2011; Lee et al. 2021; Schulenburg and Felix 2017). In the course of isolating wild-caught nematodes from their natural habitats including rotting fruits, decaying plant stems, and compost heaps, co-occurring natural microbes have also been isolated, including pathogens like microsporidia.

Of the eight *Nematocida* species isolated from wild *Caenorhabditis* and related nematodes, six have so far been shown to infect *C. elegans*. These are *N. parisii*, *Nematocida ironsii*, *Nematocida ausubeli*, *Nematocida displodere*, *Nematocida major*, and *Nematocida homosporus* (Table 5.1) (Zhang et al. 2016). In addition, one non-*Nematocida* species called *Pancytospora epiphaga* can infect *C. elegans*. *N. parisii* has been isolated from wild *C. elegans* and wild *C. briggsae* hosts from various regions in France. Notably, the ERTm5 strain originally isolated from *C. briggsae* in Kauai (Hawaii, USA) was named *N. parisii* based on large subunit ribosomal sequence (Balla et al. 2015) but was later re-named *N. ironsii* based on whole genome sequencing and molecular divergence (Reinke et al. (2017) and see below). This finding indicates that other strains assigned as *N. parisii* based on large subunit ribosomal sequence may be reassigned once their genomes are fully sequenced. After *N. ironsii*, the species with closest sequence identity to *N. parisii* is *N. ausubeli*. This species was originally called *Nematocida* sp. 1 and is described with this name in several publications (Bakowski et al. 2014b; Balla et al. 2016; Reinke et al. 2017; Troemel et al. 2008). It subsequently was named *N. ausubeli* for Fred Ausubel, a pioneer in the field of *C. elegans* host/pathogen interactions (Zhang et al. 2016). *N. ausubeli* was originally isolated from *C. briggsae* in Kerala, India, and has subsequently been isolated in wild-caught *C. elegans* from France and from Portugal; in wild-caught *C. briggsae* from Cape Verde; and in wild-caught *C. remanei* from Germany. *N. major* has been isolated from *C. briggsae* in Guadalupe and Thailand and from *C. tropicalis* in Guadalupe. This species has not yet been isolated from *C. elegans* in the wild, but it is capable of infecting *C. elegans* in the lab. Similarly, *N. homosporus* and *P. epiphaga* have both been isolated from non-*C. elegans* nematode species in the wild, but can infect *C. elegans* in the lab. The fourth species so far shown to infect *C. elegans* in the wild is *N. displodere*, which was isolated from a wild *C. elegans* in France. This species is distinct from other *Nematocida* species in that it can infect multiple tissues, in addition to the intestine, providing an opportunity to learn more about what governs tissue tropism ((Luallen et al. 2016) and see below).

While infection by all *Nematocida* species appear capable of killing *C. elegans* hosts, different species have varying levels of virulence. Side-by-side comparisons between *N. ausubeli* and *N. parisii* demonstrate that *N. ausubeli* is more virulent, as assessed by *N. ausubeli* causing more severe reduction in body size and a greater reduction in the number of eggs laid by infected hosts (Balla et al. 2016). Furthermore, *N. ausubeli* replicates more quickly inside *C. elegans* and forms spores earlier than *N. parisii*. Side-by-side comparisons of *N. displodere* and *N. parisii* virulence have not been described, but *C. elegans* begins shedding *N. parisii* spores earlier than *N. displodere* spores, likely because *N. parisii* spores are actively shed via host exocytosis from the intestine, whereas *N. displodere* spores gradually build up inside

Table 5.1 List of microsporidia species that can infect *C. elegans* in the wild or the lab

Microsporidia species	Isolated from	Infects *C. elegans*	Number of published strains	Tissues infected	Genomes sequenced	Other names	References
Nematocida parisii	*Caenorhabditis elegans, Caenorhabditis briggsae* (France)	In wild, in lab	17	Intestine	Strains ERTm1, ERTm3		Troemel et al., PLOS Biology (2008); Cuomo et al., Genome Research (2012); Zhang et al., PLOS Pathogens (2016)
Nematocida ironsii	*Caenorhabditis briggsae* (Hawaii, USA)	In wild, in lab	1	Intestine	Strains ERTm5	*N. parisii*	Balla et al., PLOS Pathogens (2015); Zhang et al., PLOS Pathogens (2016); Reinke et al., Nature Communications (2017)
Nematocida ausubeli	*Caenorhabditis elegans* (France, Portugal); *Caenorhabditis briggsae* (India, Cape Verde, Germany); *Caenorhabditis remanei* (Germany)	In wild, in lab	10	Intestine	Strains ERTm2, ERTm6	*N.* sp. 1	Troemel et al., PLOS Biology (2008); Cuomo et al., Genome Research (2012); Bakowski et al., Genome Announcement (2014b); Zhang et al., PLOS Pathogens (2016)
Nematocida displodere	*Caenorhabditis elegans* (France)	In wild, in lab	1	Intestine, coelomocytes, neurons, epidermis, muscle	JUm2807		Luallen et al., PLOS Pathogens (2016); Zhang et al., PLOS Pathogens (2016)
Nematocida major	*Caenorhabditis briggsae* (Thailand, Guadeloupe); *Caenorhabditis tropicalis* (Guadeloupe)	In lab	3	Intestine			Zhang et al., PLOS Pathogens (2016)
Nematocida homosporus	*Oscheius tipulae* (France); *Rhabditella typhae* (Portugal)	In lab	2	Intestine			Zhang et al., PLOS Pathogens (2016)
Pancytospora epiphaga	*Caenorhabditis brenneri* (Colombia)	In lab	1	Epidermis			Zhang et al., PLOS Pathogens (2016)
Nematocida minor	*Oscheius tipulae* (Czech Republic, Armenia)	No	2	Intestine			Zhang et al., PLOS Pathogens (2016)
Nematocida ciargi	*Procephalobus* sp. (Spain)	No	1	Intestine			Zhang et al., PLOS Pathogens (2016)

the animal until it bursts (Luallen et al. 2016; Szumowski et al. 2014). Further distinctions among the different *Nematocida* species genomes, life cycles, and interactions with hosts are described below.

5.3 *Nematocida* Genomes, Transcriptomes, and Proteomes

Next-generation sequencing has provided whole genome assembly for the four microsporidia species found to infect *C. elegans* in the wild (*N. parisii*, *N. ausubeli*, *N. ironsii*, and *N. displodere*). Microsporidia have the smallest known genomes among eukaryotes, with the human-infecting species *Encephalitozoon intestinalis* as one of the smallest at only 2.3 Mb (Corradi et al. 2010). The first *Nematocida* genome sequences were obtained as part of the Microsporidian Genome consortium and included two strains of *N. parisii* (ERTm1 from Paris, France, and ERTm3 from Santeuil, France) (Cuomo et al. 2012) and two strains of *N. ausubeli* (ERTm2 from Kerala, India, and ERTm6 from the Cape Verde Islands) (Bakowski et al. 2014b; Cuomo et al. 2012). *N. parisii* ERTm1 and ERTm3 each have genomes of about 4.1 Mb, with 2661 predicted genes for ERTm1 and 2770 for ERTm3. Transcriptome analysis aided annotation of the *N. parisii* genome and indicated that 2546 of the ERTm1 genes were expressed during at least one of five stages of the *N. parisii* intracellular replicative life cycle and/or during the spore stage (Cuomo et al. 2012). Genome size was more divergent between the two *N. ausubeli* sequenced strains, at 4.7 Mb with 2770 predicted genes for ERTm2 and 4.3 Mb with 2433 genes for ERTm6. Both *N. parisii* and *N. ausubeli* appear to be diploid, with the highest degree of heterozygosity seen in *N. ausubeli* ERTm2 at 1 SNP every 82 bases (Cuomo et al. 2012). Large regions in *N. parisii* and *N. ausubeli* genomes have lost heterozygosity, which is suggestive of a recent or rare recombination event as part of a sexual or parasexual cycle. Morphological evidence of sexual cycles had previously been described for microsporidia species infecting other hosts (Becnel et al. 2005).

These genome sequencing studies were part of a larger phylogenomics effort to compare diverse microsporidia genomes (Cuomo et al. 2012). This effort identified gene gains and losses specific to the Microsporidia phylum and elucidated possible strategies used for the unique life cycle of these parasites. For example, compared to other eukaryotes, microsporidia have lost the cell cycle inhibitor retinoblastoma, which appears to be one of the earliest steps in the evolution of the Microsporidia phylum and may have facilitated their rapid replication inside host cells (Cuomo et al. 2012; Haag et al. 2014). Furthermore, these studies and others at the same time (Capella-Gutierrez et al. 2012) firmly established microsporidia as being most closely related to fungi, which had previously been a topic of controversy (Keeling and McFadden 1998).

Following the efforts of the Microsporidian Genomes Consortium, the genomes of one strain each of *N. displodere* and *N. ironsii* have also been described. As mentioned earlier, *N. ironsii* ERTm5 has the same large subunit ribosomal sequence

as *N. parisii*, but whole genome sequencing revealed only 92.3% identity between ERTm5 and ERTm1 DNA, thus leading to its definition as a distinct species (Reinke et al. 2017). For comparison, *N. parisii* strains ERTm1 and ERTm3 share 99.8% genome sequence identity (Cuomo et al. 2012). The *N. ironsii* ERTm5 genome spans 4.4 Mb with 2709 predicted genes and is the closest to that of *N. parisii*. At 3 Mb, the *N. displodere* strain JUm2807 has a smaller genome than either *N. parisii*, *N. ironsii*, or *N. ausubeli* (Luallen et al. 2016). This reduction is due in part to a decrease in intergenic regions: 85.8% of *N. displodere*'s genome is protein coding, while 69.2% and 63.7% are protein coding in *N. parisii* and *N. ausubeli*, respectively. In addition, the *N. displodere* genome has only 2278 predicted genes, which is fewer than *N. parisii*, *N. ironsii*, or *N. ausubeli*. *N. displodere* proteins share only 48% amino acid sequence similarity with *N. parisii* or *N. ausubeli* proteins, supporting the assignment of this species as an outgroup in the *Nematocida* genus compared to *N. parisii* and *N. ausubeli* and also compared to other species. Of note, genome sequencing indicated that all *Nematocida* genomes contain expanded species-specific gene families, with the largest being a family with 235 members found in *N. displodere* (Luallen et al. 2016; Reinke et al. 2017). Thus, this family comprises about 10% of the protein-coding potential in the *N. displodere* genome. This gene family is characterized by the presence of a RING domain, which is a protein-protein interaction domain found in most E3 ubiquitin ligases.

To determine which microsporidia proteins might enable its obligate intracellular life cycle, localized proteomics was performed to identify *Nematocida* proteins that are "host-exposed" to the *C. elegans* host cytosol or nucleus (Reinke et al. 2017). Specifically, spatially restricted enzymatic tagging was performed by expressing the promiscuous biotin ligase APX in either the *C. elegans* intestinal cytosol or intestinal nucleus, which allows APX to attach a biotin tag to lysine resides on any nearby proteins in these cellular compartments. APX-expressing transgenic animals were then infected with either *N. parisii* or *N. ausubeli*. To identify *Nematocida* proteins located in either the intestinal cytosol or intestinal nuclei, biotin-tagged proteins were isolated by streptavidin affinity pull-down, followed by liquid chromatography and tandem mass spectrometry. Overall, this analysis identified 82 *Nematocida* proteins (72 from *N. parisii* and 10 from *N. ausubeli*) with high confidence of interacting with intestinal cell tissue, including several proteins found in the nucleus. These host-exposed proteins were enriched for those that contained transmembrane domains and/or signal sequences. In addition, most of these host-exposed proteins were found to be rapidly evolving and species-specific. In fact, only 12% of *N. parisii* host-exposed proteins have orthologues outside of the "clade" of *N. parisii*, *N. ironsii*, and *N. ausubeli*, whereas 63% of proteins as a whole in the *N. parisii* genome have orthologs outside of this clade. These host-exposed proteins appear to not just be species-specific, but also belong to large gene families. Such families appear to be a general feature of microsporidia genomes. The size and divergence of these gene families may reflect the outcome of host/pathogen arms races that drive both sides to expand and diversify the sequence of proteins directly involved in the battle for survival during intracellular replication (Lazetic and Troemel 2020).

5.4 *Nematocida* Life Cycles and Their Impact on *C. elegans* Cell Biology

The life cycles of *Nematocida* species share many similarities with other microsporidia species, including the spore stage being the transmissible form, and all parasite replication and differentiation occurring inside of host cells, before spores exit to infect new hosts (Fig. 5.1). As *N. parisii* strain ERTm1 is the best characterized of the microsporidia species infecting *C. elegans*, we will first describe its life cycle and then compare it to other species below.

N. parisii is transmitted fecal orally, with infection starting after *C. elegans* ingests infectious spores that enter the lumen of the intestine (Fig. 5.1) (Troemel et al. 2008). The *C. elegans* intestine is composed of 20 polarized epithelial cells with structural and functional similarity to intestinal epithelial cells in mammalian hosts, except that these intestinal cells are non-renewable (Dimov and Maduro 2019). While in the intestinal lumen, *N. parisii* spores fire an infection apparatus common to all microsporidia species called a polar tube, which is coiled inside of the spore before receiving a cue to fire. As the polar tube fires, a small parasite cell called a sporoplasm is injected through the tube and delivered into a *C. elegans* intestinal cell. The exact cues and mechanisms used by *N. parisii* to fire the polar tube and invade host cells are poorly understood, but a recent report describes an intestinally secreted host factor that promotes microsporidia invasion, perhaps through enabling more efficient polar tube firing (El Jarkass et al. 2022). Notably, the process of intestinal cell invasion is fast, as sporoplasms inside intestinal cells can be observed within minutes after feeding *C. elegans* infectious *N. parisii* spores (Balla et al. 2019). Like other microsporidia species, *N. parisii* sporoplasms develop into multinucleated, replicating cells called meronts, which then differentiate back into spores that exit into the lumen. From there, spores can be defecated out of the animal to infect new hosts. The intestine is the only tissue where *N. parisii* replicates, and spore exit appears to be directional. Specifically, all spores exit apically from intestinal cells into the lumen, and have not been seen to exit basolaterally into the pseudocoloemic space of the animal (Estes et al. 2011).

N. parisii sporoplasms and meronts appear to replicate in direct contact with host intestinal cytosol. In particular, they have their own cellular plasma membrane, but do not appear to have a separate host membrane surrounding them at this stage (Szumowski et al. 2014). Further, *N. parisii* causes several restructuring events in the host while going through its life cycle. Here we will only briefly summarize such events related to spore exit, as they have been previously reviewed (El Jarkass and Reinke 2020; Troemel 2016). One restructuring event is a partial loss of polarity, with apically restricted cytoskeletal component actin appearing on the basolateral side of intestinal cells (Estes et al. 2011). This relocalization may trigger another restructuring event, which is the appearance of gaps in the terminal web. The terminal web is a conserved actin and intermediate filament-rich structure found in the apical region of most polarized epithelial cells, including epithelial cells of the *C. elegans* intestine. These gaps in the terminal web appear precisely when *N. parisii*

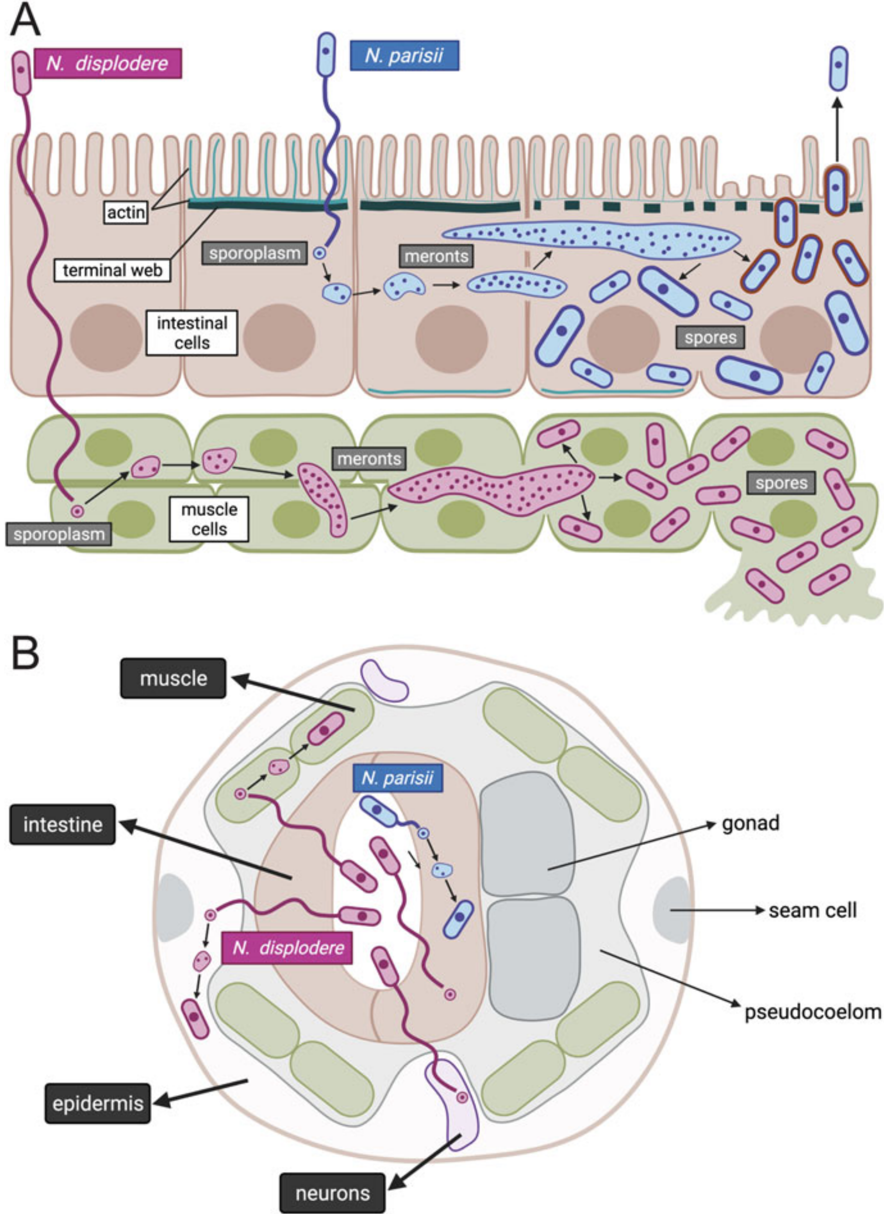

Fig. 5.1 Life cycle of *N. parisii* and *N. displodere* inside the *C. elegans* host. Longitudinal (**a**) and cross-sectional (**b**) diagrams of *Nematocida* infection of the *C. elegans* intestine, muscle, epidermis, and neurons. *N. parisii* spore in blue is shown in the intestinal lumen, firing a polar tube that delivers a sporoplasm (a membrane-bound parasite cell) into the cytoplasm of *C. elegans* intestinal cells. This sporoplasm develops in direct contact with the host cytoplasm, replicating its nuclei without undergoing cell division, to develop into a multinucleate meront. This meront can spread across intestinal cells, causing the intestinal organ to become a syncytial structure with shared cytoplasmic

meronts differentiate into spores, and thus formation of gaps may remove a barrier to enable spores to exit into the lumen (Estes et al. 2011). As meronts differentiate into spores, they enter a separate membrane-bound compartment that feeds into the host recycling endocytosis pathway. Near the apical membrane, these compartments get coated with the *C. elegans* small GTPase RAB-11, which is a host trafficking factor that facilitates fusion of these spore-containing compartments with the apical membrane to direct non-lytic exit of spores back into the intestinal lumen (Szumowski et al. 2014; Szumowski et al. 2016). Overall, these restructuring events appear to enable a large number of *N. parsii* spores to be shed from worms while minimizing damage to the host intestine.

In contrast to the intestinal-specific tropism described for *N. parisii* and other *Nematocida* species, the more recently described species *N. displodere* is able to replicate in several tissues in *C. elegans*. *N. displodere* replicates most robustly in muscle and epidermis cells, with rare replication seen in coelomocytes (phagocytic cells with no known role in immunity), neurons, and intestinal cells (Fig. 5.1) (Luallen et al. 2016). *N. displodere* bursts out of worms (*displodere* in Latin means to cause to explode), which may occur because it is not possible to non-lytically exit out of the non-intestinal tissues where *N. displodere* replicates and differentiates most efficiently. After *N. displodere* replicates in the epidermis and muscle, it differentiates into spores that exit from the animal by bursting through the *C. elegans* vulva, or egg-laying structure. *N. displodere* infection begins through feeding, and thus the pathogen appears to access all of these diverse tissues by starting from the intestinal lumen. Interestingly, the *N. displodere* polar tube is longer than that of *N. parisii* (12.6 μm vs. 4.0 μm). These observations, together with the observation that *N. displodere* sporoplasms are found inside non-intestinal tissues less than 2 minutes after feeding spores to worms, suggest that *N. displodere* spores fire their polar tubes from the intestinal lumen all the way through both the apical and basal side of intestinal cells to invade non-intestinal tissue like the epidermis and muscle, although this event has not been directly visualized (Fig. 5.1). Another notable characteristic of *N. displodere* is having only one spore size, while *N. parisii* has two distinct spore sizes. The functional significance of these spore differences is unknown. It should be noted that the morphological and life cycle differences between *N. displodere* and *N. parisii* are accompanied by

Fig. 5.1 (continued) contents. As *N. parisii* meronts develop into spores, gaps appear in the terminal web (made of actin and intermediate filaments), which is thought to remove a barrier to exit. Once *N. parisii* meronts develop into spores, they are found in separate membrane-bound compartments of unknown origin. These spores then become coated with the small GTPase RAB-11 (in red) and fuse with the apical membrane, to be released non-lytically back into the lumen. *N. displodere* spore in purple is also shown in the intestinal lumen, firing a polar tube that is longer than the polar tube of *N. parisii*. The *N. displodere* polar tube is hypothesized to reach all the way from the lumen into muscle cells, epidermal cells, and neurons to deliver sporoplasms inside of these cells. *N. displodere* sporoplasms develop into meronts, which can lead to fusion of muscle cells to form a syncytium (the epidermis is already a syncytium). In contrast to the non-lytic exit of *N. parisii*, *N. displodere* spores appear to burst out of cells

differences in genome content as described above, including the strikingly expanded gene family that has 235 members in *N. displodere*. This family has been named NemLGF2 and has only 1–3 members each in *N. parisii*, *N. ironsii*, and *N. ausubeli* genomes (Reinke et al. 2017). Perhaps this family is responsible for the distinct lifestyle and tissue tropism of *N. displodere*, which has the broadest tissue tropism described so far for any *C. elegans* microbial pathogen.

One pathogen-driven restructuring event seen in common with infections by *N. displodere*, *N. parisii*, and *N. ausubeli* is the fusion of host cells (Balla et al. 2016). This fusion occurs while meronts are replicating, with *N. parisii* able to spread across several intestinal cells after first invading only a single intestinal cell. Imaging experiments with photoconvertible GFP demonstrated that *N. parisii* growth across intestinal cells causes sharing of cytoplasmic contents, indicating that what once was 20 separate intestinal cells becomes a large syncytial organ during infection. Similar results were found with *N. ausubeli*, which also only grows in the intestine. In contrast, *N. displodere* did not spread across intestinal cells in the rare instances where it replicated there, but instead spread across muscle cells after invading a single cell. These results indicate that the ability to fuse host cells is conserved across *Nematocida* species, with each species triggering syncytial formation in the host organ where it grows best. This cell-to-cell spread by *Nematocida* meronts likely enables faster, more efficient dissemination than if they were to differentiate back into spores, exit the infected cell, and then invade uninfected host cells using the polar tube invasion strategy. These findings provide a new perspective on how microsporidia can invade host cells, using a strategy independent of spores and polar tubes.

What regulates the differentiation of microsporidia meronts into spores? Experiments using differing doses of *N. parisii* spores and differing sizes of *C. elegans* host animals indicated that sporulation is triggered when meronts reach a critical density inside the intestine (Balla et al. 2016). An RNAi screen identified predicted *C. elegans* transcription factors as host factors that regulate sporulation of *N. parisii* inside intestinal cells. These host factors include members of the Myc family of transcription factors (Botts et al. 2016). *N. parisii* replication appears to proceed normally in *C. elegans* animals defective for these factors, but *N. parisii* differentiation is compromised. The exact role for *C. elegans* Myc transcription factors in regulating *N. parisii* sporulation is unclear, but one possibility is that they regulate expression of *C. elegans* metabolites or other cues that are sensed by *N. parisii* upon high meront density within the intestine to enter the sporulation program.

5.5 *C. elegans* Natural Variation in Resistance to *Nematocida* Infections

The original *N. parisii* isolate ERTm1 came from a wild-caught *C. elegans* near Paris, France, but ERTm1 is able to infect the N2 laboratory strain and other strains of *C. elegans* as well (Troemel et al. 2008). A comparison of wild-caught *C. elegans*

strains found that the *C. elegans* Hawaiian isolate CB4856 displays higher resistance to infection with a Hawaiian isolate of *Nematocida* called *N. ironsii* ERTm5 (Balla et al. 2015). There is a lower initial colonization of *N. ironsii* inside CB4856 intestinal cells compared to N2 intestinal cells, and CB4856 intestinal cells can also clear *N. ironsii* infection over time, unlike N2, which always succumbs to infection by *N. ironsii*, as well as by other microsporidia species (Balla et al. 2015; Balla et al. 2019). Competition experiments showed that this increased resistance of CB4856 provided a fitness advantage over N2 in the presence of *N. ironsii*. Interestingly, the increased resistance and clearance (the reduction in parasite load over time) ability of CB4856 is restricted to the first larval (L1) stage of development, with no difference between N2 and CB4856 at later larval stages. Of note, the L1 stage is the only stage where infection reduces progeny production, with a greater reduction of progeny production caused by infection of N2 compared to infection of CB4856. While infection by *N. ironsii* shortens life span of N2 and CB4856 at any stage of development, this pathogen somewhat surprisingly does not reduce progeny production in older animals, despite establishing a similarly robust infection. Therefore, L1 may be the only stage where increased resistance against *N. ironsii* can promote the evolutionary success of *C. elegans*.

Quantitative genetics using recombinant-inbred lines identified four regions in the CB4856 genome associated with resistance (Balla et al. 2015). Introgressing two of these regions from the CB4856 genome into the N2 genome conferred resistance, and introgressing them from the N2 genome into the CB4856 genome conferred susceptibility, thus confirming the role of these two regions in resistance to *Nematocida* infection, acting additively. While the causative alleles in these genomic regions have not been identified, they are enriched for genes encoding components of cullin-ring E3 ubiquitin ligases. Ubiquitin ligases are enzymes that add ubiquitin tags to proteins and other targets to alter their fates, which can include autophagy-mediated degradation of intracellular pathogens in a process called xenophagy (Kuo et al. 2018). For this reason, the role of ubiquitin and the downstream process of autophagy was further analyzed in the ability of the CB4856 strain to clear *N. ironsii* (Balla et al. 2019). Ubiquitin and LGG-1/ATG8, a common marker for autophagy, were previously shown to target *N. parisii* sporoplasms in N2 animals. The level of targeting was relatively low, and ubiquitin-mediated autophagy played a modest but significant functional role in resistance against infection, although clearance was not examined in this study (N2 is not able to clear infection) (Bakowski et al. 2014a). In support of the possibility that more efficient xenophagy could explain the increased resistance of CB4856 hosts, this strain was found to have much higher levels of ubiquitin targeting sporoplasms compared to N2. For example, 84% of parasite cells were targeted at 15 hours post-inoculation (hpi) in CB4856 animals, and only 11% targeted at 15 hpi in N2 animals (Balla et al. 2019). Ubiquitin targeting correlated with parasite clearance in terms of parasite species, as analysis in CB4856 hosts showed there was high levels of targeting to *N. ironsii* cells, which are cleared, and little targeting to *N. parisii* and *N. ausubeli* parasite cells, which are not cleared. Furthermore, increased ubiquitin targeting was seen in infections of CB4856 L1 but

not L4 animals, again showing a correlation between ubiquitin targeting to parasite cells and their subsequent clearance.

Despite this strong correlation between host targeting of ubiquitin to *N. ironsii* parasite cells and their subsequent clearance, a functional role for ubiquitin and the downstream process of autophagy has not yet been shown. Ubiquitin is an essential gene, and *C. elegans* has an enormous number of ubiquitin ligases, making it difficult to test their roles. Ubiquitin ligase-mediated autophagy converges on core components however, including the ATG8 homolog LGG-1 mentioned above, which is commonly used as a marker for autophagy in *C. elegans*, as well as the other *C. elegans* ATG8 homolog LGG-2, which is most similar to LC3 in mammals. Interestingly, it was seen that LGG-2 targeting parasite cells correlated with clearance, with higher levels of LGG-2 targeting *N. ironsii* cells in CB4856 compared to *N. ironsii* cells in N2 hosts. There was no difference in LGG-1 targeting *N. ironsii* cells in CB4856 compared to N2. However, clearance still occurred normally in *lgg-2* mutants in CB4856, indicating this gene is not required for clearance. One possibility is that when LGG-2 is absent, LGG-1 can take its place. Of note, LGG-1 has redundant functions with LGG-2 for other processes in the N2 background. However, this model is difficult to test in CB4856 animals, because *lgg-1* is an essential gene in N2 (and thus likely also in CB4856), and RNAi knockdown is relatively ineffective in CB4856 animals.

While the host factors that cause clearance of *N. ironsii* over time are yet undefined, *lgg-2* was found to be required for the 50% decrease in *N. ironsii* initial colonization inside CB4856 intestinal cells compared to N2 intestinal cells (Balla et al. 2019). Deletion of *lgg-2* had no effect in N2 animals, but in CB4856 animals, it led to an increase in intracellular colonization by *N. ironsii* to the same levels as N2 animals. This result indicates a specific role for *lgg-2* in the increased resistance to intracellular colonization by *N. ironsii* in CB4856 animals. Thus, the role of different defense components can vary depending on the host strain background. Given the numerous roles for autophagy components in host defense outside of xenophagic clearance of intracellular pathogens, it will be interesting to define how *lgg-2* controls intracellular colonization of microsporidia infection.

5.6 *C. elegans* Host Transcriptional Response to Microsporidia Infection: The Intracellular Pathogen Response

To determine the *C. elegans* transcriptional response to microsporidia infection, RNA-seq transcriptomic profiling was performed on *N. parisii*-infected N2 animals isolated at five different points of infection representing *N. parisii* invasion, growth, and sporulation (Bakowski et al. 2014a). At each infection time point, there was a significant enrichment of differentially expressed genes associated with the intestine, as compared to uninfected controls. Through comparisons with other gene sets, the

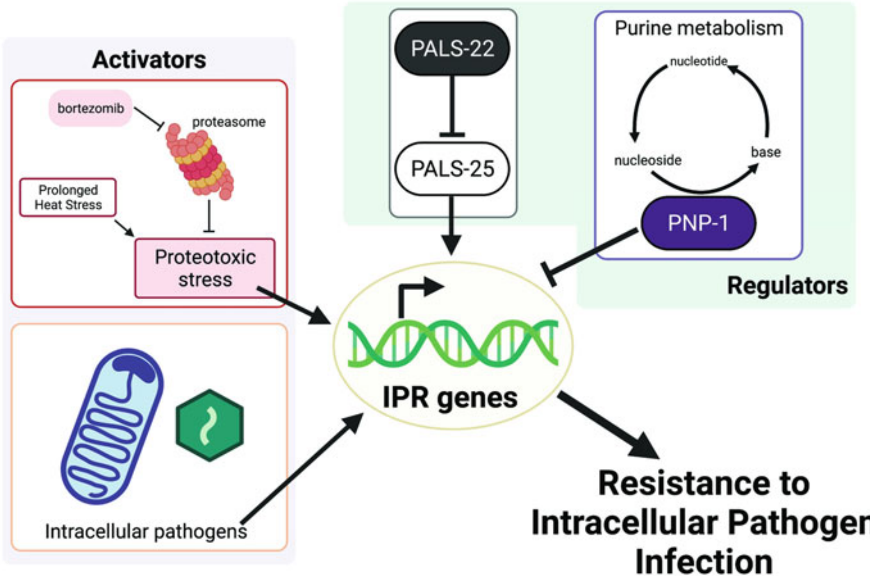

Fig. 5.2 Model of the Intracellular Pathogen Response. Intestinal intracellular pathogens, like *N. parisii* and the *Orsay virus*, upregulate mRNA expression of a common set of *C. elegans* genes called the Intracellular Pathogen Response, or IPR genes. IPR genes can also be induced by proteotoxic stress, as well as by perturbation to purine metabolism. Independently of these triggers, they are also regulated by a pair of antagonistic paralogs called PALS-22 and PALS-25. Upregulation of IPR genes leads to increased resistance to pathogen infection

genes regulated by *N. parisii* infection showed little similarity to genes regulated by bacterial extracellular pathogens or known immunity signaling pathways. However, there was a significant similarity between genes upregulated by *N. parisii* and genes upregulated by *Orsay virus* infection. *Orsay virus* is a three-gene, positive-sense, single-stranded RNA virus isolated from wild *C. elegans* in France (Felix et al. 2011). Despite their vast molecular differences, the *Orsay virus* and *N. parisii* are both obligate intracellular pathogens of the *C. elegans* intestine, suggesting they trigger a previously undefined immune response. The common gene set induced by both pathogens was termed the Intracellular Pathogen Response, or IPR (Fig. 5.2) (Bakowski et al. 2014a; Reddy et al. 2017). Analysis of a subset of IPR genes by qRT-PCR indicated that *N. ausubeli* is a weaker IPR activator than *N. parisii* in *C. elegans*, despite having a higher negative impact on host fitness than *N. parisii* (Balla et al. 2016; Zhang et al. 2016). One possible explanation is that *N. ausubeli* actively inhibits the IPR to enable a more virulent infection compared to *N. parisii*, or perhaps *N. ausubeli* is not as easily recognized by the host to induce the IPR.

What is the function of IPR genes? IPR genes are enriched for those that encode components of multi-subunit cullin-ring ubiquitin ligases (Bakowski et al. 2014a). Ubiquitin localizes to about 4–7% of *N. parisii* sporoplasms in the N2 host, and this localization was shown to be reduced in half by RNAi knockdown of the cullin *cul-*

6, a core component of cullin-ring ubiquitin ligases. Almost no ubiquitin localizes to parasite cells at later stages of *N. parisii* development, such as multinucleate meronts and spores. However, large clusters of conjugated ubiquitin appear throughout *C. elegans* intestinal cells, while *N. parisii* meronts are replicating and differentiating into spores, including sites distant from the parasite. Such ubiquitin clusters are a hallmark of perturbations in protein homeostasis (proteostasis), indicating that intracellular growth of microsporidia impairs proteostasis. Interestingly, IPR genes can also be induced by perturbations to proteostasis, such as genetic or pharmacological inhibition of the proteasome. RNA-seq analysis demonstrated that proteasome blockade induces almost all IPR genes (Reddy et al. 2019). Importantly, IPR genes are distinct from genes regulated by other stress response pathways, such as the HSF-1/heat shock response and the SKN-1/proteotoxic stress response, indicating the IPR constitutes a novel response pathway (Reddy et al. 2017, 2019).

In addition to ubiquitin ligase components, IPR genes include the *pals* genes, which are defined by a loosely conserved *pals* protein signature (Leyva-Diaz et al. 2017). The biochemical functions of *pals* genes are unknown, but *pals* stands for protein containing ALS2CR12 signature, which is found in the single *pals* gene in humans called ALS2CR12, of unknown function. There is also a single *pals* gene in mouse and none found in *Drosophila melanogaster*. The *pals* gene family expanded in the *C. elegans* genome to over 39 members, and these genes are found in clusters in the genome (Leyva-Diaz et al. 2017). 26 *pals* genes are IPR genes in that they are upregulated by both *N. parisii* and viral infection, and phylogenetic analysis indicates sequence similarity among upregulated genes (Fig. 5.3). One of these genes is *pals-5*, which is highly induced in the intestine upon infection and is used as a robust readout for the IPR through the use of a *pals-5p::GFP* reporter (Bakowski et al. 2014a).

Several forward genetic screens monitoring *pals-5p::GFP* expression have identified *C. elegans* regulators of the IPR and have provided insight into the physiological function of this pathogen response. The first regulator of IPR gene expression identified in *C. elegans* is called *pals-22*, which is a negative regulator found through a genetic screen for mutants with constitutively high *pals-5p::GFP* expression (Reddy et al. 2017). Expression of *pals-22* is not regulated by infection; rather it acts as a negative regulator of induced *pals* and all other IPR genes. *pals-22* mutants have several phenotypes, including (1) increased resistance to natural pathogens of the intestine such as *N. parisii* and the *Orsay virus*; (2) increased resistance to the natural oomycete pathogen *Myzocytiopsis humicola*, a eukaryotic pathogen that infects the epidermis; (3) increased transgene silencing; (4) increased susceptibility to *Pseudomonas aeruginosa* bacterial infection; (5) shortened life span; and (6) increased tolerance of proteotoxic stress (Fasseas et al. 2021; Leyva-Diaz et al. 2017; Reddy et al. 2017; Reddy et al. 2019). It is this last phenotype that has been characterized in the most detail, using the phenotype of thermotolerance as a readout. Here, a combination of genetics and biochemistry demonstrated that the increased thermotolerance in *pals-22* mutants is dependent on *cul-6*, acting together with other components in a multi-subunit ubiquitin ligase complex (Panek et al. 2020; Reddy et al. 2017). The target(s) of this ubiquitin ligase remain to be defined, but their

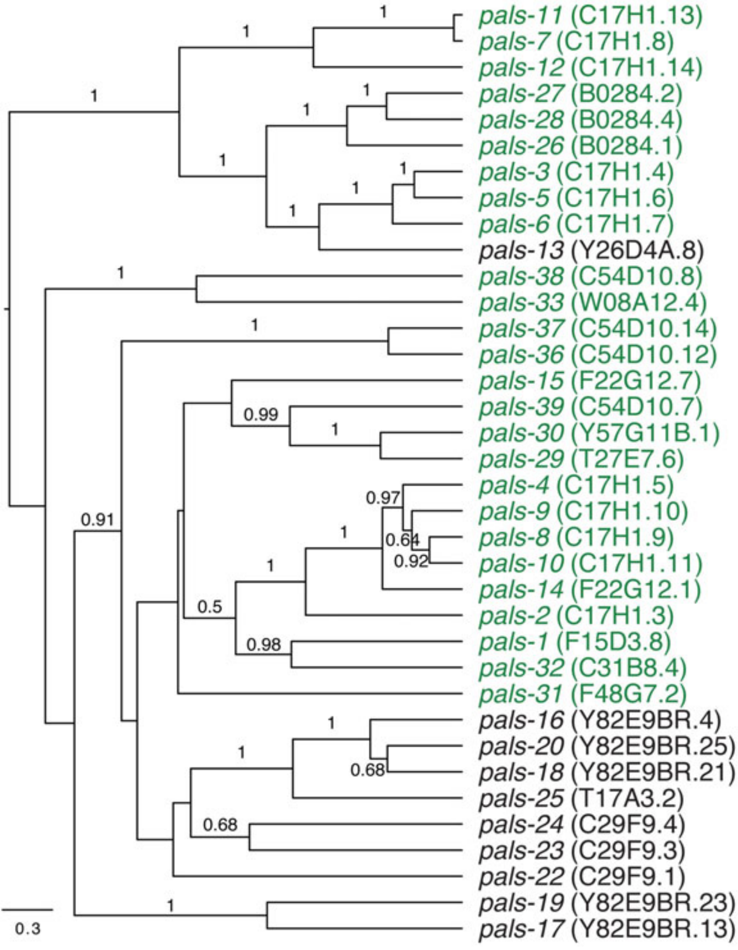

Fig. 5.3 Summary of *pals* genes. Phylogenetic tree of sequence relationships of *pals* genes. Green text indicates genes that are upregulated by both *N. parisii* and *Orsay virus* infection. Adapted with permission from Reddy et al. 2017

identification may provide novel insights into maintenance of proteostasis, especially during intracellular infection.

To better understand how *pals-22* regulates the IPR and defense against natural pathogens, suppressor screens were performed in a *pals-22* mutant background to find mutants with loss of constitutive *pals-5p::GFP* expression. These screens identified eight alleles in the gene *pals-25*, which is immediately downstream of *pals-22* in the genome, and is transcribed together with *pals-22* as part of an operon (Reddy et al. 2019). Like *pals-22*, *pals-25* expression is not induced by intracellular infection. All *pals-22* mutant phenotypes, including increased IPR gene expression, increased thermotolerance, and altered immunity, as well as reduced fitness in other

contexts, are reversed to wild-type levels by mutations in *pals-25*. Because *pals-22* and *pals-25* direct opposite phenotypes and are in the same gene family, they are termed "antagonistic paralogs," with *pals-22* acting as a negative regulator of *pals-25*, which is a downstream activator of the IPR. As PALS-22 and PALS-25 proteins are not enriched in the nucleus, they do not appear to be transcription factors, and it is not known how they regulate IPR gene transcription. By determining the overlap between genes upregulated by *N. parisii* infection, those upregulated in *pals-22* mutants, and also reversed back to wild-type levels in *pals-22 pals-25* double mutants, the IPR has been refined to include approximately 80 genes, including induced *pals* genes. Overall, these results show that *pals-25* is a global activator of *pals-5* and other IPR gene expression, but it is still unclear which induced PALS protein or other IPR proteins function to restrict microsporidia growth downstream of *pals-22/25*. Answering this question could provide better insight into how *C. elegans* controls intracellular pathogens in the intestine.

Genome analysis of 330 wild *C. elegans* strains suggests that there may be balancing selection acting at the *pals-22* and *pals-25* locus, with distinct alleles being maintained in *C. elegans* populations in the wild. Interestingly, the CB4856 Hawaiian *C. elegans* strain has predicted loss-of-function mutations in both *pals-22* and *pals-25* (Thompson et al. 2015). There are no obvious orthologs of *pals-22* and *pals-25* in the 27 other nematode genomes examined, including *C. inopinata*, the recently described sister species of *C. elegans* (Leyva-Diaz et al. 2017; Reddy et al. 2019). The *pals* gene family is smaller in other *Caenorhabditis* species compared to *C. elegans*, with eight *pals* genes in *C. briggsae*, eight in *C. brenneri*, and 18 in *C. remanei* (Leyva-Diaz et al. 2017). It is difficult to confidently assess orthology among *pals* genes in different *Caenorhabditis* species, because of the high level of sequence divergence within this gene family. For example, *C. elegans* PALS-22 and PALS-25 proteins only have 19.4% identity on the amino acid level. However, the induction of *pals* genes by intracellular infection appears to be conserved between *C. elegans* and *C. briggsae* (Chen et al. 2017). While *C. briggsae* cannot be infected by the *Orsay virus*, it can be infected by the related Santeuil virus, found naturally infecting *C. briggsae* (Felix et al. 2011). Comparison of the genes expressed upon *Orsay virus* infection in *C. elegans* and those expressed upon Santeuil virus infection in *C. briggsae* identified 58 homologous genes differentially regulated by both viruses. These genes included all 8 *C. briggsae pals* genes, with one of them only modestly induced. Taken together, these results suggest that the IPR may be an evolutionarily conserved immune response to intracellular pathogens in *Caenorhabditis* nematodes.

While *pals-22 and pals-25* control IPR gene expression and rewire *C. elegans* physiology, they are dispensable, as their deletion from the genome results in animals with wild-type phenotypes. For example, *pals-22 pals-25* double mutants still upregulate IPR gene expression upon exposure to all known triggers of the IPR, including *N. parisii* infection, virus infection, proteotoxic stress, and chronic heat stress, among other triggers (Reddy et al. 2019). Thus, *pals-22* and *pals-25* appear to have been inserted in the *C. elegans* genome as an ON/OFF switch for the IPR, but act in parallel to other factors that mediate induction (Fig. 5.2). Induction of the IPR

is best understood for viral infection, which is sensed by DRH-1, a *C. elegans* homolog of mammalian RIG-I-like receptors that sense double-stranded viral RNA and other viral replication products. DRH-1 is required for induction of IPR genes upon viral infection, but not upon other triggers (Sowa et al. 2020). How microsporidia infection and other triggers induce the IPR is unknown.

While there appear to be many independent upstream triggers of the IPR, there does appear to be a common downstream regulator through which these triggers induce gene expression. The predicted bZIP transcription factor ZIP-1 was identified in two independent RNAi-based screens as being required for inducing *pals-5p::GFP* expression, and it appears to be important for induction of this reporter upon all known IPR triggers (Lažetić et al. 2022). Interestingly, RNA-seq analysis demonstrated that ZIP-1 is required for induction of less than half of the IPR genes. This finding indicates there are other transcription factors required for regulating these *zip-1*-independent IPR genes. Despite only regulating some IPR genes, *zip-1* is required to promote resistance to *Orsay virus* and *N. parisii* infection and is particularly important in a mutant background where IPR genes are constitutively expressed. These results indicate that ZIP-1 controls IPR genes that protect against intracellular infection.

Recently, forward genetic screens identified *pnp-1* as another negative regulator of the IPR, functioning in parallel to *pals-22* and upstream of *zip-1* (Tecle et al. 2021). *pnp-1* encodes a purine nucleoside phosphorylase (PNP), which is an enzyme that acts in the purine salvage pathway to recycle guanine and adenine nucleotides. RNA-seq profiling of *pnp-1* mutants demonstrated that they have constitutive upregulation of almost all IPR genes, including the induced *pals* genes. In keeping with this constitutive upregulation of IPR genes, *pnp-1* mutants are resistant to *Orsay virus* and *N. parisii* infection, and *zip-1* is important for this resistance. Restoring expression of *pnp-1* in *C. elegans* intestinal epithelial cells rescues *pnp-1* mutant phenotypes, including IPR gene expression and resistance against *N. parisii*, indicating that purine metabolism in the intestine regulates the IPR. It would be interesting to investigate if loss of *pnp-1* in *C. briggsae* results in resistance to *Nematocida* and/or Santeuil virus infection in this host.

The phenotypes of *pnp-1* highlight the relevance of purine metabolism to obligate intracellular infections like those caused by microsporidia. Purine metabolism pathways are conserved across all domains of life and consist of the energy-costly de novo pathway and the far less energy-costly salvage pathway. PNPs function only in the purine salvage pathway and are evolutionarily conserved from bacteria to man. In contrast to the lack of *pals-22* sequence conservation, *pnp-1* sequences are highly conserved, for example, 95% homology between *C. elegans* and *C. briggsae*. PNPs convert the purine nucleoside inosine into the purine base hypoxanthine, which is then submitted to the action of various enzymes to generate purine nucleotides. Metabolomics analysis confirmed that *pnp-1* mutants have increased levels of inosine and decreased levels of hypoxanthine, as expected if *pnp-1* functions as a PNP in *C. elegans*. Considering that microsporidia are completely dependent on the

host for nucleotides, it seems plausible that perturbations in purine metabolism in the host could impair microsporidia growth. Moreover, buildup of purine metabolite precursors (such as inosine) or the absence of others (such as hypoxanthine) due to infection could be sensed by the host which then deploys defense mechanisms against infection, such as the IPR. Interestingly, human mutations in purine salvage enzymes like PNP and adenosine deaminase result in immunodeficiency due to T cell dysfunction, but little is known about the role of PNP and other purine salvage enzymes in epithelial cells, which are the common sites of virus and microsporidia infection, and where *pnp-1* functions in *C. elegans*. Perhaps purine metabolism enzyme mutations in humans provide an evolutionary advantage in the context of viral or microsporidia infection in epithelial cells, which are often the first cells to be infected.

The work with *pals-22* and *pnp-1* demonstrates that IPR induction promotes resistance within a generation. Recent work indicates that *N. parisii* infection and IPR induction can provide an immunity benefit to the progeny of infected animals (Willis et al. 2021). Thus, immunity against microsporidia can be maternally inherited in *C. elegans*. *N. parisii* infection itself is not required for inheritance of resistance, only IPR activation in the parental generation. Mothers with IPR activation, such as *pals-22* mutants, have progeny without IPR gene activation, but nonetheless these progeny are resistant to *N. parisii* infection. Several other IPR activators confer an immune benefit against *N. parisii* to the next generation, including viral infection, and mutations in another negative regulator of IPR gene expression, *lin-35*, which is a homolog of the cell cycle regulator retinoblastoma. This inherited immunity does come at a fitness cost however; progeny from infected parents are smaller, less fecund and display increased susceptibility to other types of stresses. Inherited resistance to *N. parisii* infection is observed in *C. briggsae*, indicating that this is an evolutionary conserved trait (Burton et al. 2021). It would be interesting to determine whether inherited immunity can be triggered by infection with other *Nematocida* species or by loss of *pnp-1*, and if *zip-1* is required for this process. It is not well understood how IPR activation leads to increased resistance either within a generation or across a generation, but as more is uncovered, it will be interesting to compare whether similar or different mechanisms are at play.

5.7 Perspectives and Future Directions

The power of the *C. elegans* host system has facilitated progress on several fronts of microsporidia research. For example, the ease of imaging the transparent body plan of *C. elegans* has enabled visualization of *Nematocida* species fusing host cells together during replication to create syncytial structures that facilitate spread of this pathogen. These studies were notable because they described a polar tube-independent invasion mechanism of microsporidia. The ease of isolating wild-caught *C. elegans* and related nematodes has provided a growing collection of

natural microsporidian pathogens of nematodes, which offer new models for studying important questions about microsporidia pathogenesis, such as what determines their host and tissue tropism. Localized proteomics in *C. elegans* intestinal cells highlighted how species-specific gene families from microsporidia encode proteins that directly interface with host tissue during the host/pathogen battle, which may drive the expansion and diversification of these families. Similarly, forward genetics has highlighted the expansion and diversification of species-specific gene families involved in host defense against these pathogens (Lazetic and Troemel 2020). Genetic and transcriptomic studies have identified the IPR, a shared immune/stress response pathway induced by natural microsporidian and viral infections. The IPR is regulated by metabolism of purine nucleotides, which are crucial factors stolen by both microsporidia and viruses to enable their replication. The short life cycle of *C. elegans* facilitated the discovery that activation of the IPR can confer an immune benefit to the next generation. However, many questions remain. For example, it is unknown how *C. elegans* senses microsporidia to trigger the IPR, how the IPR confers resistance to infection, and whether there is an analogous system in mammals, which lack obvious sequence orthologs of IPR regulators *pals-22/25* and many other IPR genes. In addition, the mechanisms by which *Nematocida* species fuse *C. elegans* organs into syncytia are unknown. Further elucidation of these findings and investigation of their potential roles in mammalian hosts will be exciting areas for future inquiry.

Despite such progress, research of microsporidia infections in *C. elegans* is hampered by the same problem that all microsporidia researchers face: manipulation of the microsporidia genome (Reinke and Troemel 2015). The lack of genetics or DNA transformation techniques makes it challenging to determine which *Nematocida* factors trigger the various restructuring events described above, cause disease, or activate the IPR. Heterologous expression of pathogen proteins provides one avenue to explore these questions. Here, the host-exposed *Nematocida* proteins identified through localized proteomics, including proteins from the species-specific expanded gene families, provide attractive candidates to explore (Reinke et al. 2017). Without genetic manipulation of microsporidia, we are like detectives who meticulously investigate a crime scene but never manage to specifically identify the perpetrators. Although genetics and transformation techniques are currently not available in microsporidia, the development of highly sensitive reporter systems such as nanoluciferase will provide useful tools for developing these techniques in the future (Sfarcic et al. 2019). Together with CRISPR-Cas9-mediated gene editing, novel genetic tools will help define the microsporidia factors that enable its elaborate intracellular life and the host response to these ubiquitous, fascinating parasites.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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Ethical Approval The chapter is a review of previously published accounts; as such, no animal or human studies were performed.

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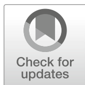
Chapter 6
Advances in the Genetic Manipulation of *Nosema bombycis*



Tian Li, Junhong Wei, and Guoqing Pan

Abstract The microsporidium *Nosema bombycis* can infect and transmit both vertically and horizontally in multiple lepidopteran insects including silkworms and crop pests. While there have been several studies on the *N. bombycis* spore, there have been only limited studies on the *N. bombycis* sporoplasm. This chapter reviews what is known about this life cycle stage as well as published studies on purification of the *N. bombycis* sporoplasm and its survival in an in vitro cell culture system. Genetic transformation techniques have revolutionized the study of many pathogenic organisms. While progress has been made on the development of such systems for microsporidia, this critical problem has not been solved for these pathogens. This chapter provides a summary of the latest research progress on genetic manipulation of *N. bombycis*.

Keywords *Nosema bombycis* · Sporoplasm · Genetic manipulation · RNAi · Transfection · In vitro cell culture systems · Silkworm pathogens

Nosema bombycis was described in 1857 by Naegeli, making it the first identified microsporidia species. *N. bombycis* infects silkworms causing pébrine, a disease that produces significant economic damage to the sericulture industry. *N. bombycis* can infect and transovarially transmit in multiple lepidopteran insects including the crop pests *Spodoptera litura* and *Helicoverpa armigera* (Fig. 6.1), suggesting that *N. bombycis* probably plays important roles in the natural ecosystem and potentially could be useful for biocontrol of crop pests (Pei et al. 2021).

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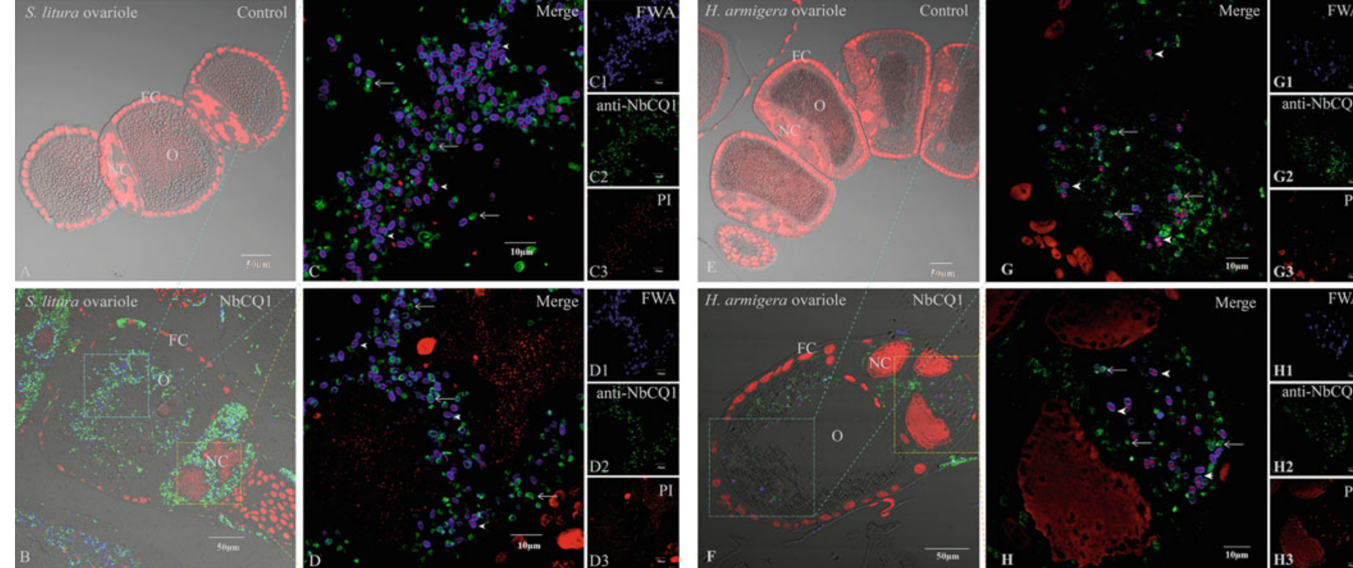

Fig. 6.1 Paraffin sections of *S. litura* and *H. armigera* ovariole infected by NbCQ1 (Pei et al. 2021). (**a**, **e**) Uninfected ovariole; (**b**, **f**) infected ovariole; (**c**, **g**) an enlarged view of the infection of oocyte; (**d**, **h**) an enlarged view of the infection of follicle cell; (C1, D1, G1, H1) NbCQ1 spores stained with FWA; (C2, D2, G2, H2) proliferative NbCQ1 labeled with antibody; (C3, D3, G3, H3) nucleus stained by PI. *FC* Follicle cell, *O* oocyte, *NC* nurse cell; Arrowhead shows mature spore; arrow shows parasite proliferation

6.1 The *Nosema bombycis* Sporoplasm

A primary step in microsporidian infection of host cells is polar tube extrusion with release of the sporoplasm from the tip of the polar tube during invasion. Inside the host cells, the sporoplasm develops into proliferating meronts. The sporoplasm is spherical or oval in shape, 1.5–2.0 μm in size, has a typical plasma membrane, and contains one to two nuclei (Avery and Anthony 1983; Cali et al. 2002; Ishihara and Hayashi 1968). The subcellular structures and functions of the various components of sporoplasm are areas of active investigation. For example, where the sporoplasm cytoplasmic membrane originates is not known, with some authors suggesting this plasma membrane comes from the polaroplast membranes in the spore. Nor is it understood how the sporoplasm plasma membrane interacts with the host cell during invasion and replication. Characterizing this cytoplasmic membrane is important for understanding the interactions of the sporoplasm with its host cell during invasion. For instance, sporoplasms from *Amerson michaelis* or *Spraguea lophii* can survive in vitro if provided with ATP (Weidner and Findley 1999), suggesting that there are transporters on the membrane that import outside nutrients. In general, however, due to their dependence on host cells for nutrients, sporoplasms cannot survive for a long time outside of host cells (Takvorian et al. 2013).

It is possible to germinate microsporidian spores in vitro allowing their sporoplasms to be released from the extruded polar tubes. Li and colleagues purified sporoplasms from germinated spores of *N. bombycis* and characterized their morphology, subcellular structure, and gene expression (He et al. 2020a). The *N. bombycis* sporoplasms were shown to be spherical and non-refractive, 3.64 ± 0.41 μm in diameter, and had two typical nuclei (Fig. 6.2). The nucleus size observed in extruded sporoplasms (0.59 ± 0.05 μm) was smaller than the nuclei seen within ungerminated spores (0.78 ± 0.07 μm), suggesting that the nucleus could be compressed during passage through the polar tube. The sporoplasm was surrounded by a single membrane and filled with homogeneous granules. Propidium iodide (PI) stained the sporoplasm nucleus, indicating that the sporoplasm membrane is permeable. Wheat germ agglutinin (WGA), which binds to sialic acid and N-acetylglucosaminyl residues, could not bind to the sporoplasm membrane, indicating that this membrane lacked these posttranslational modifications.

Majority of the time, the sporoplasm invades into the host cell during the penetration of the host cell membrane that occurs at the tip of the polar tube (i.e., that invasion occurs in the invasion synapse). However, it has been observed that some sporoplasms released outside the host cell can also initiate infection via host phagocytosis (Fig. 6.3). Sporoplasms that had been phagocytosed grew and developed into meronts inside the host cell (Fig. 6.4). This alternative route provides the pathogen with a second way to infect it host cells.

The sporoplasm is a developmental life stage between the mature spore and meronts in the microsporidian lifecycle. Changes in gene expression have been seen when microsporidia spores germinate, releasing the sporoplasm. He et al. performed an RNA-seq that compared the gene expression seen in mature spores

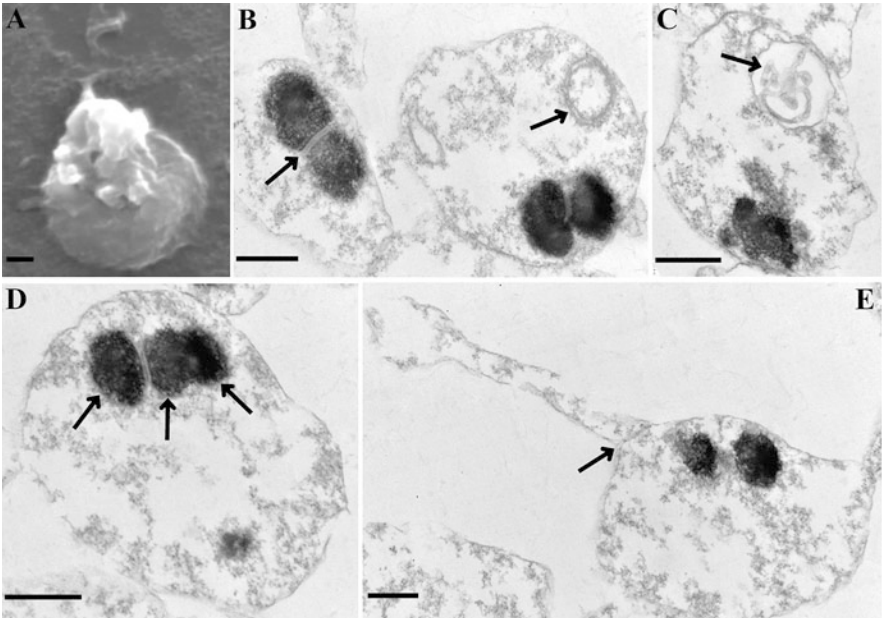

Fig. 6.2 Ultrastructure of sporoplasm (He et al. 2020a). (**a**) Scanning electron micrograph of a sporoplasm. (**b**–**e**) Transmission electron micrographs of sporoplasms. (**b**) Connection between two nuclei (indicated by black arrows). A vesicular structure consisting of a concentric ring and/or containing coiled tubules was observed in the cytoplasm (indicated by black arrows in panels **b** and **c**). A sporoplasm containing three nuclei was observed (indicated by black arrows in panel **d**). (**e**) After germination, the sporoplasm remained attached to a tubular structure surrounded by a membrane. Scale bar = 500 nm

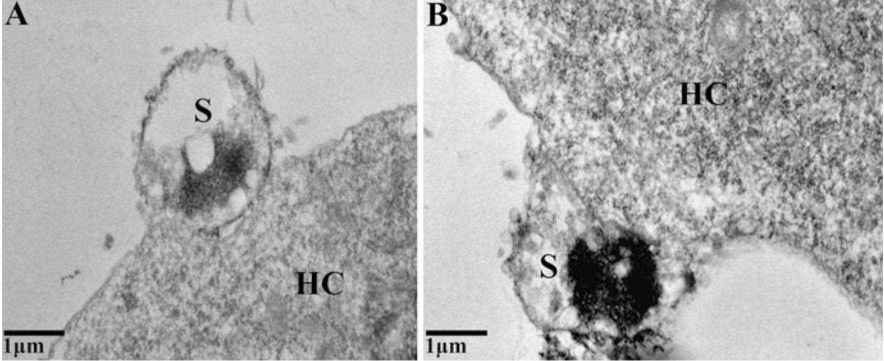

Fig. 6.3 Phagocytosis of *N. bombycis* sporoplasm by a host cell (He et al. 2020a). Transmission electron micrograph demonstrating a sporoplasm bound to an SF9 cell membrane by pseudopod-like protrusions. *S* Sporoplasm, *HC* host cell

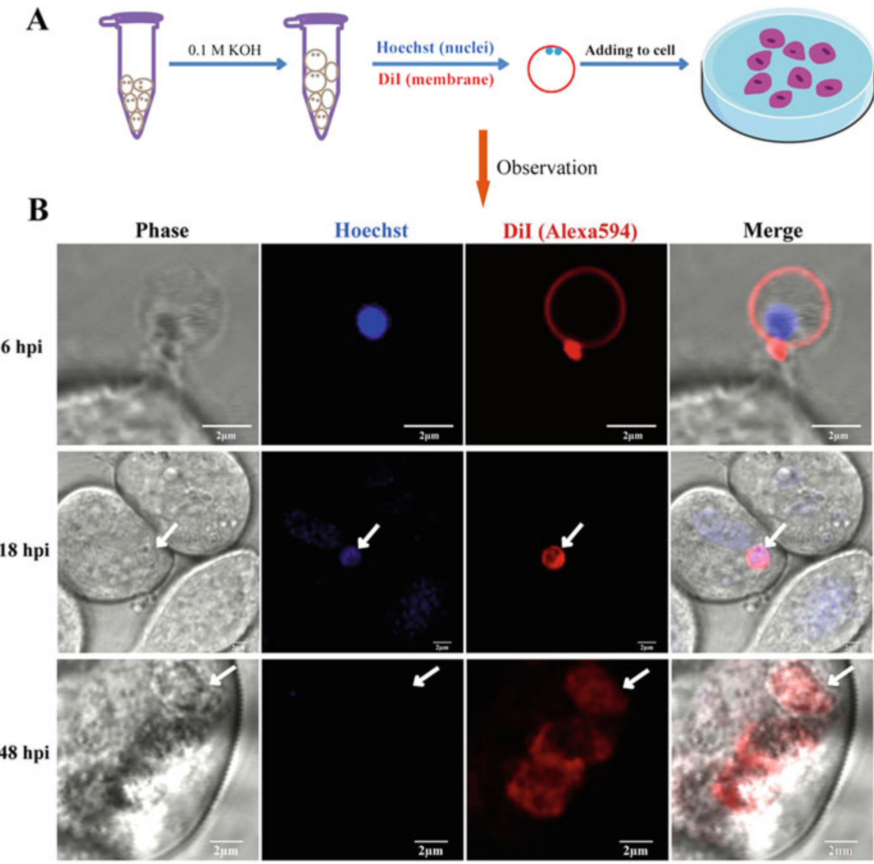

Fig. 6.4 Continuous fluorescent observation of sporoplasm infection of host cells (He et al. 2020a). (**a**) Diagram of the experimental process. (**b**) The sporoplasm was stained with Hoechst 33258 (blue) and DiI (red) and placed in a Petri dish containing the SF9 cell line at 6, 18, and 48 h post infection (hpi). The white arrow indicates a sporoplasm emitting red fluorescence

and sporoplasms from *N. bombycis* (He et al. 2020a). They identified 541 differentially expressed genes (DEGs) in the sporoplasm, including 302 upregulated genes and 239 downregulated genes. Genes involved in trehalose synthesis metabolism, glycolysis, and pentose phosphate pathway were downregulated, while some transporter genes were upregulated, including energy substance-related transporters such as ADP/ATP carrier protein, mechanical sensitive ion channel proteins, and an amino acid transporter. These findings suggest that the sporoplasm may inhibit its own carbon metabolic activity and instead obtain nutrients form the host via the transporters on membrane.

6.2 In Vitro Cultivation of the Sporoplasm of *Nosema bombycis*

Microsporidia are obligate intracellular pathogens. They can only multiply inside host cells where they proliferate by exploiting host nutrients. Studies on microsporidia, such as biochemical and physiological analysis and genetic engineering, have been severely limited by the absence of a cell free in vitro cultivation system (Reinke and Troemel 2015). Microsporidian spores are not capable of proliferation and are dormant, but in contrast, the sporoplasm is quite active and is involved in proliferation (Takvorian et al. 2020). *N. bombycis* sporoplasms can be isolated and purified from germinated spores, providing materials to set up in vitro cultivation systems. Early studies revealed that sporoplasms can be kept alive in vitro for 24 h in medium containing ATP (Weidner and Findley 1999; Weidner and Trager 1973). These studies suggested that sporoplasms may have the potential to be cultivated for a longer period of time in vitro and perhaps even to develop into meronts. He et al. described the detailed procedures for preparing sporoplasms from *N. bombycis* (He et al. 2020b). In general, *N. bombycis* spores were treated with 0.1 M KOH at room temperature for 40 min and then incubated in cell medium for 5 min to release the sporoplasms, which were then purified by Percoll gradient centrifugation. These researchers then designed a cell-free medium for sporoplasm culture (Medium TC100; 5% fetal bovine serum; 1 μM concentration of NTP and dNTP; 10 μM concentration of ATP; BmE cell cytosol; pH 6.8) and tried to culture the purified sporoplasm in vitro. They were able to keep sporoplasms alive for 5 days in this cell-free system. In contrast sporoplasms died on day 3 when maintained in isotonic PBS. Furthermore, sporoplasm DNA increased in this cell-free culture system suggesting that replication was occurring. However, transformation of sporoplasms to meronts was not observed, indicating that this cell-free culture medium lacks key factors required for this developmental transition.

6.3 Genetic Manipulation of Microsporidia

High efficiency and reliable genetic modification tools are required for microsporidia research. Genetic engineering, which has been developed for many obligate intracellular organisms such as *Plasmodium*, *Toxoplasma*, and *Cryptosporidium*, would be a key technology for advancing research on microsporidia (Goonewardene et al. 1993; Soldati and Boothroyd 1993; Vinayak et al. 2015). To fully understand the function of a gene (and its coded protein) in vivo, it is necessary to be able to manipulate the target gene in the genome. By disruption, complementation, and overexpression of a specific gene, the role of the gene played in cells can be determined by analyzing the phenotypic consequences of such manipulations. In addition, using tags such as defined epitopes and/or fluorescent proteins would significantly facilitate studies on protein localization and function in vivo.

Microsporidia expressing fluorescent protein(s) would also be an important model for studies of immune function and research on life cycles of these parasites including studies on latent infection. Reinke et al. (Reinke and Troemel 2015) have discussed several key issues in microsporidia genetic modification and provided many important ideas, and recently there has been progress emerged in this area. Herein, we shortly review successful methods of other obligate eukaryotic intracellular parasites and consider strategies for successful modification of microsporidia.

6.3.1 Genetic Modification Techniques for Intracellular Parasites

The first breakthrough involving genetic modification of an obligate intracellular eukaryotic pathogen was in the Apicomplexan *Toxoplasma gondii* in the 1990s. DNA containing the chloramphenicol acetyltransferase (CAT) gene and regulatory regions was electroporated into *T. gondii*, and successful transformation was documented by measurement of CAT enzyme activity (Soldati and Boothroyd 1993). Subsequently, both dihydrofolate reductase (DHFR) and CAT were used as drug selection markers (DHFR to confer pyrimethamine resistance and CAT to confer chloramphenicol resistance) to construct stable transformants by random integration and homologous recombination (Donald and Roos 1993; Kim et al. 1993). Restriction enzyme-mediated integration (REMI) was shown to significantly increase transformation efficiency in *T. gondii* (Black et al. 1995). More recently, genome-editing tools including TALEN (Transcription Activator-Like Effector Nucleases) and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) have been optimized for genetic manipulation in *T. gondii*, which has greatly enhanced the efficiency of targeted gene modifications (Chen et al. 2019; Sidik et al. 2014; Shen et al. 2014). The application of CRISPR/Cas9 not only significantly accelerated transformant construction speed but also enabled genome-wide screens of gene function to identify virulence factors and essential genes (Sidik et al. 2016; Young et al. 2019).

The first reported transient expression of an exogenous reporter in *Plasmodium* was achieved by electroporating infected red blood cells (Wu et al. 1995). To construct stable transformants, plasmids containing DHFR genes (providing pyrimethamine resistance) were integrated into *Plasmodium falciparum* chromosomes by homologous recombination (Wu et al. 1996). Subsequently a *PiggyBac* transposable system has been developed for high-efficiency DNA integration and random mutagenesis in *Plasmodium* (Fonager et al. 2011; Balu et al. 2005). A zinc-finger nuclease technique has also been used in *P. falciparum* and *Plasmodium vivax* for genome editing (Straimer et al. 2012; Moraes Barros et al. 2015). CRISPR methods have also been demonstrated to work effectively in *Plasmodium*, which greatly facilitated specific gene knock-in, gene knockout, and single-nucleotide

substitutions in *Plasmodium* strains (Wagner et al. 2014; Ghorbal et al. 2014; Qian et al. 2018; Mohring et al. 2019).

Development of genetic manipulation tools for *Cryptosporidium parvum* was more difficult, but eventually approaches were developed for this obligate intracellular parasite (Vinayak et al. 2015). Plasmid DNA introduced into purified *C. parvum* sporozoites by electroporation was designed to express nanoluciferase as a reporter gene and aminoglycoside phosphotransferase as an antibiotic resistant marker for paromomycin selection. Transgenic DNA was then specifically integrated into target loci via CRISPR/Cas9 technique and homologous recombination. The breakthroughs that resulted in the development of this genetic modification system for *C. parvum* relied on various factors, including screening for a strong promoter, effective transformant selection by drug resistance, codon optimization of reporter genes, evaluation of best electroporation devices and parameters, and the application of CRISPR/Cas9 systems. The development of these genetic modification tools has significantly facilitated research on *Cryptosporidium*, playing a critical role in new studies on gene function and drug target discovery (Choudhary et al. 2020).

6.3.2 Key Issues in Developing Genetic Modification Techniques for Microsporidia

Effective delivery of DNA into the target cell is the basis for all genetic manipulation methods. The commonly used delivery methods include transfection, electroporation, biolistics, chemical transformation, microinjection, *Agrobacterium* transformation, and viral transduction. *Agrobacterium* transformation requires direct attachment of the donor bacteria to the free-living receipt cell and thus is unlikely to be effective at delivering DNA into intracellular microsporidia within host cells. However, this technique has been used successfully on various fungal spores, suggesting it may be able to deliver DNA into microsporidia spores. Microinjection has been used to successfully deliver foreign DNA into living cells, egg, oocyte, or embryos, but microsporidia are generally below the size limits for effective utilization of this technique. Viral transduction needs a virus vector which could bind and enter the target cell, and to date there are no reports of a specific viruses that infect microsporidia, let alone applicable viral vector. Biolistic methods have potential in delivering DNA into microsporidia, but the specific parameters need comprehensive optimization, because all attempts in our laboratory using this technique have failed. In contrast, liposome transfection has been shown to be effective in introducing DNA into microsporidia (Fig. 6.5) (Guo et al. 2016). In addition, electroporation can also be used to transfer DNA into microsporidia (Dr. LM Weiss, personal communication).

Microsporidia have a complex life cycle, consisting of multiple intracellular stages and an environmentally resistant extracellular spore form. Which life stage

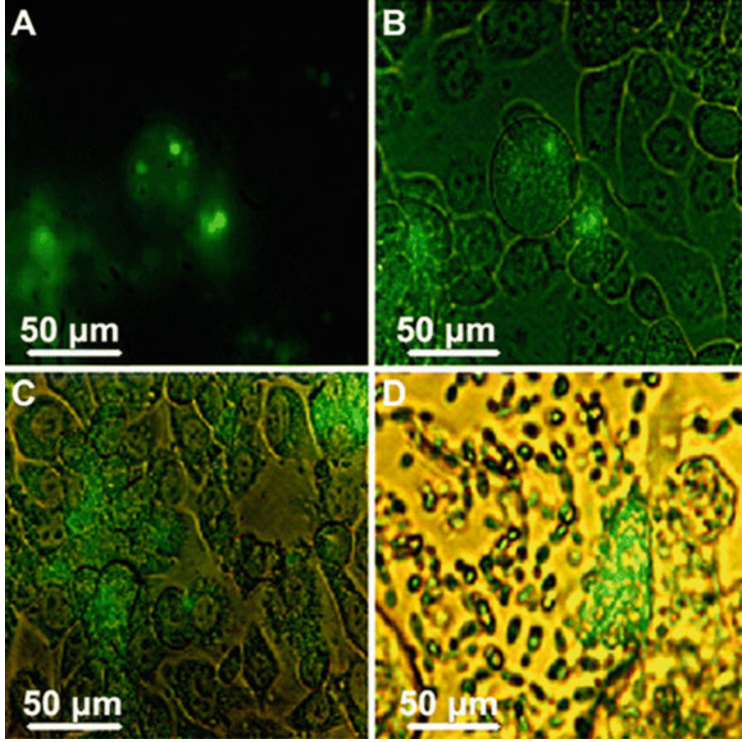

Fig. 6.5 Fluorescence observation of the *N. bombycis*-infected BmN cells transfected with pIZT/V5-His vector in vitro (Guo et al. 2016). (**a**, **b**) Cells with fluorescent particles (**a**) with fluorescent vision (**b**) and under both fluorescent and normal light. (**c**, **d**) The broken cells and released *N. bombycis* with green fluorescence under both fluorescent and normal light

is better for introducing exogenous DNA, the intracellular active stage or inactive extracellular stage? Though it is easy to purify mature spore of microsporidia, the thick spore wall of the mature microsporidia spore is difficult to penetrate by standard methods. As spore germination and polar tube extrusion require intrasporal osmotic pressure, the spore may be quite sensitive to pore-forming treatments like electroporation and biolistics, which may cause the spore to "missfire" or even fail to germinate. For genetic modification, it is probably better to choose cells in a replicative stage to deliver DNA. However, there are limited options for extracting intracellular meronts from cells, so it is difficult to directly follow the DNA delivery strategies used successfully in *T. gondii*, *C. parvum*, and *Plasmodium* spp. Meronts of some microsporidia develop in direct contact with the cytoplasm like a cellular organelle, suggesting strategies like biolistics, which have been successfully applied for transforming mitochondrial and chloroplast, may be useful to transform microsporidian meronts.

To make this problem more challenging, while some species like *Encephalitozoon* and *Nosema* can be grown in tissue culture, most microsporidian

species actually need to be grown in intact host animals. DNA delivery in these species may be more difficult, and strategies like biolistic or electroporation of infected cells may be impractical. However, injecting a plasmid DNA/lipofectin mixture into the body cavity of *N. bombycis*-infected silkworms successfully delivered DNA into the parasite, and expression of the reporter gene could be seen (Guo et al. 2016).

Liposome transfection of *N. bombycis*-infected cells or isolated mature spores could be used to introduce DNA into host cells, but current transformation rate is relatively low and requires optimizations (unpublished data). Recent progress in the in vitro cultivation of *N. bombycis* sporoplasms has provided a new option for DNA delivery (He et al. 2020a). Several features make this an attractive approach, including the sporoplasm only having a plasma cell membrane (facilitating liposome or electroporation methods), the in vitro culture system being viable for at least 5 days, DNA replication and metabolism having been shown to occur in this in vitro system, and uptake of the free sporoplasm by host cells leading to replication intracellularly.

Different approaches to genetically modify microsporidia include integration of exogenous DNA into the genomic DNA or the maintaining of the exogenous DNA as an extrachromosomal element. Currently, constructing stable integrated transformations is the preferred approach, because little is known about episomal or extrachromosomal DNA in microsporidia and no plasmid or other kind of extrachromosomal vector is available for microsporidia. Genomic analysis suggests that microsporidia possess core MRX/MRN enzyme complexes for both canonical NHEJ (Non-Homologous End Joining) and homologous recombination, but lack some enzymes for canonical NHEJ and homologous recombination, suggesting that they may use alternative pathways for DNA repair. Based on the published experience in previously mentioned obligate eukaryotic intracellular parasites, homologous recombination appears to be a good choice for stable DNA integration and target gene disruption. NHEJ may also work in microsporidia, because previous work showed that non-transposon vector could be integrated into genomic DNA of *N. bombycis* (Guo et al. 2016). Based on experience in genetic tool development in other eukaryotic intracellular parasites, the application of CRISPR/Cas9 together with homologous recombination will likely be useful for genetic modification in microsporidia. *PiggyBac* transposition system is another choice for knock-in and random mutagenesis research in microsporidia.

DNA entry and integration into the genome is a rare event, and thus selection techniques are crucial to recover transformants. Drug resistance and auxotrophic selection markers are commonly used genetic tools to screen transformants; however, auxotrophic selection markers are currently not applicable to microsporidia as little is known about auxotrophy in microsporidia. Drugs that inhibit the growth of non-transformed organisms, but not those containing the corresponding resistant gene, can be used to select for transformants. The anti-microsporidia drug fumagillin specifically inhibits methionine aminopeptidase 2 (MetAP2) and has essentially no effect on methionine aminopeptidase 1 (MetAP1) (Sin et al. 1997). Thus, MetAP1 could potentially be used as a drug resistance marker for microsporidia transformant

selection. Fluorescent or luminescent proteins may also serve as alternative screening method, but the limitation is ensuring that the proteins are expressed at high enough levels to be observed and the amount effort needed to screen for transformants. Instead, a combination of both drug selection and fluorescent or luminescent proteins would probably be the optimal approach for transformant selection, as was done for the development of transfection in *C. parvum.* Though development of genetic modification methods in microsporidia may vary between different species, success in any species will greatly facilitate research in this important but poorly understood group of obligate intracellular parasites.

Successful approaches to genetically modify obligate eukaryotic intracellular parasites often share several common features. They use electroporation for DNA delivery; a drug screen is a key step for enrichment of transformants; and they have utilized new techniques such as CRISPR/Cas9 system that greatly enhanced transformation efficiency. The impressive work on the development of transformation methods in the various apicomplexans provides a powerful proof-of-principle approach for the development of transformation systems in other obligate intracellular pathogens. For microsporidia, electroporation, transfection, electroporation, and biolistics are inferred or have been proven to be applicable DNA delivery methods. Intracellular meronts or extracellular sporoplasms are probably useful life stages to target because of the relative ease of DNA entrance and integration, while extracellular spores have a tough spore wall hindering DNA delivery, and the extrusion apparatus might be quite sensitive to mechanical disruption during DNA delivery. The use of CRISPR/Cas9 and a drug-resistant gene with large homology regions flanking target gene is probably the best DNA integration strategy at present. The development of applicable reporters, drug selection markers, and promoter sequences is a vital task for vector construction for the development of microsporidian transfection methods.

6.3.3 RNA Interference in Microsporidia

RNA interference (RNAi) is a natural mechanism for posttranscriptional gene silencing triggered by dsRNA, mediating resistance to pathogenic nucleic acids or regulation of gene expression. It has been widely used as a knockdown technology to analyze gene function in many organisms. Dicer and argonaute are two core components of the RNAi machinery; however, many microsporidia like *Encephalitozoon* and *Enterocytozoon* lack them, suggesting that use of an RNAi strategy for genetic manipulation is not applicable for all species of microsporidia. Fortunately, this pathway is still present in many other microsporidia including *Nosema*, *Trachipleistophora*, and *Vittaforma* (Heinz et al. 2012*;* Pan et al. 2013). RNAi has been widely used to block gene expression for functional genomics research; thus, we can apply RNAi as an alternative tool of gene disruption for in vivo gene function research in species which have RNAi components (Fig. 6.6).

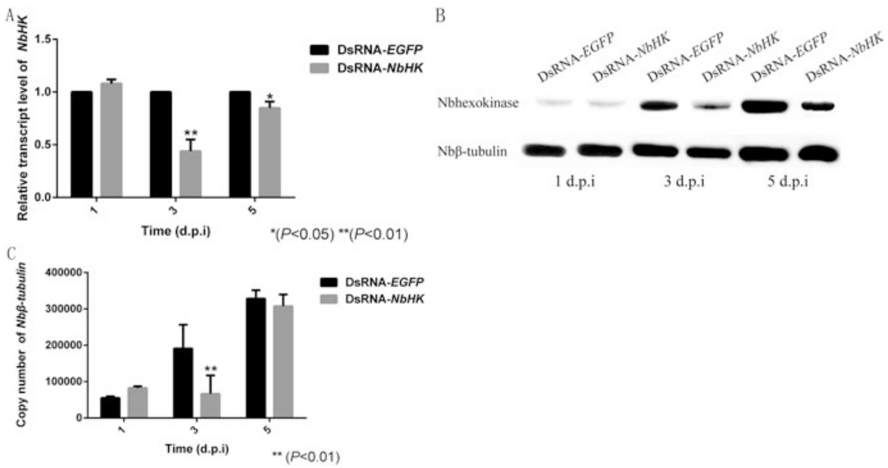

Fig. 6.6 Effect of NbHK downregulation on *N. bombycis* proliferation (Huang et al. 2018). (**a**) The transcription levels of NbHK. Complementary DNAs of the experimental (dsRNA-NbHK) and mock (dsRNA-EGFP) groups were analyzed by qPCR. (**b**) NbHK expression levels of NbHK. Total protein of experimental (dsRNA-NbHK) and mock (dsRNA-EGFP) groups were analyzed by Western blot. (**c**) The level of infection of Sf9-III. Genomic DNA was extracted from experimental (dsRNA-NbHK) and mock (dsRNA-EGFP) groups at 1, 3, and 5 d.p.i. Copy numbers of Nb-tubulin indicate the level of infection

There are publications on *Nosema* spp. infections in host species that reported introduction of exogenous dsRNA into the host organism could downregulate the expression of a targeted microsporidian specific gene. Nitzan Paldi et al. chemically synthesized dsRNA targeting *Nosema*-specific ADP/ATP transporters and fed them to experimentally infected honeybees. Ingestion of these dsRNA dramatically lowered the expression of the target genes, reduced the overall parasite load, and reduced the infection associated corresponding physiology changes in infected honey bees (Paldi et al. 2010). Pan et al. demonstrated that bacterium-expressed dsRNA targeting ADP/ATP protein encoding genes of *N. bombycis* could suppress gene expression during *N. bombycis* infection in the silkworm, leading to significantly reduced parasite counts in transfected silkworm (Pan et al. 2017). In addition, several researchers have applied RNAi techniques to gene function analysis of transferrin, NbTMP1, Hsp70, and ATP-binding cassette (ABC) transporters in microsporidia (He et al. 2019; 2020c; Zheng et al. 2021). Interfering with other targets such as polar tube protein 3 (PTP3), naked cuticle gene, and hexokinase also effectively suppressed the proliferation of *N. bombycis* and *Nosema ceranae* (Huang et al. 2018; Li et al. 2016; Rodriguez-Garcia et al. 2018). The application of RNAi techniques in *Nosema* infections has facilitated gene function research, and many researchers have applied RNAi for studies on the inhibition of the growth of microsporidia in insects. It is likely that this technique will also accelerate research in other species which have RNAi pathways (e.g., *Anncaliia*).

Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Chapter 7
Nosema apis and *N. ceranae* Infection in Honey bees: A Model for Host-Pathogen Interactions in Insects

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Abstract There has been increased focus on the role of microbial attack as a potential cause of recent declines in the health of the western honey bee, *Apis mellifera*. The *Nosema* species, *N. apis* and *N. ceranae*, are microsporidian parasites that are pathogenic to honey bees, and infection by these species has been implicated as a key factor in honey bee losses. Honey bees infected with both *Nosema* spp. display significant changes in their biology at the cellular, tissue, and organismal levels impacting host metabolism, immune function, physiology, and behavior. Infected individuals lead to colony dysfunction and can contribute to colony disease in some circumstances. The means through which parasite growth and tissue pathology in the midgut lead to the dramatic physiological and behavioral changes at the organismal level are only partially understood. In addition, we possess only a limited appreciation of the elements of the host environment that impact pathogen growth and development. Critical for answering these questions is a mechanistic understanding of the host and pathogen machinery responsible for host-pathogen interactions. A number of approaches are already being used to elucidate these mechanisms, and promising new tools may allow for gain- and loss-of-function experiments to accelerate future progress.

Keywords *Nosema* · Microsporidia · Honey bee · Infection · Host-pathogen interactions

7.1 Introduction

The western honey bee, *Apis mellifera*, is crucial to key agricultural and ecological systems, and the pollination of many important crops is dependent on this species (Potts et al. 2016). If this pollination crisis remains unsolved, it will contribute to serious nutritional deficiencies for humans (Smith et al. 2015) and significant

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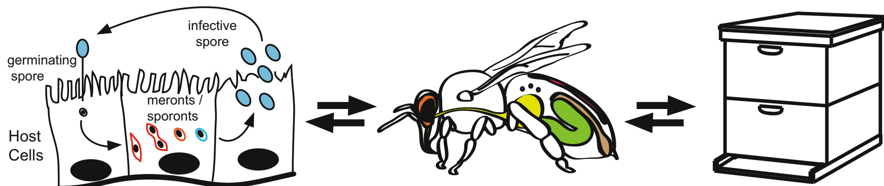

Fig. 7.1 Honey bees infected with both *Nosema spp*. display significant changes in their biology at the cellular, tissue, organismal, and colony levels

economic losses (Gallai et al. 2009) worldwide. Infection by pathogenic microbes represents one critical factor influencing honey bee declines in recent years (Goulson et al. 2015; Steinhauer et al. 2018). The microsporidian species *Nosema apis* and *N. ceranae* can cause individual pathology and mortality in honey bees that has been well documented (Fries 2014; Goblirsch 2017; Martín-Hernández et al. 2018). Such infections can also contribute to colony-level disease and collapse in conjunction with other elements, which appear to differ by region. Such factors include genetic variation on the part of both parasite and host and the presence of other stressors (e.g., climate variation or pesticide exposure) (Fries 2014; Goblirsch 2017; Martín-Hernández et al. 2018).

Honey bees infected with either *Nosema* spp. display significant changes in their biology at the cellular, tissue, and organismal levels with impacts on host metabolism, immune function, physiology, and behavior. Infected individuals lead to colony dysfunction and can contribute to colony disease in some circumstances (Fig. 7.1). Starting with the digestive tract, this review will describe some of the cellular, tissue, and organismal changes associated with infection by *Nosema* spp., with special focus on *N. ceranae*, which originated in the eastern honey bee, *Apis cerana*, and seems to have displaced *N. apis* in the western honey bee in many regions (Klee et al. 2007; Emsen et al. 2016; Gisder et al. 2017). The impacts of individual bee disease and mortality on the colony and on honey bee health more broadly have been comprehensively reviewed recently and will not be covered here (Fries 2014; Goblirsch 2017; Martín-Hernández et al. 2018).

Remarkable progress has been made in our understanding of *Nosema* disease in the years since the first report of *N. ceranae* in western honey bees. Yet many key questions remain unanswered. While there is still some debate, both *Nosema* spp. appear to display very narrow tissue tropism, being restricted to the digestive tract (Huang and Solter 2013; Higes et al. 2020). Thus, proximal changes to midgut function and the presence of a rapidly developing infectious agent must necessarily be responsible for dysfunction at the organismal level. However, the relative contribution of the host and parasite cells to these larger order changes and the molecular mechanisms through which they are achieved are incompletely understood. Conversely, the factors that influence the growth and development of cells of both *Nosema* spp. within the host environment remain unclear. Finally, our understanding of the positive and negative consequences of the observed organismal changes on both host and parasite is limited. Understanding the host and pathogen machinery

responsible for these negative changes is critical for answering these questions. A number of approaches are already being used to better understand these mechanisms, and promising new tools may allow for the gain- and loss-of-function and biochemical experiments necessary to accelerate future progress.

7.2 Changes in Digestive Tract Structure and Function After *Nosema* Infection

In worker honey bees, both *N. apis* and *N. ceranae* are restricted to infecting cells of the midgut. Typical of microsporidia, environmental spores first inject sporoplasms into host cells where they develop into a meronts that begin to rapidly proliferate. Meronts then mature into sporoblasts, which produce large numbers of primary spores and ultimately new infective environmental spores. These spores are released from the infected cell to begin the cycle anew (Solter et al. 2012). Digestive tract cells infected with *Nosema* spp. show evidence of important restructuring of subcellular structures, including aggregated ribosomes (Liu 1984) and association of microsporidia with the mitochondria in both *N. ceranae* (Higes et al. 2007) and *N. apis* (Graaf et al. 1994) infection. These cells also display extensive lysis, tissue disorganization, and cell sloughing into the lumen of the midgut (Liu 1984; Higes et al. 2007; García-Palencia et al. 2010; Dussaubat et al. 2012). In adults of insect species studied to date, epithelial cells are sloughed off into the lumen at some rate before being replaced by stem and progenitor cell proliferation and differentiation (Apidianakis and Rahme 2011). In the fruit fly, homeostatic self-renewal of the digestive tract has been shown to be critical for maintaining organ function after insult from damage or infection (Jiang et al. 2016; Guo et al. 2016b).

As an intracellular parasite with a long and complex lifecycle, *Nosema*-mediated regulation of cell turnover is likely paramount for maximum production of mature spores prior to enterocyte shedding and removal. Such regulation could occur through control of a variety of cellular processes including apoptosis and proliferation. In 2013, it was reported that midgut cells of *N. ceranae*-infected honey bees have reduced rates of apoptosis. The authors hypothesized that this reduced cell death was due to active manipulation of host cell apoptosis to allow for maximal pathogen reproduction (Higes et al. 2013). Further studies have confirmed this finding and also revealed that apoptosis suppression was most pronounced in the posterior regions of the midgut (Kurze et al. 2018). Studies have indicated that *N. apis* infection is initially restricted to the posterior section of the midgut (Fries 1988; Graaf et al. 1994), although this has not been observed in *N. ceranae* infection (Snow 2016). The mechanisms through which *N. ceranae* suppresses apoptosis are incompletely understood. One study implicated augmented expression of the *inhibitor of apoptosis protein-2* (*Iap2)* gene in cell survival (Kurze et al. 2015). Interestingly, both the suppressed apoptosis and the increased *Iap2* gene expression were lost in a strain of bees selected for *Nosema* tolerance. Another study found that a

number of other survival factors are aberrantly upregulated in infected midguts, including the Bcl2-like *Buffy* gene and the Iap family member *BIRC5* gene (Martín-Hernández et al. 2017). Alterations in the expression of apoptotic machinery have also been observed in infected midgut tissue using transcriptomic (Dussaubat et al. 2012; Holt et al. 2013; Doublet et al. 2017) and proteomic (Kurze et al. 2016a) approaches. Other microsporidia species infecting vertebrates and invertebrates are known to inhibit host cell apoptosis (Scanlon et al. 1999; Aguila et al. 2006). For example, *Nosema bombycis* reduces apoptosis in silkworm cells exposed to actinomycin D, an RNA polymerase inhibitor commonly used to induce apoptosis. Here, upregulation of *Buffy* was also implicated (He et al. 2015).

N. ceranae infection also impacts midgut proliferation. Panek et al. found that proliferation, as assessed by BrdU+ "crypts," was reduced in *N. ceranae*-infected bees (Panek et al. 2018). This decrease is correlated with altered transcription of components of a number of pathways known to regulate midgut regeneration in the fruit fly (Jiang et al. 2016; Guo et al. 2016b), such as *Hippo* (Panek et al. 2018), and *Wnt* (Dussaubat et al. 2012), as well as cell cycle genes themselves (Martín-Hernández et al. 2017). Proliferation of midgut cells in the honey bee has been shown to be influenced by age, social function, and diet (Ward et al. 2008; Willard et al. 2011). However, a more complete understanding of the pathways responsible for controlling proliferation in the honey bee midgut is vital to understand how these pathways are altered by *Nosema* infection. For example, due to their sequence divergence, the honey bee JAK/STAT pathway ligands that regulate tissue regeneration in the fruit fly midgut have been elusive and were only recently discovered as being induced by thermal stress in the honey bee midgut (Bach et al. 2021). As new players in honey bee midgut biology are characterized, their role in *Nosema* infection can be explored.

A number of other interesting alterations in midgut biology have been observed after *N. ceranae* infection. Relating to cell proliferation, Panek et al. observed that *N. ceranae* appeared to be excluded from the proliferative stem cell population (Panek et al. 2018) although a previous study did not observe similar findings (Higes et al. 2007). Such exclusion likely has a significant impact on the ability of the digestive tract to retain some functionality during the long course of a typical microsporidia infection by preventing exhaustion of regeneration potential. The peritrophic membrane (PM), a key protective feature of insect digestive tracts (Hegedus et al. 2009), also appears disorganized after *N. ceranae* infection (Dussaubat et al. 2012), though full characterization of this phenomenon is lacking. Disruptions in PM function may be important for spore production and shedding but may also modify the nature of the interactions between the midgut cells and the cells of the microbiome or even other pathogens. Several studies have also suggested that *N. ceranae* infection impairs the integrity of the epithelial layer in the midgut (Higes et al. 2007; Dussaubat et al. 2012). While formal demonstration of increased intestinal barrier permeability has not yet been provided, it would be expected to enable diverse entities from the digestive tract to enter the hemocoel and future studies should clarify its role in *Nosema* disease.

Transcriptomic and proteomic data also suggests dramatic alterations in the expression of genes involved in all major functions of the digestive tract in insects, including nutrient acquisition and processing, host defense, microbiome maintenance, chemical detoxification, and barrier function (Antúnez et al. 2009; Dussaubat et al. 2012; Chaimanee et al. 2012; Holt et al. 2013; Aufauvre et al. 2014; Doublet et al. 2017). Additionally, expressions of a number of host miRNA are altered by infection (Huang et al. 2015), although the targeted mRNA are largely unknown. Our current understanding of how digestive tract function is altered will be discussed in more details in the sections below.

Infection by* Nosema *spp. Impacts Nutrient Acquisition and Processing Infection by *Nosema* spp. causes proximal changes to midgut nutrient acquisition and processing that impact organismal metabolism. The digestive function of the midgut epithelium is compromised at early time points after infection by *N. apis*, as both trypsin and chymotrypsin levels are decreased (Malone and Gatehouse 1998). Changes to metabolism in the gut have been inferred from gene expression studies that show changes in the genes involved in transport and processing of carbohydrate molecules. In particular, genes encoding trehalose transporters and alpha-glucosidase have been found to be upregulated during infection (Dussaubat et al. 2012), while trehalase has been shown to be downregulated (Aufauvre et al. 2014). Alpha-glucosidase protein levels have also been shown to be impacted (Vidau et al. 2014). It is not currently clear which of the above changes are specific to microsporidia infection versus generic consequences that would be found with diverse stressors. For example, trehalose transporter gene expression (Bach et al. 2021) and alpha-glucosidase protein levels (Huang et al. 2013) have both been shown to be altered by stressors not related to infection state, suggesting that these changes might not be a specific to infection. Regardless, these changes are particularly interesting because trehalose is currently thought to be the most plausible source of glucose for microsporidia (Timofeev et al. 2020). As one of the so-called "Terresporidia," neither *N. apis* nor *N. ceranae* appear to possess the alternative oxidase of more basal microsporidia (Timofeev et al. 2020). Thus, it is unclear how regeneration of reduction equivalents such as NAD+ is achieved. It is possible that novel transport proteins able to move NAD+ (Dean et al. 2018) could provide one solution and the species of the *Nosema* genus indeed possess a rich transportome (Chetia et al. 2017). A recent study also found that the levels of eight proteins of the oxidative phosphorylation pathway were altered by *N. ceranae* infection (Houdelet et al. 2020), which is particularly striking in the context of the association between microsporidia and mitochondria previously discussed. RNAi studies indicate that *N. ceranae* is in fact dependent on its ATP/ADP transporters (Paldi et al. 2010), likely for commandeering host ATP as has been shown for other microsporidia (Jarkass and Reinke 2020). *N. bombycis* also possesses a putative ATP-binding cassette transporter, which is critical for parasite cell growth (He et al. 2019). Multiple metabolic enzymes of microsporidia are secreted into host cells (Senderskiy et al. 2014), presumably to modify host metabolism (Timofeev et al. 2020). Hexokinase, which catalyzes the first step in glycolysis, was first found

to be secreted by *Nematocida* spp. (Cuomo et al. 2012) and then by diverse microsporidia species (Timofeev et al. 2020). The enzymatic activity of hexokinase has been confirmed for both *N. ceranae* and *N. bombycis* (Dolgikh et al. 2019). RNAi studies demonstrated a critical role for this enzyme in *N. bombycis* growth (Huang et al. 2018b).

Infection leads to dramatic changes in metabolism in a number of tissues that are distal from the site of infection. Reduced trehalose levels are observed in the hemolymph of honey bee foragers, although glucose levels stay the same (Mayack and Naug 2010). Other metabolites including amino acids, lipid biosynthesis components, and the polyamine compound spermidine are also altered in the hemolymph of *N. ceranae*-infected bees (Aliferis et al. 2012; Jousse et al. 2020). Whole bee lipid loss is observed (Li et al. 2018), which likely represents changes in fat body lipid storage and provides one indicator of reduced energy reserves. Bees infected with *N. ceranae* are energetically stressed and have higher hunger levels (Alaux et al. 2009; Naug and Gibbs 2009; Mayack and Naug 2009; Martín-Hernández et al. 2011), which have important impacts on their physiology and behavior and overall colony health as discussed below. *N. apis* infection also impacts bee energetics, but the effect is less pronounced compared to *N. ceranae* infection (Martín-Hernández et al. 2011). Interestingly, decreased survival of bees infected with N. *ceranae* can be ameliorated by ad libitum feeding, suggesting that energetic stress may be a critical driver of mortality in infected bees (Mayack and Naug 2009). Energetic stress, as measured by hemolymph trehalose levels, is not observed in the *Nosema*-tolerant strain after *N. ceranae* infection (Kurze et al. 2016b), perhaps suggesting that mechanisms increasing tolerance to infection are as critical as those for resistance to infection (Kurze et al. 2016c)

Immune Responses Are Altered by* Nosema *Disease The immune response to microsporidiosis in invertebrates is currently thought to be composed of multiple arms. First, microsporidia infection leads to transcriptional induction of immune recognition and effector proteins, including antimicrobial peptides (AMPs). This arm has been most extensively studied in honey bees. At early time points, experimental (Schwarz and Evans 2013; Huang et al. 2016b) and natural (Li et al. 2017b) infection by *N. ceranae* induces expression of genes encoding AMPs and pattern recognition receptors (PRRs) and alters expression of components of the Imd and Toll pathways, both known to respond to pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) stimuli. After this initial phase, the expression of immune effector (e.g., AMPs) genes and other immune-related transcripts was reduced below the levels of infected controls. There appears to be significant study-related variability in immune gene expression (Antúnez et al. 2009; Dussaubat et al. 2012; Chaimanee et al. 2012; Holt et al. 2013; Aufauvre et al. 2014; Doublet et al. 2017), which may be due to differences in experimental procedures (dose, tissue sampled, or time of sampling) or strain variation on the part of either the host or the parasite. *N. bombycis* infection induces robust transcriptional activation of immune genes in *Bombyx mori*, including genes encoding AMPs and serine proteases and serpins (Ma et al. 2013), suggesting that

microsporidia infection induces some similar responses in different insect species (Pan et al. 2018). Thus far, there are only a few pieces of evidence that these responses have a protective effect on the host. *Naked cuticle* (*nkd*) is a Wnt pathway antagonist that is induced by *N. ceranae* infection and is thought to regulate immune responses. Knockdown of this gene results in reduced parasite load and induction of immune genes, such as the AMPs *abecin*, *apidecin*, and *defensin-1* and the Pattern Recognition Receptors (PRR) *PGRP-S2* (Li et al. 2016). Exposure to Pathogen-associated molecular patterns (PAMPs) increased survivorship in *N. ceranae*-infected bees, potentially by inducing a more robust immune response, although no measurement of such responses was made (Valizadeh et al. 2020). Drones from the *Nosema*-tolerant strain above also appear to have some evidence of more active immune pathways, which may influence their survival (Huang et al. 2012).

Systemic immune responses, including cellular and humoral, have not been emphasized in honey bee studies of microsporidia infection to date, presumably because of the highly restricted tissue tropism of the microsporidia infection in bees. Cellular immunity, as measured by hemocyte numbers (Alaux et al. 2010), does appear impacted by microsporidia infection, although hemocytes have been shown to phagocytose *N. apis* spores in vitro (Gilliam and Shimanuki 1967). The phenoloxidase cascade, involved in immune-mediated melanization, is similarly unaffected by *N. ceranae* infection (Alaux et al. 2010; Pasquale et al. 2013; Roberts and Hughes 2014), although serine proteases involved in melanization are upregulated by infection (Aufauvre et al. 2012; Badaoui et al. 2017). However, in silkworms, there is evidence that systemic melanization is an important mechanism of immune defense against microsporidia infection (Ma et al. 2013; Ni et al. 2020) and *N. bombycis* parasite actively suppresses melanization (Bao et al. 2019).

An intracellular immune mechanism that targets microsporidia was recently discovered in *C. elegans*. This mechanism, called the "intracellular pathogen response" (IPR), uses ubiquitin to target and clear parasite cells via autophagy (Bakowski et al. 2014; Balla et al. 2019; Reddy et al. 2019). While this specific pathway has not been found in insects, autophagy has been shown to be a critical component of immunity in the fruit fly (reviewed in (Buchon et al. 2014)), and that was recently shown to confer some protection against *N. bombycis* infection in silkworms (Hua et al. 2021). Characterization of intracellular immune mechanisms in bees that might target microsporidia represents a crucial direction for future research.

Complex Interactions Between* Nosema *spp. and the Honey bee Microbiome The microbiomes of insects play key roles in their biology (Engel and Moran 2013), and it is likely that some of the impacts of honey bee disease are caused by interactions between the honey bee microbiome and *Nosema* spp. Recent studies have shown that the gut microbiota of honey bees is more complex than that found in solitary insects (Kwong and Moran 2016) and that its composition can have a significant impact on honey bee health (Raymann and Moran 2018). The microbiome community provides benefits to the honey bee host that include metabolic contributions (Zheng et al. 2017) and immune modulation (Kwong et al. 2017). In addition, the

microbiome can regulate hormonal signaling found to be involved in some of the organismal-level processes perturbed by *Nosema* infection (described below) (Zheng et al. 2017). Thus, teasing out the relative contributions of host cell pathology and host dysbiosis in inciting *Nosema* disease will be challenging. Perturbation of the honey bee microbiota by diverse mechanisms, such as antibiotic exposure or dietary alterations, can negatively impact honey bee health. Changes in the microbiome can impact the severity and outcomes of infections by pathogenic microbes, including *Nosema* spp. Microbiome composition is correlated with infection intensity of *N. ceranae* infection in *A. mellifera* (Maes et al. 2016; Rubanov et al. 2019; Paris et al. 2020). The interaction appears to be bidirectional. Penicillin-streptomycin exposure can make bees more susceptible to *N. ceranae* infection (Li et al. 2017a). Conversely, reducing *N. ceranae* infection through RNAi increased the abundance of certain bacterial species, suggesting that infection directly or indirectly impacts the digestive tract microbiome (Huang and Evans 2020). Interestingly, recent data from *Apis cerana* also shows that the microbiota can suppress *N. ceranae* growth (Wu et al. 2020), suggesting an antagonistic relationship between *N. ceranae* and the microbiome is a more widespread phenomenon in different bee species.

7.3 *Nosema* Infection Induces Changes in Organismal Physiology and Behavior

Microsporidia infection in bees is associated with alterations in diverse tissues and organs that impact both physiology and behavior of infected individuals. Hypopharyngeal glands (HPG) are involved in producing secretions for feeding larvae in nurse bees and in foraging bees (Ahmad et al. 2021). HPG are decreased in size (Wang and Moeller 1969) and functionally deficient (Liu 1990) in adult bees infected with *N. apis* and show alterations in gene expression and enzyme activity in bees infected with *N. ceranae* (Li et al. 2019). The insect fat body is an important tissue for managing energy homeostasis (Arrese and Soulages 2010). Despite its importance as a metabolic hub for the organism, relatively little is known about the impacts of infection by *Nosema* spp. on this organ. One study found that fat body genes involved in metabolism and hormonal signaling were impacted by infection (Holt et al. 2013). *N. ceranae* infection also alters gene expression in the brain (Holt et al. 2013; McDonnell et al. 2013; Mayack et al. 2015; Doublet et al. 2016) and impacts learning and memory (Gage et al. 2017). Collectively, these tissue-/organ-level shifts act in concert to alter organismal physiology and behavior.

Taken together, organismal changes mimic an acceleration of those observed during normal honey bee development. Individuals of the nonreproductive worker caste of honey bees exhibit a phenomenon known as age polyethism, defined as the age-related division of labor for nonreproductive tasks (Seeley 1982). The most pronounced transition with this process is from nurse bees, which perform tasks inside the colony, to forager bees, involved in gathering resources from the

environment (Johnson 2010). This shift is associated with significant behavioral and physiological changes and is highly regulated to optimize colony function. Infection by either *N. apis* (Hassanein 1953; Wang and Moeller 1970; Woyciechowski and Moroń 2009) or *N. ceranae* (Goblirsch et al. 2013; Dussaubat et al. 2013; Natsopoulou et al. 2015; Lecocq et al. 2016) causes worker bees to accelerate this aging process as manifested by both physiological and behavioral changes. One 2013 study found that infected bees are twice as likely to engage in precocious foraging compared to controls (Goblirsch et al. 2013). Foragers infected by *N. ceranae* (Naug 2014; Alaux et al. 2014) and *N. apis* (Lach et al. 2015; Dosselli et al. 2016) perform less efficiently, and *N. ceranae*-infected foragers are less likely to return to the colony due to energetic stress (Kralj and Fuchs 2010) and reduced homing ability (Wolf et al. 2014). Infected workers also spend more time outside the nest engaged in risky behaviors such as robbing (Kuszewska and Woyciechowski 2014).

Multiple hormonal systems known to control worker division of labor are impacted in bees infected by *Nosema* spp. The vitellogenin (Vg)/juvenile hormone (JH) axis is critical for controlling age polyethism (Johnson 2010). JH levels are increased in bees infected with either *Nosema* spp. (Ares et al. 2012), while Vg expression is reduced (Antúnez et al. 2013; Goblirsch et al. 2013; Zheng et al. 2014; Garrido et al. 2016). Bees infected as larva have increased Vg levels as young adults, suggesting different impacts based on the age of infection (BenVau and Nieh 2017). Infected bees also show alterations in the octopamine pathway (Mayack et al. 2015), which is also known to participate in the transition to foraging (Johnson 2010), and insulin signaling (Holt et al. 2013), involved in regulating caste (Ament et al. 2008, 2010).

Interestingly, a number of other stressors are known to induce a similar precociousness in the physiological and behavioral shifts associated with the development from nurse to forager as well as the hormonal systems controlling these transitions. For example, infection by diverse pathogens and parasitization by an arthropod pest induce comparable changes (McDonnell et al. 2013; Doublet et al. 2016). One theory to explain the related outcomes from these distinct stressors is that they all impinge on nutritional status which is known to heavily influence this transition (Ament et al. 2010). Here again, additional foundational knowledge of honey bee biology will likely facilitate a better understanding of *Nosema* disease. Based on the complexity of the interorgan communication systems used in fruit flies to regulate organismal physiology and behavior (Droujinine and Perrimon 2016; Liu and Jin 2017), it is likely that other molecules yet to be characterized in honey bees integrate signals of nutrition status (and injury) at the physiological levels and couple these inputs with control of life stage transitions in bees. The *unpaired* family of JAK/STAT pathway ligands described above is also known to regulate both physiology and behavior in flies (Droujinine and Perrimon 2016; Liu and Jin 2017). Characterizing the role of the recently described honey bee homologs of these ligands (Bach et al. 2021) in interorgan communication may represent one potential link between the infected digestive tract and organismal pathology.

The behavioral changes observed in parasitized bees may be seen to have adaptive benefits to both the parasite and host. For example, by engaging in risky behavior, infected bees spend less time in the colony potentially limiting transmission to nestmates and benefitting the colony (Rueppell et al. 2010). This potentially altruistic self-removal by infected bees may represent one of the social immunity strategies found in eusocial insect species (Cremer et al. 2018). However, such behavior could result in more efficient spread to individuals of uninfected colonies, thereby benefiting the pathogen. Similarly, *N. ceranae*-infected bees appear to seek out the warmer areas in the colony, which could help energetically stressed bees manage thermoregulation or provide the higher temperatures preferred by this microsporidia species and a better opportunity to spread the pathogen (Campbell et al. 2010). Thus, the adaptive benefit, if any, and the beneficiary of such behavioral changes in infected honey bees are unclear (Wagoner et al. 2013).

Many groups have further reported shortened worker lifespans after infection by *Nosema* spp. (Higes et al. 2006; Alaux et al. 2009; Vidau et al. 2011; Goblirsch et al. 2013; Williams et al. 2014; Doublet et al. 2015). For example, Goblirsch et al. observed a 9-day reduction in worker lifespan (Goblirsch et al. 2013). However, it is important to note that other studies have not observed differences in mortality in infected bees and these inconsistencies may be due to similar factors involved in modulating the colony impacts of *Nosema* disease, such as genetic variation on the part of both parasite and host and the presence of other stressors (Goblirsch 2017; Martín-Hernández et al. 2018).

7.4 Organismal Pathogenesis Caused by *Nosema* Disease Disrupts Colony Organization and Function

Honey bee colonies are eusocial (Holldobler and Wilson 2008), and colony physiology is regulated by the collective activities of individual colony members (Friedman et al. 2020). Individual infection by *Nosema* spp. appears to cause significant disruptions in colony organization and function which likely contribute to colony-level disease. For example, *N. ceranae* infection alters pheromone production in workers (Dussaubat et al. 2010, 2013) and queens (Alaux et al. 2011). *Nosema* spp. infection has been observed to alter production of ethyl oleate, which is involved in the regulation of division of labor among workers (Dussaubat et al. 2010). Another recent study found increased levels of alarm pheromone in colonies infected with *N. ceranae* (Mayack et al. 2021). Other studies have demonstrated differences in cuticular hydrocarbon profiles (McDonnell et al. 2013; Murray et al. 2016), although it is not clear how these changes impact interactions between workers (Murray et al. 2016; Biganski et al. 2018).

In the honey bee colony, the health and function of the reproductive individuals in the colony are critical for colony success. While less studied, both drones and queens can be infected by *Nosema* spp. Thus far, individuals from the reproductive castes

display similar perturbations to worker bees in addition to important changes in biology unique to their colony function (reviewed in (Goblirsch 2017; Martín-Hernández et al. 2018)). The many impacts of microsporidia infection on the physiology and behavior of workers in addition to these effects on reproductive individuals likely contribute to colony death, especially in the presence of other stressors. The negative consequences of *Nosema* infection on bees are thought to be influenced by the presence of other stressors, including nutritional stress due to loss of appropriate forage, chemical poisoning from pesticides, changes to natural living conditions brought about through large-scale beekeeping practices, myriad environmental changes due to climate change, and infection by arthropod parasites and other pathogenic microbes. For example, *N. ceranae* infection can interact with chemical stressors, such as pesticides, on honey bees leading to synergistic effects on health and mortality (Alaux et al. 2010; Vidau et al. 2011; Wu et al. 2012; Pettis et al. 2012, 2013; Aufauvre et al. 2012, 2014; Retschnig et al. 2014; Doublet et al. 2015). In addition, chemical stressors are known to increase the prevalence or intensity of *N. ceranae* infection likely through impacts on host well-being (Wu et al. 2012; Pettis et al. 2013). Bees are also often coinfected by multiple pathogens (Runckel et al. 2011; Cornman et al. 2012), and the impacts on both pathogens and the host are likely to be complex. This topic has been recently covered in great detail (Goblirsch 2017; Martín-Hernández et al. 2018) and will not be explored further here.

7.5 Impact of Host Factors on Microsporidia Growth and Development

The parameters that influence the growth and development of *Nosema* cells within the host environment are still largely unknown. Microsporidia as a group have significantly reduced genomes relative to free-living fungi, with much of the lost coding content being found in metabolic pathways (Nakjang et al. 2013). To make up for reduced biosynthetic complexity, microsporidia are known to acquire diverse array of metabolites from host cells, often through the use of expanded families of transporters (Chetia et al. 2017), which are sometimes acquired through horizontal gene transfer (Dean et al. 2018). Recent studies of *Tubulinosema ratisbonensis* which infects fruit flies (Niehus et al. 2012) have shown that specific metabolites, namely, phosphatidic acid and related lipids, are limiting for the proliferation of the microsporidium in host cells (Franchet et al. 2019). Most studies of *N. ceranae* suggest that increased protein consumption leads to increased infection intensities as measured by spore counts (Porrini et al. 2011; Basualdo et al. 2014; Zheng et al. 2014; Fleming et al. 2015; Jack et al. 2016; Tritschler et al. 2017), suggesting that for this species, amino acids may be limiting. There is also evidence that many other host-derived nutrients are important for *N. ceranae* growth. For example, iron, which is often a limiting nutrient for diverse pathogenic microbes (Cassat and Skaar 2013), was recently observed to be reduced in forager honey bees (but not nurses) after

N. ceranae infection (Rodríguez-García et al. 2021). RNAi-mediated knockdown of *transferrin* resulted in decreased available iron and led to reduced *N. ceranae* infection intensity. Limiting iron availability may be useful as a therapeutic strategy (Rodríguez-García et al. 2021).

The life cycle of *Nosema* spp. is complex and is likely influenced by the host environment and involves multiple developmental transitions characterized by distinct morphological forms and evolving patterns of expression of both mRNA (Huang et al. 2016b) and miRNA (Huang and Evans 2016; Shao et al. 2021) transcripts. For example, many microsporidia species appear to use the "early sporulation" strategy to produce primary spores for spread within host tissues before switching to the production of the secondary or environmental spore. The presence of intracellular empty spore coats indicates that intracellular germination of spores is occurring during *N. ceranae* infection (Higes et al. 2007; García-Palencia et al. 2010) as has been shown for *N. apis* (Fries et al. 1992). Another strategy for rapid spread within the tissues without the need to progress to the environmental spore is found in microsporidia infection in nematodes, which involves the removal of lateral membranes to form syncytia (Balla et al. 2016). Here, developmental timing, in particular the switch to sporulation, is triggered by parasite density (Balla et al. 2016). This may be true for *N. ceranae* as well, as inoculation dose has been shown to play a role in infection intensity and spore production (McGowan et al. 2016). Another possible signal could be related to available host resources, which may not only impact growth directly (see above) but may influence developmental decisions. Other possible cues include cell stress, which alters the developmental program of other fungi (Boyce and Andrianopoulos 2015). One stress that is known to impact *N. ceranae* infection is temperature (Martín-Hernández et al. 2009; Higes et al. 2010; McNamara-Bordewick et al. 2019), and some evidence suggests this effect is due to changes in the development of the pathogen (Higes et al. 2010). However, detailed analysis of the stage and impact has not heretofore been possible due to the difficulty in isolating the distinct developmental forms from host cells. Similarly, the current frontline drug for treating *N. ceranae* infection, fumagillin, may also impact development. At lower doses, this drug causes hyperproduction of spores compared to spore production in untreated bees (Huang et al. 2013).

While the typical focus is on the impacts on the honey bee host, the effect of various stressors on the growth and development of *Nosema* spp. in bees is also important to understand. Many environmental factors are carefully controlled by the healthy host cell, thereby reducing variability. Adaptation to this host-controlled environment would be predicted to have a significant impact on the cell stress machinery in microsporidia. In fact, comparative genomics indicates that microsporidia have lost many of the quality control and cell stress pathways found in free-living eukaryotes (McNamara-Bordewick et al. 2019; Snow 2020), perhaps leading to a loss of the redundancy and flexibility that often allow organisms to withstand cellular stresses. However, data suggests high levels of stress resistance in *Nosema* spp. For example, although the majority of fungal species prefer the 12°–30 °C range and relatively few species tolerate temperatures higher than 35 °C (Robert and Casadevall 2009), *N. ceranae* exhibits a striking ability to grow at the

high temperatures (34–35 °C) maintained in honey bee colonies (Martín-Hernández et al. 2009; Higes et al. 2010), despite the loss of the Heat Shock Factor (Hsf) gene, encoding the canonical regulator of the heat shock response (McNamara-Bordewick et al. 2019). *N. apis*, while still missing the Hsf gene, is similar to typical fungi in its sensitivity to high temperatures (Burnside and Revell 1948; Woyciechowski and Czekońska 1999), and honey bees can recover from infection when kept at the slightly elevated temperature of 37 °C (Lotmar 1943). While the canonical cell stress response systems appear reduced, there may be adaptation of the cellular machinery to allow for novel responses to cell stress. This may be achieved through mechanisms that increase tolerance to stress. Microsporidia encode aminoacyl-tRNA synthetases without editing domains, and the wrong amino acid is added in up to 6% of cases for some codons. This diversity of protein variants may allow these parasites to adapt under different stress conditions (Melnikov et al. 2018). Another mechanism may involve manipulation of the host cell. For example, *N. ceranae* infection induces decreases in both the amount of ROS and oxidative damage (Paris et al. 2017), possibly mediated through pathogen manipulation of host cell machinery. Microsporidia genomes possess a large number of genes encoding proteins with predicted signal peptides. Many of these are likely secreted into the host cell and are thus candidates for interceding in the function of host cell processes (Cuomo et al. 2012; Campbell et al. 2013). In fact, Reinke et al. were able to use exogenous machinery expressed in host cells to label a number of host-exposed proteins from *Nematocida* species. Based on analysis of other microsporidia genomes, the authors predicted that other microsporidia species might use 6–32% of their proteome to interface with the host (Reinke et al. 2017). While such an experiment is not currently possible in honey bees, there is a need for methods to identify *Nosema* proteins responsible for host manipulation.

7.6 Conclusion and Future Perspectives

Since the discovery of *N. ceranae* infecting western honey bees, there have been impressive gains in our understanding of the effects these microsporidia have on honey bee health (Goblirsch 2017; Martín-Hernández et al. 2018). A number of questions and areas for further research are evident. As parasite growth is restricted to the digestive tract, the routes through which parasite growth and tissue dysfunction in the midgut leads to the dramatic physiological and behavioral changes at the organismal level are incompletely understood. If caused by pathogen-mediated manipulation, how are these changes effected at the molecular level? If due to disruptions in normal midgut function and host responses to this pathology, are changes specific to microsporidia infection or are they observed with diverse stressors? The critical host parameters that influence the growth and development of *Nosema* cells within the host environment also remain obscure. Relatedly, elucidating the positive and negative consequences of observed changes on both host and parasite is also critical.

A number of approaches are already being used to perform gain- and loss-of-function experiments to answer these questions. RNAi machinery exists in *N. ceranae* (Paldi et al. 2010), and RNAi has been shown to be a powerful tool in elucidating the machinery of both the parasite (Paldi et al. 2010; Huang et al. 2016a, 2018a; Rodríguez-García et al. 2018; Huang and Evans 2020) and the host (Li et al. 2016; Rodríguez-García et al. 2021). New genetic tools are being developed that could greatly facilitate investigations into the mechanisms involved in host-pathogen interactions. Recently, CRISPR/Cas9 protocols have been developed for the honey bee (Kohno et al. 2016; Kohno and Kubo 2018; Roth et al. 2019; Hu et al. 2019; Nie et al. 2021). Such tools could allow confirmation of host pathways and processes suspected to be involved in *Nosema*-induced pathogenesis. For example, loss-of-function experiments could be used to probe host factors involved in the reduced apoptosis, such as the candidate *Iap* (Kurze et al. 2015; Martín-Hernández et al. 2017).

Methods to generate genetically modified microsporidia would similarly represent an indispensable resource for making future advances (Reinke and Troemel 2015). Some critical progress has been made in culturing sporoplasm in *N. bombycis* for this purpose (He et al. 2019 2020) and in delivering genetic material (Guo et al. 2016a). A heterologous cell culture system has been established (Gisder et al. 2011; Gisder and Genersch 2015) that could facilitate biochemical studies although additional more robust cell culture models are necessary.

Such future studies could also provide much needed potential target molecules for treatment strategies for *Nosema* disease in honey bees. *N. apis* infection has long been controlled by treatment with the drug fumagillin, a methionine aminopeptidase 2 inhibitor (Heever et al. 2014). Yet, the effectiveness of fumagillin treatment for treating *N. ceranae* at the colony level appears transient. High doses of this drug impair host cell function, and evidence suggests that *N. ceranae* can escape suppression in some circumstances, although the mechanisms remain unknown (Huang et al. 2013). Most critically, the long-term availability of fumagillin is uncertain, making efforts to find alternative treatment strategies critical to protect honey bees from this parasite (Heever et al. 2014; Holt and Grozinger 2016). New treatment strategies to reduce the impact of *Nosema* disease on this pollinator species are currently being explored (see (Huntsman et al. 2021) and references therein), which could have significant positive impacts on the health of both agricultural and ecological systems.

Taxonomic Note

Nosema apis and *N. ceranae* have recently been redefined as *Vairimorpha apis* and *V. ceranae* based on a recent molecular phylogenetics analysis of the Nosema and Vairimorpha clades (Tokarev et al. 2020). For the purposes of this review the *Nosema* Genus will still be used.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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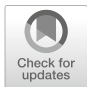
Chapter 8
The Function and Structure of the Microsporidia Polar Tube



Bing Han, Peter M. Takvorian, and Louis M. Weiss

Abstract Microsporidia are obligate intracellular pathogens that were initially identified about 160 years ago. Current phylogenetic analysis suggests that they are grouped with Cryptomycota as a basal branch or sister group to the fungi. Microsporidia are found worldwide and can infect a wide range of animals from invertebrates to vertebrates, including humans. They are responsible for a variety of diseases once thought to be restricted to immunocompromised patients but also occur in immunocompetent individuals. The small oval spore containing a coiled polar filament, which is part of the extrusion and invasion apparatus that transfers the infective sporoplasm to a new host, is a defining characteristic of all microsporidia. When the spore becomes activated, the polar filament uncoils and undergoes a rapid transition into a hollow tube that will transport the sporoplasm into a new cell. The polar tube has the ability to increase its diameter from approximately 100 nm to over 600 nm to accommodate the passage of an intact sporoplasm and penetrate the plasmalemma of the new host cell. During this process, various polar tube proteins appear to be involved in polar tube attachment to host cell and can interact with host proteins. These various interactions act to promote host cell infection.

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8.1 Introduction

Microsporidia are a diverse group of spore-forming, unicellular obligate intracellular parasites (Fig. 8.1a). The identification of the first recognized microsporidia *Nosema bombycis* was in 1857; since that initial description, over 200 genera and 1700 species of microsporidia have been identified over the past 160 years. Phylogenetic analysis suggests that microsporidia are related to fungi, being grouped with Cryptomycota as a basal branch or sister group to fungi (James et al. 2013). The genome size of the microsporidia varies from 2.18 to 51.35 Mb coding for 2000–5000 proteins. The genomic size of Encephalitozoonidae is less than 2.5 Mb, making them among the smallest eukaryotic genomes described to date (Weiss and Vossbrinck 1999; Katinka et al. 2001). Genome data for the microsporidia are available online at MicrosporidiaDB.org (https://microsporidiadb.org/micro/), which is part of the VEuPath database (https://veupathdb.org/veupathdb/) (Aurrecoechea et al. 2011).

Microsporidia can cause localized or disseminated infection in humans (Sak et al. 2011; Didier and Weiss 2011), and currently there are 17 species of microsporidia, in 10 genera, that have been reported to infect humans (Visvesvara 2002; Juarez et al. 2005). These human-infecting microsporidia are responsible for a variety of diseases, and while infections were initially thought to be restricted to immune-compromised patients, it is now known that infection can also occur in immune-competent individuals (Ramanan and Pritt 2014; Weiss 2020). The clinical manifestations of microsporidiosis are diverse, varying depending on the causal species, the host immune status, and the mode of transmission. Disease manifestations include diarrhea, keratoconjunctivitis, cholangitis, kidney and urogenital infection, myositis, ascites, hepatitis, sinusitis, disseminated infection, and asymptomatic infection (Weiss 2014, 2020; Weber et al. 2000; Desportes-Livage and Datry 2005).

Microsporidia can infect a wide range of hosts, including humans, and the manner of transmission and source of human infection vary depending on the species. Foodborne and waterborne transmission occurs (Stentiford et al. 2016a; Orlandi et al. 2002). Microsporidia spores have been identified in urine, feces, and infected animal carcasses, and these spores can eventually enter into water sources, which will contaminate recreational and potable water (Paterson and Lima 2015; Moss and Snyder 2017). Some species of microsporidia can infect both human and animals, indicating that zoonotic transmission is possible. For example, studies on urban pigeons infected by *Encephalitozoon intestinalis* and *Encephalitozoon hellem* revealed that there is no barrier to microsporidia transmission between park pigeons and humans (Haro et al. 2005).

The environmentally resistant microsporidian spore contains a unique structure, the invasion apparatus that contains a single-coiled polar tube (within the spore, the polar tube is often referred to as the polar filament) (Han et al. 2020). The spore is the

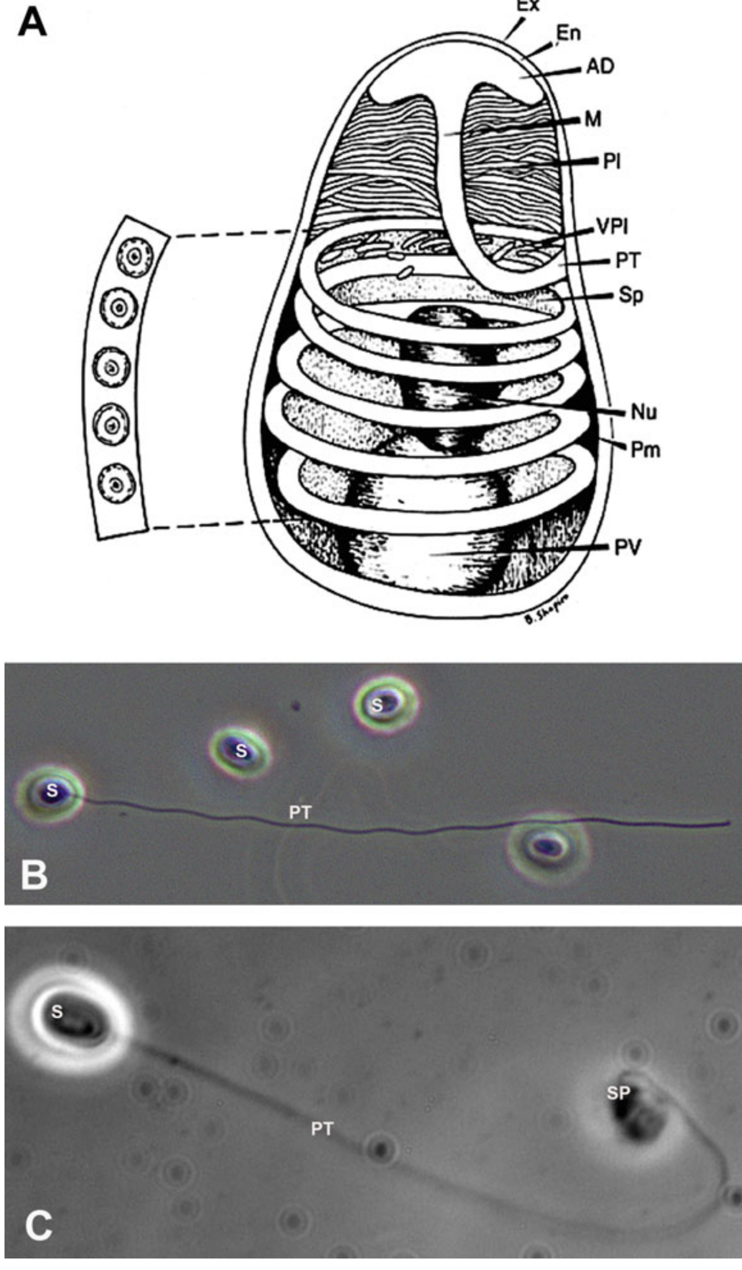

Fig. 8.1 Microsporidian spore structure and light microscope images. (**a**) Diagram of a microsporidian spore. Microsporidian spores vary in size from 1 to 12 μm. The spore coat is thinner at the anterior end of the spore and consists of an electron lucent endospore (En), electron-dense exospore (Ex), and the plasma membrane (Pm). The sporoplasm (Sp) contains ribosomes, the posterior vacuole (PV), and a single nucleus (Nu). The anchoring disc (AD) at the anterior end of the spore is the site of attachment of the polar tube. It should be noted that the polar tube is often

terminal and infectious stage of the microsporidial life cycle and the only viable stage that can survive outside of the host cell which enables it to endure harsh extracellular environmental conditions (Yang et al. 2018). Spore wall proteins are probably involved in the processes of spore adherence, signaling, and other interactions with host cells (Southern et al. 2007). The polar tube is a highly specialized structure involved in invasion of host cells (Xu and Weiss 2005). Polar tubes are found in all microsporidial species. Generally, the polar tube (often called the polar filament when it is within the spore) tightly coils within the spore and forms a spring-like structure (Jaroenlak et al. 2020). Upon appropriate environmental stimulation, the polar tube will rapidly discharge out of the spore, and the tip of the polar tube will contact and interact with the host cell serving as a conduit for the nucleus and sporoplasm passage into the new host cell (Han et al. 2017). Due to the importance of the polar tube during invasion by microsporidia, the formation of the polar tube, the structure, its protein composition, and the mechanism of polar tube eversion have drawn the interest of scientists for the last six decades (Han et al. 2020).

8.2 Structure of the Microsporidian Spore and Germination

Although diverse, all microsporidia produce a specialized, resistant, extracellular stage, the spore, which encloses and protects the infective sporoplasm and the invasion apparatus. Spores are generally small, oval- or pyriform-shaped, and range in size from 1 μm to 12 μm (Vavra 1976). Those infecting mammals are generally 1–4 μm in length (Weber et al. 1994).

Several imaging techniques have been used to identify and study the microsporidian spore and polar tube extrusion. Differential interference contrast (DIC) and phase contrast (PC) microscopy both enable the observation of live

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Fig. 8.1 (continued) called the polar filament when it is within the spore prior to germination. The anterior or straight region of the polar tube that connects to the anchoring disc is called the manubroid (M), and the posterior region of the tube coils around the sporoplasm. The number of coils and their arrangement (i.e., single row or multiple rows) is used in microsporidian taxonomic classification. The lamellar polaroplast (Pl) and vesicular polaroplast (VPl) surround the manubroid region of the polar tube. The insert depicts the polar tube coils in this figure in a cross section illustrating that the polar tube within the spore (i.e., polar filament) has several layers of different electron density by electron microscopy. Reprinted with permission from Keohane EM, Weiss LM. 1999. The structure, function, and composition of the microsporidian polar tube. pp. 196–224. *In* Wittner M, Weiss LM (ed), The Microsporidia and Microsporidiosis. ASM Press, Washington, DC (Keohane and Weiss 1999). (**b**) Differential interference contrast (DIC) microscopy image of *Anncaliia algerae* spores. One of the spores (S) has become activated, and the polar tube (PT) is in the process of extrusion. (**c**) Phase contrast microscopic image of an *Anncaliia algerae* spore (S) with the extruded polar tube (PT) and the sporoplasm (SPM) still attached to the distal end of the PT

unfixed, unstained spores. This is especially useful when studying spore activation, polar tube extrusion (Fig. 8.1b), and the transport of the infective sporoplasm (Fig. 8.1c). Scanning electron microscopy (SEM) has been very useful for obtaining three-dimensional images of the exterior surface of spores and their extruded polar tubes (Fig. 8.2a). Transmission electron microscopy (TEM) has been the imaging tool most frequently used to study spore structure and its contents, since Huger (Huger 1960) published the first TEM images of a microsporidian spore (Weiss and Bechnel 2014). This technique is particularly useful for imaging microsporidia, due to the small size of the microsporidian spore and its extensive membrane systems, infection apparatus (polar filament), and sporoplasm, all tightly packed inside the spore. When observed with TEM, microsporidian spores have an electron-dense outer spore coat overlying an inner thicker lucent coat followed by a membrane system surrounding the spore contents and polar filament coils. The spore contents include an anterior anchoring disk complex, tightly packed arrays of membrane clusters (the lamellar polaroplast and flattened tubules), and a centrally located sporoplasm, composed of scant cytoplasm containing a single nucleus or pair of abutted nuclei (diplokaryon), tightly packed ribosomes, some endoplasmic reticulum, and Golgi (Fig. 8.2b). Surrounding the central region of the spore is a coiled polar filament (termed the polar tube when extruded) (Cali and Takvorian 2014; Vavra 1976). The polar filament/polar tube when exiting the spore transports the sporoplasm through itself into a host cell. Inside of the spore, cross-sectional images of the polar filament indicate that it is a solid structure composed of alternating concentric rings of electron-dense and lucent material surrounding electron-dense material in the center (Fig. 8.2b) (Cali and Takvorian 2014; Vavra 1976). The number of coils and their contents varies in different organisms (Cali and Takvorian 2014; Vavra 1976).

Due to the density of the spore wall and extreme packing of its contents, traditional TEM studies using thin sections (60–90 nm) have not provided the images needed to fully understand the organization of the spore contents. In an attempt to better understand the internal structures and their location, our laboratory group has utilized high-voltage transmission electron microscopy (HVTEM), taking multiple tilt angle images of 400-nm-thick sections of spores (Fig. 8.3a) and generating three-dimensional (3-D) computerized models (Fig. 8.3b and c). During activation and extrusion, the internal organization of the spore undergoes massive membrane reorganization with the "solid" polar filament becoming a hollow tube as it everts and exits the spore (Cali and Takvorian 2014; Vavra 1976; Chioralia et al. 1998; Cali et al. 2002). The long (50–500 μm) narrow (90–120 nm) polar tube transfers the relatively large (1–1.5 μm) infective sporoplasm from the spore into a host cell in less than 2 seconds (Frixione et al. 1992; Jaroenlak et al. 2020).

Recently, Jaroenlak et al. (2020) utilized serial block-face scanning electron microscopy (SBFSEM) imaging of intact *Anncaliia algerae* and *Encephalitozoon hellem* spores to study their three-dimensional organization. Utilizing fixed cells cut at 50 nm, the authors imaged the cells and then stacked the images and segmented areas of interest to produce 3-D models of the spore contents. Models generated of the spore and the intact polar filament indicated that the polar filament coils in a

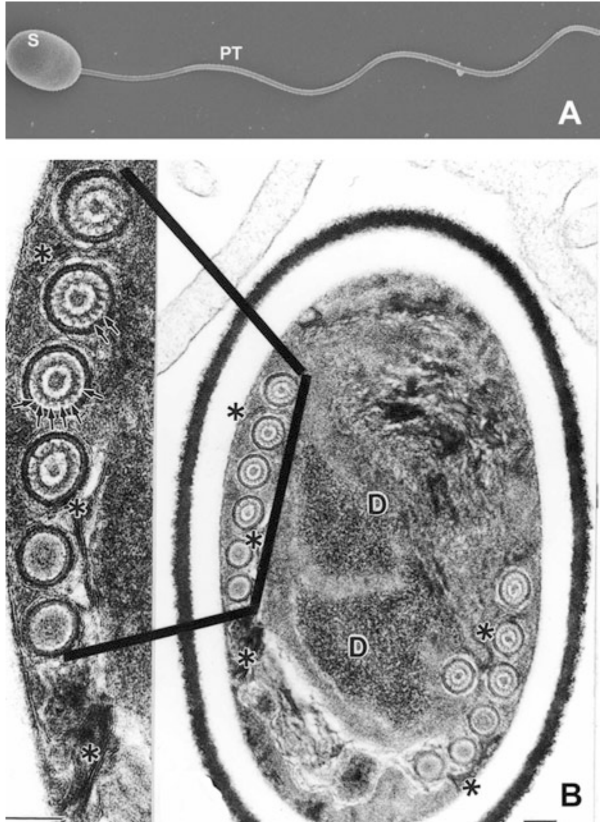

Fig. 8.2 Scanning and transmission electron microscopic images of microsporidian spores. (**a**) Scanning electron microscope image of an activated *Anncaliia algerae* spore (S) with the extruded polar tube (PT). (**b**) Transmission electron microscope (TEM) image of an *Anncaliia algerae* spore and an enlarged image of an area of the polar filament (PF). The spore has an electron-dense outer spore coat (exospore) overlying an inner thicker lucent region (endospore) followed by a membrane system surrounding the spore contents and polar filament coils (PF). The spore contents are composed of a complex of tightly packed arrays of membrane clusters and a centrally located sporoplasm, composed of scant cytoplasm containing a pair of abutted nuclei (diplokaryon), tightly packed ribosomes, and some endoplasmic reticulum. Surrounding this complex is a coiled polar filament (termed the polar tube (PT) when extruded) which becomes straight in the anterior part of the spore where it will exit when it is activated. The enlarged area of the PF coil cross sections illustrates the internal concentric rings and electron-dense particles (arrows) composing part of the internal structure of the PF. Images reprinted with permission from Cali A, Weiss LM, Takvorian PM (2002) *Brachiola algerae* spore membrane systems, their activity during extrusion, and a new structural entity, the multilayered interlaced network, associated with the polar tube and the sporoplasm. J Eukaryot Microbiol 49 (2):164–174. doi:https://doi.org/10.1111/j.1550-7408.2002.tb00361.x (Cali et al. 2002)

right-hand helix (Fig. 8.4a, b, c). The authors' live cell studies determined the polar tube extrudes and reaches its full length under 1 second and that the sporoplasm which is about seven times larger than the polar tube diameter remains intact as it passes through the polar tube (see Chap. 9).

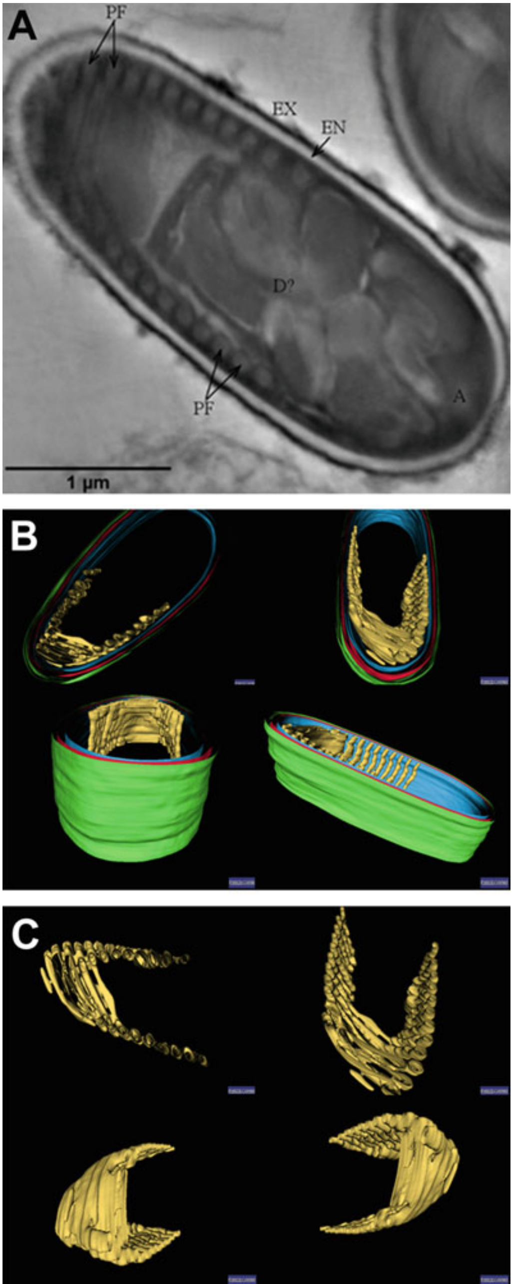

Fig. 8.3 High-voltage (1.2million KV) transmission electron microscope images and three-dimensional models generated from the images of an *Anncaliia algerae* spore. (**a**) High-voltage transmission electron microscope (HVTEM) image of a 400-nm-thick section obtained with a 1.2 million accelerating voltage. One hundred and twenty one (121) images of the section were taken at 0–60° tilt angles at two degree increments from the four aspects for a total of 120 tilt images plus the

An additional method of imaging that has been utilized recently to examine the spore and its internal contents is focused ion beam scanning electron microscopy (FIB-SEM) which can utilize the same specimen blocks used for TEM (Fig. 8.5a). The ion beam can be used to cut/mill a cross-section of the sample to obtain information from regions of interest beneath the sample surface. A complete volume of a sample can be achieved by repeated milling of thin slices and imaging of the new surfaces. The captured images of the spores can be as thin as 10 nm. The captured images are aligned, stacked, and segmented to produce 3-D models similar to those obtained by HVTEM and SBFSEM with the use of computer programs such as Amira©. FIB-SEM software can produce tomograms from the approximately 200 images of a 2-μm-thick spore when cut at 10 nm and 150 images at 15 nm cuts. FIB-SEM images (15-nm spaced "Z" stack) of activated *Anncaliia algerae* spores demonstrate the polar tube in a coil with a straight portion of the tube exiting the spore through the thin anterior portion of the exospore (Fig. 8.5b). The extruding polar tube passes through the anchoring disc-polaroplast complex of an extended "collar" of at least three layers: the erupted exospore, the anchoring disc, and the polaroplast. The 3-D color model of the spore, PT, anchoring disc complex, and posterior vacuole and membranes provides a perspective of the organization of the active extruding spore contents (Fig. 8.6a). Removal of the exospore wall and rotation of the model to show the anterior aspect of the PT and anchoring disc complex provide a view of the exiting PT passing through the complex as it uncoils and straightens (Fig. 8.6b).

8.3 Polar Filament Development and Formation

The vast majority of microsporidia undergo a three-part developmental cycle which starts with entry of the sporoplasm into a host cell. The first part is proliferation to increase the numbers of organisms, followed by sporogony, in which the "simple" proliferative cells undergo a complex morphological change into sporoblasts. These

Fig. 8.3 (continued) original zero tilt position. The images were then aligned, "Z" stack, and a volume of the images was produced. Slice number 52 of the volume is of a nonactivated *Anncaliia algerae* spore. The exospore (EX), endospore (EN), diplokaryion (D), anchoring disk (A), and polar filament (PF) are all visible. (**b**) HVTEM three-dimensional model of the spore generated from the 121 tilt series image volume segmented with Amira© software. The exospore (EX) is green, endospore (EN) is red, the spore wall inner membranes are blue, and the polar filament is gold in this rendering. The spore models are tilted to enable different viewing angles of the PF inside the spore. (**c**) HVTEM three-dimensional model of the segmented polar filament obtained from the volume in Fig. 8.3a. The model illustrates the uniform orientation of the PF. The models are tilted to enable different viewing angles of the PF. These various images were obtained by Dr. Peter M. Takvorian using equipment at the Resource for Visualization of Biological Complexity, NYS Department of Health Wadsworth Center, Empire State Plaza, P.O. Box 509, Albany, *NY* 12201, USA

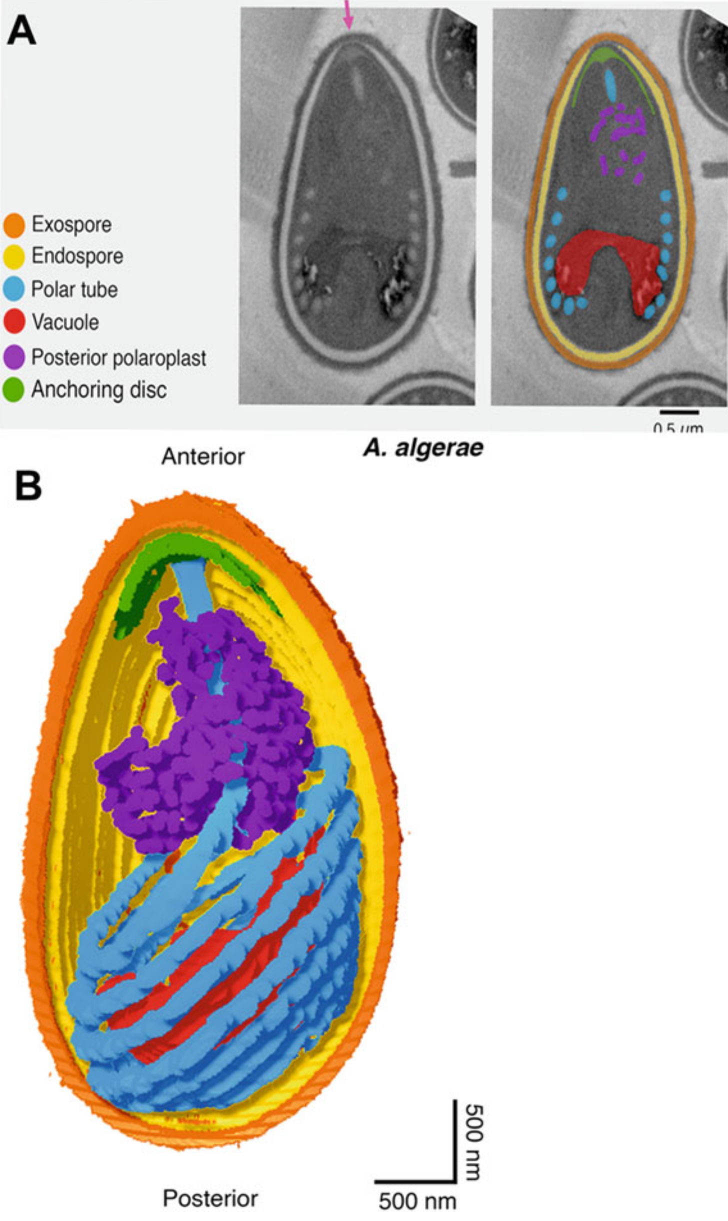

Fig. 8.4 Serial block-face scanning electron microscopy (SBFSEM) imaging of intact *Anncaliia algerae* spores. (**a**) Serial block-face scanning electron microscopy imaging of intact *Anncaliia algerae* spores**.** Samples were serially sliced at 50 nm thickness (left), and images for a representative slice are shown. (**b**) Representative SBFSEM slice highlighting segmented organelles. Original micrograph is shown (left), as well as the same image with color overlays indicating segmented organelles (right): exospore (orange), endospore (yellow), PT (blue), vacuole (red), posterior polaroplast (purple), and anchoring disc (green). Magenta arrow indicates the thinnest part of the endospore layer where the anchoring disc is localized. (**c**) Representative 3D reconstruction

cells elongate and secrete electron-dense material on their surface which will become the exospore coat, and then synthesis of the polar filament components begins. The synthesis of the polar filament occurs in sporoblasts which contain a large Golgi (Beznoussenko et al. 2007; Takvorian and Cali 1994). The morphogenesis of the polar filament is first observed in early sporoblasts as an "oval body" of membranes and dense material (Takvorian and Cali 1996). The Golgi is involved in the formation of the polar filament, and polar filament proteins are assembled and posttranslationally modified by the Golgi. Enzyme histochemical reaction products generated by both the *cis*- and *trans*-Golgi (Takvorian and Cali 1994, 1996) surrounding the forming polar filament can be observed by TEM (Fig. 8.7a). During polar filament development, it is thought that polar tube proteins (PTP) are glycosylated. This is consistent with immunogold labeling of extruded polar tubes with concanavalin A (Con A), a lectin which binds to specific sites of glycosylation (Fig. 8.7b) (Xu et al. 2004; Takvorian and Cali 1996). Eventually the filament coils around the inner aspect of the endospore, encased by a labyrinth of membranes surrounding the outer sporoplasm-limiting membrane, indicating that the polar filament is probably external to the sporoplasm contents (Cali et al. 2002).

The diameter, length, and arrangement of polar filament coils in the spore are variable and dependent on the species of microsporidia. The number of coils present can vary depending on species from 4 to approximately 30 (Weiss et al. 2014). There are two regions of the polar filament: the anterior straight portion which connects to the anchoring disk in the anterior end of the spore and the medial-posterior coiled region (Huger 1960; Vavra 1976). The average diameter of the typical polar filament is 90–120 nm in the coiled region, and the straight portion often has a somewhat larger diameter (Chioralia et al. 1998; Takvorian and Cali 1986). Generally, the straight portion will extend to the middle part of the spore and then coils; however, in some species like *Spraguea americana* (formerly *Glugea americanus)*, the straight portion will extend all the way to the posterior part of the spore, then turn anteriorly, and start to coil (Chioralia et al. 1998; Takvorian and Cali 1986; Vavra 1976) (Vavra 1976; Chioralia et al. 1998; Takvorian and Cali 1986).

8.4 The Structure of the Polar Tube

Although it has been more than 60 years since Kramer in 1960 (Kramer 1960) demonstrated the passage of the infective sporoplasm from the spore through the polar tube and Huger's (Huger 1960) first TEM images of the polar filament inside

Fig. 8.4 (continued) of an *Anncaliia algerae* spore from SBFSEM data. Each color represents an individual organelle, color code as in (**b**). Images reprinted with permission from Jaroenlak P, Cammer M, Davydov A, Sall J, Usmani M, Liang F-X, Ekiert D, Kroon G (2020a) 3-Dimensional organization and dynamics of the microsporidian polar tube invasion machinery. PLoS pathogens 16:e1008738. doi:https://doi.org/10.1371/journal.ppat.1008738 (Jaroenlak et al. 2020)

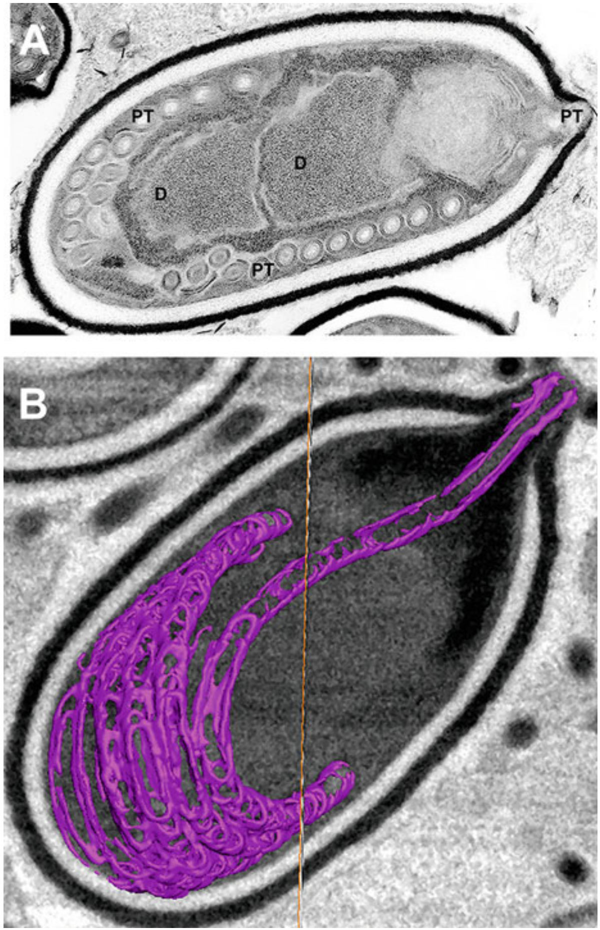

Fig. 8.5 Transmission electron microscope (TEM) image and focused ion beam scanning electron microscope image of an activated *Anncaliia algerae* spore. (**a**) TEM image of an activated *Anncaliia algerae* spore starting the extrusion process. The internal organization of the spore undergoes massive membrane reorganization while the polar filament changes, becoming a hollow tube as it everts and starts exiting the spore through the anterior anchoring disk (A) and the opening of the spore wall. The PF has translocated, and the diplokaryon (D) is in the middle the spore and in a tandem relationship. (**b**) Focused ion beam scanning electron microscope (FIB-SEM) image from a tomogram generated from 15-nm-thick "Z" stack images of an activated *Anncaliia algerae* spore with an extruding polar filament. The polar filament was segmented with Amira© software to produce a 3-D rendering. The spore image has an overlay of the uncoiling polar filament (purple) exiting the anterior thin exospore wall. The spore wall has erupted forming a collar through which the PT is exiting. The collar is composed of part of the anchoring disc, polaroplast, and exospore wall. These various images were obtained by Dr. Peter M. Takvorian with assistance from Dr. William J. Rice and Ashleigh Raczkowski using equipment at the Simons Electron Microscopy Center New York Structural Biology Center 89 Convent Avenue, NY, NY 10027

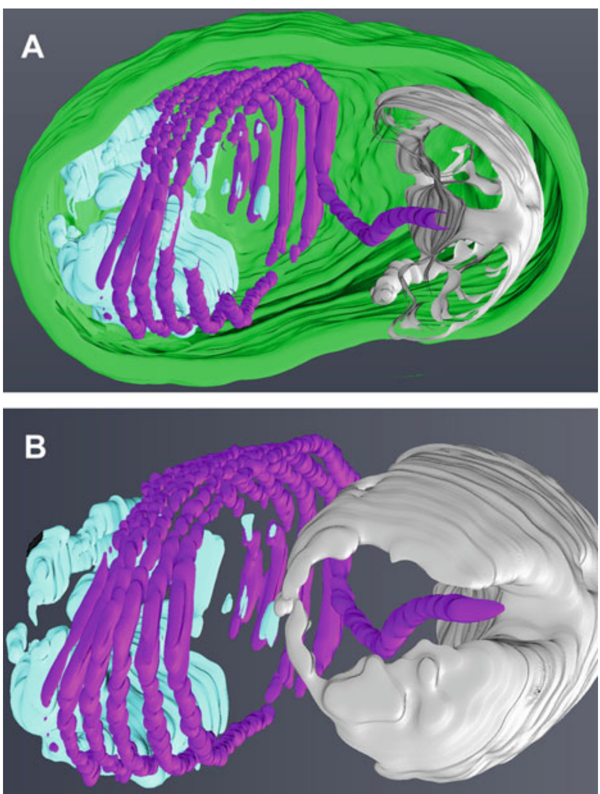

Fig. 8.6 Three-dimensional models generated from "Z" stack images obtained from focused ion beam scanning electron microscopy (FIB-SEM). (**a**) A three-dimensional model generated from the 15-nm-thick "Z" stack image volume (see Fig. 8.5b) obtained with an FIB-SEM. The image stacks of the activated spore are aligned and then segmented with Amira© software. The segmented areas are the exospore (green), the anchoring disc-polaroplast complex (silver), the polar tube (purple), and the posterior vacuole and membranes (light blue). (**b**) A three-dimensional model of the segmented spore (see Fig. 8.5b) containing internal organelles, anchoring disc-polaroplast complex (silver), the polar tube (purple), and the posterior vacuole and membranes (light blue). The removal of the exospore wall and rotation of the model to show the anterior aspect of the PT and anchoring disc complex provides a view of the exiting PT passing through the complex as it uncoils and straightens. These various images were obtained by Dr. Peter M. Takvorian with assistance from Dr. William J. Rice and Ashleigh Raczkowski using equipment at the Simons Electron Microscopy Center New York Structural Biology Center 89 Convent Avenue, NY, NY 10027

the spore, the polar tube structure, protein composition, extrusion mechanism(s), and sporoplasm transport are still enigmatic. The diameter of the extruded polar tube can increase from 100 to 600 nm during the passage of cargo and the sporoplasm, which illustrates the flexibility of the polar tube structural design (Weidner 1972; Lom and Vavra 1963; Weidner 1976). Cryo-transmission electron microscopy (CTEM) has been used to image vitreous frozen unfixed polar tubes and construct computer

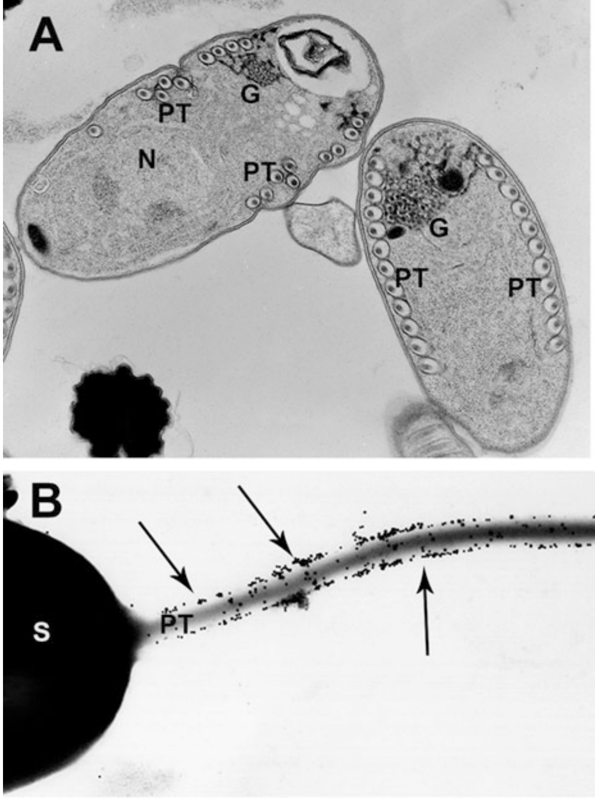

Fig. 8.7 Transmission electron microscope (TEM) image of polar filament formation and post-transcriptional glycosylation of the filament involving the Golgi. (**a**) TEM image of *Glugea stephani* sporoblasts containing developing polar filaments (PF), enzyme histochemically labeled for Golgi. The electron-dense reaction product (RP) outlines each outer layer of the filament, and fenestrated clusters of RP are present on the Golgi complex-filament interface. During PF development, the polar tube proteins are posttranslationally modified by the Golgi. (**b**) TEM image of an *Anncaliia algerae* spore with extruding polar tube. The spore was immune-gold labeled to demonstrate the presence of Con A on the polar tube. Note the large numbers of 12-nm gold particle labeling the polar tube surface. Reprinted with permission from Xu Y, Takvorian PM, Cali A, Orr G, Weiss LM (2004) Glycosylation of the major polar tube protein of *Encephalitozoon hellem,* a microsporidian parasite that infects humans. Infection and immunity 72 (11):6341–6350. doi: https://doi.org/10.1128/IAI.72.11.6341-6350.2004 (Xu et al. 2004)

generated 3-D models of the structure of extruded polar tubes (Takvorian et al. 2020). This study demonstrated that the polar tube surface is covered with fine fibrillary material (Fig. 8.8a) which are probably the sites of modified glycoproteins on the surface of the polar tube (Fig. 8.7b). These polar tube CTEM images demonstrate various structures containing masses of tightly folded or stacked membranes, assorted cargo (Fig. 8.8b), and that the polar tube has a closed tip that can form a terminal sac before the polar tube tip opens (Takvorian et al. 2020). Images of

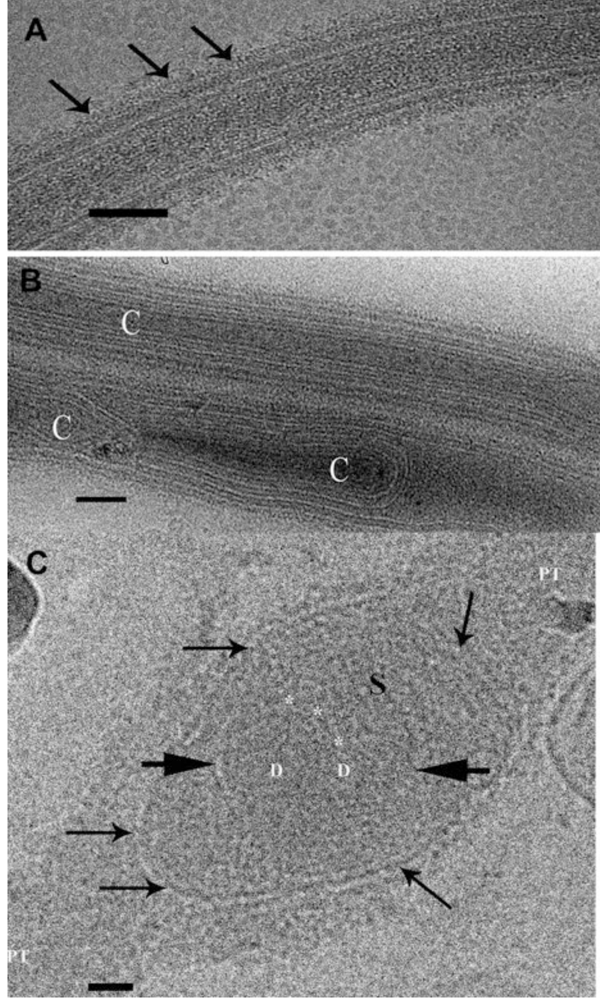

Fig. 8.8 Transmission electron microscope images of *Anncaliia algerae* extruded polar tubes that are cryogenically preserved and imaged while frozen. (**a**) Cryo-TEM image of a cryogenically preserved extruded polar tube (PT). Multiple layers of varying densities are visible. The outermost surface edge is covered with the fine fibrils (arrows). Bar is 50 nm. (**b**) Cryo-TEM image of two polar tube (PT) segments that contain various forms of material. The upper tube has multiple layers of membrane-like material arranged parallel to the orientation of the tube and surrounding a narrow long cylinder (C). The lower PT contains membrane-like and tubular structures, some of which bend around cylinders (C). Bar is 50 nm. (**c**) Cryo-TEM image of a polar tube (PT) that contains a membrane enclosed (arrows) sporoplasm (S) inside the tube. The oval- or sperm head-shaped sporoplasm has greatly distended a portion of the tube. The sporoplasm contains medium-dense material, and a membrane (short arrows) encloses two nuclei in a diplokaryon (D) arrangement. The membrane enclosed nuclear region has an indentation, and three or four small circular structures are abutted to it (*). Bar is 50 nm. Images reprinted with permission from Takvorian PM, Han B, Cali A, Rice WJ, Gunther L, Macaluso F, Weiss LM (2020) An Ultrastructural Study of the Extruded Polar Tube of *Anncaliia algerae* (Microsporidia). J Eukaryot Microbiol 67 (1): 28–44. doi:https://doi.org/10.1111/jeu.12751 (Takvorian et al. 2020)

membrane clusters, cargo, and the sporoplasm traveling through the tube confirmed that the tube can expand its diameter to accommodate the items passing through it. An image of a greatly distended segment of the tube contained a sporoplasm shaped like a sperm head (Fig. 8.8c), which indicates that the sporoplasm traverses the polar tube as a fully intact membrane-bound cellular entity (Takvorian et al. 2020). Multiple aligned and stacked images (tomogram) of a portion of polar tube were segmented, and 3-D models were generated using Amira® software, enabling visualization of the cargo inside the tube and its surface structure. The polar tube surface was covered with tufts of fibrillar material, and its lumen contained membranes, cylinders, and assorted cargo (Fig. 8.9a, b, c, d).

8.5 Protein Composition of the Polar Tube

As noted previously, it was not until 1960 when Kramer demonstrated that the function of the microsporidian polar tube was to transport the infective sporoplasm out of the spore (Kramer 1960), and in the same year, Huger (1960) published the first TEM images of the spore (Huger 1960). These two reports sparked great interest in spore internal structure(s) and the composition and structure of the polar tube. Due to the spore's resilience and resistance to breakage and the difficulty of obtaining large quantities of purified spores, polar tube research was very slow for the next 30 years, despite the widespread interest in its development, structure, and function. Keohane et al. (Keohane et al. 1994; Keohane et al. 1996b) obtained massive quantities of *Spraguea americana* (formerly *Glugea americanus*) spores from centimeter-sized cysts of the optic chiasma and spinal nerves of infected American angler fish *Lophius americanus.* The microsporidian spores were purified, mechanically disrupted with glass beads in a bead beater®, and washed in 1% SDS and 9 m urea, leaving only empty spore shells and polar tubes (Fig. 8.10a). A similar approach was used to produce a polar tube preparation from spores of various Encephalitozoonidae purified from tissue cultures (Keohane and Weiss 1999; Keohane et al. 1999; Delbac et al. 1998a; Delbac et al. 2001). Due to the unusual solubility properties of the polar tube which resist dissociation in 1% SDS and 9 M urea but dissociate in various concentrations of 2-mercaptoethanol (2-ME) or dithiothreitol (DTT), polar tube protein 1 (PTP1) was isolated from ruptured microsporidian spores by treating them with DTT to solubilize the polar tube proteins (PTPs) followed by further purification of the PTPs by reverse-phase high-performance liquid chromatography (HPLC) (Keohane et al. 1994; Keohane et al. 1996b). Subsequently, antibodies were produced to various PTPs and employed for immune localization of these various PTPs utilizing immunofluorescence microscopy (IFA) and/or immunogold electron microscopy (Immuno-EM) (Fig. 8.10b).

Studies of polar tube protein composition to date have identified six distinct PTPs (i.e., PTP1 to PTP6) that are found in most microsporidia (Table 8.1). The first PTP to be identified was PTP1 (Keohane et al. 1996a, c). Amino acid analysis of PTP1 from various microsporidia confirmed that PTP1 is a proline-rich protein containing

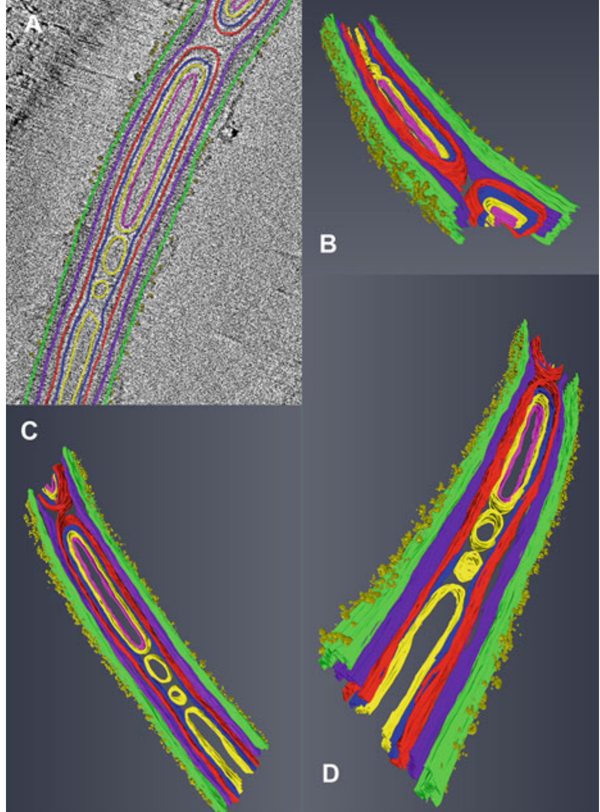

Fig. 8.9 Tomograms and three-dimensional models generated from cryo-TEM "Z" stacks of aligned images. (**a–d**) Tomogram of a portion of polar tube (PT) containing membranes, cylinders, and its surface is covered with tufts of fibrillar material. The tomogram was segmented and 3D models were generated from it using Amira© software. The colors are assigned to different structures inside and on the surface of the polar tube. The fibril tufts are visible on the surface of the PT. The models are tilted at various angles to enable observation of different internal structures and their relationships. Images reprinted with permission from Takvorian PM, Han B, Cali A, Rice WJ, Gunther L, Macaluso F, Weiss LM (2020) An Ultrastructural Study of the Extruded Polar Tube of *Anncaliia algerae* (Microsporidia). J Eukaryot Microbiol 67 (1):28–44. doi:https://doi.org/10.1111/jeu.12751 (Takvorian et al. 2020)

significant amounts of O-linked mannosylation (Keohane and Weiss 1998; Xu et al. 2003; Xu et al. 2004). High proline content is a feature of several structural proteins including collagen and elastin. To this end, the proline content of PTP1 is consistent with the hypothesis that this protein should have a high tensile strength and elasticity, as it is one of the main constituents of the polar tube and these properties would be important for discharge and passage of sporoplasm through the narrow polar tube (Keohane et al. 1996; Keohane et al. 1998; Delbac et al. 2001). Most microsporidian PTP1s have central amino acid repeat regions that are predominantly hydrophilic; however, these repeats differ in composition and number

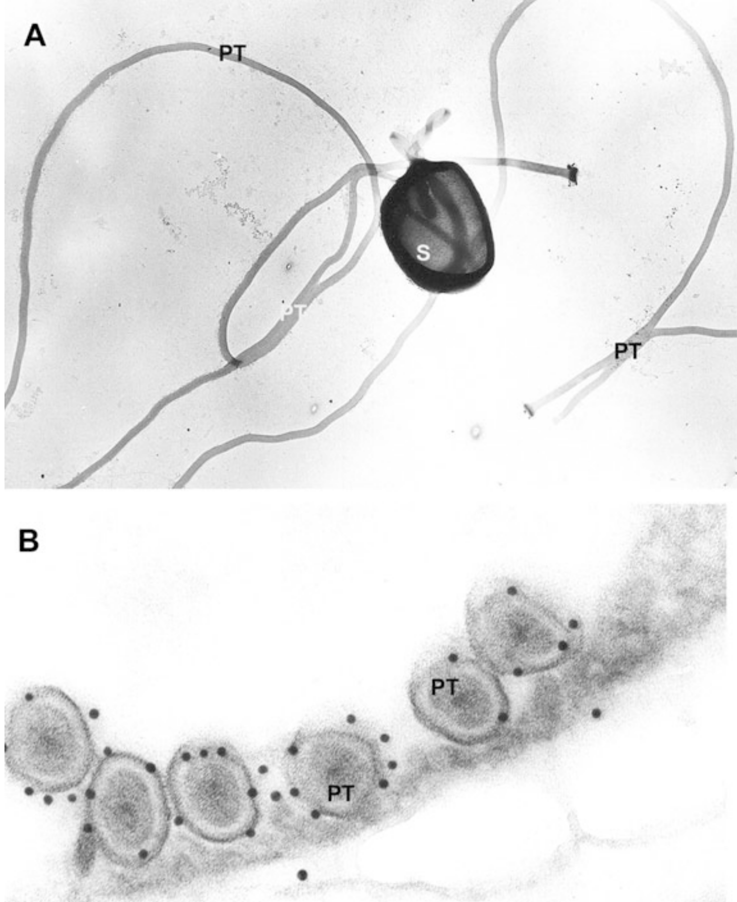

Fig. 8.10 Transmission electron microscope images of a mechanically disrupted spore and polar tubes used during production of polar tube protein antibodies. (**a**) TEM image of *Glugea americanus* (*Spraguea americana*) spores mechanically disrupted with glass beads, washed in 1% SDS and 9 M urea, and negatively stained with uranyl acetate. The spore is broken open, part of the PT is still inside it, and several intact PTs are present. (**b**) TEM image of *Glugea americanus* (*Spraguea americana*) polar filament immunogold labeled for polar tube protein 1 (PTP-1). A cross section of six PF coils is visible and 12-nm gold secondary labels the primary antibody raised against PTP-1. Most of the gold is attached to the outer PT layer. Images reprinted with permission from Keohane EM, Orr GA, Takvorian PM, Cali A, Tanowitz HB, Wittner M, Weiss LM (1996) Identification of a microsporidian polar tube reactive antibody. J. Euk Microbiol. 43(1): 26–31. (Keohane et al. 1996)

depending on the microsporidian species. The N- and C-terminus of the various microsporidian PTP1 display more conservation suggesting that these areas may have important structural or functional domains. An N terminal-signal sequence is cleaved to form the mature protein and is predicted to target PTP1 for processing

Table 8.1 Polar tube proteins (PTPs)

	PTP1	PTP2	PTP3	PTP4	PTP5	PTP6
Encephalitozoon cuniculi	395 aa **ECU06_0250**	277 aa **ECU06_0240**	1256 aa **ECU11_1440**	276 aa **ECU07_1090**	251 aa **ECU07_1080**	238 aa **ECU08_1710**
Encephalitozoon intestinalis	371 aa **Eint_060150**	275 aa **Eint_060140**	1256 aa **Eint_111330**	279 aa **Eint_071050**	252 aa **Eint_071040**	234 aa **Eint_081670** 200 aa **Eint_081680**
Encephalitozoon hellem	453 aa 413 (EhATCC) **EHEL_060170**	272 aa **EHEL_060160**	1284 aa **EHEL_111330**	278 aa **EHEL_071080**	251 aa **EHEL_071070**	225 aa **EHEL_081670**
Encephalitozoon romalae	380 aa **EROM_060160**	274 aa **EROM_060150**	1254 aa **EROM_111330**	280 aa **EROM_071050**	251 aa **EROM_071040**	231 aa **EROM_081700** 198 aa **EROM_081710**
Antonospora locustae	355 aa **ORF1050**[a]	287 aa **ORF1048**[a] 568 aa (PTP2b) **ORF1712**[a] 599 aa (PTP2c) **ORF1329**[a]	Partial sequence	381 aa **ORF969**[a]	242 aa **ORF968**[a]	nd
Paranosema grylli	351 aa	287 aa	Partial sequence	381 aa	Partial sequence	nd
Enterocytozoon bieneusi	nd	283 aa **EBI_26400**	1219 aa **EBI_22552**	nd	nd	nd
Trachipleistophora hominis	nd	291 aa **THOM_1756**	1518 aa **THOM_1479**	Partial sequence **THOM_1575**	259 aa **THOM_1161**	153 aa **THOM_2851**
Nosema ceranae	456 aa **NCER_101591**	275 aa **NCER_101590**	1414 aa **NCER_100083**	208 aa **NCER_100526**	268 aa **NCER_100527**	185 aa **NCER_100577**

Nosema bombycis[b]	409 aa **NBO_7g0016**	277 aa **AEK69415**	1370 aa **AEF33802**	222 aa **ACJZ01000169 (3927–4595)**	271 aa **ACJZ01002324 (213–1028)**	247 aa **NBO_1135G0001**
Anncaliia algerae	407 aa **KI0ABA33YN06FM1**	3 partial sequences	1203 aa **KI0APB23YG12FM1**	254 aa **KI0ANB26YM04FM1**	240 aa **KI0AGA10AA09FM1**	nd
Vittaforma corneae	nd	293 aa **VICG_01748**	Partial sequence **VICG_01948**	254 aa **VICG_01195**	204 aa **VICG_01807**	nd
Vavraia culicis floridensis	nd	291 aa **VCUG_00650**	1864 aa **VCUG_02017**	372 aa **VCUG_02471**	356 aa **VCUG_02366**	nd
Edhazardia aedis	nd	307 aa **EDEG_00335**	1447 aa **EDEG_03869** 1284 aa **EDEG_03429**	465 aa **EDEG_03857**	252 aa **EDEG_03856**	nd
Nematocida parisii	nd	251 aa **NEQG_02488**	1177 aa **NEQG_00122**	nd	nd	nd
Hamiltosporidium tvaerminnensis (Octosporea bayeri)	nd	nd	Partial sequence **ACSZ01010190**	Partial sequence **ACSZ01005588**	212 aa **ACSZ01000826**	nd

aa amino acids

nd not determined, probably because of high-sequence divergence or incomplete assembly of the genome. For PTP1, there are also some differences in the number of amino acids for different strains of *Encephalitozoon cuniculi* and *Encephalitozoon hellem* (Peuvel 2000)

Octosporea bayeri from Broad Institute (http://www.broadinstitute.org/annotation/genome/microsporidia_comparative/GenomesIndex.html)

[a]Antonospora locustae database (http://forest.mbl.edu/cgi-bin/site/antonospora01)

[b]*Nosema bombycis* (annotated sequences of *Nosema bombycis* and *Nosema antheraeae* are deposited in GenBank with the following accession numbers: ACJZ01000001-ACJZ01003558)

through the endoplasmic reticulum and Golgi complex, which is consistent with morphologic observations of polar tube development.

Proteomic and immunologic approaches resulted in the subsequent identification of PTP2 and PTP3 (Delbac et al. 2001; Peuvel et al. 2002). PTP2 is found at the same genomic locus as PTP1. In general, PTP2 is more conserved among microsporidia than PTP1, and PTP2s in various microsporidia that have been identified are similar in molecular weight, basic isoelectric point (pI), high lysine content, and conservation of cysteine residues (Delbac et al. 2001). Some microsporidia (e.g., *Antonospora locusta*, *Anncaliia algerae*, *Paranosema grylli*, and *Tubulinosema ratisbonensis*) have more than one PTP2 gene; however, it is known that the functional significance of having multiple PTP2 protein isotypes is for the biology of these organisms (Polonais et al. 2013). PTP3 was found to be solubilized by SDS treatment of polar tubes, with no requirement for a reducing agent such as DTT, indicating that cysteine bonding is not critical for this protein (Peuvel et al. 2002). It has been suggested that PTP3 might be a scaffold protein that plays an important role during the formation of polar tube by interaction with other PTPs (Peuvel et al. 2002; Bouzahzah et al. 2010). PTP1, PTP2, and PTP3 can be purified as a complex using cross-linking agents suggested that they probably interact directly with each other in the polar tube (Peuvel et al. 2002; Bouzahzah et al. 2010). Further study using yeast two-hybrid methods demonstrated that PTP1 could interact with PTP1, PTP2, and PTP3 at both its C- and N-terminal subdomains, but not its central region (Bouzahzah et al. 2010). This is consistent with the hypothesis that the PTP1 central region, which is highly variable between microsporidia species, is not involved in protein-protein interactions that are needed to form the polar tube.

PTP4 was found by proteomic analysis of purified polar tubes coupled with IFA validation of identified proteins (Han et al. 2017; Weiss et al. 2014; Han and Weiss 2017). A unique epitope of *Encephalitozoon hellem* PTP4 was found to specifically localize to the tip of the polar tube and was shown to bind to the host transferrin receptor 1 (TfR1), which is one of the key receptor proteins in the clathrin-mediated endocytosis pathway (Han and Weiss 2017). An antibody to *Antonospora locustae* PTP4 demonstrates a similar localization to the tip of the polar tube (Weiss et al. 2014). The genes for PTP4 and PTP5 are usually clustered together in microsporidian genomes (e.g., *Anncaliia algerae*, *Encephalitozoon hellem*, *Encephalitozoon intestinalis*, and *Encephalitozoon cuniculi*) which is similar to what is observed with *ptp1* and *ptp2*, suggesting that *ptp4* and *ptp5* may have been linked in either evolution or expression (Weiss et al. 2014; Han and Weiss 2017). An antibody to recombinant PTP5 localizes to the polar tube (Weiss et al. 2014). Recently, a novel polar tube protein (PTP6) was identified from *Nosema bombycis*, a pathogen of silkworms. NbPTP6 was rich in histidine and serine and has multiple glycosylation sites. Further study revealed that this new polar tube protein is capable of binding to the host cell surface indicating a potential role for PTP6 in the process of polar tube interaction with its host cell (Lv et al. 2020). PTP6 homologs can be found in the genomes of other microsporidia (Table 8.1).

In *Nosema bombycis*, several spore wall proteins (NbSWPs) that have been identified have demonstrated interactions with NbPTPs (Wu et al. 2008, 2009;

Wang et al. 2007; Li et al. 2012; Dolgikh et al. 2005; Cai et al. 2011). Using IFA, several of these NbSWPs have been shown to localize to the proximal region of the polar tube in *Nosema bombycis* (Wu et al. 2008, 2009; Wang et al. 2007; Li et al. 2012; Dolgikh et al. 2005; Cai et al. 2011). However, homologs of these proteins have either not been demonstrated in the Encephalitozoonidae, or IFA staining with antibody to potential homologs has not shown similar polar tube localization in the Encephalitozoonidae.

8.6 Glycosylation of Polar Tube Proteins

Microsporidia Golgi are networks of highly anastomosing, and often varicose, tubules and are connected or associated with either the ER or the plasma membrane where the polar tube proteins are synthesized (Fig. 8.7a) (Xu et al. 2004; Takvorian and Cali 1996; Beznoussenko et al. 2007). Glycosylation of transported proteins is one of the main functions of the Golgi. Despite microsporidia lacking the genes encoding the *N*-linked glycosylation machinery as well as the “classical mammalian” *O*-linked glycosylation genes, α-1, 2-mannosyltransferase, which is considered to be Golgi-specific, has been found in microsporidian genomes (Katinka et al. 2001; Lussier et al. 1997). Thus, during sporogony, when microsporidia transport PTPs via the Golgi, various PTPs could be mannosylated by the Golgi-specific-α-1, 2-mannosyltransferase resulting in posttranslational glycosylation of several PTPs (Fig. 8.7b) (Beznoussenko et al. 2007; Han et al. 2017; Lv et al. 2020).

PTP1 is a major polar tube component which accounts for at least 70% of the mass of the polar tube (Keohane et al. 1998). Sequence prediction based on the primary amino acid sequence of PTP1 demonstrates several potential *N*-and *O*-linked glycosylation sites (Xu et al. 2003). Analysis of EhPTP1 by mass spectrometry demonstrated it was larger than predicted by its primary amino acid sequence, and HPLC purification of native PTP1 demonstrated a sawtooth pattern when the peak is spread out with a shallow acetonitrile graduation, suggesting the presence of posttranslational modifications on PTP1 (Xu et al. 2004). A lectin overlay assay demonstrated that ConA reacts with α-mannose residues bound to PTP1 (Xu et al. 2004). In addition, PTP1 could be purified from a DTT-solubilized polar tube mixture by ConA affinity chromatography (Xu et al. 2004). These data indicate that PTP1 contains O-linked mannosylation residues to which ConA binds (Xu et al. 2004). These posttranslational modifications were further confirmed by an immunoprecipitation assay using radiolabeled mannose and glucosamine in *Encephalitozoon cuniculi* (Bouzahzah and Weiss 2010). O-mannosylation of PTP1 might be crucial for the invasion process by protecting the polar tube from degradation in the gastrointestinal tract of hosts. In many eukaryotic pathogens, protein glycan modifications are involved in host pathogen interactions and contribute to adherence and invasion (Varki 1993). Binding of PTP1 with ConA suggested that PTP1 may interact with host cell membrane mannose-binding molecules such as dectin-2, mannose-binding lectin-MBL, and C-type lectin receptors during

microsporidian infection and facilitate infectivity and adherence of the polar tube to the host cell membrane. Mannose pretreatment of RK13 host cells decreased their infection by *Encephalitozoon hellem*, consistent with an interaction between the mannosylation of PTP1 and some unknown host cell mannose-binding molecule (Xu et al. 2004). A CHO cell line (Lec1) that is unable to synthesize complex-type N-linked oligosaccharides and produces increased mannose-rich homogeneous $(GlcNAc)_2(Man)_5$ and O-linked fucose glycoforms had an increased susceptibility to *Encephalitozoon hellem* infection compared to wild-type CHO cells (Xu et al. 2004).

8.7 Interactions of the PTPs, SWPs, and Sporoplasm

The infection of microsporidia to the host cells is a unique, highly specialized invasion process that involves the spore wall (SW), polar tube (PT), and infectious sporoplasm (SP). Before germination, the polar tube coils inside the spore and connects to a mushroom-shaped anchoring disk (AD) at the anterior end of the spore (Vavra 1976). Upon appropriate environmental stimulation, the polar tube will discharge from the apical end of the spore, and the sporoplasm will flow through the hollow tube and appear as a droplet at the distal end (Fig. 8.11a, b) (Ohshima 1937; Han et al. 2017). Interactions between PTPs and SWPs are probably important in the polar tube orderly orientation, arrangement, and anchorage to anchoring disk and support the structural integrity of the spore wall.

Eight spore wall proteins have been identified from the Encephalitozoonidae. *Encephalitozoon cuniculi* spore wall protein 1 (EcSWP1), *Encephalitozoon intestinalis* spore wall protein 1 (EiSWP1), and EiSWP2 are localized to exospore, and *Encephalitozoon cuniculi* endospore wall protein 1 (EcEnP1), EiEnP1, EcEnP2, EcSWP3, and *Encephalitozoon cuniculi* chitin deacetylase (EcCDA) were found to be endosporal proteins (Southern et al. 2007; Peuvel-Fanget et al. 2006; Bohne et al. 2000; Brosson et al. 2005; Hayman et al. 2001; Xu et al. 2006). Ten spore wall proteins (NbSWP5, NbSWP7, NbSWP9, NbSWP11, NbSWP12, NbSWP16, NbSWP25, NbSWP26, NbSWP30, and NbSWP32) were identified by proteomic analysis from *Nosema bombycis* (Wu et al. 2008; Li et al. 2012; Yang et al. 2014, 2017; Wang et al. 2015; Chen et al. 2013; Wu et al. 2009; Li et al. 2009). Immunostaining indicated that NbSWP5, NbSWP7, and NbSWP9 localize to the spore wall and polar tube region (Yang et al. 2017; Li et al. 2012). Co-immunoprecipitation, liquid chromatography-tandem mass spectrometry, and yeast two-hybrid data revealed that NbSWP5 could interact with NbPTP2 and NbPTP3, while NbSWP9 could interact with NbPTP1 and NbPTP2 (Yang et al. 2017; Li et al. 2012). NbSWP7 may bind to the polar tube via the interaction with NbSWP9. NbSWP9 is a scaffolding protein which contributes to the anchoring of the polar tube to the spore wall (Yang et al. 2017). Several of these various SWPs are modified by posttranslational glycosylation involving mannosylation, and these modifications are probably important for spore adherence to mucin or host cells

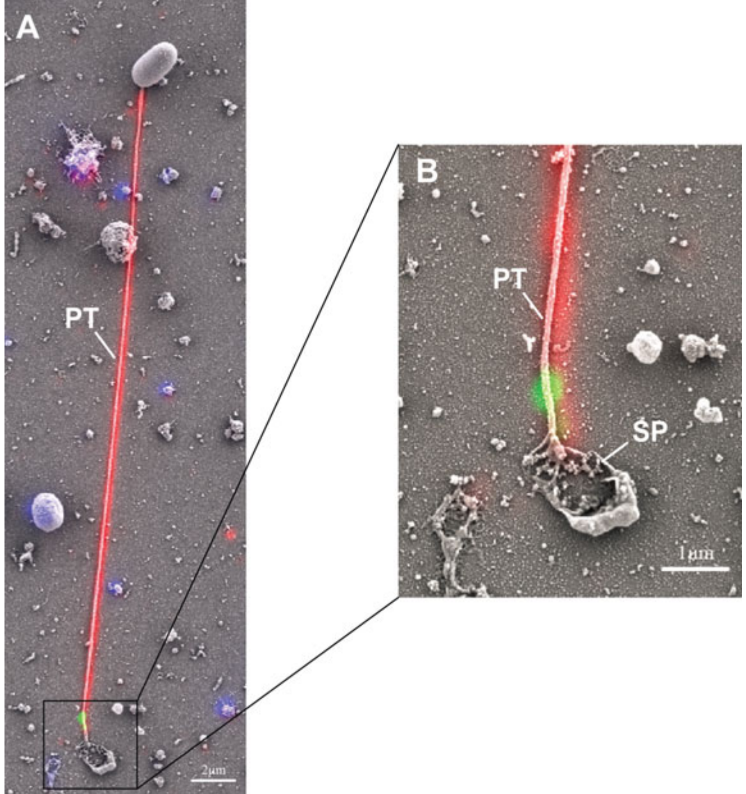

Fig. 8.11 Correlative light and electron microscopy (CLEM) analysis of germination of *Encephalitozoon hellem*. *Encephalitozoon hellem*-infected tissue cultures were incubated with rabbit polyclonal to EhPTP1 (red) and murine monoclonal to EhPTP4 (green). The fluorescence image and SEM image of the same site were taken sequentially, and the fluorescence images with labeling of EhPTP4 and the polar tube were correlated to the SEM images which demonstrated the germination of a microsporidium at high resolution. Panel (**a**) shows an extruded polar tube with EhPTP4 staining at the end of the polar tube (PT). Panel (**b**) shows the enlarged section of panel (A), and the droplet of released sporoplasm (SP) was still attaching to the tip of polar tube. Reprinted with the permission from Han B, Polonais V, Sugi T, Yakubu R, Takvorian PM, Cali A, Maier K, Long M, Levy M, Tanowitz HB (2017a) The role of microsporidian polar tube protein 4 (PTP4) in host cell infection. PLoS pathogens 13 (4):e1006341 (Han et al. 2017)

during passage of spores in the gastrointestinal tract, facilitating invasion; in support of this concept, exogenous glycosaminoglycans decreased adherence of spores to host cells (Dolgikh et al. 2007; Peuvel-Fanget et al. 2006; Hayman et al. 2005; Southern et al. 2007; Cai et al. 2011; Wu et al. 2008, 2009; Li et al. 2009; Zhu et al. 2013). SWPs may also bind to the host cell directly, and this may facilitate invasion, e.g., NbSWP26 has been shown to bind to the turtle-like protein of *Bombycis mori* (BmTLP) (Zhu et al. 2013). Ricin-B lectin-like proteins, which in *Nosema bombycis* were found to increase infection when added to cell cultures (Liu et al. 2016), are

also encoded in the genomes of other microsporidian species (Szumowski and Troemel 2015) and probably interact with carbohydrates found on host proteins facilitating spore binding.

Once polar tube germination is triggered, transportation of the sporoplasm through the polar tube is initiated when the tube has reaches ~50% extension (Jaroenlak et al. 2020). The sporoplasm then appears as a droplet at the distal end of the polar tube and remains attached to the polar tube for several minutes (Weidner 1972). Interactions between PTPs and the sporoplasm during transportation through the polar tube remain to be defined. A study utilizing correlative light and electron microscopy (CLEM) demonstrated that the droplet of released sporoplasm was still attached to the tip of polar tube during passage (Fig. 8.11a, b) (Han et al. 2017). Further study on the interactions of the polar tube and sporoplasm using yeast two-hybrid methods revealed that PTP4, which has a unique protein epitope exposed at the tip of polar tube (Han et al. 2017), could interact with sporoplasm surface protein 1 (SSP1) (Han et al. 2019). The interaction between PTP4 and SSP1 might be important as an anchor that helps to keep the sporoplasm attached to the end of polar tube after spore germination (Han et al. 2019). This interaction may be also be involved in the ability of the polar tube to coil around the sporoplasm within the intact spore, thereby establishing an anchor point for an interaction of the polar tube with the sporoplasm membrane (Han et al. 2019).

During microsporidian invasion of its host cells, EhSSP1 binds to the host cell surface at the site where the polar tube pushes into the host cell membrane (Han et al. 2019). Host cell voltage-dependent anion channels (VDAC1, VDAC2, and VDAC3) have been shown to interact with EhSSP1, and EhSSP1 co-localized with host mitochondria and the microsporidian parasitophorous vacuoles in infected cells (Han et al. 2019). Electron microscopy demonstrated that mitochondria clustered around meronts, that the outer mitochondrial membrane interacted with meronts and the parasitophorous vacuole membrane, and that VDACs were concentrated at the interface of mitochondria and parasite (Han et al. 2019). RNAi knockdown of VDAC1, VDAC2, and VDAC3 in host cells resulted in a significant decrease in the number and size of parasitophorous vacuoles and a decrease in mitochondrial parasitophorous vacuole association (Han et al. 2019). The interaction of EhSSP1 with VDAC probably plays an important part in energy acquisition by microsporidia as they lack mitochondria and are reliant on the host for ATP generation (Goldberg et al. 2008; Williams et al. 2002; Tamim El Jarkass and Reinke 2020).

8.8 The Role of the Interaction of PTPs with the Host Cell During Infection

The polar tube as the unique invasion organelle of microsporidia is the key to the success of these obligate intracellular pathogens. Despite the identification of several PTPs in the last few years, how the polar tube interacts with host cell during infection is still widely unknown. Studies on PTP1 revealed that glycosylation of PTP1 might

be functional in the parasite-host interaction by creating a "sticky" polar tube capable of adhering to the host cell membrane mannose receptors (Bouzahzah and Weiss 2010; Han et al. 2020). A recent study on the PTP4 in *Encephalitozoon hellem* identified a monoclonal antibody (mAb) which recognized an epitope of PTP4 that was exposed only at the distal end of the polar tube. Immunoprecipitation using this PTP4 mAb identified transferrin receptor 1 (TfR1), a host cell surface protein, as the potential interaction target of PTP4 during microsporidian invasion of host cells (Fig. 8.12). An interaction of PTP4 and TfR1 was further confirmed by a fluorescence co-localization assay. Alterations of the interaction between PTP4 and TfR1 either by an antibody blocking assay or by knocking out *tfr1* in host cells could significantly inhibit infection of host cells by microsporidia. These experiments suggest that for the Encephalitozoonidae (and possibly other microsporidia) PTP4 plays a crucial role in the process of invasion by interacting with host cell TfR1 (Han et al. 2017). NbPTP6 has also been shown to bind to the host cell surface, suggesting a potential role for PTP6 in the process of polar tube interaction with host cells (Lv et al. 2020).

It had been postulated that the polar tube functions like a hypodermic needle in penetration of the host cell (Weiss and Bechnel 2014; Han et al. 2020; Xu and Weiss 2005); however, electron micrographs of the polar tube interaction with its host cell demonstrated that an invagination occurs at the site of this interaction (Weiss and Bechnel 2014; Han et al. 2020). When spores are germinated in media, the sporoplasm that emerges from the polar tube tip is very delicate, swells, and breaks, suggesting that survival of the emerging sporoplasm requires a protected environment (Weiss and Bechnel 2014). It is thought that the polar tube invaginates the host cell membrane forming a microenvironment (the invasion synapse) in which final penetration occurs (Fig. 8.12). During infection, the sporoplasm is discharged from the spore through the polar tube into this invasion synapse formed by polar tube and host cell plasma membrane (Han et al. 2020). Interactions of sporoplasm surface proteins and PTPs with the host cell membrane probably occur in this invasion synapse and are likely involved in invasion, e.g., PTP4 binding to TfR1 (Han et al. 2017) and recombinant EhSSP1 has been demonstrated to bind to the host cell surface at the site where the polar tube invaginated the host cell membrane during invasion (Han et al. 2019). An ultrastructural study of *Anncaliia algerae* demonstrated that the extracellular discharged sporoplasm tightly abutted to the host plasmalemma and appeared to be in the process of being incorporated into the host cytoplasm by phagocytosis and/or endocytosis (Takvorian et al. 2005).

8.9 PTPs as Diagnostic and Genotyping Targets for Microsporidia

Microsporidia can parasitize a wide host range from invertebrates to vertebrates and have caused significant threats to human health and agricultural economic losses (Han and Weiss 2017; Stentiford et al. 2016b). The diagnosis of microsporidiosis

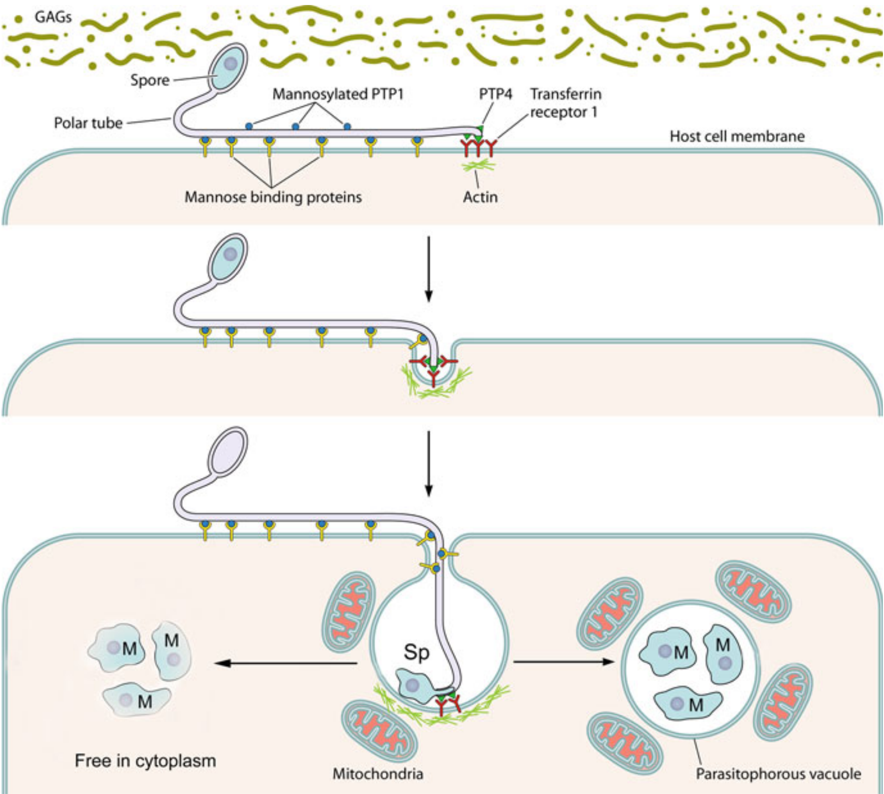

Fig. 8.12 A model of polar tube adherence and invasion. This model is based primarily on data collected using *Encephalitozoon* spp. as a model for host cell invasion. The spore wall contains spore wall proteins (e.g., EnP1, NbSWP7, NbSWP9, NbSWP11, NbSWP12, and NbSWP16) that can interact and adhere to glycosaminoglycans (GAGs) and other substances in the mucin layer (green) of the gastrointestinal track or can interact with GAGs on the surface of host cells. These interactions are probably involved in germination. As the polar tube germinates, the polar tube adheres to the host surface by interactions of polar tube protein 1 (PTP1) with host cell surface mannose-binding proteins (MBP). This allows the polar tube to form an invasion synapse by pushing into the host cell membrane. In the formation of the invasion synapse, interactions of PTP1 (and possibly PTP4) with the host cell membrane result in the establishment of a protected microenvironment for the extruded microsporidian sporoplasm which excludes the external environment. Within the invasion synapse, epitopes of polar tube protein 4 (PTP4) that are exposed at the tip of polar tube interact with transferrin receptor 1 (TfR1), and possibly other host cell interacting proteins (HCIPs), at the host cell plasma membrane triggering signaling events. During the final steps of invasion, these various interactions lead to the formation of the invasion vacuole which can include clathrin-mediated endocytosis as well as the involvement of host cell actin. The sporoplasm (and meront) possess surface proteins, such as sporoplasm protein 1 (SSP1), which interact with various host cell surface proteins tethering the sporoplasm to the plasma membrane during invasion facilitating development of the invasion vacuole. At this early stage of infection, host mitochondria are already located around the invasion vacuole. For microsporidia that develop in the cytoplasm (arrow on the left), such as *Nosema* spp. and *Anncaliia* spp., the organisms penetrate the invasion vacuole, and meronts can be seen undergoing development within the cytoplasm of the host cell. For microsporidia that develop in a parasitophorous vacuole (arrow on

usually involves the identification of spores in clinical samples using light microscopy employing stains such as chromotrope 2R, Calcofluor white (fluorescent brightener 28), and Uvitex 2B; however, these techniques do not identify the species of microsporidia causing an infection (van Gool et al. 1993; Vavra et al. 1992; Weber et al. 1992). Electron microscopy and molecular techniques can be used to identify the species of microsporidia causing an infection (Procop 2007). In general, molecular techniques are faster, more sensitive, and specific when compared with regular diagnostic methods for microsporidiosis (Procop 2007). Each of these detection methods has limitations: light microscopy depends on the professional skill and subjective judgment of technicians; electron microscopy is expensive, time-consuming, and unavailable for routine diagnosis at all laboratories (Thellier and Breton 2008); and molecular techniques such as loop-mediated isothermal amplification (LAMP), nested PCR, and qPCR while highly sensitive can produce nonspecific amplicons and false-positive results if an inappropriate diagnostic target is used (Suebsing et al. 2013; Jaroenlak et al. 2016).

PTP2 has been identified in many species of microsporidia including *Encephalitozoon cuniculi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, *Nosema ceranae*, *Nosema bombycis*, *Paranosema grylli*, and *Enterocytozoon hepatopenaei* (Polonais et al. 2005; Cornman et al. 2009; Pan et al. 2013; Kanitchinda et al. 2020). *Enterocytozoon hepatopenaei* infects Pacific white leg shrimp *Penaeus vannamei* causing growth retardation and uneven size distributions that lead to severe losses in shrimp productivity (Jaroenlak et al. 2018). The *Enterocytozoon hepatopenaei ptp2* gene shares low nucleotide sequence similarity with *ptp2* homologues from other microsporidia. A recent study on the detection of *Enterocytozoon hepatopenaei* demonstrated that *ptp2* provided high sensitivity and specificity as a target gene for assays using recombinase polymerase amplification (RPA), CRISPR-Cas12a fluorescence, and SYBR Green I fluorescence quantitative PCR methods (Kanitchinda et al. 2020; Wang et al. 2020).

Serological diagnosis using recombinant PTPs as antigens either by immunoblotting or enzyme-linked immunosorbent assay could be potential tools to evaluate the prevalence of microsporidia in populations (Xu and Weiss 2005). It was reported previously that 8% of Dutch blood donors and 5% of pregnant French women had an IgG immune response against the polar tube of *Encephalitozoon intestinalis* (Gool et al. 1997). Carbohydrate moieties of microsporidian PTP1 have been reported to be

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Fig. 8.12 (continued) right), such as *Encephalitozoon* spp., the invasion vacuole completes its internalization of the sporoplasm; it becomes a meront and starts replicating. The meront surface interacts with the invasion vacuole membrane forming electron-dense membrane structures that allows meront SSP1 to interact with voltage-dependent anion selective channels (VDAC) located on the outer membrane of the mitochondria. The interaction of SSP1 and VDAC appears to play a crucial role in association of host cell mitochondria with the invasion vacuole facilitating energy acquisition from the host cell by replicating meronts. Adapted with permission from Han B, Pan G, Weiss LM (2021) Microsporidiosis in Humans. Clin Microbiol Rev.:e0001020. doi:https://doi.org/10.1128/CMR.00010-20 (Han et al. 2021)

targeted by IgG in immunocompetent individuals, and these antibodies were shown to decrease the infectivity of microsporidia in vitro (Peek et al. 2005). In another report, the class-specific anti-polar tube antibodies found in healthy and HIV-infected individuals were examined using an ELISA assay (Omura et al. 2007). Interestingly, 36% of the individuals were IgM-positive, but no IgG or IgA reactions were detected (Omura et al. 2007).

Molecular tools have been developed and employed as tools for detailed epidemiologic studies of transmission of infection, sources of infection, and analysis of subtypes of microsporidian species. A series of markers such as the internal transcribed spacer (ITS) of ribosomal DNA, the polar tube protein 1 (PTP1) gene, and two intergenic spacers (IGS-TH and IGS-HZ) have been utilized for genotyping of microsporidia (Haro et al. 2003). These markers have been used to look at zoonotic transmission of various microsporidia by defining specific subtypes seen in different animals. In general, genotyping of microsporidia using ITS markers has not been used as a routine test in most diagnostic laboratories due to technical demands and high cost (Xiao et al. 2001b). The gene coding sequence of PTP1 of *Encephalitozoon cuniculi* and *Encephalitozoon hellem* has long central repeats of 78 bp and 60 bp, and these regions have been used to examine the genetic diversity of isolates of these microsporidia (Delbac et al. 1998b; Keohane et al. 1998). Nucleotide sequence analysis of the PTP1 gene divided 11 *Encephalitozoon cuniculi* isolates into 3 genotypes and 24 *Encephalitozoon cuniculi* isolates into 4 genotypes (Xiao et al. 2001a, b).

8.10 Conclusion and Future Perspectives

Microsporidia have been identified as pathogens that have important effects on our health, food security, and economy (Stentiford et al. 2016b). Human microsporidiosis represents a significant emerging opportunistic disease, once thought to be restricted to immune-compromised patients; however, infections in immune-competent individuals also occur (Sak et al. 2011; Didier and Weiss 2011). The polar tube is part of the unique invasion organelle of microsporidia which plays a critical role during microsporidia infection. Despite its description over 125 years ago by Thelohan (Thelohan 1894), we still do not understand the details of how this structure functions and the exact mechanism by which microsporidia enter their host cells and establish a host-pathogen relationship. Progress, however, has been made in understanding the proteins in this invasion apparatus and the interaction of these proteins with some host cell proteins. Understanding how microsporidia use host cell proteins in both invasion and egress will provide insight into their impact on hosts and enhance our current understanding of the transmission dynamics of these pathogens. Due to the importance of various PTPs such as PTP1 and PTP4 in the invasion process, drugs or antibodies which could inhibit the interaction of PTPs with host proteins should provide information needed for new therapeutic approaches to control these pathogens.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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Chapter 9
Mechanics of Microsporidian Polar Tube Firing

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Abstract As obligate intracellular parasites with reduced genomes, microsporidia must infect host cells in order to replicate and cause disease. They can initiate infection by utilizing a harpoon-like invasion organelle called the polar tube (PT). The PT is both visually and functionally a striking organelle and is a characteristic feature of the microsporidian phylum. Outside the host, microsporidia exist as transmissible, single-celled spores. Inside each spore, the PT is arranged as a tight coil. Upon germination, the PT undergoes a large conformational change into a long, linear tube and acts as a tunnel for the delivery of infectious cargo from the spore to a host cell. The firing process is extremely rapid, occurring on a millisecond timescale, and the emergent tube may be as long as 20 times the size of the spore body. In this chapter, we discuss what is known about the structure of the PT, the mechanics of the PT firing process, and how it enables movement of material from the spore body.

Keywords Polar tube · PT · PT configuration · Extruded PT · PT structures · PT firing · Spore germination · PT firing dynamics · Cargo transport · Nuclear deformation

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9.1 Introduction

Microsporidia are a group of obligate intracellular, eukaryotic parasites that are classified as a sister group to fungi (Keeling and Doolittle 1996; Lee et al. 2008). About 1500 species of microsporidia have been discovered, infecting a wide range of hosts from vertebrates including humans (Cali et al. 1993; Teachey et al. 2004) to invertebrates such as silkworms (Gupta et al. 2016). Outside of their hosts, microsporidia exist as spores, incapable of replicating on their own. One of the defining characteristics of microsporidia is the presence of a harpoon-like invasion organelle known as the polar tube (PT), which is wound as a coil resembling a spring, inside the spore (Kudo 1918; Han et al. 2020; Jaroenlak et al. 2020). Upon an appropriate stimulus, germination of the PT occurs extremely rapidly, on a millisecond timescale, making it one of the fastest known biological processes (Frixione et al. 1992; Jaroenlak et al. 2020), and visually reminiscent of nematocyst discharge, which is even faster (Nüchter et al. 2006). The exact mechanism of PT-mediated infection is not clearly understood. Some lines of evidence point toward the PT piercing through a host cell membrane to deliver its infectious cargo directly into the host (Weidner 1972; Takvorian et al. 2005), while other studies suggest that the PT may be latching onto a host cell membrane to form an invasion synapse, which enables the phagocytosis of the sporoplasm (Han et al. 2017). In some microsporidian species, the entire spore can be phagocytosed by the host cell (Couzinet et al. 2000; Cai et al. 2012). It is unclear which mode or modes of entry may lead to a productive infection. In addition to being involved in gaining entry into the host cell through the plasma membrane, it has been suggested that the PT may also be used to cross other membranes within a host cell. For example, in *Encephalitozoon cuniculi*, it has been suggested that the PT is used to escape from the phagolysosome compartment after the spore has been phagocytosed (Franzen et al. 2005). Ultimately, whether germination occurs in a phagolysosome or in the extracellular environment may vary between species, and the relative importance of these two possibilities is unclear.

Even though the PT was first described over 125 years ago (Thelohan 1894), many aspects of its structure, function, and mechanism remain unknown. The mechanism of how PT firing is triggered, along with its tremendous conformational change from a tight coil to a long linear filament, is not well understood. Furthermore, the PT organelle is evolutionarily unrelated to other biological filaments such as microtubules and actin. To add to their unique properties, PTs are extremely stable. The PT structure can resist dissociation in 1–3% SDS (Weidner 1976, 1982; Keohane et al. 1996a), 1% Triton X-100 (Weidner 1982), 1–10% H_2O_2 (Weidner 1982; Keohane et al. 1996a), 5–8 N H_2SO_4 (Weidner 1976, 1982), 1–2 N HCl (Weidner 1982), chloroform (Weidner 1982), 1% guanidine HCl (Weidner 1982), 0.1 M proteinase K (Weidner 1982), and 9 M urea (Weidner 1982; Keohane et al. 1996a), suggesting it is a hardy structure capable of resisting harsh chemical conditions. The PT can be solubilized, however, in the presence of a reducing agent such as dithiothreitol (DTT) which suggests that disulfide bonds are important

in stabilizing the structure (Weidner 1976, 1982; Keohane et al. 1996a). These properties have been exploited to purify parts of the PT that can withstand these harsh treatments, from which a few core proteins have been identified. The proteins that have been identified are known as polar tube proteins (PTPs). Four PTPs have been shown to localize along the length of the PT by immunofluorescence studies (Keohane et al. 1996b, 1998; Delbac et al. 2001; Peuvel et al. 2002; Nagajyothi et al. 2010; Lv et al. 2020), suggesting that these proteins may be involved in forming the core structure of the PT. It is likely that other proteins yet to be identified are also important in forming the PT structure and facilitating interactions between the PT and host cell membranes.

Specific conditions are important in modulating the germination of microsporidian spores, thus preventing premature and unproductive germination events in the environment outside target host cells and tissues (Undeen and Epsky 1990; Keohane and Weiss 1999). Although in vivo conditions that trigger germination are not well understood, in vitro conditions have been studied to trigger firing, and these vary among microsporidian species (Keohane and Weiss 1999; Weiss et al. 2014). Some of the factors important for initiating spore germination include changes in pH (Ishihara 1967; Undeen and Epsky 1990) and presence of certain alkali metal cations (Frixione et al. 1994) or polyanions (Pleshinger and Weidner 1985). Once fired, the emerging tube serves as a conduit to transfer cargo such as the spore nucleus into a host cell where infection is initiated.

The PT, which occupies a small fraction of the total spore volume (3.5%), must attain a balance of strength and flexibility which enables it to undergo large conformational changes into a linear tube and transport bulky spore content into target cells (Jaroenlak et al. 2020). In this chapter, we describe current knowledge on the structure of the PT, how it is triggered to fire, the firing process, and how cargo is transported through the tube.

9.2 Structural Features of the Microsporidian Polar Tube

In the dormant spore, before germination, the PT is coiled like a spring. After it has germinated, it takes the form of a linear tube. Below we discuss what is known about the organization and structure of the PT in both the pre-germinated and post-germinated states.

9.2.1 Configuration of the Polar Tube in the Dormant Microsporidian Spore

The PT is a defining feature of the phylum microsporidia, and its unique ultrastructure inside the microsporidian spore has enabled a concrete method of detecting

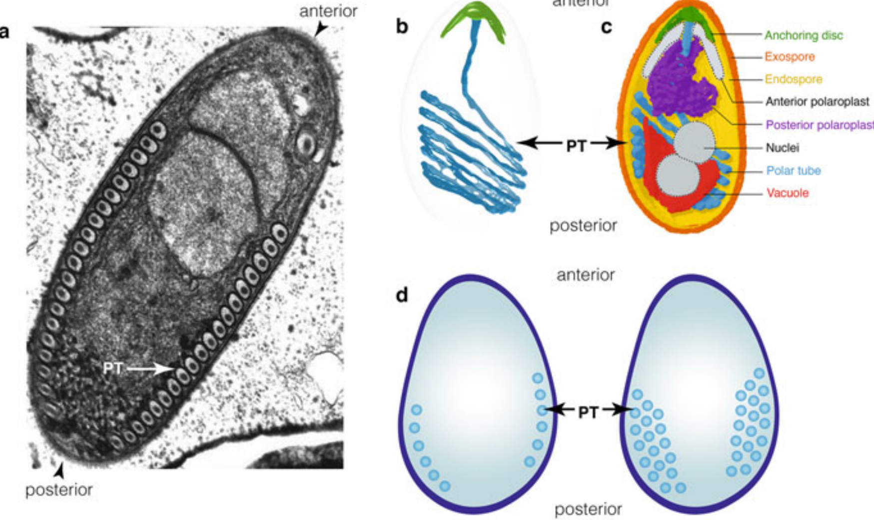

Fig. 9.1 PT configuration inside the dormant spore. (**a**) Transmission electron micrograph section of a sporoblast (developing spore) of microsporidian *Fibrillanosema crangonycis*. The closely packed circular coils at the edge of the sporoblast correspond to the PT. The PT coils appear to be made of multiple concentric layers of varying electron densities. Reprinted from Wikipedia. Permission is granted under the Creative Commons Attribution 3.0 Generic License to reuse. Original photograph taken by Leon White (Naranjo-Ortiz and Gabaldón 2019). (**b**) Representative 3D reconstruction of PT (blue) of *A. algerae*. The PT is connected to the anchoring disc (green) at the anterior end of the spore. The PT is composed of linear and coiled parts. The coiled region is a right-handed coil. Adapted from Jaroenlak et al. (2020). (**c**) Model for 3D organization of a microsporidian spore, based on TEM and a 3D reconstruction of the spore, adapted from Adapted from Jaroenlak et al. (2020). The PT and other organelles are annotated. (**d**) Two main kinds of PT arrangement can be seen across microsporidian species. The PT coils are arranged either in a single row (left) or in multiple rows (right) at the perimeter of the spore (**b**, **c**: reprinted from Jaroenlak et al. 2020. Permission is granted under the Creative Commons Attribution 4.0 Generic License to reuse)

microsporidian infection in tissues for diagnostic purposes. Genomic analyses suggest that development of the PT was one of the earliest steps in microsporidia evolution, and the PT is present in all microsporidia species discovered to date (Wadi and Reinke 2020). The PT is easily identifiable in transmission electron microscopy (TEM) sections of microsporidian spores (Fig. 9.1a) (DeGirolami et al. 1995; Xu and Weiss 2005; Wang et al. 2019). These two-dimensional sections show that the PT structure can be divided into two parts: a linear part, which emerges from the anterior region of the spore and extends toward the posterior end, and a coiled part, which usually appears as a series of circular cross sections in the posterior half of the spore (Fig. 9.1a) (Freeman et al. 2013; Han et al. 2017; Wang et al. 2019).

Recently, three-dimensional reconstructions of intact, mature microsporidian spores were generated using a technique called serial block-face scanning electron

microscopy (SBF-SEM), which provided insights into how the PT was packed in the spore. SBF-SEM is a scanning electron microscopy technique in which serial sections through a field of spores are collected in a high-throughput manner, allowing for the reconstruction and analysis of many spores in 3D. Reconstructions of the PT inside the spore are available in 3D for two species, *Anncaliia algerae* and *Encephalitozoon hellem* (Jaroenlak et al. 2020). 3D reconstructions of the PT inside *A. algerae* spores provide information on how this structure is organized. These 3D reconstructions show the following: (1) The linear part of the PT directly connects to the coiled part of the PT about halfway down the spore, resulting in one end of the PT being at the very posterior end of the spore (Fig. 9.1b). (2) The planes of the PT coils are parallel to each other but are tilted relative to the anterior-posterior (AP) axis of the spore (Fig. 9.1b, c). Such a slanted orientation may arise due to the asymmetric distribution of organelles inside the spore or due to the distribution of the machinery involved in building the PT during development. (3) The PT is a right-handed coil (Fig. 9.1b). This right-handed bias may arise as a result of the structural elements that build the PT or alternatively due to asymmetry in the machinery involved in PT assembly. These features of overall PT organization have also been observed in the 3D reconstructions of another species, *E. hellem*, which is distantly related to *A. algerae.* While the overall organization of the PT is very similar in both species, there are subtle differences in packing of the tube between these species. For instance, *E. hellem* PT coils are less tilted relative to the AP-axis than *A. algerae*, and the coils also have a smaller distance between them, resulting in a more tightly packed PT (Jaroenlak et al. 2020). These subtle differences in PT organization may have arisen to accommodate variable features between the spores of different microsporidian species such as PT length, spore shape, dimensions, and the cargo present inside the spore (e.g., the presence of a single nucleus in *E. hellem* versus two nuclei in *A. algerae*).

The PT traverses the spore, from the anterior end to the posterior end, and therefore closely interacts with several other organelles and structures in the spore (Fig. 9.1c). The linear portion of the PT is closely associated with a membranous structure called the polaroplast, while the coiled portion of the tube curves along the body of the spore, forming a cage around the nucleus (Canning et al. 2005; Toguebaye et al. 2014; Tokarev et al. 2018; Jaroenlak et al. 2020). The anterior end of the PT is connected to the spore wall via a dome-shaped structure called the anchoring disc, located at the anterior end of the spore (Lom et al. 2001; Canning and Curry 2004; Fokin et al. 2008; Jaroenlak et al. 2020). At the tip of the anchoring disc, the endospore is the thinnest, and the PT emerges from this point during the germination process (Fig. 9.1c) (Lom et al. 2001; Canning and Curry 2004; Fokin et al. 2008; Jaroenlak et al. 2020). The PT has been observed to be connected to the anterior end of the spore via the anchoring disc in two different orientations. In *A. algerae,* the PT and anchoring disc are connected at the center of the apical tip of the spore, whereas in *E. hellem*, they are most often found to be attached near the apical end, but off-center and slightly away from the AP-axis. The posterior vacuole lies within the cage created by the PT, and the vacuole membrane is interdigitated with the PT, suggesting a possible interaction between these two organelles

(Jaroenlak et al. 2020). The posterior vacuole has also been implicated in playing a role in the PT germination process (Undeen and Frixione 1990).

In some species, the circular cross sections of the coils are arranged in a single row along the edge of the plasma membrane (Canning et al. 1999), while in other species, the coils may form multiple rows along the edge of the plasma membrane (Fig. 9.1d) (Abdel-Ghaffar et al. 2012; Toguebaye et al. 2014). The PT coils may be uniform in width along its entire length (isofilar), or the coils toward the anterior part of the spore may appear wider and thicker relative to the posterior end (anisofilar) (Canning et al. 1998). While the PT is conserved in microsporidia and may serve the important purpose of being an invasion apparatus for all known microsporidia species (Wadi and Reinke 2020), it has undergone unique ultrastructural and organizational changes in various species.

One of the most obvious ways in which the PT differs across different species is in terms of its length, but the reason for this is not well understood. The number of PT coils observed in TEM sections, which is a proxy for the length of the PT, is different across species. A range of coil numbers have been observed. For instance, TEM sections of *Encephalitozoon intestinalis* show an average of five coils (DeGirolami et al. 1995; Murareanu et al. 2021), whereas TEM sections of another species, *Glugea jazanensis*, show 28–30 coils (Abdel-Baki et al. 2015; Murareanu et al. 2021). The exact number of coils observed may differ, depending on the angle at which the TEM section is cut, but the number of coils is proportional to the length of the PT for a given species and is different among species. Approximate PT lengths can be estimated using measurements from the 2D TEM sections and have been calculated in this manner for an extensive list of microsporidian species (Murareanu et al. 2021). Since these calculations are made using 2D images from thin slices cut at different angles and the volume/shape of the spore is not accurately determined, some inaccuracies may be expected. The length of the PT in its coiled state can be most accurately measured from 3D reconstructions and was found to be 20 μm for *E. hellem* and 42 μm for *A. algerae* (Jaroenlak et al. 2020). A direct comparison of measurements made from 3D data (20 μm) and calculations from 2D data (25.5 μm) for *E. hellem* suggests that calculations from 2D images can provide a useful estimate, but may not be as accurate as measurements made in 3D. After the PT has fired from the spore, the length can be measured by optical microscopy, and experimentally determined extruded PT lengths range from 2.5 to 420 μm (Murareanu et al. 2021). The reasons for variation in PT lengths across species are not well understood. One hypothesis is that PT length variation represents an adaptation to specific host cells or tissues. An observation that supports this hypothesis is that two species of microsporidia that infect different tissues in *Caenorhabditis elegans* have PT with different lengths. *Nematocida parisii*, which infects the intestine, has a shorter PT (4.03 μm, post-germination) compared to *Nematocida displodere* which infects the muscle and epidermis in addition to the intestine and has a longer PT (12.55 μm, post-germination) (Luallen et al. 2016). Although the initial site of infection for both species is the intestinal lumen of *C. elegans*, *N. displodere*'s long PT may be able to pierce through multiple layers of membranes to infect the muscle and epidermis, which lie behind the intestine,

whereas the short PT of *N. parisii* would not be able to reach these tissues (Luallen et al. 2016).

A closer look at the circular cross section of the PT shows that it is composed of multiple electron-dense and electron-lucent concentric layers, with a dark density at the center. The number of these concentric layers can vary between different coils of the same spore (Lom 1972; Sinden and Canning 1974; Chioralia et al. 1998b; Cali et al. 2002; Freeman et al. 2013; Han et al. 2017; Wang et al. 2019). It has been observed that the linear part of the PT may consist of fewer layers than the coiled part of the tube (Chioralia et al. 1998a, b).

While the exact topology of the PT is not well understood, it is expected that the PT is an extracytoplasmic structure (Lom and Vavra 1963), as the proposed structural components of the PT, the PTPs (Chap. 8), are predicted to contain N-terminal secretion signal peptide sequences. A TEM section of an *A. algerae* spore shows the PT surrounded by a membrane, separated from the spore cytoplasm, suggesting that the PT may be topologically outside the plasma membrane (Cali et al. 2002).

9.2.2 Structural Features of the Extruded Polar Tube

After firing from the spore, the coiled PT becomes a linear structure. When observed under a light microscope, the PT fires along the direction of the anterior-posterior axis and appears as a long, linear filament (Fig. 9.2a). The emerging PT is connected to the spore body through a collar-like structure that is believed to be formed by the anchoring disc in the dormant spore (Fig. 9.2b) (Lom 1972; Cali et al. 2002).

Cryo-electron tomography of the extruded *A. algerae* PT showed several different arrangements of membranes within the tube (Takvorian et al. 2020). At this resolution, the individual electron-dense layers of the tube wall can be clearly resolved (Fig. 9.2c) (Takvorian et al. 2020). Various cargos such as membranes, vesicles, and sacs containing dense particulate material can also be observed. Some images show a multilayer appearance where the PT contained a membrane-limited undulating tube within it (Weidner 1972, 1982; Weidner et al. 1995; Takvorian et al. 2020). In some images, a uniform array of lines, estimated to be 64 Å apart, was observed along the length of the PT (Fig. 9.2d). The pattern observed in a Fourier transform of this view of the PT suggests the presence of a helical structure (Takvorian et al. 2020). The diameter of the *A. algerae* PT was found to be between 100 and 135 nm, and parts of the tube containing cargo often showed a slightly increased diameter, up to 220 nm, suggesting the skeleton of the tube is flexible enough to undergo small changes in dimensions to accommodate cargo of varying sizes (Takvorian et al. 2020). The length of the PT has been observed to increase after firing (Jaroenlak et al. 2020), and its length shortens a few seconds after sporoplasm emergence (Weidner 1972; Frixione et al. 1992; Jaroenlak et al. 2020), further supporting the idea that the PT is flexible. Balanced with flexibility, rigidity is also important for the PT as it must penetrate through layers of membranes to deliver cargo into host cells.

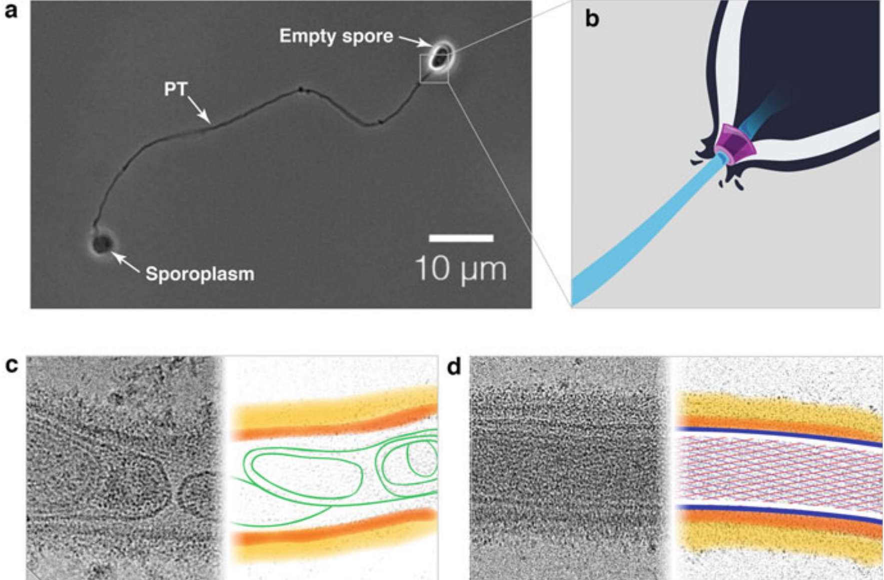

Fig. 9.2 Structural characterization of the extruded PT. (**a**) Phase-contrast micrograph of in vitro germinated *A. algerae* spore. The PT, empty spore, and released sporoplasm are annotated. (**b**) Diagram of the anterior end of the spore from where the PT emerges. The collar-like complex (purple) is thought to be formed by the structure that would previously be the anchoring disc in a dormant spore. (**c**, **d**) Cryo-electron microscopy micrographs of portions of an *A. algerae* PT. Unpublished cryo-EM images kindly provided by Louis Weiss (Takvorian et al. 2020). Distinct features observed in the micrographs have been annotated with different colors. (**c**) PT with cargo inside (green). (**d**) PT of a uniform diameter, showing a mesh-like pattern (pink) which represents the underlying helical arrangement of the structural components of the PT. Two other layers observed along the wall of the tube are annotated in orange and blue. Fuzzy pattern observed at the walls of both micrographs (yellow) represents glycosylation

Proteomic studies of the PT have identified several PTPs, discussed extensively in Chap. 8, some of which are proposed to form the structural basis of the PT. The PTPs have been predicted to contain numerous O-linked and N-linked glycosylation sites (Weidner 1976; Keohane et al. 1996a, c, 1998; Delbac et al. 2001; Nagajyothi et al. 2010; Lv et al. 2020). Genome sequencing has revealed that microsporidia can perform O-linked glycosylation and initial steps of N-linked glycosylation but are unable to form complex N-linked glycans. The presence of mannosyltransferase genes hints that the major sugar used for O-linked glycosylation would be mannose (Katinka et al. 2001; Dolgikh et al. 2007; Beznoussenko et al. 2007; Lv et al. 2020). Concanavalin A has been shown to bind the extruded PT via immunogold EM, suggesting that the PT is glycosylated (Xu et al. 2004). TEM studies on the *A. algerae* PT revealed a fuzzy surface, also suggestive of glycosylation (Takvorian et al. 2020). PT glycosylation may serve many functions for microsporidia, including increasing virulence by facilitating adhesion of the PT onto host cells and

contributing to the overall structural stability of the PT (Xu and Weiss 2005; Han et al. 2017).

9.3 Germination of Microsporidian Spores

Germination of microsporidian spores ultimately leads to the transfer of the infectious sporoplasm from the inside of the spore into the interior of a target host cell. The germination process can be divided into two distinct steps: (1) initiation, wherein the spore responds to cues from the host (or germination buffer, in vitro) and prepares for the release of the spore contents, and (2) rapid extension of the PT and cargo translocation through the tube. These two steps will be discussed in more details in the following sections.

9.3.1 Initiation of Spore Germination

Microsporidia spore germination was first described by Thelohan in 1894 (Thelohan 1894), who used nitric oxide to trigger PT discharge in vitro (Thelohan 1894). Since then, many studies have been conducted to understand conditions that trigger the germination process in several microsporidian species in vitro. Common in vitro triggers include a shift in pH (Ishihara 1967; Undeen and Epsky 1990), high salt concentration (Frixione et al. 1994), and alkali metals (Frixione et al. 1994). In vitro germination conditions for each microsporidian species have been extensively reviewed (Keohane and Weiss 1999; Weiss et al. 2014). Reported spore activation conditions diverge widely among microsporidian species, and a systematic study of in vitro triggers across species has not yet been carried out, limiting our understanding of generalized mechanisms of PT germination initiation. To some extent, differences in triggers may reflect the adaptation of microsporidia to different environments (Undeen and Epsky 1990). Since microsporidia infect a wide range of hosts such as terrestrial and aquatic animals, specific activation of spore germination could prevent an accidental firing of the PT in unproductive environments lacking their target host tissue (Keohane and Weiss 1999). In vivo triggers for spore germination still remain largely unknown. Below we summarize a few in vitro triggers for spore germination (Fig. 9.3) and how these may inform us about the first initiation step of PT firing.

9.3.1.1 In Vitro Activation by pH, Alkali Metal Cations, and Anions

pH is a common trigger for germination, and various pH conditions have been reported to promote spore germination. Optimal pH of the germination buffer is required to yield the maximum percentage of germinated spores (Pleshinger and

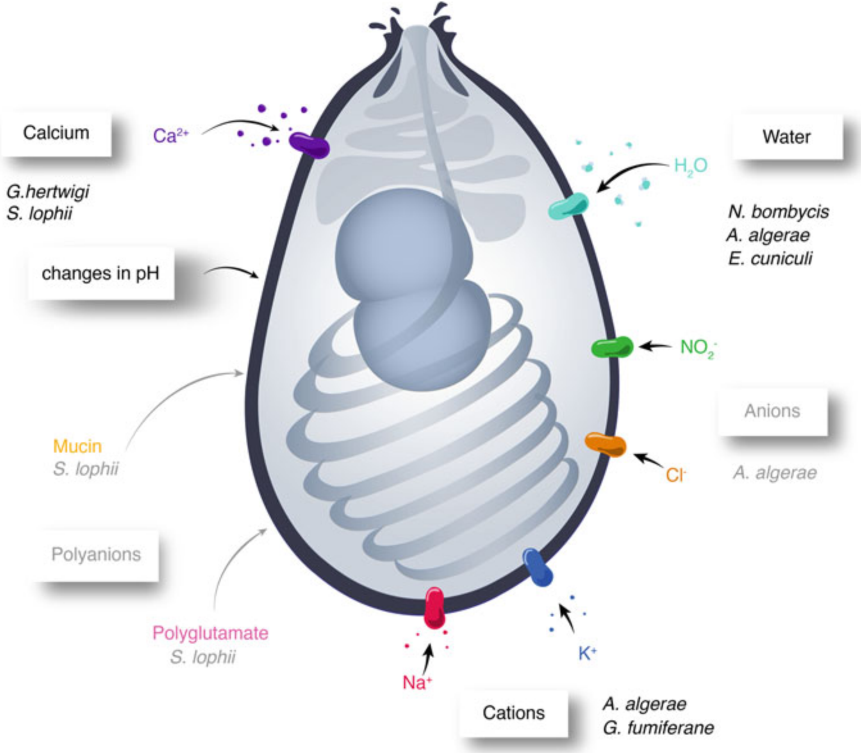

Fig. 9.3 Possible factors important for initiation of PT firing. In vitro conditions triggering spore germination of many microsporidian species are shown in white boxes. These conditions include pH, alkali cations (e.g., Na^+ and K^+), anions (e.g., Cl^- and NO_2^-), calcium (Ca^{2+}), water influx (possibly through aquaporin channels), and polyanions such as mucin and polyglutamate. Conditions listed in black indicate that they were reported to be important for triggering germination in more than one microsporidian species. Conditions listed in gray were reported to be important for triggering germination in one microsporidian species

Weidner 1985; Undeen and Epsky 1990). pH shift from neutral to alkaline is a common trigger of germination in many microsporidian species (Weidner and Byrd 1982; Pleshinger and Weidner 1985). However, other species can be triggered to germinate under acidic conditions (Korke et al. 1916; Hashimoto et al. 1976; de Graaf et al. 1993). Moreover, some species are less dependent on the pH of the germination buffer since they can germinate in both acidic and basic conditions (Hashimoto et al. 1976; Undeen 1983; Undeen and Avery 1988). The role of pH alone in the germination process is unclear and is usually studied together with other ions (Undeen and Epsky 1990; Leitch et al. 1993; Frixione et al. 1994).

Several ions have been tested for their capacity to activate spore germination in vitro (Undeen 1978; Undeen and Avery 1988; Frixione et al. 1994). Alkali metal cations such as Na^+ and K^+ have been shown to play a role in triggering spore germination in *A. algerae* (Frixione et al. 1994), while a buffer supplemented with

NaCl and other monovalent salts such as LiCl, RbCl, and CsCl has been shown to increase the percentage of spore germination in *Nosema locustae* (Undeen and Epsky 1990). The role of alkali metal ions in spore germination was further investigated by substitution of Na^+ and K^+ with choline chloride, which contains membrane impermeant cations (Frixione et al. 1994). No germination was observed in the presence of choline chloride, and germination could be restored by adding Na^+ or K^+, suggesting that cations must penetrate the spore wall and spore plasma membrane to trigger germination (Frixione et al. 1994). The effects of Na^+, K^+, and other cations on germination vary among microsporidian species (Ishihara 1967; Undeen 1978). Ions appear to be the most critical factors for spore germination in *A. algerae* (Undeen 1978), *Glugea fumiferanae* (Ishihara 1967), and *Vavraia culicis* (Undeen 1983).

Roles of anions in spore germination have been studied in the presence of Na^+ or K^+ in *A. algerae* (Undeen and Avery 1988; Frixione et al. 1994). Cl^- and NO_2^- result in ~70% germination in 0.1 M Na^+ solutions, while other anions such as SO_4^{2-} and HPO_4^{2-} produce 1% germination (Frixione et al. 1994). It is possible that SO_4^{2-} and HPO_4^{2-} have lower permeability, suggesting that they may not be able to pass the spore wall and plasma membrane layers (Frixione et al. 1994). Anions alone are likely not sufficient to trigger spore germination in *A. algerae* but may play a passive role by facilitating Na^+ and K^+ movement into the spore (Frixione et al. 1994). In *Spraguea lophii*, polyanions, such as mucin and polyglutamate, and pH shift to alkali conditions trigger spore germination (Pleshinger and Weidner 1985). The mechanism by which these polyanions act is not well understood.

9.3.1.2 In Vitro Activation by Calcium and Calmodulin

The effect of calcium in spore germination has been studied in *Glugea hertwigi* (Weidner and Byrd 1982) and *S. lophii* (Pleshinger and Weidner 1985). A calcium probe, chlortetracycline (CTC), suggests that calcium is present inside spores and is enriched at one end of the spore (Pleshinger and Weidner 1985). Similar results were observed in intact *G. hertwigi* when stained with another calcium-sensitive dye, arsenazo III (Weidner and Byrd 1982). Calmodulin has also been suggested to play a role in germination, and staining using immunoperoxidase labeling against calmodulin also showed positive signal in *S. lophii* spores (Pleshinger and Weidner 1985). It has been hypothesized that calcium displacement is related to the swelling of polaroplast in *G. hertwigi* spores (Weidner and Byrd 1982). Swelling of polaroplast has also been reported during spore germination of *Nosema michaelis* (Weidner 1972).

A few experiments have been carried out to assess the role of calcium in spore germination. First, EGTA, known to chelate calcium, reduces *S. lophii* spore germination from 70 to 5% at a concentration of 0.07 mM (Pleshinger and Weidner 1985) and completely inhibits germination at a concentration of 0.25 mM (Pleshinger and Weidner 1985). Second, treatment of *S. lophii* spores with 15 μM of verapamil, a calcium channel antagonist, inhibits spore germination by 50% (Pleshinger and

Weidner 1985). While verapamil is known to block bacterial calcium voltage-gated channels, the presence of these calcium channels and their function in *S. lophii* and other microsporidian species have not yet been characterized. Therefore, the mechanism of action of verapamil or other calcium channel antagonists in *S. lophii* is not clear. Third, treatment of *S. lophii* spores with micromolar ranges of calmodulin antagonists, trifluoperazine and chlorpromazine, inhibits spore germination (Pleshinger and Weidner 1985).

In contrast to the results described for the role of calcium in *S. lophii* spores, it has been reported that addition of high concentrations of $CaCl_2$ (0.1 M) in the germination buffer containing a calcium ionophore A23187 inhibits spore germination in *G. hertwigi* (Weidner and Byrd 1982). It is unclear whether the contrasting data reported in different species represents a difference in fundamental mechanism or in species-specific activation (Weidner and Byrd 1982; Pleshinger and Weidner 1985).

9.3.1.3 In Vitro Activation by Water and Osmolarity of the Medium

The role of osmotic pressure in germination has been investigated in different microsporidian species. Differences in the concentrations of solute molecules between the inside and outside of spores lead to movement of water across the spore wall and plasma membrane, generating an osmotic pressure (Undeen and Frixione 1990). Increase of the osmotic pressure inside the spore during germination is caused by the increase of solute concentration inside the spore, which may come from ions (Frixione et al. 1994), small molecules (Frixione et al. 1994), or degradation of polysaccharide trehalose (Undeen et al. 1987) in some microsporidian species. The inhibitory effect of an external osmotic pressure on germination was studied in *A. algerae* using hyperosmotic conditions such as polyethylene glycol (PEG) and sucrose (Undeen and Frixione 1990). Germination buffer containing high concentrations of hyperosmotic solutions (100% (w/v) of PEG 400 and 1.8 M sucrose) completely inhibited spore germination (Undeen and Frixione 1990), while lower concentrations (42.9% (w/v) of PEG 400 and 1.2 M sucrose) delayed spore germination (Undeen and Frixione 1990). This inhibition of spore germination may result from a change in osmotic pressure. Estimation of osmotic pressure from sucrose and PEG concentrations showed that the osmotic pressure equivalent to 60–70 atm is required to completely inhibit spore germination in *Nosema hyphesobraconis* (Lom and Vavra 1963) and *A. algerae* (Undeen 1978; Undeen and Frixione 1990), suggesting that an equivalent amount of pressure must be generated inside the spore during the germination process (Undeen and Frixione 1990). As osmotic pressure inside the spore increases during germination, one possible source of higher solute concentration comes from degradation of trehalose into glucose molecules in *A. algerae* spores (Undeen et al. 1987). Trehalose is an abundant carbohydrate in some microsporidian spores (Wood et al. 1970; Undeen et al. 1987; Undeen and Vander Meer 1999). After germination, trehalose levels decrease by three times compared to ungerminated spores, and the level of glucose increases ~155 times. The authors have suggested that the elevated glucose level

provides a high osmolarity environment to initiate spore germination (Undeen et al. 1987; Undeen and Vander Meer 1994). However, this phenomenon is only observed in aquatic microsporidia. There was no observed change in sugar content after germination in terrestrial microsporidia (Undeen and Vander Meer 1999). Not all microsporidia contain a gene encoding a trehalase enzyme that catalyzes trehalose to glucose molecules, suggesting that this is not a fundamentally conserved mechanism of the PT firing process.

Water influx through aquaporin has been implicated in the germination process (Frixione et al. 1997). Typically, several microsporidia such as *Encephalitozoon* spp., *Nosema bombycis*, *Hepatospora eriocheir*, and *Enterospora canceri* contain at least one aquaporin gene in their genomes (Katinka et al. 2001; Williams 2009), and aquaporins were found to be localized to the spore wall layer of *N. bombycis* spores (Chen et al. 2017). *E. cuniculi* (Ghosh et al. 2006) and *N. bombycis* (Chen et al. 2017). Aquaporins heterologously expressed in *Xenopus laevis* oocytes were shown to be functional, as both aquaporins promote rapid influx of water into *Xenopus* oocytes (Ghosh et al. 2006; Chen et al. 2017). However, treatment of *N. bombycis* spores with a polyclonal antibody against aquaporin had only a small effect on spore germination, reducing the germination rate by 6% (Chen et al. 2017), though the antibody may not readily diffuse through the spore coat. Incubation of *A. algerae* spores with 250 μM $HgCl_2$ completely inhibited spore germination (Frixione et al. 1997). $HgCl_2$ may inhibit aquaporins or other proteins containing cysteine residues, leaving open the possibility that aquaporins play a role in germination.

9.3.1.4 In Vivo Activation of the Spore

In vitro conditions for spore germination have been probed in several species. However, it is unclear how these in vitro conditions relate to physiological conditions inside the host. Typically, the major route of microsporidia infection is ingestion of mature spores by hosts (Didier and Weiss 2011; Stentiford et al. 2016). Once ingested, the spores are subjected to a variety of conditions including changes in pH and different ion concentrations within the host digestive tract, which in some cases may favor spore germination. The pH in each part of the insect digestive tract is quite different, ranging from about pH 7 to pH 10 (Dadd 1975; Troemel and Becnel 2015) (Fig. 9.4). The differences in pH may help to control the physical location in which microsporidia germination is favored. In several species, a correlation has been noted between the specific pH that favors germination and the pH in a particular region of the digestive tract. First, in *G. fumiferanae*, the optimal pH for spore germination in vitro is pH 10.8 (Ishihara 1967). Small changes in pH of the germination buffer largely affect spore germination, for example, the germination is reduced by 80% at pH 10 (Ishihara 1967). The pH of the foregut and anterior part of the midgut is estimated to be around pH 10 (Ishihara 1967) and may minimize germination, while the pH of other midgut regions such as the central midgut has a pH higher than 10, which may favor germination (Ishihara 1967; Troemel and Becnel 2015). It is possible that spore germination is inhibited until the spores are

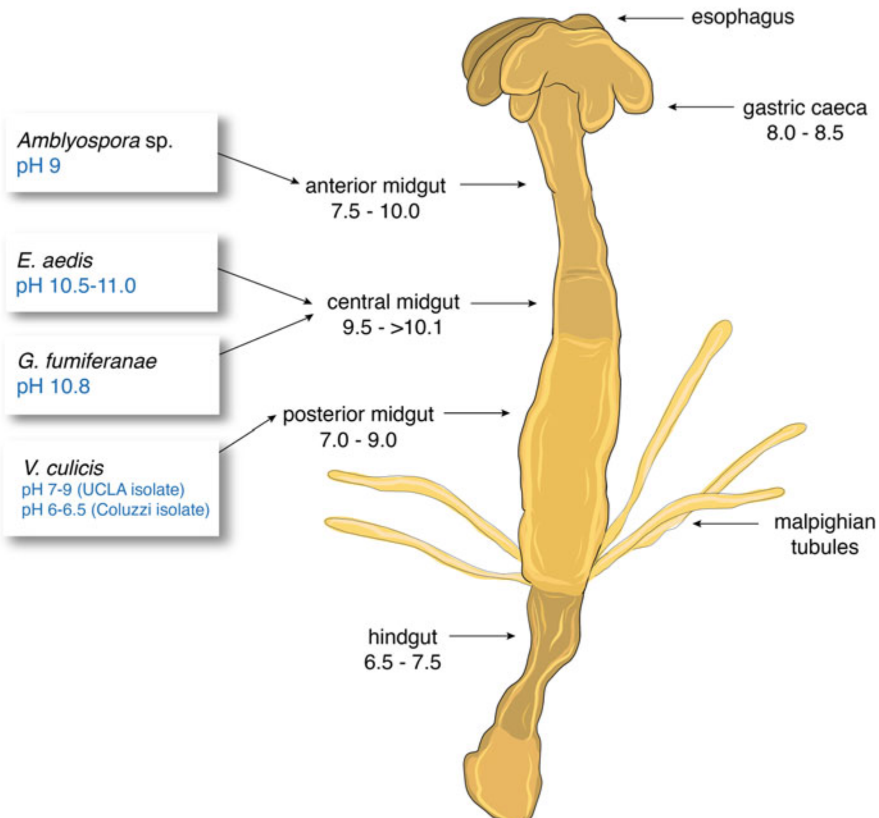

Fig. 9.4 Variation of the pH in each region of the mosquito digestive tract. Generalized representation of a mosquito digestive tract indicating the pH in each region. The pH known to trigger mosquito-infecting microsporidia is noted with each of the microsporidia species in boxes alongside the representation of the digestive tract. This figure is based on an image from Troemel and Becnel (2015)

close to the central midgut, where the pH is closer to the optimal pH for germination in vitro (Ishihara 1967). Second, the optimal pH that induces *Edhazardia aedis* spore germination in vitro has been shown to be consistent with the pH of anterior and central midgut of the mosquito digestive tract (pH 10.5–11), where *E. aedis* spores commonly germinate (Troemel and Becnel 2015).

In addition to pH, in several *Nosema* species which are terrestrial microsporidia, a dehydration step is required before stimulation of spore germination with alkali buffer, in vitro (Olsen et al. 1986; Undeen and Epsky 1990). Dehydration may reflect the environments in which spores need to pass from one host to others, changing from a wet stage in the host tissue to drier external environment (Undeen and Epsky 1990).

9.3.1.5 Summary of Factors Important for Initiation

Microsporidia spore germination is a sophisticated process consisting of several steps. Currently the sequence of activation events is not well understood. Based on available knowledge of both in vitro and physiological conditions that activate spore germination, the following triggers may be important for the initiation of spore germination (Fig. 9.3): (1) alkali metal cations (Undeen 1978; Undeen and Avery 1988; Frixione et al. 1994), (2) external stimuli such as suitable pH in the host digestive system (Ishihara 1967; Troemel and Becnel 2015), (3) ion flux across the spore wall and plasma membrane (Frixione et al. 1994), (4) calcium ions and calmodulin (Weidner and Byrd 1982; Pleshinger and Weidner 1985), and (5) osmotic pressure and the flux of water (Undeen and Frixione 1990; Frixione et al. 1997).

Physical changes that have been observed and reported during the early steps of germination are the swelling of the polaroplast (Weidner 1972; Weidner and Byrd 1982), the formation of a collar-like complex at the anterior end (Lom 1972; Schottelius et al. 2000; Takvorian et al. 2005), and the rupture of the thinnest part of the spore wall, at the anterior tip of the spore (Keohane and Weiss 1999). However, the exact order of these events remains unclear. Swelling of polaroplast has been reported to occur in *G. hertwigi* (Weidner and Byrd 1982) and *S. lophii* (Weidner 1972). The polaroplast swelling was hypothesized to happen after the calcium influx (Weidner and Byrd 1982). Considering the position of the polaroplast at the apical end of the spore, the swelling may exert a force on the spore wall, causing the thinnest part of the spore wall to rupture (Keohane and Weiss 1999). It is possible that reorganization of the polaroplast at the apical end of the spore drives the formation of the collar structure. The collar structure has been observed in several microsporidian species such as *A. algerae* (Takvorian et al. 2005), *E. hellem* (Schottelius et al. 2000; Bigliardi and Sacchi 2001), *E. intestinalis* (Takvorian et al. 2005), and *Enterocytozoon bieneusi* (Takvorian et al. 2005). The function of the collar-like structure is not known. It may potentially hold the PT in place during the spore germination (Bigliardi and Sacchi 2001).

9.3.2 PT Firing and Cargo Transport

The PT firing process occurs rapidly, on the millisecond timescale. Live-cell imaging techniques have been utilized to capture and study the kinetics of this firing process in a few microsporidian species: *A. algerae* (Frixione et al. 1992; Jaroenlak et al. 2020; Takvorian et al. 2020), *E. hellem* (Jaroenlak et al. 2020), *E. intestinalis* (Jaroenlak et al. 2020), and *E. aedis* (Troemel and Becnel 2015). The entire PT firing process from PT elongation to the passage of the sporoplasm happens in less than 500 ms for *Encephalitozoon* spp. and ~1.6 s for *A. algerae* (Jaroenlak et al. 2020). Four distinct phases are observed during this process (Fig. 9.5): (phase 1) PT elongation, during which the PT extrudes to reach its maximum length; (phase 2)

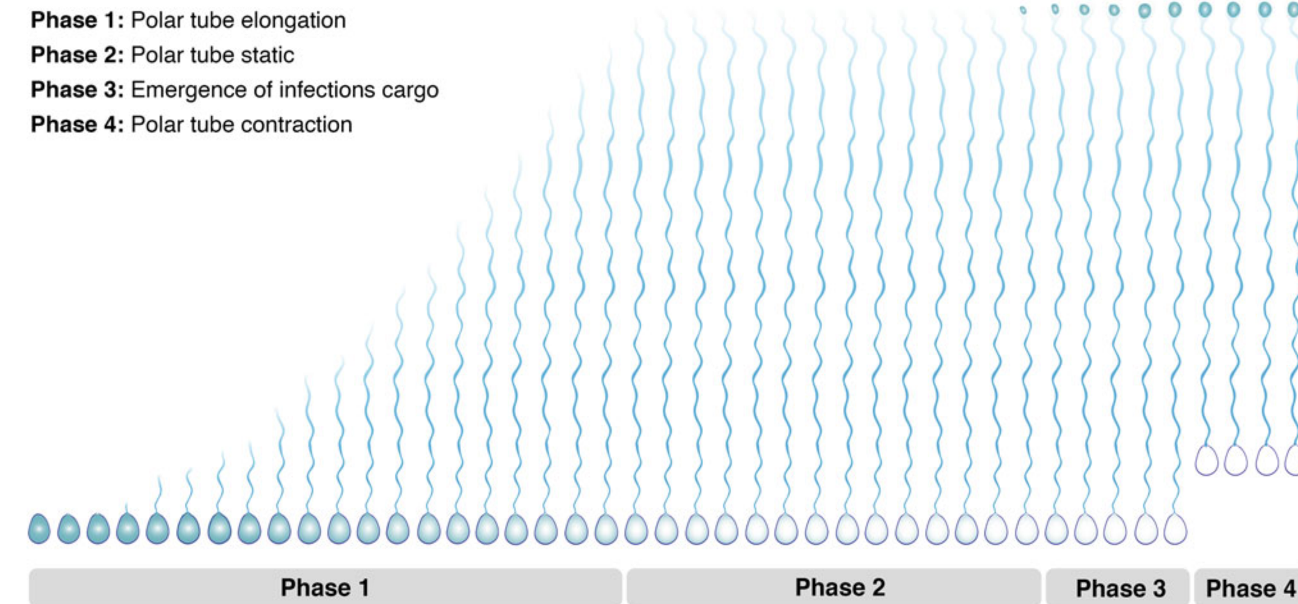

Fig. 9.5 Kinetics of the PT germination process. Four distinct phases are observed during PT germination in *A. algerae*, *E. hellem*, and *E. intestinalis*. In phase 1, the PT elongates to its maximum length. In phase 2, the PT length remains unchanged. In phase 3, the sporoplasm exits from the PT and appears as a circular compartment at the distal end of the PT. In phase 4, the PT shortens, and the degree of shortening depends on the microsporidian species

a static phase, during which the length of the PT remains unchanged and presumably cargo is transported through the tube; (phase 3) emergence of infectious sporoplasm at the distal end of the PT; and (phase 4) PT contraction after the sporoplasm is expelled (Jaroenlak et al. 2020). These phases were observed in three microsporidian species so far, namely, *A. algerae*, *E. hellem*, and *E. intestinalis* (Jaroenlak et al. 2020). Details of each phase are discussed below.

9.3.2.1 Phase 1: Elongation Phase

The PT emerges from the apical part of the spore (Frixione et al. 1992; Jaroenlak et al. 2020) and elongates to reach its maximum length (Fig. 9.5). During the elongation phase, the PT is initially observed to have a corkscrew pattern in *A. algerae* and later appears to become straighter (Frixione et al. 1992; Jaroenlak et al. 2020). The pitch of the emerging PT helix has been reported to be approximately 5.4 μm (Frixione et al. 1992). The helical pattern observed in *A. algerae* is not clearly observed in *Encephalitozoon* spp. (Jaroenlak et al. 2020), suggesting that there may be some differences in the emergence of the PT between different species. The fully extended PT is about twice as long as its length inside the spore (Weidner 1972; Jaroenlak et al. 2020), suggesting that the PT may stretch or that the protein subunits that make up the PT may undergo a conformational change during the spore germination, resulting in the change of the PT length (Jaroenlak et al. 2020).

Elongation of the PT is extremely rapid, and the time in which the PT reaches 90% of its maximum length is 200–300 ms in *Encephalitozoon* spp. and about 800 ms in *A. algerae* (Jaroenlak et al. 2020). The average maximum velocity of PT elongation ranges from 235 to 336 μm/s depending on the species (Jaroenlak et al. 2020). The velocity of PT firing is much faster than most cellular biological processes. For comparison, cargo transport by the motor protein kinesin takes place at about 0.51 μm/s (Vale et al. 1994). The average maximum acceleration varies from 1500 μm/s^2 in *A. algerae* to 5000 μm/s^2 in *Encephalitozoon* spp. (Jaroenlak et al. 2020). Overall, the kinetics of PT firing are more similar in closely related species such as *E. hellem* and *E. intestinalis* than in *A. algerae*, which is distantly related to the *Encephalitozoon* spp. (Jaroenlak et al. 2020).

9.3.2.2 Phase 2: Static Phase

After the PT extends to its maximum length, a stationary phase is observed, where no change occurs in the length of the PT. It is likely that cargo is being transported through the tube during this time. On average, phase 2 lasts for 800 ms for *A. algerae* and 60–140 ms for *Encephalitozoon* spp. (Jaroenlak et al. 2020). While the maximum length of *A. algerae* PT (~101 μm) is twice that of *Encephalitozoon* spp. (~50 μm) (Jaroenlak et al. 2020), phase 2 is six times shorter in *Encephalitozoon* spp. than in *A. algerae*. This result suggests that if cargo transport occurs during phase 2, it occurs at a faster rate in *Encephalitozoon* spp. than in *A. algerae* (Jaroenlak et al.

2020). Alternatively, there might be a delay of the onset of cargo transport in *A. algerae*.

9.3.2.3 Phase 3: Emergence of Cargo

In this phase, the cargo, or sporoplasm, emerges at the distal end of the PT as a circular structure that appears to be bound by a membrane (Fig. 9.5) (Weidner et al. 1984; Takvorian et al. 2005). Additional membrane, possibly originating from the polaroplast, is likely required to facilitate transport and to accommodate the sporoplasm as it is transported through the PT (Weidner et al. 1984). Under in vitro conditions in which the imaging experiments are carried out to monitor PT firing, the sporoplasm remains attached to the PT throughout the entire experiment (Jaroenlak et al. 2020).

9.3.2.4 Phase 4: Polar Tube Contraction

A few seconds after the sporoplasm is expelled, the PT shortens by 3–5% in *A. algerae* and *E. intestinalis* and by 24% in *E. hellem* (Fig. 9.5) (Jaroenlak et al. 2020). The shortening of the PT is observed across the entire PT, and is not a retraction of the PT back into the spore coat (Jaroenlak et al. 2020). The difference in the extent of shortening, even between closely related species such as *E. hellem* and *E. intestinalis*, suggests that there is a difference in plasticity of the PT among microsporidian species (Jaroenlak et al. 2020). PT shrinkage is possibly due to the stretching of the PT during elongation in phase 2. Once the sporoplasm emerges from the tube, the tension in the PT may be released in phase 4, allowing it to shrink. Movement of the spore body is also observed after the sporoplasm is expelled (Frixione et al. 1992).

9.3.2.5 Incomplete Germination

In addition to complete spore germination, incomplete germination has also been observed, in which sporoplasm is not ejected from the PT (Frixione et al. 1992; Jaroenlak et al. 2020). Incompletely germinated PTs can be short or normal length, but have no sign of sporoplasm emergence, suggesting that the PT can be stalled in either phase 1 or phase 2 (Jaroenlak et al. 2020). Incomplete germination has also been observed in *A. algerae*, when spores were placed in hyperosmotic solutions such as 1.2 M sucrose (Frixione et al. 1992). The PT firing kinetics slowed down, and the time required for PT elongation increased to 15–45 s (Frixione et al. 1992). Incomplete germination was observed more frequently in hyperosmotic solution compared to a normal germination buffer, suggesting that osmotic pressure may play a role in the germination process (Frixione et al. 1992).

9.4 Mechanism of Polar Tube Firing

The mechanism of PT ejection from the spore and the energy source that drives the process remain open questions in the field. However, three distinct models have been put forth over the years as possible explanations for this complex and extremely rapid process:

1. The PT could be released from the spore in a manner analogous to the release of a compressed spring (Fig. 9.6a, b). In this "jack-in-the-box" model of firing, the PT is highly deformed and strained when coiled inside the spore and may store considerable potential energy in its spring-like coils, which is released and drives its transition into a linear tube as it pushes itself out of the spore.
2. Eversion may be used to describe another model of PT firing where the PT turns inside out as it is released, analogous to turning a jacket sleeve inside out (Fig. 9.6a, c). In this model, one end of the tube is fixed in place at the apical end of the spore at the site of PT exit. Beginning at this end, turning the rest of the PT inside out would lead to the translocation of the entire PT through the fixed segment and then ultimately to outside of the spore.
3. A third possible model to describe tube extrusion is analogous to the extension of a telescope, wherein the concentric cylindrical layers that have been observed to make up the PT slide over each other and emerge from the spore one by one (Fig. 9.6a, d).

Each of these models makes predictions about how the PT may differ before and after germination. If the PT emerges via a jack-in-the-box mechanism, then it would be expected that the entire tube simply slides out of the spore during extrusion. In the simplest case, the architecture of the PT in its coiled form and in its linear form would therefore be expected to remain essentially the same (Fig. 9.6b). In contrast, the eversion and telescope models predict that the pre-germination and post-germination states would look quite different. In the eversion model of tube extrusion, the entire PT would be turned inside out, and thus one would expect to observe a complete inversion of architecture between the two forms (Fig. 9.6c). Finally, extension of the PT similar to a telescope predicts that the multilayered organization of the PT observed inside mature spores would be converted into a much simpler structure upon extension to its maximum length, ultimately being composed of just a single layer in most places (Fig. 9.6d). In addition to the overall organization of the PT pre- and post-germination, how the tube moves relative to other parts of the tube and the spore during the germination process would differ in each of these models. In the jack-in-the-box model, the entire exterior surface of the PT must move through the extracellular space, and the new exterior surface will only appear at the junction between the tube and the spore coat. The eversion model is quite different. In this model, the exterior surface of the extracellular tube does not move; all movement occurs on the tube interior as the tube turns inside out, and a new exterior surface is created only at the distal extracellular tip of the tube. The predictions of the telescope

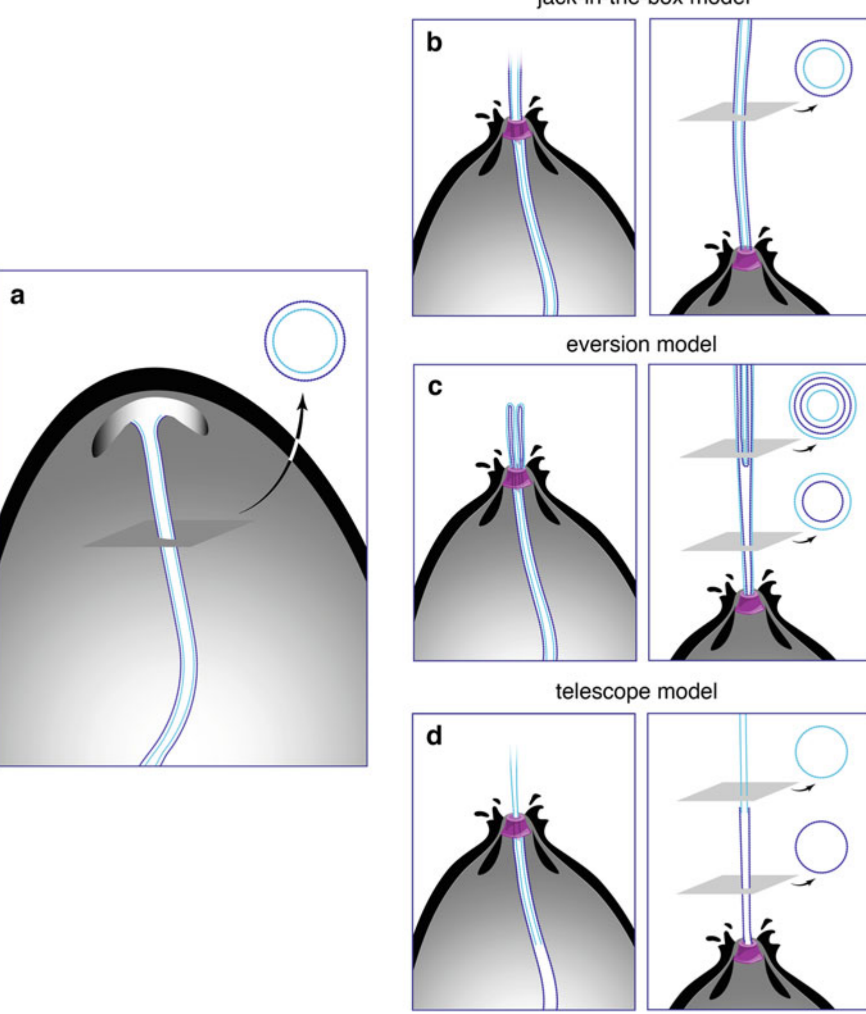

Fig. 9.6 Possible models of the PT firing mechanism. (**a**) Dormant spore in its pre-firing state, with the PT inside the spore. (**b**) Jack-in-the-box model, in which the PT emerges with the same topology as when it is inside the spore. The architecture of the tube and layers observed throughout the tube are expected to remain the same, as shown by the cross section. (**c**) Eversion model, in which the tube turns inside out as it emerges from the spore. The architecture of the inverting part of the tube is expected to contain more layers (top cross section), whereas part of the tube that has everted would have an architecture that is inverted relative to the tube in the dormant spore (bottom cross section). (**d**) Telescope model, in which the layers that constitute the PT would slide past each other during the firing process. The architecture of the fired tube is expected to be simpler than the architecture of the tube pre-firing as shown by the two cross sections. Cross sections are not drawn to scale but rather to indicate the order of the layers

model are less clear-cut depending on whether layers extend sequentially or all at once but would probably more closely resemble the jack-in-the-box model.

While no definitive mechanism of PT firing has been discovered yet, several key experiments have tackled this question:

1. As the PT extrudes, monitoring the region from which it extends is one way to differentiate between the jack-in-the-box and eversion models. If released as a "jack-in-the-box," the tip of the tube must move forward in space, resulting in extrusion of the PT at its base. On the other hand, if released by eversion, the portion of the tube released must remain in place while the leading edge extrudes. In 1982, Weidner used a pulse labeling approach to decipher where the tube may be extruding from. *G. hertwigi* spores were germinated in a solution containing latex particles and visualized 1–3 s after immersion. When observed under the microscope, the basal ends of the PTs of 10–15% of the spores were decorated with latex particles, while the distal ends of these tubes were naked. This suggests that the PT was growing at the tip rather than at the base, suggestive of the eversion model (Weidner 1982). It is unclear how the experiment was carried out in the timeframe of PT eversion, but these results are clear and intriguing. In the same study, Weidner also noted that when the PT discharges, the parts of the tube that have already emerged remain unchanged and the tube can continue to change direction at the tip (Weidner 1982). Experimental evidence for Weidner's observation came from another study by Frixione et al. in 1992, in which *A. algerae* spores were tracked at various time points using optical microscopy. It was observed that the portion of the PT that was already extruded in a wave-like pattern remains unchanged (Frixione et al. 1992). There are two possible explanations for these data: either the PT follows the same pattern because it is everting, or the lagging part of the extruding PT follows the path carved by the leading part to experience the least amount of drag force.
2. PT emergence from the spore can be triggered using specific germination conditions as described previously in this chapter. When triggered, the tube emerges as a linear filament out of the spore (Olsen et al. 1986; Frixione et al. 1992; Takvorian et al. 2005; Jaroenlak et al. 2020). However, the coiled PT can also be expelled from the spores by mechanical pressure. In this case, however, the tube emerges as a tangled mesh and does not fire to produce a linear tube as it does when triggered by a stimulus (Dissanaike and Canning 1957). If the tube emerged analogous to a spring or jack-in-the-box, we would expect the energy for the firing process to originate from the potential energy stored inside the coiled PT, and we would therefore expect that releasing the tube via pressure would cause the tube to emerge as a linear filament, similar to when it is triggered. The fact that mechanical pressure does not recapitulate other triggers for releasing the tube suggests that a simple jack-in-the-box mechanism may not be sufficient to describe the mechanism of PT firing.
3. In a study by Weidner in 1982, *S. lophii* spores were incubated with Nile red, a dye that stains lipid membranes. In live-cell imaging studies performed on these spores, it was observed that the intensity of the dye was always greatest at the

growing end of the tube. One way of interpreting these data is that the intense signal came from the presence of double-membrane cylinders at the tip which may slide over each other during eversion of the tube (Weidner et al. 1995). This interpretation could be consistent with eversion.

4. When tubes of germinating spores were observed using DIC imaging, a funnel-like shape was observed at the tip of the tube just before emergence of the sporoplasm. This funnel shape was interpreted as representing an eversion-like mechanism (Weidner et al. 1995).
5. Another intriguing piece of data comes from Lom and Vavra in 1963 while observing PT germination in *Bacillidium* spp. using a light microscope. These species are evolutionarily distant, and that is reflected in their PT structure, which, instead of having a number of coils as depicted above, has simply a linear tube, or manubrium, as described earlier in this chapter. The PT is connected to the anterior end of the spore through a wide manubrium which is then connected to a narrower polar filament. The pronounced morphological difference between these two parts of the tube serves as markers to understand how the PT might be emerging, with the manubrium marking the anterior portion and the thin filament marking the posterior portion of the PT inside the spore. After firing, the manubrium was noted as being proximal to the spore, still connected to the anterior end, while the thin polar filament was at the distal tip. One interpretation of these data is that the structure has turned inside out (eversion) as it emerged, thus inverting the relative position of the two parts of the PT (Lom and Vavra 1963).
6. The observation of incompletely germinated *G. hertwigi* spores under TEM showed that these tubes have a “tube within tube” structure. This could represent either a telescoping mechanism or an eversion mechanism (Weidner 1982).

9.5 Cargo Transport Through the Polar Tube

It remains unclear precisely what is being transferred from microsporidia to the host during spore germination and infection. It is possible that the whole cell is transported since large empty spore coats have been observed after spore germination (Weidner et al. 1984; Undeen and Frixione 1991). Another possibility is that cargo is selectively transported through the tube. It is certain, however, that the nucleus forms a large part of the cargo that is delivered to the host cell. In the extruded sporoplasm of *G. hertwigi* and *S. lophii* spores, a single nucleus and some short membranous compartments have been observed (Weidner et al. 1984), while in *A. algerae*, diplokaryon nuclei and a multilayered interlaced network (MIN), which are thought to be derived from the Golgi apparatus, have been observed (Cali et al. 2002; Takvorian et al. 2013). Currently, tools for genetic manipulation of microsporidia are not available, posing a significant barrier to studying cargo transport. A small molecule dye has been successfully used to label nuclei of *A. algerae* spores prior to germination, allowing the nucleus to be tracked during transport through the PT (Fig. 9.7a) (Jaroenlak et al. 2020). After the PT fires, both

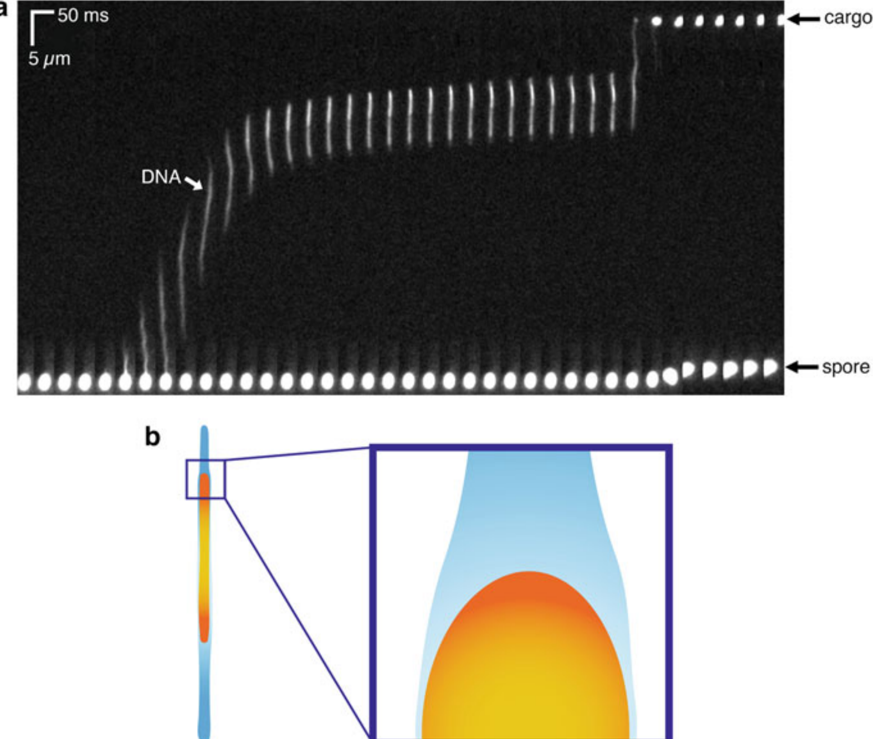

Fig. 9.7 Cargo transport through the PT. (**a**) Kymograph of nuclear transport through the PT. Live-cell imaging of the nuclear movement was performed using *A. algerae* spores with a 50-ms time interval. This image was obtained from Jaroenlak et al. (2020). (**b**) Schematic diagram of cargo (orange) transport through the PT (blue). PT bulges slightly to accommodate the cargo (see also Fig. 9.2c), while the cargo deforms substantially to fit the tube (**a**: reprinted from Jaroenlak et al. 2020. Permission is granted under the Creative Commons Attribution 4.0 Generic License to reuse)

nuclei of *A. algerae* move and rearrange inside the spore, possibly in response to initiation of PT firing (Jaroenlak et al. 2020). Live-cell imaging revealed that nuclear transport occurs ~500 ms after the PT firing is initiated, and the time that *A. algerae* PT takes to fully extend is approximately 830 ms (Jaroenlak et al. 2020). At the 500 ms time point, the PT is extended to ~50% of its length (Fig. 9.8). Therefore, the PT does not have to be fully extended prior to nuclear transport through the tube. It is unknown how nuclear transport compares in other microsporidian species.

As nuclei are transported through *A. algerae* spores, they are deformed to a large degree, presumably in order to fit through the narrow tube (Fig. 9.7a, b), and they can return to a globular shape after they emerge from the tube (Jaroenlak et al. 2020). Nuclear transport occurs on a very fast timescale, comparable to PT extension (Jaroenlak et al. 2020) (Fig. 9.8), and movement of the nuclei pauses approximately three quarters of the way through the PT (Jaroenlak et al. 2020). Frixione and colleagues have proposed that the end of the PT may remain closed during extension (Frixione et al. 1992). According to this model, once the PT is fully extended,

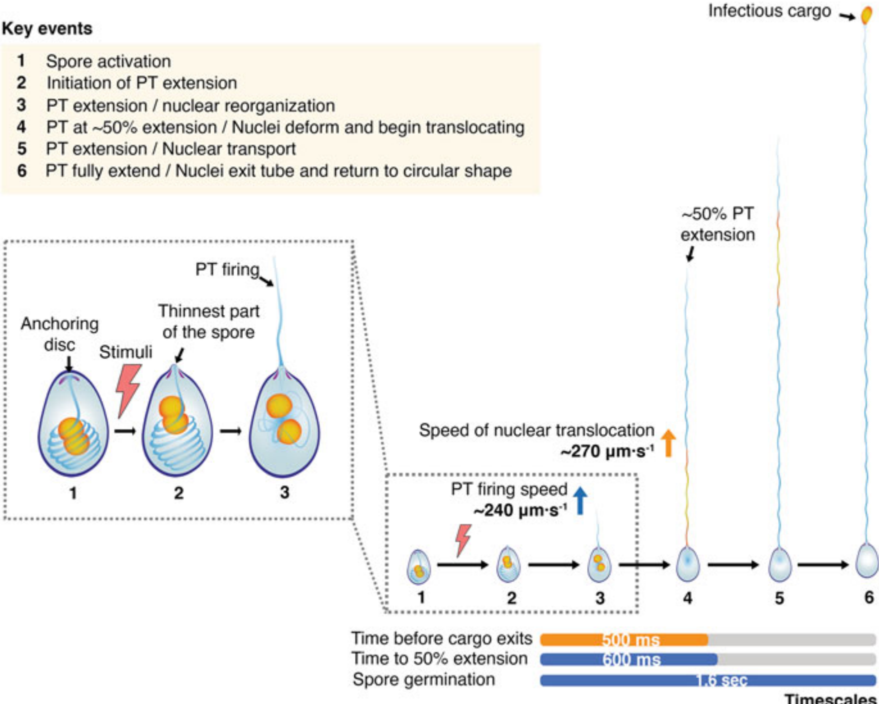

Fig. 9.8 Model for PT firing and nuclear transport. Spore germination can be triggered by several factors as shown in Fig. 9.3. Following initiation, physical changes occur in the spore, including the rupture of the thinnest part of the spore at the anterior end, where the PT is anchored to the spore via the anchoring disc. After rupture, the PT begins to fire from the spore body and elongates. Nuclei and possibly other compartments reorganize inside the spore as the PT is exiting. At the ~50% extension point, nuclei deform and travel through the PT with a comparable speed to the PT extension. Nuclei regain their circular shape after the nuclei reach the distal end of the PT. This image was adapted from Jaroenlak et al. (2020). Reprinted from Jaroenlak et al. (2020). Permission is granted under the Creative Commons Attribution 4.0 Generic License to reuse

the closed end of the PT undergoes plastic relaxation and rupture, allowing the sporoplasm to exit from the PT (Frixione et al. 1992). Thus, it is plausible that the observed pausing during nuclear translocation may be due to a delay in the opening of the distal end of the PT. Alternatively, this could potentially reflect a change in the driving forces pushing cargo through the tube or a rapid dissipation of the driving force upon initial PT firing and cargo transport (e.g., initial pressure built up inside the spore), followed by a further increase in the force to complete cargo export.

One possible driving force for cargo transport is the osmotic pressure built up in the spore during germination, which has been proposed to drive extrusion of the PT (Undeen and Frixione 1990; Frixione et al. 1992). This pressure may originate from the swelling of the vacuole, polaroplast, or other internal organelles. This pressure could be a driving force for cargo transport in any of the three models of PT firing discussed above. Another possibility is that an interplay of cohesive and adhesive forces drives sporoplasm through the already extruded tube. Cohesive forces can be

described as attractive forces between molecules of the same type, whereas adhesive forces can be described as attractive forces between molecules of different types. Cryo-EM on the extruded PT of *A. algerae* has shown that the sporoplasm makes close contact with PT walls during transport (Fig. 9.2c) (Takvorian et al. 2020). Cohesive forces exist within the membranous compartments within the sporoplasm causing it to stick to itself, and at the same time, it will have adhesive forces against the wall of the PT (which is proteinaceous). If the adhesive forces between the sporoplasm and the PT are greater than the cohesive forces it has within itself, then capillary action may play a part in driving the sporoplasm through the tube. This may also explain why the PT diameter is so small relative to the dimensions of the whole spore, as a smaller diameter would enhance capillary forces enabling the sporoplasm to traverse a longer length. Another possibility is that cohesive forces among sporoplasm components are stronger than the adhesive forces against the tube walls, and this possibly facilitates the movement as a whole through the PT (Frixione et al. 1992). Sporoplasm has been defined as a shear-thinning viscoelastic fluid which suggests that it decreases in viscosity when passing through the PT under high strain (Frixione et al. 1992).

Measurement of the nuclear deformation in *A. algerae* indicates that the aspect ratio of the nuclei is only 0.1 when the nuclei are inside the PT compared to the ratio of 0.9 when they are outside (Jaroenlak et al. 2020). These results showed that nuclei of microsporidia are extremely flexible or the forces driving nuclear transport are incredibly strong. In addition, nuclear deformation in microsporidia happens on an extremely fast timescale (~1 s) (Jaroenlak et al. 2020). The deformation of nuclei in microsporidia is reminiscent of the translocation processes in other cell types such as immune cells that migrate through tight junctions of the blood vessel (de Oliveira et al. 2016) or cancer cells that metastasize to other tissues (Reymond et al. 2013; Liu et al. 2018). However, two key differences are in the timescale of deformation and the extent of deformation. Nuclear deformation in microsporidia takes place on the millisecond timescale, while in most other cell types, it typically occurs over the course of minutes to hours (Fu et al. 2012; Denais et al. 2016; Krause et al. 2019). The aspect ratio also shows that the extent of deformation is much greater in microsporidia nuclei. Lamins, the main components of a nuclear lamina, have been reported to be an important factor for nuclear envelope rigidity in many cells (Lammerding et al. 2006). Microsporidia likely lack lamins entirely (Katinka et al. 2001; Aurrecoechea et al. 2011; Jaroenlak et al. 2020), perhaps at least in part explaining why the microsporidian nucleus can deform to such a large degree and in such a short period of time (Jaroenlak et al. 2020).

9.6 Conclusion and Future Perspectives

Microsporidia have evolved a unique way of infecting host cells using the PT. From a basic biological point of view, the PT is a fascinating organelle in terms of its structure and function. It undergoes a striking conformational change from a tight

coil to a linear tube on a millisecond timescale and allows the passage of the spore contents into a host cell, in order to initiate an infection (Fig. 9.8). It is still unclear whether the PT pierces through the host cell membrane. In this regard, it would be useful to enumerate the magnitude of the force with which the PT fires and whether this is sufficient to pierce through one or several layers of membranes. Another key question in the field is why different species of microsporidia have evolved PTs of different lengths. A few studies have hinted that PTs of different lengths may have access to different tissues. Future studies to determine the correlation between PT length and the host tissues that particular species infect would be useful in understanding the evolution of PT length. While in vitro conditions for PT germination have been intensively studied, each trigger has been studied only in one or two microsporidian species. A systematic study across different species will be helpful in understanding which triggers can be generalized across species, thus providing insights into a general mechanism of activation. Moreover, how these in vitro conditions reflect physiological conditions in the host and what the triggers are in host cells and tissues remain open questions. While PT firing kinetics are clear, it remains unclear what is happening inside the spore once PT germination has been initiated and how other organelles may be reconfigured and contribute to the firing process. Three-dimensional reconstruction of the spores during the germination process would provide an insight into the mechanism of spore germination and configuration of the PT relative to other organelles. Once fired, the PT serves as a conduit for the infectious cargo transported to the host cell. Precisely what is being transported to the host cell remains largely unknown. Development of tools to label specific compartments inside the spore would allow for a better understanding of what cargo is transported through the tube and whether any parts of the sporoplasm are excluded in this process. The mechanism of PT firing still remains a key open question in the field and is ripe for future discovery.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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Ethical Approval The chapter is a review of previously published accounts; as such, no animal or human studies were performed.

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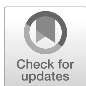
Chapter 10
Microsporidian Pathogens of Aquatic Animals



Jamie Bojko and Grant D. Stentiford

Abstract Around 57.1% of microsporidia occupy aquatic environments, excluding a further 25.7% that utilise both terrestrial and aquatic systems. The aquatic microsporidia therefore compose the most diverse elements of the Microsporidia phylum, boasting unique structural features, variable transmission pathways, and significant ecological influence. From deep oceans to tropical rivers, these parasites are present in most aquatic environments and have been shown to infect hosts from across the Protozoa and Animalia. The consequences of infection range from mortality to intricate behavioural change, and their presence in aquatic communities often alters the overall functioning of the ecosystem.

In this chapter, we explore aquatic microsporidian diversity from the perspective of aquatic animal health. Examples of microsporidian parasitism of importance to an aquacultural ('One Health') context and ecosystem context are focussed upon. These include infection of commercially important penaeid shrimp by *Enterocytozoon hepatopenaei* and interesting hyperparasitic microsporidians of wild host groups.

Out of ~1500 suggested microsporidian species, 202 have been adequately taxonomically described using a combination of ultrastructural and genetic techniques from aquatic and semi-aquatic hosts. These species are our primary focus, and we suggest that the remaining diversity have additional genetic or morphological data collected to formalise their underlying systematics.

Keywords Aquaculture · Fisheries · Ecosystem · Taxonomy · Pathobiome · Hyperparasite · Marine · Freshwater · OneHealth · Opportunistic

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10.1 Introduction

The Microsporidia (Opisthokonta: Ophisthosporidia) are obligate pathogens of protozoan, animal, and human hosts from terrestrial and aquatic environments, and many pose significant risks for food security (Stentiford et al. 2016; Bass et al. 2018). The group boasts a broad species diversity, with an estimated ~1500 members: 69.7% are considered to utilise aquatic habitats (freshwater/brackish/marine), with ~25% of these microsporidians using multiple environments, including both terrestrial and aquatic environments (Murareanu et al. 2021). The aquatic microsporidians compose the greatest diversity of the phylum and are pervasive across freshwater and marine vertebrates, invertebrates, and unicellular hosts.

The aquatic microsporidia phylogenetically branch in all existing clades (I, II, III, IV, V) (Stentiford et al. 2013a; Stentiford and Dunn, 2014; Kent et al. 2014; Vossbrinck et al. 2014). These members display a variety of morphologies and structural features, such as the needle-shaped spores of *Nadelspora canceri* from the shore crab *Carcinus maenas* (Stentiford et al. 2013b), and the long spines of *Areospora rohanae* from the southern king crab *Lithodes santolla* (Stentiford et al. 2014). The transmission of aquatic microsporidia follows the conventional horizontal and vertical methods, with some species such as *Pleistophora mulleri* (parasite of Amphipoda) being capable of both (MacNeil et al. 2003). The hosts they infect, their transmission patterns, and their persistence in the aquatic environment can contribute to ecological change and aquacultural deficit.

Aquatic ecosystems are composed of multiple host groups that harbour parasitic species, which drive their mortality, growth, and behaviour. Aquatic microsporidia have been identified to drive cannibalistic behaviour (Bunke et al. 2019), alter the host symbiome (Herren et al. 2020) and can help to restrict the population growth and impact of biological invasions (Bojko et al. 2019). Within the aquatic environment, the microsporidia have been found in hosts that inhabit the deep ocean (Sapir et al. 2014), frozen ocean (Czaker 1997), pelagic zone (Diamant et al. 2010), coastal zone (Stentiford et al. 2013b), and a similar environmental diversity within freshwater habitats (Bojko et al. 2020a).

Within this chapter, we explore microsporidian parasites in aquatic hosts. The current taxonomic list of supported microsporidia with both genetic and morphological description ($n = 202$) is included alongside any known hosts and associated environments. Host-pathogen interactions are discussed for the aquatic microsporidia, including examples of behavioural manipulation, hyperparasitism, and host-parasite interactions in wild and aquacultured communities.

10.2 Microsporidian Diversity in Aquatic Vertebrates

Aquatic vertebrates include members across the Vertebrata but primarily consist of marine/freshwater mammals (Mammalia), birds (Aves), reptiles (Testunides/Lepidosauromorpha/Crocodylomorpha), amphibians (Amphibia), and fish (Actinopterygii/Chondrichthyes/Coelacanthimorpha/Dipnoi). Each group is explored within this section, detailing the diversity of partially and adequately described microsporidians (Table 10.1; Fig. 10.1).

10.2.1 Microsporidian Parasites of Aquatic Mammals

Aquatic mammals consist of the Sirenia (manatee/dugong), Cetacea (whale, dolphin, porpoise), Mustelidae (otters), Castoridae (beavers), Phocidae (true seals, earless seals), Otariidae (sea lions, eared seals, fur seals) and Odobenidae (walrus). To date, microsporidians have been observed in dolphins, otters, and seals; however, most of these parasites are identified using generic PCR tools from faecal samples.

Faecal samples from bottlenose dolphin (*Tursiops truncatus*) local to south California and Florida were found to harbour microsporidia with between 86.5 and 100% similarity to *Tetramicra*, *Kabatana*, *Microgemma,* and *Enterocytozoon* (Fayer et al. 2008). Many of these microsporidian groups are common fish/crustacean parasites, which may be consumed by dolphins; however, *E. bieneusi* isolate (s) (~87% similarity) suggest that internal pathology should not be ruled out (Stentiford et al. 2019). Serological studies from dolphin blood samples have identified blood-borne microsporidians that appear to be *Encephalitozoon* sp. (Desoubeaux et al. 2018). Other microsporidian infections in aquatic mammal faeces include the detection of *Enterocytozoon* from otters and beavers in Maryland, USA, including some PCR detection of human-pathogenic strains of *E. bieneusi* (Sulaiman et al. 2003).

Outside of PCR detection from faecal samples, the American fur seal (*Arctocephalus australis*) has been diagnosed with Encephalitozoonosis (*Encephalitozoon* sp.) of the brain, which was originally detected from two pups from Chile (Seguel et al. 2015). Morphological techniques, including transmission electron microscopy (TEM) and histology, confirmed that the parasite infected the brain tissue, causing necrosis. Microsporidia appear capable of infecting aquatic mammals, calling for dedicated studies to uncover their underlying diversity and ecological relevance.

Table 10.1 The aquatic microsporidia, their hosts, and their environmental presence. Also included is their higher taxonomy, incorporating them into their related clade (I–V). This table includes those aquatic or semi-aquatic microsporidians that have both genetic and morphological characterisation, which is necessary for formal taxonomic identification. The environment the parasite present in is depicted by T (terrestrial), FW (freshwater), or M (marine) and if the parasite is hyperparasitic (Hyp)

Clade	Microsporidian species	Known/type host(s)		Environment	Reference
I	*Agglomerata cladocera*	Crustacea	*Daphnia* sp.	FW	Sokolova et al. (2016b)
I	*Agglomerata daphniae*	Crustacea	*Daphnia* sp.	FW	Weng et al. (2020)
I	*Alfvenia sibirica*	Crustacea	*Cyclops* sp.	FW	Sokolova et al. (2016b)
I	*Amblyospora bakcharia*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora baritia*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora bogashovia*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora bracteata*	Insecta	Mosquito	FW/T	Hazard and Oldacre (1976)
I	*Amblyospora californica*	Insecta	Mosquito	FW/T	Becnel (1992)
I	*Amblyospora canadensis*	Insecta	Mosquito	FW/T	Hazard and Oldacre (1976)
I	*Amblyospora chulymia*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora cinerei*	Insecta	Mosquito	FW/T	Andreadis (1994)
I	*Amblyospora connecticus*	Insecta	Mosquito	FW/T	Andreadis (1988)
I	*Amblyospora criniferis*	Insecta	Mosquito	FW/T	Garcia and Becnel (1994)
I	*Amblyospora excrucii*	Insecta	Mosquito	FW/T	Andreadis (1994)
I	*Amblyospora ferocis*	Insecta	Mosquito	FW/T	Garcia and Becnel (1994)
I	*Amblyospora flavescens*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora hristinia*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora indicola*	Insecta	Mosquito	FW/T	Sweeney et al. (1990)
I	*Amblyospora jurginia*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora kazankia*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)

(continued)

Table 10.1 (continued)

Clade	Microsporidian species	Known/type host(s)		Environment	Reference
I	*Amblyospora khaliulini*	Insecta	Mosquito	FW/T	Andreadis et al. (2018)
I	*Amblyospora mavlukevia*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora mocrushinia*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora modestium*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora opacita*	Insecta	Mosquito	FW/T	Andreadis (1994)
I	*Amblyospora rugosa*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora salairia*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora salinaria*	Insecta	Mosquito	FW/T	Becnel and Andreadis (1998)
I	*Amblyospora severinia*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora shegaria*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora sticticі*	Insecta	Mosquito	FW/T	Andreadis (1994)
I	*Amblyospora stimuli*	Insecta	Mosquito	FW/T	Andreadis (1994)
I	*Amblyospora timirasia*	Insecta	Mosquito	FW/T	Andreadis et al. (2012)
I	*Amblyospora weiseri*	Insecta	Mosquito	FW/T	Lukeš and Vávra (1990)
I	*Amphiacantha longa*	Protozoa	Gregarine	Hyp	Larsson (2000)
I	*Amphiamblys capitellides*	Protozoa	*Ancora sagittata*	Hyp	Larsson and Køie (2006)
I	*Andreanna caspii*	Insecta	*Ochlerotatus caspius*	FW/T	Simakova et al. (2008)
I	*Bacillidium vesiculoformis*	Oligochaeta	*Nais simplex*	FW	Morris et al. (2005a)
I	*Bryonosema plumatellae*	Bryozoa	*Bryozoan*	FW	Canning et al. (2002)
I	*Bryonosema tuftyi*	Bryozoa	*Bryozoan*	FW	Canning et al. (2002)
I	*Janacekia debaisieuxi*	Insecta	*Kiefferulus tainanus*	FW/T	Larsson (1988)
I	*Janacekia tainanus*	Insecta	*Kiefferulus tainanus*	FW/T	Liu et al. (2020)
I	*Jirovecia sinensis*	Oligochaeta	*Branchiura sowerbyi*	FW	Liu et al. (2020)

(continued)

Table 10.1 (continued)

Clade	Microsporidian species	Known/type host(s)		Environment	Reference
I	*Mitosporidium daphniae*	Crustacea	*Daphnia*	FW	Haag et al. (2014)
I	*Nematocenator marisprofundi*	Nematoda	Methane-seep nematodes	M	Sapir et al. (2014)
I	*Neoperezia chironomi*	Insecta	*Chironomus plumosus*	FW/T	Issi et al. (2012)
I	*Neoperezia semenovaiae*	Insecta	*Chironomus plumosus*	FW/T	Issi et al. (2012)
I	*Pseudonosema cristatellae*	Bryozoa	Bryozoan	FW	Canning et al. (2002)
I	*Schroedera airthreyi*	Bryozoa	Bryozoan	FW	Morris et al. (2005b)
I	*Schroedera plumatellae*	Bryozoa	*Plumatella fungosa*	FW	Morris and Adams (2002)
I	*Systenostrema alba*	Insecta	*Dragonfly Aeshna viridis*	FW/T	Sokolova et al. (2006)
I	*Trichonosema algonquinensis*	Bryozoa	*Pectinatella magnifica*	FW	Desser et al. (2004)
I	*Trichonosema pectinatellae*	Bryozoa	*Pectinatella magnifica*	FW	Canning et al. (2002)
II	*Pseudoberwaldia daphniae*	Crustacea	*Daphnia longispina*	FW	Vávra et al. (2019)
III	*Berwaldia schaefernai*	Crustacea	*Daphnia* sp.	FW	Vávra and Larsson (1994)
III	*Binucleata daphniae*	Crustacea	*Daphnia* sp.	FW	Refardt et al. (2008)
III	*Culicospora magna*	Insecta	*Culex restuans*	FW/T	Becnel et al. (1987)
III	*Culicosporella lunata*	Insecta	*Culex pilosus*	FW/T	Becnel and Fukuda (1991)
III	*Edhazardia aedis*	Insecta	*Aedes aegypti*	FW/T	Becnel et al. (1989)
III	*Episeptum anaboliae*	Insecta	*Anabolia furcata*	FW/T	Hyliš et al. (2007)
III	*Episeptum circumscriptum*	Insecta	*Hydropsyche* sp.	FW/T	Hyliš et al. (2007)
III	*Episeptum pesudoinversum*	Insecta	*Sericostoma personatum*	FW/T	Hyliš et al. (2007)
III	*Episeptum trichoinvadens*	Insecta	*Potamophylax cingulatus*	FW/T	Hyliš et al. (2007)
III	*Fibrillanosema crangonycis*	Crustacea	*Crangonyx* sp.	FW	Slothouber-Galbreath et al. (2004)
III	*Flabelliforma montana*	Insecta	*Phlebotomus ariasi*	FW/T	Canning et al. (1991)

(continued)

Table 10.1 (continued)

Clade	Microsporidian species	Known/type host(s)		Environment	Reference
III	*Gurleya daphniae*	Crustacea	*Daphnia* sp.	FW	Friedrich et al. (1996)
III	*Gurleya vavrai*	Crustacea	*Daphnia longispina*	FW	Green (1974)
III	*Hazardia milleri*	Insecta	*Culex quinquefasciatus*	FW/T	Hazard and Fukuda (1974)
III	*Hyalinocysta chapmani*	Insecta	*Culiseta melanura*	FW/T	Andreadis and Vossbrinck (2002)
III	*Intrapredatorus barri*	Insecta	*Culex fuscanus*	FW/T	Chen et al. (1998)
III	*Lanatospora costata*	Crustacea	*Megacyclops viridis*	FW/M	Vávra et al. (2016a)
III	*Larssonia obtusa*	Crustacea	*Daphnia magna, D. pulex, D. longispina*	FW	Vávra et al. (2018)
III	*Marssoniella elegans*	Crustacea	*Cyclops vicinus*	FW	Vávra et al. (2005)
III	*Parathelohania anophelis*	Insecta	*Anopheles quadrimaculatus*	FW/T	Hazard and Oldacre (1976)
III	*Parathelohania divulgata*	Insecta	*Anopholes* sp.	FW/T	Simakova and Pankova (2004)
III	*Parathelohania iranica*	Insecta	*Anopheles superpictus*	FW/T	Omrani et al. (2017)
III	*Parathelohania obesa*	Insecta	*Anopheles crucians*	FW/T	Hazard and Oldacre (1976)
III	*Parathelohania tomski*	Insecta	*Anopheles messeae*	FW/T	Andreadis et al. (2012)
III	*Senoma globulifera*	Insecta	*Anopheles messeae*	FW/T	Simakova et al. (2005)
III	*Takaokaspora nipponicus*	Insecta	*Ochlerotatus japonicus*	M/T	Andreadis et al. (2013)
III	*Thelohania contejeani*	Crustacea	*Austropotamobius pallipes*	FW	Lom et al. (2001a)
III	*Thelohania montirivulorum*	Crustacea	*Cherax destructor*	FW	Moodie et al. (2003a)
III	*Thelohania parastaci*	Crustacea	*Cherax destructor*	FW	Moodie et al. (2003b)
IV	*Agmasoma penaei*	Crustacea	*Penaeus* sp.	M	Sokolova et al. (2015)
IV	*Anncaliia azovica*	Crustacea	*Niphargogammarus intermedius*	FW	Tokarev et al. (2018)
IV	*Anncaliia algerae*	Insecta, Mammalia	*Drosophila* sp., Human, Mosquito	FW/T	Tokarev et al. (2018)

(continued)

Table 10.1 (continued)

Clade	Microsporidian species	Known/type host(s)		Environment	Reference
IV	*Anostracospora rigaudi*	Crustacea	*Artemia parthenogenetica*	M	Rode et al. (2013)
IV	*Crispospora chironomi*	Insecta	*Chironomus plumosus*	FW/T	Tokarev et al. (2010)
IV	*Enterocytospora artemiae*	Crustacea	*Artemia parthenogenetica*	M	Rode et al. (2013)
IV	*Enterocytozoon salmonis*	Fish	*Salmo salar*	FW/M	Chilmonczyk et al. (1991)
IV	*Enterocytozoon hepatopenaei*	Crustacea	*Penaeus vannamei*	M	Tourtip et al. (2009)
IV	*Enterospora canceri*	Crustacea	*Cancer pagurus*	M	Stentiford et al. (2007)
IV	*Euplotespora binucleata*	Protozoa	*Euplotes woodruffi*	FW/M	Fokin et al. (2008)
IV	*Globosporidium paramecii*	Protozoa	*Paramecium primaurelia*	FW	Yakovleva et al. (2020)
IV	*Globulispora mitoportans*	Crustacea	*Daphnia* sp.	FW	Vávra et al. (2016b)
IV	*Glugoides intestinalis*	Crustacea	*Daphnia* sp.	FW	Larsson et al. (1996)
IV	*Helmichia lacustris*	Insecta	*Chironomus plumosus*	FW/T	Tokarev et al. (2012)
IV	*Hepatospora eriocheir*	Crustacea	*Eriocheir sinensis*	FW/M	Stentiford et al. (2011)
IV	*Mrazekia macrocyclopis*	Insecta	*Kiefferulus tainanus*	FW/T	Issi (2010)
IV	*Nosema austropotamobii*	Crustacea	*Austropotamobius pallipes*	FW	Tokarev et al. (2020)
IV	*Nosema cheracis*	Crustacea	*Cherax destructor*	FW	Tokarev et al. (2020)
IV	*Nosema granulosis*	Crustacea	*Amphipod*	FW	Terry et al. (1999)
IV	*Nucleospora braziliensis*	Fish	*Oreochromis niloticus*	FW	Rodrigues et al. (2017)
IV	*Nucleospora salmonis*	Fish	*Oncorhynchus tshawytscha*	FW/M	Docker et al. (1997)
IV	*Nucleospora cyclopteri*	Fish	*Cyclopterus lumpus*	M	Freeman et al. (2013)
IV	*Obruspora papernae*	Fish	*Callionymus filamentosus*	M	Diamant et al. (2014)
IV	*Ordospora colligata*	Crustacea	*Daphnia*	FW	Larsson et al. (1997)
IV	*Parahepatospora carcini*	Crustacea	*Carcinus maenas*	M	Bojko et al. (2017a)
IV	*Paranucleospora theridion*	Fish and Crustacea	*Salmo salar*, *Lepeophtheirus salmonis*	M	Nylund et al. (2010)

(continued)

Table 10.1 (continued)

Clade	Microsporidian species	Known/type host(s)		Environment	Reference
IV	*Sporanauta perivermis*	Nematoda	*Odontophora rectangula*	M	Ardila-Garcia and Fast (2012)
V	*Areospora rohanae*	Crustacea	*Lithodes santolla*	M	Stentiford et al. (2014)
V	*Ameson herrnkindi*	Crustacea	*Panulirus argus*	M	Small et al. (2019)
V	*Ameson iseebi*	Crustacea	*Panulirus japonicus*	M	Itoh et al. (2020)
V	*Ameson metacarcini*	Crustacea	*Metacarcinus magister*	M	Small et al. (2014)
V	*Ameson michaelis*	Crustacea	*Callinectes sapidus*	M	Findley et al. (1981)
V	*Ameson portunus*	Crustacea	*Portunus trituberculatus*	M	Wang et al. (2017)
V	*Ameson pulvis*	Crustacea	*Carcinus maenas and other Brachyura*	M	Vivares and Sprague (1979)
V	*Apotaspora heleios*	Crustacea	*Palaemontes paludosus*	M	Sokolova and Overstreet (2018)
V	*Cambaraspora floridanus*	Crustacea	*Procambarus paeninsulanus, Procambarus fallax, Cambarus blackii, Cambarus schufeldti*	FW	Bojko et al. (2020a)
V	*Cucumispora dikerogammari*	Crustacea	*Dikerogammarus villosus*	FW	Ovcharenko et al. (2010)
V	*Cucumispora ornata*	Crustacea	*Dikerogammarus haemobaphes*	FW	Bojko et al. (2015)
V	*Cucumispora roeselii*	Crustacea	*Gammarus roeselii*	FW	Bojko et al. (2017b)
V	*Dasyatispora levantinae*	Fish	*Dasyatis pastinaca*	M	Diamant et al. (2010)
V	*Dictyocoela berillonum*	Crustacea	Amphipod	FW	Bacela-Spychalska et al. (2018)
V	*Dictyocoela cavimanum*	Crustacea	Amphipod	FW	Bacela-Spychalska et al. (2018)
V	*Dictyocoela deshayesum*	Crustacea	Amphipod	FW	Bacela-Spychalska et al. (2018)
V	*Dictyocoela duebenum*	Crustacea	Amphipod	FW	Bacela-Spychalska et al. (2018)

(continued)

Table 10.1 (continued)

Clade	Microsporidian species	Known/type host(s)		Environment	Reference
V	*Dictyocoela gammarellum*	Crustacea	Amphipod	FW	Bacela-Spychalska et al. (2018)
V	*Dictyocoela muelleri*	Crustacea	Amphipod	FW	Bacela-Spychalska et al. (2018)
V	*Dictyocoela roeselum*	Crustacea	Amphipod	FW	Bacela-Spychalska et al. (2018)
V	*Dictyocoela diporeiae*	Crustacea	Amphipod	FW	Bacela-Spychalska et al. (2018)
V	*Facilispora margolisi*	Crustacea	Copepoda	M	Jones et al. (2012)
V	*Fusasporis stethaprioni*	Fish	*Gymnocorymbus ternetzi*, *Paracheirodon axelrodi*	FW	Lovy et al. (2021)
V	*Glugea hertwigi*	Fish	*Osmerus mordax*	FW	Weissenberg (1911)
V	*Glugea gasterostei*	Fish	*Gasterosteus aculeatus*	FW/M	Sprague (1977)
V	*Glugea americanus*	Fish	*Lophius americanus*	M	Takvorian and Cali (1986)
V	*Glugea arabica*	Fish	*Epinephelus polyphekadion*	M	Azevedo et al. (2016)
V	*Glugea eda*	Fish	*Caesio striata*	M	Mansour et al. (2020)
V	*Glugea jazanensis*	Fish	*Lutjanus bohar*	M	Abdel-Baki et al. (2015)
V	*Glugea pagri*	Fish	*Pagrus major*	M	Su et al. (2014)
V	*Glugea plecoglossi*	Fish	*Plecoglossus altivelis*	M	Sprague (1977)
V	*Glugea sardinellensis*	Fish	*Sardinella aurita*	M	Mansour et al. (2016)
V	*Glugea stephani*	Fish	*Pleuronectes platessa*	M	Sprague (1977)
V	*Glugea anomala*	Fish	*Caesio striata*	M	Weissenberg (1911)
V	*Heterosporis anguillarum*	Fish	*Anguilla japonica*	FW/M	Lom et al. (2000)
V	*Heterosporis sutherlandae*	Fish	*Perca flavescens*, *Esox lucius*, *Sander vitreus*	FW	Phelps et al. (2015)
V	*Hyperspora aquatica*	Protozoa	*Martelia cochillia*	M/Hyp	Stentiford et al. (2017)

(continued)

Table 10.1 (continued)

Clade	Microsporidian species	Known/type host(s)		Environment	Reference
V	*Ichthyosporidium giganteum*	Fish	*Ctenolabrus rupestris*	M	Sanders et al. (2012)
V	*Ichthyosporidium weissii*	Fish	*Clevelandia ios*	M	Sanders et al. (2012)
V	*Inodosporus octospora*	Crustacea	*Palaemon serratus*	M	Stentiford et al. (2018)
V	*Kabatana newberryi*	Fish	*Eucyclogobius newberryi*	M	McGourty et al. (2007)
V	*Kabatana takedai*	Fish	*Oncorhynchus masou*	M	Lom et al. (2001b)
V	*Loma acerinae*	Fish	*Gobiidae*	FW/M	Lom and Pekkarinen (1999)
V	*Loma embiotocia*	Fish	*Cymatogaster aggregata*	FW/M	Shaw et al. (1997)
V	*Loma psittaca*	Fish	*Colomesus psittacus*	FW/M	Casal et al. (2009)
V	*Loma branchialis*	Fish	*Melanogrammus aeglefinus*	M	Brown et al. (2010)
V	*Loma kenti*	Fish	*Microgadus tomcod*	M	Brown et al. (2010)
V	*Loma lingcodae*	Fish	*Ophiodon elongatus*	M	Brown et al. (2010)
V	*Loma morhua*	Fish	*Gadus morhua*	M	Brown et al. (2010)
V	*Loma pacificodae*	Fish	*Gadus macrocephalus*	M	Brown et al. (2010)
V	*Loma richardi*	Fish	*Anoplopoma fimbria*	M	Brown et al. (2010)
V	*Loma salmonae*	Fish	*Oncorhynchus tshawytscha*	M	Brown et al. (2010)
V	*Loma wallae*	Fish	*Gadus chalcogrammus*	M	Brown et al. (2010)
V	*Microgemma caulleryi*	Fish	*Hyperoplus lanceolatus*	M	Leiro et al. (1999)
V	*Myospora metanephrops*	Crustacea	*Metanephrops challengeri*	M	Stentiford et al. (2010)
V	*Myosporidium spraguei*	Fish	*Lota lota*	FW	Jones et al. (2017)
V	*Myosporidium merluccius*	Fish	*Merluccius* sp.	FW	Baquero et al. (2005)
V	*Myosporidium ladogensis*	Fish	*Lota lota*	FW	Jones et al. (2020)
V	*Nadelospora canceri*	Crustacea	*Carcinus maenas*	M	Stentiford et al. (2013b)

(continued)

Table 10.1 (continued)

Clade	Microsporidian species	Known/type host(s)		Environment	Reference
V	*Ovipleistophora diplostomuri*	Crustacea, Fish, Platyhelminth	*Procambarus bivittatus, Lepomis macrochirus, Posthodiplostomum minimum*	FW/Hyp	Lovy and Friend (2017); Bojko et al. (2020b)
V	*Ovipleistophora mirandellae*	Fish	*Gymnocephalus cernuus*	FW	Pekkarinen et al. (2002)
V	*Ovipleistophora arlo*	Crustacea	*Palaemon serratus*	M	Stentiford et al. (2018)
V	*Panopeispora mellora*	Crustacea	*Dyspanopeus sayi*	M	Bojko et al. (2021)
V	*Paradoxium irvingi*	Crustacea	*Macrobrachium nipponense*	M	Stentiford et al. (2015)
V	*Perezia nelsoni*	Crustacea	*Farfantepenaeus aztecus*	M	Vivares and Sprague (1979)
V	*Pleistophora beebei*	Fish	*Brachyhypopomus beebei*	FW	Casal et al. (2016)
V	*Pleistophora hyphessobryconis*	Fish	*Danio rerio, Puntius tetrazona*	FW	Li et al. (2012)
V	*Pleistophora mulleri*	Crustacea	*Gammarus duebeni*	FW	Terry et al. (2003)
V	*Pleistophora aegyptiaca*	Fish	*Saurida tumbil*	M	Abdel-Ghaffar et al. (2012)
V	*Pleistophora ehrenbaumi*	Fish	*Anarhichas lupus*	M	Sprague (1977)
V	*Pleistophora finisterrensis*	Fish	*Micromesistius poutassou*	M	Leiro et al. (1996)
V	*Pleistophora hippoglossoideos*	Fish	*Hippoglossoides platessoides*	M	Sprague (1977)
V	*Pleistophora typicalis*	Fish	*Myoxocephalus scorpius*	M	Sprague (1977)
V	*Potaspora aequidens*	Fish	*Aequidens plagiozonatus*	FW	Videira et al. (2015)
V	*Potaspora macrobrachium*	Crustacea	*Macrobrachium nipponense*	M	Ding et al. (2016b)
V	*Potaspora morhaphis*	Fish	*Potamorhaphis guianensis*	M	Casal et al. (2008)
V	*Pseudokabatana alburnus*	Fish	*Culter alburnus*	FW	Liu et al. (2019)
V	*Pseudoloma neurophilia*	Fish	*Danio rerio*	FW	Matthews et al. (2001)
V	*Spraguea gastrophysus*	Fish	*Lophius* sp.	M	Casal et al. (2012)
V	*Spraguea lophii*	Fish	*Lophius* spp.	M	Weissenberg (1911)

(continued)

Table 10.1 (continued)

Clade	Microsporidian species	Known/type host(s)		Environment	Reference
V	*Tetramicra brevifilum*	Fish	*Cyclopterus lumpus*	M	Matthews and Matthews (1980)
V	*Thelohania butleri*	Crustacea	*Pandalus jordani*	M	Brown and Adamson (2006)
V	*Triwangia caridinae*	Crustacea	*Cardinia formosae*	FW	Wang et al. (2013)
V	*Unikaryon legeri*	Platyhelmintha	*Meigymnophallus minutus*	M/Hyp	Canning and Nicholas (1974)
V	*Unikaryon panopei*	Platyhelmintha	*Microphallus* sp. (from *Panopeus herbstii*)	M/Hyp	Sokolova et al. (2021)
V	*Vavraia culicis* (*var. floridensis*)	Insecta	*Several mosquito species*	FW/T	Vavra and Becnel (2007)
IV/V	*Hamiltosporidium magnivora*	Crustacea	*Daphnia* sp.	FW	Haag et al. (2011)
IV/V	*Hamiltosporidium tvaerminnensis*	Crustacea	*Daphnia magna*	FW	Haag et al. (2011)
Unknown	*Metchnikovella incurvata*	Protozoa	*Polyrhabdina* sp.	M/Hyp	Sokolova et al. (2013)
Unknown	*Metchnikovella dogieli*	Protozoa	*Selenidium pygospionis* (from *Pygospio elegans*)	M/Hyp	Paskerova et al. (2016)
Unknown	*Metchnikovella spiralis*	Protozoa	Five gregarine species	Hyp	Sokolova et al. (2014)

10.2.2 Microsporidian Parasites of Aquatic Birds

Aquatic birds, or birds that feed in aquatic environments, are present across the Aves and are common hosts of opportunistic microsporidians (Slodkowicz-Kowalska et al. 2006). Most screening methods applied to date are conducted on faecal samples to search for pathogens with a risk to humans, with one of the most recent screening studies involving the detection of *E. bieneusi* in the whooper swan (*Cygnus cygnus*) (Wang et al. 2020). Aside from *Enterocytozoon* spp. and *Encephalitozoon* spp., little alternative diversity has been identified from aquatic birds. This includes a lack of microsporidians whose host range involves only birds.

10.2.3 Microsporidian Parasites of Aquatic Reptiles

Aquatic reptiles include members within the Testudines (turtles and terrapins), Lepidosauromorpha (lizards and snakes), and the Crocodylomorpha (crocodiles

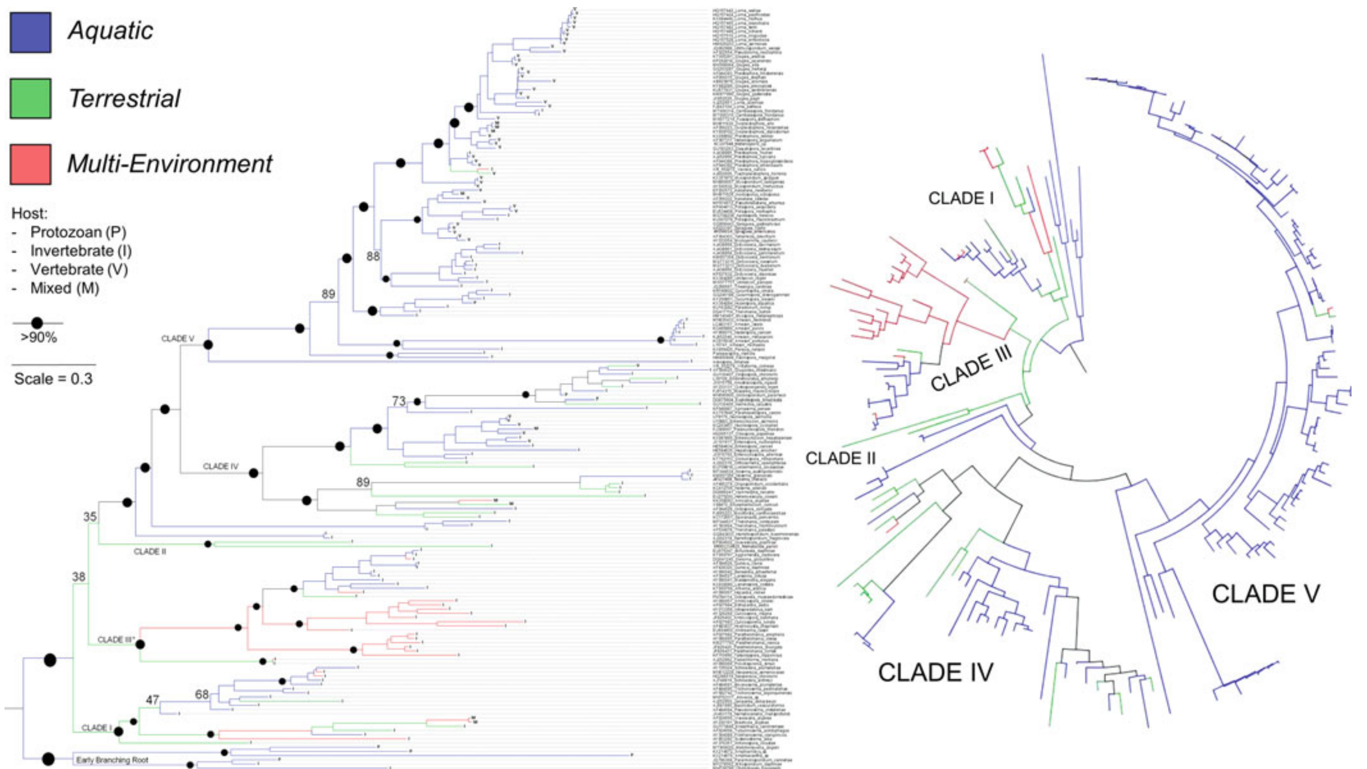

Fig. 10.1 An 18S maximum-likelihood phylogenetic tree including all aquatic and several terrestrial microsporidian species. The tree includes 182 individuals and was developed from 3125 columns of the aligned 18S genes, including gap regions. The evolutionary model is GTR+F+I+G4 and included 1000 bootstrap replicates. The tree displays each species' environmental origin as well as host group: protozoan (P), invertebrate (I), vertebrate (V), mixed (M). The clade-level (I, II, III, IV, V) grouping is also noted on the earliest relevant branch

and alligators). To date, microsporidian parasites have been noted from crocodiles, water lizards, aquatic snakes, and loggerhead turtles from aquatic environments, with multiple more from terrestrial reptilian groups (Sokolova et al. 2016a).

The first microsporidian identified from an aquatic reptile was *Pleistophora atretii* from the freshwater snake, *Atretium schistsum*, in India (Narasimhamurti et al. 1982). Since this initial finding, unidentified microsporidians have been noted in the water dragon (*Intellagama leueurii*) and yellow-bellied sea snake (*Pelamis platura*) in Australia (Reece and Hartley 1994); *Encephalitozoon hellem* from Australian crocodiles (*Crocodylus johnstoni*) (Scheelings et al. 2015); *Heterosporis* sp. in Australian marine snakes (Gillett et al. 2016); and a Clade III microsporidian in a loggerhead sea turtle from Atlantic Canada (Martinson et al. 2018). In all cases, these have been lab-reared or single detection studies, providing little data on the ecological consequences of infection.

10.2.4 Microsporidian Parasites of Amphibians

The Amphibia are a group of freshwater vertebrates with a global distribution. Microsporidia have been noted from toads, tree frogs and salamanders (Vergneau-Grosset and Larrat 2016). Toads (*Bufo* spp. and *Xenopus* spp.) have been identified with *Alloglugea bufonis* from *Bufo marinus* tadpoles (Paperna and Lainson 1995) and *Plistophora myotrophica* in *Bufo* and *Xenopus* (Canning et al. 1964). The tree frogs *Rana muscosa* and *Phyllomedusa bicolor* have uncharacterised microsporidian parasites (Graczyk et al. 1996; Pessier 2014). Finally, *Vavraia* sp. and other uncharacterised microsporidia have been documented to infect salamanders (Gamble et al. 2005; Yu et al. 2019). In all cases, these have been lab-reared or single detection studies, providing little data on the ecological or aquacultural consequences of infection.

10.2.5 Microsporidian Parasites of Fish

Fishes harbour the greatest diversity of vertebrate-infecting microsporidian parasites (Kent et al. 2014). Over 122 species across >20 genera are identified, with ~56 species from 19 genera fully described using genetic, histological, and ultrastructural/developmental data (Table 10.1; Fig. 10.1). Most fish-infecting genera (*Glugea*, *Loma*, *Tetramicra*, *Spraguea*, *Ichthysporidium*, *Pleistophora*, *Ovipleistophora*, *Heterosporis*, *Kabatana*, *Pseudokabatana*, *Dasyatispora*, *Nucleospora*, *Paranucleospora*, *Potaspora*, *Pseudoloma*, and *Fusasporis*) are described pathologically (Kent et al. 2014; Liu et al. 2019; Lovy et al. 2021). All these genera fall into the Clade V (Marinosporidia) or Clade IV (Terresporidia) groups within accepted microsporidian taxonomic limits (Vossbrinck et al. 2014).

Broadly, all the above genera sit within Clade V, excluding *Paranucleospora* and *Nucleospora* spp. (Clade IV), which target the nuclei of host cells. The Clade V fish-infecting groups infect a range of tissue and organ types, detailed by Kent et al. (2014). The most recently described genera, *Pseudokabatana* and *Fusasporis*, are also Clade V microsporidia. *Pseudokabatana alburnus* is the only described species from this genus and infects the liver of *Culter alburnus* local to the Jiangxi Province in China (Liu et al. 2019). Although closely related, this species and members of the genus *Kabtana* differ in their tissue tropism. This infection has not been detected in cultured fish but was first isolated from a wild population.

Fusasporis stethaprioni is a non-xenoma-forming species that can cause a systemic infection in black and cardinal tetra (*Gymnocorymbus ternetzi*; *Paracheirodon axelrodi*) (Lovy et al. 2021). The infection was noted from quarantined ornamental fish in the USA, and the closest known microsporidian relative exists within the *Glugea*. This species is considered an early branching relative to the *Glugea*, suggesting it may respond to the same treatment procedures applied to other *Glugea* infections (Kent et al. 2014). The fish-infecting microsporidia are described in further detail in Chap. 11 of this book.

10.3 Microsporidian Diversity in Aquatic Invertebrates

Invertebrate diversity far outweighs vertebrate diversity, and this is also the case for invertebrate-infecting microsporidian parasites. Hosts identified to date belong to the: Arthropoda; Platyhelminthes; Annelida; Mollusca; Bryozoa; Cnidaria; Rotifera; Acanthocephala; Cephalorhyncha; Dicyemida; Gastrotricha; Kinorhyncha; Phoronida; and Porifera (Stentiford et al. 2013a; Murareanu et al. 2021). Overall, adequately described microsporidian parasites of aquatic and terrestrial invertebrates' number ~86 genera and an estimated 208 species. The number of microsporidian species in aquatic and semi-aquatic invertebrates totals 137 (~68 genera) (66%).

10.3.1 Microsporidian Diversity in Aquatic Insects and Arachnids

Currently, 58 species of microsporidian have been adequately described from aquatic or semi-aquatic insects. These include members from the Clade I (*Amblyospora*, *Andreanna*, *Janacekia*, *Neoperezia*, *Systenostrema*), Clade III (*Culicospora*, *Culicosporella*, *Edhazardia*, *Episeptum*, *Flabelliforma*, *Hazardia*, *Hyalinocysta*, *Intrapredatorus*, *Parathelohania*, *Senoma*, *Takaokaspora*), Clade IV (*Anncallia*, *Crispospora*, *Helmichia*, *Mrazekia*) and Clade V (*Vavraia culicis*) (Table 10.1; Fig. 10.1).

Clade I microsporidian parasites of insects include 36 species in five separate genera. The *Amblyospora* includes ~30 of these described species—more than any other microsporidian genus to date. *Andreanna caspii* and the 30 *Amblyospora* spp. infect mosquitoes (Simakova et al. 2008), and many share a complex lifecycle between both copepods (Crustacea) and mosquitos, a lifecycle that has not been noticed for *A. caspii* but is common in the *Amblyospora* (Andreadis et al. 2018). These complex lifecycles allude to an interesting ecological position, which is also noted in Clade IV and Clade V microsporidians of trematodes, fish, humans, and crustaceans (Stentiford et al. 2019). *Janacekia debaisieuxi*, *Janacekia tainanus*, *Neoperezia chironomi*, *Neoperezia semenovaiae* infect chironomid flies, and *Systenostrema alba* infects the dragonfly, *Aeshna viridis* (Sokolova et al. 2006; Issi et al. 2012; Weng et al. 2021). The chironomid-infecting species form a close phylogenetic cluster, termed the 'Bacillidium-Janacekia-Jirovecia-Pseudonosema-Schroedera-Neoperezia-Bryonosema-Trichonosema cluster' (Weng et al. 2021). Recently, *Systenostrema alba* was isolated from the lungs of COVID-19 and asthma patients, as well as a parasite of dragonfly larvae (Sokolova et al. 2006; Stavropoulou et al. 2021).

Clade III parasites are the more diverse clade of insect-infecting microsporidians, including 11 genera and 18 species that also infect mosquitoes and caddisflies (Table 10.1). Many of these Clade III genera also house species that infect terrestrial insects, and the phylogenetic lineage of this group is distinct from the Clade I parasites of these same hosts (Fig. 10.1). One genus, *Episeptum*, is thought to use a secondary host group and is a genetic oddity, considered to be Clade III members (Hyliš et al. 2007). All other genera include microsporidian parasites that may regulate host dipteran populations (Becnel and Johnson 2000).

Clade IV microsporidian parasites of insects are represented by four genera and four species (Table 10.1; Fig. 10.1). *Crispospora chironomi* and *Helmichia lacustris* infect chironomid flies; *Mrazekia macrocyclopis* infects caddisflies; and *Anncaliia algerae* infects *Drosophila* spp. and mosquitoes, as well as humans (Table 10.1). *Vavraia culicis* (and subspecies) is the only adequately described Clade V microsporidian of insects and infects several mosquito species (Vavra and Becnel, 2007). This parasite causes female *Culex pipiens* to mature more quickly than uninfected or male *C. pipiens*, although infection also results in smaller females (Agnew et al. 1999).

Finally, microsporidian pathogens are rarely described from arachnids. *Napamichum* sp. is the only aquatic microsporidian that has been observed, which parasitizes the water mite, *Limnochares aquatica* (Larsson 1990). This species has no accompanying genetic data.

10.3.2 Microsporidian Parasites of Aquatic Crustaceans

Microsporidian diversity is greatest within the Crustacea and includes 72 adequately described species from 43 genera across Clades I–V: *Agglomerata*, *Alfvenia*,

Mitosporidium, and *Amblyospora* (Clade I); *Pseudoberwaldia* (Clade II); *Berwaldia*, *Binucleata*, *Fibrillanosema*, *Gurleya*, *Lanatospora*, *Larssonia*, *Marssoniella*, and *Thelohania* (Clade III); *Agmasoma*, *Anncaliia*, *Anostracospora*, *Enterocytospora*, *Enterocytozoon*, *Enterospora*, *Globulispora*, *Glugoides*, *Hamiltosporidium*, *Hepatospora*, *Nosema*, *Ordospora*, *Parahepatospora* and *Paranucleospora* (Clade IV); *Ameson*, *Areospora*, *Cambaraspora*, *Cucumispora*, *Dictyocoela*, *Facilispora*, *Indosporous*, *Myospora*, *Nadelspora*, *Ovipleistophora*, *Panopeispora*, *Paradoxium*, *Perezia*, *Pleistophora*, *Potaspora*, and *Triwangia* (Clade V) (Table 10.1). This list does not include observations missing genetic or ultrastructural data, listed by Stentiford and Dunn (2014).

Clade I and II microsporidian parasites are comparatively rare in crustaceans but have been identified from copepods (*Amblyospora* spp. and *Alfvenia sibirica*) and *Daphnia* spp. (*Agglomerata cladocera*, *Agglomerata daphnia*, *Mitosporidium daphniae* and *Pseudoberwaldia daphniae*) (Table 10.1). Clade III microsporidians number 11 species that infect Crustacea, including daphnids, amphipods, crabs, copepods, and crayfish (Table 10.1). The *Thelohania* of Clade III will eventually be redescribed to another genus, since the original *Thelohania butleri* is a Clade V species. This group causes significant ecological harm for endangered crayfish (Anderson et al. 2021; Fig. 10.1).

Clade IV includes a broader diversity of crustacean-infecting parasites, several of which are pathogens of aquacultured hosts, and several more are thought to pose a zoonotic risk (Stentiford et al. 2019). Fourteen genera hold 17 adequately described species that infect amphipods, mysids, shrimp, crabs, daphnids, and crayfish, as well as several other animal taxa (Table 10.1). *Enterospora* spp. and *Paranucleospora theridion* are particularly noteworthy due to their presence within the nucleus of the host cell (Stentiford and Bateman 2007; Stentiford et al. 2007; Nylund et al. 2010). Others, such as *Enterocytozoon hepatopenaei*, are limiting pathogens of shrimp aquaculture (Tourtip et al. 2009).

Clade V harbours the greatest diversity of crustacean-infecting microsporidian parasites, accounting for 16 genera and 33 species (Table 10.1). Decapods (crab, shrimp, lobster, crayfish) harbour the majority; however, amphipods (Peracarida) host 13/32 of the Clade V species. The recently formalised *Dictyocoela* genus is the most diverse Clade V group, containing eight species (Bacela-Spychalska et al. 2018), followed by the *Ameson,* with six adequately described species. Clade V parasites of crustaceans commonly infect the musculature of the host and are considered to prioritise horizontal transmission (Stentiford and Dunn 2014). Some species have complex lifecycles, such as the *Ovipleistophora*, which are present in a range of hosts (Sokolova and Overstreet 2020).

10.3.3 Microsporidian Parasites of the Mollusca

No microsporidian parasites have been formally characterised from molluscs, despite several interesting discoveries. Microsporidian observations have been

made from mussels and scallops, using both genetic and pathological tools, but not in combination. The detection of bioaccumulated, human-infecting microsporidians highlights a risk to the human food chain (Lucy et al. 2008). Other discoveries include mollusc-specific microsporidian pathogens, such as *Steinhousia mytilovum*, which infests the oocytes of *Mytilus galloporvincialis* (Sagristà et al. 1998), and microsporidiosis in queen scallops *Aequipecten opercularis* from the UK (Lohrmann et al. 1999). Phylogenetic understanding of these mollusc-specific groups is pending but will advance the field once determined, revealing a better interpretation of human-mollusc sources of microsporidian infection.

Several microsporidian parasites have been identified from molluscan symbionts. These include observations from the squid mesozoan (*Kantharella antarctica*), which harbours the microsporidian hyperparasite, *Wittmannia antarctica* (missing genetic data) (Czaker 1997). Also noteworthy are protozoan hosts, which include four phyla that harbour microsporidian (hyper)parasites: Apicomplexa; Cercozoa; Amoebozoa; and Ciliophora (Murareanu et al. 2021). To date, these discoveries have been made from aquatic protozoans, some of which are parasites of molluscs. There are seven microsporidian parasites of Protozoa that are members of six genera: *Amphiacantha*; *Amphiamblys*; *Euplotespora*; *Globosporidium*; *Hyperspora*; and *Metchnikovella*. Only the *Hyperspora* genus holds microsporidia of mollusc protozoan parasites.

Martleia cochillia (Paramyxea) is a common parasite of marine European cockles. This protist harbours the microsporidian pathogen *Hyperspora aquatica* (Stentiford et al. 2016), which phylogenetically groups alongside the crustacean-infecting microsporidians in Clade V, such as the *Cucumispora* (Fig. 10.1). Protozoa, such as *Marteilia* spp., may act as a vectoring mechanism for the movement of microsporidia between molluscan hosts; however, this hitch-hiking strategy requires further investigation (Stentiford et al. 2016).

10.3.4 Microsporidian Parasites of Aquatic Helminths and Annelids

Around 60 helminths have been identified to carry microsporidian parasites (Murareanu et al. 2021). Eleven of these include examples from aquatic environments. Most of these findings are not taxonomically recognised, such as the ‘microsporidum’ (hyper)parasites of multiple Rotifera, *Tubifex* sp. (Oligochaeta), *Podocotyloides magnatestis* (Trematoda) and *Acanthocephaloides propinquus* (Acanthocephala) (Buron et al. 1990; Cable and Tinsley 1992; Oumouna et al. 2000; Gorbunov and Kosova 2001; Morris et al. 2005a; Toguebaye et al. 2014; Liu et al. 2020). Accepted species from annelidan hosts include *Bacillidium vesiculoformis* from *Nais simplex* (Morris et al. 2005a) and *Jirovecia sinensis* from the freshwater *Branchiura sowerbyi* (Liu et al. 2020); both of which have supporting ultrastructural and genetic data. The marine nematode *Odontophora rectangular* and

multiple methane-seep nematodes, and the trematode parasites: *Meigymnophallus minutus* (parasite of marine cockle, *Cardium edule*), *Microphallus* sp. (parasite of *Panopeus herbstii*) and *Posthodiplostomum minimum* (parasite of freshwater bluegill sunfish, *Lepomis macrochirus*), host hyperparasitic microsporidia that are complete with genetic, developmental, and ultrastructural data.

The trematodes appear to be parasitized by a diversity of microsporidian groups, many of which still need classification (Sokolova and Overstreet 2020). The primary species that are classified include *Ovipleistophora diplostomuri*, *Unikaryon panopei*, and *Unikaryon legeri* (Canning and Nicholas 1974; Lovy and Friend 2017; Sokolova et al. 2021). *Unikaryon* spp. have been identified from trematodes alone, whereby *O. diplostomuri* has been identified to infect the tissues of crayfish (muscle), fish (liver) and trematodes (Lovy and Friend 2017; Bojko et al. 2020b).

Nematodes constitute a recent boom for the discovery of microsporidian parasites, two of which are taxonomically accepted from aquatic nematodes (Ardila-Garcia and Fast 2012; Sapir et al. 2014). These include *Sporanauta perivermis* in *O. rectangular* and *Nematocenator marisprofundi* from methane-seep nematodes. Discoveries from helminths to date are made from across the microsporidian clades, highlighting a wide polyphyletic diversity.

10.4 Emerging and Persistent Microsporidians of Aquaculture Species

Most studies on microsporidian pathogens of aquatic animals relate to wildlife. However, the retainment of aquatic animal populations in constrained spatial habitats over prolonged time periods for the purposes of aquaculture has offered a unique insight into microsporidian host-pathogen interaction, pathology, transmission, and epidemiology. Previous syntheses have proposed microsporidians as 'pathogens of opportunity' (Weiss and Becnel 2014). In aquaculture, not only are dense populations of animals available for infection with microsporidian pathogens present in water, feeds, and other transient hosts within the pond setting but, captive populations may also reside in sub-optimal conditions in which environmental-, nutritional-, and genetic-immunosuppression exposes them to the perils of this opportunism (Stentiford et al. 2019).

Perhaps the highest-profile example of this has been the emergence of *Enterocytozoon* group microsporidia (EGM) and particularly, *Enterocytozoon hepatopenaei* (EHP), in the Asian penaeid shrimp farming sector over the past decade (Tourtip et al. 2009; Stentiford et al. 2019; Fig. 10.2). Here, a parasite rarely detected in *Penaeus monodon* (original shrimp species farmed in Asia during the 1990s) emerged as the agent of a significantly more prevalent and pathogenic disease in *Penaeus vannamei* (the predominant species farmed across the region). The emergence of EHP as a high prevalence disease over the past decade has coincided with co-emergence of several other important pathogens in shrimp (e.g. AHPND),

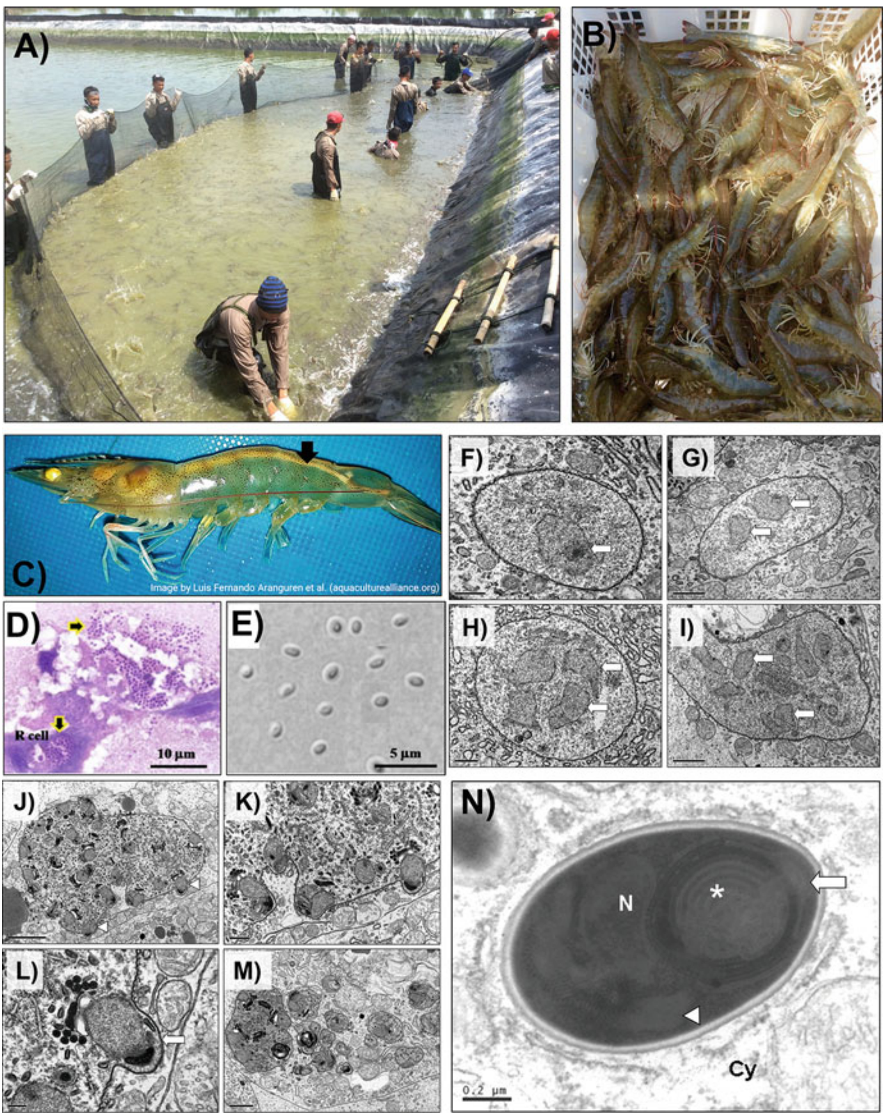

Fig. 10.2 *Enterocytozoon hepatopenaei*, a high-profile aquaculture disease. (**a**, **b**) Densely stocked *Penaeus* sp. farmed for meat in Thailand. (**c**) An example specimen displaying the classic gross pathology of 'white faeces syndrome'. The gut is visible and white (arrow). (**d**, **e**) Histological and wet-prep spores from the gut (Tourtip et al. 2009). (**f**) Uninucleate meront with central nucleus (arrow). Scale = 0.5 μm. (**g**) Bi-nucleate meront. No indication of diplokaryon (arrows). Scale = 1 μm. (**h**) Tetra-nucleate meront with central nuclei (arrows). Scale = 0.5 μm. (**i**) Multinucleate plasmodium with centralised nuclei (arrows) and presence of membranous processes. Scale = 1 μm. (**j**) Multinucleate plasmodium with assembled pre-sporoblasts. Pre-sporoblasts with nucleus, anchoring disk, polar filament and posterior vacuole are emitted from the plasmodium (arrow heads). Scale = 2 μm. (**k**) Higher power image of sporoblast production from sporogonal plasmodium (arrow heads). Scale = 0.5 μm. (**l**) Individual sporoblasts were emitted from the plasmodium with anchoring disk facing outwards and polar filament inwards. The sporoblast membrane was generated from the sporogonal membrane (arrow). Scale = 0.2 μm. (**m**) Liberated

most of which appear to be disorders of the gut—leading to either direct mortality, growth disorders affecting yield and profit or, syndromic conditions in which multiple pathogens may be implicated in the observed condition (Kooloth Valappil et al. 2021).

EHP is closely related to the human pathogen *Enterocytozoon bieneusi*, which emerged as an opportunistic co-infection during the AIDS pandemic of the 1980s (Stentiford et al. 2016). The parallels in pattern of emergence between EHP and *E. bienuesi* may not be immediately apparent—however, *E. bienuesi* is the only terrestrial taxon within the otherwise exclusively aquatic EGM, relying on co-pathogen (HIV) induced immunosuppression to infect the host gut and cause debilitating diarrheal illness in affected human hosts (Stentiford et al. 2019). Recently, EHP and co-hosted bacterial agents have been implicated in so-called 'White Faeces Syndrome' (WFS) in shrimp—a pathology that leads to shed faecal strings floating on the surface of shrimp ponds and generally poor outcomes for farms in which WFS is observed (Prachumwat et al. 2021).

Given that *E. bienuesi* has now been detected in a wide range of immunosuppressed human populations, in addition to those infected with HIV (Stentiford et al. 2016), it is likely that EGM microsporidia exploit immunosuppressed hosts (specifically the gut epithelial cells) rather than drive immunosuppression per se. Their presence in penaeid shrimp farming is considered a sentinel of underlying poor immunological health in affected populations—control options likely require a need to eliminate the pathogen from farm systems and optimise the immunological health of the farmed stock (Stentiford et al. 2019). The availability of sensitive and specific diagnostic assays for EHP (Jaroenlak et al. 2016) and full genome data for the pathogen (Wiredu Boakye et al. 2017) offer potential tools to develop control protocols for EHP in these systems.

Other gut-infecting microsporidians have also caused disease in farmed crustacean species. *Hepatospora eriocheir* has been detected at high prevalence in both wild *Eriocheir sinensis* from their non-native range (Stentiford et al. 2011) and from farmed populations of this host in China, where it induces hepatopancreatic necrosis disease (HPND) (Ding et al. 2016a). Taxonomic studies on this parasite have shown that very similar (likely identical) strains can be detected in divergent crustacean host groups from marine and brackish habitats (Bateman et al. 2016). Further, closely related taxa have been detected in non-crustacean invertebrates (Robbins et al. 2021), underlining the potential for host promiscuity in these gut-infecting microsporidian taxa.

Once again, the susceptibility of the host (and the population in which it resides) may be as important as the pathogenicity of specific pathogen taxa in the disease

Fig. 10.2 (continued) sporoblasts (arrows) in direct contact with host cell cytoplasm (asterisk) Scale = 1 μm. (**n**) The anchoring disk (arrow), polaroplast (asterisk), and spore nucleus (N) are clearly visible though the polar filament was not clearly depicted in any of the spore stages observed (triangle). Spores lay in direct contact with the host cell cytoplasm (Cy). Scale = 0.2 μm. Images **d**–**n** are reproduced with permission from Tourtip et al. (2009)

outcomes observed in wild and captive populations. As demonstrated elsewhere within this chapter, it is likely that we will continue to uncover novel diversity both within the EGM and in closely related genera (Bojko et al. 2017a, b). Further, there is growing recognition that regardless of the taxonomy of the host in which members of the EGM and related taxa are initially detected, significant potential exists for multi-trophic transmission, including between invertebrate and vertebrate host taxa (Nylund et al. 2010). In this respect and given their potential to exploit immunologically weakened hosts of diverse taxonomy, it will be important to remain open minded about the potential host range of the EGM. Are they host-specific pathogens or, may specific hosts display susceptibility to any taxa that display a fundamental propensity to infect gut epithelial cells in immune-suppressed hosts? As aquatic biomes are impacted by environmental forcing factors (e.g. climate change), which alter the susceptibility of (predominantly) poikilothermic hosts to disease, we may expect to see further emergence of EGM and related microsporidian-related diseases, some of which will impact yield in aquaculture and fisheries sectors (Stentiford et al. 2016).

10.5 Microsporidian Pathogens in Wild Aquatic Communities

Aquatic animal health is an important element of sustainable fisheries and aquaculture (Stentiford et al. 2016) but is additionally important when attempting to understand ecological resilience in the face of biological invasion or climate change (Shields 2019; Chinchio et al. 2020). In wild aquatic communities, animals are in direct contact with co-habiting species and their parasites. These ecosystems can include microsporidians capable of a variety of transmission methods and a broad host range, including some species with complex hyperparasitic lifecycles. Changes to these ecosystems, which are a valuable component of each animals' evolutionary history, can be consequential for microsporidian geographic range, prevalence, host range, and their influence upon their host's survival and behaviour (Bojko et al. 2019, 2020c).

A functional and biodiverse ecosystem includes parasites, which can alter host population size, among other features (Hudson et al. 2006). Microsporidian parasites can be both opportunistic and mortality-inducing, resulting in a single species being capable of transmitting through, and reducing population sizes of, several host groups (Andreadis et al. 2018). Microsporidian-associated impacts on fish populations are explored in detail in Chap. 11. In invertebrates, microsporidian infections in aquatic animals have been shown to alter their behaviour, including cannibalistic and predatory tendencies (Bunke et al. 2019). Host-parasite interactions between microsporidia, hosts, and the resulting consequences for ecosystem function and maintenance are therefore interconnected (Hudson et al. 2006).

Microsporidia have been identified from a diverse array of aquatic environments and host groups (Stentiford et al. 2013a; Murareanu et al. 2021). The Microsporidia appear extremely resilient, whereby it is uncommon for microsporidia to be absent from aquatic invertebrate populations. Many of these parasites display a wide host range, infecting a spectrum of host species, in addition to being adapted to a hyperparasitic lifestyle (Fig. 10.3). Some microsporidian parasites have a host range that can include species across the Animalia and may be vital for the

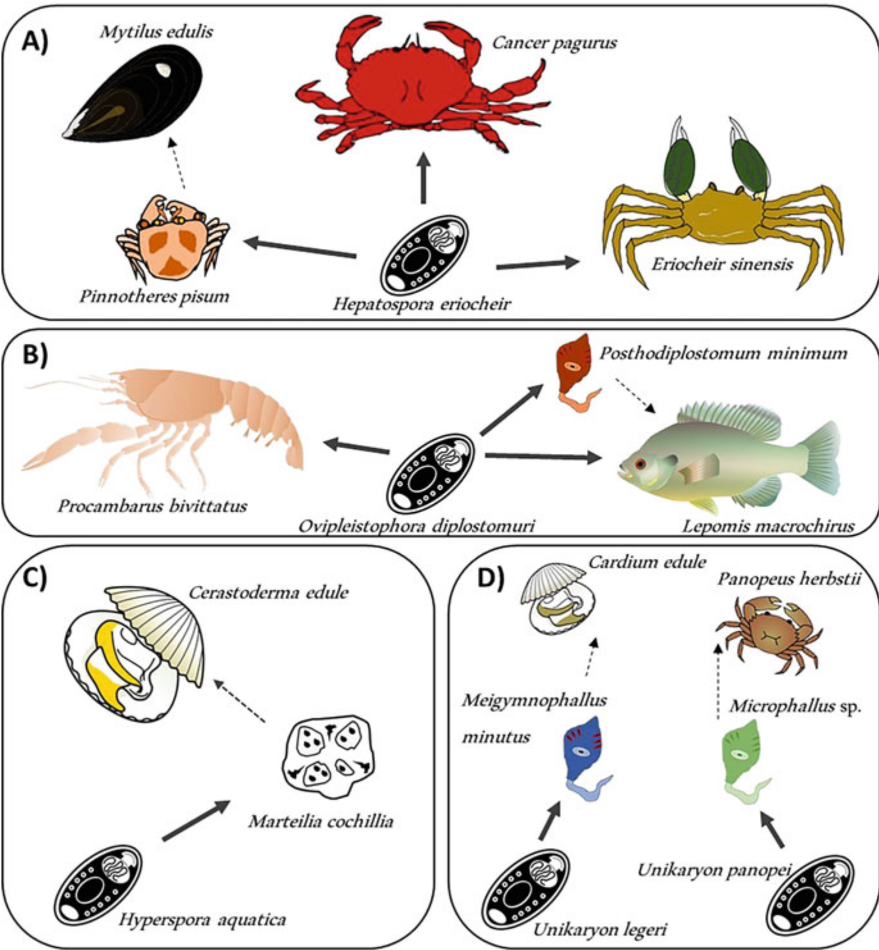

Fig. 10.3 Examples of hyperparasitism and host range among aquatic microsporidian species. (**a**) *Hepatospora eriocheir* has been identified in three crustacean hosts, one of which is a parasite of the blue-lipped mussel, *Mytilus edulis*. (**b**) *Ovipleistophora diplostomuri* directly infects crayfish, fish, and trematode hosts. Trematode infections are present in the fish host and may convey parasite transmission. (**c**) *Hyperspora aquatica* infects the protozoan (Ascetosporea) *Marteilia chochillia*, a parasite of bivalve molluscs. (**d**) Two *Unikaryon* spp. each infect digenean trematode hosts, which in turn infect either mollusc or crustacean hosts

completion of their lifecycle. Recent data on *Ovipleistophora* spp. suggests that fish, crustaceans, and platyhelminths can all become infected (Bojko et al. 2020a; Sokolova et al. 2021; Fig. 10.3). *Amblyospora* spp. development includes both insect and crustacean host groups (Andreadis et al. 2018). Finally, recent discoveries are highlighting that closely related parasites can infect very different host groups: the *Cambaraspora* is part of the Glugeidae, which was once thought to only infect fish hosts (Bojko et al. 2020a).

Hyperparasitic examples include *Hepatospora eriocheir*, *Unikaryon* spp. and *Hyperspora aquatica* (Fig. 10.3). *Hepatospora eriocheir* infects three different species of crab, which each share very different aquatic niches. *Cancer Pagurus* is a marine coastal species, *Eriocheir sinensis* (type host) is a freshwater/brackish species and a global invader, and finally, *Pinnotheres pisum* is a crustacean parasite of native UK mussel species—each crustacean host has been found to harbour *H. eriocheir* (Bateman et al. 2016), which may have been introduced via the mitten crab invasion of the UK. The second examples, *Unikaryon legeri* and *U. panopei*, are both hyperparasites of digeneans (Trematoda) that infect marine crustaceans (Sokolova et al. 2021). Finally, *H. aquatica* is a parasite of *M. cochillia* (Paramyxida) that has been found to infect a range of molluscan hosts (Stentiford et al. 2017).

Changes to microsporidian presence/absence and abundance have been suggested as reasons for increasing host population sizes. Often, microsporidia survive in the environment for long periods, infect multiple hosts, and can lay latent in hosts for generations, meaning that it is rare for Microsporidia to become extinct in a functioning ecosystem. However, biological invasions provide an insight into how the loss of microsporidia can affect host population size and success (Bojko et al. 2013). Biological invasions are one of the main causes of parasite translocation across geographic regions. To date, ~12% of the pathogen profile of >320 invasive non-native crustaceans are composed of microsporidia (Bojko et al. 2020c).

The killer shrimp, *Dikerogammarus villosus*, is an invasive species across Europe. UK populations of this host have lost their native microsporidian parasite, *Cucumispora dikerogammari* (Bojko et al. 2013). Populations in the UK are some of the most impactful and ecologically damaging, potentially due to the loss of this parasite group. In parallel, the sister species *Dikerogammarus haemobaphes* has been introduced with its microsporidian parasite, *Cucumispora ornata*—a parasite that supresses its hosts' activity and induces host mortality (Bojko et al. 2019). This comparison suggests that the loss of microsporidia within aquatic ecosystems could result in increased population growth of the host species, potentially resulting in ecosystem imbalance.

10.6 Future Perspectives on Aquatic Microsporidia

Aquatic microsporidian diversity is broad, including 202 adequately described species that can be phylogenetically, ultrastructurally, and pathologically compared. Herein, we provide a breakdown of aquatic microsporidian diversity, including an

aquatic-focal phylogenetic tree, and provide examples of prolific aquaculture diseases in addition to microsporidian-related impacts on aquatic ecosystems.

Aquatic vertebrates appear to hold the least microsporidian diversity and would benefit from additional metagenomic and pathology screening for these parasites. Those that are known require greater and more detailed taxonomic understanding. On the other hand, terrestrial and aquatic invertebrate and protozoan microsporidian diversity is building quickly, with >210 adequately described species. Our next steps include the collection of genomic data for our diverse array of microsporidia, providing a broader capacity to compare pathological, ultrastructural, and ecological variation across the five clades.

Those microsporidians that pose a significant risk to aquatic animal health require greater host-pathogen understanding. Immunosuppression in aquatic animals appears to be of specific concern to aquaculture and wild fisheries, and the underlying cause of these issues remains largely unknown but may relate directly to the animal's microbiome and pathobiome, which can be altered by stocking density and simulated environment (Prachumwat et al. 2021). Ecological perspectives can help to better understand the natural origins of infection, which includes the study of ever-changing ecological systems that are under pressure from invasion and a changing climate.

In conclusion, both our understanding of microsporidian parasite diversity and the impact of these heavily reduced eukaryotes on aquaculture and aquatic ecosystems requires greater application of novel pathological and omics technologies. Such data will provide more factor-encompassing case studies that will shed light on the important effects of these parasites across aquatic animal health.

Conflict of Interest

Conflict of Interest The authors declare that there is no conflict of interest.

Compliance with Ethical Standards The chapter is a review of previously published accounts, as such, no animal or human studies were performed.

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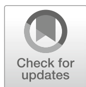
Chapter 11
Recent Advances with Fish Microsporidia



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Abstract There have been several significant new findings regarding Microsporidia of fishes over the last decade. Here we provide an update on new taxa, new hosts and new diseases in captive and wild fishes since 2013. The importance of microsporidiosis continues to increase with the rapid growth of finfish aquaculture and the dramatic increase in the use of zebrafish as a model in biomedical research. In addition to reviewing new taxa and microsporidian diseases, we include discussions on advances with diagnostic methods, impacts of microsporidia on fish beyond morbidity and mortality, novel findings with transmission and invertebrate hosts, and a summary of the phylogenetics of fish microsporidia.

Keywords *Glugea anomala* · Molecular phylogenetics · Maternal transmission · *Desmozoon lepeophtherii* · *Ovipleistophora diplostomuri*

11.1 Introduction

Microsporidian infections have been recognized in wild fishes for at least 100 years. Starting In the early 1900s, with the increase in disease investigations of captive fishes, there was a concurrent increase in the description of novel microsporidia species and associated diseases. Dyková (2006) provided a review of Microsporidia

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in fish, and 8 years later we reviewed the subject (Kent et al. 2014). Here we focus on advances in this field since 2013. In the last decade, finfish aquaculture has greatly expanded, both within the traditional species, such as salmon, carp, tilapia, and sea bream, as well as other species, such as native fishes within specific local regions. Aquaculture now generates approximately the same tonnage as wild-caught species (FAO Yearbook 2018). At the same time, the use of fish as a laboratory model, especially zebrafish *Danio rerio*, has continued to grow at a rapid rate. Concurrently, the importance of *Pseudoloma neurophilia,* the most common pathogen of zebrafish, has increased. The hobby pet fish industry representing hundreds of marine and freshwater species has also expanded, and a new, deadly microsporidium has been reported in popular tetra species (Lovy et al. 2021). In addition to the discovery of new microsporidian species and diseases in fish, several aquatic Microsporidia beyond *Desmozoon lepeophtherii* (jn. syn. *Paranucleospoa theirdon*) have been discovered to alternate between fish and invertebrate hosts (Lovy and Friend 2017; Stentiford et al. 2018; Bojko et al. 2020).

We provide new findings on fish Microsporidia, including discussion of new taxa and new hosts or diseases caused by previously described taxa (Table 11.1). We also update information on life cycles involving invertebrates and maternal/vertical transmission, impacts of infections beyond morbidity (e.g., behavior), and new information on treatments and diagnostic approaches. Additionally, we review the phylogeny of fish Microsporidia, including new taxa described since 2013.

11.2 Fish Models for Microsporidia

In regard to Microsporidia, zebrafish (*Danio rerio*) and three-spine stickleback (*Gasterosteus aculeatus*) offer three host/pathogen models: *Pseudoloma neurophilia* and *Pleistophora hyphessobryconis* in zebrafish, and *Glugea anomala* in sticklebacks. Here we discuss these two fish species and the problems and opportunities that these infections pose in research settings. The benefit of aquatic models, led by the use of zebrafish, as part of an integrative approach to improving human health is being recognized by the scientific community (Phillips and Westerfield 2014). Although teleost fishes live in water and are morphologically very different from mammals, they possess many characteristics that are shared amongst all vertebrates. Indeed, at the cellular and molecular levels, fish share much in common with mammals. For example, 70% of genes in the zebrafish genome correspond with human genes, and importantly, 84% of genes known to be associated with human disease have a zebrafish counterpart (Howe et al. 2013). However, small fish also provide a distinct usefulness and economy for in vivo research compared to rodents, in that they are less expensive and require less space in a research facility than rodents on a per animal basis (Sullivan and Kim 2008; Lescak and Milligan-Myhre 2017).

The zebrafish is a tropical freshwater fish that belongs to the family Cyprinidae. In the wild these fish typically are found in floodplains in India, occupying

Table 11.1 Recent reports on microsporidia of fishes. GenBank No. refer to new sequences used in phylogenetic analyses (Fig. 11.1)

Parasite	Host	Tissue location/ pathology	Reference	GenBank No.
Marine—Captive				
Microsporidium aurata	*Sparus aurata*	Macroscopic cysts in coelom	Morsy et al. (2013)	KF022044
Glugea pagri	*Pagrus major*	Coelomic cavity, chronic mortality	Su et al. (2014)	
Enterospora neurophilia	*Sparus aurata*	Intestinal epithelium, emaciation, mortality	Palenzuela et al. (2014)	JX101917
Desmozoon lepeophttherii	*Salmo salar*	Systemic, branchitis, peritonitis, stunted growth, and mortality	Weli et al. (2017)	AJ4313166.2
Tetramicra brevifilum	*Cylopterus lumpus*	Macroscopic cysts in the muscle	Scholz et al. (2017)	AF 364303
Nuclospora cyclopeteri	*Cylopterus lumpus*	Visible xenomas in various organs, skin, gills	Freeman et al. (2013)	LF KC203457
Enterocytozooid	*Epinephelus lanceolatus X E. fuscoguttatus*	Intestine, thinning of intestine, emaciation, high mortality	Xu et al. (2017)	KR263870
Marine—Wild				
Glugea arabica	*Epinephelus polyphekadion*	Coelomic cavity, intestine, macro-scopic cysts (xenoma)	Azevedo et al. (2016)	KT005391.1
Glugea eda	*Caseio striata*	Coelomic cavity, intestine, macro-scopic cysts (xenoma)	Mansour et al. (2020)	MK568064
Glugea jazanensis	*Lutjanus bohar*	Coelomic cavity, intestine, macro-scopic cysts (xenoma)	Abdel-Baki et al. (2015a)	KP262018
Glugea nagelia	*Cephalopholis hemistiktos*	Coelomic cavity, intestine, macro-scopic cysts (xenoma)	Abdel-Baki et al. (2015b)	KJ802012
Glugea sardinellenisis	*Sardinella auriata*	Coelomic cavity, intestine, macro-scopic cysts (xenoma)	Mansour et al. (2016)	KU577431
Glugea serranus	*Serranus atricauda*	Coelomic cavity, intestine, macro-scopic cysts (xenoma)	Casal et al. (2016a)	KU363832
Heterosporis lessinanus	*Saurida undosumis*	Macroscopic cysts in muscle	Al Quraishy et al. (2019)	

(continued)

Table 11.1 (continued)

Parasite	Host	Tissue location/ pathology	Reference	GenBank No.
Obruspora papernae	*Callionymus filamentosus*	Macroscopic xenomas in ovary, reduced fecundity	Diamant et al. (2014)	HG005137
Microgemma tilanpasiri	*Boleophthalmus dussumieri, Trypauchen vagina*	Liver, granulomatous lesions	Freeman et al. (2015) Vandana et al. (2021)	MN733420
Freshwater—Captive				
Fusasporis stetaprioni	*Gymnocorymbus ternetzi, Paracheirodon axelrodi*	Low, chronic mortality, poor breeding, secondary infection with parasitic ciliates. Systemic infections,	Lovy et al. (2021)	MW077214
Pleistophora hyphessobryconis	*Leiarius marmoratus* × *Pseudoplatystoma reticulatum*	Muscle, other clinical presentations	Winters et al. (2016)	
Nucleospora braziliensis	*Oreochromis niloticus*	Intestine, with systemtic chages	Rodrigues et al. (2017)	MW491352
Nucleospora sp.	*Danio rerio*	Kidney, lymphoid neoplasm	Kent et al. (2021)	
Enterocytozoid	*Oncorhynchus tshawtyscha*	Intestine, inflammation of lamina propria, and atrophy of epithelium	Kent et al. pers. comm.	
Freshwater—Wild				
Pseudokabatana alburnus	*Cultur alburuns*	Xenomas in liver	Liu et al. (2019)	MF974572
Myosporidium ladogensis	*Lota lota*	Muscle, myodegeneration	Jones et al. (2017)	
Myosporidium spraguei	*Sander lucioperca*	Muscle, myodegeneration	Jones et al. (2017)	KX351970
Potaspora aequidens	*Aequidens plagiozonatus*	Muscle, macroscopic cysts caused by xenoma	Videira et al. (2015)	KP404613
Microsporidium sp.	*Salvelinus alpinus*	Somatic muscle, replacement, extensive opaque lesions	Jalenques (2021)	MW429835
Heterosporis sutherlandae	*Perca flavescens, Esox lucius, Stizostedion vitreum*	Muscle, macroscopic lesions	Phelps et al. (2015)	
Heterosporis lessepsianus	*Saurida lessepsianus*	Muscle, macroscopic lesions	Al Quraishy et al. (2019)	MF769371

(continued)

Table 11.1 (continued)

Parasite	Host	Tissue location/ pathology	Reference	GenBank No.
Microsporidium sp.	*Salvelinus alpinus*	Somatic muscle, replacement, extensive opaque lesions	Jalenques et al. (2021)	MW429835
Glugea gasterosteri	*Gasterosteus aculeatus*	Xenomas in internal organs	Tokarev et al. (2015)	KM9977990
Glugea sp.	*Cottus bairdii*	Xenomas	Ryan and Kohler (2016)	KU885381
Glugea sp.	*Cottus cognatus*	Xenomas	Ryan and Kohler, (2016)	KU885382
Pleistophora beebei	*Brachyhypopomus beebei*	Muscle, macroscopic lesions	Casal et al. (2016a, b)	KX099692
Ovipleistophora diplosomuri	*Lepomis macrochirus*	Well as the liver and other coelomic organs in the fish host for the worm, bluegill sunfish *Lepomis macrochirus* (Lovy and Friend 2017)	Lovy and Friend (2017)	
Microsporidia from invertebrates linked to fish				
Ovipleistophora diplostomuri	*Posthodiplostomum minimum* in *Lepomis macrochirus*, *Procambarus bivittatus*	Infection of outer metacercarial cyst capsule of *Posthodiplostomum minimuminimum*	Bojko et al. (2020); Lovy and Friend (2017)	KY809102
Inodosporus octospora	*Palaemon serratus*	Has similar rDNA sequence with a *Kabatana* sp. *Gobiusculus flavescens,*	Stentiford et al. (2018)	MH911629
Ovipleistophora arlo	(*Palaemon serratus*)	Sequence suggests a likely fish host	Stentiford et al. (2018)	MH911630

slow-moving waters with a wide range of temperatures up to 40 °C (Spence et al. 2008). Outside of their native habitat, zebrafish have long been popular as pets in home aquaria and now in research settings. The use as a biomedical model was first advanced by Dr. George Streisgner, University of Oregon (Eisen 2020), a geneticist who studied embryo development. He determined that zebrafish embryos were an ideal model as they can be viewed and manipulated at all stages of development. The ZFIN website (http://zfin.org) now lists approximately over 1000 laboratories that employ zebrafish, and an NIH RePORTER website search using the term "zebrafish" revealed a list of 1172 active grants using this model. The field was initially led by

Fig. 11.1 Microsporidium infection in muscle of Arctic char *Salvelinus alpinus*. Courtesy of Marion Jalenques

investigations in developmental genetics, in which experimental endpoints involved primarily embryos or larval fish. Adult zebrafish are now used extensively as models throughout the biomedical research arena. These include areas as varied as infectious disease susceptibility and immune system function (Torraca et al. 2014), aging (Gilbert et al. 2014), toxicology (Tanguay 2018), and behavior (Gerlai 2019). Currently, the zebrafish model is being used as a model for cancer, (Völkel et al. 2018; Hason and Bartůněk 2019; Yan et al. 2019), as well as for the novel SARS-CoV-2 virus and other human and zebrafish pathogens (Sullivan et al. 2017; Kraus et al. 2020; Galindo-Villegas 2020).

11.2.1 Microspordia in Zebrafish

Several infectious microsporidial diseases have been frequently documented in laboratory zebrafish (Sanders et al. 2012b; Kent and Sanders 2020), most commonly *Pseudoloma neurophilia*. As with all translational animal models, underlying infections can impact research, causing non-protocol induced variation (Kent et al. 2012; Szilagyi 2016)**.** *P. neurophilia* occurs in about 50% of zebrafish laboratories (Kent and Sanders 2020), and while some infected fish show clinical disease, such as emaciation or spinal deformities, most infected fish appear largely asymptomatic. The parasite targets the central nervous system, with aggregates of spores conjugating in the hindbrain, spinal cord, ventral white matter, and the spinal nerve roots. Many infections also result in encephalitis, meningitis, or chronic multifocal myositis (Spagnoli et al. 2015b). Within the rhombencephalon gray matter, infection targets the reticular formation and the griseum centrale. Given the location of the infection, Spagnoli et al. (2015a) suggested that the infection may affect zebrafish startle responses and anxiety. Three studies, two conducted by Spagnoli et al. (2015b, 2016a) and one conducted by Midttun et al. (2020a), evaluated the effects of infection on a variety of behavior endpoints. Results generally supported consistent changes in behavior, resulting in a stress- and anxiety-prone phenotype. The same group also demonstrated that *P. neurophilia* infections cause alterations in

brain transcriptome profiles, a finding that carries broad implications for the biology of the parasite and how it operates within the zebrafish host (Midttun et al. 2020b).

Given how widespread *P. neurophilia* is in research laboratories, studies using zebrafish behavioral endpoints should avoid using infected fish (Szilagyi 2016). However, the ease of transmission of *P. neurophilia* in the laboratory (Kent and Bishop-Stewart 2003; Sanders et al. 2013) also provides an opportunity to investigate host–pathogen relationships focused on diseases of the central nervous system and behavior. Gratacap and Wheeler (2014) suggested potential zebrafish models for eukaryotic pathogens including *P. neurophilia*, and Spagnoli et al. (2016a) showed that infection with this parasite impacted normal zebrafish behavior, resulting in reduced interfish distance in shoals. The relationships between *P. neurophilia* infection and zebrafish behavior suggest that this host–pathogen model could be used for neurological research and development of therapeutics. In addition to behavior, the CNS has fundamental control of the heart rate, and damage has been connected with heart arrhythmias and other electrical problems (Tahsili-Fahadan and Geocadin 2017). The Cardiac Autoregulation and Arrhythmias Laboratory (Dalhousie University, Canada) employs the zebrafish to study the electrical function of the heart (Stoyek et al. 2018) and it was recently discovered that severe *P. neurophilia* infections were associated with neural lesions that led to bradycardia and some profound arrhythmias. Future studies will concentrate on elucidating links between the infection and these anomalies. If this association exists, then perhaps the parasite can be used to study the effects of specific changes in the hind brain and spinal cord on arrhythmias and other electrical endpoints in the heart.

Current research on microsporidial infections in zebrafish supports the paradigm that Microsporidia are particularly common in immune compromised individuals. Studies investigating *P. neurophilia* (Ramsay et al. 2009) and *Loma salmonae* (Lovy et al. 2008) have demonstrated that immune suppression, induced by either corticosteroids or crowding, increased the severity of microsporidiosis in the fish host. Gamma irradiation, a commonly used immunosuppressant, was shown to cause more severe and disseminated infections of *P. neurophilia* in zebrafish, even though neither irradiation nor *P. neurophilia* alone caused mortalities (Spagnoli et al. 2016b). The susceptibility of immune-compromised zebrafish to microsporidial infections is relevant to researchers who utilize gamma-irradiated zebrafish for xenograft of tumors in cancer research (Fazio et al. 2020) and for those who study correlations between immune status and microsporidian infections in other species.

11.2.2 **Glugea anomala** ***in Three-Spine Stickleback***

The three-spine stickleback is small teleost fish native to coastal regions of the northern hemisphere. These fish emerged as an important model organism in studies on behavior, host–parasite interactions, immunology, and evolutionary biology (Barber and Scharsack 2010; Barber 2013; Milligan-Myhre et al. 2016; Lescak and Milligan-Myhre 2017; Norton and Gutiérrez 2019; Kirschman et al. 2020).

The stickleback evolutionary history consists of colonization of a wide range of aquatic habitats, both marine and freshwater, and has been associated with adaptive divergence among derived populations (Reynolds et al. 1995). Due to their unique population genetic structure, recent studies have utilized stickleback to understand host–microbe interactions in the gut microbiome (Barber and Scharsack 2010). More pertinent to understanding microsporidiosis, sticklebacks also exhibit characteristics that have made them invaluable to parasitology and infectious disease research, including their tractability as a laboratory model (Kurtz et al. 2004; Barber 2013; Milligan-Myhre et al. 2016; Lescak and Milligan-Myhre 2017; Kirschman et al. 2020).

Glugea anomala is a common microsporidium found in wild sticklebacks and is reported to cause massive xenomas in the skin and viscera, often leading to death of the host (Dyková and Lom 1978; Kent et al. 2014). Through physical trauma or disturbance, xenomas rupture and spores are released into the water to infect neighboring hosts, therefore proliferation via horizontal transmission depends on host proximity. Ward et al. (2005) demonstrated that sticklebacks infected with *G. anomola* increase their shoaling behavior, while Petkova et al. (2018), expanded on this by demonstrating that infected individuals are more social than uninfected fish. Collectively, these studies demonstrate an association between *G. anomola* infection and host behavior that influences the parasite's ability to proliferate.

Morozińska-Gogol (2015) reported that changes in parasite communities may be a potential cause of population decline in wild three-spined stickleback in the Puck Bay area of Poland. In this study, initial surveys conducted in the 1990s revealed more than 20 parasite species in stickleback populations. More recent surveys identified only eight parasite species, one of which is *G. anomala*. Microsporidial infections such as *G. anomala* may alter physiological condition and behavior of sticklebacks (Ward et al. 2005), thereby potentially contributing to the selective consumption of infected individuals by predators, or in some circumstances, contributing to co-infections with other opportunistic pathogens which may further contribute to host mortality. In regards to infectious disease and immunology, Kurtz et al. (2004) demonstrated that sticklebacks with low MHC diversity exhibited more severe *G. anomala* infections. Importantly, these studies highlight how microsporidial infections contribute to parasite community dynamics which can ultimately influence behavior, subsequent disease, and mortality of stickleback hosts. As evident in the stickleback model, behavioral and immunological systems are tightly linked (Barron et al. 2015) and these relationships can be further elucidated through the use of these teleost models.

11.3 Life Cycles: Invertebrate Hosts and Maternal Transmission

11.3.1 Invertebrate Hosts

Desmozoon lepeophtherii This species was the first microsporidium to be identified in both a fish and invertebrate host. It was described from the parasitic copepod *Lepeophtheirus salmonis* infecting farmed salmon (Freeman and Sommerville 2009). Shortly thereafter, Nylund et al. (2010) described *Paranucleospora theridion* infecting both the copepod and its salmonid host. Comparison of the SSU rDNA sequences between *P. theridion* and *D. lepeophtherii* revealed that they are essentially identical, which led to a naming controversy that required adjudication by the International Commission of Zoological Nomenclature. Although Nylund et al. (2009) used the name *P. theridon* in a publication earlier than Freeman and Sommerville (2009), it was not in a peer-reviewed journal. According to the International Code of Zoology (ICZN) “Criteria of Publication (Article 8)” rules of priority, the appropriate name for this unusual microsporidium would be *P. theridion*, but the ICZN later ruled that *Desmozoon* has priority over *Paranucleospora* because Nylund et al. (2009) did not provide a description or deposit any kind of type material or DNA sequence (Freeman and Sommerville 2011).

Ovipleistophora diplostomuri This unique microsporidium infects the cyst wall of metacercariae of *Posthodiplostomum miniumum* as well as the liver and other coelomic organs in the fish host, bluegill sunfish *Lepomis macrochirus* (Lovy and Friend 2017). The microsporidium also was reported in infecting the muscle of freshwater crayfish *Procambarus bivittatus* (Bojko et al. 2020).

Oviplesitophora arlo and Inodosporus octospora Various lines of evidence indicate that these Microsporidia infect both crustacean and fish hosts (Stentiford et al. 2018). Synonymy was demonstrated between *Indosporus octospora*, a parasite that infects the common prawn (*Palaemon serratus*) and *Kabatana* sp., a fish parasite infecting two-spot goby (*Gobiusculus flavescens*). Analysis of the SSU rDNA sequence for *O. arlo* places it within the *Ovipleistophora* genus, which is known to infect ovarian tissues of freshwater fish hosts.

Obruspora papernae Diamant et al. (2014) suggested that this microsporidium of fish may use the ectoparasitic copepod *Lernanthropus callionymicola* as an alternative host based on the presence of *O. papernae* DNA in this copepod.

11.3.2 Maternal Transmission

Maternal transmission of pathogens occurs in a wide variety of hosts, with many examples amongst both freshwater and marine fishes (Funkhouser and Bordenstein 2013). Transmission to progeny either by the intraovum route or by spores released from the ovarian stroma at spawning is both examples of maternal transmission of fish Microsporidia. Both forms of transmission occur with *P. neurophilia* of zebrafish (Sanders et al. 2013), but the latter route appears to be the most common. However, Crim et al. (2017) found that embryos of only 1 of 26 spawns from positive fish were positive by PCR, further supporting the findings of Sanders et al. (2013) that maternal transmission does occur, but it is a somewhat rare event. However, given that research zebrafish are frequently spawned throughout the year, it is not surprising that the infection is so widespread in zebrafish facilities.

Other Microsporidia cause massive infections in ovarian stroma including *Pleistophora hyphessobryconis* (Sanders et al. 2010), *Fusporis stethaprioni* (Lovy et al. 2021, *Obruspora papernae* (Diamant et al. 2014), *Glugea* sp. in sculpins (Ryan and Kohler 2016) and *Ichthyosporidium weissi* (Sanders et al. 2012a), and thus for these species extra-ovum maternal transmission is possible. In addition, eggs from one infected female sculpin tested positive for *Glugea* spp. by PCR (Ryan and Kohler 2016), suggesting a similar situation as observed with *P. neurophilia* in which intraovum infection, though rare, also occurs. Spores of fish microsporidia are resistant to chlorine and iodine at levels commonly used for disinfecting eggs (Ferguson et al. 2007; Shaw et al. 1999), hence spores of species that infect ovaries are difficult to remove at spawning even if they are outside the egg (see below).

11.4 Treatment and Disinfection

Several recent reports describe the treatment of microsporidiosis in humans (Weiss 2015; Han and Weiss 2018), but we are unaware of such reports for microsporidial infections in fish since our review in 2014 (Kent et al. 2014). Regarding disinfectants, Ferguson et al. (2007) showed that spores of *P. neurophilia* and *G. anomala* were not completely killed using levels of chlorine or iodine safe for fish embryos. Peneyra et al. (2017) followed with a more recent study using the presence of parasite DNA as an indicator of survival. They found that Wescodyne (iodine) soak and Wescodyne Plus spray were able to consistently eradicate *P. neurophilia* spores from containers that they used for spawning fish, but the concentrations used would be lethal to zebrafish embryos.

As with many other animals, frozen fish sperm is often used to establish new stocks. Given the prevalence of *P. neurophilia* in zebrafish research facilities, Norris et al. (2018) investigated the ability of *P. neurophilia* spores to survive freezing. Two separate experiments with spores in sperm cryopreservation yielded very similar results, showing some, but reduced, survival of spores by using three

different viability assays: SYTOX stain, Fungi-Fluor stain, and presence of a spore vacuole. Without cryopreservant, spores showed no survival at –20 °C and only 2% at –196 °C.

11.5 Impacts Beyond Morbidity

In addition to recent discoveries of new host–pathogen relationships and diseases, recent Microsporidia studies have elucidated host impacts beyond overt disease and pathology, such as behavioral changes, immune status, gene expression, growth, and fecundity.

11.5.1 Immune Status and Gene Expression

Cooler temperatures may reduce the overall immune integrity of some fishes, and it has been demonstrated that heavy infections of *Enterospora nucleophila* in gilthead seabream in the winter contribute to multifactorial "winter syndrome" (Palenzuela et al. 2014; Sitjà-Bobadilla et al. 2016). *Glugea* in sticklebacks and *P. neurophilia* in zebrafish have been also associated with alterations in immune function and gene expression (described above under Sect. 11.2).

11.5.2 Growth

Stentiford et al. (2016) review the impacts of microsporidia on the global food chain, including concerns regarding reduced growth and productivity caused by microsporidiosis in fish. Microsporidia infections have long been known to reduce growth and body condition in fishes. Speare et al. (1998) showed that *Loma salmonae* causes a temporary reduction in the growth rate of rainbow trout. *P. neurophilia* in zebrafish often presents as emaciation, and infected fish are smaller than controls (Ramsay et al. 2009). A recent study demonstrated that females infected with *P. neurophilia* weighed less than control fish (Sanders et al. 2020), and this reduced weight was associated with smaller gonads in females, potentially reducing the fecundity of infected females (Ramsay et al. 2009).

11.5.3 Fecundity

A growing list of Microsporidia taxa preferentially infect the ovaries of fish hosts (Table 11.1), and some of these result in decreased breeding success (Lovy et al.

2021). This list now includes diverse taxa such as *Obruspora papernae* (Diamant et al. 2014), *Ichthyosporidium weissi* (Sanders et al. 2012a, b), *Ovipleistophora ovarie* (Phelps and Goodwin 2008), and *Fusasporis stethraprioni* (Lovy et al. 2021). These recent reports suggest that many females have reduced fecundity due to massive infections replacing much of the ovary or causing severe inflammation.

11.5.4 Behavior

As discussed under Sect. 11.2, three recent studies demonstrated that *P. neurophilia* affects zebrafish behavior (Spagnoli et al. 2015a, 2016a; Sanders et al. 2016; Midttun et al. 2020a), while Petkova et al. (2018) reported that *Glugea*-infected sticklebacks were more social than uninfected fish, and numerous studies have shown the direct behavioral changes caused by the parasite (Ward et al. 2005). Together, these studies confirm a link between parasite infection and host behavior, which may improve the horizontal transmission of *Glugea* in stickleback populations.

11.5.5 Product Quality

Muscle-infecting microsporidia often cause unsightly, opaque, macroscopically visible foci in somatic muscle, diminishing the quality of flesh destined for human consumption or causing externally visible opacities in ornamental fishes (Fig. 11.1). Many of these infections were reported before 2013, e.g. *Kabatana* in salmonids, *Myosporidium merluccisu* in hake, *Pleistophora hyphessobryconis* in ornamental fishes and laboratory zebrafish (Lom 2002, Lom and Nilsen 2003; Kent et al. 2014). More recent reports of this phenomenon, such as massive infections in Artic char *Salvelinus alpinus* (Jalenques et al. 2021) are summarized in Table 11.1 and discussed below with each category of fish (e.g., freshwater or marine). In general, these infections seldom cause overt morbidity, but the value of the fish is reduced when a large percent of the somatic muscle or associated connective tissue is replaced by parasites.

11.6 Diagnostics

A wide range of diagnostic methods has been used to identify Microsporidia in fish. Diagnosis of *P. neurophilia* in zebrafish has largely been based on histopathology, as this is the primary method for detection since it was first identified as a threat in the early 2000s (Sanders et al. 2013; Sanders et al. 2020). More recently, molecular assays have allowed for the detection of the parasite in all stages of the parasite

development and in both the larval and adult zebrafish life cycle (Sanders and Kent 2014).

Several PCR assays were developed for fish Microsporidia before 2013, including *Loma salmonae* and *Nucleospora salmonis* in salmon (Barlough et al. 1995; Brown and Kent 2002) in salmon, as well as for *P. neurophilia* in zebrafish tissues (Whipps and Kent 2006; Sanders and Kent 2011). More recently, multiplex high-throughput PCR assays for detection of multiple pathogens in salmon tissues have been developed (Fluidigm BioMark Platform) which test for *L. salmonae*, *N. salmoni*s, *Desmozoon salmonis,* and other important salmonid pathogens and are used for high-throughput microbial monitoring in salmon (Laurin et al. 2019; Bass et al. 2017). However, recent reports of the detection of many of these Microsporidia in new hosts and new geographic locations have not been verified with follow-up sequencing, thus the positive detections could potentially be due to novel parasites for which sequences are not available (Nekouei and Miller 2019).

Additionally, there have been two reports using in situ hybridization (ISH) to identify early developmental stages of microsporidia in fish since 2013. With ISH, Sanders and Kent (2014) demonstrated that *P. neurophilia* infections are initiated in the intestine, followed by a brief, disseminated infection, with subsequent development in the central nervous system. Ahmed et al. (2019) used an ISH probe to identify presporogonic stages of *E. nucleophila* in gilthead seabream, while also using a calcofluor white stain for mature spores. Consistent with the observations with *P. neurophilia* in zebrafish, Ahmed et al. (2019) noted a frequent disassociation between the presence of abundant spores and the distribution of infection by earlier stages. Earlier studies used ISH to identify early developmental stages of the gill microsporidium *Loma salmonae* in the intestine, blood, and heart (Sánchez et al. 2001; Speare et al. 1998).

Many of the molecular assays for fish pathogens require lethal sampling, but with advances in eDNA assays and other molecular technologies, researchers have recently developed novel nonlethal testing methods to detect both macro- and microorganisms in water (Uthicke et al. 2018, Rusch et al. 2018; Norris et al. 2020). In zebrafish facilities, these include testing tank and spawn water as well as detritus for *P. neurophilia* (Sanders et al. 2013; Crim et al. 2017; Miller et al. 2019), although these tests were found not to be sensitive for testing tank water for *P. neurophilia*. Schuster et al. (2021) developed a sensitive assay to detect *P. neurophili*a in tank water samples using droplet digital PCR (ddPCR), which is commonly used in environmental diagnostics (Uthicke et al. 2018; Chen et al. 2021). For this assay, tank water is filtered through a 0.45 μm filter and subjected to sonication to disrupt the spores, releasing DNA content. Because of the resistant nature of the microsporidian spore, the parasite requires adequate disruption of spores to expose target DNA (Sanders and Kent 2011, Schuster et al. 2021). Using this assay, performance was evaluated in various sample types from aquaria holding *P. neurophilia*-infected zebrafish. By implementing a novel multi-state occupancy model to evaluate relationships between habitat, sampling method, distribution, and abundance, it was determined that static tanks are most informative for population-level diagnostics. The assay is reported to reliably detect down to 77 spores/L, and

when testing water from tanks in which tanks that were static for 8 hours, the estimated detection probability was 83–94%, which is significantly improved with a subsequent sampling of tanks or if tanks are abundantly infected with parasites.

Previous studies have suggested that a combination of diagnostics methods should be implemented for routine surveillance of *P. neurophilia* (Kent and Sanders 2020; Kent et al. 2020; Caballero-Huertas et al. 2021), with emphasis on early detection (Murray et al. 2011). Combining traditional histopathology, tissue-specific PCR, and the newly developed eDNA assay for *P. neurophilia*, trends in disease transmission dynamics at a population level can be elucidated for targeted therapeutics or interventions. Additionally, these assays can be used to elucidate relationships at the host–parasite interface and clarify the transmission dynamics and disease mechanisms that drive the success of microsporidian parasites.

11.7 Molecular Phylogenetics and Taxonomy

Our understanding of the phylogenetic history and taxonomic relationships of the Microsporidia have advanced considerably over the last 10 years as sequences from additional isolates have become available. The original phylogeny of microsporidian parasites of fishes by Lom and Nilsen (2003) has been refined by modifications from more recent reviews (Stentiford et al. 2013; Vossbrinck et al. 2014) and studies focused on specific categories of Microsporidia such as muscle infecting taxa (Jalenques et al. 2021, Phelps et al. 2015; Winters et al. 2016), members of the Enterocytozooidae (Diamant et al. 2014; Freeman et al. 2013; Palenzuela et al. 2014, Stentiford et al. 2019), *a*nd studies of new genera (e.g., *Fusasporis*) (Lovy et al. 2021). Fish infecting microsporidians are found primarily in Clade 5 of the phylogeny described by Vossbrinck et al. (2014), with a few members, including some more recent species, found in the *Enterocytozoon* group Microsporidia (EGM) in Clade 4B. Within these clades, the fish infecting microsporidia can be further categorized into five phylogenetic groupings as proposed by Lom and Nilsen (2003). While some of these groups appear to reflect higher-level taxonomic rankings, this is not true for all. However, the phylogenetic relationships represented by these groups provide useful context when discussing the fish-infecting microsporidia (Fig. 11.2).

Group 1 This group contains members of the family, Pleistophoridae including members of the genera *Pleistophora*, *Heterosporis*, and *Ovipleistophora.* The genera *Myosporidium, Dasyatispora*, and certain *Microsporidium* spp. may possibly be included in this group. Many species in this group are muscle infecting microsporidia that do not form xenomas. Members of the genus *Ovipleistophora* were originally believed to infect primarily ovarian tissues of freshwater teleost hosts. However, this tissue tropism has been challenged by the recently described *Ovipleistophora diplostomuri* (Lovy and Friend 2017), a microsporidium that forms aggregates within the fibroblasts surrounding the metacercarial wall of the digenean

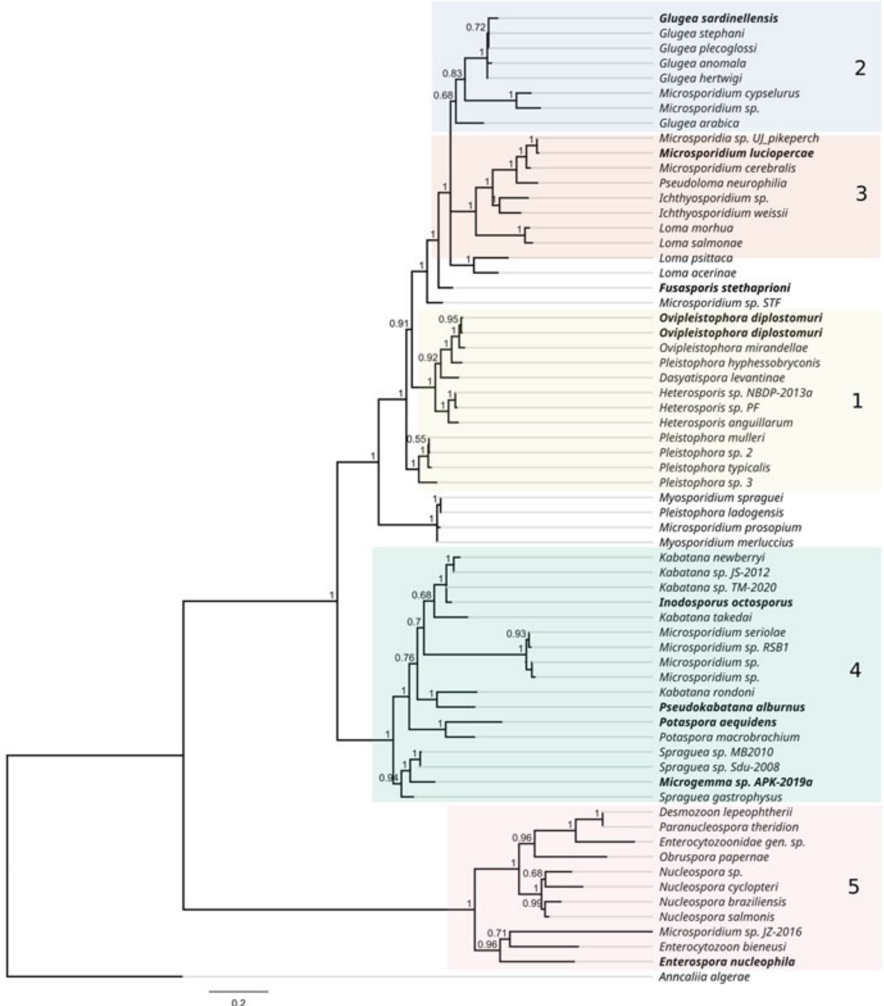

Fig. 11.2 ssrRNA sequences were obtained from taxa representing the major lineages of fish infecting microsporidia from the NCBI National Center of Biotechnology Information non-redundant nucleotide database. A multiple sequence alignment was constructed using MUSCLE (Edgar 2004) and ambiguous end regions of the aligned sequences were removed. Phylogenetic inference was performed using MrBayes v.3.2.6 (Huelsenbeck and Ronquist 2001) as implemented in Geneious Prime (v.2021.0.3) using a general time-reversible substitution model (GTR) of nucleotide substitution with gamma-distributed rate variation across sites and a proportion of invariable sites (G+I) with inverse gamma rate variation, a chain length of 1,000,000 and a 100,000 burn-in. *Anncaliia algerae* AY230191) was selected as an outgroup taxon. Sequences representing recently described species or initial sequence submissions of previously described species are in bold.

parasite, *Postdiplostomum minimum*, infecting its bluegill host. In addition, Stentiford et al. (2018) described *Ovipleistophora arlo*, a novel species infecting the skeletal muscle of the common prawn, *Palaemon serratus*. The discovery of this close relative of fish-infecting microsporidia within the genus *Ovipleistophora* along with another, *Inodosporus octospora*, closely related to *Kabatana,* in Crustacea suggests fairly recent trophic transfer of microsporidian parasites between these and fish hosts based on genetic relatedness.

Group 2 Glugeidae Thélohan, 1892. This group contains the genus *Glugea*, whose members are characterized by the formation of grossly visible xenomas in various tissues. Several new species of *Glugea* have been described recently, likely due in part because infections are easy to identify without microscopy. Members of this group have a very high level of genetic identity and form a distinct clade.

Group 3 This group includes the genera *Loma, Pseudoloma,* and *Ichthyosporidium*. *Loma* remains a paraphyletic genus with *Loma acerinae* and *Loma psittaca* appearing basal to the clade containing *L. salmonae* and *L. morhua*. While these two species form xenomas, the clade also contains *Pseudoloma neurophilia* from zebrafish and *Microsporidium cerebralis*, from Atlantic salmon *Salmo salar* (Brocklebank et al. 1995). These two species are closely related based on ssrRNA sequences (Fig. 11.1) and infect neural tissue. However, both form aggregates of spores in the CNS rather than developing xenomas.

Group 4 Group 4 has historically been composed of members of the genera *Spraguea, Microgemma,* and *Kabatana* as well as a few members of the provisional *Microsporidium* genus. Recently described novel genera, *Pseudokabatana* (Liu et al. 2019) and *Potaspora* (Videira et al. 2015) may also be placed in this group. The genus *Inodosporus* and its sole member *I. octosporus* (Overstreet and Weidner 1974, Azevedo et al. 2000) also appears in this group. However recent phylogenetic evidence suggests that *Inodosporus* and *Heterosporis* are synonymous (Stentiford et al. 2018).

Group 5 This group contains members of the family Enterocytozoonidae (Cali and Owen 1990), including the fish-infecting microsporidian genera *Nucleospora, Enterospora, Obruspora,* and *Desmozoon/Paranucleospora*. Stentiford et al. (2019) and Palenzuela et al. (2014) provide recent reviews of the family. Most currently described members of the Enterocytozoonidae primarily infect cells of the gastrointestinal tract, with the notable exception being *Obruspora papernae* (Diamant et al. 2014). The members that develop within the nuclei of host cells form a clade. Again, the one exception here is *O. papermae*, providing further support for this organism to represent a novel genus. *Enterocytozoon* and *Enterospora* are paraphyletic when *Enterocytozoon hepatopanaei* include in phylogenetic analyses (Palenzuela et al. 2014; Stentiford et al. 2019), suggesting that there are several members of this group that remain to be identified. A novel enterocytozooid (*Enterocytozoon schreckii*) from salmon that was recently discovered (Couch et al. 2022) fails to resolve this discrepancy. The salmon parasite shares characteristics consistent with *Enterocytozoon:* infecting enterocytes, no

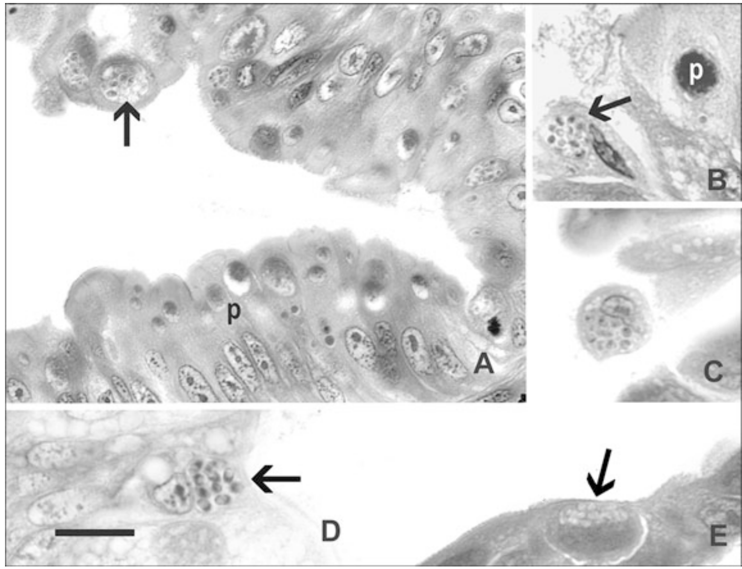

Fig. 11.3 Enteroctyozooid microsporidum in enterocytes of adult Chinook salmon *Oncorhynchus tshawytscha*. (**a**, **b**, **d, e**) Aggregates of spores within epithelial cells (arrows). p: presporogonic forms (sporagonial plasmodia or meronts). (**c**) slough enterocyte replete with spores. (**a–c, e**) Hematoxylin and eosin. (**d**) Geimsa. Bars = 10 μm (Couch et al. 2022)

intranuclear development (Figs. 11.3 and 11.4). However, the parasite appears to develop within an interfacial envelope with in enterocytes of a fish host, which is more consistent with *Enterospora*. *Enterocytozoon hepatopenaei* from shrimp is most closely related to *Enterospora nucleophilia* from fish. It is likely that this will be resolved by the addition of more novel species to this group.

Genomes The decreasing cost of next-generation sequencing and computational power has resulted in a dramatic increase in genomic information for many taxonomic groups. However, limitations to in vitro culture and challenges of purification of most members of the Microsporidia have resulted in a more modest increase in sequenced genomes from this group. This is especially true of the Microsporidia that infect fishes. Only 2 of the 35 genomes currently available on the online informatics tool, MicrosporidiaDB (Aurrecoechea et al., 2011), are from fish-infecting microsporidia: *Pseudoloma neurophilia* (Ndikumana et al., 2017) and *Spraguea lophi* (Campbell et al., 2013). Genomic information is critical to our understanding of the taxonomy and biology of these highly specialized parasitic organisms. More resources are needed to sequence and analyze additional genomes from the fish-infecting microsporidia.

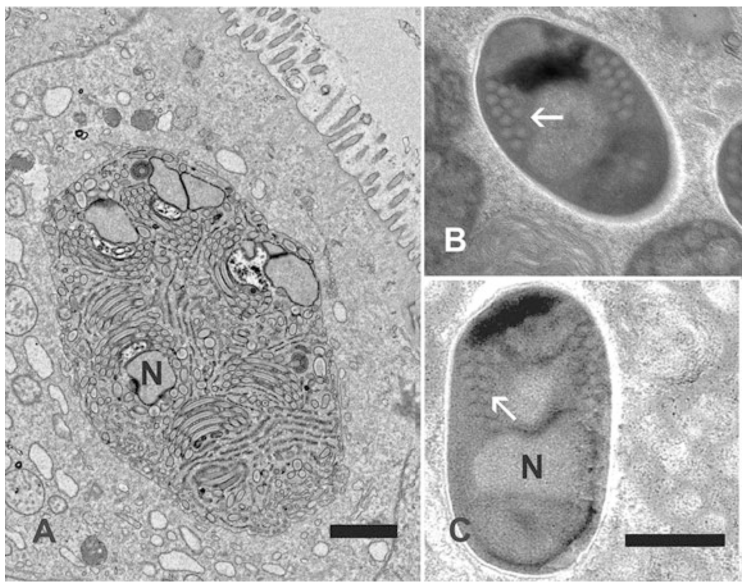

Fig. 11.4 Electron microscopy of enterocytozooid from salmon enterocytes. (**a**) Sporaginal plasmondium. N: nucleus. Bar = 1.0 um. (**b**, **c**) Developed spores. Note 10 turns in polar tube (arrows). Bar = 0.5 μm. Courtesy of Peter Takvorkian, Rutgers University and Leslie Cummins, Albert Einstein College of Medicine (Couch et al. 2022)

11.8 New Microsporidia Taxa, Hosts, and Diseases

The expansion of finfish aquaculture has raised concerns regarding the impacts of parasites, including Microsporidia, on cultured and wild fishes (Herrero et al. 2018; Boerlage et al. 2020). Since 2013, several novel microsporidian taxa, noteworthy infections in new hosts, and new disease manifestations by previously described taxa have been reported in fishes (Table 11.1). In this section, we summarize some of these important recent discoveries.

11.8.1 Novel Microsporidia Species in Marine Aquaculture

Cage culture of food fishes in coastal marine environments has dramatically increased over the last decade in both the quantity and variety of fish species (FAO Yearbook 2018). Concurrently, new concerns have arisen regarding infectious diseases in marine aquaculture, including microsporidiosis.

***Microsporidium aurata*, *Glugea pagri* and *Enterospora neurophilia* in Sea Bream** Sea bream species are now commonly reared in seawater net pens in coastal regions throughout the Mediterranean Sea and Asia (FAO Yearbook

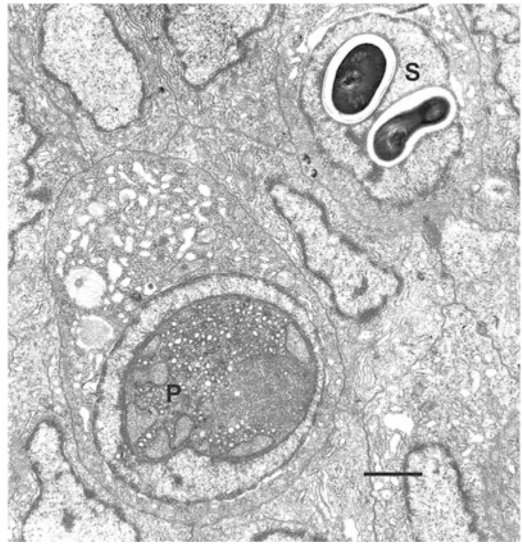

Fig. 11.5 *Enterospora nucleophila* in enterocytes of bream *Sparus auratus*. Sporagonial plasmodia (P) and spores (S) in nucleus. Bar = 1 μm. Courtesy of P. Takvorian, Rutgers University

2018). Several microsporidia in the genera Pleistophora (Faye et al. 1990; Athanassopoulou 1998) and *Glugea* (Mathieu-Daude et al. 1992; Abela et al. 1996) have long been known to infect wild and cultured sea bream. In the last decade, two novel pathogenic microsporidia have been described in gilthead seam bream (*Sparus aurata*), *Microsporidium aurata,* and *Enterospora nucleophilia*, and Glugea pagri was described from red bream Pagrus major.

Microsporidium aurata was infects from sea bream reared in Egypt, and this xenoma-forming species forms large, white cysts in the muscle (Morsy et al. 2013) thereby diminishing the market value of infected fish. The parasite also infects the intestine and may have detrimental effects such as reduced growth or feeding efficiency. The parasite is related to *Microsporidium prosopium*, which infects the muscle of the whitefish *Prosporium willamsoni* in western Canada (Kent et al. 1999) (Fig. 11.1).

Enterospora nucleophila infects the intestinal epithelium of gilthead seabream (Fig. 11.5), primarily in enterocytes, but also rodlet cells and eosinophilic granule cells (Palenzuela et al. 2014). Infections cause cellular and nuclear alterations, including thickened cell membranes. Consistent with intestinal damage, infections cause reduced growth and chronic mortalities. *E. nucleophila* is a member of the family Enterocytozooidae, and shares characteristics with members of both Nucleospora and Enterocytozoon. Consistent with the former, it develops within nuclei of a fish host, but like *Enterocytzoon beinusi*, it also undergoes cytoplasmic development and targets enterocytes. Recent phylogenetic analysis indicates that these genera are paraphyletic as we discuss under Sect. 11.7. Picard-Sánchez et al. (2021) showed that *E. nucleophila* is horizontally transmitted by various routes, including cohabitation with infected fish and exposure of naïve fish to effluent from tanks with infected fishor oral and anal gavage.

Glugea pagri was described in red bream, a fish that is commonly reared in netpens off the southern coast of China. The infection was first identified in association with mortality at an aquaculture facility, and necropsy revealed massive infection with large cysts (xenomas) replacing much of the organs within the coelomic cavity (Su et al. 2014). Histology revealed that the xenomas targeted the intestinal muscularis, and as seen with some other *Glugea* species, two different spore sizes occur within these xenomas.

Microsporidia of salmon Seawater netpen aquaculture of salmon, primary Atlantic salmon (*Salmo salar)* has rapidly expanded since the 1980s. Before 2013, several microsporidian species were recognized as important pathogens, including *Loma salmonae, L. fontinalis, Glugea truttae, Nucleospora salmonis, Microsporidium cerebralis, and Desmozoon lepeotherii* (jn. syn *Paranucleospora theirdon*) (Lom 2002; Kent et al. 2014). Since its original discovery as a parasite of sea lice *Leophtheirius salmonis*, *D. lepeotherii* has been recognized as a common ectoparasite of farmed Atlantic salmon. The infection is associated with hyperplasia and inflammation of the gill epithelium, skin lesions and hemorrhage, and loss of scales (Nylund et al. 2009, 2010). More recently, Weli et al. (2017) reported that *D. lepeotherii* is capable of causing serve, systemic infections characterized by either acute or chronic branchitis, peritonitis, stunted growth, and mortality. A combination of histopathology, in situ hybridization, calcofluor-white staining, and real-time PCR were used to describe disease progression in farmed Atlantic salmon in Norway. Using these data, Weli et al. (2017) were able to differentiate chronic inflammatory lesions in the coelom caused by the parasite from those caused by commonly used vaccines with oil adjuvants that are delivered intraperitoneal.

***Tetramicra breviflium* and *Nucleospora cyclopeteri* in Lumpfish** Lumpfish *Cyclopterus lumpus* are a source of caviar, and they are often housed with Atlantic salmon as cleaner fish to remove ectoparasites such as sea lice *Lepeophtherius salmonis*. The number of lumpfish used by the salmon farming industry has increased significantly since 2008, and thousands of lumpfish are now housed in salmon netpens in Norway (Dahle et al. 2020). Female lumpfish are also harvested in a coastal spring fishery for their eggs for caviar. Two microsporidia causing disease in lumpfish held in captivity have recently been recognized: *Nucleospora salmonis* (Freeman et al. 2013) and *Tetramicra breviflium* (Scholz et al. 2017), in addition to *Nucleospora cyclopteri* has long been known to infect both captive and wild fish (Mullins et al. 1994; Freeman et al. 2013) and causes lymphoid proliferative disease similar to *N. salmonis* (Kent et al. 2014). *N. cyclopteri* infection can be lethal to lumpfish, as immature leukocytes in various organs are infected resulting in a disseminated lymphoid proliferative disorder. As with infections in salmon, lumpfish show bilateral exophthalmos and pallor of the gills and other organs indicative of anemia. Infected fish also consistently show marked enlargement of the kidney (renomegaly). Alarcón et al. (2016) reported clinical disease in farmed lumpfish infected with a variety of parasites, including *N. cyclopteri*. Systemic inflammatory lesions and kidney nodules were observed in moribund fish. Fish also exhibited

skeletal infections by *Kudoa islandica*, but it was concluded that the main cause of morbidity was the microsporidium.

Tetramica brevifilium has broad host specificity; it was first described in wild turbot (*Scophthalmus maximus*) (Matthews and Matthews 1980), and then in black anglerfish *Lophius budegassa* by Maíllo et al. (1998). Most recently, *T. brevifilium* was detected in wild-caught lumpfish held in captivity (Scholz et al. 2017). Macroscopically visible xenomas occurred in essentially all the visceral organs, skin, gills, and eye. Affected fish showed exophthalmos and abdominal distension.

Enterocytozooidae in Groupers Groupers are marine fishes commonly reared in relatively small seawater netpens and sold live in markets. A member of the Enterocytozooidae infects the intestine of hybrids of *Epinephelus lanceolatus* × *Epinephelus fuscoguttatus.* (Xu et al. 2017). Like many other members of this group, this novel pathogen targets enterocytes. Of note, infected cells are markedly hypertrophied, and the contents are replaced with numerous spores. Like *Enterospora*, it develops in both the nucleus and the cytoplasm. Using rDNA sequence, Xu et al. (2017) demonstrated that this novel species falls within the Enterocytozooidae, somewhat related to *E. bieneusi*. Typical of enteric infections, fish are anorexic, emaciated, and mortalities can reach 90%. Intestines are edematous, thin-walled, and transparent. This infection is a serious threat to this aquaculture enterprise as it infects nursery stocks that are sent to numerous locations in China and other countries.

11.8.2 Novel Microsporidia Species from Wild Marine Hosts

Many of the first reports of Microsporidia in fishes were in commercially important marine fishes, particularly those causing macroscopically visible lesions in the flesh, gills, or coelomic organs (Kent et al. 2014). Since 2013, several new taxa and infections have been reported (Table 11.1).

New *Glugea* Species in Wild Marine Fishes With their large, macroscopic xenomas, members of the genus *Glugea* are one of the most frequently reported Microsporidia in parasite surveys of wild fishes (Lom 2002; Kent et al. 2014). Since 2013, at least six new species have been described: (1) *Glugea arabica* infects the camouflage grouper *Epinephelus polyphekadion* in the Red Sea. Infections present as small, blackish xenomas adhering to the intestinal wall (Azevedo et al. 2016). (2) *Glugea eda* was described in the Red Sea off the coast of Saudi Arabia by Mansour et al. (2020). This species infects the striated fuslier *Caseio striata*, and like other members of the genus, causes visible xenomas in the coelomic cavity. (3) *Glugea jazanensis* was discovered during a survey of the microsporidian fauna of the two-spot red snapper *Lutjanus bohar* from the Red Sea off the coast of Saudi Arabia. Infection was reported as dark brownish spherical xenomas in the body cavity attached to the mesenteries (Abdel-Baki et al. 2015a). (4) *Glugea nagelia* was described in a survey of yellowfin hind *Cephalopholis hemistiktos* caught in the Red

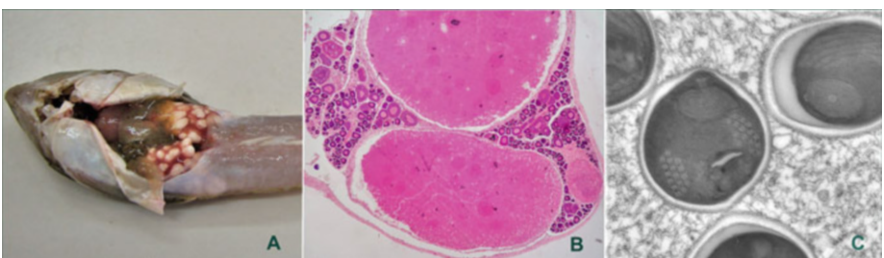

Fig. 11.6 *Obruspora paperna.* (**a**) Ovaries of replaced by numerous, coalescing xenomas. (**b**) Histologic section showing two large xenomas replete with spores in ovary. (**c**) Electron microscopy showing subsherical spores with three rows of polar tubes. Courtesy of Arik Diamant, Israel

Sea coast off Saudi Arabia (Abdel-Baki et al. 2015b). As is typical of the genus, the infection presented as macroscopically visible xenomas on the intestinal wall. (5) *Glugea sardinellensis* was described by Mansour et al. (2016) in the round sardinella *Sardinella aurita* off the Tunisian coast. This species caused very large cysts within the coelomic cavity. (6) *Glugea serranus* was reported in blacktail comber, *Serranus atricauda* off the Madeira Archipelago (Casal et al. 2016a).

Heterosporis lesspsianus forms white cysts in the muscle of the lizardfish *Saurida lesspsianus* from the Red Sea (Al Quraishy et al. 2019). This species is similar to *Heterosporis saurida* from *Saurida undosquamis*.

Obruspora papernae is an unusual microsporidian causing massive infections in the ovaries of the blotchfin dragonet *Callionymus filamentosus* in the Mediterranean Sea. This species was described by Diamant et al. (2014). This fish is exotic to the area by making its way through the Suez Canal. The parasite causes large, xenoma-like masses (Fig. 11.6), but rDNA sequence places it in the Enterocytozooidae (see Molecular phylogenetics Sect. 11.7). Consistent with other members of the family, sporogenesis occurs within a sporogonial plasmodium. Prevalence of infection approached 80% in females. Males showed no signs of infection even when parasite DNA was detected. *O. papernae* rDNA was also found in the ectoparasitic copepod *Lernanthropus callionymicola*, suggesting that this parasite may be added to the list of fish Microsporidia that infect their copepod parasites.

Microgemma tilanpasiri was first described infecting the liver of the burrowing goby *Trypauchen vagina* from Malaysia (Freeman et al. 2015). Subsequently, a very similar parasite (likely the same species) was described in mudskippers *Boleophthalmus dussumier*, also in the family Gobiidae, from the north-west coast of India. The infected fish were part of a histologic survey, and the parasite xenomas were observed in the liver of 11% of the fish (Vandana et al. 2021).

11.8.3 Novel Microsporidia Species in Freshwater Aquaculture

Freshwater aquaculture, particularly pond culture of carp species, has expanded significantly in recent years, concurrent with the expansion of marine aquaculture. Marine and freshwater aquaculture now accounts for about half of overall food fish and shellfish production by weight (FAO Yearbook 2018). We also include ornamental and research fish in this category.

Nucleospora braziliensis was the first *Nucleospora* species identified in Brazil and in tilapia (Rodrigues et al. 2017). The species was detected in farmed Nile tilapia *Oreochromis nilticus*. The main lesions observed included congestion of the stomach, hepatomegaly, and splenomegaly (Table 11.1). There was a high prevalence of the microsporidia reported, which raises questions regarding the host specificity and origins of *N. braziliensis* given that Nile tilapia are exotic to Brazil.

Pleistophora hyphessobryconis has long been known to cause neon tetra disease in ornamental fish (Kent et al. 2014), but it was recently found to infect the skeletal muscle of a non-ornamental fish, the hybrid jundiara (*Leiarius marmoratus x Psuedoplatystoma reticulatum*) in a commercial aquaculture facility (Winters et al. 2016). This finding suggests even broader host specificity beyond the aquarium fish trade (Winters et al. 2016). As with ornamental species, massive multifocal opaque lesions were observed throughout the flesh. Histologically, the microsporidium has a disease presentation of which we would expect of *P. hyphessobryconis, as* skeletal muscle was replaced by inflammatory cells and masses of microsporidian spores.

Fusasporis stethaprioni is an unusual and highly pathogenic microsporidium that causes disseminated infections in two tetra species that are popular aquarium fishes, black tetra *Gymnocorymbus ternetzi* and cardinal tetra *Paracheirodon axelrodi* (Lovy et al. 2021) (Fig. 11.7). These infections were noted in captive fishes reared in Florida, but we have observed the same infection in wild-caught cardinal

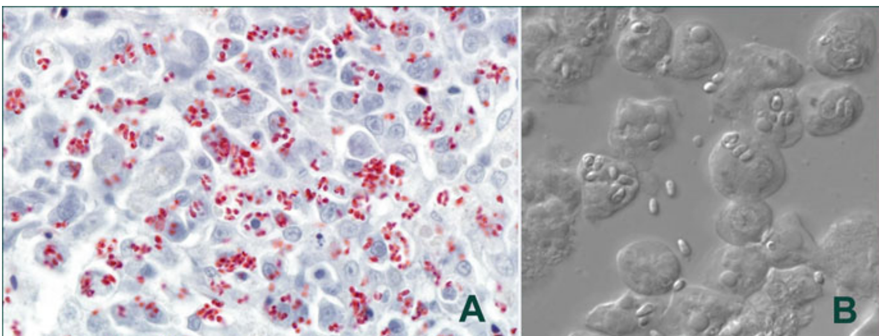

Fig. 11.7 *Fusasporis stethaprioni.* (**a**) Histologic section of liver from a black *tetra Gymnocorymbus ternetzi* with numerous spores, stained with Luna stain. (**b**) Normarksi phase contrast with spores. Courtesy of J. Lovy, Office of Fish and Wildlife Health and Forensics, New Jersey

tetras that had been recently imported to the United States. Infected fish exhibit high mortality and reduced breeding. Severe infections with numerous spores in small aggregates occur throughout the visceral organs, particularly in the liver and ovaries. Presently, the infection has only been documented in tetras (family Characidae). However, given the virulence of this microsporidium and the widespread geographic distribution of its hosts, a better understanding of host specificity would be valuable to clarify the risk to native fish species.

***Nucleospora* sp.** was recently detected in a lymphoid lesion of a laboratory zebrafish (Kent et al. 2021). This novel species develops in intranuclear inclusions consistent with a *Nucleospora* sp. As seen with *N. salmonis* in salmon, the spores occurred within proliferating, immature lymphoid cells (Morrison et al. 1990).

Pseudoloma neurophilia. See Fish Models Sect. 11.2.

11.8.4 Novel Microsporidia in Wild Freshwater Fishes

Several new microsporidia have been described from wild fishes in freshwater over the last decade, most of which cause macroscopic pathological changes.

Pseudokabatana alburnus is a new genus and species that was described from the liver of topmouth culter *Culter alburuns* in China (Liu et al. 2019). The parasite develops in hepatocytes, ultimately forming rather large xenomas. rDNA sequence showed that this parasite was a distinct taxon, branching off independently with *Kabtana rondoni*.

***Microsporidium* sp. in Arctic char** is a unique microsporidium found in the skeletal muscle tissue of Arctic char *Salvelinus alpinus* from two different lakes in Nunavik (QC, Canada) (Jalenques et al. 2021) (Fig. 11.1). Arctic char is an important fishery for traditional and subsistence foods for Canada's Inuit population and this microsporidium was initially described after reports of digestive disorders surfaced following consumption of infected fish. Histological analysis revealed that the microsporidia detected in fish muscle tissues appeared primarily as free spores and less frequently as sporophorous vesicles in the muscle. Although it is unlikely to be the cause of the digestive disorders reported, the novel microsporidium was found to be highly prevalent in Arctic char, thus the impact of this pathogen on char populations warrants further investigation.

A similar disease was reported in mountain whitefish *Prosopium williamsoni* infected with *Microsporidium prosopium* in which infected individuals exhibited extensive white macroscopic lesions throughout the musculature of the fish (Kent et al. 1999). However, unlike the *Microsporidium* sp. infecting Arctic char, *M. prosopium* did not occur within mature muscle fibers and no sporophorous vesicles form, as spores are free in the cytoplasm.

Heterosporis sutherlandae has widespread geographic distribution and broad host specificity. This novel *Heterosporis* species has been identified in yellow perch (*Perca flavencens*), northern pike (*Escox lucicus*), and walleye (*Stizostedion vitreum*) in the Great Lakes region of Minnesota and Wisconsin (Phelps et al.

2015). This disease is important to the sport and commercial fishing, as the intracellular proliferation of spores results in the destruction and necrosis of host skeletal muscle tissue. As with other microsporidia that infect skeletal muscle of fish, *H. sutherlandae* may pose a significant threat to native fishes, although to date there is no documentation of significant population-level effects. Currently, no PCR assays have been developed for this microsporidium, therefore surveillance of this widespread pathogen warrants has been limited.

Myosporidium spraguei **and** ***Microsporidium lucioperca*e** are two new species of Microsporidia identified in the skeletal muscle of freshwater fishes, pike-perch *Sander lucioperca* and burbot *Lota Lota* in Finland (Jones et al. 2017). These two fish species, important to local sport fishers, are commonly found in waterways of Finland and were described from freshwater lakes. *M. spraguei* forms xenomas in pike perch, and molecular studies revealed its presence in burbot. *M. luciopercae* is found in the skeletal muscle of both pike perch, a non-xenoma forming microsporidium, with sporogony occurring in sporophorus vesicles; however, mature spores are found in direct contact with sarcoplasm. Sports fisherman have reported no obvious changes to fish infected with *M. spraguei*, however with *M. luciopercae*, there is an obvious loss of structure and fillets resemble more of a "ground meat," as mature spores are in direct contact with the sarcoplasm, which causes myocyte degeneration and necrosis.

Pleistophora beebei Casal et al. (2016b) described this species in the skeletal muscle of the abdominal cavity, in the Amazonian fish *Bracyhypopomus beebei* (Casal et al. 2016a, b). As with other members of the genus, this microsporidium forms cysts-like masses in the muscle. Unique characteristic of this species is that it forms two spore types; with large spores at about 8 μm in length compared to small spores at about 5 μm. Nevertheless, it was still placed in the genus *Pleistophora*, largely based on rDNA sequence comparisons.

Glugea anomala Numerous papers and reviews of this parasite in sticklebacks have been published since its description over a hundred years ago (Kent et al. 2014). Recent papers have been focused on behavior changes, which are discussed above in our Sects. 11.2 and 11.5.4.

Glugea gasterostei Tokarev et al. (2015) described this second species of *Glugea* from stickleback, *Gasterosteus aculeatus.* In addition to differences in rDNA sequence, they noted that the parasite targeted internal organs, rather than the skin as seen with *G. anomala*.

Glugea **spp. from sculpins** Ryan and Kohler (2016) describe *Glugea* sp. in two species of sculpins in northern USA. The formation of xenomas associated with the body wall, fat body, gonads, and kidneys. Infections range from mild to very heavy, with variable xenoma numbers and sizes. Female sculpin experience heavier infections and more frequent infections of the gonads relative to males (see Maternal transmission Sect. 11.3.2).

Compliance with Ethical Standards
1. **Conflict of Interest**: The authors declare that there is no conflict of interest.
2. **Funding**: none
3. **Ethical Approval**: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Note that no live animals were involved as new data on the salmon microsporidium were obtained from carcasses donated by the Oregon Department of Fish and Wildlife.

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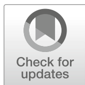
Chapter 12
Chronic Infections in Mammals Due to Microsporidia



Bohumil Sak and Martin Kváč

Abstract Microsporidia are pathogenic organism related to fungi. They cause infections in a wide variety of mammals as well as in avian, amphibian, and reptilian hosts. Many microsporidia species play an important role in the development of serious diseases that have significant implications in human and veterinary medicine. While microsporidia were originally considered to be opportunistic pathogens in humans, it is now understood that infections also occur in immune competent humans. *Encephalitozoon cuniculi, Encephalitozoon intestinalis,* and *Enterocytozoon bieneusi* are primarily mammalian pathogens. However, many other species of microsporidia that have some other primary host that is not a mammal have been reported to cause sporadic mammalian infections. Experimental models and observations in natural infections have demonstrated that microsporidia can cause a latent infection in mammalian hosts. This chapter reviews the published studies on mammalian microsporidiosis and the data on chronic infections due to these enigmatic pathogens.

Keywords Microsporidia · Latency · Mammals · Infection · Recurrent infection · Epidemiology · Transmission

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12.1 Introduction

Microsporidia are parasitic eukaryotic obligate intracellular pathogens. Microsporidia infect a broad range of invertebrates and vertebrates and have been demonstrated to infect all groups of animals, from protists to humans. Nearly 1400 species in over 200 genera have been reported so far (Cali et al. 2017). Microsporidia were originally considered primitive organisms that were classified as protozoa, based on several features such as the presence of 70S ribosomes (16S and 23S rRNA subunits) and their lack of centrioles as well as respiratory organelles (hydrogenosomes and mitochondria) (Shadduck and Greeley 1989; Hirt et al. 1999). However, based on comparisons of molecular markers, it was gradually concluded that microsporidia should be classified as highly reduced eukaryotes closely related to fungi (Edlind et al. 1996; Keeling and Doolittle 1996). Recent information suggests that they are related to the Cryptomycota and are therefore either a basal group to fungi or a sister taxon to fungi.

The infectious form of microsporidia is its resistant spore that can survive in the environment for a long time. In the presence of a host signal, the spore expels its polar tube and infects its host cell. The spore injects an infectious sporoplasm into the host cell cytoplasm through its polar tube and then the sporoplasm inside the cell undergoes extensive proliferation by either merogony (binary fission) or schizogony (multiple fission). By merogonia/sporogonia microsporidia then develop into mature spores either in the cytoplasm or within a parasitophorous vacuole. During merogony/sporogony, a thick spore wall which provides resistance to adverse environmental conditions forms around the spores. As the spore number increases, filling the host cell cytoplasm, the limiting cell membrane is disrupted causing the release of spores into the environment. These released mature spores can then infect new cells continuing the infectious life cycle of the parasite.

12.2 Microsporidiosis

Many microsporidia species play an important role in the development of serious diseases, especially in insects and fish. For example, microsporidia are the cause of silkworm pébrine, honeybee nosemosis, porcelain crayfish disease, and numerous fish infections. Microsporidia can also infect mammals and birds including companion, laboratory and food producing animals as well as humans. Originally, microsporidia were considered to only be opportunistic infections associated with the AIDS pandemic, where in the mid-1980s they caused life-threatening diarrhea and systemic disease. Gradually, however, hand in hand with improving detection methods and greater scientific and clinical attention, microsporidia are now recognized to cause infections in the broader human population including children, travelers, elderly and organ transplant recipients. Moreover, individuals with high

exposure to animals and to contaminated soil and water have been demonstrated to have an increased risk for microsporidiosis (Sak et al. 2010b).

12.3 Immunity

The host immune response to infection by microsporidia includes both adaptive and innate immune responses (see Chap. 13). The innate response includes immediate nonspecific defense against pathogens, often induces inflammation, and the cells involved (i.e., dendritic cells, macrophages, and natural killer cells) help transition the innate response to an adaptive immune response. The adaptive immune response includes both cell-mediated and humoral responses. IFN-γ is a critical cytokine that mediates partial protection, even in the absence of CD4+ and CD8+ T-lymphocytes. IFN-γ enhances the cytotoxic activity of natural killer cells and activates macrophages to effectively kill phagocytized microsporidia spores via an oxidative burst mechanism (Didier and Shadduck 1994; Didier et al. 2010). Although T-cell-dependent B-cell activation for antibody production is considered partially effective for protection against microsporidiosis (Sak et al. 2006; Salát et al. 2006), it is generally accepted that the major protective immune response is due to T-cell-mediated cytotoxic CD8+ T-lymphocytes (Braunfuchsová et al. 2001). Furthermore, the activity of these CD9+ T-cells does not appear to be dependent upon CD4+ T-cells (Khan et al. 1999; Moretto et al. 2000).

12.4 Routes of Transmission

12.4.1 Horizontal Transmission

It is generally accepted that the usual route of transmission of microsporidiosis is through the fecal-oral route, as spores are passed in the urine or feces of infected individuals and thus contaminate the environment, mostly water sources. Spores of *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis,* and *Encephalitozoon cuniculi* have been documented in surface waters in France and the USA (Dowd et al. 1998; Cotte et al. 1999; Fournier et al. 2000), irrigation water in Mexico and USA (Thurston-Enriquez et al. 2002), drinking water in Spain (Izquierdo et al. 2011; Galvan et al. 2013b), and wastewater in Ireland (Cheng et al. 2011) and China (Li et al. 2012). One microsporidiosis outbreak has been documented as being linked to contaminated food consumption (Decraene et al. 2012). While few studies have reported on foodborne transmission of microsporidia, fresh produce such as strawberries, raspberries, lettuce, celery, parsley, and oranges including juices produced locally in Poland, Costa Rica, Sweden, and Egypt have been demonstrated to contain *Enterocytozoon bieneusi*, *Encephalitozoon cuniculi,* and/or *Encephalitozoon intestinalis* (Calvo et al. 2004; Jedrzejewski et al. 2007; Decraene et al. 2012).

Recently *Encephalitozoon cuniculi* and *Enterocytozoon bieneusi* have been identified in the milk of both dairy cows and goats (Lee 2008; Kváč et al. 2016) and the possibility of *Encephalitozoon cuniculi* transmission with pasteurized cow's milk as well as fermented pork products and fresh goat cheese has been experimentally documented (Kváč et al. 2016; Sak et al. 2019). Besides these routes of transmission, infection in the respiratory tract of hosts suggests that airborne transmission of microsporidiosis by contaminated aerosols is feasible. Respiratory infections have been reported with *Encephalitozoon hellem*, *Encephalitozoon intestinalis*, *Encephalitozoon cuniculi,* and *Enterocytozoon bieneusi* (Botterel et al. 2002; Orenstein et al. 2005; Kicia et al. 2018a, 2019).

12.4.2 Vertical Transmission

Vertical transmission from mother to offspring has been reported in infections due to *Encephalitozoon cuniculi* in rodents, carnivores, horses, rabbits, mice, and non-human primates (van Rensburg et al. 1991; Didier et al. 1998; Snowden et al. 1998; Baneux and Pognan 2003; Kotková et al. 2018). Human vertical transmission has not been demonstrated. Current reports of suspected transplacental transmission of *Encephalitozoon cuniculi* which have been published are based on circumstantial evidence such as serological diagnosis (Anver et al. 1972; Owen and Gannon 1980; van Rensburg et al. 1991). According to Owen and Gannon (1980) monitoring the levels of antibody titers in nurslings provides no clear evidence that there was a placental transfer as maternal antibodies can be passed on to the young in the milk of their mothers.

12.5 Diagnosis of Microsporidia

The diagnosis of microsporidiosis has been made using tissue samples, fluids (e.g., urine and feces), and serum (Garcia 2002). If spores are only detected in feces from a single sample, it cannot be definitively excluded that such spores were present in food or water and are just being passaged through the gastrointestinal tract (Bornay-Llinares et al. 1998); however, long-term shedding of spores in feces clearly indicates infection (Sak et al. 2010a).

Molecular, microscopic (light and electron) and serological methods are proven techniques for the detection of microsporidia. Light microscopy using either microsporidia specific stains or genus specific antibodies enables the detection of microsporidia, but does not allow species determination. Electron microscopy or molecular tests, however, can be used for species determination (Garcia 2002). Serology can indicate infection and can be species specific (Garcia 2002; Mathis et al. 2005); however, serology does not distinguish between active or prior infections. Serology is useful for epidemiologic investigations to determine the infection

prevalence in a population. Molecular methods (e.g., FISH, PCR, sequencing, or RFLP) allow the identification of microsporidia at a species/genotype level (Slodkowicz-Kowalska et al. 2006). These molecular techniques are valuable in determination of epidemiological indicators such as the spreading dynamics of a pathogen, pathogen origin, pathogen transmission pathways, and pathogen host specificity (Haro et al. 2005; Mathis et al. 2005).

12.6 Microsporidia in Mammals

Two groups of microsporidia that infect mammalian hosts can be distinguished: (1) species using mammals as their true hosts, and (2) a group of opportunistic microsporidia that have some other primary host that is not a mammal, but are able to infect mammals "accidentally." Species considered to be true mammalian microsporidia include *Enterocytozoon bieneusi, Encephalitozoon cuniculi*, *Encephalitozoon intestinalis* (previously called *Septata intestinalis*) (Wasson and Peper 2000). A limited number of studies have examined the source and mode of transmission of species from the "accidental" pathogenic microsporidia seen in humans/mammals. Species rarely found in mammals and having other known or presumable host include *Anncaliia* spp. (*A. algerae*, *A. connori,* and *A. vesicularum*), *Encephalitozoon hellem, Vittaforma corneae, Endoreticulatus* spp., *Nosema ocularum, Microsporidium* spp. (*M. africanum* and *M. ceylonensis*), *Trachipleistophora* spp. (*T. anthropopthera* and *T. hominis*), *Tubulonosema acridophagus,* and *Pleistophora ronneafiei*. Several of these microsporidians were detected in single cases, mostly in humans.

Gastrointestinal tract infection with diarrhea is the typical manifestation of microsporidiosis due to true mammalian microsporidia. Infective spores are produced in the alimentary tract and infection may be limited to one portion of the gut or include several intestinal tissue types, including gut epithelia and sometimes muscles. However, the target tissues represent a species-specific interaction with its host. Systemic microsporidiosis acquired by ingesting spores is also initiated in gut epithelial cells followed by microsporidian invasion of other visceral tissues such as brain, spleen, lung, liver, and sinuses. In microsporidia species considered to be non-mammalian, mostly ocular infections, sinusitis and myositis and rarely disseminated infection, urinary and intestinal infection have been described.

The observed clinical syndromes depend on the particular species causing the infection. *Enterocytozoon bieneusi* infection can cause diarrhea, malabsorption, and cholangitis. *Encephalitozoon cuniculi* infection can cause disseminated disease, encephalitis, and hepatitis. *Encephalitozoon intestinalis* can cause disseminated infection, diarrhea, and superficial keratoconjunctivitis. *Encephalitozoon hellem* can cause disseminated infection, respiratory disease, superficial keratoconjunctivitis, sinusitis, and prostatic abscesses. *Nosema*, *Vittaforma*, and *Microsporidium* infection can cause stromal keratitis following ocular trauma. *Anncaliia algerae*, *Tubulonosema*, *Pleistophora*, *Endoreticulatus*, and *Trachipleistophora* can cause

myositis, and *Trachipleistophora*, *Tubulonosema*, and *Anncaliia* are also associated with disseminated disease and encephalitis.

12.6.1 Encephalitozoon *spp.*

Three *Encephalitozoon* species have been identified as human pathogens: (1) *Encephalitozoon cuniculi* (Levaditi et al. 1923), which has a wide mammalian host range (Canning and Lom 1986) and is found worldwide in domestic rabbits and in distinct geographic areas in carnivores and monkeys; (2) *Encephalitozoon hellem*, which was distinguished from *E. cuniculi* in 1991 (Didier et al. 1991a) and is seen in avian hosts; and (3) *Encephalitozoon intestinalis* (originally designated *Septata intestinalis* (Cali et al. 1993)), which has been found in the feces of farm animals in Mexico as well as gorillas in Africa. These three *Encephalitozoon* spp. are essentially morphologically identical by light microscopy. While *Encephalitozoon intestinalis* can be morphologically distinguished from *Encephalitozoon cuniculi* and *Encephalitozoon hellem* by electron microscopy, these last two species are indistinguishable by this method. Molecular tests (e.g., PCR) can, however, be used to identify all three species.

12.6.1.1 *Encephalitozoon cuniculi* Levaditi, Nicolau, Schoen 1923

As the first microsporidian identified in mammal, *Encephalitozoon cuniculi* was detected by Wright and Craighead (1922) in the brain, kidneys, and spinal cord of a rabbit with motor paralysis and was named due to syndromes caused in rabbits in 1923 by Levaditi et al. (1923) and this name was used for the parasites found in rabbits and rodents for decades. In 1964, based on similarities with microsporidium found in insects, the name *Nosema cuniculi* was proposed and widely used for several years (Lainson et al. 1964); however, from the beginning of the 1970s the return to original nomenclature was recommended (Shadduck 1969).

Encephalitozoon cuniculi is the best-studied microsporidian species of mammals, as it was the first microsporidian isolated for in vitro culture from a mammalian host (i.e., a rabbit) (Shadduck 1969), thereby providing a source of spores for developing diagnostic methods and for studies on experimental infections in laboratory animals, which have successfully imitated infections in natural hosts (Gannon 1980; Kotková et al. 2013).

Encephalitozoon cuniculi develops intracellularly within a parasitophorous vacuole bounded by a membrane of presumed host origin. All developmental stages are monokaryotic, having one single unpaired nucleus. Meronts divide repeatedly by binary fission and usually remain in the vacuolar membrane. Meronts are round to ovoid structures, measuring from 2 to 6 by 1–3 μm, and lie close to the vacuolar membrane. Sporonts appear free in the center of the vacuole and develop a thick surface coat which becomes the exospore of spores. Sporonts divide by bisporous

sporogony into two sporoblasts which mature into spores. Mature spores measure 2.0–2.5 by 1.0–1.5 μm, and the polar filament has five to seven coils in a single row (Canning and Lom 1986). The genome of *Encephalitozoon cuniculi* is compact consisting of approximately 2.9 megabases (MBS) in 11 chromosomes, a total of 1997 potential protein-coding genes only (Katinka et al. 2001).

Encephalitozoon cuniculi has a broad mammalian host range and has also been reported occasionally in birds (Hinney et al. 2016). Natural mammalian infections have been reported in hosts that include rodents, lagomorphs, carnivores, livestock, and human and non-human primates (Weber et al. 1994; Didier et al. 1998). This microsporidia species lacks the organ/cell specificity and infects a wide spectrum of host cells which includes epithelial cells, macrophages, kidney tubule cells, and vascular endothelial cells (Gannon 1980). In mammals this organism has a predilection for the brain and kidney, but has been found in most tissues (Gannon 1980). Recently, a role for the involvement of immune cells connected with pro-inflammatory immune responses (e.g., trafficking macrophages and other phagocytic cells such as dendritic cells, neutrophils, monocytes, and eosinophils) in the expansion of infection in host tissues has been suggested based on occurrence of microsporidia in inflamed tissues (Kicia et al. 2018b) and the targeted migration seen following experimental induction of inflammation towards inflammation foci (Brdičková et al. 2020).

Four different genotypes/strains (I–IV) have been determined on the basis of biochemical, immunological, and molecular variations according to the number of short repeats in the ribosomal internal transcribed spacer (ITS) region. While not strictly host specific, these genotypes were originally isolated from different animals. While genotypes I–III are cultured in vitro and commonly used for experimental purposes, genotype IV is not yet available and little is known about its biological properties. *Encephalitozoon cuniculi* genotype I was isolated from rabbits (Shadduck 1969), genotype II was isolated from a mouse and has been found to naturally infect blue foxes (*Alopex lagopus*) (Didier et al. 1995), genotype III was isolated from domestic dogs (Shadduck et al. 1978), and genotype IV was identified in a renal transplant recipient, cats, and dogs (Talabani et al. 2010; Nell et al. 2015). Experimental studies on model hosts have revealed different virulence, host survival, and progression of the infection with *Encephalitozoon cuniculi* genotypes I–III. The infection extent of genotype I was comparable to genotype II and the infection onset speed and mortality rate of genotype I were similar to genotype III (Kotková et al. 2013, 2017, 2018; Sak et al. 2017, 2020b, 2021).

Encephalitozoon cuniculi in Rabbits

Since the early 1920s, encephalitis and neurological syndromes have been described in domestic rabbits (Wright and Craighead 1922; Doerr and Zdansky 1923; Goodpasture 1924); however, there are only limited reports of this infection in wild lagomorphs (Wilson 1979; Chalupský et al. 1990; Thomas et al. 1997; Zanet et al. 2013; Bartova et al. 2015; Martinez-Padilla et al. 2020). As a high percentage

of serologically positive rabbits do not show any clinical symptoms, encephalitozoonosis is now widely recognized as naturally occurring in domestic rabbits, mostly as chronic latent infection (Halánová et al. 2003; Valenčáková et al. 2012; Abu-Akkada et al. 2015a). In experimental models, antibodies against *Encephalitozoon cuniculi* develop within 3 weeks of infection (Cox et al. 1979). Antibody titers remain high for months following infection and can persist for years with fluctuating levels (Waller et al. 1978; Scharmann et al. 1986). Compared to mice and horses, it is not known whether rabbits shed spores during the chronic stage of infection. Infected rabbits can on occasion manifest neurologic signs of infection that include torticollis, opisthotonosis, ataxia, hyperesthesia, or paralysis as well as other findings such as weight loss and failure to thrive that can lead to death (Robinson 1954; Kunstyr and Naumann 1985). Necropsy lesions may be seen in the renal cortex (focal, irregular, depressed areas), however, typically gross lesions are absent (Flatt and Jackson 1970). Histologically, abnormalities can be seen in tissue sections from brain (focal nonsuppurative meningoencephalitis with astrogliosis and perivascular lymphocytic infiltration) and kidney (focal to segmental lymphocytic-plasmacytic interstitial nephritis with variable amounts of fibrosis); despite these histological findings, it can be difficult to find microsporidia in these areas of inflammation (Cox and Gallichio 1978). In contrast, in areas with little inflammatory response, one may be able to observe intracellular clusters of proliferating microsporidia as well as spores. Occasionally, microsporidia may be seen histologically in other tissues from infected rabbits such as the lung, heart, eye, and liver (Cox et al. 1979; Stiles et al. 1997). Data on symptomatic cases is summarized in Table 12.1.

Encephalitozoon cuniculi in Rodents

With the widespread use of laboratory rodents in research before the establishment of modern management techniques (i.e., gnotobiotic derivation, animal barrier facility and barrier caging) *Encephalitozoon cuniculi* was one of the most common and important sources of interference with experimental results in the last century (Canning 1967; Shadduck and Pakes 1971; Majeed and Zubaidy 1982; Canning and Lom 1986; Boot et al. 1988; Illanes et al. 1993). *Encephalitozoon cuniculi* genotype II was originally isolated directly from a naturally infected mouse (Koudela et al. 1994). However, despite a high serological prevalence of infection among colonies of laboratory rodents reaching up to 80% (Chalupský et al. 1979; Gannon 1980), *Encephalitozoon cuniculi* infection of immune competent rodents, as in rabbits, is usually subclinical and animals rarely show clinical signs except focal granulomas or parasites in the tissues (Nelson 1962). The fact that microsporidiosis in murine hosts mimics the course of human infection and studies of immune responses in immune competent and immune deficient strains of laboratory mice (and other rodents) provided a foundation for understanding microsporidiosis in mammals (Braunfuchsová et al. 2001, 2002; Khan et al. 2001; Moretto et al. 2004; Salát et al. 2004, 2006; Sak et al. 2006; Moretto et al. 2010). Clinical signs include

Table 12.1 Clinical cases of *Encephalitozoon cuniculi* in rabbits (*Oryctolagus cuniculus*)

Country	Clinical signs, pathology	Reference
Australia	Glomerulosclerosis, hyalinosis	Packham et al. (1992)
Austria	Neurological symptoms, uveitis renal failure	Kunzel et al. (2008)
Austria	Encephalitis, nephritis	Csokai et al. (2009)
Canada	Head tilt, limb paresis, nephritis	Nast et al. (1996)
Egypt	Vestibular disease, paresis, paralysis, cataracts, uveitis, abortions	Morsy et al. (2020)
Germany	Encephalitis, lethargy, paresis	Doerr and Zdansky (1923)
Germany	Head tilt, torticollis	Kunstyr and Naumann (1985)
Italy	Kidney lesions, brain lesions	Maestrini et al. (2017)
Italy	Uveitis	Giordano et al. (2005)
Japan	Neurological impairment, head tilt, partial paralysis	Furuya et al. (2001)
Japan	Ataxia, torticollis, stunted growth, hind-limb asthenia	Fukui et al. (2013)
Mexico	Cerebral lesions, renal lesions	Rodriguez-Tovar et al. (2016)
Poland	Paralysis, paresis, torticollis	Zietek et al. (2014)
Slovakia	Ocular signs, anorexia, ataxia, asthenia of hind-limbs	Valenčaková et al. (2008)
Turkey	Ataxia, aggressiveness, seizures, and circling	Ozkan and Alcigir (2018)
UK	Cataract	Ashton et al. (1976)
USA	Neurological, renal, or ocular signs	Harcourt-Brown and Holloway (2003)
USA	Uveitis	Pilny (2012)
USA	Brain lesions	Goodpasture (1924)
USA	Paralysis	Wright and Craighead (1922)

wasting, lethargy, and death. Multifocal miliary white spots are seen on necropsy in the lungs, liver, heart, and spleen, and hepatosplenomegaly may also be observed (Heřmánek et al. 1993; Koudela et al. 1993; Didier et al. 1994). By histological examination, infection causes multifocal miliary granulomas with various amounts of cell debris and suppurative necrosis, and this has been observed in spleen, lung, liver, heart, brain, kidney, peritoneum, and pancreas (Koudela et al. 1993; Didier et al. 1994). However, most of the experimental studies on microsporidia in mice have focused their evaluations on the prolongation of host survival time and the actual burden of microsporidia in asymptomatic model hosts remained poorly characterized. Based on recent results quantifying the amount of spores in infected animals, a progressive, sometimes lifelong chronic course was revealed not only in immune deficient, but also in immune competent mice lacking any clinical signs (Kotková et al. 2013, 2017, 2018; Sak et al. 2017, 2020a). Moreover, in these studies only temporary elimination of microsporidia was observed in laboratory mice following albendazole treatment, and immune suppression mediated by dexamethasone resulted not only in reactivation of infection with dissemination in chronically infected animals, but also in reactivation of undetectable infection in animals

Table 12.2 Clinical cases of *Encephalitozoon cuniculi* in rodents

Country	Host (scientific name)	Clinical signs, pathology	Reference
Canada	Vancouver Island marmot (*Marmota vancouverensis*)	Neurological disease, meningoencephalomyelitis	Milnes et al. (2018)
Canada	Muskrat (*Ondatra zibethicus*)	Granulomatous encephalitis	Wobester and Schuh (1979)
Czech Republic	Steppe lemming (*Lagurus lagurus*)	Weight loss, conjunctivitis, hind limb paresis	Hofmannová et al. (2014)
Germany	Barbary striped grass mouse (*Lemniscomys barbarus*)	Pneumonia, encephalitis, splenomegaly	Kitz et al. (2018)
Poland	Guinea pig (*Cavia porcellus*)	Torticollis, encephalitis	Wilczynska et al. (2020)
USA	Arctic lemming (*Dicrostonyx stevensoni*)	Circling, torticollis, posterior paralysis	Cutlip and Beall (1989)

considered to be fully cured (Kotková et al. 2013). These data corresponded to that seen when screening of wild and pet rodents, where only limited clinical cases were reported (Table 12.2), although *Encephalitozoon cuniculi* was reported in numerous species worldwide, such as house mice, rats, shrews, capybaras, beavers, and voles using molecular screening or serology testing (Muller-Doblies et al. 2002; Fayer et al. 2006; Fuehrer et al. 2010; Valadas et al. 2010; Sak et al. 2011c; Tsukada et al. 2013; Danišová et al. 2015; Meredith et al. 2015; Perec-Matysiak et al. 2019). Rodents are an important reservoir of zoonotic pathogens, such as microsporidia, as they occupy a wide range of habitats and comprise about 40% of the mammalian diversity (Mathis et al. 2005).

Encephalitozoon cuniculi in Carnivores

Encephalitozoon cuniculi typically causes sporadic infections that present as fulminating disease in neonatal carnivores, mostly in dog and fox puppies and kits (Shadduck et al. 1978). Natural infection with *Encephalitozoon cuniculi* has been most thoroughly described in domestic dogs and farm-raised foxes as high neonatal mortality among blue fox caused by endemic encephalitozoonosis which resulted in economic losses for the fur industry in Scandinavian countries (Nordstoga and Westbye 1976). Moreover, *Encephalitozoon cuniculi* has been occasionally identified as a cause of neurological, renal, or ocular disease in dogs (Table 12.3). In utero infection is suspected as the mode of transmission in fox kits and dog pups (Mohn et al. 1974), resulting in ataxia, posterior weakness, tremors, reduced appetite and stunted growth, convulsions, blindness with progression to circling, and aggressive behavior (Szabo and Shadduck 1987; Mcinnes and Stewart 1991). Necropsy lesions can be observed in the cortex and renal pelvis (on cross section) and distended edematous meninges can be seen (Nordstoga and Westbye 1976). Histologic lesions in dogs and foxes include nephritis, pneumonia, hepatitis, and meningoencephalitis (Szabo and Shadduck 1987). This disease is often accompanied by ocular vasculitis

Table 12.3 Clinical cases of *Encephalitozoon cuniculi* in carnivores

Country	Host (scientific name)	Clinical signs, pathology	Reference
Argentina	Dog (*Canis familiaris*)	Nephritis, encephalitis	Postma et al. (2018)
Austria	Cat (*Felis catus*)	Cataract, uveitis	Benz et al. (2011)
Austria	Cat (*Felis catus*)	Stunted growth, encephalomyelitis	Rebel-Bauder et al. (2011)
Austria	Cat (*Felis catus*)	Uveitis	Csokai et al. (2010)
Austria	Snow leopard (*Panthera uncia*)	Cortical cataract, uveitis	Scurrell et al. (2015)
Chile	South American fur seal (*Arctocephalus australis*)	Encephalitis	Seguel et al. (2015)
China	Artic blue fox (*Alopex lagopus*)	Anepithymia, convulsion, blindness, renal lesions	Meng et al. (2014)
Czech Republic	Stone marten (*Martes foina*)	Encephalitis	Hůrková and Modrý (2006)
Czech Republic	European otter (*Lutra lutra*)	Neurological disorder	Hůrková and Modrý (2006)
Czech Republic	Arctic blue fox (*Alopex lagopus*)	Ataxia, vision disorders, clonic spasms	Persin and Dousek (1986)
Finland	Arctic blue fox (*Alopex lagopus*)	Renal and brain lesions	Akerstedt et al. (2002)
Germany	Dog (*Canis familiaris*)	Anorexia, polyuria, polydipsia, azotemia	Engelhardt et al. (2017)
Hungary, Serbia	Dog (*Canis familiaris*)	Cataract, uveitis	Nell et al. (2015)
Ireland	Red foxes (*Vulpes vulpes*)	Encephalitis	Murphy et al. (2007)
Norway	Mink (*Neovison vison*)	Brain and spinal cord lesions, cataract	Bjerkas (1990)
Norway	Arctic blue fox (*Alopex lagopus*)	Brain and spinal cord lesions	Bjerkas (1987), Bjerkas and Nesland (1987)
Norway	Mink (*Neovison vison*)	Renal lesions, nephritis, and vasculitis	Zhou et al. (1992)
Norway	Arctic blue fox (*Alopex lagopus*)	Cataract	Arnesen and Nordstoga (1977)
Norway	Arctic blue fox (*Alopex lagopus*)	Arterial lesions	Nordstoga and Westbye (1976)
Norway	Arctic blue fox (*Alopex lagopus*)	Stunted growth, neurological disorder	Mathis et al. (1996)
South Africa	Dog (*Canis familiaris*)	Blindness, ataxia, meningo-encephalitis, nephritis, hepatitis	Basson et al. (1966)
South Africa	Dog (*Canis familiaris*)	Encephalitis, nephritis	Mcinnes and Stewart (1991)
South Africa	African wild dogs (*Lycaon pictus*)	Encephalitis, nephritis	Van Heerden et al. (1989)

(continued)

Table 12.3 (continued)

Country	Host (scientific name)	Clinical signs, pathology	Reference
South Africa	Dog (*Canis familiaris*)	Blindness, retarded growth, nephritis	Botha et al. (1979)
South Africa	Dog (*Canis familiaris*)	Fading syndrome, encephalitis	Stewart et al. (1979)
Sweden	Arctic blue fox (*Alopex lagopus*)	Encephalitis	Berg et al. (2007)
USA	Dog (*Canis familiaris*)	Encephalitis, nephritis	Snowden et al. (2009)
USA	Dog (*Canis familiaris*)	Encephalitis, nephritis	Plowright and Yeoman (1952)
USA	Dog (*Canis familiaris*)	Nephritis, encephalitis, and segmental vasculitis	Shadduck et al. (1978)
USA	Dog (*Canis familiaris*)	Neurological disorder	Snowden et al. (1999)
USA	Dog (*Canis familiaris*)	Encephalitis, nephritis	Cole et al. (1982)
USA	Cat (*Felis catus*)	Corneitis	Buyukmihci et al. (1977)
USA	Black-footed ferret (*Mustela nigripes*)	Nephritis, pneumonia, encephalitis	Ter Beest et al. (2019)

and the development of lens cataracts resulting in vision deficits (Arnesen and Nordstoga 1977). Surviving animals or bitches producing infected litters are usually asymptomatic and can shed parasites in the urine (Mcinnes and Stewart 1991; Snowden et al. 2009) demonstrating the presence of latent chronic infection in these animals. The parents of infected animals are typically unaffected and serologically positive; however, the pups or kits can have variable mortality and morbidity rates (Shadduck et al. 1978). Recently, *Encephalitozoon cuniculi* has been recognized as an important feline infection which can cause ocular and central nervous system infections with symptoms. Based on limited number of epidemiological surveys performed in carnivores, encephalitozoonosis, based on seroprevalence or molecular detection of parasites in feces on asymptomatic animals, is higher than anticipated on clinical cases (Hersteinsson et al. 1993; Halánová et al. 2003; Hinney et al. 2016; Piekarska et al. 2017; Duzlu et al. 2019).

Encephalitozoon cuniculi in Other Mammals

Infection caused by *Encephalitozoon cuniculi* has been reported in several species of other mammals such as domestic animals, livestock, and pets. Most of the reports are based on serology or molecular detection in subclinical cases in swine/boar (Deplazes et al. 1996; Reetz et al. 2009; Malčeková et al. 2010; Němejc et al. 2014), sheep (Malčeková et al. 2010; Abu-Akkada et al. 2015a), cattle (Halánová et al. 1999; Malčeková et al. 2010; Abu-Akkada et al. 2015a), goat (Cislakova et al. 2001; Abu-Akkada et al. 2015a), buffaloes (Sak et al. 2013; Abu-Akkada et al.

Table 12.4 Clinical cases of *Encephalitozoon cuniculi* in other mammals

Country	Host (scientific name)	Clinical signs, pathology	Reference
Hungary	Horse (*Equus ferus*)	Placentitis, abortion	Szeredi et al. (2007)
Ireland	Horse (*Equus ferus*)	Meningoencephalitis	Hollyer et al. (2014)
South Africa	Horse (*Equus ferus*)	Nephritis	van Rensburg et al. (1991)
USA	Horse (*Equus ferus*)	Placentitis, abortion	Patterson-Kane et al. (2003)

Table 12.5 Clinical cases of *Encephalitozoon cuniculi* in monkeys

Country	Host (scientific name)	Clinical signs, pathology	Reference
Germany	Cotton-top tamarin (*Saguinus oedipus*)	Enteritis, hepatitis,	Reetz et al. (2004)
Japan	Squirrel monkey (*Saimiri sciureus*)	Brain lesions	Asakura et al. (2006)
North America	Cotton-top tamarin (*Saguinus oedipus*)	Hepatitis, meningoencephalitis, pneumonia, nephritis	Juan-Salles et al. (2006)
North America	Emperor tamarin (*Saguinus imperator*)	Hepatitis, pneumonia, nephritis, myocarditis	Packham et al. (1992)
Not specified	Red-bellied titi (*Callicebus moloch*)	Enteritis	Seibold and Fussell (1973)
Switzerland	Emperor tamarin (*Saguinus imperator*)	Generalized infection, systemic vasculitis	Guscetti et al. (2003)
USA	Squirrel monkeys (*Saimiri sciureus*)	Meningoencephalitis, pneumonia, nephritis	Zeman and Baskin (1985)
USA	Goeldi's monkey (*Callimico goeldii*)	Arteritis, aortitis	Davis et al. (2008)
USA	Squirrel monkey (*Saimiri sciureus*)	Encephalitis	Anver et al. (1972)
USA	Squirrel monkey (*Saimiri sciureus*)	Neurological disorder	Brown et al. (1973)

2015a), and horses (Levkutová et al. 2004; Goodwin et al. 2006; Wagnerová et al. 2012; Cray et al. 2014; Laatamna et al. 2015), but clinical reports are limited to horses (Table 12.4). Additionally, disseminated encephalitozoonosis resulting in nephritis and encephalitis was documented under experimental conditions in mini Shetland ponies (Wagnerová et al. 2013).

Encephalitozoon cuniculi in Non-human Primates

Monkeys

Encephalitozoon cuniculi infection has been reported to occur in cotton-top and emperor tamarins, squirrel monkeys, red-bellied titis, and Goeldi's monkeys (Table 12.5). These infections occurred in neonates or infants that acquired the infection in utero from subclinically infected mothers demonstrating that latent infection can occur in primates. Additionally, a seroprevalence survey has been

performed in a group of squirrel monkeys which demonstrated a 94% positivity rate (Shadduck and Baskin 1989). In adult monkeys infection by *Encephalitozoon cuniculi* is typically subclinical, but offspring of these animals have microscopic lesions in their central nervous system, lung, kidney, and liver. On histological examination, multifocal granulomas can be observed in both white and gray matter, along with vasculitis and meningoencephalitis (Zeman and Baskin 1985; Guscetti et al. 2003; Juan-Salles et al. 2006).

Monkeys were widely used around the world in research because of their psychological and physiological similarity to humans as model host for various diseases including microsporidia (Snowden et al. 1998) and experiments with *Encephalitozoon cuniculi* infection have been performed mainly on rhesus macaques (*Macaca mulatta*) and vervet monkeys (*Cercopithecus pygerythrus*). In order to simulate the immunodeficiency associated with HIV in humans, some macaques were experimentally infected with Simian Immunodeficiency Virus (SIV). While immune competent animals developed a serologic response without any clinical signs of encephalitozoonosis, SIV-immunocompromised animals succumbed to a wasting disease (Didier et al. 1994). In contrast, vervet monkeys (*Chlorocebus pygerythrus*), infected either orally or intravenously, displayed no clinical signs, but necropsy granulomatous lesions were identified in their liver, kidney, and brain. Moreover, vertical transmission was documented when infecting late pregnant females (Vandellen et al. 1989).

Great Apes

Great apes share a wide range of pathogenic microorganisms with humans, perhaps due to their close evolutionary relationship and genomic similarity (Raoult 2012; Keita et al. 2014). Apes could, therefore, be a source of emerging human pathogens, and vice versa humans could be a source of pathogens in apes. Currently, gorillas and orangutans are the most endangered great apes, primarily due to habitat encroachment by anthropogenic activities, such as logging, conversion of forested land to plantations, forest fires and road building, and trade and hunting (Gillespie et al. 2008; Wich et al. 2008; Zommers et al. 2013).

Although many studies have addressed behavior, ecology, and conservation of great apes, studies on parasitofauna, especially microsporidia, are scarce. Molecular detection of microsporidia species/genotypes in great apes from captive facilities (i.e., sanctuaries and Zoological parks) revealed up to a 40% prevalence of *Encephalitozoon cuniculi* in captive western lowland gorillas (*Gorilla gorilla gorilla*), 31% in chimpanzees (*Pan troglodytes*), and 21% in bonobos (*Pan paniscus*) (Sak et al. 2011d). Moreover, among free-ranging great apes, *Encephalitozoon cuniculi* was detected in the western lowland gorilla (*Gorilla gorilla gorilla*; 7.5%) (Sak et al. 2013), mountain gorilla (*Gorilla beringei beringei*; 11%) (Sak et al. 2014), Sumatran orangutan (*Pongo abelii*; 21%), and Bornean orangutan (*Pongo pygmaeus*; 1%) (Mynařová et al. 2016). However, in contrast to New World monkeys (e.g., squirrel monkeys, emperor and cotton-top tamarins), neither captive nor free-ranging apes exhibited any clinical signs of

microsporidiosis. Overall, this indicates that latent chronic microsporidiosis is commonly found in apes.

Encephalitozoon cuniculi in Humans

Before the advent of the AIDS epidemic in the mid-1980s, microsporidiosis in humans was rarely reported and mostly associated with immune deficient children presenting with neurological disease. *Encephalitozoon cuniculi* (Matsubayashi et al. 1959; Margileth et al. 1973; Bergquist et al. 1984) was the reported etiologic agent for these rare cases. With the onset of HIV/AIDS pandemic, microsporidia were recognized as causing opportunistic infections in immunologically compromised patients with fewer than 100 CD4+ T-lymphocytes per 1 μl of blood (Orenstein 1991; Weber et al. 1994). The spectrum of disease includes cerebral, gastrointestinal, ocular, pulmonary, muscular, nasal, and systemic infections resulting in enteritis, hepatitis, peritonitis, pancreatitis, pneumonia, encephalitis, cystitis, nephritis, keratoconjunctivitis, sinusitis, and rhinitis (Terada et al. 1987; Lowder 1993; De Groote et al. 1995; Mertens et al. 1997; Orenstein et al. 1997). *Encephalitozoon cuniculi* in humans was shown to infect similar tissues to what had been seen in infected rabbits (i.e., myocytes, microglia, and renal endothelial and epithelial cells) (Mertens et al. 1997; Weber et al. 1997).

Immune competent individuals who became infected with microsporidia developed persistent infections with few clinical signs of disease and parasite replication appeared to be under control of the host immune system. It has been suggested that *Encephalitozoon cuniculi* infection remains asymptomatic as long as parasite multiplication and the host immune response are balanced (Gannon 1980). There is convincing serologic evidence that clinically asymptomatic humans are exposed to microsporidia (Bergquist et al. 1984; Hollister and Canning 1987; Hollister et al. 1991; Van Gool et al. 1997; Kučerová-Pospísilová and Ditrich 1998; Abu-Akkada et al. 2015b). Human infection has been seen with all of the known *Encephalitozoon cuniculi* genotypes. Whereas infection with genotype I and II is common, occurrence of genotype III and IV in humans is rare. It is probable that human infection with *Encephalitozoon cuniculi* is predominantly zoonotic (Shadduck et al. 1979; Didier 2005; Mathis et al. 2005; Didier and Weiss 2011). However, understanding the extent of these latent infections is complicated by the fact that microsporidia are often overlooked and not diagnosed despite their relatively high occurrence (Sak et al. 2011a; Wesolowska et al. 2019). This underdiagnosis increases the possibility of hidden infections causing significant damage and various nonspecific pathologies. An example of this is a case report in a patient with diabetes with hemiparesis and epilepsy, but no known immune suppression, of a brain abscess that contained both *Streptococcus intermedius* and *Encephalitozoon cuniculi* (Ditrich et al. 2011). *Encephalitozoon cuniculi* has also been detected in periprosthetic tissues in patients with hip joint endoprosthesis suffering from periprosthetic osteolysis (Kicia et al. 2018b). A positive correlation has been seen in experimental models between the induction of inflammation and the amount of *Encephalitozoon cuniculi* observed in

inflammation foci (Brdičková et al. 2020). This is consistent with the idea that immune cells can act as vehicles transporting *Encephalitozoon cuniculi* purposefully across the whole host body towards sites of inflammation.

As Table 12.6 shows, it would be worth considering the role of asymptomatic graft donors as a potential source of infection for immune suppressed recipients. The only documented case of such acquired extra-intestinal microsporidiosis described has been by Latib et al. (2001) in renal graft in a 39-year-old transplant recipient suffering from pyrexia and deteriorating graft function, whose renal function was recovered following albendazole treatment. However, the question of whether the remaining recipients already had the infection before transplantation or acquired it together with the graft will remain unanswered until graft donors are tested for the presence of microsporidia as they are now tested for viral and bacterial infections (Fischer et al. 2013).

12.6.1.2 *Encephalitozoon hellem* (Didier et al. 1991a)

Although *Encephalitozoon hellem* represents one of the common human microsporidian parasites, its broadest distribution can be found among birds (Black et al. 1997; Pulparampil et al. 1998; Rosell et al. 2016), suggesting that human infection may be due to exposure to infected birds (Childs-Sanford et al. 2006). However, concurrent *Encephalitozoon hellem* infection of both humans and their companion birds has yet to be definitively documented in the literature. *Encephalitozoon hellem* is morphologically and ultrastructurally identical to *Encephalitozoon cuniculi* and its recognition as a separate species was based on the immunologic and molecular analysis of this organism (Didier et al. 1991a, b; Vossbrinck et al. 1993). Like *Encephalitozoon cuniculi*, meronts of *Encephalitozoon hellem* are attached to the parasitophorous vacuole membrane and all stages are monokaryotic. Mature spores are detached in the vacuole lumen measuring 1.0–1.5 × 2.0–2.5 μm. In addition, the spores contain about six to eight turns of the polar filament and monokaryotic nuclei (Wright and Craighead 1922).

This species was first described as a human pathogen from three AIDS patients with keratoconjunctivitis (Didier et al. 1991a). Additional cases in immune compromised humans have also been described from the eye, urogenital tract, respiratory tract, and as fatal disseminated systemic infections causing bronchitis, prostatitis, nephritis, and cystitis (Weber et al. 1993; Scaglia et al. 1994, 1998; Schwartz et al. 1994; Kotler and Orenstein 1998) (Table 12.7). Antibodies to *Encephalitozoon hellem* were detected in asymptomatic immune competent humans as well (Kučerová-Pospísilová and Ditrich 1998), however, the study group consisted of people at risk for HIV/AIDS infection living on the margins of society (e.g., prostitutes, alcoholics, and intravenous drug abusers). Non-human primates, carnivores, and rodents can also be infected by this species, but clinical disease has been rarely described (Didier et al. 1994; Sak et al. 2011c, d; Lallo et al. 2012). There are two reports describing clinical microsporidiosis caused by *Encephalitozoon*

Table 12.6 Clinical cases of *Encephalitozoon cuniculi* in human

Country	Immune status	Clinical signs, pathology	Reference
Cuba	AIDS	Genital microsporidiosis	Torres et al. (2013)
Czech Republic	Immune competent	Brain abscess	Ditrich et al. (2011)
France	AIDS	Disseminated infection	Fournier et al. (2000)
Germany	HIV	Pulmonary infection	Weitzel et al. (2001)
Germany	AIDS	Disseminated infection	Franzen et al. (1995)
Italy	AIDS	Disseminated infection	Tosoni et al. (2002)
Mexico	Renal transplant recipient	Cough, fever, diarrhea, abdominal pain	Gamboa-Dominguez et al. (2003)
Not specified	AIDS	Hepatitis	Terada et al. (1987)
Not specified	AIDS	Hepatic failure	Sheth et al. (1997)
Not specified	AIDS	Peritonitis	Zender et al. (1989)
Poland	Renal transplant recipient	Respiratory diseases	Kicia et al. (2019)
Poland	Endoprosthesis recipient	Disseminated infection, periprosthetic osteolysis	Kicia et al. (2018b)
South Africa	Renal transplant recipient	Pyrexia, diarrhea	Ladapo et al. (2014)
South Africa	Renal transplant recipient	Pyrexia, deteriorating graft function	Latib et al. (2001)
Spain	AIDS	Fever, weight loss, asthenia, abdominal pain, diarrhea	Del Aguila et al. (2001)
Switzerland	HIV	Brain lesions	Weber et al. (1997)
Switzerland	Idiopathic CD4+ T-lymphocytopenia	Iris tumor	Kodjikian et al. (2005)
UK	AIDS	Keratoconjunctivitis, sinusitis	Metcalfe et al. (1992)
UK	AIDS	Renal failure	Hollister et al. (1995)
USA	AIDS	Disseminated infection	Croppo et al. (1997)
USA	Lung transplant recipient	Nephritis	Levine et al. (2013)
USA	Transplant recipients	Encephalitis	Smith et al. (2017)
USA	Transplant recipients	Fever, myalgia, fatigue	Hocevar et al. (2014)
USA	Bone marrow transplantation	Pulmonary infection	Orenstein et al. (2005)
USA	Renal transplant recipient	Disseminated infection	Mohindra et al. (2002)

(continued)

Table 12.6 (continued)

Country	Immune status	Clinical signs, pathology	Reference
USA	AIDS	Disseminated infection, fever	Mertens et al. (1997)
USA	AIDS	Renal failure, cough	De Groote et al. (1995)

Table 12.7 Clinical cases of *Encephalitozoon hellem* in humans

Country	Immune status	Clinical signs, pathology	Reference
France	Leukemia	Diarrhea, weight loss, fever, urinary problems	Nevez et al. (2015)
India	Not specified	Keratitis	Joseph et al. (2006)
Italy	AIDS	Disseminated infection	Scaglia et al. (1994)
Italy	AIDS	Pulmonary infection	Scaglia et al. (1997)
Switzerland	AIDS	Conjunctivitis, disseminated infection	Weber et al. (1993)
Switzerland	AIDS	Sinusitis	Deplazes et al. (1998)
UK	AIDS	Nasal infection	Hollister et al. (1993)
USA	AIDS	Disseminated infection	Didier et al. (1996)
USA	AIDS	Keratoconjunctivitis, fever, pneumonia	Schwartz et al. (1993b)
USA	AIDS	Keratoconjunctivitis	Didier et al. (1991a)
USA	AIDS	Conjunctivitis	Schwartz et al. (1993a)
USA	AIDS	Disseminated infection	Schwartz et al. (1992)
USA	AIDS	Prostatic abscess	Schwartz et al. (1994)

Table 12.8 Clinical cases of *Encephalitozoon hellem* in non-human mammals

Country	Animal species (scientific name)	Clinical signs, pathology	Reference
Belgium	European brown hare (*Lepus europaeus*)	Kidney lesions	De Bosschere et al. (2007)
USA	Egyptian fruit bat (*Rousettus aegyptiacus*)	Disseminated infection	Childs-Sanford et al. (2006)

hellem in naturally infected hares and bats (Table 12.8). Lethal *Encephalitozoon hellem* infection was only observed in experimentally infected immune deficient monkeys and immune deficient mice (Didier et al. 1994).

Intraspecific genotypic variability in *Encephalitozoon hellem* was initially based on the sequence of ITS rRNA genes dividing different isolates into 3 genotypes named 1 to 3 (Mathis et al. 1999b), but was later also characterized in two intergenic spacers (IGS-TH and IGS-HZ) and in the gene for PTP1 (polar tube protein 1),

which allowed the description of new genotypes: the former genotype 1 was divided into 1A, 1B, 1C, genotype 2 was divided into 2A and 2B, and genotype 3 was renamed 2C (Xiao et al. 2001; Haro et al. 2003).

12.6.1.3 *Encephalitozoon intestinalis* Cali, Kotler, Orenstein, 1993

Encephalitozoon intestinalis (originally named *Septata intestinalis* in 1993 (Cali et al. 1993) and reclassified as *Encephalitozoon intestinalis* in 1995 (Hartskeerl et al. 1995)) causes chronic diarrhea in patients with AIDS. *Encephalitozoon intestinalis* is considered the second most prevalent *Encephalitozoon* species in humans, occurs worldwide, and has been successfully maintained in long-term tissue cultures. Although *Encephalitozoon intestinalis* is morphologically similar to *Encephalitozoon cuniculi* and *Encephalitozoon hellem*, this species could be distinguished from the other two species by the special characteristics of its parasitophorous vacuole being prominently lobed and containing septa forming incomplete chambers enclosing individual parasites within the vacuole. All stages are monokaryotic, mature spores measure 2.0 × 1.2 μm, contain 4–7, but usually 5, polar tubule coils. In addition, the spores contain about six to eight turns of the polar filament (Cali et al. 1993).

Encephalitozoon intestinalis has been shown to occur in several, predominantly domestic, mammalian species such as cattle, goat, donkey, pig, dog, cat, rabbit or mountain gorillas, coatis, red ruffed and ring-tailed lemurs (Bornay-Llinares et al. 1998; Graczyk et al. 2002; Jamshidi et al. 2012; Lallo et al. 2012; Slodkowicz-Kowalska et al. 2012; Velasquez et al. 2012; Duzlu et al. 2019; Pekmezci et al. 2019; Deng et al. 2020b; Martinez-Padilla et al. 2020). Zoonotic transmission was proposed to be an important source in human infections (Bornay-Llinares et al. 1998; Graczyk et al. 2002; Didier 2005; Valenčáková et al. 2006; Malčeková et al. 2010). However, although experimental murine systemic and intestinal *Encephalitozoon intestinalis* infections can occur in several strains of immune suppressed mice (Achbarou et al. 1996; El Fakhry et al. 1998; El Fakhry et al. 2001; Salát et al. 2002, 2004), spontaneous infection in rodents due to *Encephalitozoon intestinalis* has rarely been described (Tsukada et al. 2013; Danišová et al. 2015).

Even though *Encephalitozoon intestinalis* was initially recognized as an enteric infection associated with diarrhea in AIDS patients, this species can also disseminate to the lungs, eyes, gallbladder, and urinary tract causing enteritis, colitis, hepatitis, cholangitis, sinusitis, cholecystitis, bronchitis, keratoconjunctivitis, and cystitis (Molina et al. 1995; Sobottka et al. 1995; Willson et al. 1995; Lowder et al. 1996; Soule et al. 1997). Moreover, latent infection with *Encephalitozoon intestinalis* seems to occur more frequently and the true prevalence of the parasites may be much higher than previously reported (Franzen et al. 1996; Ndzi et al. 2016). Genotypic variations in *Encephalitozoon intestinalis* were demonstrated but have not been applied to broader surveys (Galvan et al. 2013a) (Table 12.9).

Table 12.9 Human *Encephalitozoon intestinalis* infections

Country	Immune status	Clinical signs, pathology	Reference
Argentina	AIDS	Vomiting, diarrhea, weight loss	Velasquez et al. (2012)
Austria	Immune competent	Diarrhea	Wichro et al. (2005)
Democratic Republic of Congo	AIDS	Diarrhea	Wumba et al. (2010)
France	Immune competent	Diarrhea	Raynaud et al. (1998)
France	AIDS	–	Ombrouck et al. (1996)
France	AIDS	Diarrhea, fever, cholangitis, sinusitis, bronchitis, conjunctivitis	Molina et al. (1995)
Gabon	Unknown	Diarrhea	Oyegue-Liabagui et al. (2020)
Iran	Chemotherapy	–	Ghoyounchi et al. (2019)
Italy	AIDS	Renal infection	Boldorini et al. (1998)
Pakistan	Hepatocellular carcinoma	Diarrhea	Yakoob et al. (2012)
Slovenia	Immune competent	Diarrhea	Hasanagic (2006)
Turkey	Chemotherapy	Diarrhea	Hamamci et al. (2015)
Turkey	Chemotherapy	Diarrhea	Cetinkaya et al. (2016)
USA	AIDS	Cholangiopathy	Sheikh et al. (2000)
USA	AIDS	Diarrhea, nephritis, cholecystitis	Cali et al. (1993)
USA	AIDS	Diarrhea	Orenstein et al. (1992a)
USA	AIDS	Diarrhea	Orenstein et al. (1992b)
USA, Africa, Europe	AIDS	Diarrhea	Cali et al. (1991)
Venezuela	HIV	–	Rivero-Rodriguez et al. (2013)

12.6.1.4 *Enterocytozoon bieneusi* Desportes, Le Charpentier, Galian, Bernard, Cochand-Priollet, Lavergne, Ravisse, Modigliani, 1985

Enterocytozoon bieneusi was first detected by Modigliani et al. (1985) and described in detail by Desportes et al. (1985). This species is the most commonly reported cause of diarrhea in patients with AIDS (Messaoud et al. 2021). While the exact prevalence of *Enterocytozoon bieneusi* in the general population is unknown, in various studies from 2 to 50% of HIV-infected patients with severe immunodeficiency, CD4+ cell counts below 100/μl and otherwise unexplained diarrhea have been demonstrated to be infected, depending on the study group and method of diagnosis (Field et al. 1993; Kotler and Orenstein 1994). Little progress on understanding of this infection has been made because of the inability to propagate *Enterocytozoon bieneusi* in vitro and in vivo, which limits the source of parasite spores to the stools of infected human patients or animals (Sheoran et al. 2005). The early intracellular stages contain two nuclei in diplokaryotic arrangement. These stages grow into multinucleate plasmodia containing numerous bodies of heterogeneous material, which differentiate simultaneously to several polar tubes coiled to three coils in two rows each. When the extrusion apparatus is fully differentiated, the sporogonial plasmodium divides into monokyryotic sporoblasts, which are in direct contact with the host cytoplasm and transform into mature spores measuring 1.1–1.6 by 0.7–1.0 μm (Desportes et al. 1985; Sheoran et al. 2005).

The parasite usually infects the apical surface of intestinal enterocytes, but has also been occasionally detected in lamina propria cells of small-bowel biopsy specimens, gallbladder, biliary tree, pancreatic duct, liver cells, and tracheal, bronchial and nasal epithelia (Weber et al. 1992; Schwartz et al. 1995; Del Aguila et al. 1997; Matos et al. 2012) (Table 12.10). In persons with normal immunity *Enterocytozoon bieneusi* infection generally leads to self-limited diarrhea or no obvious symptoms (Mathis et al. 2005; Decraene et al. 2012). Asymptomatic carriage of this organism, as demonstrated by stool examination, has been observed in immune competent humans. It is not known if this infection can be latent and if relapses can occur with immune suppression, but latent infection is likely, as some reports have described chronic diarrhea in patients following solid organ transplantation and immune suppression (Rabodonirina et al. 1996; Guerard et al. 1999; Gumbo et al. 1999; Goetz et al. 2001).

Enterocytozoon bieneusi is also common in animals, with an ever-expanding host range. Natural asymptomatic infection with *Enterocytozoon bieneusi* was detected in swine, cattle, goats, rabbits, dogs, and cats (Deplazes et al. 1996; Breitenmoser et al. 1999; Del Aguila et al. 1999; Mathis et al. 1999a; Rinder et al. 2000; Dengjel et al. 2001; Buckholt et al. 2002; Lores et al. 2002; Fayer et al. 2003; Santín et al. 2004). Moreover, this microsporidium was described in simian immunodeficiency virus-infected macaques, beavers, foxes, otters, and raccoons (Mansfield et al. 1997; Dengjel et al. 2001; Green et al. 2004) (Table 12.11). Currently, it is not clear how *Enterocytozoon bieneusi* is transmitted between animals and humans, but

Table 12.10 Human *Enterocytozoon bieneusi* infections

Localization/ clinical signs	Immune status	Country	Reference
Asymptomatic	Immune competent	Slovakia	Halánová et al. (2013)
	Immunodeficient	Iran	Tavalla et al. (2017)
Diarrhea; Intestinal disorder	Immune deficient; transplant recipient; newborn; HIV	Australia; Cameroon; Congo; France; Germany; Haiti; Iran; Nigeria; Peru; Poland; Portugal; Russia; Spain; Thailand; Tunisia; USA	Rabodonirina et al. (1996), Liguory et al. (1998), Guerard et al. (1999), Gumbo et al. (1999), Goetz et al. (2001), Liguory et al. (2001), Sulaiman et al. (2003a), Bern et al. (2005), Sarfati et al. (2006), Raccurt et al. (2008), Pagornrat et al. (2009), Stark et al. (2009), Abdelmalek et al. (2011), Galvan et al. (2011), Sokolova et al. (2011), Akinbo et al. (2012, 2013), Chabchoub et al. (2012), Lobo et al. (2012), Wumba et al. (2012), Agholi et al. (2013a, b), Kicia et al. (2016), Karimi et al. (2020), Messaoud et al. (2021)
	Immune competent; not specified	Australia; China; Iran; Mozambique; Nigeria; Uganda	Tumwine et al. (2002), Ayinmode et al. (2011), Zhang et al. (2011, 2018e), Gong et al. (2019), Karimi et al. (2020), Muadica et al. (2020)
Respiratory tract	HIV/AIDS; transplant recipient	Chile; France; Poland; Spain	Del Aguila et al. (1997), Weitzel et al. (2001), Botterel et al. (2002), Tumwine et al. (2002), Sodqi et al. (2004), Kicia et al. (2014)
Urinary tract	HIV/AIDS; renal transplant recipient	Poland	Wesolowska et al. (2019)
Not specified	HIV; AIDS; immune deficient; transplant recipient	Australia; Cameroon; China; Gabon; Germany; Malawi; Netherlands; Nigeria; Poland; Thailand; UK; Vietnam	Rinder et al. (1997), Sadler et al. (2002), Leelayoova et al. (2006), Espern et al. (2007), Stark et al. (2009), ten Hove et al. (2009), Maikai et al. (2012), Bednarska et al. (2013), Wang et al. (2013)
	Immune competent; not specified	Czech Republic; Germany; Malawi; Myanmar; Netherlands; Thailand	Dengjel et al. (2001), ten Hove et al. (2009), Sak et al. (2011b), Prasertbun et al. (2019), Shen et al. (2020)

Table 12.11 Asymptomatic *Enterocytozoon bieneusi* infections in mammals

Animal species	Country	Reference
African lion (*Panthera leo*)	South Korea	Hwang et al. (2021)
Alashan ground squirrels (*Spermophilus alashanicus*)	China	Xu et al. (2020)
Alpaca (*Vicugna pacos*)	China	Ma et al. (2019)
Baboon (*Papio anubis*)	Kenya	Li et al. (2011)
Bat (*Rhinolophus ferrumequinum*; *Murina leucogaster*; *Miniopterus schreibersii*; *Eidolon helvum*)	Nigeria; South Korea	Lee et al. (2018), Li et al. (2019b)
Beach marten (Martes foina)	Spain	Santín et al. (2018)
Beaver (*Castor canadensis*)	USA	Sulaiman et al. (2003b)
Black bear (*Ursus americanus*; *Ursus thibetanus*)	China; USA	Guo et al. (2014), Wang et al. (2020b)
Black lemur (*Eulemur macaco flavifrons*)	Poland	Slodkowicz-Kowalska et al. (2007)
Camel (*Camelus bactrianus*; *Camelus dromedarius*)	Algeria; China	Baroudi et al. (2018), Qi et al. (2018)
Cat (*Felis catus*)	Australia; Brazil; Colombia; Czech Republic; Germany; Iran; Japan; Poland; Portugal; Slovakia; Spain; Switzerland; Turkey	Mathis et al. (1999a), Dengjel et al. (2001), Lobo et al. (2006), Santín et al. (2006), Abe et al. (2009), Jamshidi et al. (2012), Askari et al. (2015), Kváč et al. (2017), Dashti et al. (2019), Pekmezci et al. (2019), Prado et al. (2019), Zhang et al. (2019a), Vahedi et al. (2020)
Cattle (*Bos taurus*)	Argentina; Australia; China; Germany; Iran; Portugal; South Africa; South Korea; Thailand; Turkey; USA	Rinder et al. (2000), Dengjel et al. (2001), Fayer et al. (2003), Santín et al. (2004), Sulaiman et al. (2004), Santín et al. (2005), Lobo et al. (2006), Fayer et al. (2007), Lee (2007, 2008), Abu Samra et al. (2012), Fayer et al. (2012), Santín et al. (2012), Del Coco et al. (2014), Askari et al. (2015), Tang et al. (2018), Udonsom et al. (2019), Zhang et al. (2019b), Bilgin et al. (2020), Hwang et al. (2020), Tao et al. (2020), Yildirim et al. (2020b)
Chinchilla (*Chinchilla lanigera*)	China	Qi et al. (2015)
Chipmunk (*Tamias striatus*; *Eutamias asiaticus*)	China; USA	Guo et al. (2014), Deng et al. (2018)
Civet (*Paguma larvata*)	China	Yu et al. (2020)

(continued)

Table 12.11 (continued)

Animal species	Country	Reference
Deer (*Odocoileus virginianus*; *Cervus elaphus*; *Dama dama*; *Rusa unicolor*; *Cervus Nippon*; *Hydropotes inermis argyropus*; *Capreolus pygargus*; *Moschus berezovskii*; *Elaphurus davidianus*)	Australia; China; South Korea; USA	Guo et al. (2014), Santín and Fayer (2015), Zhao et al. (2017), Song et al. (2018), Zhang et al. (2018c), Amer et al. (2019), Xie et al. (2019), Tao et al. (2020)
Dog (*Canis lupus*)	Australia; China; Colombia; Iran; Japan; Portugal; Spain; Switzerland	Del Aguila et al. (1999), Mathis et al. (1999a), Lores et al. (2002), Lobo et al. (2006), Santín et al. (2008), Abe et al. (2009), Zhang et al. (2011), Jamshidi et al. (2012), Askari et al. (2015), Dashti et al. (2019), Delrobaei et al. (2019), Zhang et al. (2019a)
Donkey (*Equus asinus*)	Algeria; China	Laatamna et al. (2015), Li et al. (2020)
European badger (*Meles meles*)	Spain	Santín et al. (2018)
Fox (*Vulpes vulpes*, *Alopex lagopus*)	China; Spain; USA	Sulaiman et al. (2003b), Zhao et al. (2015), Santín et al. (2018), Ma et al. (2020), Zhang et al. (2021)
Giant panda (*Ailuropoda melanoleuca*)	China	Li et al. (2018)
Gibbon (*Nomascus leucogenys*)	China	Zhong et al. (2017a)
Goat (*Capra aegagrus hircus*)	China; Spain; Thailand	Lores et al. (2002), Udonsom et al. (2019), Zhou et al. (2019), Zhang et al. (2020)
Golden snub-nosed monkey (*Rhinopithecus roxellana*)	China	Karim et al. (2014), Yu et al. (2017)
Guinea pig (*Cavia porcellus*)	China; Peru	Cama et al. (2007), Wang et al. (2020a)
Hedgehog (*Erinaceus amurensis*)	China	Gong et al. (2021)
Himalayan marmot (*Marmota himalayana*)	China	Xu et al. (2020)
Horse (*Equus ferus*)	Algeria; China; Colombia; Czech Republic; Turkey	Santín et al. (2010), Wagnerová et al. (2012), Laatamna et al. (2015), Zhao et al. (2019), Li et al. (2020), Yildirim et al. (2020a)
Kangaroo (*Macropus Rufus*)	China	Zhong et al. (2017b)
Kudo (*Tragelaphus strepsiceros*)	Portugal	Lobo et al. (2006)

(continued)

Table 12.11 (continued)

Animal species	Country	Reference
Llama (*Lama glama*)	Germany	Dengjel et al. (2001)
Macaques (*Macaca mulatta*; *Macaca fascicularis*)	China; USA	Green et al. (2004), Ye et al. (2012), Karim et al. (2014), Ye et al. (2014), Zhong et al. (2017a), Chen et al. (2019), Zhao et al. (2020a)
Mink (*Neovison vison*)	China	Cong et al. (2018), Zhang et al. (2018b, 2021)
Mongoose lemur (*Eulemur mongoz*)	Poland	Slodkowicz-Kowalska et al. (2007)
Mountain gorilla (*Gorilla beringei beringei*)	Rwanda	Sak et al. (2014)
Mouse (*Mus musculus*; *Peromyscus* sp.; *Apodemus flavicollis*; *Apodemus agrarius*)	Czech Republic; Germany; Poland; Slovakia; USA	Sak et al. (2011c), Guo et al. (2014), Danišová et al. (2015), Perec-Matysiak et al. (2015)
Muskrat (*Ondatra zibethicus*)	USA	Sulaiman et al. (2003b), Guo et al. (2014)
Mustang (*Equus caballus*)	USA	Wagnerova et al. (2016)
Orangutan (*Pongo abelii, Pongo pygmaeus*)	Indonesia	Mynařová et al. (2016)
Porcupine (*Atherurus macrourus*)	China	Zhao et al. (2020b)
Rabbit (*Oryctolagus cuniculus*; *Sylvilagus floridanus*)	China; Iran; Spain; USA	Guo et al. (2014), Askari et al. (2015), Zhang et al. (2016), Deng et al. (2020b), Martinez-Padilla et al. (2020)
Raccoon (*Procyon lotor*)	Iran; USA	Sulaiman et al. (2003b), Guo et al. (2014), Javanmard et al. (2020)
Raccoon dog (*Nyctereutes procyonoides*)	China	Zhao et al. (2015), Zhang et al. (2021)
Rat (*Rattus norvegicus*; *Rhizomys sinensis*; *Rattus tanezumi*; *Leopoldamys edwardsi*; *Niviventer confucianus*; *Rattus andamanensis*; *Rattus losea*)	China	Wang et al. (2019, 2020a), Zhao et al. (2020b)
River otter (*Lontra canadensis*)	USA	Sulaiman et al. (2003b), Guo et al. (2014)
Sheep (*Ovis aries; Ovis ammon*)	China; Ethiopia; Iran; Sweden; Turkey	Stensvold et al. (2014), Askari et al. (2015), Ye et al. (2015), Zhang et al. (2018a), Peng et al. (2019), Wegayehu et al. (2020), Yildirim et al. (2020b), Zhang et al. (2020)

(continued)

Table 12.11 (continued)

Animal species	Country	Reference
Short-tailed weasel (*Mustela erminea*)	USA	Guo et al. (2014)
Squirrel (*Sciurus vulgaris*; *Sciurus carolinensis*; *Callosciueus erythraeus*)	China; USA	Guo et al. (2014), Deng et al. (2020a), Zhao et al. (2020b)
Swine (*Sus scrofa*)	China; Czech Republic; Germany; Japan; Malaysia; Slovakia; South Korea; Switzerland; Thailand; USA	Deplazes et al. (1996), Breitenmoser et al. (1999), Rinder et al. (2000), Dengjel et al. (2001), Buckholt et al. (2002), Jeong et al. (2007), Sak et al. (2008), Leelayoova et al. (2009), Reetz et al. (2009), Abe and Kimata (2010), Zhang et al. (2011), Li et al. (2014, 2019a), Udonsom et al. (2019), Valenčáková and Danišová (2019), Ruviniyia et al. (2020)
Visayan warty pig (*Sus cebifrons negrinus*)	Poland	Slodkowicz-Kowalska et al. (2007)
Vole (*Microtus pennsylvanicus*; *Myodes gapperi*; *Myodes glareolus*)	Poland; USA	Guo et al. (2014), Perec-Matysiak et al. (2015)
Water buffalo (*Bubalus bubalis*)	Turkey	Yildirim et al. (2020b)
White-headed langur (*Trachypithecus poliocephalus*)	China	Karim et al. (2014)
White-headed marmoset (*Callithrix geoffroyi*)	Portugal	Lobo et al. (2006)
Wild boar (*Sus scrofa*)	Austria; Czech Republic; Poland; Slovakia; Spain	Němejc et al. (2014), Dashti et al. (2020)
Yak (*Bos grunniens*)	China	Ma et al. (2017), Zhang et al. (2018a)

contaminated fomites, water, and food have been implicated as important vehicles of *E. bieneusi* transmission (Decraene et al. 2012; Guo et al. 2014).

Molecular studies have resulted in the identification of phenotypic and/or genetic variability within *Enterocytozoon bieneusi* enabling researchers to address the issue of its zoonotic potential (Chap. 14). Sequencing of the ribosomal RNA internal transcribed spacer (ITS) element is the standard method for genotyping (Katzwinkel-Wladarsch et al. 1996; Mansfield et al. 1997; Tzipori et al. 1997). Molecular characterizations of *Enterocytozoon bieneusi* at ITS locus have identified nearly 500 genotypes, some of them only based on single sequence, in 11 phylogenetic groups with different host ranges have been established using different and partially

overlapping nomenclatures (Guo et al. 2014; Zhang et al. 2018d; Li et al. 2019c). Groups 1 and 2 include most potential zoonotic genotypes, while groups 3–11 contain mostly host-specific genotypes (Li et al. 2019c; Li and Xiao 2019). Some nomenclatures have been used in single studies only and many studies have examined only certain carrier species, most of them domestic animals or humans.

Summation

The mystery of subclinical microsporidiosis mostly in immune competent individuals is complicated by the observation that gross routine examination commonly fails to demonstrate microsporidia as these organisms are sparsely distributed, likely to be disseminated throughout multiple organs, and are hard to identify by light microscopy in tissue sections. Diagnosis rests primarily upon providence in many cases, when a doctor specifically requests microsporidian diagnostic testing, however, such tests are not routinely available to the majority of clinical diagnostic laboratories. Moreover, the non-specificity of symptoms that can be associated with a wide range of conditions often makes the diagnosis of these pathogens difficult.

As is apparent from the above lists, microsporidian parasites have caused diverse diseases in a wide range of mammalian hosts. Although some host species preferences can be observed in some genotypes of *Encephalitozoon* spp. and *Enterocytozoon bieneusi*, microsporidia belonging to these genera are truly host-specific. The potential clinical manifestation of symptoms does not depend only on microsporidian species/genotype involved, but mainly on the host immune status and the host species affected. While *Enterocytozoon bieneusi* causes mostly intestinal infection, *Encephalitozoon* spp., especially *Encephalitozoon cuniculi*, tend to disseminate and the rarely detected species of microsporidia cause superficial infections. It is suggested that during life, everyone has many opportunities to come into contact with microsporidia. Most infected individuals do not become ill and may appear clinically healthy for many years and microsporidia can, in some cases, act as commensal organisms until the balance of the immune system is disrupted, as demonstrated by the inexhaustible amount of serological or molecular studies revealing a worldwide range of exposed asymptomatic mammalian species. When there is a weakening of a balanced host–parasite interface due to immune dysregulation (as occurs in AIDS treatment with immune suppressive drugs, or due to other immune suppressive diseases, or exposure to environmental immune suppressive agents) symptomatic microsporidiosis can develop. Surprisingly, some animal species tend to be more susceptible to infection and to develop specific clinical signs more frequently.

Microsporidia are not motile and short distance dispersal in the host is limited to a unique mechanism of invasion of host cells that involves a highly specialized structure, the 10–50 μm long polar tube, which is responsible for the delivery of this organism to the host cell. However, Encephalitozoonidae are able to replicate and produce mature spores in resident and trafficking macrophages. Such immune cells can be attracted to another part of the host body by chemokines produced in response to other immune response, such as inflammation, bacterial or viral infection and can serve as vehicles for microsporidia dissemination. The infection can thus

spread throughout the host's body in a few days or weeks and reach destinations that are significant distances from the primary point of entry into the host's body. However, we cannot discriminate if the presence of microsporidia, especially *Encephalitozoon* spp. in an infection site is the primary cause of a manifesting problem or if microsporidia were transferred to a place secondarily due to an immune response induced by another primary stimulus and like true opportunistic parasites, they simply seize the opportunity and worsen the course of an existing disease.

Microsporidia represent an enigmatic group of parasites that have surprised the scientific community around the world with their capabilities and flexibility for decades. Although we already know a lot about them, there are still many questions that need to be answered to understand the complexity of their biology.

Compliance with Ethical Standards

Conflict of Interest: The authors declare that there is no conflict of interest.

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Ethical Approval: The chapter is a review of previously published accounts, as such, no animal or human studies were performed.

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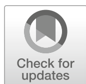
Chapter 13
Immune Response to Microsporidia



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Abstract Microsporidia are a group of pathogens, which can pose severe risks to the immunocompromised population, such as HIV-infected individuals or organ transplant recipients. Adaptive immunity has been reported to be critical for protection, and mice depleted of T cells are unable to control these infections. In a mouse model of infection, CD8 T cells have been found to be the primary effector cells and are responsible for protecting the infected host. Also, as infection is acquired via a peroral route, CD8 T cells in the gut compartment act as a first line of defense against these pathogens. Thus, generation of a robust CD8 T-cell response exhibiting polyfunctional ability is critical for host survival. In this chapter, we describe the effector CD8 T cells generated during microsporidia infection and the factors that may be essential for generating protective immunity against these understudied but significant pathogens. Overall, this chapter will highlight the necessity for a better understanding of the development of CD8 T-cell responses in gut-associated lymphoid tissue (GALT) and provide some insights into therapies that may be used to restore defective CD8 T-cell functionality in an immunocompromised situation.

Keywords Microsporidia · Innate immunity · Cell-mediated immunity · CD8 T cells · CD4 T cells · γδ T cells · IL12 · IL21 · IFNγ

13.1 Introduction

Recognized by Pasteur in 1870, microsporidia are infective to numerous species of animals, both vertebrates and invertebrates, including humans (Smith 1999) and remain mostly understudied parasites. This group of pathogens in a normal host usually is asymptomatic but often causes severe disease in immunocompromised hosts, such as patients with HIV infection (Didier and Weiss 2011). As far as a protective immune response is concerned, very limited studies are available, and the

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majority of reports are related to *Encephalitozoon cuniculi*, a microsporidian pathogen which can be cultured in the laboratory and also can cause severe symptoms in humans.

The role of cell-mediated immunity in protection against microsporidiosis was identified early on in studies of these pathogens as mice lacking T cells succumbed to infection (Didier et al. 1994; Hermanek et al. 1993). A few years later, it was reported that among the T-cell subsets, CD8 T cells play a primary effector role against these pathogens (Braunfuchsova et al. 2001; Khan and Moretto 1999). An important finding was the essential role of CD8-mediated cytotoxic response in these infections, something that is not usual for a nonviral pathogen (Khan et al. 1999). These observations were established using mice that lacked the perforin gene, and these animals exhibited increased susceptibility to the infectious challenge and a decreased ability to control the infection (Khan et al. 1999; Moretto et al. 2007). Interestingly, studies have indicated that the role of CD4 T cells, the other major T-cell subset, during *Encephalitozoon cuniculi* challenge was highly dependent on the route of infection (intraperitoneal or oral). When challenged via an intraperitoneal route, CD8-mediated protection in the absence of conventional CD4 T cells was unaffected (Khan et al. 1999; Moretto et al. 2000); however, this subset played a more synergistic role in mice with peroral infection (Moretto et al. 2004). CD8 T-cell depletion alone results in mortality during intraperitoneal infection (Khan et al. 1999), while a combinatorial role of both CD4 and CD8 T cells was required during peroral infection (Moretto et al. 2004). Moreover, recent studies in our laboratory have demonstrated that inflammatory cytokine responses observed to be induced during intraperitoneal infection are significantly lower when mice receive microsporidia via the oral (i.e., natural) route (Moretto and Khan unpublished). The oral infection model with microsporidia in mice can provide important insights into the development of CD8 T-cell immunity against other oral pathogens that fail to evoke an inflammatory response. All-inclusive factors responsible for the development of CD8 T-cell immunity against microsporidia are fundamental to the understanding of protective immunity.

13.2 Microsporidia

Microsporidia are spore-forming parasites that have been categorized with fungi, and a wide range of hosts exhibit susceptibility to these pathogens. They are present everywhere, and among the approximately 1400 known species, 14 have been attributed to human infections (Didier and Weiss 2011). Among these species identified as a cause of human infections are *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, and *Encephalitozoon cuniculi*. Infection is acquired primarily via an oral route (Didier and Weiss 2011) either by food or exposure to contaminated water (e.g., drinking water, wastewater, and recreational water) (Izquierdo et al. 2011). There is an example of an extensive outbreak of waterborne infection due to a lake contamination (Cotte et al. 1999), and

recently a food-borne outbreak linked to microsporidial infection has been reported (Decraene et al. 2012). Due to these risks, microsporidia are included in both the Drinking Water Contaminant Candidate List of the US Environmental Protection Agency (EPA) (http://www2.epa.gov/ccl/contaminant-candidate-list-3-ccl-3) and the Category B Priority Pathogens List of the Biodefense and Emerging Infectious Diseases from the National Institute of Allergy and Infectious Diseases (http://www.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/Pages/CatA.aspx).

The spread of microsporidia infection can be species-dependent, and the pathogen can be localized or disseminated leading to a wide variety of symptoms such as diarrhea, hepatitis, encephalitis, nephritis, and keratoconjunctivitis (Didier and Weiss 2006). Microsporidia mostly cause problems in immunosuppressed patients such as those suffering from HIV infection (Didier 2005). The range of infection among HIV patients in various countries (e.g., Thailand, Russia, Venezuela, etc.) has varied from 13 to 80%, and infection continues to be reported despite the availability of antiretroviral therapy (Chacin-Bonilla et al. 2006; Sokolova et al. 2011; Viriyavejakul et al. 2009). In addition to those individuals suffering from HIV infection, in the recent past, there has been an increased occurrence of microsporidiosis in people receiving solid organ transplants (Hocevar et al. 2014; Kicia et al. 2014; Ladapo et al. 2014). Microsporidiosis has also been described in travelers, children, and the elderly (Muller et al. 2001; Lores et al. 2002; Halanova et al. 2013). The diagnosis of these pathogens is hampered by their small size, and disease often can remain undiagnosed. However, in recent years due to enhanced awareness and improved methodology for detection, the number of cases of microsporidiosis has increased as compared to earlier estimations.

Screenings of populations for the prevalence of microsporidiosis have revealed some interesting findings. In studies conducted with healthy children in Slovakia, it was observed that 30% of the children tested were passing microsporidia spores in their stools (Halanova et al. 2013). In another study conducted in Japan, serological studies showed the presence of microsporidian-specific IgM in 30% of healthy subjects (Omura et al. 2007). It is notable, in that study, that serum IgM levels in the adult population were significantly lower compared to individuals who were 20 years lower in age. It has been recently reported that continuous exposure to various animals increases the risk of acquiring microsporidiosis (Sak et al. 2011). This is based on the observation that 14 out of 15 individuals with this type of animal exposure exhibited antibodies to microsporidia, although none of them had clinical symptoms.

It had been thought that microsporidiosis could be a chronic infection and evidence for the presence of chronic persistent infection has recently emerged. The treatment of mice carrying non-detectable levels of microsporidia with corticosteroids led to reactivation of latent infection and its spread to various tissues (Kotkova et al. 2013). These findings are consistent with earlier reports of frequent relapses of infection occurring in patients with AIDS who discontinued anti-microsporidian therapy once their acute infection resolved (Carr et al. 1998; Molina et al. 1998). It has also been suggested that chemotherapeutic treatment in cancer patients can precipitate intestinal microsporidiosis due to the immunosuppressive effects of

these drugs. However, in one study, spores were detected in 21.9% of the stool samples from cancer patients, but interestingly no difference was seen in the percentage of infected patient between those receiving cancer treatment and newly diagnosed patients who had yet to begin cancer treatment (Lono et al. 2008).

13.3 Innate Immune Response Against Microsporidia

Although most of the microsporidia species except *Enterocytozoon bieneusi* that are pathogenic to humans can be maintained in cell culture, the majority of immunological studies conducted in an animal model have been conducted with *Encephalitozoon cuniculi*. Until the development of adaptive immunity in the infected host, innate immune mechanisms maintain the first line of defense and are essential to control the spread of infection (Beutler 2004; Buchmann 2014). Although adaptive immunity is critical for host protection against microsporidia infection (Ghosh and Weiss 2012), innate immune cells, including macrophages, dendritic cells, and to a certain extent NK cells, all play an important role in protective immunity against these pathogens (Mathews et al. 2009). Reactive nitrogen species, reactive oxygen species, and molecules secreted by macrophages are important in controlling *Encephalitozoon cuniculi* infection (Didier 1995). Although mice lacking these molecules are able to survive the challenge, they bear a higher pathogen burden in their peritoneum (Didier et al. 2010). In addition, the importance of innate immune responses in priming adaptive immunity against *Encephalitozoon cuniculi* should not be excluded.

Another immune cell type which plays a fundamental role in priming naïve T cells is dendritic cells (DC). In addition to macrophages, DC are critically important in the control of invading intracellular pathogens (Guermonprez et al. 2002). The importance of IFNγ producing DC in the elicitation of the gut immune response against oral *Encephalitozoon cuniculi* infection has been clearly demonstrated (Guermonprez et al. 2002). Murine DC lacking IFN-γ failed to induce an intraepithelial lymphocyte (IEL) response and led to ineffective suppression of the infection (Moretto et al. 2007, 2012). Similarly, IL-12, another important cytokine produced primarily by DC (Heufler et al. 1996), has been reported to be critical for survival against intraperitoneal challenge (Khan et al. 1999), and an inability to produce this cytokine led to the development of a suboptimal CD8 T-cell response in mice with *Encephalitozoon cuniculi* infection (Moretto et al. 2010). Studies conducted in our laboratory have also revealed that IL-12 is induced very early during *Encephalitozoon cuniculi* infection, underlining its importance in the initiation of innate immunity (Lawlor et al. 2010). In these studies, it was also noted that DC activation is dependent on TLR4, a molecule responsible for the upregulation of costimulatory molecules such as CD80, CD86, and MHC class II, which are needed for the induction of CD8 T-cell immunity. NK cells are an important component of the innate immune defense (Vivier et al. 2008), but are not highly essential for protection against *Encephalitozoon cuniculi* infection. Increased NK cell activity in

the murine model *Encephalitozoon cuniculi* infection was observed (Niederkorn et al. 1983), but the response was not maintained during later stages of infection (Khan et al. 1999).

13.4 T-Cell Response to Microsporidia Infection

T cells play a predominant role in the immune protection against microsporidiosis (Schmidt and Shadduck 1983). Early studies conducted with T-cell-deficient animals demonstrated their susceptibility to *Encephalitozoon cuniculi* infection (Didier et al. 1994; Hermanek et al. 1993) and adoptive transfer of immune T cells to susceptible animals protected them from an infection which was otherwise lethal (Schmidt and Shadduck 1984). In this scenario, the role of humoral immunity can be discounted as transfer of hyper-immune sera failed to confer protection in recipient mice. In subsequent years, we have demonstrated that among the T-cell subsets, CD8 T cells play a primary role in protective immunity as CD8 knockout mice were unable to withstand intraperitoneal infection (Khan et al. 1999). Furthermore, the transfer of immune CD8 T cells to immunocompromised mice led to their protection, suggesting that protection against intraperitoneal challenge is linked to these cells (Braunfuchsova et al. 2001, 2002; Khan and Moretto 1999). In contrast, mice lacking the CD4 T-cell compartment were able to resist high challenge doses of microsporidia (Moretto et al. 2000), suggesting a limited role for these cells in controlling intraperitoneal *Encephalitozoon cuniculi* infection. Surprisingly, a lack of CD4 T cells did not compromise the CD8 T-cell immune response against intraperitoneal *E. cuniculi* infection discounting its role in any helper activity (Moretto et al. 2000). An intriguing observation was that mice lacking the TCR γδ subset developed compromised CD8 T-cell immunity and their ability to control an intraperitoneal infection with *Encephalitozoon cuniculi* was significantly diminished (Moretto et al. 2001). Furthermore, immune CD8 T cells isolated from mice lacking γδ T cells lost their ability to transfer protection to susceptible animals, unlike wild-type donors. These studies identified a previously undescribed helper potential for γδ T cells against intracellular infection. A helper role for γδ T cells in stimulating CD8 T-cell immunity has also been reported in Epstein-Barr virus human infection (Landmeier et al. 2009). In these studies, autologous γδ T cells facilitated the expansion of peptide-specific CD8 T-cell effectors. These findings have strong therapeutic implications for the management of microsporidiosis in HIV-infected patients where their CD8 response due to the lack of robust CD4 T-cell immunity is severely compromised.

Encephalitozoon cuniculi infection generates strong antigen-specific CD8 T-cell effectors in normal immunocompetent hosts, which according to earlier nomenclature were referred to as short-lived effector CD8 T cells (SLEC). These terminal effector CD8 T cells are identified by their KLRG1 expression, which is not present in the memory T-cell population. During recent years, the ability of T cells to conduct multiple functions (referred to as polyfunctionality) has been described as

one of the hallmarks of vigorous protective immunity against intracellular pathogens, especially in viral infections (Betts et al. 2006; Precopio et al. 2007; Seder et al. 2008). Studies on this topic were conducted in our laboratory, and we observed that the CD8 terminal effector population exhibited polyfunctional characteristics during acute *Encephalitozoon cuniculi* infection as shown by their ability to exhibit simultaneous upregulation of granzyme B, IFNγ, and TNFα in response to antigenic stimulation, underlining their importance in controlling the infection (Bhadra et al. 2014). It is very interesting to note that CD8 T-cell cytolytic ability is a key function for protection against *Encephalitozoon cuniculi* infection, since mice lacking perforin gene succumb to infection due to the accumulation of high pathogen load (Khan and Moretto 1999; Khan et al. 1999; Moretto et al. 2007). Nevertheless, besides the cytotoxic ability of CD8 T cells, cytokine production by T cells also contributes to protection against microsporidia infection (Khan and Moretto 1999; Achbarou et al. 1996; Braunfuchsova et al. 1999).

13.5 Gut CD8 T-Cell Response Against *E. cuniculi* Infection

Almost all earlier studies conducted on murine models of *Encephalitozoon cuniculi* infection, including those mentioned above, were performed using an intraperitoneal route of infection. As microspordial infection is normally acquired via oral route, the intestine and the gut-associated lymphoid tissue (GALT) are essential components of the host immune defense, protecting the body from foreign antigens and pathogens (Ruth and Field 2013). Therefore, it is highly essential to evaluate oral infection and the gut immune response elicited against these groups of pathogens to determine its role in combating infection.

Interestingly, unlike what was observed with intraperitoneal infection, with oral infection, the intestinal CD4 population played an important synergetic role in combination with CD8 T cells, and mortality of the animals as a result of infection was observed only when both the subsets were subjected to antibody-mediated depletion (Moretto et al. 2004; Salat et al. 2002). These findings were corroborated by other studies in which adoptive transfer of either CD4 or CD8 T-cell subset alone led to the survival of SCID mice against another microsporidia species, *Encephalitozoon intestinalis* (Salat et al. 2002). Conversely, immunocompromised mice were unable to control the infection when administered splenocytes depleted of both CD4 and CD8 T-cell subsets.

Gut immune responses can play a critical role in protection against pathogens acquired via oral route, and intraepithelial lymphocytes (IEL) represent one of the first lines of defense against gut pathogens and are an important component of the GALT. This lymphoid tissue is primarily comprised predominantly of CD8 T cells (CD8αα and CD8αβ) and a minor CD4 population, i.e., IEL are a heterogeneous population (Hayday et al. 2001). Studies conducted in our laboratory demonstrated that CD8 IEL localized in the lining of the gut were recruited very early during *Encephalitozoon cuniculi* infection and displayed strong polyfunctional properties

against pathogen-infected targets (Moretto et al. 2004). The antigen specificity of the IEL population generated in response to *Encephalitozoon cuniculi* infection was confirmed by their ability to secrete IFNγ, to exhibit cytotoxic effect against infected targets, and to confer partial protection to immunosuppressed host against a lethal challenge (Moretto et al. 2004). Among the CD8 subsets, the CD8αβ IEL were able to partially control microsporidial infection after adoptive transfer to an immunocompromised host (Moretto et al. 2007). Similar to splenic CD8 T cells, CD8αβ IEL were able to upregulate several cytotoxic responses (perforin, CD95L, and granzyme B) at early stages of infection. Additionally, protection against oral infection was predominantly dependent on perforin production, as IEL from perforin knockout mice failed to protect immunocompromised animals against a lethal challenge infection with microsporidia (Moretto et al. 2007). It has been reported that gut flora played an essential role in the maintenance of a robust gut immune response to *Encephalitozoon cuniculi* via engagement of the toll-like receptor 9 (TLR9) (Hall et al. 2008). Furthermore, mice lacking the TLR9 gene displayed increased frequencies of $CD4^{+}Foxp3^{+}$ regulatory T cells which play a well-described role in limiting the immune responses against infectious agents (Vignali et al. 2008). Understanding the role of this cell population in limiting the gut immune response against microsporidia, especially in the development and maintenance of IEL immunity in this tissue, will be very interesting.

Reports recently emerged that suggest that microsporidial infection can subsist in a latent form (Kotkova et al. 2013; Stentiford et al. 2016) that puts an elderly population at increased risk. In this regard, experimental studies performed in our laboratory have demonstrated that older mice exhibit increased susceptibility which can be attributed to a suboptimal mucosal CD8 T-cell response (Moretto et al. 2008). T cells from isolated mesenteric lymph nodes (MLNs) from 9-month-old mice displayed lower proliferative and cytotoxic capabilities as compared to younger animals. Moreover, unlike the cells from younger mice, T cells isolated from the infected older animals were unable to transfer protection to SCID mice. The defect in the mucosal T-cell immunity in the older animals was linked to suboptimal dendritic cell functionality. Unlike their splenic counterparts, in response to antigenic stimulation, intestinal DC failed to upregulate IL-12 expression or exhibit increased levels of costimulatory molecules (Moretto et al. 2008). In a subsequent study, aged animals displayed a severe defect in the generation of effector CD8 T-cell responses, and with increasing age, they lose their polyfunctional activity (Bhadra et al. 2014). Remarkably, the defect in the effector CD8 T-cell response in the aged animals is not cell-intrinsic as adoptive transfer of effector CD8 T cells from aged mice exhibits normal polyfunctional ability when transferred to younger recipients.

13.6 CD8 T-Cell Immunity Against *Encephalitozoon cuniculi: The Role of Cytokines*

The role of Th1 cytokines in controlling the spread of *Encephalitozoon cuniculi* infection is well documented, and the Th1 signature cytokine IFNγ is important for immune-protection against the pathogen. It has been convincingly demonstrated that both knockout mice and antibody depleted wild-type animals succumb to infection with microsporidia, regardless of the route of challenge (Khan and Moretto 1999; Moretto et al. 2007; Salat et al. 2004). Infection with *Encephalitozoon cuniculi* induces IFNγ producing CD4 and CD8 T cells both at systemic and local levels (Moretto et al. 2000, 2004). This cytokine was detected in the serum and splenic T-cell subsets around day 14 postinfection, which corresponds to the peak of the effector CD8 T-cell response (Khan and Moretto 1999; Braunfuchsova et al. 1999). IFNγ has been reported to be critical for the generation of robust CD8 T-cell immunity, and blockade of this cytokine neutralizes its beneficial effect on infection (Moretto et al. 2010). As was expected, when CD8 T cells from IFNγ-deficient mice are transferred adoptively to susceptible animals, they fail to protect mice from a lethal challenge with microsporidia. Interestingly, when animals are infected via an intraperitoneal route, the protective role of the CD8 T-cell subset was dependent on the ability of γδ T cells to produce IFNγ (Moretto et al. 2001). The importance of producing this cytokine was conclusively proven as administration of recombinant INFγ to γδ TCR-deficient animals protected them by restoring CD8 T-cell immunity against microsporidia.

Dendritic cells isolated from IFNγ knockout mice exhibited functional defects, and they were unable to upregulate IL-12 in response to *Encephalitozoon cuniculi* stimulation (Moretto et al. 2007). In vitro studies demonstrated that CD8 IEL cultured with DC from IFNγ knockout animals exhibited poor functional abilities and were unable to express the homing receptors needed for relocation to the gut mucosal sites. Moreover, IEL isolated from IFNγ-deficient infected animals failed to confer protection against *Encephalitozoon cuniculi* infection when adoptively transferred to immunocompromised animals (Moretto et al. 2007).

IL-2, an initiator of Th1 cytokine response, is essential for upregulation of T-bet, a transcription factor needed for IFNγ production, and therefore plays an essential role in the development of the CD8 T-cell response (Szabo et al. 2000). Intriguingly, the importance of this cytokine in *Encephalitozoon cuniculi* infection is dependent on the route of infection. In the case of intraperitoneal infection, IL-12p40 knockout mice exhibited severe susceptibility to intraperitoneal infection (Khan and Moretto 1999; Salat et al. 2004), and these animals displayed serious defects in expansion of the CD8 T-cell response (Moretto et al. 2010). Addition of exogenous IL-12 corrected these defects, but surprisingly the mechanism of restoration of CD8 T-cell immunity was independent of IFNγ, since exogenous treatment with the cytokine did not alter the deficiency. In contrast, IL-12 knockout animals challenged perorally with either *Encephalitozoon cuniculi* or *Encephalitozoon intestinalis* (Salat et al. 2004) survived the infectious challenge undermining the importance of this

cytokine against the natural oral route of infection. Based on these findings, the possibility of an unknown third signal cytokine playing a role in priming of the CD8 T-cell response cannot be completely ruled out.

Studies conducted in our laboratory have established the immunosuppressive role of TGFβ, another cytokine of high interest in an aging model of *Encephalitozoon cuniculi* infection (Bhadra et al. 2014). The loss of CD8 T-cell polyfunctionality in response to *Encephalitozoon cuniculi* infection in older animals could be attributed to highly elevated levels of TGFβ1, as cell-intrinsic blockade of this cytokine signaling cascade restored function in aged animals to the levels observed in young mice. Interestingly, the production of TGFβ in *Encephalitozoon cuniculi*-infected animals was independent of T cells, and treatment with anti-CD3 antibodies did not alter the levels of this cytokine. Obviously, the source of TGFβ during *Encephalitozoon cuniculi* infection needs to be identified so that they can be targeted to enhance the protective immunity against the pathogen.

13.7 Role of CD4 Help in the Development of CD8 T-Cell Immunity Against *Encephalitozoon cuniculi* Infection

Although the role of CD4 T cells is considered critical in the development and maintenance of CD8 T-cell immunity (Laidlaw et al. 2016), the role of this subset in relation to infection with microsporidia has not been described. Indirect evidence about the importance of CD4 T cells in the development of CD8 T-cell immunity is provided by the finding that depletion of both CD4 and CD8 T-cell subsets is necessary for triggering susceptibility to the oral *Encephalitozoon cuniculi* infection (Moretto et al. 2004). The importance of the CD4 T-cell subset in protective immunity against microsporidia is strengthened by unpublished observations from our laboratory that demonstrate a downregulated CD8 T-cell response in the gut of wild-type animals depleted of CD4 T cells (Khan I and Harrow D, unpublished data). This defect was characterized by lower cytotoxicity and IFNγ expression by CD8 T cells in the gastrointestinal tissue.

Undoubtedly, CD4 T cells provide much needed help for the elicitation of CD8 T-cell immunity in several infectious models; however, the precise nature of this help is still debated. One of the important mechanisms that describes the helper role of CD4 T cells is that of naïve CD4 T cells differentiating into Th1 cells upon antigenic stimulation, which have the ability to secrete a wide array of cytokines. These various cytokines, in turn, induce the differentiation and expansion of CD8 T cell and enhance the CD8 T-cell cytolytic ability and survivability (Swain et al. 2012). In addition to their ability to produce cytokines, CD4 T cells can be involved in the "licensing" of antigen-presenting cells via CD40-CD40L interactions, which increase their ability to deliver costimulatory signals during the priming of the CD8 T-cell response (Smith et al. 2004). CD4+ T cells from *Encephalitozoon cuniculi*-infected animals secrete IFNγ in response to antigenic stimulation (Moretto et al.

2000), and this cytokine has been shown to be essential for host protection (Khan and Moretto 1999; Salat et al. 2004). Interestingly, although CD4 T cells are also known to produce IL-2, a key cytokine linked to CD8 T-cell activation and expansion (Malek and Castro 2010), this cytokine was undetectable for at least the first 30 days following oral infection (Khan I, unpublished data).

CD4 T cells can also produce IL-21, a member of the common gamma chain family that is closely related to IL-2 (Parrish-Novak et al. 2000). Extensive studies carried out with the lymphocytic choriomeningitis virus (LCV) model have demonstrated that in the absence of IL-21 signaling an initial CD8 T-cell response can be generated; however, this response subsequently crashes as antiviral CD8 T cells begin to lose their effector capabilities (Elsaesser et al. 2009; Frohlich et al. 2009; Yi et al. 2009). Our laboratory has observed an elevated IL-21 response coincides with the peak of the effector CD8 T-cell immunity generated to *Encephalitozoon cuniculi* infection and that absence of signaling for this cytokine led to an impaired CD8 T-cell immunity (Moretto and Khan 2016). These findings are supported by another study that demonstrated that under IL-2 deprivation conditions, IL-21 might act as the major survival factor promoting the T-cell immune response (Khattar et al. 2014). In addition, it has been reported that IL-21 can synergize with two other common gamma chain members, IL-7 and IL-15, in order to promote expansion and in some cases antitumor functions of CD8 T cells (Liu et al. 2007; Nguyen and Weng 2010). However, similar to IL-2, these cytokines were not detected after *Encephalitozoon cuniculi* infection (Moretto M. unpublished data). Interestingly, during oral microsporidia infection, IL-21 was critical for the generation of an optimal effector CD8 T-cell immunity. IL-21 expression peaks during acute infection and is associated with an elevated IL-21^{+} CD4 T subset which exhibits a phenotypic resemblance to T follicular helper cells. The effector KLRG1^{+} CD8 response was sharply decreased in IL-21R knockout mice, as these cells exhibited reduced functional properties, although they retained the ability to proliferate (Fig. 13.1). IL-21 played a cell-intrinsic role in the generation of effector CD8 T cells, as stronger defects were observed in the IL-21-deficient compartment from the bone marrow chimeric mice (IL-21R knockout/wild-type). These findings are noticeably different from those reported in viral infections where IL-21 has been primarily associated with the generation and maintenance of CD8 memory response. The ability of IL-21 to induce a CD8 effector response during oral *Encephalitozoon cuniculi* infection may be linked to the recently described role of the master transcription factor T-bet in the development of IL-21 producing follicular T-helper cell response (Wang et al. 2019). The IL-21-mediated CD8 T-cell effector response may also be attributed to its ability to upregulate this transcription factor in CD8 T cells which, in turn, differentiate into cytotoxic cells (Sutherland et al. 2013). Additionally, the role of IL-21 in increased expression of BLIMP1, a transcription factor involved in CD8 T-cell terminal differentiation, may also contribute to the development of CD8 effector response against the pathogen (Kwon et al. 2009). Although our studies established a role for IL-21 in the development of CD8 T-cell effectors (Moretto and Khan 2016), the mechanisms involved need to be further elucidated, and the role of this cytokine in the development of polyfunctional

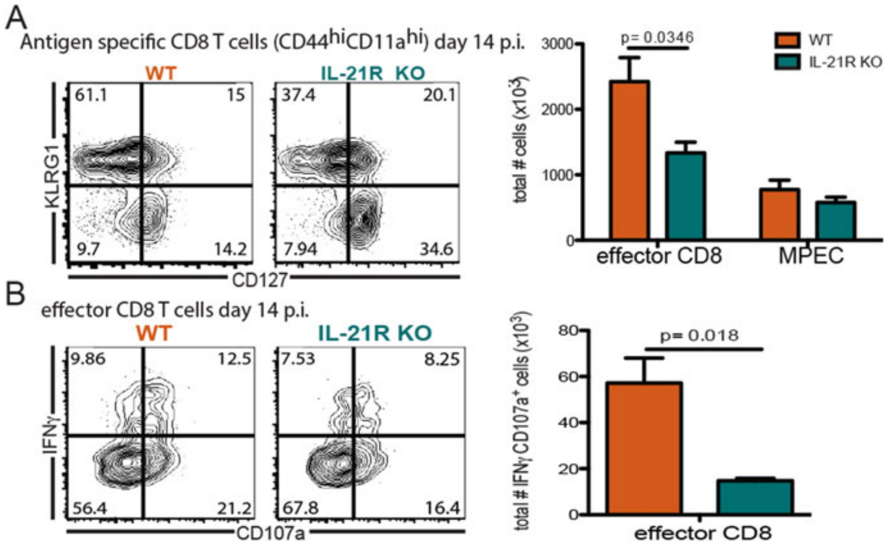

Fig. 13.1 Impaired effector CD8 T-cell response in absence of IL-21 signaling. (**a**) Effector CD8 T cells ($KLRG1^+CD127^-$) and MPEC ($KLRG1^-CD127^+$) among antigen-specific ($CD44^{hi}CD11a^{hi}$) CD8 T cells from the spleen of C57BL/J and IL-21R KO mice at day 14 postoral infection. (**b**) Polyfunctionality ($IFNgCD107a^+$) of effector CD8 T cells from C57BL/J and IL-21R KO mice at day 14 postoral infection

effector CD8 T-cell immunity both in the peripheral and gut compartment also needs to be investigated.

Although IL-21 induces an adequate CD8 T-cell effector immunity which can control oral infection due to microsporidia, the long-term response does not seem as robust as the one developed after an intraperitoneal infection with microsporidia (Moretto M and Khan I, unpublished observations). Based on our preliminary studies, it can be hypothesized that the CD8 effector immunity generated in response to IL-21 is slow to contract which may delay the development of a strong protective CD8 T-cell memory. This defect could be attributed to the lack of IL-2 during oral infection (Moretto and Khan 2016), as this cytokine is believed to be critical for the generation of a strong CD8 T-cell memory response (Kaech and Ahmed 2001) (Fig. 13.2). It will be both interesting and important to determine if exogenous treatment with IL-2 can lead to the generation of an optimal CD8 memory against this oral pathogen.

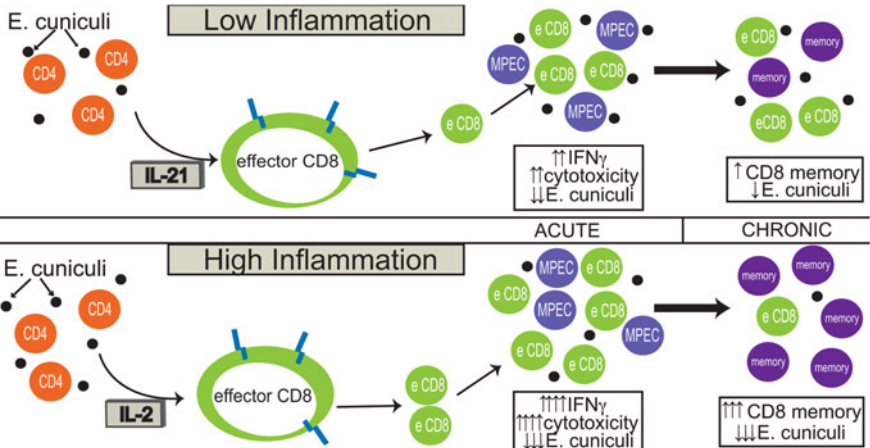

Fig. 13.2 Immune response in oral and intraperitoneal *Encephalitozoon cuniculi* infection. In oral (i.e., natural) *Encephalitozoon cuniculi* infection (low inflammatory environment), IL-21-mediated effector CD8 T-cell response leads to suboptimal memory CD8 T-cell development and inefficient control of the pathogen. In comparison, experimental intraperitoneal infection induces a high inflammation environment characterized by IL-2 production which results in robust effector CD8 T-cell response, strong CD8 T-cell memory development, and a well-controlled chronic infection

13.8 Conclusions and Future Perspectives

Based on the current information, we believe that natural/oral *Encephalitozoon cuniculi* infection induces a low inflammatory response characterized by early IL-21 production. IL-21 consecutively induces a suboptimal CD8 effector immunity which is able to control the primary infection, although it is not sufficient to clear the pathogen leading to a chronic situation. The chronic persistence of the pathogen in the host impairs the contraction of effector response which, in turn, causes a delay in the development of memory CD8 T-cell immunity. The available literature so far establishes that CD8 T-cell effectors act as essential frontline soldiers that are responsible to keep the microsporidial infection under control and prevent its spread to other tissues. Apparently, due to their ability to produce a wide variety of cytokines, CD4 T cells must be playing an important role in the development, expansion, and maintenance of CD8 T-cell immunity against microsporidia. In an immunocompromised situation (e.g., advanced HIV infection) when the number of CD4 T cells is diminished, the CD8 T-cell population will take a serious hit leading to reactivation and dissemination of the pathogen. Studies related to the nature of CD4 T-cell help required for the development and maintenance of CD8 immunity against this significant group of opportunistic pathogens are critical for the development of future immunotherapeutic agents for the management of microsporidiosis.

Compliance with Ethical Standards 1. **Conflict of Interest**: The authors declare that there is no conflict of interest.

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3. **Ethical Approval**: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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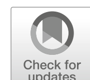
Chapter 14
A Perspective on the Molecular Identification, Classification, and Epidemiology of *Enterocytozoon bieneusi* of Animals



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Abstract The microsporidian *Enterocytozoon bieneusi* is an obligate intracellular pathogen that causes enteric disease (microsporidiosis) in humans and has been recorded in a wide range of animal species worldwide. The transmission of *E. bieneusi* is direct and likely occurs from person to person and from animal to person via the ingestion of spores in water, food, or the environment. The identification of *E. bieneusi* is usually accomplished by molecular means, typically using the sequence of the internal transcribed spacer (*ITS*) region of nuclear ribosomal DNA. Currently, ~820 distinct genotypes of *E. bieneusi* have been recorded in at least 210 species of vertebrates (mammals, birds, reptiles, and amphibians) or invertebrates (insects and mussels) in more than 50 countries. In this chapter, we provide a perspective on (1) clinical aspects of human microsporidiosis; (2) the genome and DNA markers for *E. bieneusi* as well as molecular methods for the specific and genotypic identification of *E. bieneusi*; (3) epidemiological aspects of *E. bieneusi* of animals and humans, with an emphasis on the genotypes proposed to be zoonotic, human-specific, and animal-specific; and (4) future research directions to underpin expanded molecular studies to better understand *E. bieneusi* and microsporidiosis.

Keywords Microsporidia · *Enterocytozoon bieneusi* · Genotypes · Genetic variants · Human · Animal · Zoonosis · Epidemiology · Taxonomy · Phylogeny

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14.1 Introduction

More than 1500 species of microsporidia have been recorded (Vávra and Lukeš 2013). At least 17 species have been identified in humans, of which *Enterocytozoon bieneusi* is the commonest (Fayer and Santín-Durán 2014). Currently, *E. bieneusi* appears to be fungus, but its precise classification is still controversial (Adl et al. 2005; Vávra and Lukeš 2013), with a proposal that it is sister to fungi (Karpov et al. 2014).

E. bieneusi is an obligate intracellular pathogen which is transmitted via the oral route. This species usually infects the intestine and can cause severe or chronic diarrhea, colic, vomiting, and/or wasting, although infection can be asymptomatic (Didier and Weiss 2006; Santín-Durán 2015; Stentiford et al. 2016). Transmission can be from person to person (Gumbo et al. 1999; Leelayoova et al. 2005) or between animals and people (Tzipori et al. 1997; Kondova et al. 1998; Cama et al. 2007), through water or the environment contaminated with *E. bieneusi* spores (Ben Ayed et al. 2012; Galván et al. 2013), food (Jedrzejewski et al. 2007; Decraene et al. 2012; Li et al. 2019a; Yildirim et al. 2020), or contact with infected individuals (Leelayoova et al. 2005; Pagornrat et al. 2009). Thus, *E. bieneusi* is recognized as a Category B Priority Pathogen by the National Institute of Allergy and Infectious Diseases (NIAID) (Didier and Weiss 2006) and a "contaminant candidate" waterborne microorganism by the US Environmental Protection Agency (EPA 1998).

The accurate identification of *E. bieneusi* is central to studying its epidemiology. As conventional microscopic, serological, and immunological methods do not allow an unequivocal identification to the species or subspecies level or a specific diagnosis of infection, molecular methods have been established to enable genetic characterization of this microorganism. Currently, more than 819 distinct *E. bieneusi* genotypes have been recorded in over 210 species of animals—including mammals, birds, reptiles, and some invertebrates in more than 50 countries (cf. Fayer and Santín-Durán 2014; Santín-Durán 2015; Li et al. 2019c, 2020c; Li and Xiao 2021; Zhang et al. 2021). However, for many countries, little is known about the molecular epidemiology of *E. bieneusi* of animals and humans and risk factors associated with microsporidiosis.

In this chapter, we provide a perspective on (1) clinical aspects of human microsporidiosis; (2) the genome and DNA markers as well as PCR-based methods available for the genetic identification of *E. bieneusi*; (3) aspects of the molecular epidemiology of *E. bieneusi*, with an emphasis on genotypes that are proposed to be zoonotic; and (4) future work that might be tackled to underpin, expanded molecular studies to better understand *E. bieneusi* and microsporidiosis.

14.2 Microsporidiosis: Clinical Aspects

Like many other microsporidia, *E. bieneusi* is transmitted directly; it has an infectious phase (spore) as well as proliferative and sporogonic phases within host cells (reviewed by Zhang et al. 2021), with the spore surviving in the environment (Vávra

and Larsson 2014). Usually, *E. bieneusi* infects the duodenum and jejunum (Shadduck and Orenstein 1993), although respiratory infection has been reported (e.g., Botterel et al., 2002; Graczyk et al., 2007; Kicia et al., 2016). In the small intestine, hyperplasia of the crypts and atrophy of the villi are observed histologically (Shadduck and Orenstein 1993), but many elements of the pathogenesis of microsporidiosis are still unclear (Santín-Durán 2015).

In immunocompetent people, microsporidiosis is often self-limiting within a few weeks or months (Cama et al. 2007), but sometimes asymptomatic infection can persist (Nkinin et al. 2007; Sak et al. 2011). By contrast, in immunodeficient or immunosuppressed people, such as transplant recipients and HIV/AIDS patients, *E. bieneusi* is an opportunistic pathogen and can cause life-threatening microsporidiosis (Desportes et al. 1985; Canning and Hollister 1990; Eeftinck Schattenkerk et al. 1991; Sing et al. 2001; ten Hove et al. 2009; Lanternier et al. 2009; Didier and Weiss 2011; Kicia et al. 2016; Santín-Durán 2015; Greigert et al. 2018; Desoubeaux et al. 2019; Messaoud et al. 2021).

In such immunocompromised patients, chemotherapy with albendazole, fumagillin, or nitazoxanide, is indicated, but toxicity and/or other adverse side effects can be significant issues (Bicart-Sée et al. 2000; Molina et al. 2002; Didier et al. 2004, 2006; Didier and Weiss 2006; Zhang et al. 2005; Pomares et al. 2012; Bednarska et al. 2013; Godron et al. 2013; Han and Weiss 2018; Saffo and Mirza 2019; Maillard et al. 2021). Thus, alternative treatments need development and evaluation. Preventative measures include sound hygiene, washing of fruit/vegetables, limiting animal-human contact, and the drinking of bottled or boiled water (Santín-Durán 2015). From a water industry perspective, it is wise to monitor *E. bieneusi* infection in animals, which live around or within drinking water catchment areas, so that authorities are informed as to whether animals might carry genotypes known to infect humans.

14.3 Genome and Ribosomal DNA Regions

The *E. bieneusi* draft genome (representing six chromosomes) is ~3.9 Mb in size (Akiyoshi et al. 2009). A total of 3804 genes has been predicted, 1702 of which code for proteins (Akiyoshi et al. 2009). This microsporidian seems to lack a number of metabolic/biological pathways, but the current draft genome is somewhat fragmented (Akiyoshi et al. 2009; Keeling et al. 2010), making detailed pathway and comparative genomic/genetic analyses challenging. Nonetheless, an analysis of ribosomal DNA regions in the genome showed that the internal transcribed spacer (*ITS*) does not consist of two regions (i.e., *ITS-1* and *ITS-2* separated by a 5.8S gene)—the 5.8S and large subunit (*LSU*) genes are fused (Akiyoshi et al. 2009).

Most molecular investigations of *E. bieneusi* conducted to date have indicated that the sequences of the *LSU* and *ITS* regions are suitable specific and genotypic DNA markers (respectively) for epidemiological investigations (Santín and Fayer

2009, 2011). The *ITS* region of *E. bieneusi* (240–245 bp) differs in size from *ITS*s of other human-pathogenic microsporidia, such as *Nucleospora cyclopteri*, *Nucleospora salmonis*, and *Obruspora papernae* (see Docker et al. 1997; Freeman et al. 2013; Diamant et al. 2014), and species of *Encephalitozoon* have *ITS* regions of <50 bp (Rinder et al. 1999).

14.4 Specific and Genotypic Identification

As conventional diagnostic tools (such as microscopy) do not allow accurate identification of *E. bieneusi*, molecular genetic techniques have been established and are broadly utilized for specific and genotypic identification (reviewed by Santín and Fayer 2011; Santín-Durán 2015; Zhang et al. 2021). Most of these methods rely on one-step or nested PCR-based amplification and/or sequencing of *ITS*, *LSU*, and/or *SSU* (Zhang et al. 2021). PCR-coupled sequencing provides the highest resolution, because nucleotide sequences are defined that can be used for comparisons with reference sequences in public databases (e.g., GenBank). Other PCR-based techniques, such as quantitative PCR (qPCR) and multilocus sequence typing (MLST), are also employed by some laboratories for specific detection, molecular characterization, and/or diagnosis (reviewed by Zhang et al. 2021).

The MLST technique (Feng et al. 2011) uses the microsatellite markers MS1, MS3, MS4, and MS7 for analyses (Li et al. 2013, 2019c; Du et al. 2015; Tian et al. 2015; Zhao et al. 2015; Deng et al. 2016, 2017; Wang et al. 2016; Zhang et al. 2016; Zhong et al. 2017a). This method allows the phenetic characterization of some variants of *E. bieneusi*, but not all, which is why *ITS* is also sometimes employed, in combination, for genotypic classification (e.g., Deng et al., 2017; Zhong et al., 2017a; Desoubeaux et al., 2019). However, PCR-based sequencing of *ITS* from genomic DNAs isolated from fecal, food, soil, or water samples is the commonest approach (Santín-Durán 2015; Zhang et al. 2021). For nested PCR amplification and/or sequencing of *ITS*, various primer pairs have been employed. In our experience, primer pairs EBITS1 (5′-GCT CTG AAT ATC TAT GGC T-3′)/ EBITS2.4 (5-ATC GCC GAC GGA TCC AAG TG-3′) and EBITS3 (5′-GGT CAT AGG GAT GAA GAG-3′)/EBITS4 (5′-TTC GAG TTC TTT CGC GCT C-3′) (Buckholt et al. 2002) perform the best. Some researchers have employed alternative primer sets to amplify and sequence *SSU* or *LSU* (cf. Buckholt et al. 2002; Santín and Fayer 2009; Zhang et al. 2011) as well as regions in the genes *ck1* and *swp1* to characterize genetically unique taxa (e.g., within the "canine outlier" group; Ou et al. 2021). The combined use of these and other markers could be beneficial to evaluate the specific status of *E. bieneusi* genotypes in a phylogenetic context (Zhang et al. 2021).

14.5 Host Records for *E. bieneusi* Genotypes

When *E. bieneusi* was first studied, the *ITS* region was identified as a useful genotypic marker because of the extent of sequence variation within this species (Santín et al. 2010). In 2009, at the *Tenth International Workshop on Opportunistic Pathogens*, a naming system for *E. bieneusi* genotypes was agreed upon (Santín and Fayer 2009, 2011). Since that time, improvements were made to the criteria (Zhang et al. 2021) in an effort to address some inconsistencies. Clearly, there has been a substantial expansion in the number of studies exploring genetic variation within *E. bieneusi* over the past years (Fig. 14.1; Supplementary Tables 14.1 and 14.2). For instance, in 2005, $\leq$ 100 genotypes were recognized (Drosten et al. 2005) compared with 573 in April 2019 (Zhang et al. 2021), and by June 2021, 819 genotypes had been recorded. In the last few years, China has "led the world" in the reporting of novel *E. bieneusi* genotypes, which needs to be taken into account when appraising trends as well as geographical and host occurrences of genotypes. The number of novel genotypes published from China increased from 50 in 2019 to 106 in 2020, with a total of 465 at the end of June 2021 (compared with 56 in the USA). If this trend continues, thousands of novel genotypes might be recorded within the next decade. How useful the continual recording of *ITS* genotypes remains to be established. Clearly, there needs to be more of an emphasis on this use of many more genetic markers and phenotypic characteristics (e.g., Li and Xiao, 2021; Ou et al., 2021) for the accurate definition of *E. bieneusi* and its genotypes.

Globally, *E. bieneusi* has been identified in humans, a range of other mammals, as well as birds, amphibians, reptiles, and some invertebrates in at least 50 countries

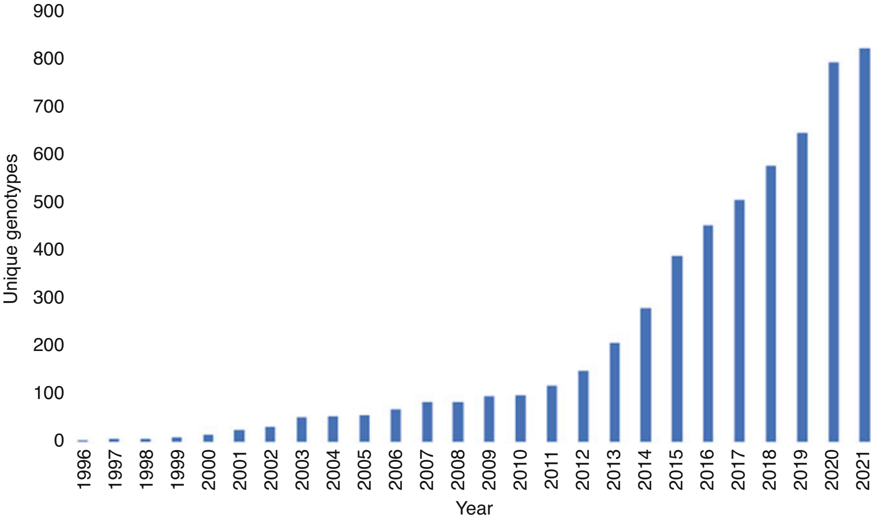

Fig. 14.1 The cumulative number of distinct *Enterocytozoon bieneusi* genotypes reported in the published literature from 1996 to 30 June 2021 (cf. Supplementary Table 14.1)

(Supplementary Table 14.1). Currently, 819 unique genotypes are recognized in the peer-reviewed literature (for which full-length *ITS* sequences are publicly available). To date, 126 genotypes have been recorded exclusively in humans from 23 countries, 614 in animals, and 58 in both humans and animals (suggesting zoonotic potential) (Supplementary Table 14.1). Genotypes D (in 91 host species, 40 countries), EbpA (55, 14), EbpC (43, 15), Type IV (31, 25), BEB6 (29, 11), O (18, 5), J (21, 11), CM4 (15, 1), Peru6 (22, 10), I (17, 12), Peru8 (15, 7), Peru11 (13,11), and BEB4 (8, 10) are most commonly found in animals including humans (Zhang et al. 2021) (Table 14.1; Supplementary Table 14.1).

14.5.1 Humans and Nonhuman Primates

Currently, at least 184 *E. bieneusi* genotypes have been recorded in people in 31 countries of the developed and developing world (Table 14.1; Supplementary Table 14.1). At least 126 genotypes are unique to humans, most ($n = 118$) of which have only been recorded once or a few times. Genotypic uniqueness appears to be highly dependent on the number of surveys conducted over time. Genotype B is the only genotype that has been found many times in different studies from 10 countries, suggesting that it is a global and ubiquitous human-specific genotype. Genotype B belongs to Group 1 (Zhang et al. 2021) and is closely related to many other genotypes with zoonotic potential. Understanding why genotype B is specific to humans could be a key to understanding this host affiliation, indicating that this genotype would be a candidate for comparative genomic investigations of *E. bieneusi* genotypes.

Of the 58 genotypes recognized as "zoonotic" (reported in both humans and animals at least once), the majority are D, EbpC, Type IV, EbpA, BEB6, I, and J (Table 14.1; Supplementary Table 14.1). However, the zoonotic genotypes with highest number of cases reported in humans are D, A, and Type IV. Interestingly, zoonotic genotypes account for at least 53% of all of the genotypes in animals (Table 14.1; Supplementary Table 14.1). In a recent study in Australia, Zhang et al. (2018c) indicated that the *E. bieneusi* genotypes detected in humans (i.e., ALP1, Ind4, hum_q1, and hum_q2) in Tasmania, Victoria, New South Wales, South Australia, Western Australia, and Queensland appear to be zoonotic. Interestingly, the ALP1 genotype identified, for the first time, in Australians (Zhang et al. 2018c) was also detected in farmed alpacas in a distinct study (Koehler et al. 2018), suggesting that this genotype might be transmissible from alpacas to humans.

In nonhuman primates, 84 *E. bieneusi* genotypes have been recorded, including some commonly found in humans (e.g., D, EbpC, and Type IV), indicating that nonhuman primates might act as reservoirs for zoonotic *E. bieneusi*. Major genotypes are D, CM4, and EbpC. At least 56 novel genotypes have been identified in primates in 18 published studies (Table 14.1; Supplementary Table 14.1), 6 and 12 of which investigated wild and captive primates, respectively. Notable studies of western lowland gorillas in the wild discovered an array of novel genotypes (Sak

Table 14.1 Summary of published *Enterocytozoon bieneusi* genotypes categorized according to animal groups, with information on the number of novel genotypes, predominant genotypes, country of origin, and number of original publications (cf. Supplementary Table 14.1)

Animal group		Host(s)	No. of genotypes (novel)	Predominant genotypes	No. of countries	No. of published studies
Primates		Human	184 (151)	D, A, Type IV	31	82
		Unique to human	126 (126)	B	23	48
		Nonhuman primates	84 (56)	D, CM4, EbpC	9	22
Companion animals		Dogs	40 (25)	PtEb IX, D	10	19
		Cats	25 (13)	D, Type IV, PtEb IX	15	19
Agricultural animals	Equids	Horse	62 (41)	Horse 1, Horse 2, D	6	12
		Donkey	19 (11)	D, NCD-2	2	4
	Suids	Pig	120 (96)	EbpC, EbpA, H	13	36
	Ruminants	Goat	41 (20)	BEB6, CHG3	4	11
		Sheep	69 (46)	BEB6, CM7	6	20
		Deer	77 (59)	BEB6, EbpC	5	15
		Cattle, yak, water Buffalo	97 (69)	I, J, BEB4, D, EbpC, Type IV	16	48
		Alpaca	13 (5)	ALP1, P, Type IV	3	6
		Camel	15 (7)	Macaque1	2	3
Wildlife—captive or pet	Canids	Fox, raccoon dog	24 (9)	Wildboar3, D	5	14
	Felids	Civet, lion, meerkat, tiger	20 (12)	D	3	6
	Mustelids	Including American river otter, badger, ermine, mink badger	19 (7)	EbpC, Peru11	3	6
	Suids	Wild boar	28 (14)	EbpC, EbpA, D	8	5
	Ursids	Including Asiatic black bear, giant panda, sun bear	24 (13)	CHB1, SC02	2	10
	Rodents	Including beaver, mice, rats, squirrels	88 (55)	D, group1-like, Type IV	6	21

(continued)

Table 14.1 (continued)

Animal group		Host(s)	No. of genotypes (novel)	Predominant genotypes	No. of countries	No. of published studies
	Lagomorphs	European rabbit, eastern cottontail	19 (7)	I, J	3	7
	Chiroptera	Including common bent-wing bat, straw-colored bat	9 (6)	I, Type IV	2	2
	Eulipotyphla	Amur hedgehog	4 (4)	No dominant genotype	1	1
	Marsupials	Including red kangaroo, swamp wallaby, wombat	9 (8)	CHK1, MWC_m2	2	3
	Birds	Including chicken, dove, parrots, pet birds, waterfowl	46 (16)	A, D, EbpA, Peru6	11	25
	Reptiles	Snakes	14 (9)	EbpC	1	2
	Amphibians	Bullfrog	20 (16)	No dominant genotype	1	1

et al. 2013, 2014). Other studies of primates in zoos (Karim et al. 2014a, 2015) suggest that such genotypes were introduced into a captive setting from the wild.

14.5.2 Companion Animals

In cats, *E. bieneusi* has been recorded at a prevalence of 1.4–31.3% in Portugal, Germany, Switzerland, Poland, the Czech Republic, Slovakia, Turkey, China, Thailand, Japan, Brazil, and Colombia (reviewed by Zhang et al. 2018c). To date, 25 *E. bieneusi* genotypes have been recorded in cats in 19 published studies, with D, Type IV, and PtEb IX predominating (Table 14.1; Supplementary Table 14.1).

In dogs, *E. bieneusi* has been recorded at a prevalence of 2.5–15.5% in Portugal, Spain, Switzerland, Poland, Iran, China, Japan, Colombia, the USA, and Australia (reviewed by Zhang et al. 2019b). At least 40 genotypes (including A, D, EbpA, EbpC, O, Peru 5, Peru 8, PigEBITS5, PtEb VI, and PtEb VII) have been recorded in canines and/or other mammals. PtEb IX and D are most common in dogs, and PtEb IX appears to be affiliated exclusively with dogs and cats. However, it was recently recorded in the European badger (Santín et al. 2018) and whooper swan (Wang et al. 2020), which may indicate pseudoparasitism following the consumption of dog or cat feces. PtEb IX clusters with 13 "novel" genotypes (CD7, CD8, GD1, GD2, GD3, GD4, GD5, GD6, NED4, NED3, SCD-1, WW8, and WW9), which were often referred to as "outliers" in early studies (Thellier and Breton 2008) due to their genetic distinctiveness from other *E. bieneusi* genotypes. Using sequence data for two additional gene markers (*ck1* and *swp1*) and *SSU*, Ou et al. (2021) compared PtEB IX with genotypes from other groups and suggested that this genotype represents a novel species of *Enterocytozoon.*

14.5.3 Agricultural Animals

Equids In horses, *E. bieneusi* has been recorded in 12 studies in 6 countries, including the Czech Republic (Wagnerová et al. 2012), Turkey (Yildirim et al. 2020), Algeria (Laatamna et al. 2015), Colombia (Santín et al. 2010), China (Deng et al. 2016; Qi et al. 2016; Zhao et al. 2019; Li et al. 2020a), and the USA (Wagnerová et al. 2016) at a prevalence of 6.8–30.9%. There are at least 62 genotypes in horses (41 novel), with genotypes "Horse 1," "Horse 2," and D being most common (Table 14.1; Supplementary Table 14.1). BEB6, CS-4, CZ3, D, EbpA, EbpC, G, O, Peru 8, SC02, and WL15 ($n = 11$) are recognized as having zoonotic potential. There have been four studies of *E. bieneusi* from donkeys (*Equus africanus asinus*)—three from China and one from Algeria. Of the 19 genotypes recorded, 11 are "novel" (Table 14.1). Of note is genotype NCD-2, which was detected in three studies conducted in five provinces of Northern China (Yue et al. 2017; Li et al. 2020a; Zhao et al. 2020).

Suids In pigs, 120 *E. bieneusi* genotypes (95 being novel) have been recorded in 36 studies, with EbpC, EbpA, and H being most common. The reported prevalence in pigs ranges from 10 to 94% in Europe (the Czech Republic, Germany, Spain), Asia (Korea, Thailand, Japan, and China), South America (Brazil and Peru), and the USA (reviewed by Zhang et al. 2021), after the discovery of *E. bieneusi* in Switzerland (Breitenmoser et al. 1999).

In some studies, the prevalence was reported to be higher in pre-weaned/weaned pigs than in growers ($P < 0.05$) (Li et al. 2014a–c; Wan et al. 2016; Shi et al. 2018; Wang et al. 2018; Zou et al. 2018), although a study (Li et al. 2014b) did estimate a higher prevalence in weaners in piglets. Husbandry practices, the health status of herds, host immune status, gender and/or age, and environmental conditions likely significantly influence prevalence rates on farms (Sak et al. 2008). Of the 28 genotypes of *E. bieneusi* recorded from wild boars, genotypes D and EbpC were most common (Table 14.1; Supplementary Table 14.1), and Li et al. (2020c) remarked that domestic pigs are a prime reservoir for human infection with genotype D, EbpC, or Type IV.

Ruminants and Camelids Information on *E. bieneusi* genotypes from small ruminants has expanded significantly. In goats, 41 genotypes (20 being novel) have been recorded, with genotypes BEB6 and CHG3 being most common (Supplementary Table 14.1). In China, *E. bieneusi* has been recorded at a prevalence of 4.1–29.7% in goats (Li et al. 2019e; Zhang et al. 2019a), with Shi et al. (2016) discovering 16 novel genotypes, an unexpectedly high number, raising questions about the validity of their data and findings. In *sheep*, 69 genotypes (including 46 novel ones) of *E. bieneusi* have been recorded in countries including Ethiopia, Sweden, Turkey, China, and Brazil, with BEB6 and CM7 being most commonly reported. Prevalence rates of *E. bieneusi* varied markedly from 3.4 to 77.8% (Ye et al. 2015; Li et al. 2019e), depending on animal age group and farm management practices. In *deer*, 77 genotypes (59 being novel) have been recorded, with the majority of studies conducted on farms in China (8), 2 from zoos and 5 studies of wild deer in Australia, China, Korea, and the USA (Table 14.1; Supplementary Table 14.1). To date, genotypes BEB6 and EbpC predominate in deer.

In cattle, *E. bieneusi* was first recorded in Germany (Rinder et al. 2000) and then in 15 other countries, including Brazil, China, Korea, Turkey, South Africa, and the USA, with prevalence varying from 2.0 to 52% (Valenčáková and Danišová 2019; Zhao et al. 2020). Currently, 97 genotypes have been recorded in agricultural bovids, including cattle, water buffalo, and yak (69 of which are "novel" and first detected in bovids); genotypes I, J, and BEB4 have been most commonly reported (48 studies). Potentially zoonotic genotypes include D and Type IV, and, for example, I and J, previously assumed to be cattle-specific, have been recorded in humans (Table 14.1; Supplementary Table 14.1). The discussion about *E. bieneusi* genotypes I and J being ruminant-specific continues in some recent publications, even though these genotypes have been recorded previously in bats, birds, canids, felids, primates, pangolins, pigs, rabbits, ursids, and rodents (Supplementary Table 14.1).

Recent studies of *E. bieneusi* in farmed cattle and alpacas in Australia (Koehler et al. 2018; Zhang et al. 2019c) recorded three (ALP1, APL3, and P) and six (BEB4, I, J, TAR_fc1, TAR_fc2, and TAR_fc3) genotypes, some of which are recognized to have zoonotic potential. The prevalence rates in cattle (10.4%) and alpacas (9.9%) were higher than those estimated for cats (2.9%) and dogs (4.4%) studied in Australia (Zhang et al. 2019b). Genotype ALP1, first recorded in alpacas in Peru (Gómez Puerta 2013), has since been found in alpacas (Koehler et al. 2018) and humans (Zhang et al. 2018b) in Australia as well as in alpacas in China (Ma et al. 2019). As many as 15 genotypes of *E. bieneusi* have been recorded in domesticated camels in Algeria and China, seven being novel and genotype "Macaque1" being prominent (Qi et al. 2016; Baroudi et al. 2017; Zhang et al. 2019a).

14.5.4 Mammalian Wildlife

E. bieneusi has been recorded in more than 135 mammalian wildlife species, with 611 genotypes characterized exclusively from animals and 58 with zoonotic potential from both humans and animals (Table 14.1; Supplementary Table 14.1). Many wildlife surveys conducted involved wildlife on farms or in zoos as well as animals (both endemic and translocated species) in the wild. The provenance of samples from these animals needs to be considered when interpreting the findings from such surveys.

Numerous studies have detected *E. bieneusi* in wildlife living in the vicinity of drinking water reservoirs, suggesting that such animals transmit *E. bieneusi* to humans via spore-contaminated drinking water (Sulaiman et al. 2003; Guo et al. 2014). For instance, Ye et al. (2012) detected *E. bieneusi* in a lake where rhesus monkeys bathed; these primates harbored the same "zoonotic" genotypes (EbpC, Peru11, and Type IV) as found in water. As rhesus monkeys are often in contact with people in nature reserves or parks (Ye et al. 2012), this finding raises a public health concern. A wastewater study in China (Fan et al. 2021) found 11 known genotypes (D and Type IV predominantly) and three "novel" genotypes. It was suggested that mice and rats in the sewer system were contributing to the genotypic diversity detected (Fan et al. 2021). Further aspects of waterborne transmission have been reviewed (Li et al. 2020c).

Canids Of the 14 published studies of *E. bieneusi* in foxes (*Vulpes vulpes* and *Vulpes lagopus*) and raccoon dogs (*Nyctereutes procyonoides*), 1 was from a zoo, 5 involved wild animals, and 8 investigated "fur farms" in China (reviewed by Guo et al. 2021; Table 14.1; Supplementary Table 14.1). In total, 24 genotypes of *E. bieneusi* were discovered in these animals, with genotypes D and "Wildboar3" being most common and nine being "novel" (Table 14.1; Supplementary Table 14.1). Genotype Wildboar3 has been identified on fur farms in China but also in a wild red fox from Spain (Santín et al. 2018), in wild boar in regions of Europe (Němejc et al. 2014; Leśniańska et al. 2016), and in an eastern grey kangaroo

in Australia (Zhang et al. 2018b). Results of studies of these and other canids from farms in China are further summarized by Guo et al. (2021).

Felids The masked palm civet (*Paguma larvata*) is also farmed for pelts in China; one study reported 13 *E. bieneusi* genotypes from this animal, 9 of which were novel (Yu et al. 2020). Various other felids, including lions, tigers, and meerkats, have been studied in zoos, with genotype D being dominant (Table 14.1; Supplementary Table 14.1).

Mustelids In three studies investigating *E. bieneusi* in farmed mink (*Neovison vison*) in China, ten *E. bieneusi* genotypes have been reported. New genotypes (NCM-1 and NCM-2) were found in two separate studies (Zhang et al. 2018a; Cong et al. 2018), along with Peru11 and EbpC. Wild mustelids (including North American river otters, ermine, European badgers, and Beech marten) have also been studied in the USA and Spain (Sulaiman et al. 2003; Guo et al. 2014; Santín et al. 2018), with three "novel" *E. bieneusi* genotypes (Table 14.1; Supplementary Table 14.1).

Ursids Studies of captive *bears* ($n = 10$), farmed bears in China (4), and wild bears in zoos (5) have discovered 25 *E. bieneusi* genotypes, with CHB1 and SC02 being common in both agricultural and farmed settings and 13 being novel (Table 14.1; Supplementary Table 14.1).

Rodents and Lagomorphs *E. bieneusi* has been recorded in at least 36 species of rodents in 23 studies published on aspects of wildlife, pets, zoo medicine, experimental animals, and agricultural animals (i.e., bamboo rats) conducted in the Czech Republic, Poland, Iran, China, and the USA. In total, 88 genotypes (55 novel) have been recorded to date, with D, Type IV, and "Group 1-like" predominating (Supplementary Table 14.1). Seven studies involving lagomorphs—mostly captive European rabbits (*Oryctolagus cuniculus*) in China—have characterized 19 (7 novel) genotypes (Table 14.1; Supplementary Table 14.1).

Chiroptera and Eulipotyphla There have been studies of bats from Korea (Lee et al. 2018) and Nigeria (Li et al. 2019b). The two genotypes of *E. bieneusi* (Bat1 and Bat2) characterized from straw-colored fruit bats in Nigeria are of interest, as they represent Group 5 (Zhang et al. 2021) and are genetically very distinct from genotypes within Groups 1 and 2. Other genotypes representing Group 5 have been discovered in humans from China, rhesus macaques from China and Bangladesh, a whooper swan from China, and an olive Baboon from Kenya (Li et al. 2011; Karim et al. 2020; Wang et al. 2020; Zhang et al. 2021; see Table 14.1; Supplementary Table 14.1). The one study of hedgehogs (*Erinaceus amurensis*) captured in the wild in China described four "novel" *E. bieneusi* genotypes (Gong et al. 2021).

Marsupials Of the three published studies, two involved captive macropods in China (Li et al. 2015; Zhong et al. 2017b) and one investigated wild kangaroos, wallabies, and wombats (Zhang et al. 2018b). In total, nine genotypes were found (eight being novel) (Table 14.1; Supplementary Table 14.1). Of particular interest

was the novel clade of distinct *E. bieneusi* genotypes (CSK2, MWC_m2, MWC_m3, and MWC_m4) which, like genotype PtEB IX (an "outlier") from dogs, do not genetically match any members of the nine other groups of *E. bieneusi* and thus might need reclassification as a distinct species of *Enterocytozoon* (Zhang et al. 2018b). Genotype CSK2 from a captive red kangaroo in China also grouped with these novel genotypes, suggesting that this genotype was translocated, along with its hosts, from Australia to China (Zhang et al. 2018b).

14.5.5 Birds

E. bieneusi has been recorded in a variety of bird species (cf. Santín-Durán 2015). *E. bieneusi* was first reported in chickens at a prevalence of 25% (2/8) (Reetz et al. 2002), and genotype J was identified. Subsequently, *E. bieneusi* was identified in captive (farmed, caged, or zoo) birds (Reetz et al. 2002; Lobo et al. 2006; Müller et al. 2008; Kašičková et al. 2009; Lallo et al. 2012; Galván-Díaz et al. 2014; Li et al. 2014c, 2015; da Cunha et al. 2016, 2017; Tavalla et al. 2018; Deng et al. 2019; Feng et al. 2019; Pekmezci et al. 2020; Dong et al. 2021; Pekmezci et al. 2021) and feral or wild birds (Haro et al. 2005, 2006; Pirestani et al. 2013; Perec-Matysiak et al. 2017; Liu et al. 2020; Wang et al. 2020). Currently, *E. bieneusi* is known to occur in 59 bird species (Table 14.1; Supplementary Table 14.1) at a prevalence ranging from 44.0% in rock pigeons (Zhao et al. 2016) to $< 1\%$ (1/112) in blue peafowl (Li et al. 2015). In total, 46 *E. bieneusi* genotypes have been recorded in birds, 22 of which have been detected in other animals (the commonest being A, D, EbpA, and Peru6) and 9 being recorded from humans and considered "zoonotic" (Supplementary Table 14.1). In pigeons (*Columba livia*), genotype Peru6 was identified in six of eight studies in countries including China, Brazil, Portugal, and Turkey. In agricultural poultry, such as chickens and geese, the dominant genotypes recorded were Peru6 and D (Supplementary Table 14.1). Other studies, notably those of Kašičková et al. (2009) and Deng et al. (2019), investigating pet birds showed that genotypes A, D, EbpA, and Peru6 predominated (Table 14.1; Supplementary Table 14.1).

14.5.6 Reptiles and Amphibians

Zoonotic genotypes and four unique, novel *E. bieneusi* genotypes were characterized from two species of captive snakes—the oriental rat snake (*Ptyas mucosus*) and the Indian cobra (*Naja naja*) (Karim et al. 2014b). Although these snakes may have harbored *E. bieneusi*, it is possible that their food source (rodents) may have contained genotypes Type IV and Henan-V (Karim et al. 2014b). A more recent study of captive snakes also in China (Li et al. 2020b) identified eight genotypes (five being novel). By contrast, a unique survey of bullfrogs (*Lithobates catesbeianus*) produced for culinary purposes (Ding et al. 2020) suggested the

presence of as many as 20 genotypes, 16 of which were novel. The unexpected diversity of genotypes reported may raise a question about the validity of the data (Table 14.1; Supplementary Table 14.1).

14.5.7 Invertebrates

There have been very few studies of *E. bieneusi* from invertebrates. One study aimed to examine flies (*Musca domestica*, *Chrysomya megacephala*, and *Sarcophaga* spp.) which were trapped on a farm (with dairy cattle and rodents) to establish whether these insects might transmit *E. bieneusi* to the other hosts (Yu et al. 2019). Another study in Ireland investigated anthropozoonotic parasites of zebra mussels (*Dreissena polymorpha*) from fresh water. *E. bieneusi* was detected in 4 of the 19 (21%) mussels studied (Graczyk et al. 2004), but it is unclear whether mussels are natural hosts of *E. bieneusi* because they are filter feeders and might, thus, accumulate *E. bieneusi* from water or the environment (e.g., Géba et al., 2020).

14.6 Critical Appraisal of the Classification/Relationships of *E. bieneusi* Genotypes

Most molecular epidemiological studies of *E. bieneusi* have utilized PCR-based sequencing of the *ITS* and comparative/phylogenetic analyses. While nested PCR-based sequencing of the *ITS* appears to provide a suitable diagnostic and analytical tool for such studies, there have been challenges in inferring the phylogenetic relationships of genotypes and genetic groups of *E. bieneusi* using *ITS* sequence data alone (Zhang et al. 2021). Although *ITS* has been recognized as the "best" marker to identify and characterize genotypes of *E. bieneusi*, its sequence is short; hence, the phylogenetic signal for such a short DNA region is limited (Zhang et al. 2021). Given this limitation, it has been challenging to get robust support for the genotypic groupings of *E. bieneusi*, such that many published studies did not report on levels of statistical support for such groupings.

In a recent article (Zhang et al. 2021), we focused on improving the naming and grouping of *E. bieneusi* genotypes based on *ITS* sequence data sets. Endeavoring to retain the grouping system proposed by Li et al. (2019d), so as to avoid confusion, we conducted independent phylogenetic analyses of complete *ITS* sequences representing hundreds of unique genotypes ($n = 571$) reported in all published studies to evaluate the relationships of *E. bieneusi* genotypes and the validity of groups. The results of this effort classified *E. bieneusi* into two major (Groups 1 and 2) and seven minor groups (Groups 3–9); this classification system is based on statistical support, obtained using Bayesian inference (BI), neighbor-joining (NJ) and maximum likelihood (ML) tree-building methods, branch lengths, and

topology (see Fig. 4 and Supplementary Figs. 1–4 in Zhang et al. 2021). To display the groups, we presented a complete alignment of all available *ITS* sequences; we showed that the patterns of nucleotide alterations can usually be used to distinguish particular groups and subgroups from one another, employing genotype "HLJ-III" (GenBank no. KJ475404) as the reference.

This classification scheme for *E. bieneusi* succeeded in retaining the previous grouping system and provides an improved basis for future molecular epidemiological work. However, it is clearly evident that the use of *ITS* sequence data alone does not provide adequate phylogenetic signal to achieve robust groupings throughout the tree. We propose that the *SSU* region should also be sequenced for any genotypes within minor groups to assist in assessing their taxonomic status by phylogenetic analysis, as conducted recently (cf. Ou et al. 2021). However, we believe that a panel of additional phylogenetically informative DNA markers is needed to better resolve the relationships of *E. bieneusi* genotypes.

14.7 Proposed Areas for Future Research

14.7.1 Complete the Genome of E. bieneusi

Defining a high-quality reference genome for *E. bieneusi* will facilitate the identification of additional gene markers needed to better infer *E. bieneusi* genotypes and groups using phylogenetic tools. As the *E. bieneusi* draft genome (~3.9 Mb; Akiyoshi et al. 2009; Keeling et al. 2010) is fragmented, it would be beneficial to achieve a chromosome-level genome assembly. This might be achievable using both short- and long-read sequencing technologies, as has been shown recently for large parasite genomes (e.g., Young et al. 2021). Long-read technologies, such as single-molecule real-time (SMRT) sequencing (Pacific Biosciences, PacBio) and Oxford Nanopore Technologies (Goodwin et al. 2016; Kchouk et al. 2017; Malla et al. 2019), are now being widely used. As sequencing technologies continue to improve, future platforms should overcome throughput and read-length issues, and should produce high-quality sequence data at low cost (cf. Van Dijk et al. 2014; Tan et al. 2015).

14.7.2 Define Additional Genetic Markers

Some previous investigations have attempted to evaluate a range of markers and techniques for the identification and/or classification of *E. bieneusi* genotypes. For example, Xiao's research team established a multilocus sequence typing (MLST) approach using repetitive sequence regions (Feng et al. 2011; Li and Xiao 2019; Li et al. 2019c,d), but these markers can be applied only to some genotypes of *E. bieneusi* (predominantly in Group 1 and some within Group 2; cf. Zhang et al.

2021), thus compromising analyses of all groups (Li et al. 2013, 2016, 2017, 2020c; Deng et al. 2018). Markers in genes encoding heat-shock protein 70 (*hsp-70*) (Germot et al. 1997; Hirt et al. 1997; Arisue et al. 2002), large subunit of RNA polymerase II (*RPB1*) (Hirt et al. 1999), α- and β-tubulins (Edlund et al. 1996; Keeling and Doolittle 1996; Keeling et al. 2000, 2003), glutamyl- and glutaminyl-tRNA synthases (Brown and Doolittle 1999), translation elongation factor 1α (*EF-1α*) (Tanabe et al. 2002, 2005), and TATA-box binding protein (*TBP*) (Fast et al. 1999) have also been used to study the taxonomic relationship between microsporidia and fungi. However, to date, no attempt has been made to utilize these genes as barcodes for *E. bieneusi* and its genotypes. Future work might assess these markers and search for a range of new and informative (e.g., single-copy genes) in the *E. bieneusi* genome. The use of a large panel of markers is likely to provide a significantly increased signal, which is required to define robust phylogenetic groupings within *E. bieneusi*.

14.7.3 Develop Improved Methods for Genetic Characterization: Metabarcoding

Whole-genome-based or ribosomal RNA gene-targeted metabarcoding would also be useful for genetic analyses of *E. bieneusi* (see Goodwin et al. 2016). Although whole-genome sequencing (employing Ion Torrent technology) was used to explore pathogens in water in Haiti and successfully detected *E. bieneusi* in three geographical locations (Roy et al. 2018), this approach is costly and impractical for analyses of large sample sizes. In contrast, targeted, PCR-based sequencing is more commonly used for analyses of large numbers of biological or environmental samples (Tan et al. 2015; Zahedi et al. 2018, 2019; Stensvold et al. 2020). The latter approach is practical, but it is critical that (1) primers, (2) PCR and sequencing conditions, (3) sequence depth/coverage, and (4) inclusion of sample replicates are evaluated prior to analytical or diagnostic application, as emphasized by Zahedi et al. (2019).

Following optimization, the targeted sequencing of an expanded panel of informative genetic markers, including *ITS*, would likely allow detailed investigations of *E. bieneusi*. A future focus could be to establish and optimize a "next-generation" targeted sequencing and informatics platform for the simultaneous genetic characterization of *Enterocytozoon*, *Giardia*, and *Cryptosporidium* in biological matrices (including feces, water, food, and the environment) for epidemiological investigations and/or for water quality management. At least 58 *E. bieneusi* genotypes have been recorded in both humans and other animals worldwide, and include genotypes BEB6, D, EbpA, EbpC, I, J, O, Peru8, and Type IV, which have also been detected in water samples (e.g., Ben Ayed et al. 2012; Li et al. 2012; Hu et al. 2014; Fan et al. 2021; Table 14.1; Supplementary Table 14.1).

14.8 Concluding Remarks

Current evidence shows that genetic variation within *E. bieneusi* is vast. How this variation relates to the species status of *E. bieneusi* is still unclear, because, to date, only a very small number of genetic markers have been employed to explore the relationships among genetic variants (genotypes or operational taxonomic units) within this "taxon" (i.e., *E. bieneusi*). However, we do know that a subset of genotypes appears to be zoonotic—being transmissible from animals to humans and vice versa. Clearly, advances in genomic sequencing and bioinformatic technologies bode well for the completion of nuclear genomes of representative genotypes of *E. bieneusi*. These advances would underpin the discovery of a large set of new, informative DNA markers for comprehensive epidemiological studies using metabarcoding and phylogenetic tools. In turn, these would provide insight into the dynamics of transmission of particular *E. bieneusi* genotypes among host species, risk factors linked to microsporidiosis, the resilience of *E. bieneusi* spores in water and the environment, and the virulence/pathogenicity of genotypes presently recognized as "potentially zoonotic". Clearly, better knowledge and understanding of *E. bieneusi* and its epidemiology using advanced tools should contribute significantly to improving the prevention and control of microsporidiosis.

Compliance with Ethical Standards
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3. **Ethical Approval**: The chapter is a review of previously published works; no animal or human studies were performed.

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