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Microsporidia infections in *Caenorhabditis elegans* and related nematodes

Gaotian Zhang

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École Normale Supérieure et
East China Normal University

Microsporidia infections in *Caenorhabditis elegans* and related nematodes

**Microsporidies, *Caenorhabditis elegans*, et autres nématodes :
biologie et caractérisation de leurs interactions**

Ecole doctorale n°515

COMPLEXITE DU VIVANT

Spécialité GENOMIQUE

**Soutenue par Gaotian ZHANG
le 23 02 2017**

**Dirigée par Marie-Anne FELIX
et Xiaoming WANG**

COMPOSITION DU JURY :

M. DELBAC Frédéric
Université Blaise Pascal
Rapporteur

M. SCHULENBURG Hinrich
Christian-Albrechts-Universität zu Kiel
Rapporteur

M. PINTARD Lionel
Institut Jacques Monod
Président du jury

Mme. LEBRETON Alice
École Normale Supérieure
Membre du jury

M. WANG Tianhou
East China Normal University
Membre du jury

Mme. FELIX Marie-Anne
École Normale Supérieure
Membre du jury

M. WANG Xiaoming
East China Normal University
Membre du jury



Microsporidia infections in *Caenorhabditis elegans* and related nematodes

Gaotian ZHANG

Director: Marie-Anne FELIX

Codirector: Xiaoming WANG

ABSTRACT

Microsporidia are fungi-related intracellular pathogens that infect a great variety of animals, including the nematode *Caenorhabditis elegans*. The first microsporidia isolated from wild *C. elegans* was named *Nematocida parisii* in 2008. *C. elegans* and *N. parisii* have been used as a powerful model for the study of host-pathogen interactions. However, it was unclear how widespread and diverse microsporidia infections are in *C. elegans* or other related nematodes in the wild.

By sampling rhabditid nematodes worldwide, we established a collection of 47 nematodes that displayed putative microsporidia infections. We characterized molecularly these infections and determined that *N. parisii* (or *N. ironsii*) is the most common microsporidia infecting *C. elegans* in the wild. We further described and named six new *Nematocida* species. In addition, we defined two new genera of nematode-infecting microsporidia, named *Enteropsectra* and *Pancytospora*, which are genetically distinct from *Nematocida*. Further investigations showed that these microsporidia are diverse in terms of spore size and shape, host tissue tropism, host cell intracellular localization, cellular exit route, host specificity pattern, etc. Overall, these findings illustrate the widespread and diverse microsporidia infections in *C. elegans* and related nematodes in the wild.

We further assayed the natural variation of *C. elegans* in sensitivity to *N. ausubeli* infection, by comparing 10 *C. elegans* strains using food consumption tests. Two *C. elegans* strains, JU1249 and JU2825, displayed the largest sensitivity differences, which were suggested to be a result of the different tolerance between the two strains. These two strains are proven to be good candidates for future studies on the genetic loci associated with *C. elegans* sensitivity variation to microsporidian infections. Furthermore, I observed an exciting effect of host-pathogen interaction. Microsporidia infection is able to suppress the progressive decline in fertility in some *C. elegans* with the mortal germline phenotype (Mrt).

KEYWORDS: Microsporidia, *C. elegans*, diversity, host-pathogen interaction, host specificity, sensitivity, natural variation

RESUME

Les microsporidies sont des pathogènes intracellulaires obligatoires apparentés aux champignons. Elles infectent de nombreux animaux, dont le nématode *Caenorhabditis elegans*. La première microsporidie isolée d'une souche de *C. elegans* sauvage a été nommée *Nematocida parisii*. L'interaction entre *N. parisii* et *C. elegans* est devenue un puissant modèle pour l'étude des interactions hôte-pathogène. Cependant, ce modèle a été récemment découvert et de nombreux détails sur son écologie et sa biologie restaient inconnus. Notamment, nous ignorions l'incidence et la diversité des infections microsporidiennes chez *C. elegans* et autres nématodes dans la nature.

A partir d'une collection de nématodes, de la famille des Rhabditidae, échantillonnés dans le monde entier, j'ai recensé un panel de 47 nématodes présentant des symptômes d'infection par des microsporidies. J'ai caractérisé moléculairement la diversité de ce parasite infectant ces nématodes et déterminé que *N. parisii* est la microsporidie la plus souvent responsable des infections chez *C. elegans* dans la nature. J'ai également décrit et nommé six nouvelles espèces de *Nematocida*. Au cours de mes travaux, j'ai aussi défini deux nouveaux genres de microsporidies génétiquement distincts de *Nematocida*, appelés *Enteropsectra* et *Pancytospora*. Mes travaux ont de plus détaillé la diversité qui existe chez les microsporidies parasites de nématodes. Ces microsporidies présentent des différences en terme de taille et forme de leurs spores, de leur tropismes tissulaire et intracellulaire chez l'hôte, de leur voie de sortie des cellules hôtes mais aussi de spectre d'hôtes. Mes résultats ont démontré que, dans la nature, les infections de *C. elegans* et autres nématodes par les microsporidies sont répandues et diverses.

De plus, j'ai estimé la variation naturelle pour la sensibilité de *C. elegans* à l'infection par *N. ausubeli*. J'ai notamment comparé 10 souches naturelles de *C. elegans* en utilisant des tests de consommation alimentaire. Deux souches de *C. elegans*, JU1249 et JU2825, présentaient des niveaux contrastés de sensibilité, ce que j'ai interprété comme étant une différence de niveau de tolérance aux infections. Ces deux souches se sont révélées être de bons candidats pour une future caractérisation des loci génétiques associés à la variation de sensibilité de *C. elegans* aux infections microsporidiennes. Enfin, j'ai observé un effet surprenant de l'infection de *C. elegans* par les microsporidies. En effet, la présence du pathogène est capable de supprimer le

déclin progressif de la fécondité à haute température chez certaines lignées de *C. elegans*.

MOTS CLES: Microsporidies, *C. elegans*, diversité, interactions hôte-pathogène, spectre d'hôtes, sensibilité, variation naturelle

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Introduction

Organisms are driven to evolve continuously by selective pressures from the ever-changing environment (Darwin, 1859). The natural environment includes abiotic and biotic factors. Abiotic factors refer to physical and chemical parameters, such as light, water, temperature. Biotic factors describe all the intraspecific and interspecific interactions between organisms, including those interacting preys and predators, hosts and pathogens, competitors, mutualists. (Woolhouse et al. 2002; Morgan and Koskella, 2011). Though both abiotic and biotic factors could provide selective pressures on species, the latter were highlighted as a fundamental driver of the perpetual evolutionary change (Van Valen, 1973; Brockhurst et al. 2014). The interacting species in biotic interactions may keep adapting to each other and coevolve antagonistically. Among the diverse biotic interactions, host-pathogen interactions probably provide the best case to study counter-adaptations and antagonistic coevolution.

Several host model organisms, such as mice, zebrafish, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Drosophila melanogaster* have been used to study the evolutionary consequences, the mechanisms and the molecular basis of host-pathogen interactions. Among these model organisms, the nematode *C. elegans* has its unique advantages to facilitate studies on hosts and pathogens. Major attributes of *C. elegans* include the small size, the simple growth conditions, the rapid generation time, the genetic tractability and the availability of a great number of genetic mutants (Sulston et al., 1983; Kamath et al., 2003; Stiernagle, 2006). In the last 50 years, *C. elegans* has proven to be a powerful tool for studies on basic biological processes, as well as for investigations on host-pathogen interactions (Kurz and Ewbank, 2000; Zhang and Hou, 2013; Cohen and Troemel, 2015).

C. elegans has been found susceptible to a wide variety of pathogens, including several human pathogens. The first identified natural intracellular pathogen of *C. elegans* is *Nematocida parisii*, which defined a new genus and species of microsporidia (Troemel et al. 2008). The Microsporidia are obligate pathogens with

over 1,400 described species that can infect about half of the metazoan phyla (Vavra and Lukeš, 2013). Microsporidian infections were found quite prevalent in wild *Caenorhabditis* and related nematodes (Troemel et al. 2008; Félix and Duvéau, 2012). Together, *C. elegans* and microsporidia provide an ideal model for the study of host-pathogen interactions and coevolution.

In the **Introduction**, first, I shall briefly present the origin, morphology and life cycle of microsporidia; In the second part, I will present the natural history of *C. elegans* and related nematodes, and the interactions of *C. elegans* with diverse microbes in the natural habitat; third, I will describe the previous and recent studies on the interactions between *C. elegans* and *Nematocida parisii*; in the last part, I will present the background of my PhD project.

I. What are Microsporidia?

The Microsporidia are obligate intracellular parasites that are related to fungi. As a large group of strictly intracellular pathogens, microsporidia species have evolved to be highly adapted to the parasitic life style. They are highly reduced in morphology, metabolism and genome (Vavra and Ronny Larsson, 2014). Within the host cytoplasm (or the nuclear), microsporidia proliferate rapidly and generate large numbers of infectious spores. Spores are the life stage that survives outside hosts, even in harsh conditions. Microsporidia are typically transmitted horizontally, while other transmission modes also exist (Cali and Takvorian, 2014). Microsporidia use the polar tube, a unique highly specialized invasion structure inside the spore, to inject their sporoplasms (the cytoplasmic content and the nucleus of the spore) into the intracellular milieu of their hosts (Vavra and Ronny Larsson, 2014). Microsporidia are found to be pathogens to a vast variety of hosts, ranging across about half of metazoan phyla (Vavra and Lukeš, 2013). Microsporidia infections can cause serious damages in veterinary and agricultural settings, as well as in immunocompromised humans. Furthermore, as one of the most frequently observed pathogens of animals, microsporidia might play significant roles in evolutionary processes (Altizer et al., 2003; Vijendravarma et al., 2009).

I.1. Microsporidia are diverse and important parasites

The study of microsporidia began in the 19th century, when an epidemic disease destroyed the silkworm industry across Europe (Franzen, 2008). Pasteur observed microsporidian spores in the affected silkworms and helped to save the silkworm industry (Franzen, 2008). Today, over 200 genera and over 1,400 species in the

microsporidia phylum have been described, and new species are being identified each year (Becnel et al., 2014; Szumowski and Troemel, 2015). Microsporidia are ubiquitous pathogens infecting a large variety of hosts, including all vertebrate orders, most invertebrates and protists (Keeling and Fast, 2002).

Many microsporidia are of economic importance. Microsporidia infections in some beneficial insects such as honey bees and silkworms cause significant economic losses (Fries, 2014). Fish is another type of microsporidia host with economic importance: to date, 120 microsporidia species have been isolated from fish (Kent et al., 2014). Livestock such as cattle, pigs and goats, pets such as dogs, cats and birds have all been reported to carry microsporidia infections (Fayer and Santin Duran, 2014). More importantly, microsporidia are also opportunistic pathogens to human. To date, 17 microsporidia species are known to infect humans (Fayer and Santin Duran, 2014). The most clinically significant microsporidia species is *Enterocytozoon bieneusi*, which infects the epithelium of the gastrointestinal tract and causes chronic diarrhea and wasting in HIV patients (Akiyoshi et al., 2009). Human microsporidiosis cases were also known to cause ocular infection, nervous system infection, musculoskeletal infection, etc. (Weiss, 2014). Taken together, microsporidia are pathogens with considerable economic and clinical importance.

I.2. Origin of microsporidia

It has long been challenging to place microsporidia in the tree of eukaryotes: microsporidia are highly reduced at many levels compared to other eukaryotes, but they possess a unique complex polar tube as the infection apparatus (Keeling, 2014). The debate about the phylogenetic placement of microsporidia has been going on for decades. Firstly, microsporidia have been proposed to be members of Cnidosporidia, based on their similarities of infection machineries with Helicosporidia and

Myxosporidia. However, the similarities previously known as “homologous” features were later found to be the result from convergent evolution. Secondly, based on their lack of some fundamental eukaryotic characters (including mitochondria, 9 + 2 microtubule structures) and the phylogeny of eukaryotic microbes using small subunit ribosomal DNA (SSU rDNA), microsporidia were suggested to be very ancient eukaryotes, a lineage of Archezoa. However, several mitochondrial-derived genes and mitochondrial remnant “mitosomes” were later found in microsporidia, suggesting that mitochondria went through reduction in the microsporidia lineage (Germot et al. 1996; Katinka et al. 2001; Keeling, 2014; Corradi, 2015). In addition, new phylogenetic analysis based on several protein-coding genes revealed that microsporidia are linked to fungi, which was later supported by phylogenomic analysis (Keeling and Doolittle, 1996; Fast et al., 1999; Capella-Gutiérrez et al., 2012). Now, it is generally accepted that microsporidia are related to fungi (Keeling, 2014).

However, the problem about the phylogenetic placement of microsporidia has not been solved completely: what is the exact nature of the relationship between fungi and microsporidia? Are microsporidia a subclade of fungi or a sister clade to fungi? Within the fungal phylogeny, *Rozella*, a monospecific fungal genus, was placed together with microsporidia as a group in a deep-branching position (Jones et al., 2012; James et al., 2013). This *Rozella*-Microsporidia group might be considered “sister” to other fungi species. Currently, microsporidia are suggested to be members of Cryptomycota, a hyperdiverse phylum encompassing *Rozella*, Aphelids and probably a large number of poorly described species (Figure 1) (James et al., 2013; Corsaro et al., 2014; Keeling, 2014).

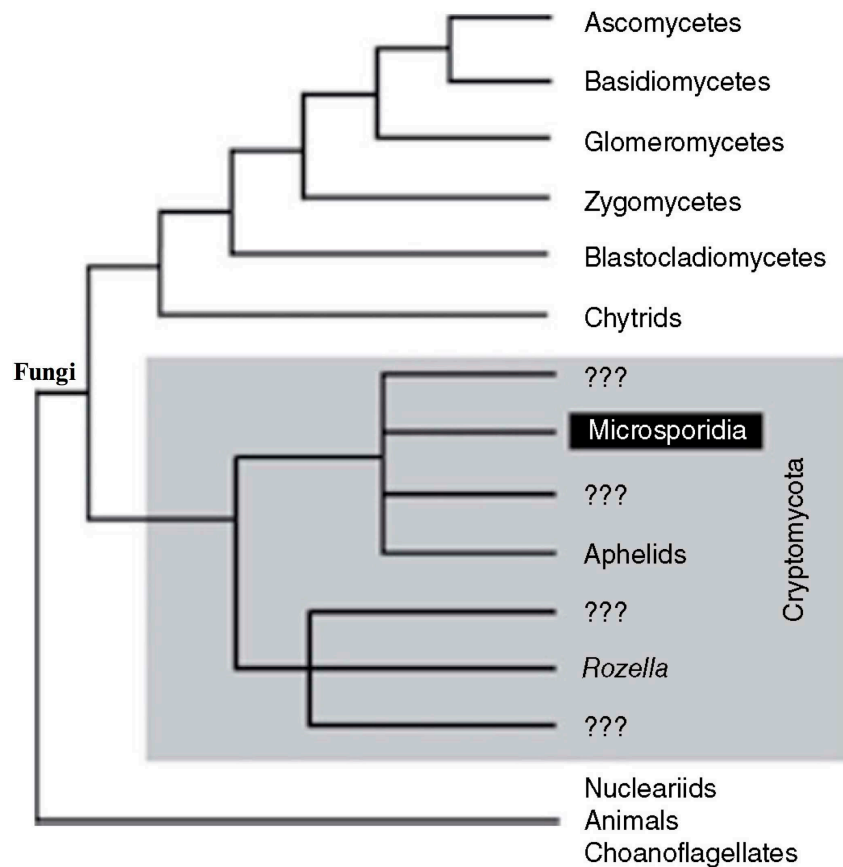


Figure 1. Schematic phylogenetic tree showing the position of microsporidia. The phylogeny is based on the analysis of multiple protein-coding genes. Adapted from Keeling (2014).

I.3. Structure and life cycles of microsporidia

I.3.1. Spore structure

Spore characters formed the basis of microsporidia species recognition and classification. Most microsporidia spores are only a few micrometers long, and can be oval, pyriform, rodlike or spherical in shape (Vavra and Ronny Larsson, 2014). The spore has a thick resistant wall with two layers, called exospore and endospore (Figure 2A, B). Besides the cytoplasmic content and an individual nucleus (or two apposed nuclei) of the spore, the other important part within a spore is the infection apparatus, which is unique to microsporidia and has no obvious homolog in other

organisms (Keeling, 2014). The infection apparatus system consists in a polar tube, a polaroplast and a posterior vacuole (Weiss et al., 2014). The polar tube is threadlike and usually much longer than the length of the spore (Figure 2C). One end of the polar tube is connected to a mushroom-shaped anchoring disk (AD) at the anterior end of the spore. The rest of the polar tube coils around the polaroplast. The number of spore coils has been an important character in species description (Figure 2A) (Weiss et al., 2014). With this unique apparatus, microsporidia are able to inoculate their hosts efficiently in only a few seconds (Frixione et al., 1992).

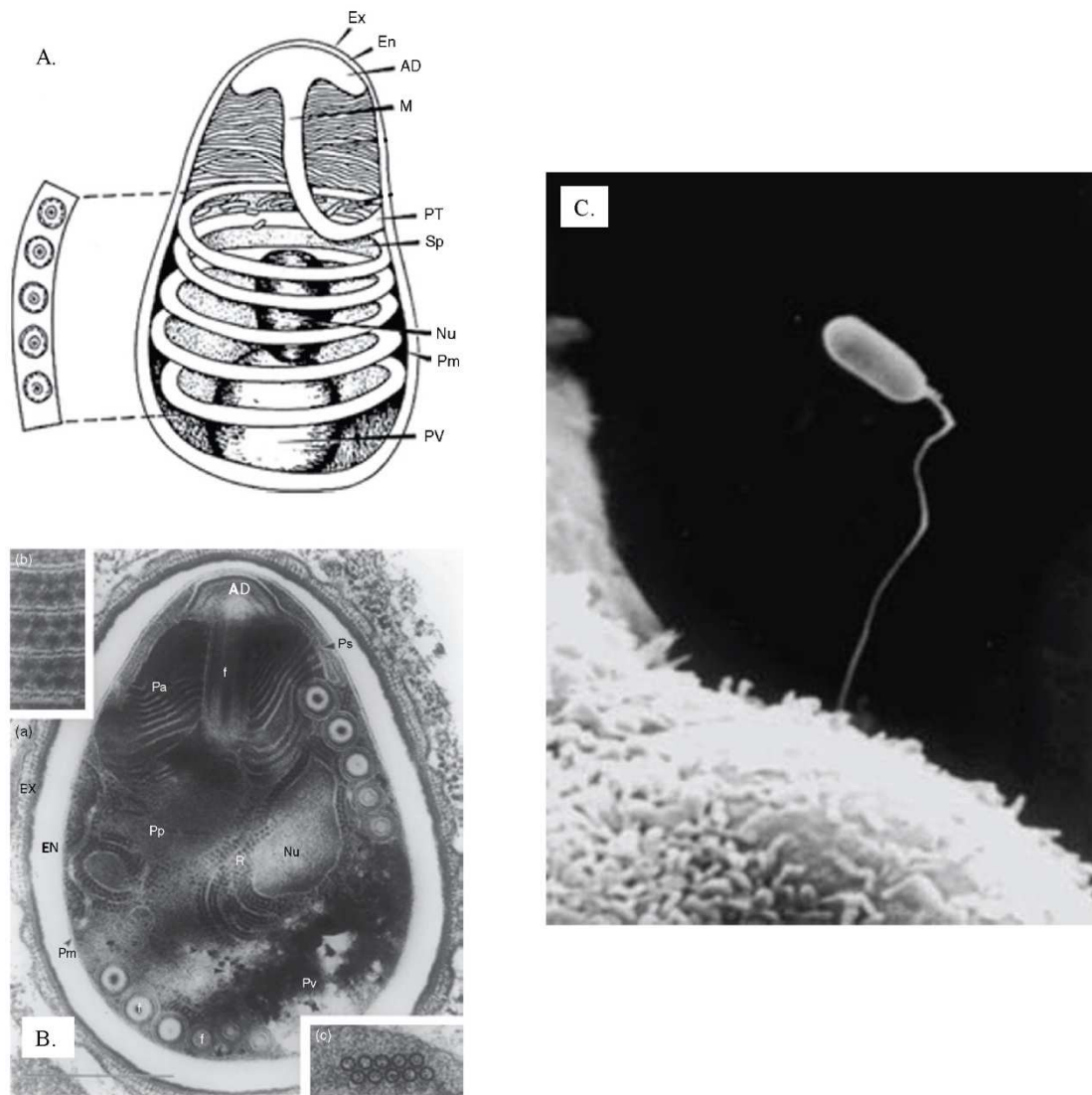


Figure 2. Morphology and structure of microsporidian spores.

A. Diagram of a microsporidian spore. The spore coat consists of an electron-dense exospore (Ex), an electron-lucent endospore (En), and a plasma membrane (Pm). The sporoplasm (Sp) contains a single nucleus (Nu), the posterior vacuole (PV), and ribosomes. The polar tube (PT) is attached to the anterior end of the spore by an anchoring disk (AD) and is divided into two regions: the manubroid or straight portion (M) and the posterior region forming five coils around the sporoplasm. The insert depicts a cross section of the polar tube coils (five coils in this spore). **B.** Ultrastructure of the mature spore. (a) Longitudinally sectioned spore exhibiting the characteristic organelles (arrowhead indicates vacuolar membrane) (*Episeptum inversum*); (b) polyribosomes attached to membranes (*Napamichum dispersus*); (c) circularly arranged polyribosomes (*Hamiltosporidium magnivora*). Scale bars = 0.5 μm (a); 100 nm (b, c). Abbreviations are the same as in A., with, in addition: f, polar tube; Pa, anterior polaroplast region; Pp, posterior polaroplast region; Ps, polar sac; R, polyribosomes. **C.** Scanning electron micrograph of extruded polar tube of a spore of *Encephalitozoon intestinalis* piercing and infecting Vero E6 green monkey kidney

cells in tissue culture. Adapted from Vavra and Ronny Larsson (2014); Weiss et al. (2014).

1.3.2. Life cycles

The life cycle of microsporidia includes extracellular and intracellular phases. In the extracellular phase, microsporidia spores are typically transmitted horizontally through host ingestion and normally infect the hosts through the gut (Vavra and Ronny Larsson, 2014). With chemical and/or physical stimulations, spores germinate. The polaroplast is activated to initiate the evagination of the polar tube. The polar tube forms a channel by extruding from the spore and penetrating into the host cell membrane. At the same time, typically, the posterior vacuole expands to push the infective sporoplasm through the polar tube, into the host cytoplasm (Franzen, 2004; Cali and Takvorian, 2014; Vavra and Ronny Larsson, 2014). Then microsporidia start the intracellular phase of their life cycle.

The intracellular microsporidia develop through the meront stage and the sporont stage, until the formation of mature spores (Figure 3). The sporoplasm injected into the host cell first develops into a meront. Meronts may go through nuclear division and immediate cell division by binary fission. Without immediate cytokinesis, multinucleate meronts will form by multiple rounds of nuclear division in one cell, and eventually divide by plasmotomy, to cells containing one nucleus or double paired nuclei (called diplokaryon). Once meronts have secreted electron-dense material on the external face of the plasma membrane, they enter the sporont stage. The electron-dense material keeps accumulating on the outer face of the sporont, and forms a thickened wall. Depending on the microsporidia species, sporonts may divide once or many times. Multinucleate cells may also form and divide by plasmotomy at this stage. The final division product of the sporont is called sporoblast. The sporoblast continues to grow and becomes a mature spore (Cali and Takvorian, 2014; Vavra and Ronny Larsson, 2014).

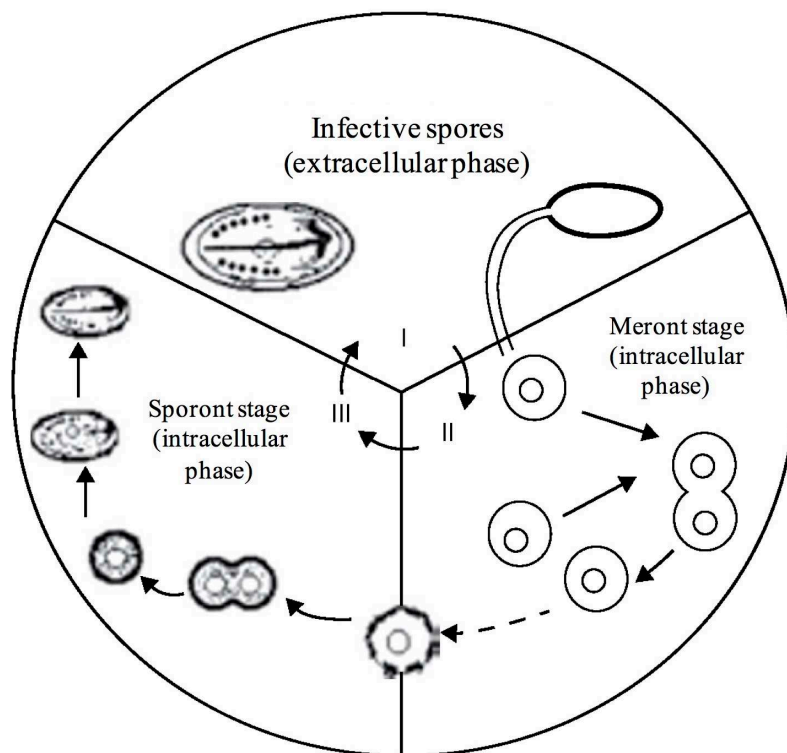


Figure 3. Diagram of a typical microsporidia life cycle.

The life cycle of microsporidia includes extracellular and intracellular phases. Spores are the only life stage that survives outside hosts. The intracellular phase contains the meront stage and the sporont stage. Adapted from Cali and Takvorian (2014).

I.4. The five clades in microsporidia

With a phylogeny based on SSU rDNA, five major clades within microsporidia have been proposed (Figure 4) (Vossbrinck et al., 2014). Though exceptions exist, the groupings of species in different clades largely correspond to their hosts and habitats (Figure 4) (Vossbrinck et al., 2014). Clade I and Clade III mainly contain microsporidia with hosts from freshwater; Clade II and Clade IV microsporidia have terrestrial animals as their hosts; Clade V microsporidia were primarily isolated from

organisms living in marine and freshwater habitats (Figure 4) (Vossbrinck et al., 2014).

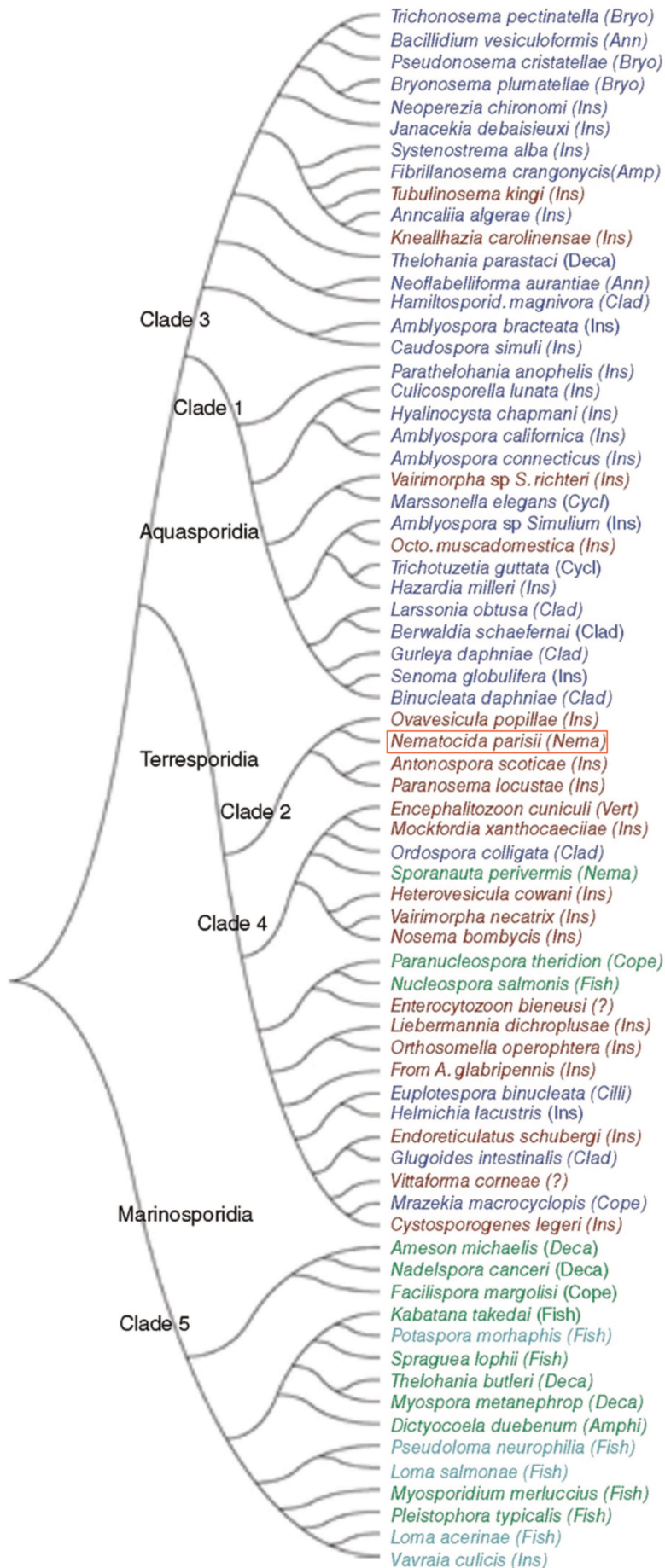


Figure 4. Phylogeny of 71 microsporidia species.

The phylogeny is based on SSU rDNA. Font color indicates the host's habitat: blue for freshwater, brown for terrestrial, green for marine and blue green for hosts that spend part of their life cycle in both marine and freshwater habitats. *Nematocida parisii*, the natural pathogen of *C. elegans*, is marked by a red frame. Adapted from Vossbrinck et al. (2014).

II. *Caenorhabditis elegans* and other related nematodes

The nematode *Caenorhabditis elegans* is a small, free-living nematode that lives across many of the humid temperate areas of the world (Kiontke et al., 2011; Andersen et al., 2012; Frézal and Félix, 2015). In the 1960s, Sydney Brenner first adopted *C. elegans* as a model organism for investigation of a great variety of biological phenomena (Brenner, 1974). Brenner took the Bristol strain N2 as the standard *C. elegans* reference strain and cultured it on agar plates seeded with *Escherichia coli* in the lab (Brenner, 1974; Ankeny, 2001). Ever since then, *C. elegans* has proven to be a powerful tool to study basic biological processes such as apoptosis and gene silencing, as well as biomedical issues such as Alzheimer's disease (Drake et al., 2003; Kaletta and Hengartner, 2006; Seth et al., 2013; Yee et al., 2014; Frézal and Félix, 2015).

The natural ecology of *C. elegans* has also become an emerging research area (Félix and Braendle, 2010; Frézal and Félix, 2015; Petersen et al., 2015). In the natural habitat of *C. elegans* and other related nematodes, biotic factors, in particular the pathogens, may significantly shape the evolutionary process of the nematodes. In this part, I will first present the natural history of *C. elegans* and other related nematodes; then I will describe the interactions of *C. elegans* with its natural pathogens.

II.1. Natural history of *C. elegans*

II.1.1. Natural habitat

The natural history of *C. elegans* was a mystery for a long time. During the first phase, from the 20th century to the beginning of the 21st century, *C. elegans* had been regarded and presented as “a soil nematode”. In fact, only a few *C. elegans* isolates

had been sampled from compost heaps and rich soil with humus, and most sampled *C. elegans* were in the dauer stage, which is an arrested larvae stage (Hodgkin and Doniach, 1997; Barrière and Félix, 2005b, 2007, 2014). The second phase began only recently: large feeding and reproducing populations of *C. elegans* and other nematodes have been collected in rotting fruits and other decomposing plant materials, where *C. elegans* takes bacteria and small eukaryotes as its food sources (Félix and Braendle, 2010; Kiontke et al., 2011; Félix and Duveau, 2012). These rotting substrates in humid temperate areas are now regarded as the natural habitats of *C. elegans* and other related nematodes (Figure 5) (Félix and Duveau, 2012; Frézal and Félix, 2015).

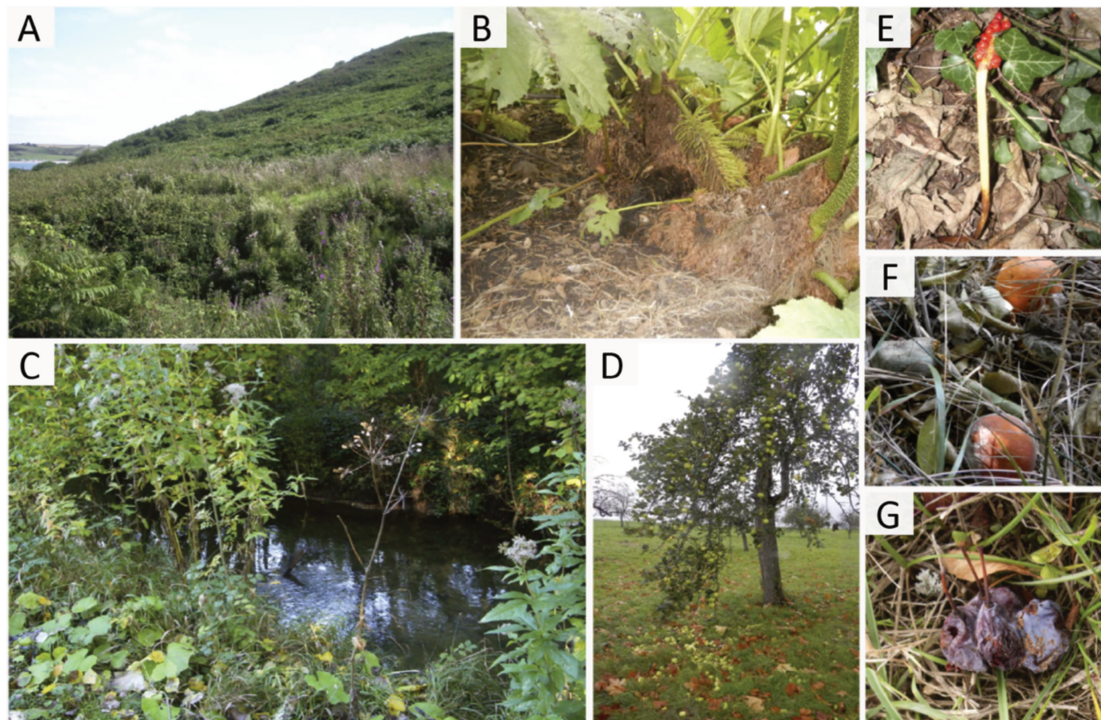


Figure 5. The natural habitats of *C. elegans* at different scales.

(A–D) Landscapes that correspond to the macroscale *C. elegans* habitat; all are relatively humid areas where *C. elegans* has been found: (A) wet shrubland; (B) urban garden; (C) riverbank; and (D) fruit trees. (E–G) Bacteria-rich decomposing vegetal substrates, corresponding to the microscale *C. elegans* habitat: (E) Arum stem; (F) oranges and (G) plums. Adapted from Frézal and Félix (2015).

Probably due to the fact that the food is ephemeral and patchily distributed in space, the *C. elegans* populations have a boom-and-bust life style in the wild (Félix and Duveau, 2012; Frézal and Félix, 2015). With a successful migration to a food source, a single hermaphrodite dauer may bloom into a huge population.

II.1.2. Sex and reproduction

C. elegans has two sexes: the self-fertilizing hermaphrodites (XX) and the facultative males (XO) (Figure 6). Adult males are slender and slightly shorter than adult hermaphrodites (Figure 6A, C). A selfing hermaphrodite can produce about 300 progenies, of which in average about 0.1% are males and all the other are hermaphrodites. Through mating, a hermaphrodite and a male can produce more than 1000 offsprings, with half male progeny and half hermaphrodite progeny (Zarkower 2006). Selfing of hermaphrodites allows the maintenance of homozygous *C. elegans* through generations, whereas mating of the two sexes facilitates genetic variation by recombination. Homozygosity can resume after some rounds of selfing.

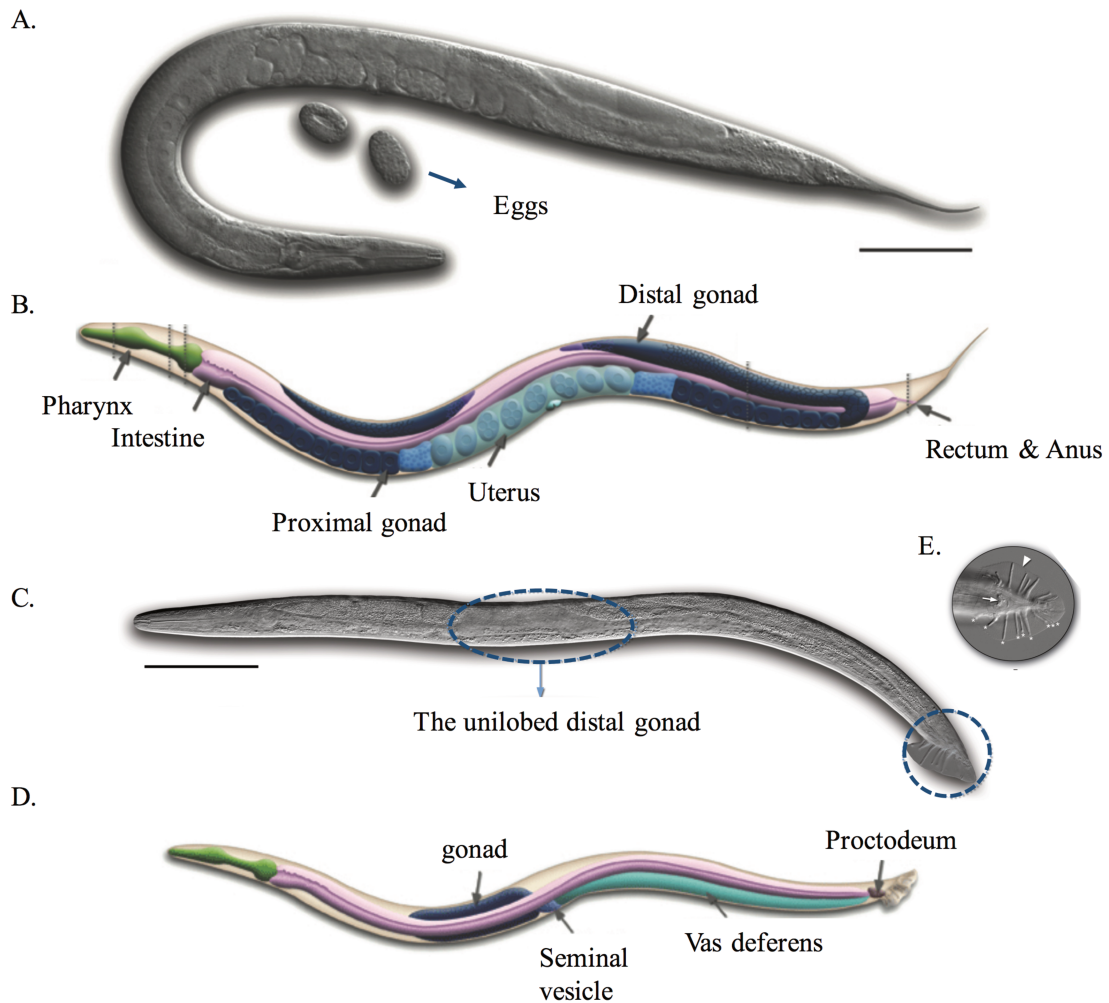


Figure 6. *C. elegans* hermaphrodite and male adults.

A. DIC image of an adult hermaphrodite, left lateral side. Scale bar 0.1mm. **B.** Schematic drawing of anatomical structures of hermaphrodite, left lateral side. **C.** DIC image of an adult male, left lateral side. Scale bar 0.1mm. **D.** Schematic drawing of anatomical structures of male, left lateral side. **E.** The adult male tail, ventral view. Arrow indicates the cloaca; arrowhead indicates the fan. Adapted from Hall and Altun (2009).

II.1.3. Life cycle and development

C. elegans has a short life cycle of 3.5 days at 20 °C and a life span of about 2-3 weeks, when well-fed in the laboratory (Félix and Braendle, 2010). The life cycle of *C. elegans* starts with the fertilized embryo, which develops through the embryonic stage and four larval stages before becoming a new mature hermaphrodite that is

capable of laying eggs, or becoming an adult male capable of fertilizing hermaphrodite adults (Figure 7).

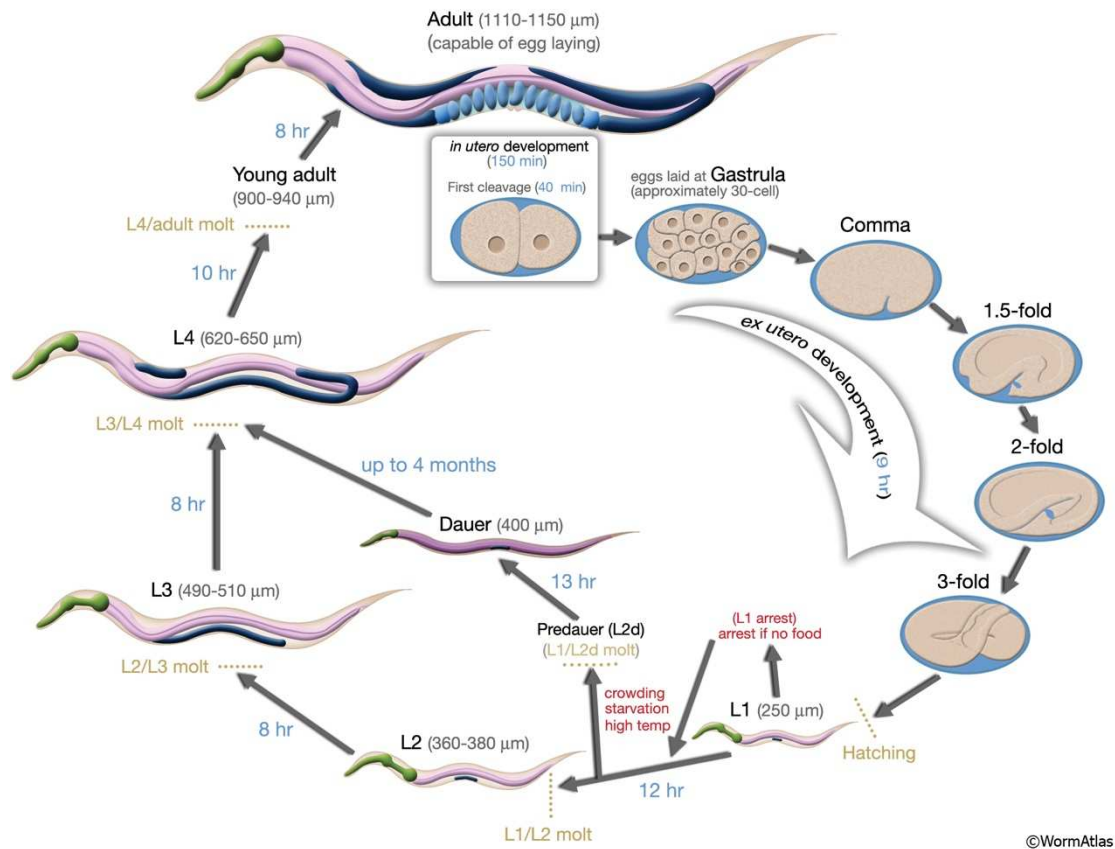


Figure 7. Life cycle of *C. elegans* at 22°C.

0 min is the fertilization. Numbers in blue along the arrows indicate the length of time the animal spends at a certain stage. First cleavage occurs at about 40-minutes post-fertilization. Eggs are laid outside at about 150-minutes post-fertilization. The length of the animal at each stage is marked next to the stage name in micrometers (μm). From Hall and Altun (2009).

II.1.3.1. Embryonic stage

Embryogenesis in *C. elegans* mainly includes cell proliferation and organogenesis. At 22° C, the first cell cleavage occurs 40 minutes after fertilization in the uterus. Eggs with 30 cells are laid at about 150 minutes. At the end of proliferation, the embryo

continues its cell divisions for 350 minutes to generate about 550 essentially undifferentiated cells, with three germ layers: ectoderm, mesoderm and endoderm. Then the organogenesis and morphogenesis stage will last for 6 to 8 hours, after which the embryo develops into an animal with fully differentiated tissues and organs. Here, the new animal is able to move and pump inside the eggshell. At about 800 minutes, the embryo hatches (Hall and Altun, 2009).

II.1.3.2. Post-embryonic stage

Food is vital for all the processes in all the stages in the post-embryonic development of *C. elegans*. In the presence of enough food, the cell division resumes in 3 hours post-hatching (Ambros, 2000; Hall and Altun, 2009). The animal passes four larval stages (L1-L4) and reaches the adult stage in 40-50 hours at 20-25°C (Hall and Altun, 2009). During the larval stages, additional cell divisions of blast cells occur and different systems develop. The larva molts at the end of each stage, with a stage-specific cuticle being shed and resynthesized.

Without food, newly hatched larvae can survive for 6-10 days, but their development is arrested. After food becomes available, these arrested L1 larvae can return to normal development. The dauer larva stage, the other arrested state and an alternative L3 developmental stage of *C. elegans*, occurs if the environmental conditions are not favorable (Figure 7) (Cassada and Russell, 1975; Hu 2007). Limited food source and high population density are the two most significant factors to induce dauers. The dauer larva is very thin and has a thick cuticle that resists to harsh environments. Dauers are capable of surviving for several months without food (Cassada and Russell, 1975). The active nictating behavior of dauers facilitates their migration to new food sources. When the environment becomes favorable for further growth, dauers first undergo the L3 development events, then molt to L4 and continue to develop into reproductive adults. If starvation happens in the L4 stage, some *C. elegans* may enter the adult reproductive diapause (ARD) state. In the reproductive

period in adulthood, if food is absent, the adult hermaphrodites stop laying eggs and may undergo a “bagging” process, in which the embryos hatch inside the uterus and the newly hatched larvae consume the hermaphrodite parent (Angelo and Van Gilst, 2009; Seidel and Kimble, 2011).

II.1.4. Basic anatomy of C. elegans

C. elegans is a tiny roundworm, only 1 mm as an adult. It has an unsegmented, cylindrical transparent body that tapers at both ends. There are several tissues inside the body of *C. elegans*, including the epidermis, the muscles, the nervous system, the alimentary system, the reproductive system (Figures 6, 8) (Altun and Hall, 2009; Corsi et al., 2015). Among the different tissues of *C. elegans*, the epidermis and the intestine are normally used by pathogens as invading routes and tropisms (details in **II.3.2.**). Here, I will describe the anatomy of the alimentary system in more detail.

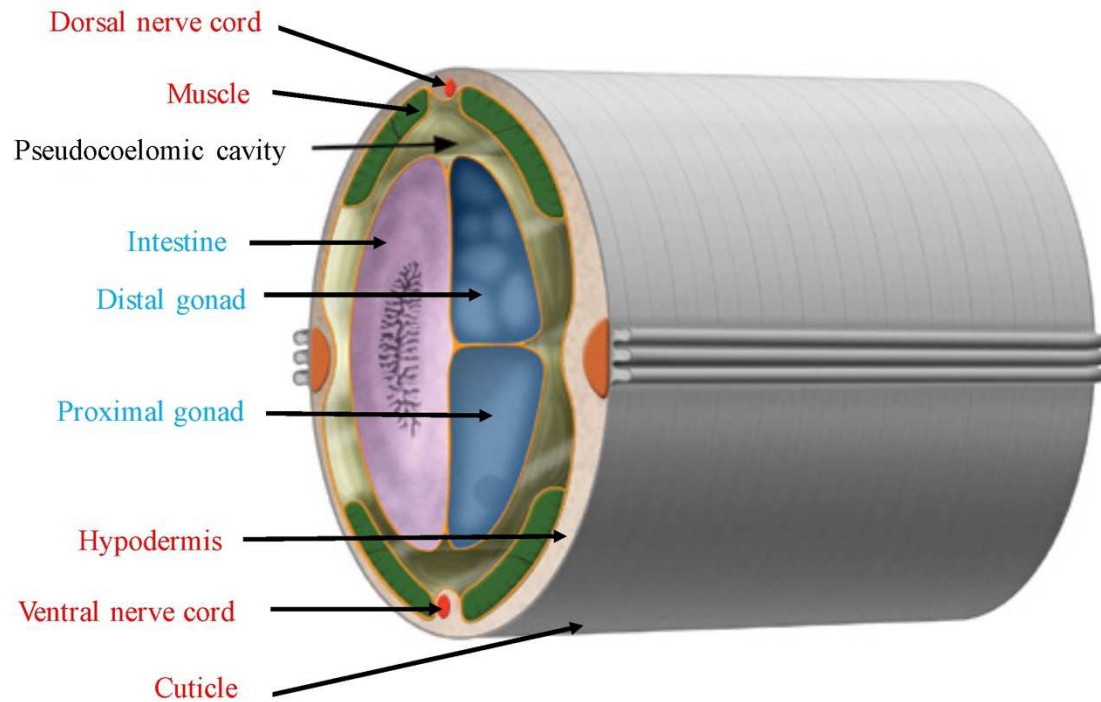


Figure 8. Posterior region of Nematode body.

Diagram of the cross section of the posterior region of nematode body. Adapted from Hall and Altun (2009).

II.1.4.1. The alimentary system

The alimentary tract of *C. elegans* consists of the pharynx, the intestine, the rectum and the anus (Figure 6B). *C. elegans* is a filter-feeder. It pumps food such as bacteria into the pharyngeal lumen. Then the posterior bulb of the pharynx grinds bacteria and transports them into the intestinal lumen (Figure 9A). This grinding behavior may protect the *C. elegans* from some pathogens, as defective grinder mutant *C. elegans* seemed more susceptible to pathogen-induced death (Aballay and Ausubel, 2002).

The intestine is a long tube with a lumen inside, which are formed by nine intestinal rings (int I-IX) of twenty large epithelial cells (Figure 9B, C). The anteriormost ring I is comprised of four cells while each of the remaining eight rings consists of two cells

(Figure 9B). The intestinal cells are polarized with two plasma membrane domains: the apical domain and the basolateral domain (McGhee 2007; Sato et al., 2014). Similar to the morphological characteristics of mammalian intestinal cells, many microvilli extend into the lumen from the apical side of *C. elegans* intestinal cells (Figure 9D, E). This brush border of microvilli is anchored in a conserved structure called the terminal web of actin (with ACT-5 protein) and intermediate filaments (with IFB-2 protein) (MacQueen et al., 2005; McGhee 2007).

The primary function of the intestine is food digestion and nutrient absorption. Nutrients are transferred into intestinal cells through the apical membranes, by endocytosis or special transporters (McGhee, 2007). Numerous granules in intestinal cells store nutrients such as lipids, proteins and carbohydrates. As the intestine is a major route for pathogens to invade into the body of *C. elegans*, the intestinal cells can also act in the defense against pathogens by shedding microvilli and activating the innate immunity pathways (Schulenburg et al., 2004; McGhee, 2007). The rectum and the anus are at the end of the digestive tract, where the final intestinal content wastes are expelled to the outside (Figure 6).

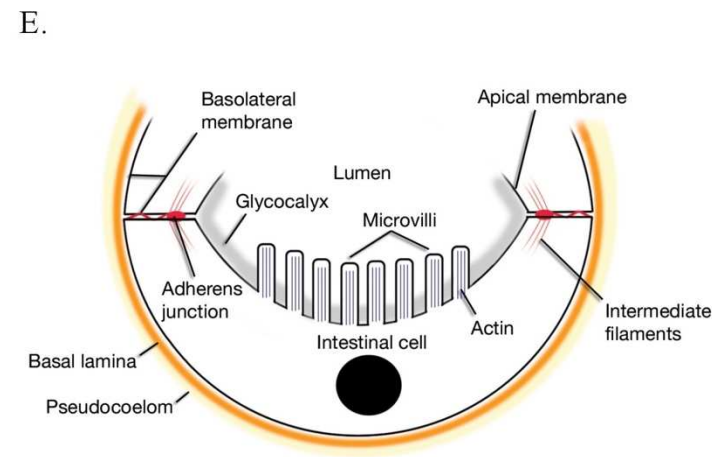
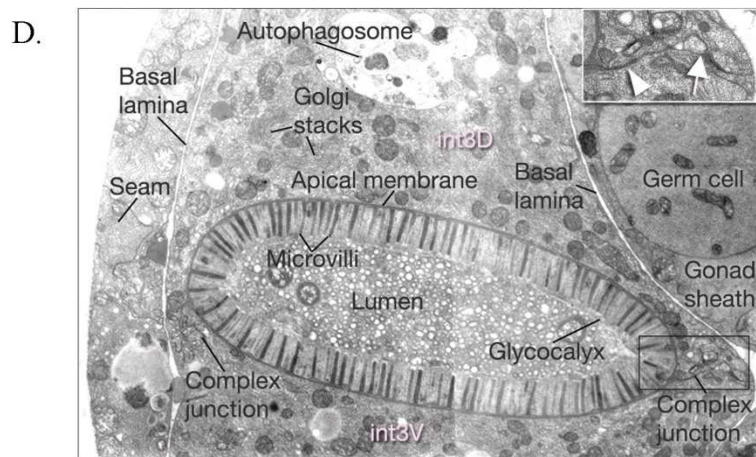
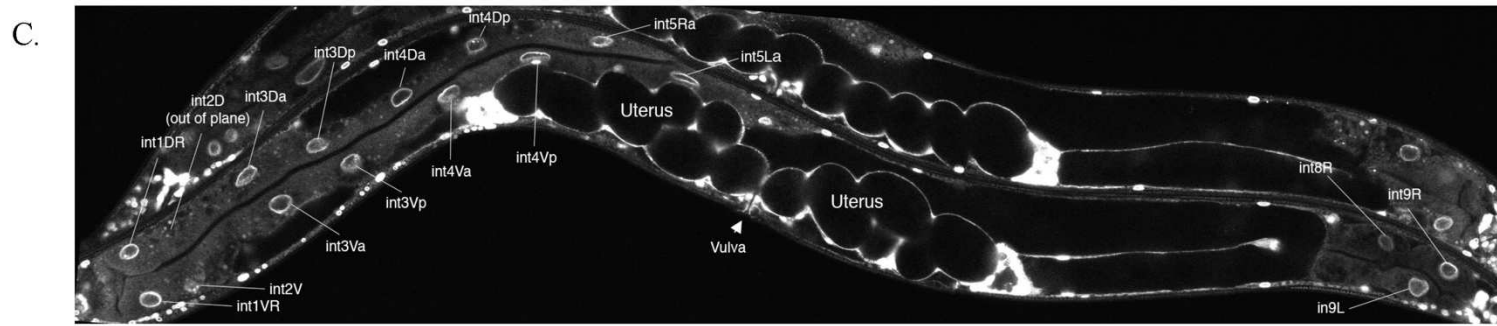
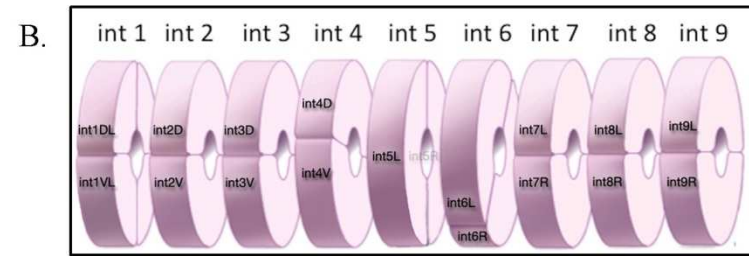
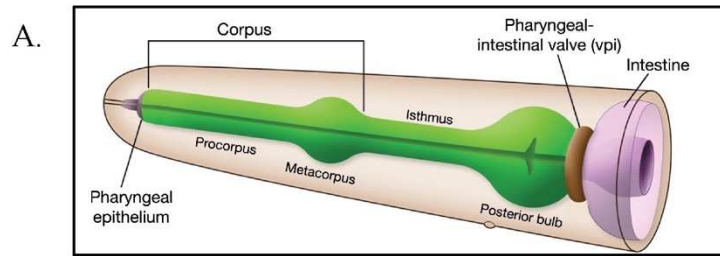


Figure 9. The anatomy of pharynx and intestine in *C. elegans*.

A. Graphic rendition of the pharynx. The posterior bulb, also called the terminal bulb, is connected to the intestine through the pharyngeal-intestinal valve. **B.** Intestinal rings in adult. The intestine has a left-handed twist of 180° around the longitudinal body axis. Cells in the nine rings are indicated depending on positions in the body plan: Left (L), Right (R), Dorsal (D), Ventral (V). **C.** Epifluorescent image of an adult transgenic animal expressing the reporter gene *emr-1::GFP*, where the *emr-1* gene encodes a homolog of the human integral nuclear membrane protein emerin (Howe et al., 2015). **D.** Ultrastructure of the third intestinal cell ring. A complex junction (arrow) next to an adherens junction (arrowhead) seals the two intestinal cells to each other (inset). **E.** Diagram of a cross section through an intestinal cell. Adapted from Altun and Hall (2009a, 2009b).

II.1.5. A model organism in the wild

Researchers have made fruitful discoveries with the *C. elegans* reference strain N2 or mutants in the N2 genetic background. However, as N2 has been maintained under similar laboratory conditions for several decades, this strain has evolved and become distinct from its wild progenitor (Sterken et al., 2015). For instance, in the standard culture plates, wild *C. elegans* display a clumping behavior: wild isolates tend to aggregate with each other, especially on the border of the *E. coli* lawn (Figure 10, right) (Gray et al., 2004; Barrière and Félix, 2005a). Wild *C. elegans* clump together, because they prefer a 7-14% oxygen concentration in the microenvironment. By clumping at the border of the bacterial lawn, they can reduce the local O₂ concentration (Cheung et al., 2005; Rogers et al., 2006). However, N2 does not show this clumping behavior (Figure 10, left). This phenotypic polymorphism was further attributed to a single nucleotide polymorphism on the *npr-1* (gene *npr-1* encodes a neuropeptide Y-like G-protein-coupled receptor) (de Bono and Bargmann, 1998; Barrière and Félix, 2005a; McGrath et al., 2009).

To have a more comprehensive understanding of the *C. elegans* species, other wild *C. elegans* strains have been used. The most widely studied natural *C. elegans* strain is

CB4856, which was collected from Hawaii. This strain is genetically distant to the N2 strain. CB4856 also shows a great variety of polymorphisms compared to other strains (Koch et al., 2000; Andersen et al., 2012; Vergara et al., 2014). By comparing different strains, the genetic basis of several traits has been found. For instance, after mating, males of CB4856 and some other natural *C. elegans* isolates deposit a copulatory plug that covers the hermaphrodite vulva, which is known as the plugging phenotype. The plug can decrease the mating success of subsequent males. However, some other wild *C. elegans* strains and N2 display the non-plugging phenotype. Mapping approaches between CB4856 and N2 revealed that the plugging phenotype variation results from the polymorphism of the gene *plg-1* (Hodgkin and Doniach 1997; Palopoli et al., 2008).

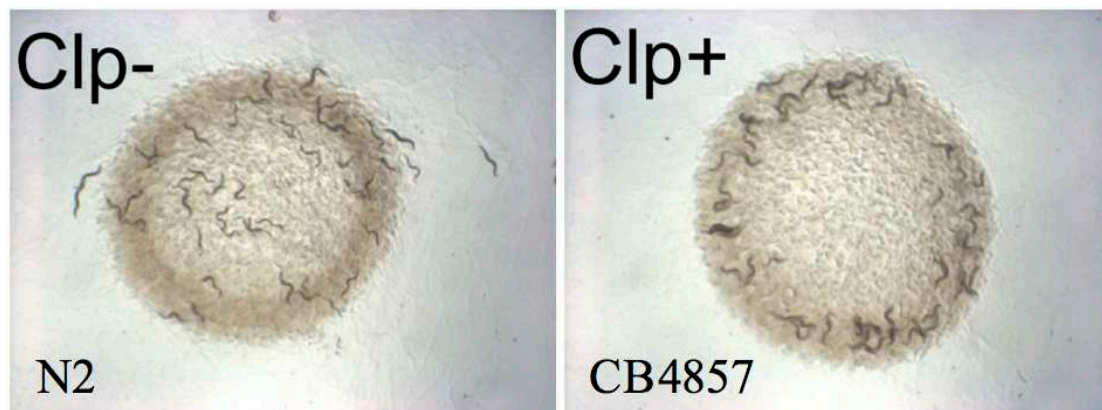


Figure 10. Phenotypic polymorphisms between N2 and wild *C. elegans* strains. N2 (left) and CB4857 (right) display distinct clumping (Clp) behavior. Adapted from Barrière and Félix (2005a).

II.2. Other nematodes

C. elegans is the best-known nematode species, but the phylum Nematoda is an extremely diverse group of animals (Figure 11). About 30,000 species have been described in Nematoda, the total number of which is estimated up to a million (Kiontke and Fitch, 2013). Nematodes are probably the most highly ecologically and physiologically adaptable metazoans, since they were found everywhere, including desert soil, polar ice, tropical rainforest and deep sea (Félix et al., 2013; Kiontke and Fitch, 2013). Nematodes are not just free-living species like *C. elegans*. Pathogenic nematodes can infect almost all other multicellular organisms, including most plants, arthropods and vertebrates (Blaxter, 2011; Kiontke and Fitch, 2013). I will further describe some free living nematodes that are closely related to *C. elegans*.

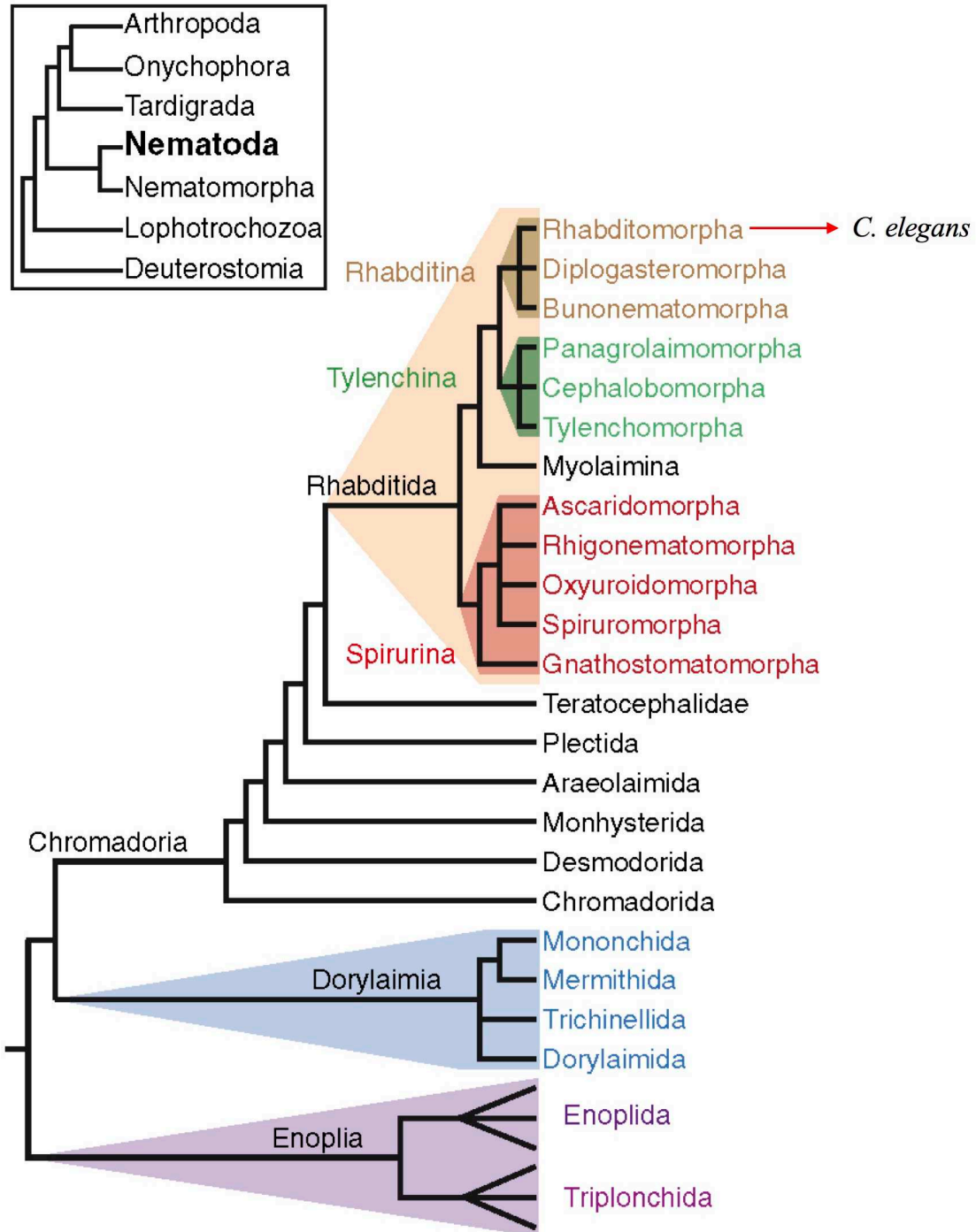


Figure 11. Phylogeny of Nematoda.

The position of nematodes in the animal tree is indicated in the inset. The main panel is the phylogeny of Nematoda, which contain three large monophyletic groups: Enoplia, Dorylaimia and Chromadoria. *C. elegans* belongs to the infraorder Rhabditomorpha (suborder Rhabditina, order Rhabditida, family Rhabditidae), which

is indicated by an arrow. The phylogeny of Nematoda is based on SSU rDNA. Adapted from Kiontke and Fitch (2013).

II.2.1. Free-living rhabditid nematodes

C. elegans and its close relatives are in the family Rhabditidae (Figures 11, 12). Most rhabditids are bacteriophagous nematodes, but this group also includes a group of vertebrate parasites such as the human hook worm *Necator americanus* (Kiontke and Fitch, 2005; Blaxter, 2011). In addition to *C. elegans*, this group also includes several “satellite models”, such as various other *Caenorhabditis* species, *Oscheius tipulae* and *Pristionchus pacificus*, which facilitate comparative biological studies (Kiontke and Fitch, 2005; De Ley, 2006). There are two major clades in rhabditids: “Pleiorhabditis” and “Eurhabditis”, and the latter clade contains *Caenorhabditis* and *Oscheius* (Figure 12) (Kiontke and Fitch, 2005).

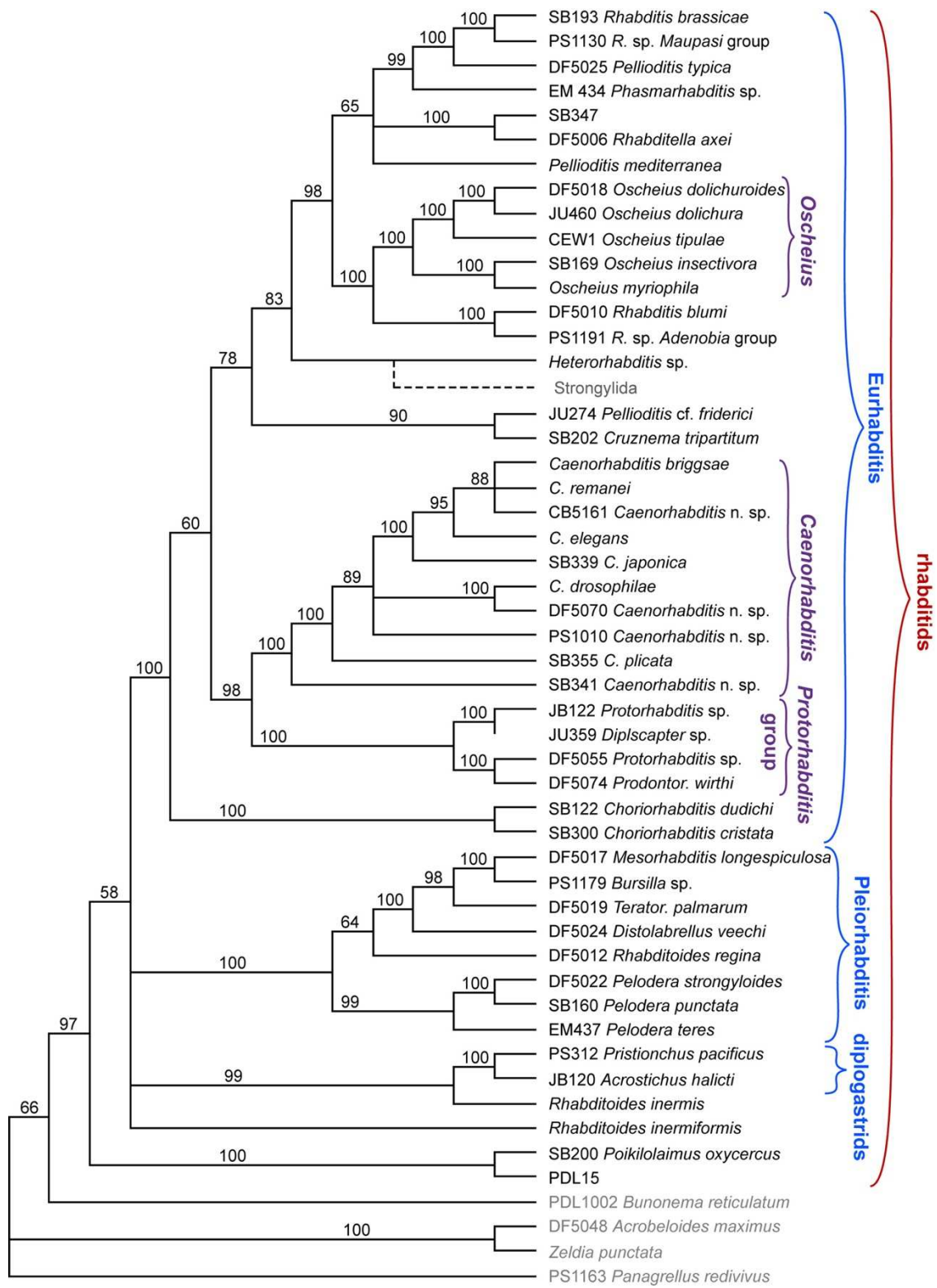


Figure 12. Phylogeny of rhabditid nematodes.

There are two major clades in rhabditids: Pleiorhabditis and Eurhabditis. The latter clade includes the *Oscheius* genus and the *Caenorhabditis* genus. The phylogeny is based on SSU rDNA and large subunit ribosomal DNA (LSU rDNA). From Kiontke and Fitch (2005).

II.2.2. The *Caenorhabditis* genus

Due to the improved knowledge on the wild micro-habitats of *Caenorhabditis* and intensive worldwide sampling, many new *C. elegans* isolates and new *Caenorhabditis* species have been discovered. To date, 27 *Caenorhabditis* species have been isolated from different areas around the world (Figure 13) (Kiontke et al., 2011; Félix et al., 2014; Cutter, 2015; Frézal and Félix, 2015). Some *Caenorhabditis* are cosmopolitan: *Caenorhabditis briggsae* is the most widespread, found abundantly in both tropical and temperate areas; most *C. elegans* and all *Caenorhabditis remanei* have been found in temperate regions (Figure 13). *Caenorhabditis brenneri* and *Caenorhabditis tropicalis* have been found exclusively in tropical areas (Kiontke et al., 2011; Cutter, 2015). Among these 27 species, some species have been found in the same location, for instance, *C. elegans* and *C. briggsae* have been found coexisting, even in the same apple in Orsay, France (Félix and Duveau, 2012); *C. elegans* and *C. remanei* have been found coexisting in North Germany (Petersen et al., 2014).

The species in the genus *Caenorhabditis* can be divided into three groups: *Elegans* group, *Drosophilae* group and *Japonica* group (Figure 13). Most collected strains belong to the *Elegans* group. *C. elegans* and *C. briggsae* are the two most highly sampled species (Figure 13) (Cutter, 2015). These two species are quite similar in morphology, life style, behavior and genome (about 104Mb) (Stein et al., 2003; Baird, 2006; Gupta et al., 2007). Nevertheless, there are many species-specific behaviors and genes between *C. elegans* and *C. briggsae*. For instance, similar to N2, *C. briggsae* has a non-clumping behavior on standard culture plates (Barrière and Félix, 2014). Relatively, *C. briggsae* prefers higher temperature, while *C. elegans* prefers lower temperature (Félix and Duveau, 2012). This temperature preference of *C. briggsae* may due to a recent expansion of *C. briggsae* from tropical to temperate areas, which provides a good example to study species distribution, gene flow and genome evolution (Baird, 2006; Cutter et al., 2006, 2010a, 2010b).

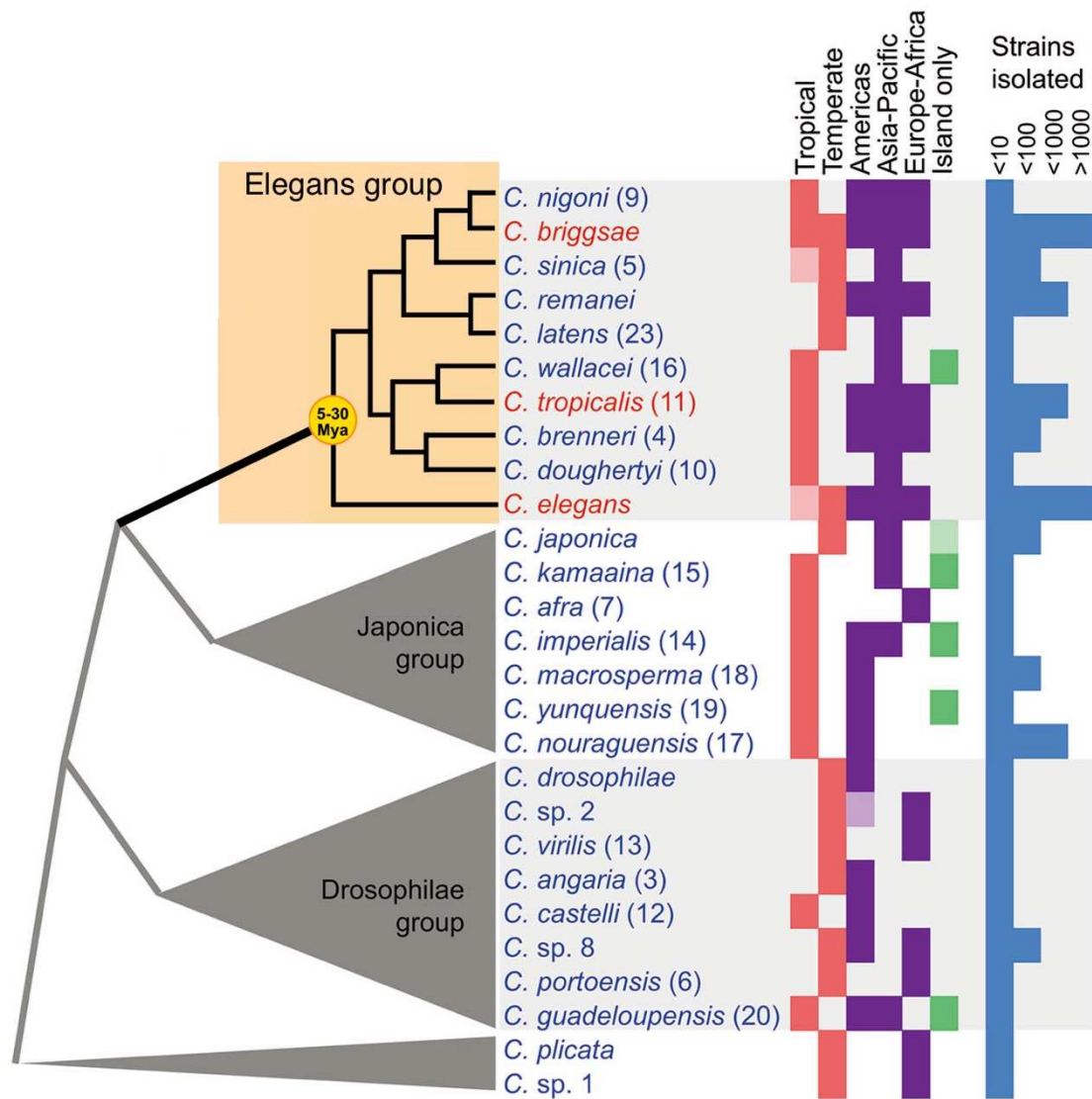


Figure 13. Phylogeny of *Caenorhabditis*.

The main panel shows the phylogeny of the major *Caenorhabditis* groups with 27 species (former numerical identifiers in parentheses). The three species with selfing mode of reproduction are in red, while the remaining known *Caenorhabditis* species are male and female. Geographic sampling locations and number of isolated strains are shown on the right of the phylogeny. Adapted from Cutter (2015); Frézal and Félix (2015).

II.2.3. *Oscheius tipulae*

Oscheius tipulae is another model organism. Morphologically, the animals have a darker color, smaller size and longer rectum, compared to *C. elegans* (Félix, 2006; Barrière and Félix, 2014). *O. tipulae* shares many similarities with *C. elegans*, such as the reproduction mode of self-fertilizing hermaphrodites and facultative males, and the culture condition in the laboratory. It has been used in comparative studies with *C. elegans*, especially on vulva development (Félix et al., 2000; Dichtel-Danjou and Félix, 2004).

Except for very dry or very cold areas, *O. tipulae* is widely distributed at different spatial scales all over the world. Dauer stage *O. tipulae* was found abundant in soil, composts and invertebrates (Baïlle et al., 2008); *O. tipulae* in proliferative stages was also found in rotting fruits and stems (Félix and Duveau, 2012). *O. tipulae* has two morphologically indistinguishable close relatives: *Oscheius* sp. 2 and *Oscheius* sp. 3 (Figure 14) (Félix et al., 2001). However, these two *Oscheius* species were only found in limited locations (Félix et al., 2001; Baïlle et al., 2008). Interestingly, a study on the natural populations of *O. tipulae* showed a relatively high genetic diversity, which may be better suited for further studies on molecular evolution (Baïlle et al., 2008).

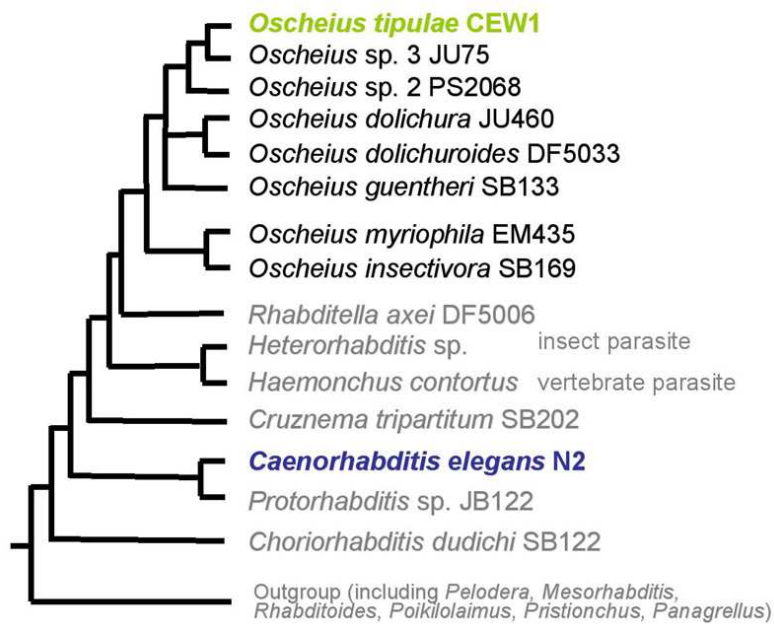


Figure 14. Phylogenetic relationships in the *Oscheius* genus.
From Félix (2006).

II.3. Interactions of *C. elegans* with diverse microorganisms

In the wild, *C. elegans* has a great variety of interactions with several other rhabditid nematodes (such as *C. briggsae*, *C. remanei* and *O. tipulae*), diverse animals (arthropods in particular) and many microorganisms (such as bacteria, fungi). For instance, since the food sources are transient, intraspecific competition for food among bacterivore nematodes may be substantial (Félix and Braendle, 2010).

Diplogastrid nematodes, such as *Pristionchus* species, may be predators of *C. elegans* and other bacterivore nematodes (Félix and Duveau, 2012; Barrière and Félix, 2014).

Some mollusks and arthropods may serve as dispersal vectors for *C. elegans* and related nematodes, while other arthropods such as mites may act as vector as well as predator (Félix and Braendle, 2010; Félix and Duveau, 2012; Cutter, 2015; Frézal and

Félix, 2015). The most notable interactions that *C. elegans* has are with microorganisms. The various microbes may act as food, commensal microorganisms, pathogens or predators, to *C. elegans* and other nematodes (Petersen et al., 2015). In the following parts, I will describe the interactions of *C. elegans* with the non-pathogenic and pathogenic microbes.

II.3.1. The interaction of C. elegans with non-pathogenic microbes

In the laboratory, N2 is usually fed with *E. coli* strain OP50. N2 may not carry any live microbes in its gut due to *E. coli* being disrupted by the animal's grinder. In the gut of wild *C. elegans*, however, a live bacterial flora could often be observed (Félix and Braendle, 2010; Félix and Duveau, 2012). This is not a surprise, as wild *C. elegans* live in the environments that are full of decaying organic material, which are food resources for microbes. However, this also means that some microbes may be able to pass the grinder of *C. elegans*. Using high-power light microscope, assemblages of bacteria and fungi can be seen colonizing the rotting fruits and stems (Félix and Duveau, 2012). Wild *C. elegans* may encounter a great variety of microbes through its whole life history.

A very recent study revealed that the most abundant bacteria in the natural environment of *C. elegans* belong to phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria* (Samuel et al., 2016). Several studies also characterized the microbial community that is directly associated with *C. elegans*, which was dominated by *Proteobacteria* such as unclassified *Enterobacteriaceae* and members of *Pseudomonas*, *Stenotrophomonas*, *Ochrobactrum*, *Sphingomonas* and *Xanthomonadaceae* (Montalvo-Katz et al., 2013; Berg et al., 2016; Dirksen et al., 2016).

C. elegans displays different responses to different microbial communities: *C. elegans* populations proliferate better in an alpha-Proteobacteria rich environment, but generate more dauers in a detrimental *Bacteroidetes* rich community (Samuel et al., 2016). Some associated microbes may also be able to increase the defensive ability of *C. elegans* in the presence of pathogens. For example, *Bacillus megaterium* and *Pseudomonas mendocina* can enhance the resistance of *C. elegans* to the pathogen *Pseudomonas aeruginosa* (Montalvo-Katz et al., 2013; Dirksen et al., 2016).

More importantly, *C. elegans* uses some bacteria and yeast in its natural habitat as food resources (Félix and Duvéau, 2012; Frézal and Félix, 2015). The exact food source of bacteria or yeast species of *C. elegans* in the wild is unclear, but different diets can affect *C. elegans* life history traits such as longevity and growth rate (Macneil and Walhout, 2013). Some microbes may decrease the growth rate or longevity of *C. elegans*, while other microbes may show directly pathogenic effects. It may be difficult to disentangle pathogenic effects from bad dietary effects, but it is clear that overlaps exist between dietary, commensal and pathogenic microbes (Macneil and Walhout, 2013; Clark and Hodgkin, 2014). The non-pathogenic microbes colonizing the gut may improve the resistance of *C. elegans* to its true pathogens.

II.3.2. The interaction of C. elegans with its pathogens

Diverse pathogens are able to infect *C. elegans*. In the wild environment, several microbes have been characterized as natural pathogens to *C. elegans* and other nematodes (Figure 15). These pathogens infect *C. elegans* mainly via two different routes: the external surfaces or the oral route. For instance, the fungus *Drechmeria coniosporia* and the bacterium *Leucobacter* species adhere to the cuticle (Félix and Duvéau, 2012; Hodgkin et al., 2013; Frézal and Félix, 2015). The fungus

Harposporium sp., the bacteria *Serratia marcescens* and *Microbacterium nematophilum*, the Orsay virus and the microsporidia *Nematocida parisii* infect *C. elegans* via the oral route (Hodgkin, 2000; Troemel et al., 2008; Félix et al., 2011; Félix and Duveau, 2012; Parsons and Cipollo, 2014). The latter two pathogens further induce intracellular infections in intestinal cells. In addition to its natural pathogens, *C. elegans* has been found susceptible to a large number of “universal” pathogens, including some human pathogens (Kurz and Ewbank, 2000; Sifri et al., 2005). Gram-negative bacterial pathogens such as *Pseudomonas aeruginosa*, *Salmonella enterica* and *Salmonella typhimurium*, Gram-positive bacterial pathogens *Enterococcus faecalis*, *Staphylococcus aureus* and *Bacillus thuringiensis* could all cause intestinal infections and kill *C. elegans* (Figure 15) (Aballay and Ausubel, 2002; Darby, 2005).

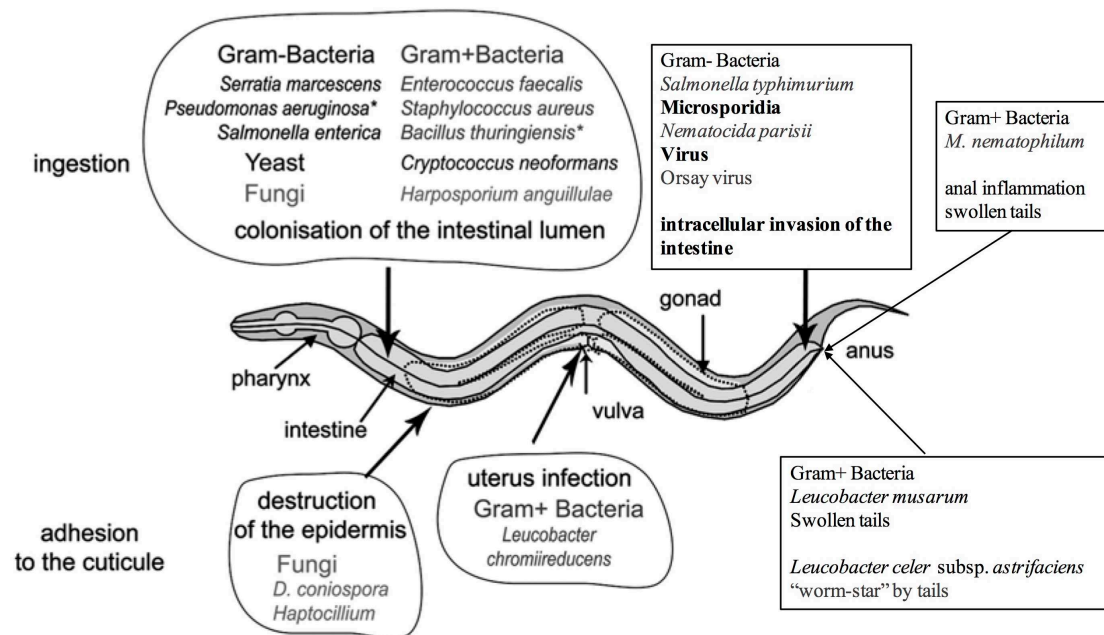


Figure 15. Pathogens of *C. elegans* and their infection routes and tropisms.
Adapted from Engelmann and Pujol (2010).

C. elegans with its pathogens, especially its natural pathogens in contact with which its immune system evolved, makes a powerful model for host-pathogen studies. In the following part of this chapter, I will first present briefly the different defensive responses of *C. elegans*. Then I will describe in more detail the interactions of *C. elegans* with its natural pathogens: the newly found *Leucobacter* species and the newly discovered intracellular viral infections in *Caenorhabditis*. The studies on the interaction between microsporidia and *C. elegans* will be presented in detail in Chapter III.

II.3.2.1. Defense of *C. elegans* against pathogens

To defend itself, *C. elegans* mainly uses three strategies: the avoidance behavior response, physical barriers and the innate immunity (Schulenburg et al., 2004; Engelmann and Pujol, 2010).

A. Behavioral avoidance

C. elegans is a bacterivore with a foraging behavior. In a natural microbe-rich habitat, it is vital for *C. elegans* to recognize non-pathogenic food from harmful pathogens. *C. elegans* has 60 ciliated sensory neurons, functioning in chemosensation and mechanosensation, which may enable the recognition of *C. elegans* to different microbes (Allen et al., 2015). For instance, while some *S. marcescens* attract *C. elegans*, the cyclic pentapeptide biosurfactant serrawettin W2 expressed by *S. marcescens* strain Db10 leads to an avoidance behavior of *C. elegans* (Pujol et al., 2001; Pradel et al., 2007). This innate avoidance of *C. elegans* was also observed in the presence of *M. nematophilum* (Yook and Hodgkin, 2007). Despite these innate repulsive responses, *C. elegans* also showed aversive learning avoidance to *P. aeruginosa* and *S. marcescens*. Though initially *C. elegans* was attracted to the odors produced by *P. aeruginosa* and *S. marcescens*, it could modify its olfactory preference and learn to avoid the pathogenic lawns (Zhang et al., 2005; Beale et al., 2006). The avoidance behavior of *C. elegans* to *P. aeruginosa* was also reported to be

affected by the mechanosensation of *C. elegans* (Chang et al., 2011). The sensory system mediates both the food seeking and pathogen avoidance behaviors (Meisel, 2014).

B. Physical barriers

C. elegans has a strong collagenous cuticle, which protects itself from environmental threats, especially from the invasion of some pathogens. In addition, the pharyngeal grinder could destroy live pathogens before infection occurs. However, some pathogens could break the physical barrier of *C. elegans*. For instance, spores of the fungal pathogen *D. coniospora* mainly adhere to the cuticle in the head or around the vulva of *C. elegans*, and then penetrate the cuticle. *S. marcescens* can disrupt the grinder, enter and colonize the intestine of *C. elegans*; spores of microsporidia and some other pathogens resist to the pharyngeal grinding (Darby, 2005; Engelmann and Pujol, 2010; Taffoni and Pujol, 2015).

C. Innate immunity

C. elegans monitors physiological perturbations such as epidermal injury, DNA damage, inhibition of transcription or translation, mitochondrial dysfunction. The surveillance on homeostasis could act as a trigger for the initiation of immune defense (Taffoni and Pujol, 2015; Ewbank and Pujol, 2016; Pukkila-Worley, 2016).

Several signaling pathways have been shown to be involved in innate immunity in *C. elegans*, such as the mitogen-activated protein kinase (MAPK) pathways, the DAF-2/DAF-16 insulin-like signaling pathway, the transforming growth factor β (TGF- β) pathway. (Ewbank 2006; Engelmann and Pujol, 2010; Kim and Ewbank, 2015). The diverse signaling pathways control the expression of many immune effectors, including the antimicrobial peptides (AMP), the caenacins (CNC), lysozymes, lectins. (Engelmann and Pujol, 2010; Kim and Ewbank, 2015).

II.3.2.2. *Leucobacter* species

Leucobacter are Gram-positive, non-motile bacteria that have been isolated from diverse environments, including chromium-rich soil and water, foodstuffs, nematodes (Morais et al., 2006; Muir and Tan 2007; Shin et al., 2011; Ge et al., 2013). Recently, a new subspecies of *Leucobacter celer* and a new species named *Leucobacter musarum* with its two subspecies were isolated from wild *Caenorhabditis* (Hodgkin et al., 2013). The *Leucobacter celer* subsp. *astrifaciens* CBX151 and the *Leucobacter musarum* subsp. *musarum* CBX152 were isolated co-infecting a *C. tropicalis* strain (JU1635) from Cape Verde. This infected *C. tropicalis* strain showed a swollen tail response that referred to as the Dar (Deformed Anal Region) phenotype, and bacterial coating over the cuticle (Figure 16A). The *Leucobacter musarum* subsp. *japonicus* was isolated from a wild *C. elegans* strain (JU1088) in Kakegawa, Japan. This infected *C. elegans* strain exhibited the Dar phenotype (Hodgkin et al., 2013; Clark and Hodgkin, 2015).

The *Leucobacter* species displayed diverse interactions with *C. elegans*. On the surface of an agar plate, the *L. celer astrifaciens* CBX151 infection can inhibit the movement and growth of *C. elegans*, but is not lethal. In liquid culture, *L. celer astrifaciens* CBX151 infection induces formation of “worm-stars”, where *C. elegans* individuals aggregate by their tails, and then leads to death of most trapped animals (Figure 16B). However, within the first hour after the star formation, some trapped *C. elegans* individuals are able to escape the worm-stars, albeit with broken tail spikes. These wounded *C. elegans* will be healed later and be able to survive. Additionally, a small proportion (5-10%) of L4 stage *C. elegans* individuals could escape the star even later (24 hours after star formation) through a form of autotomy. By autotomy, an anterior “half-worm” could escape and remain viable for several days, with the possibility to have a few selfing progenies (Hodgkin et al., 2013, 2014). On the other hand, *L. musarum musarum* CBX152 is lethal to *C. elegans* on the agar plate only at

higher temperature. At 25° C, *L. musarum musarum* CBX152 infection causes large vacuoles that distort the internal organs and distend the whole body of *C. elegans*, resulting in death of most animals within 24-48 h (Figure 16C). At lower temperature, *L. musarum musarum* CBX152 causes the Dar phenotype in *C. elegans* (Figure 16D).

The Dar phenotype was also observed in *C. elegans* infected with *Microbacterium nematophilum*, a Gram-positive bacterium that specifically infects *C. elegans* and some other nematodes (Hodgkin, 2000). Previously, through chemical and transposon mutagenesis, several *C. elegans* mutants that are resistant to *M. nematophilum* were selected (Gravato-Nobre et al., 2005; Yook and Hodgkin 2007). Most of these mutants were also found to resist to *L. musarum musarum* CBX152 (Hodgkin et al., 2013). Mutants such as *bus-2*, *bus-4* were shown to have an altered surface glycosylation, which may affect the adhesion of *L. musarum musarum* CBX152 to the cuticle of *C. elegans* (Gravato-Nobre et al., 2011).

However, on the agar surface, *L. celer astrifaciens* CBX151 can efficiently kill the *C. elegans* mutants that resist to *L. musarum musarum* CBX152. Furthermore, *C. elegans* isolates primarily infected with *L. celer astrifaciens* CBX151 seemed more resistant to *L. musarum musarum* CBX152 infection. Altogether, these observations revealed the complementary virulence between the two *Leucobacter* pathogens, which may generate opposing selective forces on *C. elegans* (Hodgkin et al., 2013, 2014).

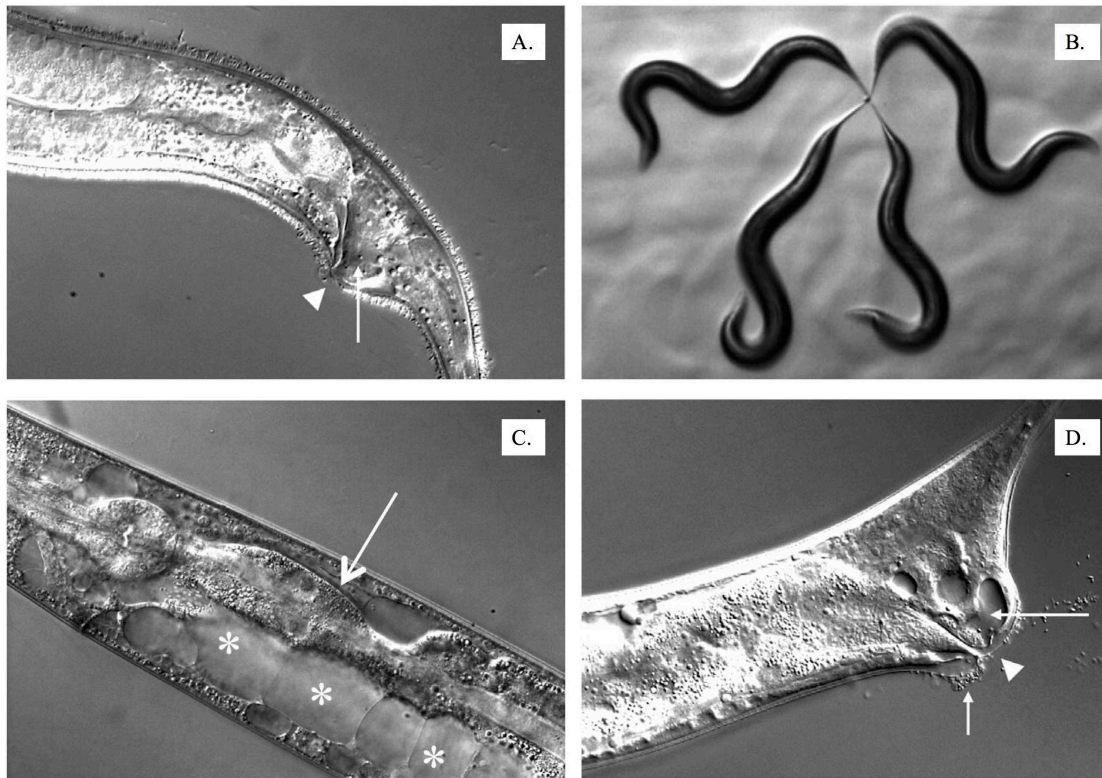


Figure 16. *Leucobacter* infections in *Caenorhabditis*.

A. The swollen tail of a *C. tropicalis* JU1635 individual, with bacterial coating on the surface. The arrow indicates the swollen tail. **B.** *L. celer* subsp. *astrifaciens* CBX151 induced a “worms-star” of a quartet of *C. elegans* individuals held together by their tails. **C.** Dying *C. elegans* after 20-hour exposure to *L. musarum* subsp. *musarum* CBX152. The anterior gut (arrow) is compressed by swollen vacuoles (asterisks) in the body cavity. **D.** The swollen tail of *C. elegans* infected with *L. musarum* subsp. *musarum* CBX152. Long arrow indicates the swollen tail; short arrow indicates the bacteria adhered around the anus. Arrowhead indicates the anal opening in A. and D. Adapted from Hodgkin et al. (2013).

II.3.2.3. Orsay virus

In 2011, the Orsay virus and the Santeuil virus were identified as the first known viruses that naturally infect *C. elegans* and *C. briggsae*, respectively (Félix et al., 2011). One year later, a third *Caenorhabditis* virus, Le Blanc virus, was characterized, also infecting *C. briggsae*, like the Santeuil virus (Franz et al., 2012). All of these three novel viruses are positive strand RNA viruses that are distantly but most closely related to Nodaviruses (Félix et al., 2011; Franz et al., 2012; Jiang et al., 2014).

Fluorescent in situ hybridization (FISH) and immunofluorescence assays (IFA) both determined that all the three viruses have the worm's intestinal cells as their primary tropism (Félix et al., 2011; Franz et al., 2014). *C. elegans* and *C. briggsae* with these viral infections display abnormal morphologies in their intestinal cells: the intestinal granules and nuclei may disappear; the intestinal cytoplasm may become fluid; intestinal cells may fuse (Figure 17).

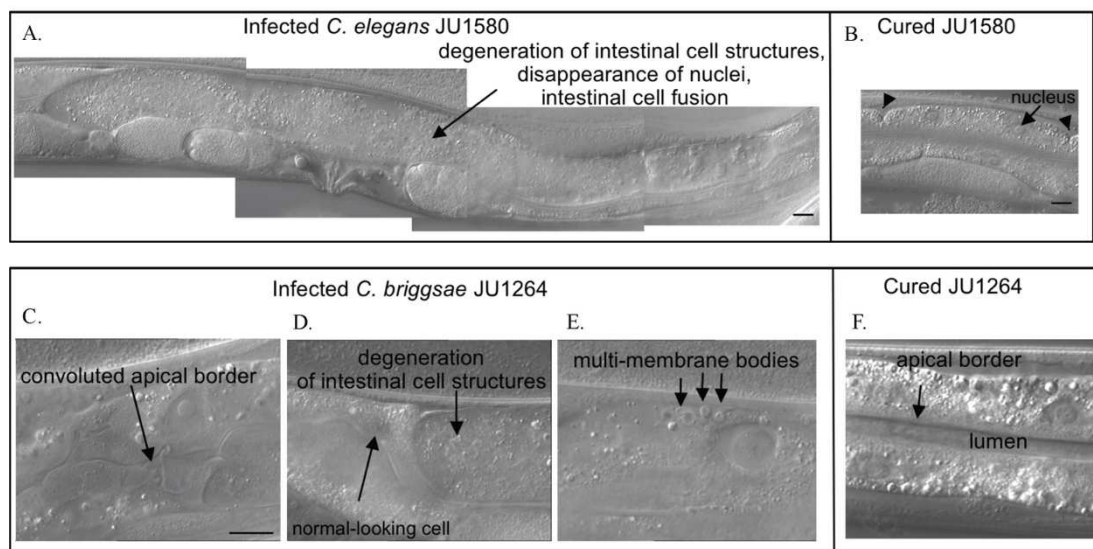


Figure 17. Viral infections in intestinal cells of wild *Caenorhabditis* isolates.

(A-B) *C. elegans* JU1580. **A.** An adult hermaphrodite infected with the Orsay virus displays an abnormally large intestinal cell that is probably due to cell fusion. **B.** An uninfected JU1580 adult. Antero-posterior boundaries between intestinal cells were indicated by arrowheads. (C-E) *C. briggsae* JU1264 infected with the Santeuil virus show diverse infection symptoms: convoluted apical intestinal border (**C**), degeneration of intestinal cell structures (**D**), presence of multi-membrane bodies (**E**). **F.** An uninfected JU1264 adult. Scale bars are 10µm. Adapted from Félix et al. (2011).

In the original study on the Orsay virus and *C. elegans*, comparisons between sequenced small RNA libraries of *C. elegans* populations with or without the infection of Orsay virus suggested that *C. elegans* produces small RNAs in response

to the viral infection. The antiviral role of RNA interference (RNAi) in the immunity of *C. elegans* was further revealed: compared to N2, the RNAi-deficient mutants showed increased levels of viral RNA abundance (Félix et al., 2011). A more recent study determined the roles of the gene *drh-1* in the recognition of the RNA virus and in the sensitivity variation of *C. elegans* to the viral infection (Ashe et al., 2013). The *drh-1* gene encodes a RIG-I-like helicase, which is a homologue of the mammalian viral recognition protein RIG-I. Using a genome-wide association mapping in 97 wild *C. elegans* isolates of the viral loads upon Orsay virus infection, a 159 base-pair deletion in the *drh-1* gene of *C. elegans* JU1580 was identified as a major determinant of the viral sensitivity in *C. elegans*. This deletion was further found to impair the fitness of JU1580 and *drh-1* mutants in the presence of viral infection: upon the infection of Orsay virus, JU1580 and *drh-1* mutants displayed delayed and decreased total progeny compared to uninfected isolates. However, the detrimental deletion of *drh-1* was found in many wild isolates of *C. elegans*, which may result from the low prevalence of Orsay virus in the wild, or a linkage between the *drh-1* deletion and a beneficial mutation, or a conditional deleterious effect of carrying the intact *drh-1* gene. Further experiments with double mutants determined that DRH-1 is an upstream factor of the antiviral siRNA pathway. DRH-1 might use a similar mechanism to RIG-I to recognize the RNA of the Orsay virus (Ashe et al., 2013).

III. Microsporidia infections in *C. elegans* and related nematodes

Historically, nematode-infecting microsporidia were primarily reported in the parasitic nematodes. *Protospirura muris*, a parasitic nematode of mouse, was reported to carry an infection by *Thelohania reniformis* (Kudo and Hetherington, 1922).

Nosema mesnili and *Plistophora schubergi* are able to infect an entomopathogenic nematode, *Neoaplectana carpocapsae*, as well as insect hosts (Veremtchuk and Issi, 1970; Poinar and Hess, 1988; Bakowski et al., 2014a). The first restricted nematode-infecting microsporidia was called “*Microsporidium*” *rhabdophilum* (undefined genus), which was isolated from a nonparasitic nematode *Oscheius (Rhabditis) myriophila* (Poinar and Hess, 1986; Bakowski et al., 2014a).

In 2008, three wild-caught *C. elegans* strains from France and one wild *C. briggsae* strain from India were reported to carry microsporidia infections (Troemel et al., 2008). A new microsporidian genus, *Nematocida*, was established with two species: *Nematocida parisii* and the undescribed *Nematocida* sp. 1. The genus *Nematocida* was placed in the Clade II of the microsporidia phylum phylogeny (Figure 4). In the phylogenetic analysis, the closest genus to *Nematocida* is *Ovavesicula*, with the type species *Ovavesicula popilliae* (Figure 4) (Vossbrinck and Andreadis, 2007; Troemel et al., 2008). *O. popilliae* infects the Japanese beetle, *Popillia japonica*, and forms oval-shaped spores (Andreadis and Hanula, 1987). *Nematocida*, on the other hand, literally means nematode killer (Troemel et al., 2008). Both *N. parisii* and *N. sp. 1* are transmitted horizontally and infect the intestinal cells of *C. elegans* or *C. briggsae*, with rod-shaped spores of two distinct sizes (Troemel et al., 2008). Further sampling revealed that microsporidian infections are quite prevalent among wild *Caenorhabditis* and related nematodes (Félix and Duvéau, 2012). These *Nematocida*

pathogens are lethal threats to *C. elegans* individuals and other related nematode species.

In addition to *Caenorhabditis*, a free-living marine nematode, *Odontophora rectangular* was found infected with a novel microsporidia species, named *Sporanauta perivermis* (Ardila-Garcia and Fast, 2012). Later, a deep sea nematode *Desmodora marci* was found infected with a microsporidium, which was named *Nematocenator marisprofundi* (Sapir et al., 2014). Neither of these two nematode-infecting microsporidia are closely related to *Nematocida* species. *S. perivermis* was placed in the Clade IV of the microsporidia, while *N. marisprofundi* was in none of the described clade, but in the basal position of the microsporidia phylum. *S. perivermis* infects host's muscle and hypodermis, and a vertical transmission mode was suggested (Ardila-Garcia and Fast, 2012). *N. marisprofundi* was shown to cause infection in the host's body wall muscles. *N. marisprofundi* does not seem to be transmitted through the fecal-oral route or the vertical transmission mode, as their spores have never been observed in the nematode intestines or nematode eggs. However, *N. marisprofundi* spores were observed in the reproductive organs of *D. marci* males and females, which suggested that the transmission is through the mating behavior (Sapir et al., 2014).

In the following parts, I will further present the interactions of *C. elegans* with *Nematocida* species.

III.1. *Nematocida parisii*

The very original *N. parisii* strain ERTm1 was isolated from the wild *C. elegans* strain CPA24 near Paris, France (Troemel et al., 2008). Wild *C. elegans* strains JU1247 and JU1395, *C. briggsae* strain JU2055 were also naturally infected with *N.*

parisii (*N. parisii* strains named ERTm3, ERTm4, ERTm5 respectively) (Troemel et al., 2008; Balla et al., 2015). In both host species, *N. parisii* is transmitted horizontally through the fecal-oral route, with meronts and two distinct sizes of spores appearing in the host intestinal cells (Figure 18). The small spore is $2.18 \pm 0.15 \mu\text{m}$ long and $0.8 \pm 0.08 \mu\text{m}$ wide, with one observed polar tube coil; while the large spore is $3.17 \pm 0.22 \mu\text{m}$ long and $1.31 \pm 0.15 \mu\text{m}$ wide, with up to five visible polar tube coils (Figure 19 F, G). Transmission electron microscopy (TEM) suggests that *N. parisii* is monokaryotic, as only one nucleus or multiple unpaired nuclei have been observed in its meront. Molecular identification and phylogenetic analysis using small subunit rDNA characterized *N. parisii* and placed it in the Clade II of the microsporidia phylum (Figure 4) (Troemel et al., 2008).

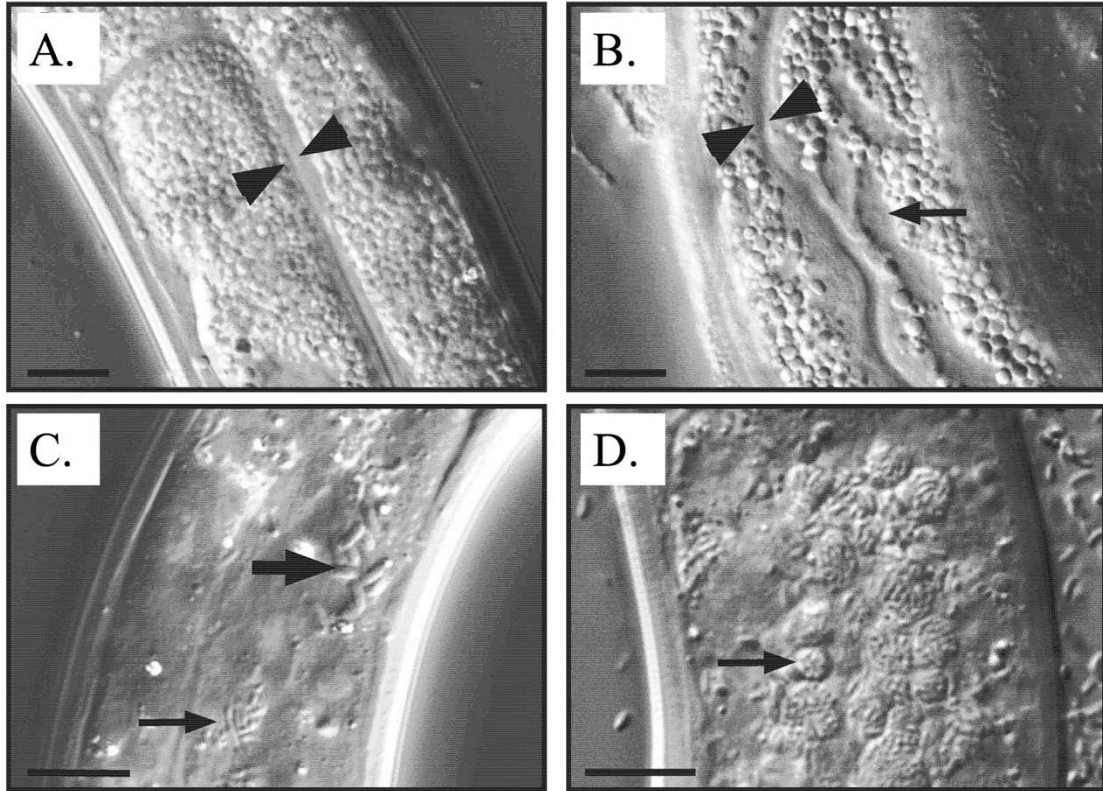


Figure 18. *N. parisii* infection symptoms in *C. elegans*.

A. The intestine of an uninfected *C. elegans*. **B.** Host intestinal granules are displaced by *N. parisii* meronts, which appear as “grooves” (arrow) in the host intestinal cells. The intestinal lumen is indicated by arrowheads in A and B. **C.** Large and small rod-shaped spores are indicated by larger and smaller arrow, respectively. **D.** Vesicles containing spores are indicated by an arrow. Scale bar is 10µm. Adapted from Troemel et al. (2008).

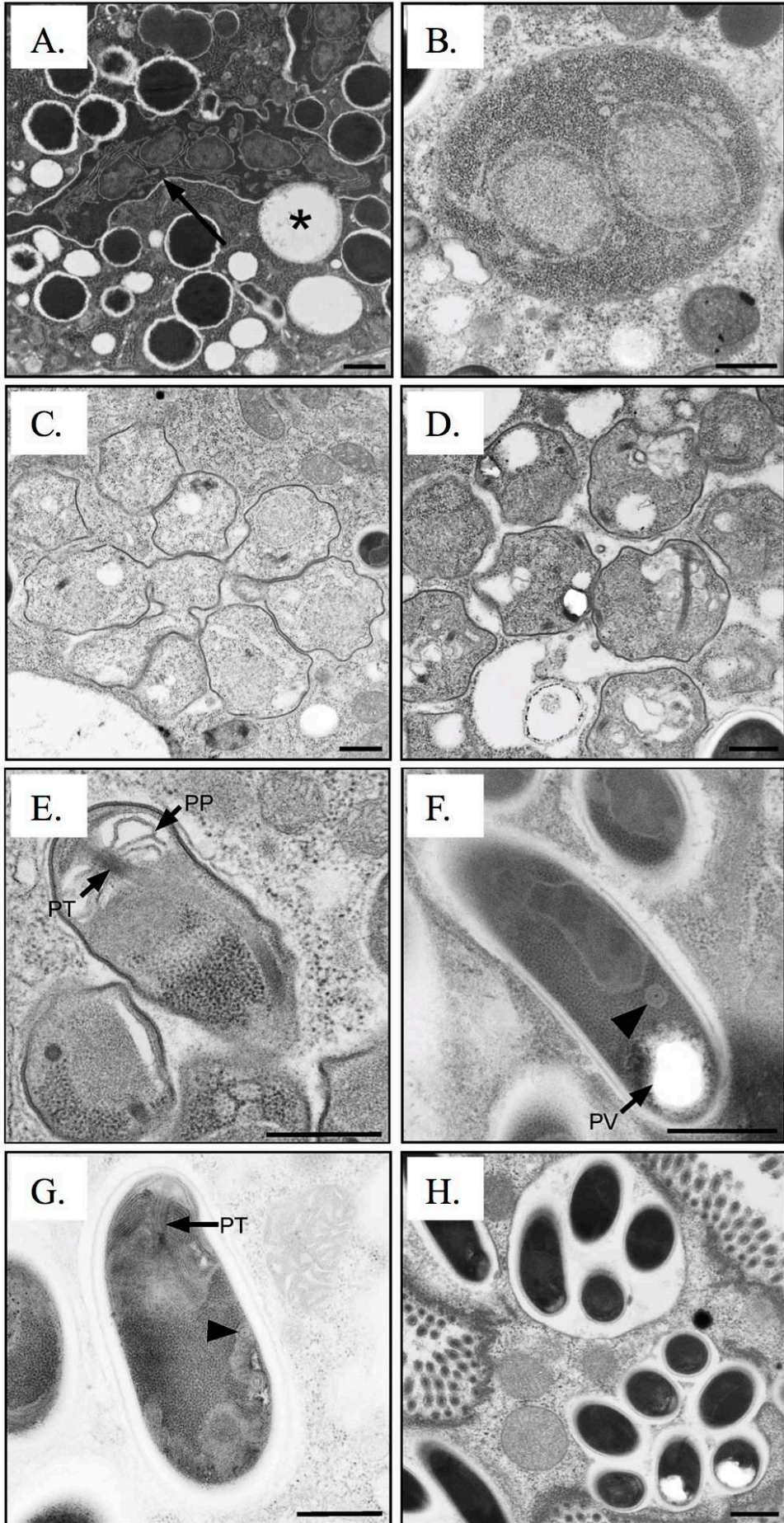


Figure 19. Ultrastructure of *N. parisii* infection in the *C. elegans* intestine.

A. An irregular shaped multinucleate meront indicated with an arrow. A host vesicle is marked with an asterisk. Scale bar is 1 μ m. **B.** A later stage meront with two nuclei and more regularly shaped plasma membrane. **C.** and **D.** are sporonts with electron-dense membranes. **E.** Developing *N. parisii* spores. **F.** A small sized spore. **G.** A larger sized spore. Arrowheads indicate cross-sections of polar tube coils in F and G. **H.** Vesicles of spores. B-H, scale bar is 500 nm. PP refers to the polaroplast membrane; PV refers to the posterior vacuole. Adapted from Troemel et al. (2008).

III.1.1. Life cycles of *N. parisii* and symptoms in the host

Ingestion of *N. parisii* spores by *C. elegans* begins the infection (Figure 20). *N. parisii* spores can survive the pharyngeal grinding and reach the intestinal lumen of *C. elegans*. Though the injecting process has not been observed directly yet, *N. parisii* spore, like other microsporidia species, probably uses the polar tube to inject its sporoplasm into the host intestinal cells.

Before becoming newly mature spores, two different developmental stages of *N. parisii* could be observed under transmission electron microscopy: the meront stage and the sporont stage. At 25 °C, within the first day after inoculation, the injected *N. parisii* sporoplasm replicates and develops into a meront, which causes displacement of gut granules and “grooves” in the *C. elegans* intestinal cells (Figure 18B). Early stage meronts are irregularly shaped and later stage meronts are more regularly shaped. Both may contain one or several nuclei (Figure 19A, B). The multinucleate meronts of *N. parisii* may divide by plasmotomy to cells containing one nucleus (Figure 20). Meronts develop into sporonts, which possess electron-dense membranes (Figure 19C, D). Multinucleate sporonts may also form and eventually divide by plasmotomy. Small spores appear around 40 hours after inoculation (Figure 19E, F). Large spores are observed later than small spores (Figure 18C, 19G); discrete vesicles filled with spores appear at the end (Figures 18D, 19H) (Troemel et al., 2008).

Newly formed mature spores of *N. parisii* exit from the host cell into the intestinal lumen soon after their formation. The spores will be released to the outside environment presumably through the defecation process of *C. elegans* (Figure 20) (Troemel et al., 2008; Estes et al., 2011).

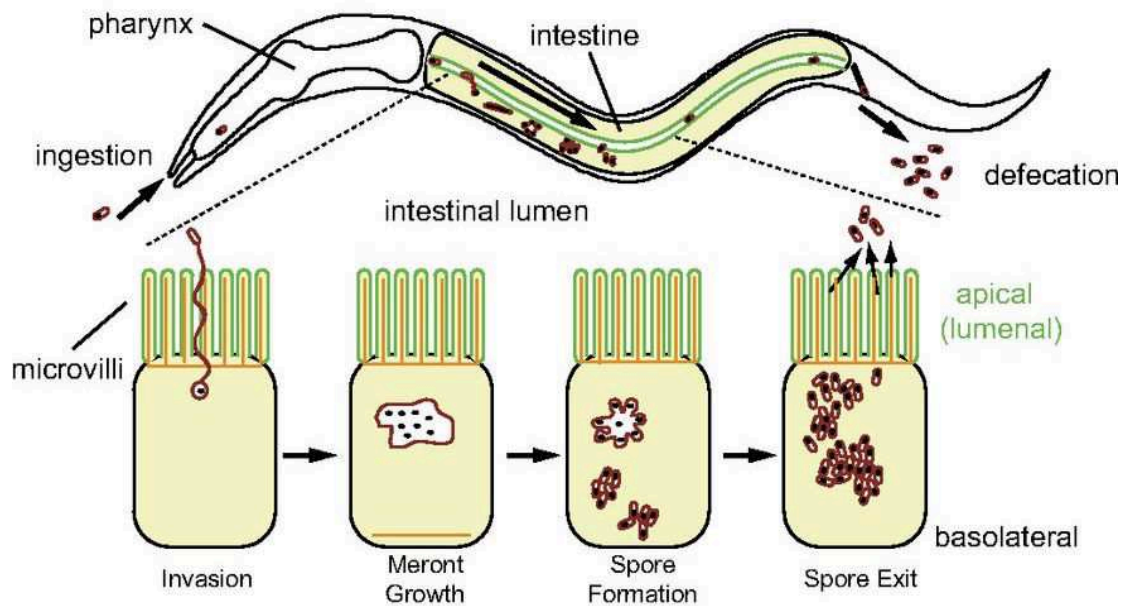


Figure 20. Diagram of life cycle of *N. parisii* infection in *C. elegans*.

N. parisii infects *C. elegans* intestinal cells (yellow), where it grows intracellularly through the meront and sporont stages. Newly formed spores exit apically into the lumen and then are defecated by the animal. The actin-rich microvilli are anchored into a terminal web (actin is labeled orange). Adapted from Szumowski et al. (2014).

N. parisii proliferates rapidly in *C. elegans*, with a doubling time about 2.9h to 3.3h during the meront stage (Cuomo et al., 2012). A single infected *C. elegans* can shed thousands of spores per hour to the environment (Estes et al., 2011). *N. parisii* can be rapidly transmitted across an entire *C. elegans* population. Infected *C. elegans* individuals with only small sized spores can infect other individuals, forming spores of both sizes in recipient *C. elegans* (Troemel et al., 2008). Infected *C. elegans* with

only a small number of spores inside its intestinal cells is able to infect other uninfected *C. elegans*, whereas infected *C. elegans* with only meronts is not infectious (Troemel et al., 2008).

On the host side, compared to healthy *C. elegans*, infected *C. elegans* has a paler color appearance, a thinner and smaller body size. *N. parisii* infection could also lead to a high rate of early death, but infected *C. elegans* could show grossly normal behaviors even when their intestinal cells are fully filled with *N. parisii* spores, reflecting the tolerance of *C. elegans* to *N. parisii* infection (Troemel et al., 2008). A recent study showed that in the presence of very high doses of *N. parisii* spores, *C. elegans* N2 in the L1 stage may enter a larval developmental arrest stage (Luallen et al., 2015).

III.1.2. Exit mechanism from C. elegans intestinal cells

N. parisii employs a specific non-lytic mechanism to exit from *C. elegans* intestinal cells to the luminal space, while minimizing damages to the host (Estes et al., 2011; Troemel, 2016). The *N. parisii* spore exit strategy can be divided into two successive phases:

1) *N. parisii* infections restructure the terminal web of the host intestinal cell, by first altering the localization of apically-restricted actin. ACT-5, a specialized actin isoform that is localized to the microvilli and the terminal web in uninfected *C. elegans*, is localized on the basolateral side of intestinal cells of infected *C. elegans* (Figure 20, 21A), while the levels of the apical actin often appeared to decrease. The rearrangement of the actin may serve as a trigger to induce IFB-2, a component of the intermediate filament in the terminal web, to reorganize and cause gaps in the terminal web (Figure 21) (Troemel et al., 2008; Estes et al., 2011). The actin rearrangement and the formation of terminal web gaps were determined to be a

discrete, highly regulated and necessary event, likely facilitating spore exiting by removing a barrier (Estes et al., 2011).

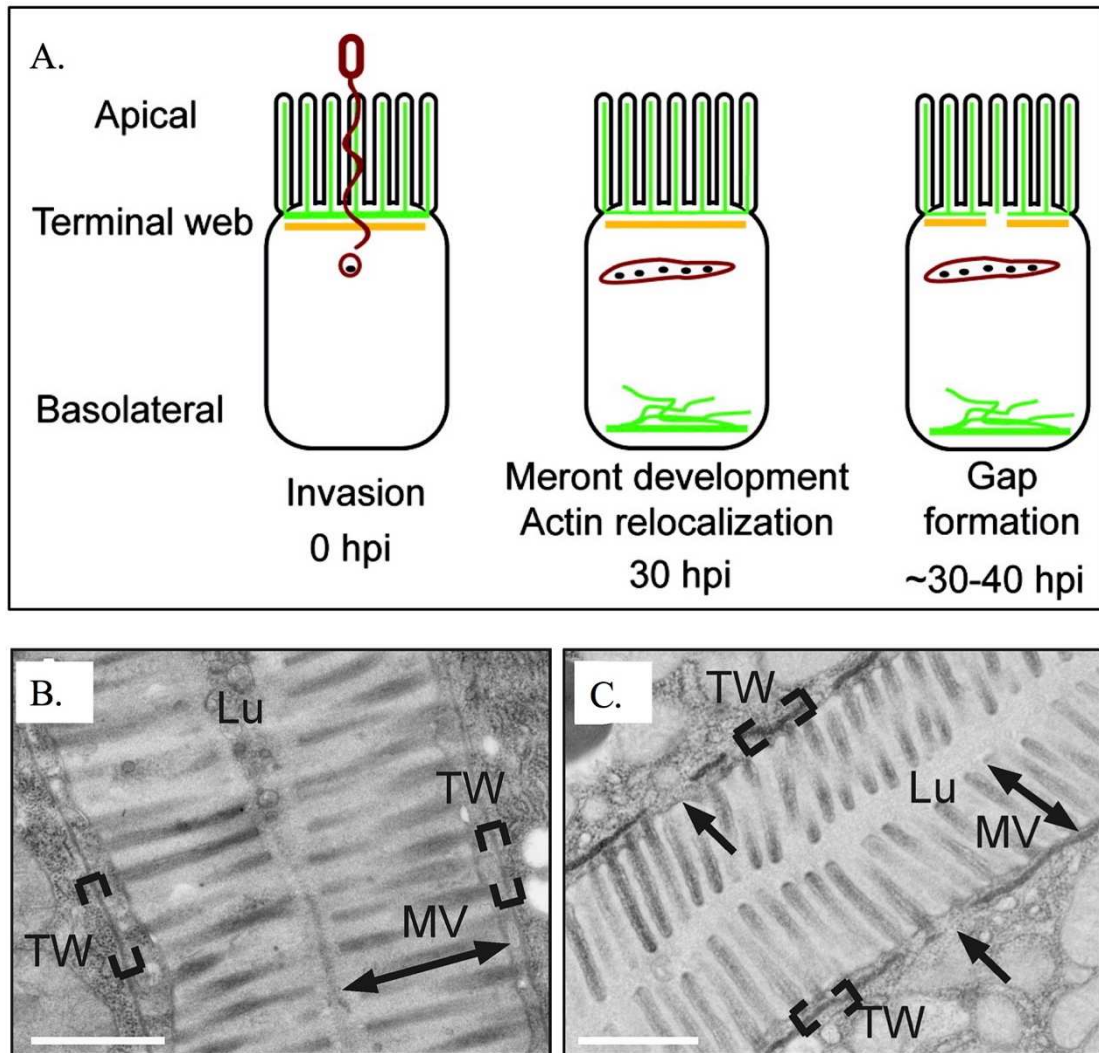


Figure 21. Phase I of *N. parisii* exit from *C. elegans* intestinal cells.

A. Diagram of Phase I of *N. parisii* exit. Green is ACT-5 and orange is IFB-2. Pathogen cells are depicted in red and nuclei depicted in black. **B.** Transmission electron microscopy shows the intact terminal web (TW) in uninfected *C. elegans* intestine. **C.** Gaps (arrows) in the terminal web of *C. elegans* infected with *N. parisii*. Double arrows indicate microvilli (MV). Lu indicates lumen. In B and C, scale bar is 1 μ m. Adapted from Troemel et al. (2008); Szumowski et al. (2012).

2) *N. parisii* spores exit from *C. elegans* intestinal cells through a RAB-11-mediated directional exocytosis mechanism: in the meront stage, *N. parisii* is in direct contact with the host intestinal cytoplasm, but transmission electron microscopy showed that a newly formed *N. parisii* spore is surrounded by an additional membrane (AM), defining a spore-containing compartment (SCC) (Figure 22B) (Szumowski et al., 2014). The localization of the host small GTPase protein RAB-11 to this compartment is required for SCCs to traffic to the apical side of the host cell and then fuse to the host membrane (Figure 22A). Other host GTPase proteins such as RAB-5 and RAB-10 are also important to the fusion process (Szumowski et al., 2014). After the SCCs fuse with the apical membrane (Figure 22C, D), spores gain access to the intestinal lumen, and acquire an apical membrane transporter protein PGP-1 marker (Szumowski et al., 2014). Then the spores are able to exit from the host cell into the lumen (Figure 22A). The newly shed spores in the lumen do not possess the additional membrane (Estes et al., 2011).

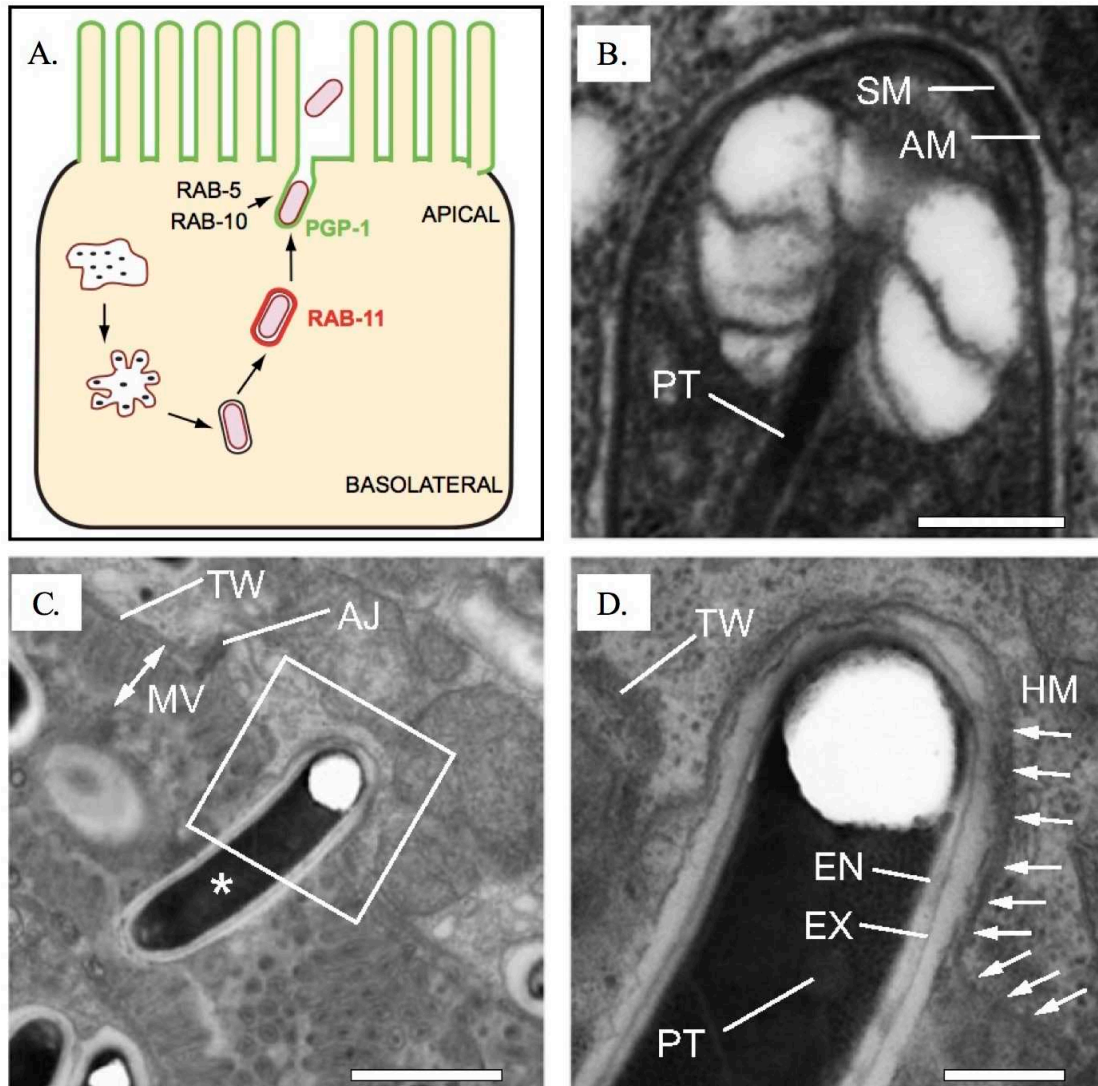


Figure 22. Phase II of *N. parisii* exit from *C. elegans* intestinal cells.

A. Diagram of Phase II of *N. parisii* exit. See text for details. **B.** Transmission electron microscopy of an immature *N. parisii* spore. The spore membrane (SM) is surrounded by an additional membrane (AM). **C.** A mature spore (asterisk) spanning the luminal membrane. **D.** Magnification of box from C. Arrows indicate the continuous host apical membrane (HM) that surrounds the spore. (B–E) AJ, apical junction; EN, endospore; EX, exospore; MV, microvilli; PT, polar tube; TW, terminal web. Scale bars: B and D, 500 nm; C, 1 μ m. Adapted from Szumowski et al. (2014).

In summary, *N. parisii* reorganizes the *C. elegans* intestinal cytoskeleton, then fuses to the host apical membrane in a GTPase-dependent mode, and finally exits host cells without causing cell lysis or any other lethal damages to the host.

III.1.3. Defensive response of C. elegans to N. parisii

All postembryonic stages of *C. elegans* are susceptible to *N. parisii* infection, except for the non-feeding dauer stage, at which the animals still can be a carrier of *N. parisii* (Troemel et al., 2008). However, neither the PMK-1 p38 MAPK immune pathway nor the DAF-2/DAF-16 insulin-like immune pathway of *C. elegans* showed any substantial roles in defense against *N. parisii* infection. In *C. elegans*, many genes upregulated upon *P. aeruginosa* and *D. coniospora* infections do not present clear expression changes when *C. elegans* is infected with *N. parisii* (Troemel et al., 2008). RNA-seq analysis showed that the transcriptional response of *C. elegans* to *N. parisii* infection is similar to that upon intracellular infection by the Orsay virus, but very different from some extracellular infections such as *P. aeruginosa* and *D. coniospora* (Bakowski et al., 2014b). Taken together, *C. elegans* may have a distinct defensive response to *N. parisii* infection compared to its immune responses to other fungal or bacterial pathogens.

C. elegans appears to be able to monitor the functioning and homeostasis of core cellular processes, and then trigger the expression of defensive genes upon any perturbation or disruption of these processes. Core process surveillance pathways could help *C. elegans* to recognize pathogen invasion. For instance, the *C. elegans* ubiquitin-proteasome system (UPS) can trigger defensive responses, such as the ubiquitin-mediated responses, upon *N. parisii* and Orsay virus infections.

Upregulation of Skp1–Cull–F-box protein (SCF) ligase components could help to restrict microsporidia growth. However, this ubiquitin-mediated defense is only able to target a small percentage of *N. parisii* cells in their early developmental stage, which may partially be due to the fact that *N. parisii* is able to suppress the host UPS defensive machinery. The proteasome and autophagy pathways also promote host defense against *N. parisii* (Bakowski et al., 2014b).

A recent study showed that natural variation exists among six *C. elegans* wild isolates in their sensitivity upon *N. parisii* infection, as the different mortality rates among these strains are positively correlated with the pathogen loads (Figure 23A, B). This result indicates variation in host resistance rather than tolerance (Balla et al., 2015). Fecundity was further compared between N2 and CB4856. The fecundity of infected *C. elegans* seemed to depend on the developmental stage of the *C. elegans* individuals when they were inoculated. Indeed, when the inoculation of *N. parisii* occurred at the L4 stage, neither N2 nor CB4856 showed significant fecundity reduction, compared to uninfected individuals. However, when the inoculation was initiated at the L1 stage, the fecundity of N2 could drop drastically compared to CB4856. The L1 stage animals of CB4856 showed an enhanced defensive ability to control and clear *N. parisii* infection. This clearing ability of CB4856 L1 larvae helps to maintain a relatively normal brood size and gain a reduced mortality rate (Balla et al., 2015) (Figure 23C, D). Though this increased immune ability of the strain CB4856 is restricted to the L1 stage, it provides a selective advantage over N2 (Balla et al., 2015). The *npr-1* gene, though known to be responsible for several phenotypic variations in growth, physiology and response to some other pathogens between CB4856 and N2, does not appear to have a role here for the L1-stage specific enhanced resistance in CB4856 (Andersen et al., 2014; Balla et al., 2015; Nakad et al., 2016). Again, this indicated that *C. elegans* may employ a distinct immune response to defend itself against *N. parisii*.

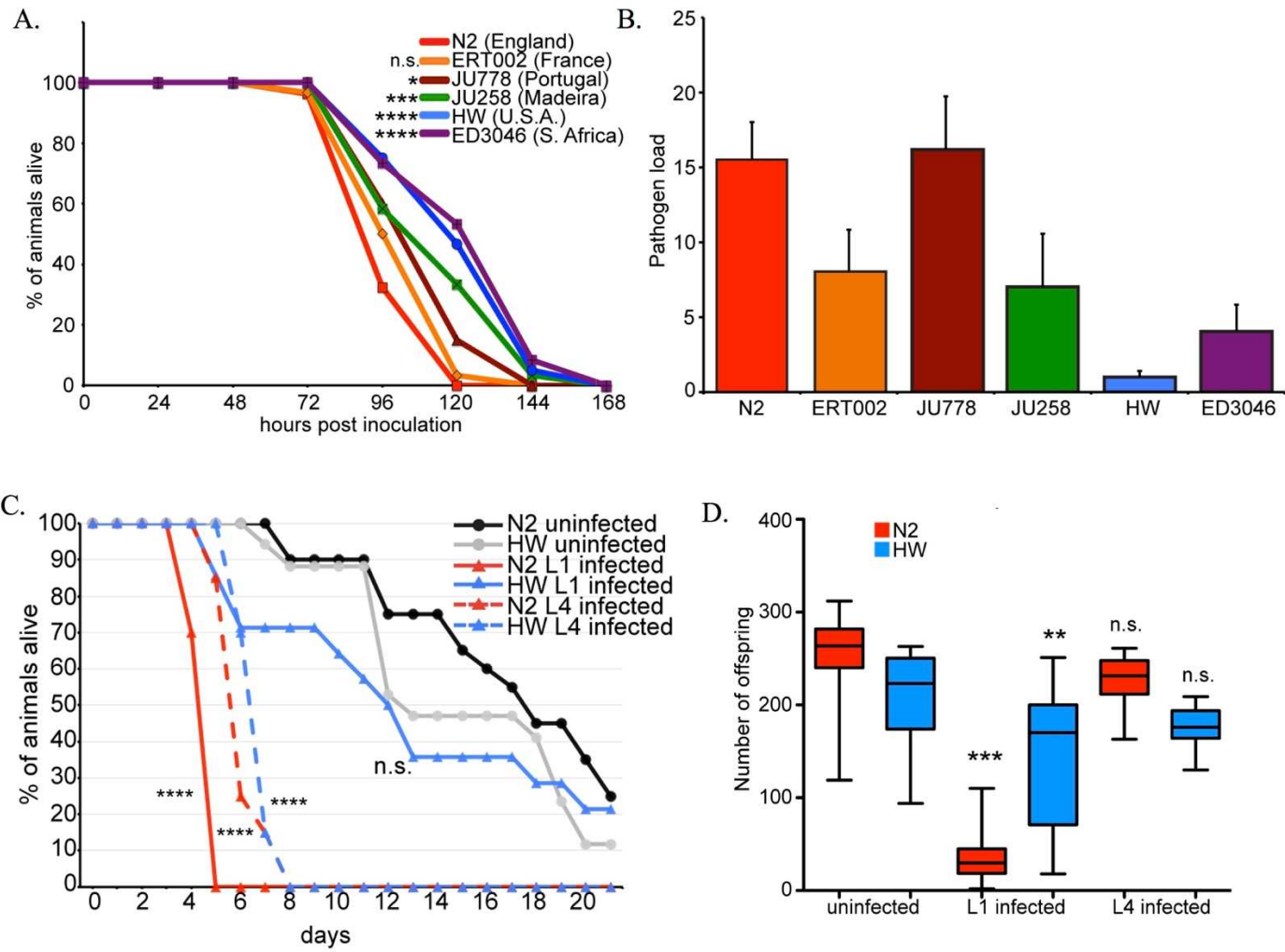


Figure 23. Natural variation among different *C. elegans* strains in the response to *N. parisii* infection.

A. Survival curves of six *C. elegans* strains upon infection by *N. parisii*. **B.** Normalized pathogen load of each strain from (A) at 30 hpi, measured by qRT-PCR targeting an *N. parisii* SSU rRNA. **C.** Survival curves of uninfected animals, and infected animals inoculated at the L1 or L4 stage. **D.** Lifetime fecundity of uninfected animals, and infected animals inoculated at the L1 or L4 stage. Adapted from Balla et al. (2015).

III.2. *Nematocida* sp. 1

Nematocida sp. 1 was reported in the initial article on *N. parisii* (Troemel et al., 2008). Its SSU rDNA sequence is approximately 95% identical to the same region of *N. parisii*. *N. sp. 1* and *N. parisii* were placed together as sister species in the phylogeny of microsporidia (Troemel et al., 2008). The first *N. sp. 1* strain (ERTm2) was found from a wild *C. briggsae* strain JU1348 collected in Kerala, India (Troemel et al., 2008). The second *N. sp. 1* strain ERTm6 was isolated from another wild-caught *C. briggsae* strain JU1638 in Cape Verde Islands. Though both *N. sp. 1* strains were found in *C. briggsae*, *N. sp. 1* is also capable of infecting *C. elegans* (Bakowski et al., 2014c). *N. sp. 1* has a similar life cycle and developmental stages to *N. parisii* (Troemel et al., 2008).

III.3. *Nematocida* genomics

Similar to other microsporidia, genomes of *Nematocida* species are quite small and compact. The *N. parisii* strains ERTm1 and ERTm3 share 99.8% identical genomes, that have 4.1 Mb in size. The *N. sp. 1* strain ERTm2 has a larger genome of 4.6 Mb, with only 68.3% average identity to the *N. parisii* ERTm1 genome (Cuomo et al., 2012). The other *N. sp.1* strain ERTm6, however, possesses a genome of about 4.28 Mb (Bakowski et al., 2014c). The genomes of both species are compact, with 72.8% of ERTm1 and 67.48% of ERTm6 genome sequences predicted to be coding, and a mean distance between coding sequences of 418 and 579 bp, respectively (Cuomo et al., 2012; Bakowski et al., 2014c). In the *N. parisii* ERTm1 genome, no introns and no untranslated regions (UTRs) were predicted in protein-coding genes, or unusually short UTRs (Cuomo et al., 2012).

Genome sequence analyses further indicated that *Nematocida* may have experienced a rare or recent recombination event: all four *Nematocida* strains of *N. parisii* and *N. sp. 1* were found

likely diploid and heterozygous, but in the *N. sp. 1* ERTm2 genome, loss of heterozygosity (LOH) regions were also found (Cuomo et al., 2012; Bakowski et al., 2014c; Troemel, 2016). *N. sp. 1* has a higher rate of heterozygosity and polymorphism than *N. parisii*. *N. sp. 1* ERTm2 has an average of one single nucleotide polymorphism (SNP) every 82 bases. However, a single large homozygous region in each of the scaffolds 1, 2 and 4 was found in the genome of ERTm2, which suggested that LOH has occurred in the three scaffolds. Further experiments showed that the LOH event did not occur in ERTm2 in the lab, as the ERTm2 isolate serially passaged for 14 weeks showed a nearly identical pattern of polymorphism to the original isolate. These findings together suggested that *Nematocida* may have a rare sexual cycle or mitotic recombination events during its life history in the wild.

Phylogenomic analysis with fungi showed that all microsporidia shared 882 highly conserved core genes, 137 of which were identified to be microsporidia-specific genes (Cuomo et al., 2012). *Nematocida* are found to be quite divergent from other microsporidian species, with a very large *Nematocida*-specific gene family in both *N. parisii* (ERTm1 and ERTm3) and *N. sp. 1* (ERTm2). In the genome of *N. parisii* ERTm1, a total of 2,661 genes were predicted, while the predicted number is 2,443 for *N. sp. 1* ERTm6 (Cuomo et al., 2012; Bakowski et al., 2014c). Pfam protein domain analysis showed that, similar to other microsporidia, the *Nematocida* genome encodes the nucleoside phosphate transporter (Npt) proteins and the nucleoside H⁺ symporter, but lost the retinoblastoma (RB) tumor suppressor gene. Two Npt transporters were suggested to be encoded in the *Nematocida* genome. The Npt transporters may help import host nucleotides into the pathogen. *Nematocida* hexokinase genes were also found to have a predicted secretion signal to the host cell cytoplasm, which may boost host synthesis of building blocks that later promote the rapid growth of *Nematocida* (Cuomo et al., 2012).

IV. The background of my PhD thesis

Hosts and parasites are tightly coupled during their life time and may coevolve antagonistically. Hosts evolve better remote detection and avoidance ability, while parasites evolve better seeking skills; hosts evolve resistance to control or clear infection, while parasites evolve higher virulence to proliferate on/in the host. These reciprocal interactions could exert strong selection pressures on both partners and thus drive antagonistic coevolution. Host-parasite coevolution contributes to the diversity in both hosts and parasites (Schulte et al. 2013; Bever et al. 2015). Meanwhile, host-parasite co-evolution and adaptation may also lead to specific interactions between hosts and parasites (Barrett et al. 2009; Antonovics et al. 2013). Patterns of specificity may be found at either the interspecific or the intraspecific level for either the host or the parasite. To study the specificity and the molecular mechanisms underlying host-parasite interaction and coevolution is crucial to understand the defense and infection mechanisms themselves as well as their evolutionary dynamics.

C. elegans and its natural intracellular parasite *Nematocida parisii* provide an excellent model system to study host-parasite interactions and coevolution (Troemel et al., 2008; Hodgkin and Partridge 2008; Balla and Troemel 2013). Studies with *C. elegans* and *N. parisii* have already made many progresses. However, it is still unclear how widespread and diverse microsporidia infections are in *C. elegans* or other related nematodes in the wild. The discovery of the rotting substrates as the natural habitats of *C. elegans* and other related nematodes provided the opportunities to address more ecological questions in the wild nematode populations (Félix and Braendle, 2010; Kiontke et al., 2011). Among the samples of wild nematode isolates, the prevalence of microsporidia infection was further observed (Félix and Duveau, 2012).

When I began my PhD studies in October 2013 in the Felix Lab, there were dozens of wild nematode strains, including several species, that displayed the putative microsporidia infection. Furthermore, natural variation in the sensitivity of different wild *C. elegans* strains to the microsporidian infection was also observed preliminarily.

During the process of my PhD project, I first characterized these microsporidia by their molecular and morphological features. Then, at the interspecific level, I studied the host-parasite specificity between different nematodes and microsporidia; at the intraspecific level, I

investigated the variation in sensitivity of different wild *C. elegans* to the same microsporidia infection, with the further goal of determining the loci involved in *C. elegans* host sensitivity variation. Overall, my PhD project focused on the host-pathogen interactions of *C. elegans* and other nematodes with microsporidia.

RESULTS

I. A large collection of novel nematode-infecting microsporidia and their diverse interactions with *Caenorhabditis elegans* and other related nematodes

In the Felix Lab collection of wild nematodes, 47 strains of ten species, mainly including *Caenorhabditis* and *Oscheius* species, were noted to display putative microsporidia infections. In preliminary observations using Nomarski microscopy, spores and meronts were all found in the intestinal cells of these putative infected nematode strains, except for one *C. brenneri* strain where spores were mainly located in the epidermis. We studied these natural infections in the wild nematode isolates. Here, we describe our discoveries of novel microsporidia species and genera infecting wild nematode isolates. In all cases, infections were found to be horizontally transmitted. We also determined their host specificities. I summarize below the main findings, followed by the article that was recently published in PLOS Pathogens.

To characterize these putative microsporidian infections in the nematodes, we first amplified and sequenced two genes (SSU rDNA and β -tubulin genes). Phylogenetic trees were constructed using these sequences and published sequences from species belonging to the microsporidia phylum. In addition to *Nematocida parisii* and *Nematocida ausubeli* (formerly called *N. sp. 1*), we have found six new *Nematocida* species. Furthermore, we have discovered two new genera, named *Enteropsectra* and *Pancytospora*.

The morphology (spore sizes, spore shapes, spore distribution in the host cells) of all the new microsporidia species were examined by Nomarski microscopy. The similarities and differences in the morphological features matched their groupings according to the phylogenetic analysis. Using transmission electron microscopy (TEM), we discovered that *N. ausubeli* and *E. longa* use different mechanisms for the exit of the spores out of their host intestinal cells.

Since each microsporidia species was isolated from a limited number of host species, we further investigated the host specificity of different microsporidia. Cross-inoculation tests were performed using eight microsporidia species and four nematode species. The host range differs depending on the microsporidia species. For example, *Nematocida homosporus* could

infect the two *Caenorhabditis* species and the two *Oscheius* species. However, *Enteropsectra longa* only infected *Oscheius* sp. 3. The host specificity of different microsporidia further revealed the diverse interactions between microsporidia and nematodes. Inter- and intraspecific host specificity exists among different nematode-infecting microsporidia.

Among the different nematode species, *C. elegans* has been the best studied. 19 *C. elegans* wild strains were naturally infected with *N. parisii* or *N. ausubeli*. *C. elegans* was also found susceptible to *N. major*, *N. homosporus* and *P. epiphaga* in the infection tests. We further used two *C. elegans* strains carrying transgenic reporter genes upregulated upon *N. parisii* infection, to compare the host response to the infections by five microsporidia species. Interestingly, the results suggested that *C. elegans* may have a different transcriptional response to the infection by *N. ausubeli* than to the infections by the other tested *Nematocida* species.

Overall, our study considerably enriched the knowledge on nematode-infecting microsporidia and their diverse interactions. We found novel microsporidia species and genera in wild nematode isolates. These nematode-infecting microsporidia display different host specificities. As *C. elegans* has proven to be a successful model organism, and microsporidia are obligate intracellular pathogens of *C. elegans* and other nematodes, different microsporidia and nematodes provide an ideal system to study host-pathogen interactions. Our study enlarged the available resources and provided a wide variety of natural nematode-microsporidia host-pathogen pairs.

My contributions in this study:

I contributed the majority of assays in this study, except the TEM part (Figure 5, 8; Supplemental Figure 1, 6), for which we collaborated with Martin Sachse and Marie-Christine Prevost from Pasteur Institute in Paris, and I contributed to the fixations and observations. The assays with transgenic *C. elegans* strains (Figure 9) were performed with Robert J. Luallen and Emily R. Troemel from University of California at San Diego.

RESEARCH ARTICLE

A Large Collection of Novel Nematode-Infecting Microsporidia and Their Diverse Interactions with *Caenorhabditis elegans* and Other Related Nematodes

Gaotian Zhang^{1,2}, Martin Sachse³, Marie-Christine Prevost³, Robert J. Luallen⁴, Emily R. Troemel⁴, Marie-Anne Félix^{1*}

1 Institut de Biologie de l'Ecole Normale Supérieure, CNRS, Inserm, ENS, PSL Research University, Paris, France, **2** School of Life Sciences, East China Normal University, Shanghai, China, **3** Ulpapole, Institute Pasteur, Paris, France, **4** Division of Biological Sciences, Section of Cell and Developmental Biology, University of California San Diego, La Jolla, California, United States of America

* felix@biologie.ens.fr



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Abstract

Microsporidia are fungi-related intracellular pathogens that may infect virtually all animals, but are poorly understood. The nematode *Caenorhabditis elegans* has recently become a model host for studying microsporidia through the identification of its natural microsporidian pathogen *Nematocida parisii*. However, it was unclear how widespread and diverse microsporidia infections are in *C. elegans* or other related nematodes in the wild. Here we describe the isolation and culture of 47 nematodes with microsporidian infections. *N. parisii* is found to be the most common microsporidia infecting *C. elegans* in the wild. In addition, we further describe and name six new species in the *Nematocida* genus. Our sampling and phylogenetic analysis further identify two subclades that are genetically distinct from *Nematocida*, and we name them *Enteropsectra* and *Pancytospora*. Interestingly, unlike *Nematocida*, these two genera belong to the main clade of microsporidia that includes human pathogens. All of these microsporidia are horizontally transmitted and most specifically infect intestinal cells, except *Pancytospora epiphaga* that replicates mostly in the epidermis of its *Caenorhabditis* host. At the subcellular level in the infected host cell, spores of the novel genus *Enteropsectra* show a characteristic apical distribution and exit via budding off of the plasma membrane, instead of exiting via exocytosis as spores of *Nematocida*. Host specificity is broad for some microsporidia, narrow for others: indeed, some microsporidia can infect *Oscheius tipulae* but not its sister species *Oscheius* sp. 3, and conversely some microsporidia found infecting *Oscheius* sp. 3 do not infect *O. tipulae*. We also show that *N. ausubeli* fails to strongly induce in *C. elegans* the transcription of genes that are induced by other *Nematocida* species, suggesting it has evolved mechanisms to prevent induction of this host response. Altogether, these newly isolated species illustrate the diversity and ubiquity of microsporidian infections in nematodes, and provide a rich resource to investigate host-parasite coevolution in tractable nematode hosts.

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Author Summary

Microsporidia are microbial parasites that live inside their host cells and can cause disease in humans and many other animals. The small nematode worm *Caenorhabditis elegans* has recently become a convenient model host for studying microsporidian infections. In this work, we sample *Caenorhabditis* and other small nematodes and 47 associated microsporidian strains from the wild. We characterize the parasites for their position in the evolutionary tree of microsporidia and for their lifecycle and morphology. We find several new species and genera, especially some that are distantly related to the previously known *Nematocida parisii* and instead closely related to human pathogens. We find that some of these species have a narrow host range. We studied two species in detail using electron microscopy and uncover a new likely mode of exit from the host cell, by budding off the host cell plasma membrane rather than by fusion of a vesicle to the plasma membrane as in *N. parisii*. We also find a new species that infects the epidermis and muscles of *Caenorhabditis* rather than the host intestinal cells and is closely related to human pathogens. Finally, we find that one *Nematocida* species fails to elicit the same host response that other *Nematocida* species do. These new microsporidia open up many windows into microsporidia biology and opportunities to investigate host-parasite coevolution in the *C. elegans* system.

Introduction

Microsporidia are fungi-related obligate intracellular pathogens, with over 1400 described species [1,2]. Interest in these organisms started 150 years ago when researchers, especially Louis Pasteur, studied silkworm disease that was caused by a microsporidian species later named *Nosema bombycis* [3]. In the past decades, microsporidia have attracted more attention when they were revealed to be a cause of diarrhea in immunocompromised patients and were further demonstrated to have a high prevalence in some areas in immunocompetent patients and healthy individuals [4–6].

Microsporidia are transmitted between hosts through a spore stage. Inside the microsporidian spore is found a characteristic structure called the polar tube, which at the time of infection can pierce through host cell membranes and introduce the sporoplasm (the spore cytoplasm and nucleus) into host cells [1,7]. These obligate intracellular pathogens are known to infect a wide range of hosts among protists and animals, especially insects, fish and mammals [1]. Even though nematodes constitute a huge phylum with over 25,000 described species, very few studies on microsporidian infections in nematodes have been reported so far [1].

The free-living nematode *Caenorhabditis elegans* has been used as a major biological model species over the last 50 years [8]. However, until the past decade, little was known about its biology and ecology in its natural environment and no natural pathogens were isolated until *C. elegans* could be readily isolated from natural environments. *C. elegans* is now known to be found in compost heaps, rotting fruits (apples, figs, etc.) and herbaceous stems, as well as with diverse carrier invertebrates (snails, isopods, etc.) [9–11]. *C. elegans* coexists with a variety of prokaryotic and eukaryotic microbes, including both its food and pathogens, which likely have a large impact on its physiology and evolution [12–15].

With an improved understanding of the natural history of *Caenorhabditis* [16,17], dramatically increased number of various wild rhabditid nematode strains and species have been isolated and identified. *C. elegans*' close relatives such as *Caenorhabditis briggsae* or *Caenorhabditis*

remanei are isolated from similar environments [18]. *Oscheius tipulae*, a very common bacterivorous nematode species, also in family Rhabditidae, can be readily isolated from soil and rotting vegetal matter [10,19,20], as well as its closest known relative *Oscheius* sp. 3, with which it cannot interbreed [21]. Interest in these rhabditid nematodes concerns not only the evolution of genomic and phenotypic characters, but also their inter- and intraspecific interactions and co-evolution with other organisms, especially with various microbes in their natural habitats. While nematodes feed on bacteria and small eukaryotes, some microbes take nematodes as their food source [13,14,16]. Among them, microsporidia are obligate intracellular parasites and thus are in particularly tight association with their hosts.

The microsporidian *Nematocida parisii* was the first found natural intracellular pathogen of *C. elegans*, which we isolated from a wild *C. elegans* sampled near Paris, France [22]. *Nematocida* sp. 1 (described here as *Nematocida ausubeli*) was further isolated from a wild *Caenorhabditis briggsae* strain in India [22]. A microsporidian species isolated in *C. elegans* was found to infect the epidermis and muscles and was named *Nematocida disploedere* [23]. Two microsporidia infecting marine nematodes have also been described, namely *Sporanauta perivermis* [24] and *Nematocenator marisprofundi* [25,26]. However, the extent and diversity of microsporidia infections in nematodes remained sparsely described.

Here we describe a collection of 47 terrestrial nematode strains that we isolated from the wild with a microsporidian infection. The microsporidia can be grown in the laboratory in their host using *C. elegans* culture conditions and stored frozen with their nematode host. They are all transmitted horizontally. In this set, we found that *N. parisii* and *N. ausubeli* (formerly called *N. sp. 1*) are in association with further host species and display a wider geographical distribution than so far reported [22]. *N. parisii* is the most common *C. elegans*-infecting species we found in the wild. We further discovered new nematode-infecting microsporidian species. From our phylogenetic analysis using small subunit (SSU) ribosomal DNA and β -tubulin sequences, five new microsporidia species were placed in the *Nematocida* genus, while the others defined two new genera in the microsporidian clade often designated as Clade IV, which includes human pathogenic microsporidia such as *Enterocytozoon bieneusi* and *Vittiforma corneae*. The similarities and differences in the morphological features of these microsporidia matched their groupings by sequence similarity. We therefore describe two new microsporidian genera, *Enteropsectra* and *Pancyctospora*, and nine new species in these two genera and *Nematocida*. We further examined *Nematocida ausubeli* and *Enteropsectra longa* by electron microscopy, which allowed us to observe different mechanisms for their exit from host intestinal cells, through a vesicular pathway for *N. ausubeli* (as described for *N. parisii*; [27]), but surprisingly through membrane budding for *E. longa*. Concerning specificity of infection, we find cases of tight specificity between host and pathogen. We also find that *N. ausubeli* fails to strongly induce the transcription of genes that are induced in *C. elegans* by *N. parisii* infection. Overall, our study points to strong and diverse interactions between wild rhabditid nematodes and microsporidia, and provides a platform for further study of these infections.

Results

A large collection of microsporidian-infected nematode cultures

Our worldwide sampling of bacterivorous nematodes was primarily aimed at isolating *Caenorhabditis* species and, to a lesser degree, *Oscheius* species. From this sampling, we identified a subset of strains with a pale body color (Fig 1A), some of which, upon morphological examination using Nomarski microscopy [22], appeared infected with microsporidia. In total, we collected 47 nematode strains (S1 Table) displaying putative microsporidian infections,

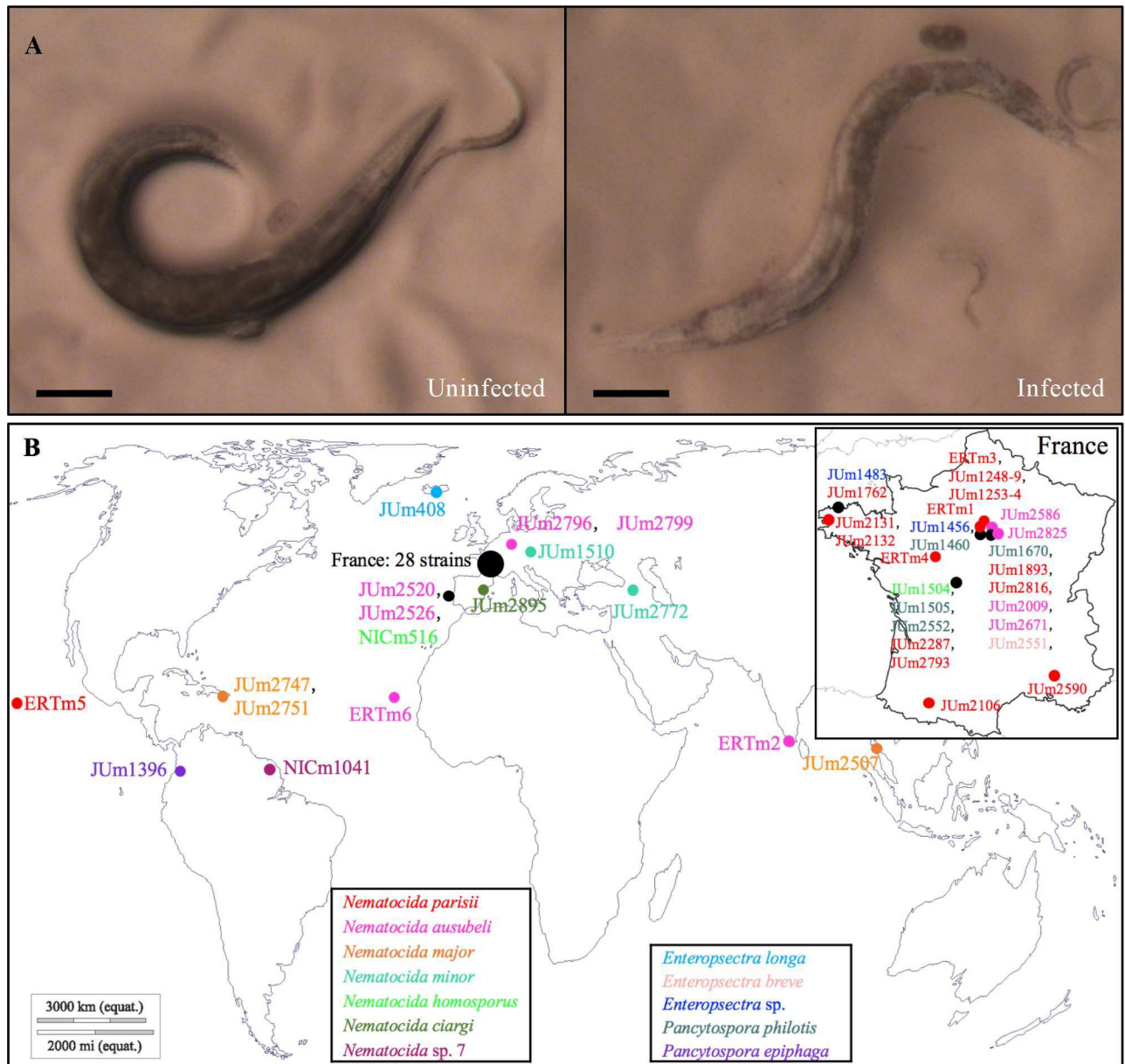


Fig 1. Isolation of nematode-infecting microsporidia. A. Morphological screen for infected worms (here *C. elegans* JU2816). Compared to uninfected worms, infected adult worms have a paler body color. Note that the pale body color may result from many environmental conditions, and thus these animals were further screened by Nomarski optics for microsporidian infections. Scale bar: 0.2 mm. B. Geographic distribution of our collection of nematode-infecting microsporidia. Sampling locations are represented by differently colored symbols based on microsporidian species. Black symbols were used when different microsporidian species were found in the same location. The world map is cited from http://www.d-maps.com/carte.php?num_car=3267&lang=en. The France map is cited from http://www.d-maps.com/carte.php?num_car=2813&lang=en.

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comprising 10 nematode species from different parts of the world (Tables 1 and 2; Fig 1B). The microsporidia strain JUm2807 was isolated during these sampling efforts and described elsewhere as *Nematocida dispodere*, and is not considered here [23].

The unidentified microsporidian strains were characterized by sequencing of PCR fragments of the SSU rDNA and β -tubulin genes. We were able to amplify 45 SSU rDNA sequences (most 1390 bp long) and 32 β -tubulin sequences (most 1210 bp long) (S1 Table).

Table 1. Collection of wild nematode-infecting microsporidia strains

A. <i>Nematocida parisii</i> infection				
Host	Strain	Country / Year	Sample composition	Spore feature
<i>Caenorhabditis elegans</i>	ERTm1^a	France / 2004	compost	2S ^c
	ERTm3 ^a	France / 2007	rotting apple	2S ^c
	JUm1248 ^{a,b}	France / 2007	rotting apple	n. d.
	JUm1249 ^b	France / 2007	rotting apple	2S
	JUm1253 ^b	France / 2007	rotting apple	n. d.
	ERTm4 ^a	France / 2008	compost	2S ^c
	JUm1762 ^b	France / 2009	rotting stem	2S
	JUm1893 ^b	France / 2009	rotting apple	2S
	JUm2106	France / 2011	compost	2S
	JUm2131	France / 2011	rotting stem	2S
	JUm2132	France / 2011	rotting stem	2S
	JUm2287	France / 2013	compost	2S
	JUm2816	France / 2014	compost	n. d.
<i>Caenorhabditis briggsae</i>	JUm1254	France / 2007	rotting apple	2S
	ERTm5^{a,d}	Hawaii / 2011	rotting fruit	2S
	JUm2590	France / 2013	rotting apple	2S
	JUm2793	France / 2014	compost	n. d.
B. <i>Nematocida ausubeli</i> (<i>N. sp.</i> 1) infection				
Host	Strain	Country / Year	Sample composition	Spore feature
<i>Caenorhabditis elegans</i>	JUm2009	France / 2010	rotting apples	2S
	JUm2520	Portugal / 2013	rotting fruit	2S
	JUm2526	Portugal / 2013	rotting fruit	2S
	JUm2586	France / 2013	rotting pear	2S
	JUm2671	France / 2011	rotting apple	2S
	JUm2825	France / 2014	rotting stem	2S
<i>Caenorhabditis briggsae</i>	ERTm2^a	India / 2007	rotting mix	2S ^c
	ERTm6 ^a	Cape Verde / 2009	soil	2S
	JUm2799	Germany / 2014	rotting fruits	2S
<i>Caenorhabditis remanei</i>	JUm2796	Germany / 2014	rotting stems	n. d.

The reference strain of each newly found species is in bold. 2S: two distinct sizes of spores; n. d.: not determined.

a Strains and names published previously

b 18S sequences courtesy of Aurore Dubuffet and Hinrich Schulenburg

c Morphological feature described in [22]

d Note that a recent article describes a new species for this strain based on genome divergence [28].

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We first blasted the sequences in GenBank for initial grouping, then built phylogenetic trees and calculated interspecific genetic distances, based on our sequences and the sequences of related species from GenBank. We present below the grouping and phylogenetic distribution of new microsporidia strains, starting with those closest to *N. parisii*.

N. parisii and *N. ausubeli* are commonly found in *Caenorhabditis* nematodes

Molecular sequences of microsporidia in ten wild *C. elegans* strains and four *C. briggsae* strains showed $\geq 99\%$ SSU rDNA and $\geq 97\%$ β -tubulin sequence identities to *N. parisii* sequences in

Table 2. Collection of other microsporidia species infecting wild nematodes

Microsporidia	Nematode host	Strain	Country / Year	Sample composition	Spore feature
<i>Nematocida</i>	<i>C. briggsae</i>	JUm2507	Thailand / 2013	rotting fruit	2S
<i>major</i> n. sp.	<i>C. briggsae</i>	JUm2747	Guadeloupe / 2014	rotting fruit	2S
	<i>C. tropicalis</i>	JUm2751	Guadeloupe / 2014	rotting fruit	n. d.
<i>Nematocida</i>	<i>Oscheius tipulae</i>	JUm1510	Czech Republic / 2008	rotting apple	2S
<i>minor</i> n. sp.	<i>O. tipulae</i>	JUm2772	Armenia / 2014	rotting fruit	n. d.
<i>Nematocida</i>	<i>O. tipulae</i>	JUm1504	France / 2008	rotting stem	1S
<i>homosporus</i> n. sp.	<i>Rhabditella typhae</i>	NICm516	Portugal / 2013	rotting apple	1S
<i>Nematocida</i>	<i>Procephalobus</i> sp.	JUm2895	Spain / 2015	rotting fruit	1S
<i>ciargi</i> n. sp.					
<i>Nematocida</i>	<i>C. sp. 42</i>	NICm1041	French Guiana / 2014	flower	n. d.
<i>sp. 7</i>					
<i>Enteropsectra</i>	<i>Oscheius</i> sp. 3	JUm408	Iceland / 2002	compost	1S, LT, AP
<i>longa</i> n. sp.					
<i>Enteropsectra</i>	<i>O. tipulae</i>	JUm2551	France / 2013	rotting apple	1S, SR, AP
<i>breve</i> n. sp.	<i>O. tipulae</i>	JUm1483	France / 2008	rotting plum	1S, SR, AP
	<i>O. tipulae</i>	JUm1456	France / 2008	rotting fruit	n. d.
<i>Pancytophora</i>	<i>O. tipulae</i>	JUm1505	France / 2008	rotting apple	LT
<i>philotis</i> n. sp.	<i>O. tipulae</i>	JUm1460	France / 2008	rotting snail	LT
	<i>O. tipulae</i>	JUm1670	France / 2009	rotting apple	LT
	<i>O. tipulae</i>	JUm2552	France / 2013	rotting stem	LT
<i>Pancytophora</i>	<i>C. brenneri</i>	JUm1396	Colombia / 2008	rotting fruit	LT
<i>epiphaga</i> n. sp.					

The reference strain of each newly found species is in bold. 2S: two distinct sizes of spores; 1S: one size of spores
 LT: long, thin rod; SR: small rod (see dimensions in Table 3); AP: form spores first along the apical side of the
 intestinal cells. n. d.: not determined

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GenBank. In the global phylogenetic analysis of microsporidia, these 14 sequences form a group with previously reported sequences of *N. parisii* strains ERTm1, ERTm3 and ERTm5 [26] (Fig 2). The *N. parisii* isolates were all found in Europe (note however that the sampling is highly biased towards Europe, especially France), with the exception of the previously reported ERTm5 (JUm2055), isolated from a *C. briggsae* strain sampled in Hawaii (Fig 1B; Table 1A) [29]. Note that a recent article assigns this strain to a new species based on genome divergence [28].

Eight other microsporidian strains showed $\geq 99\%$ SSU rDNA and $\geq 95\%$ β -tubulin sequence identities to the corresponding genes of the unnamed *Nematocida* sp. 1 in GenBank (Table 1B), previously reported in *C. briggsae* [22,30]. This *N. sp. 1* group is most closely related to *N. parisii* in the microsporidian phylogeny and the sequences of both SSU and β -tubulin genes gave the same grouping (Figs 2 and 3; S2 Fig; Table 3). Because of these new samples of *N. sp. 1* and their phylogenetic difference and genetic distance to the *N. parisii* group, here we describe *N. sp. 1* as *Nematocida ausubeli* n. sp. (see Taxonomy section after the Discussion). Whereas *N. ausubeli* was so far only reported from *C. briggsae* (India, Cape Verde [30]), we also found it in *C. elegans* and *C. remanei*, in France, Portugal and Germany (Table 1B; Fig 1B), thus broadening its geographic and host range to several species of the *Ele-gans* group of *Caenorhabditis* from Europe.

The remaining 20 microsporidia strains that we identified are distributed among several other species, including some species in another clade (see below). Thus the dominant

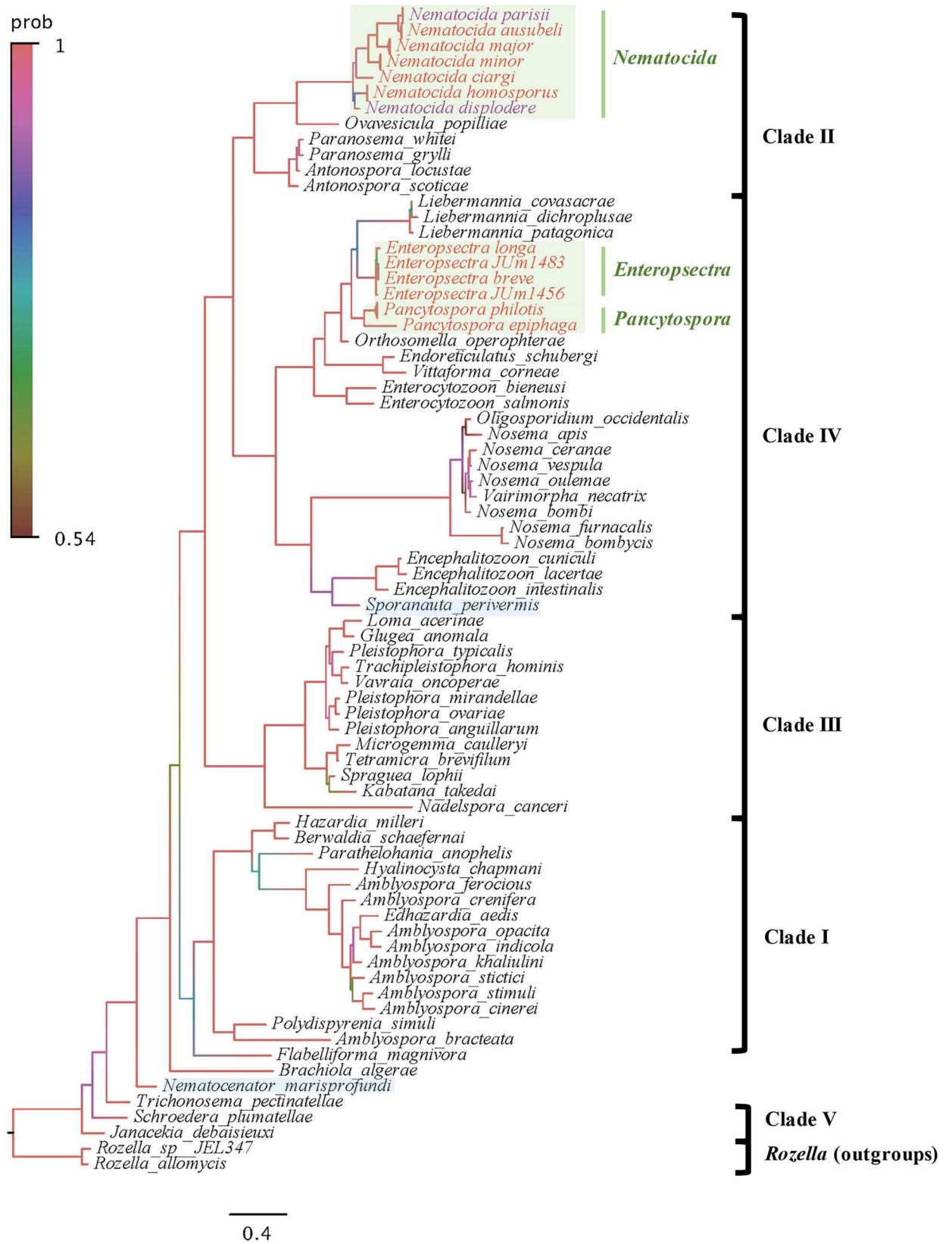


Fig 2. Bayesian inference SSU rDNA phylogeny of microsporidia species. SSU rDNA sequences from 45 nematode-infecting microsporidia species and 60 other microsporidia species in the databases were used. The tree was generated using MrBayes v3.2.2 and refined by FigTree v1.4.2. Model Kimura 2-Parameter (K2P) was applied. Branch colors show the posterior probability, with the corresponding color code shown on the left. The light green boxes designate microsporidia infecting terrestrial nematodes and light-blue rectangles designate those infecting marine nematodes. Scale bar indicates expected changes per site. Branches of species with more than one strain were compressed.

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microsporidia species in our collection of *Caenorhabditis* and other nematodes are *Nematocida parisii* and *Nematocida ausubeli* n. sp., with 17 and 10 strains, respectively. They were found in several species of the *Elegans* group of *Caenorhabditis* nematodes.

Diversity of *Nematocida* species

Of the remaining 19 microsporidian strains, nine had a *Nematocida* species as their top blast hit in GenBank, with similarity between 81% ~ 86% of SSU rDNA and 74% ~ 84% of β -tubulin genes. In terms of host and geographical ranges, these microsporidia were found in two *C. briggsae* strains (Thailand and Guadeloupe), one *C. tropicalis* strain (Guadeloupe), one *C. sp.* 42 strain (NIC1041 from French Guiana), three *Oscheius tipulae* strains (France, Czech Republic, and Armenia), one *Rhabditella typhae* strain (Portugal) and one *Procephalobus* sp. strain (JU2895 from Spain). In the phylogenetic analysis of SSU rDNA, the corresponding sequences formed a single clade with *N. parisii* and *N. ausubeli*, with *Ovavesicula popilliae* as sister group within Clade II of the microsporidian phylum (see Fig 2) [34]. In addition, the JU2807 strain that has been recently described as *Nematocida displodere* [23] is distinct from all of them.

From phylogenetic analysis and genetic distance of SSU rDNA genes, these *Nematocida* strains form four groups. These putative new *Nematocida* species have a mean genetic distance among them of at least 0.06 (Table 3), while their intra-specific genetic distances are all 0.00 (when several strains were isolated). This inter-group distance is also greater than the distance between *N. parisii* and *N. ausubeli*. Hence we describe them below as four new species: *Nematocida minor*, *Nematocida major*, *Nematocida homosporus* and *Nematocida ciargi* n. spp. (see Taxonomy section).

In terms of the phylogenetic relationships within the *Nematocida* genus in the SSU rDNA tree, the first outgroup clade to *N. parisii* + *N. ausubeli* was formed by JU2751, JU2747 and JU2751, corresponding to *N. major* (Fig 2). The second branch out was formed by JU1510 and JU2772, described here as *N. minor*. *N. ciargi* JU2895 was placed in a basal position to the clade formed by *N. parisii*, *N. ausubeli*, *N. major* and *N. minor* (Fig 2). At the base of the *Nematocida* genus, the most externally branching sequences appeared to be those of *N. displodere* JU2807, and of *N. homosporus* JU1504 and NICm516. All topologies were highly supported, except for the node defining the latter clade of *N. homosporus* and *N. displodere* (Fig 2). In the phylogenetic tree based on both genes (SSU rDNA and β -tubulin), *N. ciargi* was placed at the base of *Nematocida* genus, while *N. displodere* and *N. homosporus* still formed one clade (Fig 3). The phylogenetic tree only based on β -tubulin sequences supported the grouping of strains and overall their relative positions (S2 Fig), except that the relative placement of *N. displodere* and *N. ciargi* was exchanged. The β -tubulin phylogeny has one more branch formed by NICm1041, numbered provisionally *N. sp.* 7, for which we failed to amplify the SSU rDNA fragment. Whole-genome analysis could be performed in the future to refine these placements.

The *Nematocida* consensus phylogeny is shown in Fig 3 next to the phylogenetic relationships of the nematode hosts in which they were naturally found (see below for further specificity tests). Although the numbers of samples and species are too low for rigorous testing, the

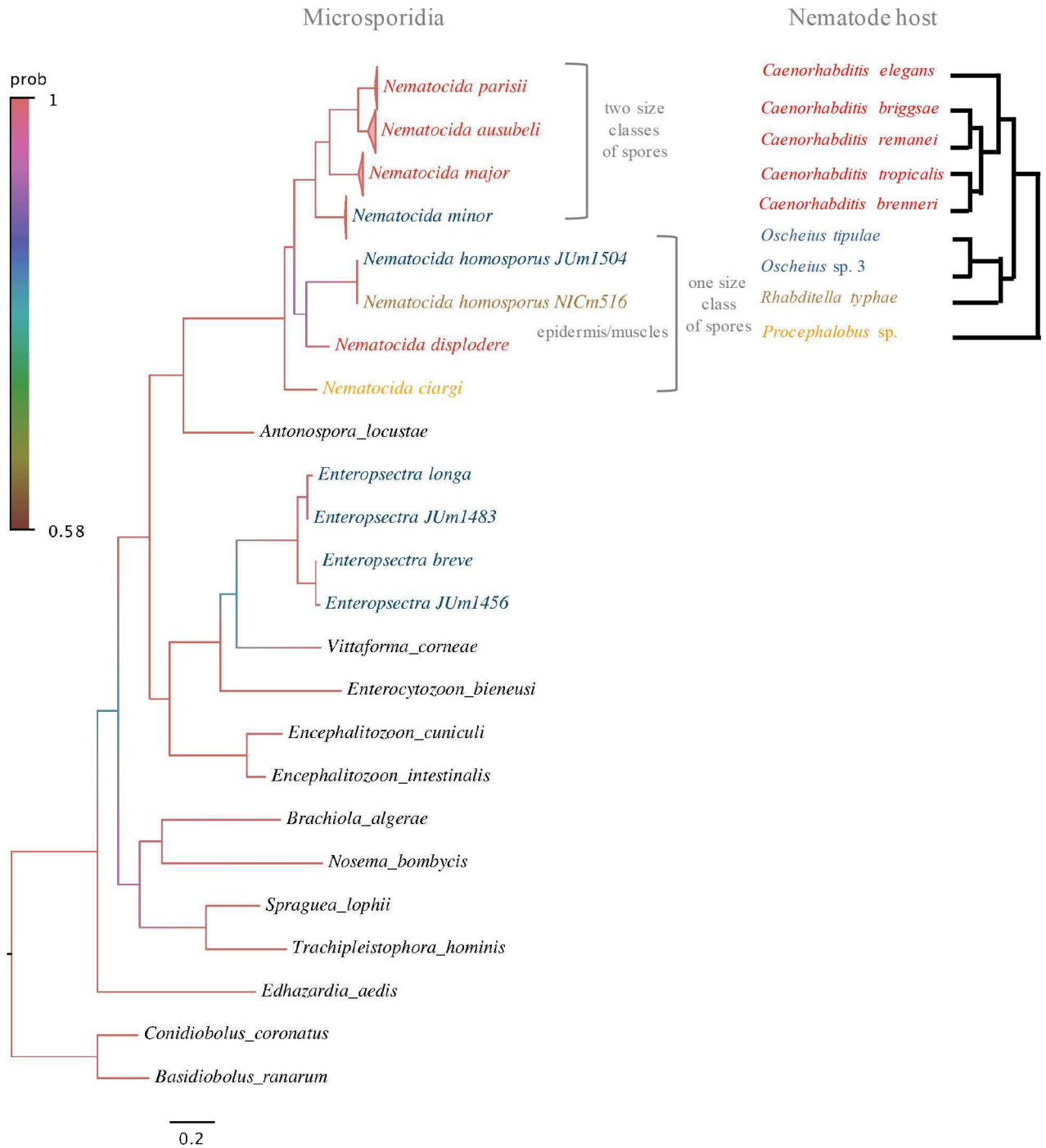


Fig 3. Bayesian inference phylogeny of concatenated SSU rDNA and β -tubulin sequences of 22 microsporidia species, and comparison with the nematode host phylogeny. Bayesian inference phylogeny (left) based on 49 sequences concatenated from SSU rDNA and β -tubulin genes of 22 microsporidia species. Model General time reversible (GTR) was applied. The branches were colored and annotated as in Fig 2. On the right is a diagram (generated based on phylogenies from [18,21,31–33]) showing the relative position of nematode species found with microsporidia infections. Nematode-infecting microsporidia pathogens and their hosts were colored based on host genus. Correspondent positions of nematode-infecting microsporidia and nematodes on their phylogenies indicate a possible coevolution of nematodes and their natural pathogenic microsporidia.

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Table 3. Molecular distances of microsporidia SSU rDNA.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 <i>Nematocida parisii</i> (17) ^a	0.000	0.007	0.024	0.026	0.029	0.025	0.025	0.044	0.058	0.055	0.056	0.052	0.051	0.061	0.053	0.059	0.053	0.053	0.050	0.066	0.068
2 <i>N. ausubelli</i> n. sp. (10) ^a	0.017	0.000	0.026	0.026	0.029	0.026	0.027	0.042	0.057	0.054	0.055	0.052	0.050	0.060	0.053	0.059	0.052	0.052	0.049	0.064	0.067
3 <i>N. major</i> n. sp. (3) ^a	0.138	0.154	0.000	0.022	0.026	0.025	0.023	0.044	0.061	0.064	0.062	0.057	0.058	0.068	0.055	0.060	0.049	0.055	0.049	0.063	0.057
4 <i>N. minor</i> n. sp. (2) ^a	0.163	0.169	0.121	0.000	0.022	0.017	0.018	0.038	0.062	0.060	0.060	0.053	0.058	0.062	0.055	0.060	0.051	0.048	0.047	0.059	0.059
5 <i>N. homosporus</i> n. sp. (2) ^a	0.182	0.188	0.154	0.122	0.000	0.011	0.014	0.034	0.057	0.055	0.055	0.054	0.052	0.061	0.049	0.056	0.051	0.049	0.041	0.055	0.058
6 <i>N. displodere</i> (1) ^a	0.151	0.165	0.142	0.082	0.043	/	0.010	0.033	0.057	0.055	0.055	0.054	0.052	0.062	0.049	0.053	0.048	0.046	0.040	0.051	0.053
7 <i>N. ciargi</i> n. sp. (1) ^a	0.151	0.165	0.130	0.096	0.062	0.034	/	0.031	0.052	0.051	0.051	0.050	0.048	0.059	0.046	0.052	0.045	0.041	0.038	0.048	0.049
8 CLADE_II (5) ^c	0.345	0.335	0.326	0.289	0.249	0.236	0.222	0.122	0.040	0.039	0.039	0.039	0.035	0.045	0.035	0.040	0.038	0.039	0.031	0.037	0.065
9 <i>Enteropsectra longa</i> n. sp. (1) ^a	0.435	0.436	0.447	0.455	0.413	0.417	0.384	0.318	/	0.004	0.002	0.017	0.020	0.023	0.021	0.036	0.045	0.049	0.049	0.054	0.076
10 <i>Enteropsectra breve</i> n. sp. (1) ^a	0.420	0.421	0.463	0.439	0.398	0.403	0.370	0.311	0.005	/	0.002	0.016	0.019	0.022	0.021	0.037	0.046	0.048	0.049	0.056	0.077
11 <i>Enteropsectra species</i> (2)	0.424	0.424	0.451	0.443	0.402	0.406	0.374	0.312	0.004	0.004	0.008	0.017	0.019	0.023	0.021	0.036	0.045	0.048	0.049	0.054	0.076
12 <i>Orthosomella operophtherae</i> (1) ^a	0.392	0.393	0.423	0.395	0.389	0.394	0.362	0.307	0.091	0.084	0.089	/	0.017	0.022	0.023	0.035	0.047	0.046	0.048	0.058	0.084
13 <i>Pancytospora philotis</i> n. sp. (4) ^a	0.384	0.378	0.418	0.427	0.374	0.378	0.346	0.269	0.115	0.108	0.113	0.091	0.005	0.021	0.020	0.048	0.052	0.048	0.054	0.070	0.098
14 <i>Pancytospora epiphaga</i> n. sp. (1) ^a	0.460	0.452	0.490	0.451	0.450	0.455	0.429	0.352	0.141	0.133	0.139	0.124	0.112	/	0.023	0.049	0.056	0.056	0.061	0.068	0.110
15 <i>Liebermannia</i> spp. (3) ^b	0.405	0.406	0.404	0.406	0.351	0.353	0.329	0.284	0.118	0.118	0.120	0.133	0.123	0.152	0.011	0.037	0.042	0.047	0.051	0.056	0.080
16 CLADE_IV (19) ^c	0.510	0.512	0.511	0.512	0.480	0.464	0.455	0.383	0.330	0.331	0.329	0.324	0.405	0.423	0.332	0.280	0.039	0.049	0.038	0.039	0.060
17 CLADE_III (12) ^c	0.443	0.443	0.413	0.428	0.428	0.409	0.387	0.355	0.384	0.395	0.389	0.398	0.430	0.465	0.371	0.409	0.223	0.038	0.037	0.040	0.061
18 CLADE_I (14) ^c	0.462	0.456	0.459	0.420	0.417	0.396	0.353	0.347	0.404	0.396	0.400	0.389	0.400	0.462	0.404	0.474	0.371	0.169	0.039	0.043	0.068
19 CLADE_? (3) ^c	0.417	0.411	0.400	0.394	0.352	0.337	0.319	0.272	0.414	0.420	0.416	0.402	0.446	0.483	0.432	0.392	0.371	0.358	0.255	0.028	0.053
20 CLADE_V (3) ^c	0.477	0.471	0.449	0.447	0.403	0.380	0.361	0.310	0.419	0.434	0.423	0.441	0.502	0.513	0.433	0.380	0.373	0.385	0.260	0.127	0.039
21 <i>Rozella</i> spp. (2) ^b	0.482	0.478	0.408	0.417	0.410	0.362	0.346	0.473	0.543	0.548	0.541	0.598	0.658	0.712	0.587	0.525	0.528	0.561	0.433	0.322	0.042

The lower left part shows the mean genetic distances between groups and the upper right part is the standard error (SE), with Kimura 2-Parameter model+G, 1000 bootstraps. Mean intra-species, intra-genus or intra-clade divergences are shown in the diagonal if available, with the number of sequences indicated in the parentheses after the name of each group (a: species; b: genus; c: larger clade). For a detailed table with all strains, see [S3 Table](#).

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data are at least consistent with the intestinal microsporidia species branching through continuous co-evolution with their nematode host. For example, all intestinal *Nematocida* species found in *Caenorhabditis* species form one clade, with a first outgroup including *Oscheius* and *Rhabditella* pathogens and a distant outgroup infecting the distant outgroup *Procephalobus* (Fig 3). The exception is *N. displodere* that was found a single time, in *C. elegans*, and corresponds to a change in tissue tropism.

Lifecycle of new *Nematocida* species

As with previously isolated *Nematocida*, the newly identified microsporidia appeared to be transmitted horizontally, because a bleaching treatment [35] of infected gravid adults eliminated the infection in the culture and reinfection could be obtained by exposure to spores in the environment. All *Nematocida* microsporidia stages described here were found exclusively in the intestinal cells and were not detected in the germ line.

As previously described for *N. parisii* [22], two main stages could be distinguished by Nomarski optics. First, the meront stage appeared as areas of infected intestinal cells devoid of storage granules. These areas were first small circular regions, then extending to longer grooves. Second, rod-shaped sporoblasts and spores appeared in the intestinal cell cytoplasm. In host cells that were heavily infected with *N. parisii* and some other species, groups of spores inside vesicles could be seen [22], possibly derived from spore re-endocytosis [36]. In this study, as described before [22], all *N. parisii* and *N. ausubeli* infections displayed two distinct classes of spore size (Table 1; Fig 4A and 4B; S3A Fig).

N. major and *N. minor* also displayed two spore size classes. *N. major* formed slightly longer but thinner spores than *N. parisii*. *N. minor* showed however much smaller spores, for each class taken separately (Tables 2 and 4; Fig 4C and 4D). In contrast, *N. homosporus* and *N. ciargi* only have a single class of spore size, with *N. homosporus* spores having an intermediate size (2.00 ± 0.22 μm long, 0.72 ± 0.12 μm wide) and *N. ciargi* spores having a smaller size (1.39 ± 0.20 μm long, 0.59 ± 0.13 μm wide). Spore vesicles were observed more frequently with *N. homosporus* or *N. ciargi* infections than with other *Nematocida* infections (Fig 4E and 4F).

N. ausubeli being the most commonly found parasite of *C. elegans* besides *N. parisii*, we further chose to study its lifecycle by electron microscopy. The ultrastructure by electron microscopy and the deduced lifecycle of *N. ausubeli* overall resembled those of *N. parisii*, with possible differences outlined below. High-pressure freezing/freezesubstitution allowed better to visualize lipid membranes compared to room temperature preparation methods. We observed meronts, which are separated from the host cell by a single membrane bilayer, likely pathogen-derived (Fig 5A and 5B; S1A and S1B Fig). Their cytoplasm appeared packed with ribosomes. Some meronts displayed an elongated shape and contained several nuclei (Fig 5B and 5L). The membrane enclosing the meronts appeared to darken progressively and intracellular membrane compartments developed, likely corresponding to the progressive transition to a sporont stage (Fig 5C). We further observed sporogony, whereby individual sporoblasts with a single nucleus are formed, each surrounded by a membrane (Fig 5D and 5E; S1A and S1D Fig). We did not observe any nuclear division at this stage (unlike in *Enteropsectra longa*, where they were easily found; see below). We observed progressive stages of sporogenesis, including formation of the anchoring disk, polaroplast membranes, polar tube, posterior vacuole and spore coat (Fig 5E–5G; S1C, S1D, S1F and S1G Fig).

In the final stages of sporogenesis and in mature spores that corresponded to the small size class observed in light microscopy, two polar tube coil cross-sections could usually be observed (Fig 5H and 5J; S1L Fig). A single large spore could be found, which displayed three polar tube coil sections on either side of the spore (six sections in total; Fig 5K). Thus, the tube coiled

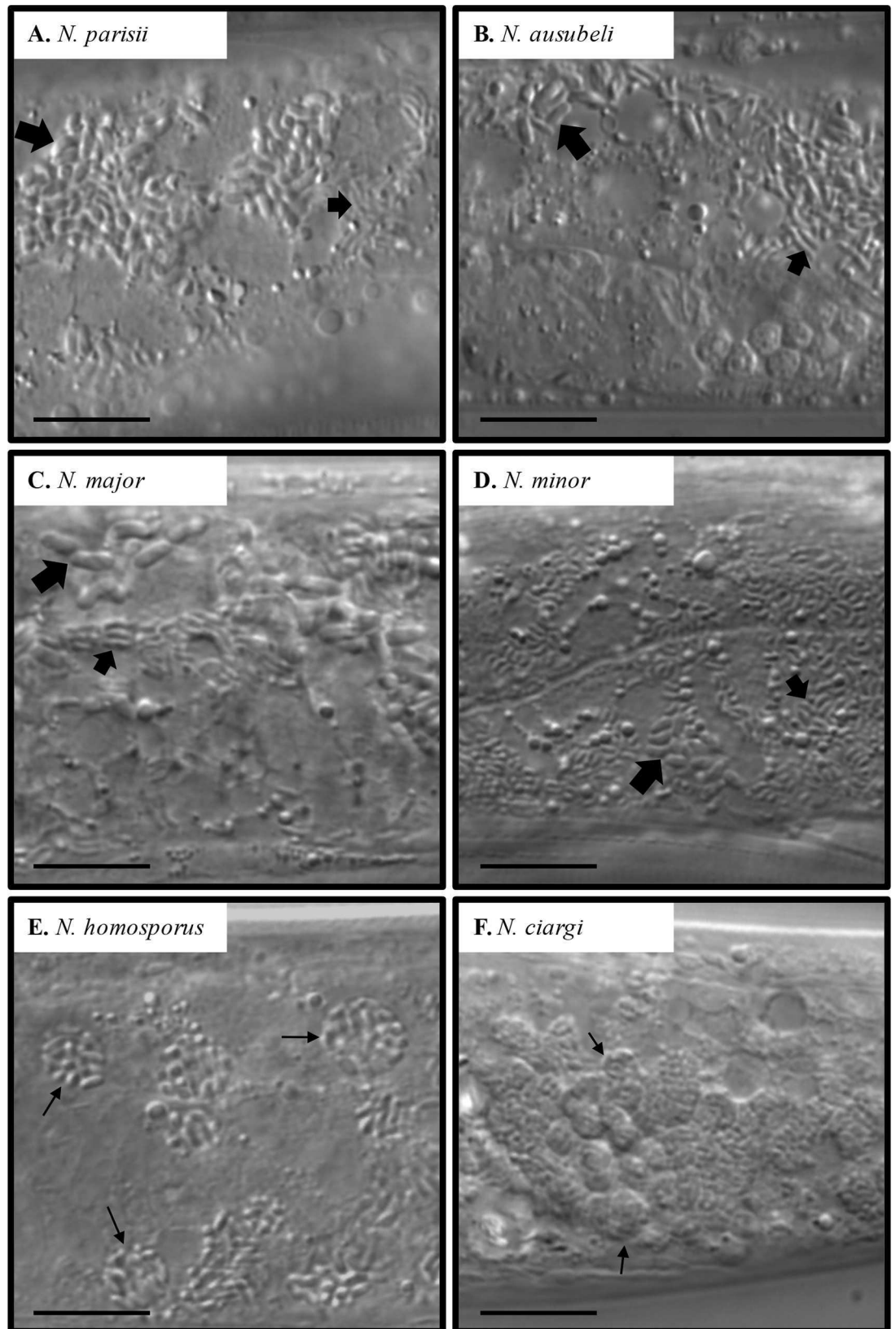


Fig 4. Spore morphology of the different *Nematocida* species by Nomarski optics. **A.** Wild *Caenorhabditis elegans* strain JU1249, with *Nematocida parisii* infection. **B.** Wild *C. elegans* strain JU2520, with *Nematocida ausubeli* infection. **C.** Wild *C. briggsae* strain JU2747, with *N. major* infection. **D.** Wild *Oscheius tipulae* strain JU1510, with *N. minor* infection. **A ~ D,** large and small spore classes are indicated by larger and smaller arrows, respectively. Spores in each class are smaller than those in A-C in the corresponding class. **E.** Wild *Rhabditella typhae* strain NIC516, with *N. homosporus* infection. A single class of spore size is observed, often clustered inside vesicles as indicated with arrows. **F.** Wild *Procephalobus* sp. strain JU2895, with *N. ciargi* infection. A single class of spore size is observed, often clustered inside vesicles as indicated with arrows. Scale bar: 10 μ m.

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several times in large spores, instead of once in the small spores. In *N. parisii*, five polar tube sections were reported on one side of the large spores [22]; it is thus possible that large spores of *N. ausubeli* harbor fewer polar tube coils than those of *N. parisii* (because a single large spore was found in each species, it is however difficult to conclude). The anchoring disk defines the anterior pole of the spore. Below the anchoring disk, the polar tube is lined on either side by polaroplast membranes (visible in Fig 5F and 5G). A polar tube cross-section with several layers could be seen in Fig 5J and the posterior turn of the polar tube in S11 Fig.

Table 4. Spore sizes of each nematode-infecting microsporidia species, as determined by Nomarski optics.

Nematode-infecting Microsporidia	Spore sizes (μ m)	
	Length	Width
<i>Nematocida parisii</i> ^a	3.17 \pm 0.22	1.31 \pm 0.15
	2.18 \pm 0.15	0.80 \pm 0.08
<i>Nematocida displodere</i> ^b	2.38 \pm 0.26	1.03 \pm 0.18
<i>Nematocida ausubeli</i> JUm2009 ^d	2.80 \pm 0.29 (2.21 ~ 3.26) ^c	0.96 \pm 0.14 (0.72 ~ 1.30)
	2.04 \pm 0.32 (1.36 ~ 2.92)	0.53 \pm 0.10 (0.28 ~ 0.80)
<i>Nematocida major</i> JUm2507	3.44 \pm 0.23 (3.04 ~ 3.79)	1.20 \pm 0.21 (0.90 ~ 1.53)
	2.35 \pm 0.23 (2.02 ~ 2.66)	0.57 \pm 0.14 (0.41 ~ 0.77)
<i>Nematocida major</i> JUm2747	3.29 \pm 0.25 (2.87 ~ 3.69)	1.20 \pm 0.23 (0.79 ~ 1.60)
	2.21 \pm 0.26 (1.85 ~ 2.60)	0.53 \pm 0.06 (0.44 ~ 0.63)
<i>Nematocida minor</i> JUm1510	1.93 \pm 0.18 (1.54 ~ 2.23)	0.83 \pm 0.13 (0.51 ~ 1.09)
	1.31 \pm 0.17 (1.09 ~ 1.73)	0.55 \pm 0.12 (0.35 ~ 0.73)
<i>Nematocida homosporus</i> JUm1504	2.07 \pm 0.30 (1.69 ~ 2.50)	0.77 \pm 0.12 (0.56 ~ 0.94)
<i>Nematocida homosporus</i> NICm516	1.95 \pm 0.14 (1.67 ~ 2.16)	0.69 \pm 0.11 (0.56 ~ 0.91)
<i>Nematocida ciargi</i> JUm2895	1.39 \pm 0.20 (1.54 ~ 2.24)	0.59 \pm 0.13 (0.41 ~ 0.84)
<i>Enteropsectra longa</i> JUm408	3.76 \pm 0.38 (3.15 ~ 4.99)	0.49 \pm 0.06 (0.35 ~ 0.68)
<i>Enteropsectra breve</i> JUm2551	1.81 \pm 0.22 (1.34 ~ 2.14)	0.66 \pm 0.12 (0.42 ~ 0.90)
<i>Pancytospora philotis</i> JUm1505	3.46 \pm 0.48 (2.42 ~ 4.65)	0.42 \pm 0.06 (0.25 ~ 0.52)
<i>Pancytospora epiphaga</i> JUm1396	3.71 \pm 0.31 (2.99 ~ 4.22)	0.80 \pm 0.09 (0.56 ~ 0.92)

^a [22]

^b [23]

^c Values of length and width of each microsporidia spore are given with the average \pm SD, followed with range of values in the parentheses.

^d Note some overlap in the measurements between the two classes of spore size for this species. The two classes of spore size are spatially segregated in all of the *Nematocida* species displaying two classes (see Fig 4A–4D, S3A Fig). When measuring spore size, we first assigned each to a group of large or small spores. In *N. ausubeli*, the measurements overlap between spores of the two classes, reflecting either our misassignment of the spore to a class, measurement error (spores in an oblique position, which we tried to avoid) or true overlap.

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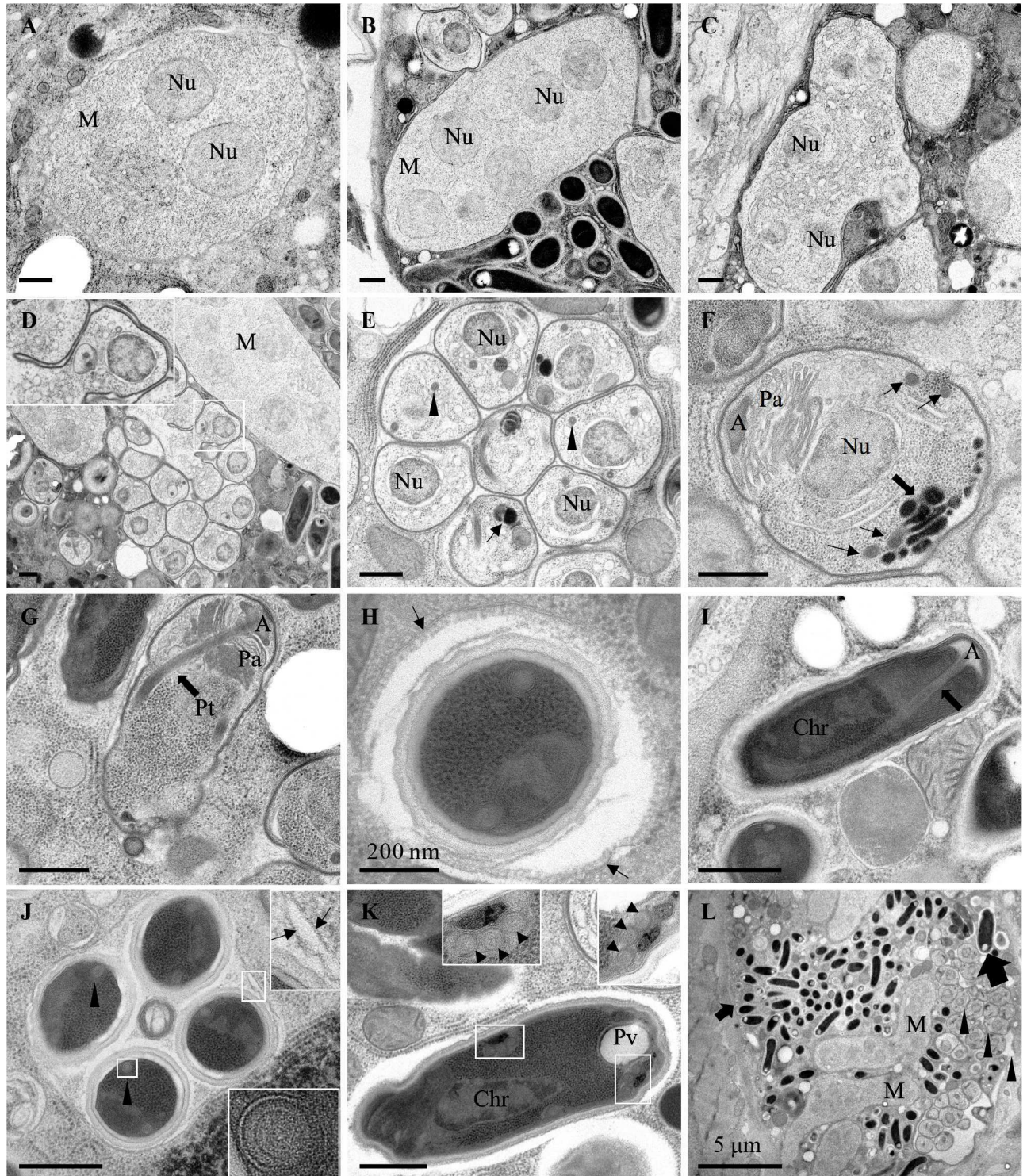


Fig 5. Ultrastructural observations of *Nematocida ausubeli*. Transmission electron micrographs of *N. ausubeli* strain JUm2009 after high-pressure freezing/freeze substitution. **A.** *N. ausubeli* meront with two nuclei. **B.** A multinucleated meront. **C.** Late stage meront. **D.** Formation of sporoblasts by polysporous sporogony. **E.** Cluster of sporonts after sporogony; the arrowheads indicate the nascent polar tube and the arrow indicates the dense membrane structure. **F.** Sporoblast with a maturing anchoring disk and the dense membrane structure on the future posterior side of the spore (large arrow). Four nascent polar tube coil cross-sections (arrows) are visible, suggesting that this sporoblast may form a spore of large size. **G.** Late stage sporoblast. The arrow indicates the polar tube. **H.** Mature spore with surrounding additional membrane (arrows). The internal side of this membrane is coated. **I.** Mature spore with polar tube indicated by arrow. The

anchoring disk and the membranes of the polaroplast are visible on the anterior side, chromatin and ribosomes on the posterior side. **J.** Cross-section of a spore vesicle containing four spores, each showing two polar tube sections (arrowheads). The upper inset shows two membranes around the vesicle (indicated by arrows). The lower inset shows an enlarged multilayered polar tube. **K.** A large size spore, with two insets showing the posterior vacuole and at least three polar tube coils (three cross-sections on either side of the spore, arrowheads). **L.** Lower magnification view of several *N. ausubeli* infection stages in host intestinal cells. Large arrow and small arrow indicate large spore and small spore, respectively. The large spore is that shown in panel K in another plane of section. Arrowheads indicate sporonts. Two multinucleate meronts are indicated. Scale bar is 500 nm, unless indicated otherwise. A, anchoring disk; Chr, chromatin; M, meront; Nu, nucleus; Pa, anterior polaroplast; Pp, posterior polaroplast; Pt, polar tube; Pv, posterior vacuole.

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The mature spore was seen to contain a posterior vacuole on the side opposite to the anchoring disk (Fig 5K; S1H–S1J Fig). This vacuole seemed to develop from a dense membrane compartment of the sporoblast (S1C and S1D Fig). The spores displayed an external coat with several layers (Figs 5H, 5J, 5K and 6A; S1I–S1L Fig).

The spores in the host cytoplasm appeared either isolated, or clustered within a large vesicle. Some isolated spores were surrounded by an additional membrane outside the spore coat and the inner face of this membrane appeared coated (Fig 5H). Unlike in *N. parisii* [27], we could not see the additional membrane around all spores. Fig 6A shows a spore apparently exiting the host cell through exocytosis (although we cannot rule out that such images correspond to endocytotic events). Spores in the lumen were not surrounded by any additional membrane (Figs 5K, 5L and 6A).

When spores were clustered in a vesicle, two membranes could be observed around them (Fig 5J, and other instances).

Nematode-infecting microsporidia in Clade IV

Whereas the *Nematocida* genus is in Clade II of the microsporidia [22,34], the remaining nine microsporidia strains in our collection were placed in Clade IV, which, unlike Clade II, contains several human-infecting microsporidia (Fig 2). This clade assignment was based on SSU rDNA sequences, which had closest (88–89%) identities to the insect parasite *Orthosomella operophterae* (host: moth *Operophtera brumata*) (Table 2). Only four β -tubulin sequences could be obtained, and these were closest (75% ~ 76% identity) to *Vittaforma corneae*, a human-infecting microsporidia species and a close relative of *Orthosomella operophterae* (whose β -tubulin sequence is not available), consistent with rDNA analysis. We thus isolated nematode-infecting microsporidia that are in a distinct evolutionary branch compared to *Nematocida* and are closer relatives of the human-infecting microsporidia.

Eight out of the nine strains in this group have *Oscheius* species as their nematode host and infect their gut: seven of them from different locations in France were found in *O. tipulae*, while JUm408 was found in *Oscheius* sp. 3 [21] from Iceland. The ninth strain, JUm1396, was isolated from a *C. brenneri* strain and is the only one in this set to infect non-intestinal tissues.

In the phylogenetic analysis, these nine strains separated into two groups, corresponding to the two new genera described below, *Enteropsectra* and *Pancytospira* (see section on Taxonomy) (Fig 2; S4 Fig). The first group included four strains, JUm408, JUm1456, JUm2551 and JUm1483, which were phylogenetically placed as a sister group to *Liebermannia* species (with hosts such as grasshoppers) (Fig 2). In the β -tubulin phylogeny, *Enteropsectra* strains also showed a sister relationship with the group of *V. corneae* and *Enterocytozoon bieneusi*, a human intestinal parasite (S2 Fig). However, with β -tubulin, JUm408 and JUm1483 formed a branch, JUm1456 and JUm2551 another branch, which was different from their SSU rDNA phylogenetic position. Based on molecular sequences, spore morphology and host specificity (below), we describe two species in the *Enteropsectra* genus, *E. longa* (type strain JUm408) and *E. brevis* (type strain JUm2551), and do not assign the two other strains to a species. *E. longa*

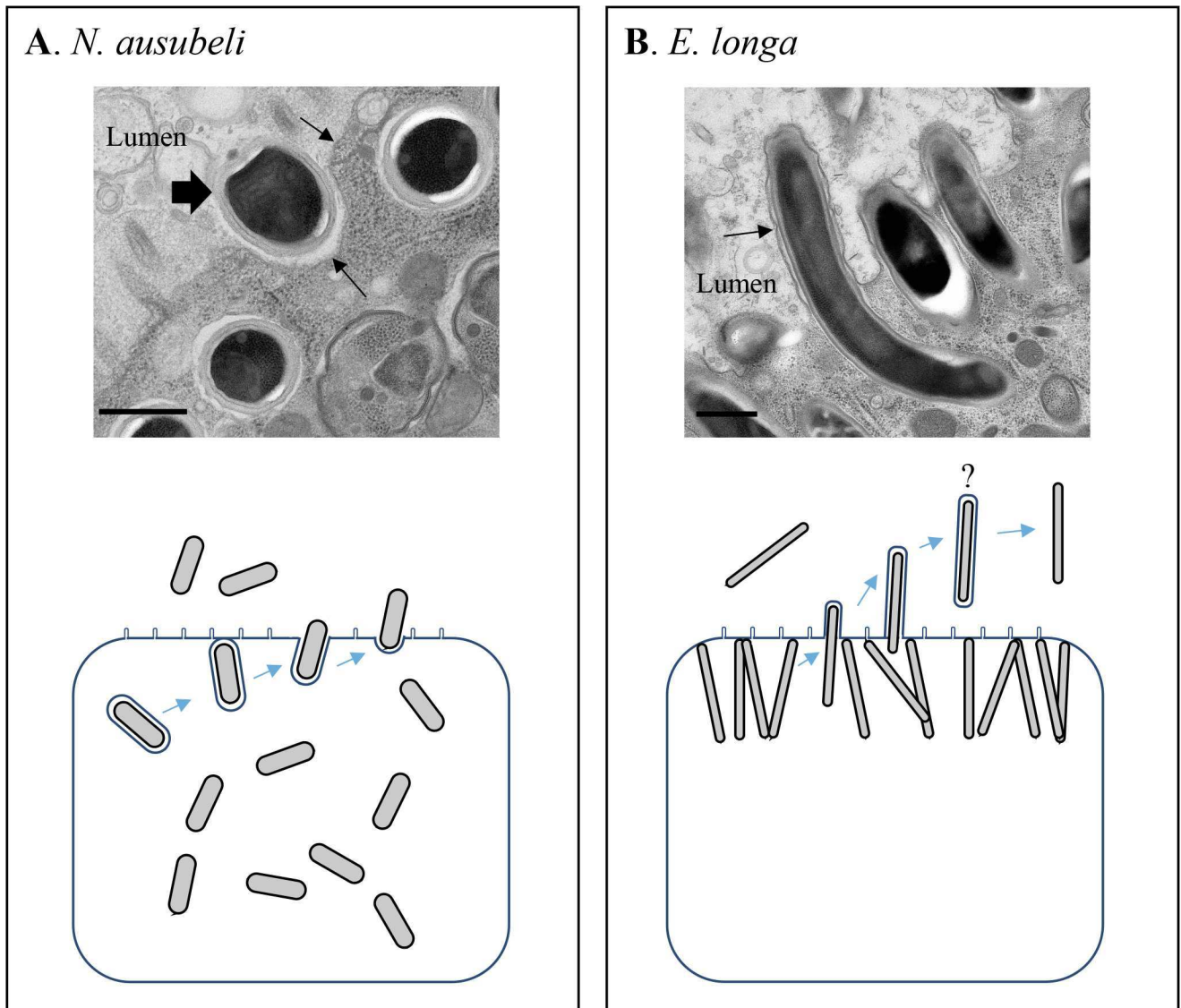


Fig 6. Cell exit modes of *Nematocida ausubeli* and *Enteropsectra longa*. **A. *Nematocida ausubeli*.** The top panel is an electron microscopy image of a *Nematocida ausubeli* spore (large arrow) exiting from the intestinal cell into the lumen. Arrows indicate the apical membrane of the host intestinal cell. The hypothetical diagram below illustrates the exit of *N. ausubeli* spores from intestinal cells by exocytosis. As in *N. parisii* [27,36], spores appear surrounded by a membrane that fuses with the apical membrane of the host intestinal cell, resulting in the release of spores. We also observed apparently mature spores without an additional membrane and do not know whether they will later acquire a membrane or exit in another manner. Earlier stages were omitted here for simplicity. **B. *Enteropsectra longa*.** The top panel is an electron micrograph of *Enteropsectra longa* spores exiting from the intestinal cell into the lumen, with the host intestinal cell membrane folding out around the *E. longa* spores (arrow). The diagram below illustrates the exit of *E. longa* spore from the intestinal cell. The host intestinal cell membrane folds out around the spore until the whole spore exits the cell, after which the host membrane around the spore seems to disappear. Meronts and sporoblasts are not represented in either panel. Scale bars: 500 nm.

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and *E. breve* strains have a small mean SSU genetic distance of 0.005 (Table 3) but differ in spore size and host specificity (see below). While *E. longa* and *E. breve* form a sister group to *Liebermannia* species on the SSU rDNA phylogeny, they have a smaller mean genetic distance to *O. operophtherae* (0.08) than to *Liebermannia* (0.11).

The second new clade of nematode-infecting microsporidia includes the five remaining strains and showed strong support as sister lineage to the clade formed by *Enteropsectra* and

Liebermannia species, with *O. operophtherae* as outgroup (Fig 2). Based on molecular sequences, host and tissue specificity, we describe two new species: *Pancytospora philotis* (JUm1505 as type strain, JUm1670, JUm2552), found in the *Oscheius* gut, and *P. epiphaga* (JUm1396) from a *C. brenneri* strain from Colombia that caused an epidermis and muscle infection (Fig 7; S5 Fig).

Tissue tropism and lifecycle of nematode-infecting Clade IV microsporidia species

As with *Nematocida*, all of the infections by Clade IV microsporidian strains mentioned above appeared to be transmitted horizontally, as bleaching of the nematode culture eliminated the infection. The *Enteropsectra* strains and *P. philotis* were only observed to infect the intestine of *Oscheius* nematodes. By contrast, *P. epiphaga* (JUm1396) was found to infect epidermis and muscles of *C. brenneri* (Fig 7D; S5D, S5E and S5F Fig), thus sharing its tissue tropism with *N. displodere*, although on a different evolutionary branch. *P. epiphaga* could also infect *C. elegans* (N2 reference background) (S7F Fig) and *C. briggsae* (AF16).

A striking feature of *Enteropsectra* strains is their cellular localization within the nematode intestinal cells: *Enteropsectra* were all observed to form their spores on the apical side of the epithelial cell at first, while meront stages could be seen in a more basal position (Figs 7A, 7B, 7E; 8L). This polarization within the host intestinal cell was not observed in infections of *P. philotis* nor of any *Nematocida* species (Table 2; Figs 4 and 7).

The *Enteropsectra* and *Pancytospora* species displayed quite different sizes and shapes of spores from those of *Nematocida* species and we did not see any spore-containing vesicles in these microsporidian infections. They all show a single class of spore size. Though apart in the phylogenetic analysis, *E. longa* (JUm408) and *P. philotis* share similar dimensions of spores, which are particularly long and thin: *E. longa* (JUm408) spores measure $3.76 \pm 0.38 \mu\text{m}$ by $0.49 \pm 0.06 \mu\text{m}$, while *P. philotis* spores measure $3.46 \pm 0.48 \mu\text{m}$ long by $0.42 \pm 0.06 \mu\text{m}$. These spores are even longer than the largest spores and thinner than the smallest spores in *Nematocida*. In stark contrast, *E. breve* (JUm2551) form small rod-shaped and crescent-shaped spores (Fig 7B; Table 4).

Because of the striking difference in spore distribution, we further analyzed by electron microscopy the type species of the *Enteropsectra* genus, *Enteropsectra longa* (JUm408) in *Oscheius* sp. 3 JU408. The meront stage appeared overall similar to that of *Nematocida* species: the early stages displayed a cytoplasm packed with ribosomes and very few membranes (Fig 8A); elongated multinucleated meronts could also be observed (Fig 8B). The parasite membrane then progressively darkened, indicating the transition to the sporont stage (Fig 8C–8F). Figures of intranuclear mitosis could be seen at this stage, with intranuclear microtubules and spindle plaques at the nuclear membrane (Fig 8E; S6A Fig). Signs of sporogenesis then developed, with a nascent polar tube (Fig 8F–8H; S6B Fig). The spore membrane and nascent wall appeared wrinkled (Fig 8G) before becoming smooth in mature spores (Fig 8H–8J). The spore wall with its endospore and exospore layers could be clearly observed (Fig 8J). An anchoring disk formed (Fig 8I and 8K), but the polaroplast membranes were less developed than in *Nematocida* species. In most spores, the polar tube presented a single section (Fig 8G, 8H and 8J; S6D Fig). The polar tube however could be seen to turn on the posterior side of the spore (S6C Fig) and occasionally two polar tube sections could be counted in the same spore section (S6E and S6F Fig). The polar tube thus likely folds back anteriorly on the posterior side of the spore on a short part of its length.

By electron microscopy, we observed a potential key difference in the exit mode of the spores between *Enteropsectra longa* (JUm408) on one hand, and *N. parisii* and *N. ausubeli* on

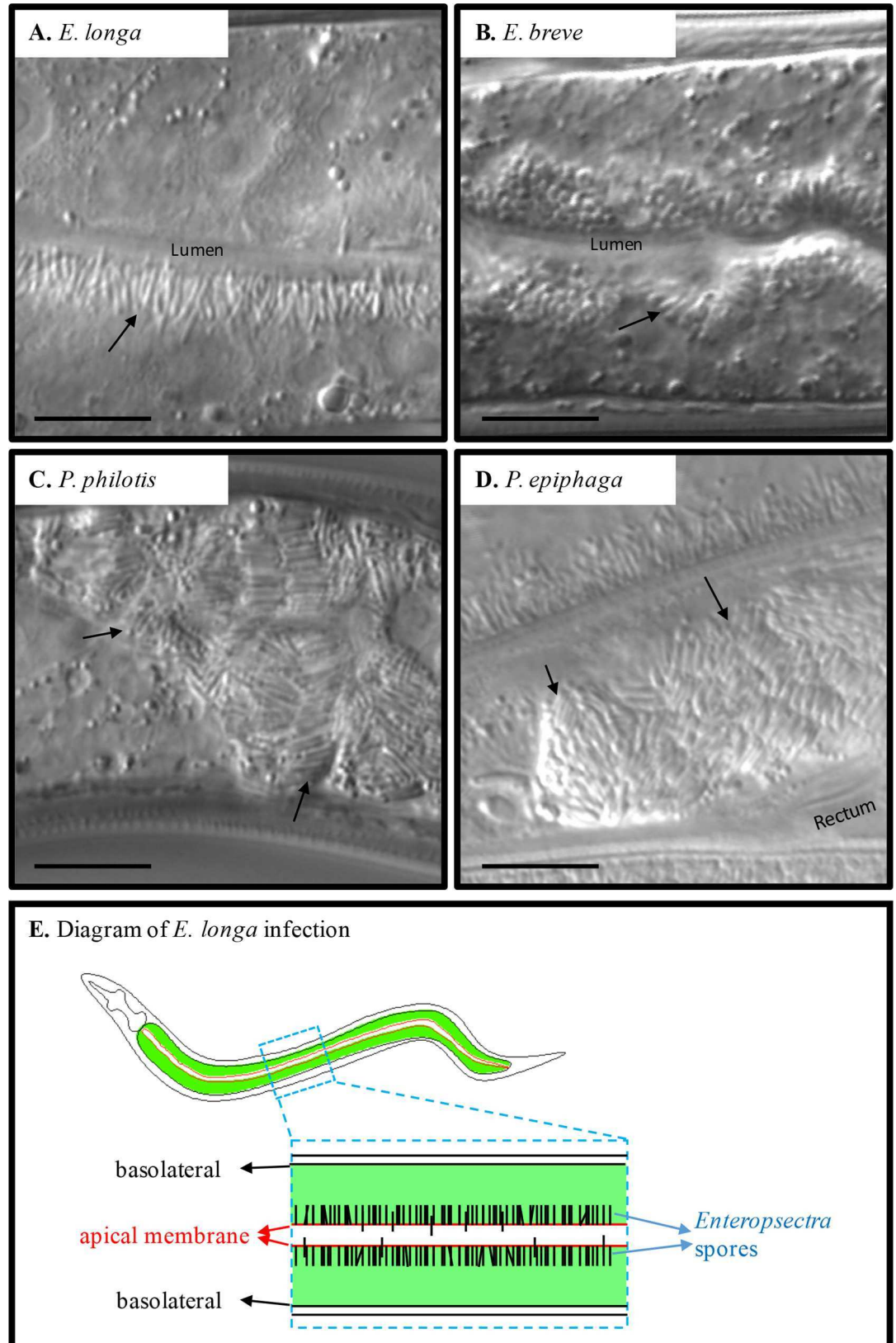


Fig 7. Spore morphology of *Enterospectra* and *Pancytospora* species by Nomarski optics. A. Wild *Oscheius* sp. 3 strain JU408, with *Enterospectra longa* infection. Gut lumen was indicated. Arrow indicates spores, here long and thin spores that are aligned along the apical side of intestinal cells. B. Wild *O. tipulae* strain JU2551, with *Enterospectra breve* infection. Arrow indicates spores, here small spores along the apical side of intestinal cells. C. Wild *O. tipulae* strain JU1505, with *Pancytospora philotis* infection. Spores are found throughout the intestinal cell. D. Wild *Caenorhabditis brenneri* strain JU1396, with *Pancytospora epiphaga* infection. Spores are seen in the epidermal cells in the tail that does not contain any gut tissue (posterior to the rectum). Anterior is to the right. The "fur" on the outside of the cuticle is formed by unidentified bacteria (see [37] for another example). Scale bar: 10 μ m in A-D. E. Two-dimensional diagram of *Oscheius* sp. 3 intestine infected with *Enterospectra longa*. The intestine is formed of polarized epithelial cells. *Enterospectra longa* starts to form spores along the apical side of the intestinal cells.

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the other hand. First, the sporoblasts and mature spores of *E. longa* were never seen to be surrounded by an additional membrane outside the spore wall, precluding exocytosis as an exit route. Second, the spores were seen to protrude on the apical side of the host cell, pushing out the host cell membrane like a finger in a glove (Fig 6B; S6G–S6I Fig). We further focused on spore sections in the intestinal lumen and saw both spores with a surrounding membrane (S6I Fig) and spores without any membrane (Fig 8K).

On the host side, rough endoplasmic reticulum was often seen to wrap around sporoblasts, yet never encircling them fully (Fig 8G). The host cell nuclei presented a characteristic nucleolar structure, which became organized in long tubules (often appearing circular in cross-sections; S6J and S6K Fig). On one occasion, microsporidian spores were observed within the host intestinal cell nucleus, whose nucleolus had apparently further degenerated (S6L Fig).

Host specificity: natural associations and laboratory infection tests

The pattern of natural association revealed an apparent specificity of a given microsporidian species for a nematode genus, mostly *Caenorhabditis* versus *Oscheius* in our collection. Strikingly, *N. parisii*, *N. ausubeli* and *N. major* infections were found in *Caenorhabditis* species, while *N. minor*, *N. homosporus* and Clade IV microsporidia species infections were all found in *Oscheius* species and not in *Caenorhabditis* (or, for *N. homosporus*, in *Rhabditella*, a closer relative of *Oscheius* compared to *Caenorhabditis*; Fig 3). The notable exception in Clade IV was the epidermal *P. epiphaga* JU1396, found in *C. brenneri*. These results suggested a pattern of host-pathogen specificity between nematode and nematode-infecting microsporidia.

We further complemented these natural associations with infections performed in the laboratory. To test for the capacity of a given microsporidia strain to infect a given host, uninfected nematode cultures (cleaned by bleaching) were exposed to microsporidian spores. We used clean spore preparations from seven microsporidian species (see Materials and Methods), namely *N. parisii*, *N. ausubeli*, *N. major*, *N. homosporus*, *E. longa*, *E. breve* and *P. philotis*. On the host side, we focused on four nematode species of two genera: *C. elegans*, *C. briggsae*, *O. tipulae* and *O. sp. 3*, all of which reproduce through self-fertilizing hermaphrodites and facultative males [19,20]. We favored wild strains that had been found naturally infected with microsporidia and were thus not generally resistant to microsporidian infections (Table 5).

N. parisii (JU2816) infected more than 50% of *C. elegans* (N2, JU2009) and *C. briggsae* (JU2747, JU2793) individual animals at 72 hpi. However, no microsporidian infection symptom was observed in *O. tipulae* (JU1483, JU170) nor *O. sp. 3* (JU408, JU75) at 72 and 120 hpi (Table 5). *O. tipulae* strains JU1504, JU1510 and JU2552 were also exposed to *N. parisii* spores, and none of them became infected either. These infection results indicated that *N. parisii* was unable to infect *O. tipulae* nor *O. sp. 3* (Table 5).

Specificity of *N. ausubeli* (JU2009) slightly differed from that of *N. parisii*. By 72 hpi, half of all *Caenorhabditis* animals and about 30% of *O. sp. 3* showed signs of infection. None of *O. tipulae* worms were infected even at 120 hpi (Table 5). However, when we made a new *N.*

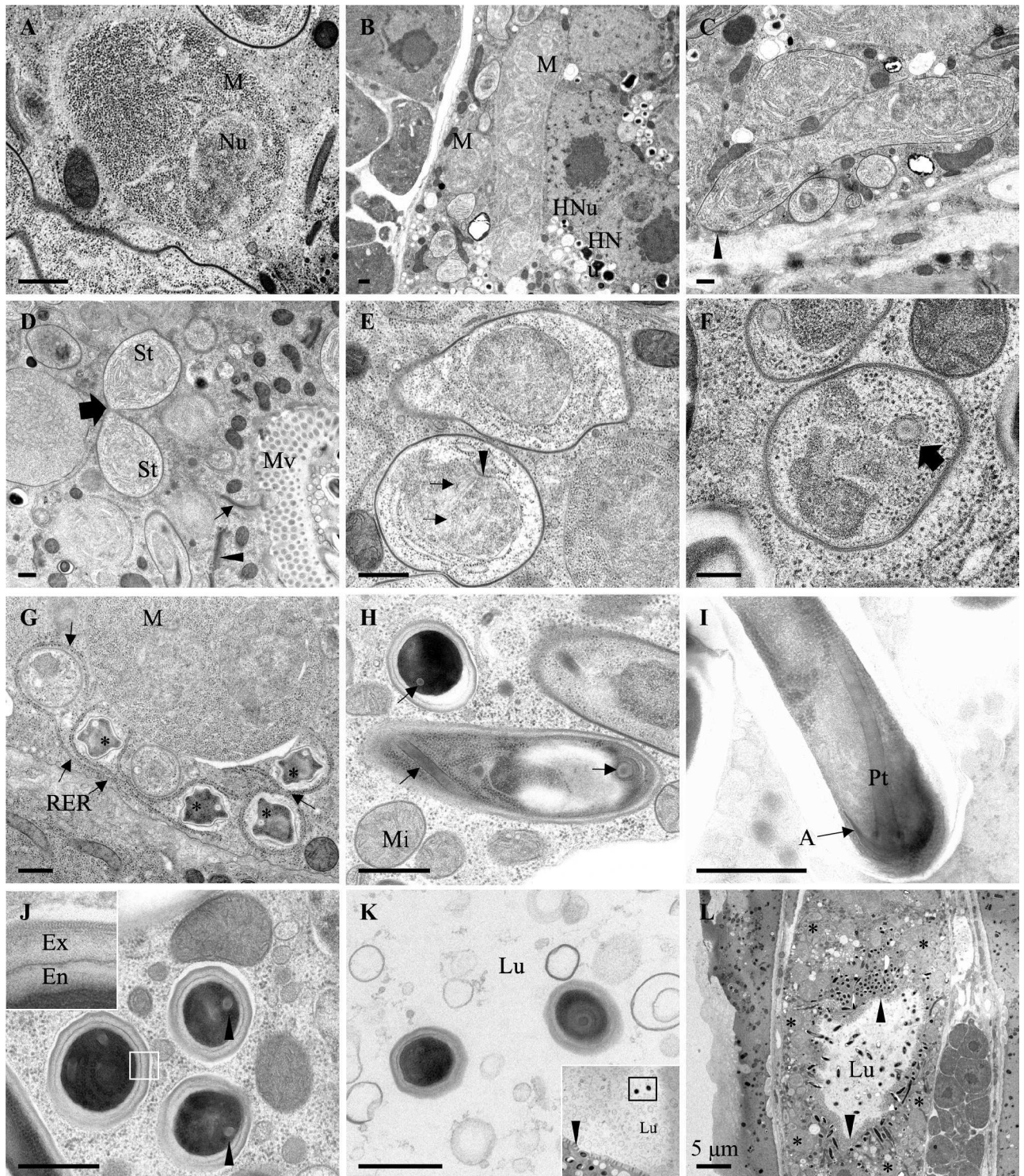


Fig 8. Ultrastructural observations of *Enteropsectra longa*. Transmission electron micrographs of *E. longa* strain JUm408 after high-pressure freezing/freeze substitution. **A.** *E. longa* meront. A nucleus is visible in the cytoplasm full of ribosomes. **B.** Lower magnification with a multinucleated meront. Two meronts are indicated, one with a single nucleus in the plane of section (left) and one with several nuclei (right). Two host nuclei are visible on the right, with a dark nucleolus. Intestinal cells contain two nuclei. **C.** Early sporonts with an electron-dense coat indicated by arrowhead. **D.** Sporont undergoing a cell division (big arrow); small arrow indicates junction of host intestinal cells; the arrowhead indicates a host Golgi apparatus. **E.** Mitotic spindle (arrows designate microtubules) in a sporont; the spindle plaque is indicated by an arrowhead. **F.** Nascent polar tube (arrow) in a sporoblast. **G.** Wrinkled sporoblasts (*). Arrows indicate the host rough

endoplasmic reticulum folding around the microsporidia. **H.** Late stage sporoblast in the center, mature spore on the top left; arrows indicate polar tubes. **I.** Mature spore with the anterior part of the polar tube, including the anchoring disk. **J.** Cross-section of mature spores. The exospore and endospore layers are shown in the inset. Arrowheads indicate polar tubes. **K.** Two mature spores in the intestinal lumen that do not show an additional membrane around them. Low magnification inset shows the positions of the two spores in the lumen and arrowhead indicates host microvilli. **L.** Low magnification view of cross-section of host, with the intestinal lumen in the center. *E. longa* spores (arrowheads) concentrate around the apical membrane of intestinal cell, while meronts and early sporonts are on the basal side. Scale bar is 500 nm, unless indicated otherwise. A, anchoring disk; Chr: chromatin; Ex, exospore; En, endospore; Lu, lumen; M, meront; Mi: host mitochondrion; Mv, host microvilli; Nu, nucleus; HNu host nucleus; Pt, polar tube; RER, rough endoplasmic reticulum; St: sporont.

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ausubeli (JUm2009) spore preparation and used it directly for infection tests, *O. tipulae* strains JU1510 and JU2552 could be infected, but the preparation lost its ability to infect *O. tipulae* over storage at -80°C (see [Materials and Methods](#)). We conclude that *O. tipulae* was far less susceptible to infection than *C. elegans*, *C. briggsae* and *O. sp. 3* to *N. ausubeli* infection.

The host spectrum of *N. major* (JUm2747) was quite similar to that of *N. parisii*: it infected *C. elegans* (N2, JU2009) and *C. briggsae* (JU2507, JU2793), but not *O. tipulae* (JU1483, JU170) nor *O. sp. 3* (JU408, JU75) ([Table 5](#)). *N. homosporus*, however, could infect both *Caenorhabditis* and both *Oscheius* species and thus appeared as the most generalist ([Table 5](#)). Yet *O. tipulae* seemed relatively less sensitive than *C. elegans*, *C. briggsae* and *O. sp. 3* to *N. homosporus* infection.

Enteropsectra spp. and *Pancytospora philotis* showed different and even opposite specificities compared to the four tested *Nematocida* species. Indeed, none could successfully infect any tested *Caenorhabditis* strains at 120 hpi. Within the two *Oscheius* species, specific interactions were further observed. *Enteropsectra longa* (JUm408) only infected *O. sp. 3* strains (JU408, JU75), but not *O. tipulae* (JU1483, JU2551), while *E. breve* (JUm2551) infected all four *O. tipulae* and *O. sp. 3* strains ([Table 5](#)). *Pancytospora philotis* (JUm1505) only infected *O. tipulae* (JU1483, JU1505), but not *O. sp. 3* strains (JU408, JU75). Since *O. sp. 3* is the closest known species to *Oscheius tipulae*, *E. longa* and *P. philotis* are examples of narrow specialization in the host-parasite interaction. We also found that *C. elegans* N2 could be infected with *P. epiphaga* (JUm1396), showing epidermal and muscle infection ([Table 5](#); [S7F Fig](#)).

The spore morphology of a given microsporidian species was maintained in different nematode species, indicating that host genotype does not affect this pathogen phenotype. For instance, *O. tipulae* (JU1510) infected with *N. ausubeli* (JUm2526) displayed two sizes of spores in its intestinal cells as upon *Caenorhabditis* infection by *N. ausubeli* ([S7A Fig](#)). *Oscheius sp. 3* (JU408) infected with *Enteropsectra breve* (JUm2551) formed small rod-shaped or crescent-shaped spores along the apical side of the worms' intestinal cells, as upon *O. tipulae* infections ([S7D Fig](#)). *C. elegans* N2 infected with *P. epiphaga* (JUm1396) formed long and thin spores in the epidermis and muscles, as upon *C. brenneri* infection ([S7F Fig](#)).

N. ausubeli elicits a less robust host transcriptional response than other *Nematocida* species, despite establishing a robust infection

Given the capacity of all *Nematocida* species to infect *C. elegans*, we next sought to compare the *C. elegans* response to infection among our newly isolated microsporidia species. *N. parisii* infection in *C. elegans* has been shown to induce a broad transcriptional response [38]. Among genes that were highly upregulated at all infection timepoints were *C17H1.6* and *F26F2.1*, two genes of unknown function. Two transgenic *C. elegans* strains, ERT54 and ERT72, were generated as transcriptional reporters for these two genes and have been previously shown to be strongly induced in early *N. parisii* and *N. displodere* infection [38]. We tested these reporter strains with our new microsporidia species by placing them onto plates with a culture of infected worms and microsporidian spores, then monitoring GFP expression at different

Table 5. Nematode-infecting microsporidia specificity.

		<i>N. parisii</i> (JUm2816)	<i>N. ausubeli</i> (JUm2009)	<i>N. major</i> (JUm2747)	<i>N. homosporus</i> (NICm516)	<i>E. longa</i> (JUm408)	<i>E. breve</i> (JUm2551)	<i>P. philotis</i> (JUm1505)	<i>P. epiphaga</i> (JUm1396)
Species strain	Replicate	infected/ total	infected/ total	infected/ total	infected/ total	infected/ total	infected/ total	infected/ total	infected/ total
<i>C. elegans</i>	1	14/20	8/20	11/19	4/12	0/20	\	0/20	10/18 ^a
N2	2	17/20	8/17	10/19	8/18	0/20	\	0/20	15/19 ^b
<i>C. elegans</i>	1	10/19	10/20	14/20	2/20	0/20	0/20	0/20	\
JU2009	2	11/19	5/19	5/20	5/15	0/20	\	0/20	\
<i>C. briggsae</i>	1	10/17	6/13	\	\	0/20	0/20	0/20	\
JU2747	2	14/19	7/17	\	\	0/20	\	0/20	\
<i>C. briggsae</i>	1	\	\	13/20	4/15	\	\	\	\
JU2507	2	\	\	12/20	9/15	\	\	\	\
<i>C. briggsae</i>	1	11/17	11/20	12/20	9/21	0/20	\	0/20	\
JU2793	2	12/20	13/20	14/19	10/18	0/20	\	0/20	\
<i>O. tipulae</i>	1	\	\	\	13/16	\	\	\	\
JU1504	2	\	\	\	13/17	\	\	\	\
<i>O. tipulae</i>	1	\	\	\	\	\	6/14	11/20	\
JU1505	2	\	\	\	\	\	\	7/20	\
<i>O. tipulae</i>	1	0/20	0/20	0/20	0/17	0/20	4/17	3/20	\
JU1483	2	0/20	0/20	0/20	1/14	0/20	5/15	8/17	\
<i>O. tipulae</i>	1	0/20	0/20	0/20	1/14	\	\	\	\
JU170	2	0/20	0/20	0/20	4/20	\	\	\	\
<i>O. tipulae</i>	1	\	\	\	\	0/20	9/19	\	\
JU2551	2	\	\	\	\	0/20	7/15	\	\
<i>O. sp. 3</i>	1	0/20	8/17	0/20	9/20	11/17	3/18	0/20	\
JU408	2	0/20	3/17	0/20	9/17	16/16	5/18	0/20	\
<i>O. sp. 3</i>	1	0/20	8/18	0/20	11/17	12/16	3/18	0/20	\
JU75	2	0/20	4/15	0/20	11/19	12/15	3/13	0/20	\
<i>R. typhae</i>	1	\	\	\	5/12	\	\	\	\
NIC516	2	\	\	\	3/13	\	\	\	\

Experiments in the same column are in parallel. The same batch of spores was used on the four nematode species, each with two different strains and two replicates each. At 72 hours post inoculation (hpi) or 120 hpi at 23°C, nematodes found with meronts and/or spores in their intestinal cells were considered infected; otherwise, they were considered uninfected.

^a Infection were checked at 72 hpi, out of 18 worms, 10 were found infected with meronts, but not spores.

^b Infection were checked at 96 hpi, out of 19 worms, 15 were found infected with meronts and spores.

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timepoints in the reporter strains, as well as monitoring microsporidian meront and spore formation. As expected, *N. parisii*, *N. ausubeli*, *N. major* and *N. homosporus* could all infect these reporter strains, forming meronts and spores, and induce reporter GFP expression. By contrast, *E. longa* and *Enteropsectra* JU1483 failed to show evidence of proliferative infection and did not robustly induce reporter expression (Fig 9A and 9B; S2 Table). Most interestingly, while *N. parisii*, *N. major* or *N. homosporus* consistently induced the GFP reporters, different strains of *N. ausubeli* (JU2009, ERTm2, ERTm6; Fig 9; S2 Table) did not, although this species did robustly infect and proliferate within the *C. elegans* intestine.

To verify that this differential induction of the GFP reporters matched the transcripts of the endogenous genes, we conducted qRT-PCR after controlled *N. parisii* (ERTm1) and *N. ausubeli* (ERTm2) infections of N2 using purified spore preparations that were normalized for an

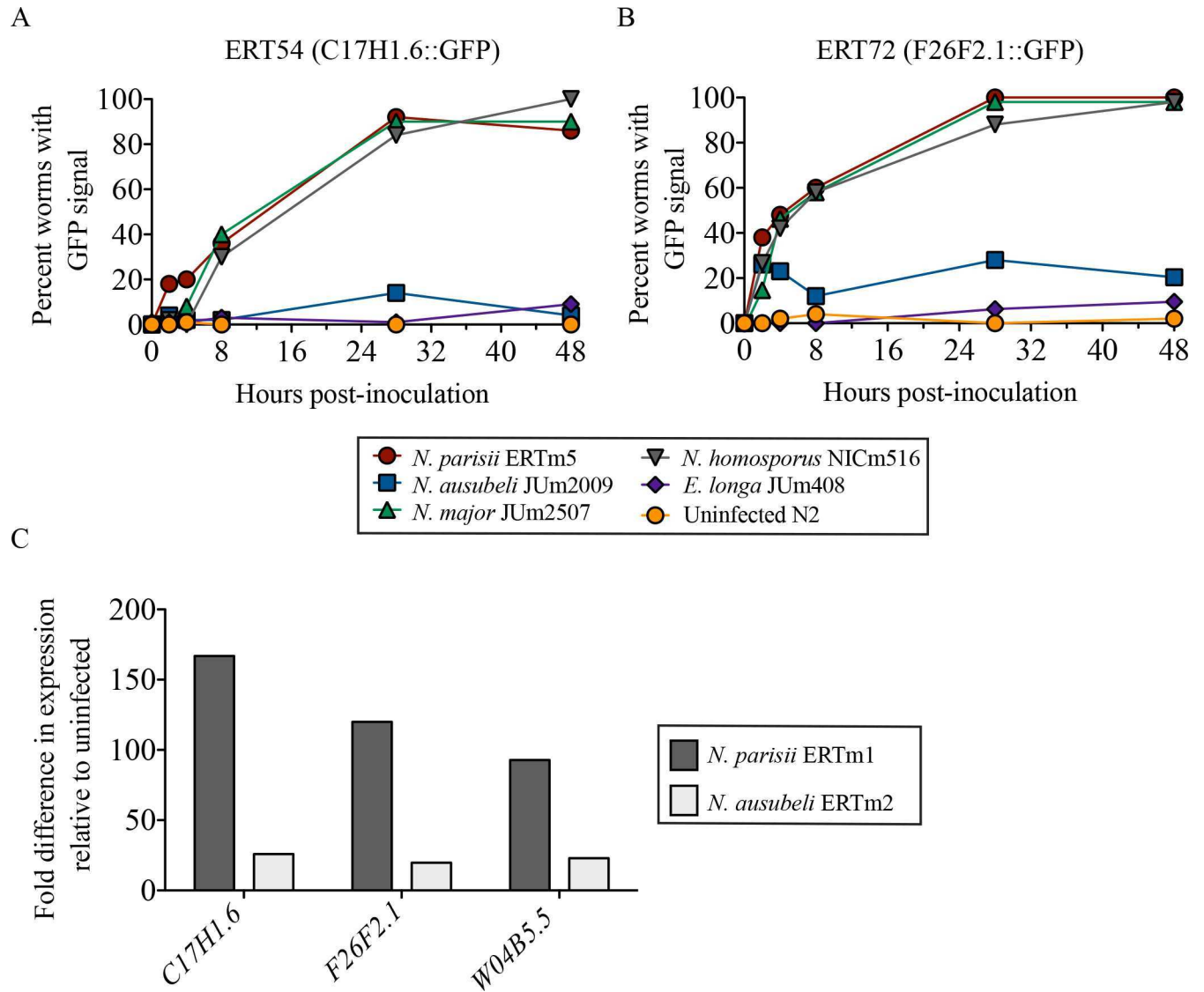


Fig 9. Responses of *C. elegans* strains with transcriptional reporters *C17H1.6p::GFP* and *F26F2.1p::GFP* to exposure by different microsporidia. Strains ERT54 carrying *C17H1.6p::GFP* (A) and ERT72 carrying *F26F2.1p::GFP* (B) were analyzed for GFP induction at different time points after infection with different microsporidia and the proportion of animals with GFP induction is shown. GFP was reproducibly induced in ERT54 and ERT72 upon infection with *N. parisii*, *N. major* and *N. homosporus*, while GFP signal was rarely observed in ERT54 and ERT72 inoculated with *N. ausubeli* or *E. longa* or the negative control. *N. ausubeli* did infect the *C. elegans* reporter strains, as monitored by DIC as in Table 5. C. Transcript levels for three genes were measured after 4 hours of infection of N2 *C. elegans* by *N. parisii* (ERTm1) and *N. ausubeli* (ERTm2). The fold increase in transcript level was measured relative to uninfected N2 levels. Infection dose was normalized between *Nematocida* by successful invasion events counted as intracellular sporoplasms at 4 hpi. To independently compare the microsporidian doses in parallel to the transcript quantification, we also measure the levels of *Nematocida* SSU rRNA after 4 hours of infection of *C. elegans* in the same experiment: we found that the rRNA level measured after infection with ERTm2 was 1.25-fold higher than that with ERTm1.

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equivalent level of invasion (see Materials and Methods). Indeed, we saw that both *C17H1.6* and *F26F2.1* transcripts, along with another gene highly induced by *N. parisii* infection *W04B5.5* [39], were induced approximately 6-7-fold lower upon *N. ausubeli* infection compared to *N. parisii* infection (Fig 9C), while the levels of pathogen rRNA (indicative of pathogen load) remained similar. Thus, *N. ausubeli* infection caused a much reduced host response compared to other *Nematocida* species (Fig 9; S2 Table), despite causing an equivalent, or even more robust infection [40]. Considering the phylogenetic relationships of the *Nematocida*

species (Figs 2 and 3; S2 Fig), this evolutionary change can be polarized: *N. ausubeli* seems to have lost the capacity to activate these transcriptional reporters as strongly as its closest relatives, or has acquired the capacity to inhibit their activation. Thus, although morphologically quite similar and both are able to infect *C. elegans*, *N. parisii* and *N. ausubeli* elicit distinct host responses.

Discussion

Independent evolutionary branches of nematode parasitism by microsporidia

Microsporidia are ubiquitous obligate intracellular pathogens that have agricultural and medical significance, but have been difficult to study in the laboratory. Our study provides a collection of microsporidia that can infect bacterivorous nematodes and can easily be studied in the laboratory in their natural hosts and in related species. These rhabditid nematode-infecting microsporidia seem to have more than one origin within the Microsporidia phylum: at least one origin within Clade II and one or two within Clade IV. We thus here enlarge considerably the spectrum of microsporidia that can be cultured in nematodes, including some that are genetically close to human pathogens in Clade IV.

Environmental SSU rDNA microsporidian sequences have been reported from soil, sand and compost samples from North America [41]. (The corresponding species have not been named.) Some of them branch in the SSU phylogeny in the vicinity of the nematode-infecting microsporidia that we isolated (S4 Fig). Specifically, some branch close to *Nematocida homosporus* and some may be outgroups to *Nematocida* or further species of the genus. In Clade IV, one is closely related to the *Pancytospora epiphaga* JUm1396 sequence.

The clades of nematode-infecting microsporidia that we describe have close relatives that infect arthropods, especially insects. This relationship may be due to deep co-evolution (arthropods and nematodes being close relatives on the animal phylogeny), or to the fact that nematodes share their habitats and interact with insects by using them as hosts or carriers [16], which may have facilitated a host shift or a complex lifecycle with several hosts. The microsporidia described here can be cultured continuously in their nematode hosts, but we cannot rule out the possibility that some of them may use non-nematode hosts as well, including insects. Of note, all of them use a horizontal mode of transmission, despite the fact that many instances of vertical transmission of microsporidia in arthropods, molluscs and fish are known [42,43]. In addition, *Nematocida* species are diploid with evidence of recombination and thus possibly a sexual cycle [30,39], which might occur in another host.

N. parisii, *N. ausubeli* and *N. major* are relatively common pathogens of *Caenorhabditis* but not of *Oscheius*

Our results suggest that infections by *N. parisii* and *N. ausubeli* are quite common in wild *Caenorhabditis* strains, especially in *C. elegans* and *C. briggsae*. In our collection, *N. parisii*, *N. ausubeli* and *N. major* infections were found in 30 strains of four *Caenorhabditis* species. Though we have a sampling bias towards France, *N. ausubeli* was found in Asia, Europe and Africa, while *N. parisii* was found mostly in France and once (ERTm5) from Hawaii. *N. major* was only found from three *Caenorhabditis* strains of *C. briggsae* and *C. tropicalis*, all of which were sampled in tropical areas, despite the fact that that we have sampled many hundreds of *C. elegans* isolates and that *N. major* can easily infect *C. elegans* in our specificity infection tests (Table 5). A possibility is that *N. major* may be preferentially distributed in the tropics rather than temperate zones, where *C. elegans* are mostly found (Table 2, Fig 1B) [16].

In addition to *C. elegans* and *C. briggsae* strains, we also have a relatively large collection of microsporidia-infected *Oscheius* strains (10 *O. tipulae* strains and one *O. sp.* 3 strain). However, none of these strains was found with *Nematocida* or *N. major* infections. In line with their natural associations, *N. parisii* and *Nematocida major* were not able to infect any *Oscheius* strains in the laboratory. These specializations may be due to long-term coevolution and adaptation processes [44].

In addition, one new microsporidian species infecting *Caenorhabditis* was found in clade IV, *Pancytopora epiphaga*. As this Clade IV microsporidian species can infect *C. elegans*, it would be interesting to develop its study as a model system for Clade IV species infection.

Diverse microsporidia infect *Oscheius* species

Microsporidian species that naturally infect *Oscheius* species are diverse (Fig 10, green entries). *N. minor*, found from two *O. tipulae* strains, forms two distinct sizes of spores, similar to *N. parisii*, *N. ausubeli* and *N. major*. *N. homosporus* was found from one *O. tipulae* strain and one *R. typhae* and is the only species tested here that is able to infect species of three genera *Caenorhabditis*, *Oscheius* and *Rhabditella*, suggesting that *N. homosporus* may be a relatively less specific pathogen for rhabditid nematodes.

The Clade IV *Oscheius*-infecting microsporidia are separated into two groups: *Enteropsectra* species, and *Pancytopora philotis*. None of those could infect *Caenorhabditis* and their host specificity is even narrower, distinguishing between *Oscheius tipulae* and its sister species *Oscheius sp.* 3. The SSU rDNA genetic distances between *E. longa* and *E. brevis* are quite small and two other closely related *Enteropsectra* strains are also available (Tables 2 and 3). Overall, *Enteropsectra* and the *Tipulae* group of *Oscheius* species [19,21] provide an interesting case to study the evolution of a narrow host specificity.

Evolutionary changes in tissue tropism

Although microsporidia are known to be able to adopt either a horizontal or a vertical transmission [42,45], we here only observed infection in somatic tissues and transmission was horizontal. Most of the infections occurred in host intestinal cells, while two independent instances showed infections elsewhere. As reported previously, *Nematocida displodere* can infect many tissues and cells in *C. elegans*, including the epidermis, muscle, coelomocytes and neurons, although it appears to invade all cells by firing its polar tube from the intestinal lumen [23]. The second independent case is *Pancytopora epiphaga* can be seen in the epidermis, coelomocytes and muscles. Whether it also enters the nematode's cells through the gut remains to be studied.

Cellular exit strategies

The most striking variation we observed concerns the cellular exit strategies of the spores (Fig 6). *Nematocida parisii* spores acquire an additional membrane around the spore wall and thus exit through a vesicular pathway, using the host exocytosis machinery [27]; in addition, clusters of spores with two additional membranes were observed. If the process is similar in *N. ausubeli* to that in *N. parisii*, the spore clusters may correspond to re-endocytosis of spores from the lumen [36] or perhaps to autophagy of internal spores using the apical plasma membrane. Of note, the host rough endoplasmic reticulum could often be seen to form concentric patterns in the intestinal cell cytoplasm (S1B and S1E Fig), sometimes wrapping around the sporoblasts (Fig 5E). Whether the reticulum may be a precursor for the additional membranes through an autophagic pathway [46,47], is an alternative possibility.

Pathogen \ Host	Nematocida						Enteropsectra		Pancytospora	
	<i>N. parisii</i>	<i>N. ausubeli</i>	<i>N. major</i>	<i>N. minor</i>	<i>N. homosporus</i>	<i>N. ciargi</i>	<i>E. longa</i>	<i>E. breve</i>	<i>P. philotis</i>	<i>P. epiphaga</i>
<i>Caenorhabditis elegans</i>	Mosaic Green	Mosaic Green	Plain Green	White	Plain Green	White	Red	Red	Red	Plain Green
<i>Caenorhabditis briggsae</i>	Mosaic Green	Mosaic Green	Mosaic Green	White	Plain Green	White	Red	Red	Red	White
<i>Caenorhabditis tropicalis</i>	White	White	Mosaic Green	White	White	White	White	White	White	White
<i>Caenorhabditis remanei</i>	White	Mosaic Green	White	White	White	White	White	White	White	White
<i>Caenorhabditis brenneri</i>	White	White	White	White	White	White	White	White	White	Mosaic Green
<i>Oscheius tipulae</i>	Red	low sensitivity	Red	Mosaic Green	Mosaic Green	White	Red	Mosaic Green	Mosaic Green	White
<i>Oscheius</i> sp. 3	Red	Plain Green	Red	White	Plain Green	White	Mosaic Green	Plain Green	Red	White
<i>Rhabditella typhae</i>	White	White	White	White	Mosaic Green	White	White	White	White	White
<i>Procephalobus</i> sp.	White	White	White	White	White	Mosaic Green	White	White	White	White

Fig 10. Summary of the interactions between rhabditid nematodes and microsporidia in the wild and in laboratory. A mosaic green color means that the corresponding natural infection was found. Plain green means that the infection worked in the laboratory and red means that the infection did not work in the laboratory. White: not determined.

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By contrast, in *Enteropsectra longa*, the sporoblasts and mature spores were never seen surrounded by an additional membrane, which rules out exocytosis as an exit route. Instead, the

spores pushed out and deformed the apical plasma membrane of the host intestinal cell (Fig 6; S6 Fig). Whether the final release step was by pinching of the plasma membrane at the base or by rupturing it is unclear, although the former is more probable, given that the intestinal cells were not seen to leak out. We observed spore sections in the lumen, far from any intestinal cells in the corresponding section, with either an additional membrane around them or none. A possible scenario is that the spores are first released with a membrane, and that the membrane then disintegrates (Fig 6B). Yet because we did not follow by serial sectioning the length of the spores, we cannot know for sure that those with a membrane were not still attached to the epithelial cell. We thus cannot rule out an alternative mechanism whereby the spores are released through a hole in the plasma membrane—although given spore size, this latter exit mechanism would likely lead to host cell rupture and death, an event that was never observed. Of note, another exit mode was noted in the human gastrointestinal microsporidia *Enterocytozoon bienersi* (in Clade IV like *E. longa*), whereby the infected cell itself is extruded in the lumen [48–50]. Presumably, the cell then rapidly dies and the spores are released by disintegration of the enterocyte plasma membrane. In the present case of *Enterospectra longa*, the epithelial intestinal cell remains overall intact and only the spore exits, possibly with the surrounding enterocyte plasma membrane that then disintegrates.

A diverse collection of natural host/microsporidia pairs in *C. elegans* and related wild-caught nematodes

Beyond access to a diversity of microsporidia, our collection of host-parasite combinations also provides a resource for defining the genetic basis of host resistance. Most current work on *C. elegans* and *N. parisii* is performed using the *C. elegans* reference strain N2 and the *N. parisii* ERTm1 isolate, yet this strain combination has been shown to lead to a very strong infection where the host does not mount an effective defense response (e.g. in comparison with *C. elegans* CB4856; [29]), thus making it a difficult system in which to identify immune defense pathways. The present collection offers many further possibilities of genetic screens using induced mutations or natural genetic variation for resistance pathways.

Conclusion

Overall, we here considerably enlarged the resources and knowledge on the microsporidia infecting bacteriovorous terrestrial nematodes. These microsporidia are diverse in terms of phylogenetic relationships, spore size and shape, the presence of vesicles containing spores, host specificity pattern, host tissue tropism, host cell intracellular localization and cellular exit route.

Materials and Methods

Nematode sampling, isolation and microsporidia strains

Hundreds of samples, mostly from rotting fruits, rotting stems and compost, were collected worldwide over several years, and nematodes were isolated as described [11]. The nematode species was identified as described [11,33], using a combination of morphological examination (dissecting microscope and Nomarski optics), molecular identification (18S, 28S or ITS rDNA) and mating tests by crossing with close relatives. Isogenic nematode strains were established by selfing of hermaphrodites or for obligate male–female species from a single mated female. Individuals of strains showing a paler intestinal coloration (Fig 1A) were examined by Nomarski optics. Strains with meronts and spores in the intestinal cells or elsewhere were labeled as suspected to harbor a microsporidian infection. Each nematode strain was then frozen and stored at -80°C .

For this study, these frozen nematodes were thawed and maintained on nematode growth media (NGM) seeded with *E. coli* OP50 at 23°C. The microsporidian strain was identified after the strain identifier of its host nematode strain (itself identified according to *C. elegans* community rules; <http://www.wormbase.org/about/userguide/nomenclature>), with an additional "m" between the letters and the numbers for the microsporidia. For instance, a microsporidian strain from the nematode strain JU1762 was named JUm1762. Previously published nematode-infecting microsporidian strains keep their names: ERTm1 (from strain CPA24), ERTm2 (from JU1348), ERTm3 (from JU1247), ERTm4 (from JU1395), ERTm5 (from JU2055) and ERTm6 (from JU1638) [26,29,30,39,51] (Tables 1 and 2). ERTm4 was previously reported to correspond to *N. parisii* infection [29], but as no sequence data was available in GenBank, we also sequenced the SSU rDNA and β -tubulin genes for this study.

SSU rDNA and β -tubulin gene sequencing

We sought to amplify by PCR and sequence fragments of two microsporidian genes (SSU rDNA and β -tubulin genes) from all potentially infected rhabditid isolates. Ten infected worms were placed in a PCR tube with 10 μ l single worm lysis buffer (1X PCR buffer (Dream-Taq Buffer 10X, Thermo Fisher), 1 mM MgCl₂, 0.45% Tween 20 and 170 ng/ μ l proteinase K), which was then treated at 60°C for 60 min, followed by 95°C for 15 min. This DNA extract was then used as DNA template. To amplify microsporidian SSU rDNA, primers v1f (5'-CA CCAGGTTGATTCTGCCTGAC-3') and 1492r (5'-GGTTACCTTGTACGACTT-3') [52] were used to amplify strains JUm408, JUm1254, JUm1483, JUm1504, JUm2009, JUm2106, JUm2131, JUm2132, JUm2287, JUm2520, JUm2526, JUm2551, JUm2586, JUm2590, JUm2671 and NICm516. We used v1f and 18SR1492 (5'-GGAAACCTTGTTACGACTT-3') to amplify sequences of JUm1456, JUm1460, JUm1505, JUm1510, JUm1670, JUm2552, JUm2793, JUm2796, JUm2799, JUm2816, JUm2825 and JUm2895. We designed a new pair of primers SPF (5'-GATACGAGGAATTGGGGTTTG-3') and SPR (5'-GGGTACTGGAAATTCGGT GTT-3') for JUm2507, JUm2747, JUm2751 and JUm2772. We failed to amplify SSU rDNA for JUm1501 and NICm1041.

To amplify the microsporidian β -tubulin gene, newly designed forward primer β n1F (5'-A CAAACAGNCARTGYGGNAAYCA-3') and reverse primer β n1R (5'-TGCTTCAGTRAA YTCCATYTCRTCCAT-3') were used. To obtain the β -tubulin gene sequence of JUm2551 and JUm1456, nested PCR was performed using first primers β n1F and β n1R then β nOF (5'-C CGGACAATATCGTCTTTGG-3') and β nOR (5'-CAGCTCCTGAATGCTTGTTG-3') (S1 Table). PCR products showing a positive signal by gel electrophoresis were sequenced on both strands on ABI 3730XL sequencing machines (MWG). SSU rDNA of five additional *N. parisii* strains (JUm1248, JUm1249, JUm1253, JUm1762, JUm1893) were provided by Aurore Dubuffet and Hinrich Schulenburg. The results were analyzed using Geneious v7.1.7 [53] and compared by BLAST with the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Note that some PCR products could not be amplified (S1 Table). Especially, we failed to amplify the β -tubulin gene in several *Oscheius* infections. Both genes fail to amplify for the putative microsporidian infection of the *O. tipulae* strain JU1501 and this infection could not be characterized.

SSU rDNA and β -tubulin gene sequences have been submitted to GenBank under accession numbers KX352724-KX352733, KX360130-KX360167 and KX378155-KX378171 (S1 Table).

Phylogenetic analysis

SSU rDNA and β -tubulin gene sequences of microsporidia from this study were analyzed with those of other published microsporidian species and fungi (*Rozella* spp. for SSU rDNA, *Basidiobolus ranarum* and *Conidobolus coronatus* for β -tubulin and concatenated sequences of

both genes) as outgroups (Figs 2 and 3; S2 Fig) [34]. For phylogenetic analysis of SSU rDNA genes, 28 out of 33 sequences obtained from this study were compared with 11 sequenced *Nematocida* isolates (ERTm1, ERTm2, ERTm3, ERTm5, ERTm6, JUm1248, JUm1249, JUm1253, JUm1762, JUm1893, and JUm2807), JUm1396, 60 other microsporidian species chosen from all five major clades of microsporidia [34] and two *Rozella* species [54]. For analysis of β -tubulin genes, only sequences from the six *Nematocida* species (ERTm1, ERTm2, ERTm3, ERTm5, ERTm6 and JUm2807) and 18 other published microsporidian species were available to be compared with our 32 sequences (S1 Table). To phylogenetically analyze both genes together, we concatenated the two genes of stains ERTm1–6, JUm2807, our 30 strains, 10 other microsporidia species and two outgroups (*B. ranarum* and *C. coronatus*). Sequences were aligned using Geneious v7.1.7 with default parameters and further aligned manually and concatenated if available. The alignments were imported to MEGA 6 [55] to estimate the best DNA evolution models and compute mean genetic distances (1000 bootstrap replicates). Bayesian inference phylogenies were constructed using Mesquite v3.04 [56] and MrBayes v3.2.2 [57], with the same DNA models as above [58] and refined by FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

High-pressure freezing and transmission electron microscopy

Worms were frozen in M9 buffer [35] supplemented with 20% BSA (Type V) in the 100 μ m cavity of an aluminium planchette, Type A (Wohlwend Engineering, Switzerland) with a HPM 010 (BalTec, now Abra Fluid AG, Switzerland). Freeze substitution was performed according to [59] in anhydrous acetone containing 2% OSO₄ + 2% H₂O in a FS 8500 freeze substitution device (RMC, USA). Afterwards samples were embedded stepwise in Epon. To achieve a good infiltration of spores, the infiltration times in pure resin were prolonged for 48 h compared to the published protocol. After heat polymerization thin sections of a nominal thickness of 70 nm were cut with a UC7 microtome (Leica, Austria). Sections were collected on 100 mesh formvar coated copper grids and poststained with aqueous 4% uranylacetate and Reynold's lead citrate. Images were taken with a Tecnai G2 (FEI, The Netherlands) at 120 kV and equipped with a US4000 camera (Gatan, USA).

Spore size measurements

Spore size was measured as described [22]. Briefly, infected nematodes were photographed by Nomarski optics and spores were measured using the Image J software [60]. We only took into account spores with a clear outline within the focal plane. In species with two spore size classes, large spores are less numerous than small ones and they are found in groups. When measuring, the spores were first assigned to a size class, in part based on the spatial clustering of large spores. 20 spores were measured for each spore type; except *N. ausubeli*, for which 42 small ones and 40 large ones were measured.

Microsporidia spore preparation

For the microsporidian spore preparation, we first tried the methods previously established for *N. parisii* and *N. ausubeli* [22,51]. Because wild nematodes naturally live in habitats with various microbes [16,17], the microsporidia-infected nematode cultures generally originally contained other microbes, such as bacteria, fungi, or even viruses. In order to obtain a relatively pure microsporidian spore preparation, we treated the nematode cultures repeatedly with antibiotics (100 μ g/ml gentamycin, 50 μ g/ml Ampicillin, 50 μ g/ml Kanamycin, 20 μ g/ml Tetracycline, and 50 μ g/ml streptomycin), monitoring the presence of non-*E. coli* bacteria and fungi on the plate. Nematode strains do not lose the microsporidian infection after antibiotic

treatment. After antibiotic treatment, if the appearance of a plate with infected worms looks like those with bleached worms, we considered the plate to be clean and the infected worms were used to extract clean spores. Even though inconspicuous microbes may still be carried over, as we know so far, none of them could prevent the worms from getting infected with microsporidia nor induce similar symptoms as microsporidia.

Antibiotic-cleaned worms without other detectable microbes were harvested in 2-ml microfuge tube and autoclaved silicon carbide beads (1.0 mm, BioSpec Products, Inc.) were added. The tube was then vortexed for 5 min at 2,500 rpm and the lysate of worms filtered through a 5 μ m filter (Millipore) to remove large worm debris. Spore concentration was quantified by staining with chitin-staining dye direct yellow 96 (DY96).

This method worked well on *N. major* and *N. homosporus*, but spores of Clade IV species extracted this way could not infect any worms. To prepare infectious spores of these species, we used instead a plastic pestle to crush worms manually, and stored these spore preparations at 4°C.

Nematocida species spore preparations could generally be stored at -80°C for later infection tests. However, storage at -80°C could affect the infection efficiency of these spore preparations. Indeed, when we made a fresh *N. ausubeli* (JU2009) spore preparation and used it directly for infection tests, it could infect *O. tipulae* strains JU1510 and JU2552, with meronts and spores found in their intestinal cells at 120hpi. One month later, we used the same batch that had been stored at -80°C to infect *C. elegans* (N2), *O. tipulae* (JU1483, JU170, JU1510 and JU2552). At 120 hpi, 100% of N2 adult worms were infected, while none of the *O. tipulae* strains became infected. These results suggested that this spore preparation became less infectious after being frozen and stored at -80°C for one month, which did not compromise infection in *C. elegans* but did compromise infection of *O. tipulae*. For further specificity tests, spore preparations of *N. major*, *N. homosporus* and Clade IV species were then used within two hours after extraction, without freezing.

Infection assays

20 uninfected L4 or young adults (i.e. prior to first egg formation) were transferred to a 6 cm NGM plate seeded with *E. coli* OP50. 5 million microsporidian spores in 100 μ l distilled water were placed on the *E. coli* lawn. The cultures were then incubated at 23°C. The infection symptoms of 20 adults were checked by Nomarski optics at 72 hours after inoculation. If no infection symptoms were found at this timepoint, they were scored a second time at 120 hours post-inoculation.

Assays with reporter strains

Two transgenic *C. elegans* strains, ERT54 *eyJIs8[C17H1.6p::gfp; myo-2p::mCherry]* and ERT72 *eyJIs15[F26F2.1p::gfp; myo-2::mCherry]* were used in infection assays to test infection specificity and transcriptional response of *C. elegans* to different microsporidian infections. These two lines express a constitutive fluorescent Cherry marker in the pharyngeal muscles and induce GFP upon infection with *N. parisii* [38]. In the first qualitative assay (23°C), we focused on the ERT54 strain. First, 10 L4 stage animals from seven naturally infected strains (*C. elegans* JU1762 with *N. parisii* infection, *C. elegans* JU1348 with *N. ausubeli*, *C. briggsae* JU2507 with *N. major*, *O. tipulae* JU1504 with *N. homosporus*, *R. typhae* NIC516 with *N. homosporus*, *O. tipulae* JU1483 with *Enteropsectra*, *Oscheius* sp. 3 JU408 with *E. longa*) were transferred to new plates and cultured for two days, in order to release microsporidian spores onto the plates. Then 10 L4 stage worms of the ERT54 strain were added onto these plates and onto a clean plate as control. Two days post-inoculation (dpi), a chunk was transferred to new plate to

prevent starvation. One day later (3 days dpi), GFP expression of ERT54 animals (visualized using the Cherry reporter in the pharynx) and infection symptoms were scored. 20 worms showing GFP expression (if any, else the Cherry marker was used) were picked and transferred to a new clean plate. GFP expression was monitored on 8 dpi and 14 dpi. In the second quantitative assay (23°C), first, 10 L4 stage animals from five naturally infected strains (*C. briggsae* JU2055 with *N. parisii* infection, *C. elegans* JU2009 with *N. ausubeli*, *C. briggsae* JU2507 with *N. major* infection, *R. typhae* NIC516 with *N. homosporus* infection, *Oscheius* sp. 3 JU408 with *E. longa* infection) and uninfected *C. elegans* reference strain N2 (as negative control) were transferred to new plates and cultured for three days. Then 200 L4 stage worms of ERT54 or ERT72 were added. GFP expression of 50 worms (if possible) of reporter strains was monitored at five different timepoints (2 hours post inoculation (hpi), 4 hpi, 8 hpi, 28 hpi, 48 hpi) and infection symptoms were scored at 48 hpi.

qRT-PCR of reporter transcripts

For measurements of transcripts levels by quantitative RT-PCR (qRT-PCR) (primers used see [S2 Table](#)), 3000 synchronized N2 *C. elegans* L1 larvae were infected for 4 hours at 25°C with 5.0×10^5 ERTm1 (*N. parisii*) spores and 1.5×10^6 ERTm2 (*N. ausubeli*) spores. Prior analysis of serial spore dilutions determined that these ERTm1 and ERTm2 spore doses resulted in an average of 1 sporoplasm per L1 larva at 4 hpi at 25°C as measured by FISH to *Nematocida* rRNA. At 24 hpi, animals were harvested and RNA was isolated by extraction with Tri-Reagent and bromochloropropane (BCP) (Molecular Research Center). cDNA was synthesized from 175 ng of RNA with the RETROscript kit (Ambion) and quantified with iQ SYBR Green Supermix (Bio-Rad) on a CFX Connect Real-time PCR Detection System (Bio-Rad). Transcript levels were first normalized to the *C. elegans* *snb-1* gene within each condition. Then transcript levels between conditions were normalized to uninfected N2 for *C. elegans* transcripts or normalized to ERTm1 rRNA for *Nematocida* rRNA.

Taxonomic section

Rationale for the description of new microsporidia taxa. We describe here two new genera and nine new species of microsporidia based on rDNA and β -tubulin sequences and phenotypic analyses.

The rDNA (and β -tubulin, when we could amplify it) sequences could be readily grouped in three distinct clades, one including *Nematocida parisii* and many of our strains in microsporidia clade II, and the two other clades in microsporidia clade IV (Figs 2 and 3; [S2 Fig](#)).

All described microsporidian species infecting nematodes are reviewed in [26]. Previously described species with associated SSU rDNA sequences are *Nematocida parisii* [22], *Nematocida disploedere* [23], *Sporanauta perivermis* [24] and *Nematocenator marisprofundi* [25], the two latter infecting marine nematodes. Compared to the species studied here, *S. perivermis* is found in another group of clade IV, while *N. marisprofundi* appears as a distant outgroup [25] ([Fig 2](#)). Our strains are thus all distinct from the two latter species. In addition, two species were reported without any associated molecular sequence [26]. The first one, *Thelohania reniformis*, infected the intestine of a parasitic nematode with a single class of spores of a size exceeding in length and/or width any of those we describe [26]. The second species, of an undefined genus ("*Microsporidium*" *rhabdophilum*), infected the pharyngeal glands, hypodermis and reproductive system of *Oscheius myriophila* [61], and does not match in tissue tropism and spore morphology any of the present species.

The biological species concept cannot be used in describing these microsporidia as their sexual cycle is unknown and thus we cannot test their crossing ability. Microsporidia species

have been classically delimited through their morphology and their association with a host. In recent years, DNA sequences have further helped to assess phylogenetic relationships among microsporidia, and to assign strains to a species when morphology was not sufficient [62]. Among our strains, as a first example, two close groups of strains in the *Nematocida* clade correspond to *N. parisii* and *N. sp. 1* in [22], respectively. These groups are consistently distinct from each other by molecular analysis of rDNA and β -tubulin genes (Table 3; S3 Table) but do not appear very different from spore size and general morphology ([22], this work). Their molecular distance (0.017) is consistent with molecular distances between microsporidian species and even greater than other examples of interspecific distance for this gene [63]. Their whole genome sequence [39] further shows that the two species are wide apart, with only 62% amino-acid identity between protein orthologs, while strains of the same species are much closer, such as 0.2% difference at the nucleotide level between *N. parisii* ERTm1 and ERTm3 and 1 SNP every 989 bp for *N. sp. 1* ERTm2 and ERTm6 [30,39]. We therefore formally describe here *N. sp. 1* as a new species and call it *N. ausubeli* n. sp.

Concerning the other strains in the *Nematocida* clade, given their greater molecular distance to each other, we define four other *Nematocida* species that are also distinct from *N. displodere* [23]. In this case, each of them further shows a distinct spore morphology (Table 4; Fig 4). No other described microsporidian species to our knowledge has a similar sequence nor host distribution. We thus describe them below as four new species of *Nematocida*, namely *N. major* n. sp. (two sizes of spores, each slightly larger than the respective class in *N. parisii* and *N. ausubeli*), *N. minor* n. sp. (two sizes of spores, each smaller than the respective class in *N. parisii* and *N. ausubeli*), *N. homosporus* n. sp. (a single class of spores) and *N. ciargi* n. sp. (a single class of spores, particularly small), each with their reference strain. The two latter species were not found in *Caenorhabditis* nematodes but in other bacteriovorous terrestrial nematodes. We could not amplify the SSU gene of *Nematocida* "sp. 7" NICm1041 and therefore refrain from formally describing this putative new species.

The remaining strains of microsporidia in our sampling do not belong to clade II but to clade IV. By blast of the rDNA sequence, they are closest to *Orthosomella operophtherae*, an insect pathogen, and by phylogenetic analysis they form two clades. One clade includes JUm408, JUm1456, JUm1483 and JUm2551, and is sister to *Liebermannia* species (also arthropod parasites)—but not particularly close in molecular distance (*Orthosomella* is closer). The other clade includes five strains (JUm1505, JUm1460, JUm1670, JUm2552 and JUm1396) and appears as an outgroup to the four strains + *Liebermannia* spp. Based on the host phylum, the molecular distances and the monophyletic clade groupings, we describe here two new genera named *Enteropsectra* n. gen. for the first group of four strains (type JUm408), and *Pancyctospora* n. gen. for the second independent clade (type JUm1505).

In *Enteropsectra* n. gen., we isolated four strains. Based on genetic distance (Table 3; S3 Table), spore morphology (Figs 7 and 8; S6 Fig; Table 4), and host specificity (Table 5) of JUm408 and JUm2551, we define two species: *E. longa* JUm408 with large spores (type species of the genus) and *E. breve* JUm2551 with small spores. We do not assign a species name to the two other strains (JUm1456 and JUm1483) as their molecular relationship depends on the gene (SSU rDNA versus β -tubulin). For example, JUm1483 show small spores, was found infecting *Oscheius tipulae* and groups with JUm2551 by SSU rDNA, but its β -tubulin sequence is closer to JUm408. We thus prefer to abstain assigning a species name to this strain.

In *Pancyctospora* n. gen., we isolated five strains. Based on genetic distance (Table 3; S3 Table), host specificity and tissue tropism (Fig 7; S5 Fig; Table 5), we define two species: *Pancyctospora philotis* n. sp. (type species of the genus) infects *Oscheius tipulae* intestine, while *Pancyctospora epiphaga* n. sp. was found to infect *Caenorhabditis brenneri* epidermis and muscles.

Nomenclatural acts. The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix “<http://zoobank.org>” The LSID for this publication is: urn:lsid:zoobank.org:pub:0C31D734-FE13-49F9-8318-ADC6714F316E. The electronic edition of this work was published in a journal with an ISSN.

Taxonomic descriptions. Phylum Microsporidia Balbiani 1882

***Nematocida ausubeli* n. sp. Zhang & Félix 2016.** LSID urn:lsid:zoobank.org:act:6-D7E3D0D-3348-4885-BF64-DF1EE1B7EEBA

The type strain is ERTm2. Two strains ERTm2 and ERTm6 have been submitted to the American Type Culture Collection (ATCC, <https://www.atcc.org>) as PRA-371 and PRA-372, respectively. The type host is *Caenorhabditis briggsae* [64,65], strain JU1348, which was isolated from a mixed sample of decaying vegetal matter (rotting fruits, leaf litter, soil, bark, flowers). The type locality is Periyar Natural Preserve, Kerala, India. The species was also found in *C. briggsae* in Germany and Cape Verde, and *Caenorhabditis elegans* and *Caenorhabditis remanei* in Europe. The ribosomal DNA sequence, deposited to Genbank under Accession JH604648. The genome of the reference strain has been sequenced [39] (accession AERB01000000). The spores are ovoid and measure 2.80 x 0.96 μm (ranges 2.21–3.26 x 0.72–1.30) for the large class and 2.04 x 0.53 μm (ranges 1.36–2.92 x 0.28–0.80) for the small class. Infection is localized to the host intestinal cells. Transmission is horizontal, via the oral-fecal route. The species is named to honor Dr. Frederick Ausubel and his work on innate immunity of *C. elegans*.

***Nematocida major* n. sp. Zhang & Félix 2016.** LSID urn:lsid:zoobank.org:act:4D7C3F14-187A-4AD1-A62F-DA79BED716E0

The type strain is JUm2507. The type material is deposited as a live frozen culture of the infected host at ATCC and in the collection of the corresponding author (MAF; <http://www.justbio.com/worms/index.php>). The type host is *Caenorhabditis briggsae* [64,65], strain JU2507, isolated from rotting figs. The type locality is Khao Sok National Park, Thailand. The species was also found in Guadeloupe in *C. briggsae* and *Caenorhabditis tropicalis*. The ribosomal DNA sequence, deposited to Genbank under Accession KX360148. The spores are ovoid and measure 3.4 x 1.2 μm (ranges 2.9–3.8 x 0.8–1.6) for the large class and 2.3 x 0.54 μm (ranges 1.8–2.7 x 0.41–0.77) for the small class. Infection is localized to the host intestinal cells. Transmission is horizontal, presumably via the oral-fecal route. The species is named after the large size of its spores.

***Nematocida minor* n. sp. Zhang & Félix 2016.** LSID urn:lsid:zoobank.org:act:646590B-C-E5A8-4FD5-9026-96B276A4D159

The type strain is JUm1510. The type material is deposited as a live frozen culture of the infected host at ATCC and in the collection of the corresponding author (MAF; <http://www.justbio.com/worms/index.php>). The type host is *Oscheius tipulae* [66], strain JU1510, isolated from compost. The type locality is Hluboka nad Vlatavou near Budweis, Czech Republic. The species was also found in *O. tipulae* in Armenia. The ribosomal DNA sequence, deposited to Genbank under Accession KX360147. The spores are ovoid and measure 1.9 x 0.83 μm (ranges 1.5–2.2 x 0.5–1.1) for the large class and 1.3 x 0.55 μm (ranges 1.1–1.7 x 0.35–0.73) for the small class. Infection is localized to the host intestinal cells. Transmission is horizontal, presumably via the oral-fecal route. The species is named after the small size of its spores.

***Nematocida homosporus* n. sp. Zhang & Félix 2016.** LSID urn:lsid:zoobank.org:act:C959C7AD-DC01-4391-8B18-1B5D02F7349B

The type strain is JUm1504. The type material is deposited as a live frozen culture of the infected host at ATCC and in the collection of the corresponding author (MAF; <http://www.justbio.com/worms/index.php>). The type host is *Oscheius tipulae* [66], strain JU1504, isolated from a rotting *Arum* stem. The type locality is Le Blanc (Indre), France. The species was also found in the nematode *Rhabditella typhae* in Portugal. The ribosomal DNA sequence, deposited to Genbank under Accession KX360153. The spores are ovoid and measure 2.0 x 0.72 μm (ranges 1.7–2.7 x 0.56–0.94). Infection is localized to the host intestinal cells. Transmission is horizontal, presumably via the oral-fecal route. The species is named after the single class of spore size that can be observed in the host.

***Nematocida ciargi* n. sp. Zhang & Félix 2016.** LSID urn:lsid:zoobank.org:act:77EF241F-463D-443A-819D-C32B1BC49332

The type strain is JUm2895. The type material is deposited as a live frozen culture of the infected host at ATCC and in the collection of the corresponding author (MAF; <http://www.justbio.com/worms/index.php>). The type host is *Procephalobus* sp. strain JU2895 (Cephalobina), isolated from rotting citrus fruits. The type locality is Barcelona, Spain. The ribosomal DNA sequence, deposited to Genbank under Accession KX360152. The spores are ovoid and 1.4 x 0.59 μm (ranges 1.5–2.2 x 0.41–0.84). Infection is localized to the host intestinal cells. Transmission is horizontal, presumably via the oral-fecal route. The species is named after its type locality, close to the Centre de Regulació Genòmica in Barcelona, Spain.

***Enteropsectra* n. gen. Zhang & Félix 2016.** LSID urn:lsid:zoobank.org:act:33CE2667-0109-44DA-9878-34FAD4A2F96B

This is a novel microsporidian lineage within microsporidian clade II (ref), with *Orthosomella*, *Liebermannia* as the closest relatives, based on SSU rDNA phylogenetic analyses. The type species is *Enteropsectra longa* n. sp. Zhang & Félix 2016. The genus is named *Enteropsectra* (feminine, from the Greek "psectra": brush) after the morphological aspect of the spores at the apical side of the intestinal cells of the nematode host, resembling a bottle brush.

***Enteropsectra longa* n. sp. Zhang & Félix 2016.** LSID urn:lsid:zoobank.org:act:FC304EDC-B2CA-4486-BF8F-095BE0B60E45

The type strain is JUm408. The type material is deposited as a live frozen culture of the infected host at ATCC and in the collection of the corresponding author (MAF; <http://www.justbio.com/worms/index.php>). The type host is *Oscheius* sp. 3 [21], strain JU408, isolated from a soil sample. The type locality is the Botanical garden of Reykjavik, Iceland. The ribosomal DNA sequence, deposited to Genbank under Accession KX360142. The spores have the shape of a long and thin rod, measuring 3.8 x 0.49 μm (ranges 3.1–5.0 x 0.35–0.68). The polar tube makes one turn at the posterior part of the spore; one or two polar tube sections can be seen in transmission electron microscopy when the spore is cut transversally. Infection is observed in the host epidermis and does not affect the intestinal cells. The spores do not seem to be enclosed as groups of spores in a vesicle. Transmission is horizontal, presumably via the oral-fecal route. The species is named after the long size of its spores.

***Enteropsectra breve* n. sp. Zhang & Félix 2016.** LSID urn:lsid:zoobank.org:act:236607CA-8C44-414D-916C-802C7C67600D

The type strain is JUm2551. The type material is deposited as a live frozen culture of the infected host at ATCC and in the collection of the corresponding author (MAF; <http://www.justbio.com/worms/index.php>). The type host is *Oscheius tipulae* [66], strain JU2551, isolated from a rotting apple. The type locality is an apple orchard in Orsay (Essonne), France. The ribosomal DNA sequence, deposited to Genbank under Accession KX360145. The spores are ovoid and measure 1.8 x 0.66 μm (ranges 1.3–2.1 x 0.42–0.90). Infection is observed in the host intestine and does not affect the intestinal cells. Transmission is horizontal, presumably via the oral-fecal route. The species is named after the short size of the spores.

***Pancytospora* n. gen. Zhang & Félix 2016.** LSID urn:lsid:zoobank.org:act:1ADAC856-ED00-49C9-9906-634BAF38B355

This is a novel microsporidian lineage within microsporidian clade IV, with *Orthosomella*, *Liebermannia* and *Enteropsectra* n. gen. as the closest relatives, based on SSU rDNA phylogenetic analyses. The type species is *Pancytospora philotis* n. sp. Zhang & Félix 2016. The genus is named *Pancytospora* (feminine) after the distribution of the spores throughout the cells.

***Pancytospora philotis* n. sp. Zhang & Félix 2016.** LSID urn:lsid:zoobank.org:act:5EA01A65-F5F4-4EE6-A1B6-2726F9CE8579

The type strain is JUm1505. The type material is deposited as a live frozen culture of the infected host at ATCC and in the collection of the corresponding author (MAF; <http://www.justbio.com/worms/index.php>). The type host is *Oscheius tipulae* [66], strain JU1505, isolated from a rotting peach. The type locality is Le Blanc (Indre), France. The species was also found in *O. tipulae* in other locations in France. The ribosomal DNA sequence is deposited to Genbank under Accession KX360131. The spores have the shape of a long and thin rod, measuring 3.5 x 0.42 μm (ranges 2.4–4.7 x 0.25–0.52). Infection is localized to the host intestinal cells. Transmission is horizontal. The species is named after its specificity to *Oscheius tipulae* (abbreviation *Oti*).

***Pancytospora epiphaga* n. sp. Zhang & Félix 2016.** LSID urn:lsid:zoobank.org:act:41573063-B494-41C3-B0A8-2417DFEB3DCC

The type strain is JUm1396. The type material is deposited as a live frozen culture of the infected host at ATCC and in the collection of the corresponding author (MAF; <http://www.justbio.com/worms/index.php>). The type host is *Caenorhabditis brenneri*. The type locality is a private garden in the vicinity of Medellin, Colombia. The ribosomal DNA sequence, deposited to Genbank under Accession KX424959. The spores are ovoid and measure 3.71 x 0.80 μm (ranges 2.99–4.22 x 0.56–0.92). Infection is observed in the host epidermis and does not affect the intestinal cells. Transmission is horizontal. The species is named after the site of infection in the nematode, mostly the epidermis.

Supporting Information

S1 Fig. Ultrastructural observations of *Nematocida ausubeli*. Transmission electron micrographs of *N. ausubeli* strain JUm2009. **A.** Lower magnification view of several *N. ausubeli* infection stages in host intestinal cells (same animal as in Fig 5D). The large arrow indicates sporonts. The small arrow indicates spores. Two multinucleate meronts are indicated. **B.** A multinucleate meront and a multilayered figure of host rough endoplasmic reticulum (arrow). **C.** Sporont, with a nascent posterior vacuole (arrow) next to the dense membrane apparatus. Arrowheads indicate the nascent polar tube. **D.** Formation of sporonts in a polysporous sporogon. The small arrow indicates the nascent posterior vacuole and the large arrow indicates the dense membrane apparatus that appears to act as a primordium of the posterior vacuole. **E.** Rings of host rough endoplasmic reticulum. **F.** Different *N. ausubeli* infection stages in host intestinal cells. The large arrow indicates sporoblasts. The small arrow indicates spores. One meront with a single nucleus is indicated. Microvilli of host intestinal cell are indicated with arrowhead. **G.** Six clustered sporonts. The intestinal cell appears reduced in width. The large arrows indicate microvilli; the small arrows indicate nascent anterior and posterior polaroplasts. **H.** Mature spore with posterior vacuole shown in inset. **I.** Posterior vacuole in a mature spore. **J.** Detail of the posterior side of a spore, with the posterior vacuole. **K.** Mature spore in the lumen, arrowhead indicates microvilli. **L.** Cross-section of two spores in the intestinal lumen. Scale bar is 500 nm, unless indicated otherwise. B, bacterium; Lu, intestinal lumen; M, meront; Nu, nucleus; Pt, polar tube; Pv, posterior vacuole; RER, rough endoplasmic reticulum. (TIF)

S2 Fig. Bayesian inference phylogeny of β -tubulin gene sequences. β -tubulin gene sequences from 38 nematode-infecting microsporidia species and 18 other microsporidia species were used. Model General time reversible (GTR) was applied. The branches were colored and annotated as above. This tree shows the position of *Nematocida* sp. 7. (TIF)

S3 Fig. Meront morphology of the different *Nematocida* species by Nomarski optics. **A.** Wild *Caenorhabditis elegans* strain JU1249, with *Nematocida parisii* infection. **B.** Wild *C. elegans* strain JU2520, with *Nematocida ausubeli* infection. **C.** Wild *C. briggsae* strain JU2747, with *N. major* infection. **D.** Wild *Oscheius tipulae* strain JU1510, with *N. minor* infection. **E.** Wild *Rhabditella typhae* strain NIC516, with *N. homosporus* infection. **F.** Wild *Procephalobus* sp. strain JU2895, with *N. ciargi* infection. Meronts are indicated by arrows in all images. Scale bar: 10 μ m (TIF)

S4 Fig. Bayesian inference phylogeny of SSU rDNA including the sequences of unidentified microsporidia species from environmental samples. SSU rDNA Bayesian inference phylogeny with the sequences of Fig 2 and nine microsporidian sequences (S1 Table) found in environmental samples in soil, sand and compost samples [41]. Model Kimura 2-Parameter (K2P) was applied. The branches were colored and annotated as Figs 4 & 5. (TIF)

S5 Fig. Meront morphology of *Enteropsectra* and *Pancytospora* species and tissue tropism of *Pancytospora epiphaga* by Nomarski optics. **A.** Wild *Oscheius* sp. 3 strain JU408, with *Enteropsectra longa* infection. **B.** Wild *O. tipulae* strain JU2551, with *Enteropsectra breve* infection. **C.** Wild *O. tipulae* strain JU1505, with *Pancytospora philotis* infection. One intestinal cell (below) was filled with spores, but only meronts seen in the other cell (up) close to it. **D.** Wild *Caenorhabditis brenneri* strain JU1396, with *Pancytospora epiphaga* infection, in the epidermis. Meronts are indicated by arrows in all images. **E.** Wild *Caenorhabditis brenneri* strain JU1396, with *Pancytospora epiphaga* infection in the pharyngeal region. Spores are indicated by arrows. **F.** Wild *Caenorhabditis brenneri* strain JU1396, with *Pancytospora epiphaga* infection. The large arrow indicates spores in the coelomocyte, while the small arrow indicates spores in the epidermis. Arrowheads indicate uninfected intestinal cells that are filled with gut granules. Scale bar: 10 μ m (TIF)

S6 Fig. Ultrastructural observations of *Enteropsectra longa*. Transmission electron micrographs of *E. longa* strain JUm408. **A, B.** Organization of various microsporidian stages in a host cell; the large and small arrows indicate apical and basal membranes of the host intestinal cells, respectively. The spores are on the apical side of the cell (and are not well infiltrated in these sections). In panel A, on the lower right is seen a nuclear division of a sporont. The mitotic spindle is indicated by an arrowhead. In panel B, the longitudinal section of the polar tube of a spore is indicated by an arrowhead. The white halo that can be seen between the mature spore wall and the cytoplasm is not due to the presence of a membrane but to incomplete infiltration during the preparation of the samples for electron microscopy. Such light-appearing areas are also seen occasionally on the internal side of the spore wall in both observed species. **C.** Mature spore with inset indicating the turn of the polar tube on the posterior end of the spore. Anchoring disk is indicated by arrow. **D-F.** Cross-section of *E. longa* spores with arrowheads indicating polar tubes. Most cross-sections show a single section of the polar tube. In E,F are shown the two cases where the polar tube was cut twice, likely close to the posterior end of the spore. **G.** Exit of spore. **G, H.** Exit of spores from the intestinal cell apical side into the lumen. The host cell apical membrane (black arrows) folds around *E. longa* spores. Microvilli are indicated by arrowheads. The posterior vacuole is indicated by a white arrow in panel G. **I.** Two mature spores are each

surrounded by an additional membrane (arrowheads), while the third one (right) does not. Inset at low resolution shows the positions of the two spores in the lumen in the corresponding section. **J, K.** Host intestinal cell nucleolus. The tubular substructures were not observed in the control uninfected animals. These structures have a width of approximately 250 nm and appear to be formed by ribosomal precursors. **L.** A nucleus filled with spores with a piece of degenerated nucleolus (arrow). The arrowhead indicates the nuclear membrane. Invasion of the host nucleus by the microsporidia was observed only once and the host nucleus is not the only place for sporogenesis in this species. Scale bar is 500 nm, unless indicated otherwise. A, anchoring disk; Ex, exospore; En, endospore; HNu host nucleus; HNI host nucleolus; Lu, lumen; M, meront; My, microvilli; Nu, microsporidian nucleus; Pt, polar tube; S: spore; St: sporont. (TIF)

S7 Fig. Spore morphology of microsporidia infection in different hosts by Nomarski optics. **A.** *Oscheius tipulae* strain JU1510, with *Nematocida ausubeli* JUm2526 infection. Refer to [Fig 4B](#) for morphology in the infection of a natural host species. Large and small spore classes are indicated by larger and smaller arrows, respectively. **B.** *Oscheius* sp. 3 strain JU75, with *N. homosporus* NICm516 infection. Refer to [Fig 4E](#) for morphology in a natural host species. Spore vesicles are indicated by arrows. **C.** *Oscheius* sp. 3 strain JU75, with *Enteropsectra longa* JUm408 infection. Refer to [Fig 7A](#) for morphology in a natural host species. Long thin spores along the apical membrane of host intestinal cells are indicated by arrows. **D.** *Oscheius* sp. 3 strain JU408, with *Enteropsectra breve* JUm2551 infection. Refer to [Fig 7B](#) for morphology in a natural host species. Small rod spores along the apical membrane of host intestinal cells are indicated by arrows. **E.** *Oscheius tipulae* strain JU170, with *Pancytospora philotis* JUm1505 infection. Refer to [Fig 7C](#) for morphology in a natural host species. Long thin spores concentrated in intestinal cells is indicated by arrow. **F.** *C. elegans* N2, with *Pancytospora epiphaga* JUm1396 infection. The arrowhead indicates intestinal granules. Refer to [Fig 7D](#) for morphology in a natural host species. Scale bar: 10 μ m. (TIF)

S1 Table. Accession numbers for SSU rDNA and β -tubulin sequences.
(XLSX)

S2 Table. ERT54 and ERT72 transcriptional reporter induction by various microsporidia.
(XLSX)

S3 Table. SSU rDNA pairwise distances of all the sequences used for phylogenetic analysis in [Fig 2](#) and genetic distances in [Table 3](#).
(XLSX)

S1 Datafile. Alignment with 116 SSU rDNA sequences used for [Figs 2 and 3](#); [S6 Fig](#); [Table 3](#); [S3 Table](#).
(NEX)

S2 Datafile. Alignment with 58 β -tubulin sequences used for [Fig 3](#); [S2 Fig](#). Sequences were aligned using Geneious v7.1.7 with default parameters and further aligned manually and concatenated if available.
(NEX)

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Author Contributions

Conceptualization: MAF ERT.

Funding acquisition: MAF GZ ERT RJL.

Investigation: GZ MS MCP RJL.

Methodology: GZ MS RJL ERT MAF.

Resources: MAF.

Supervision: MAF ERT.

Visualization: GZ RJL MAF.

Writing – original draft: GZ MAF ERT RJL MS.

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Supplemental Files

Figure S1

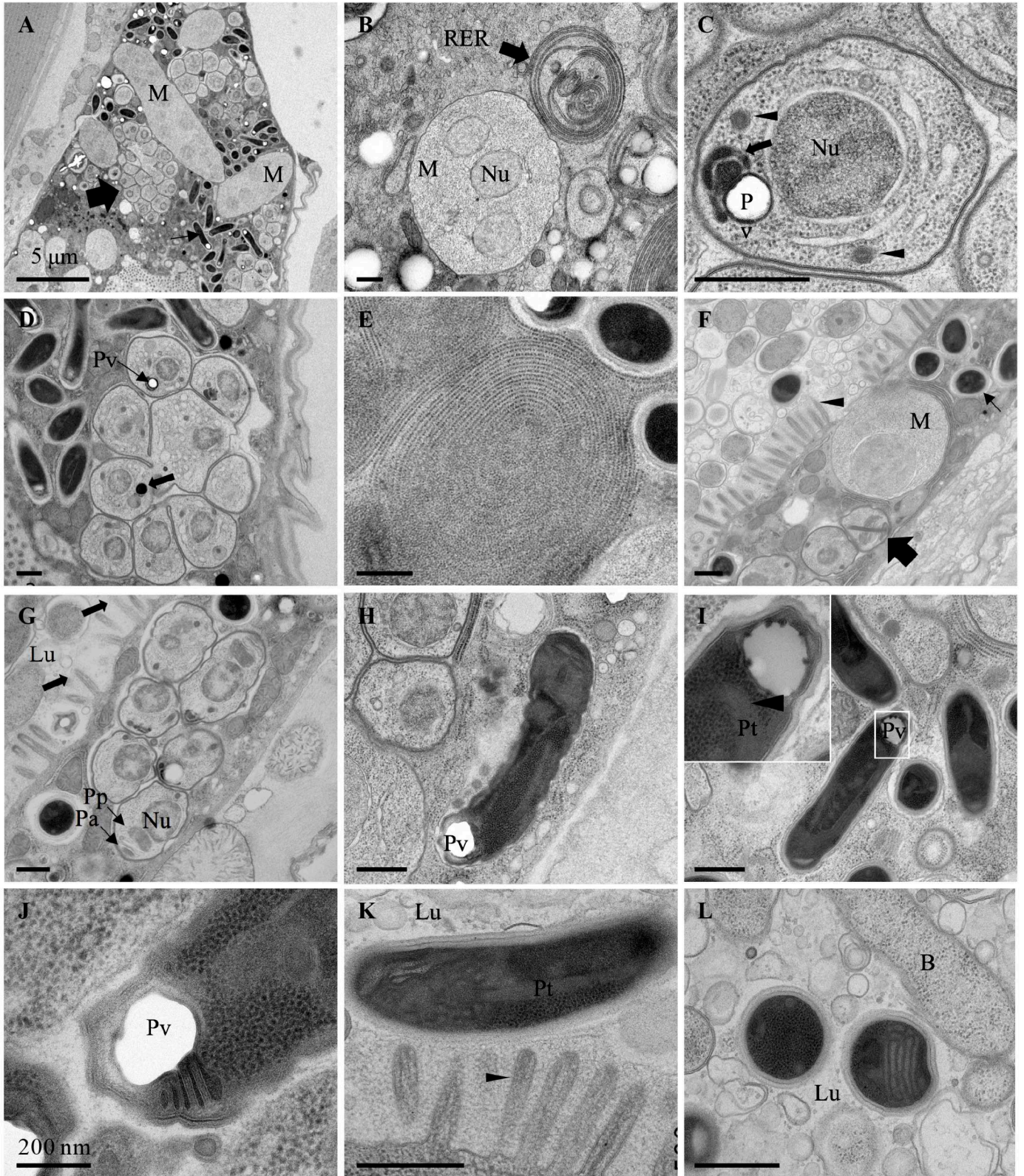


Figure S2

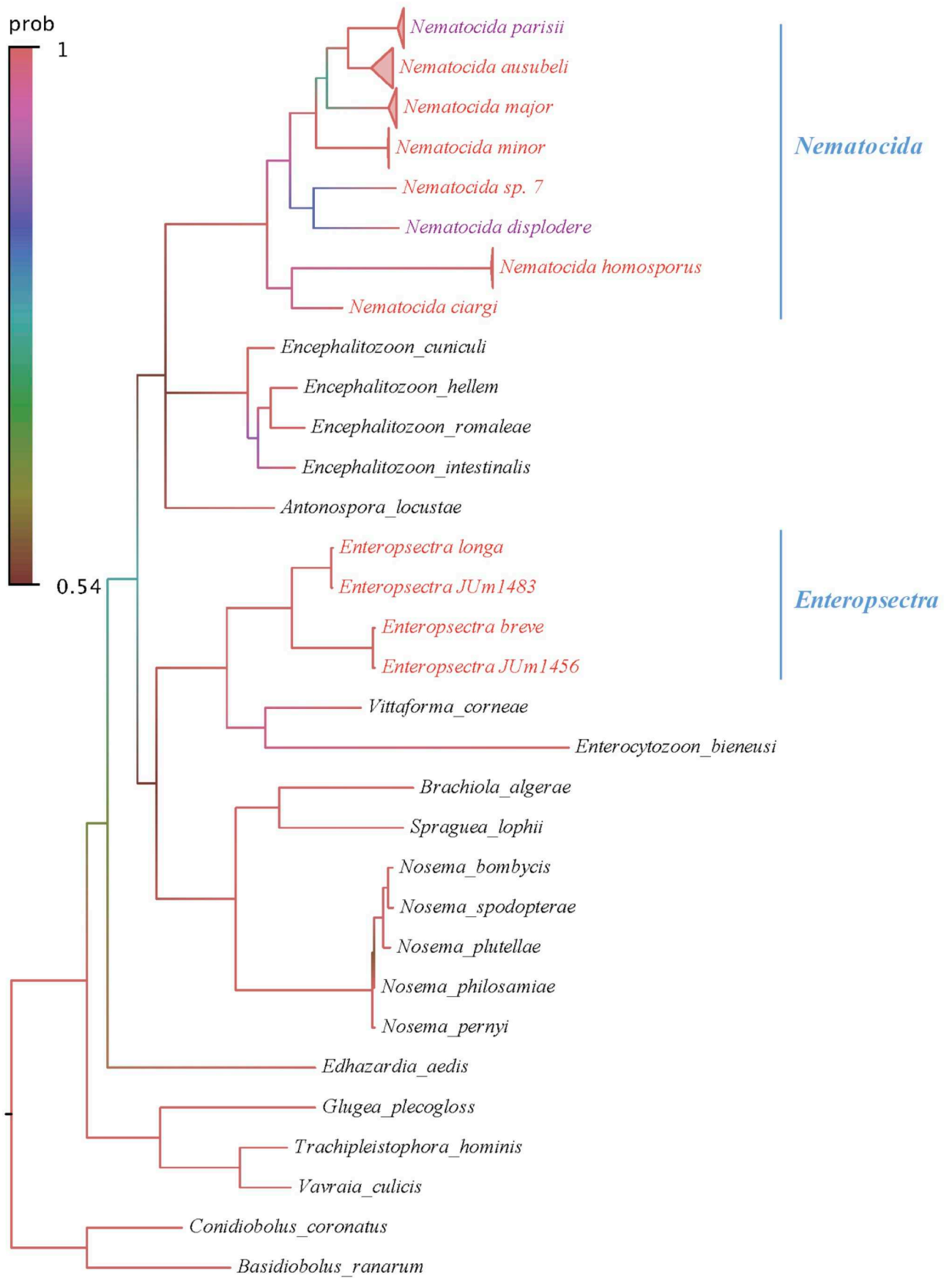


Figure S3

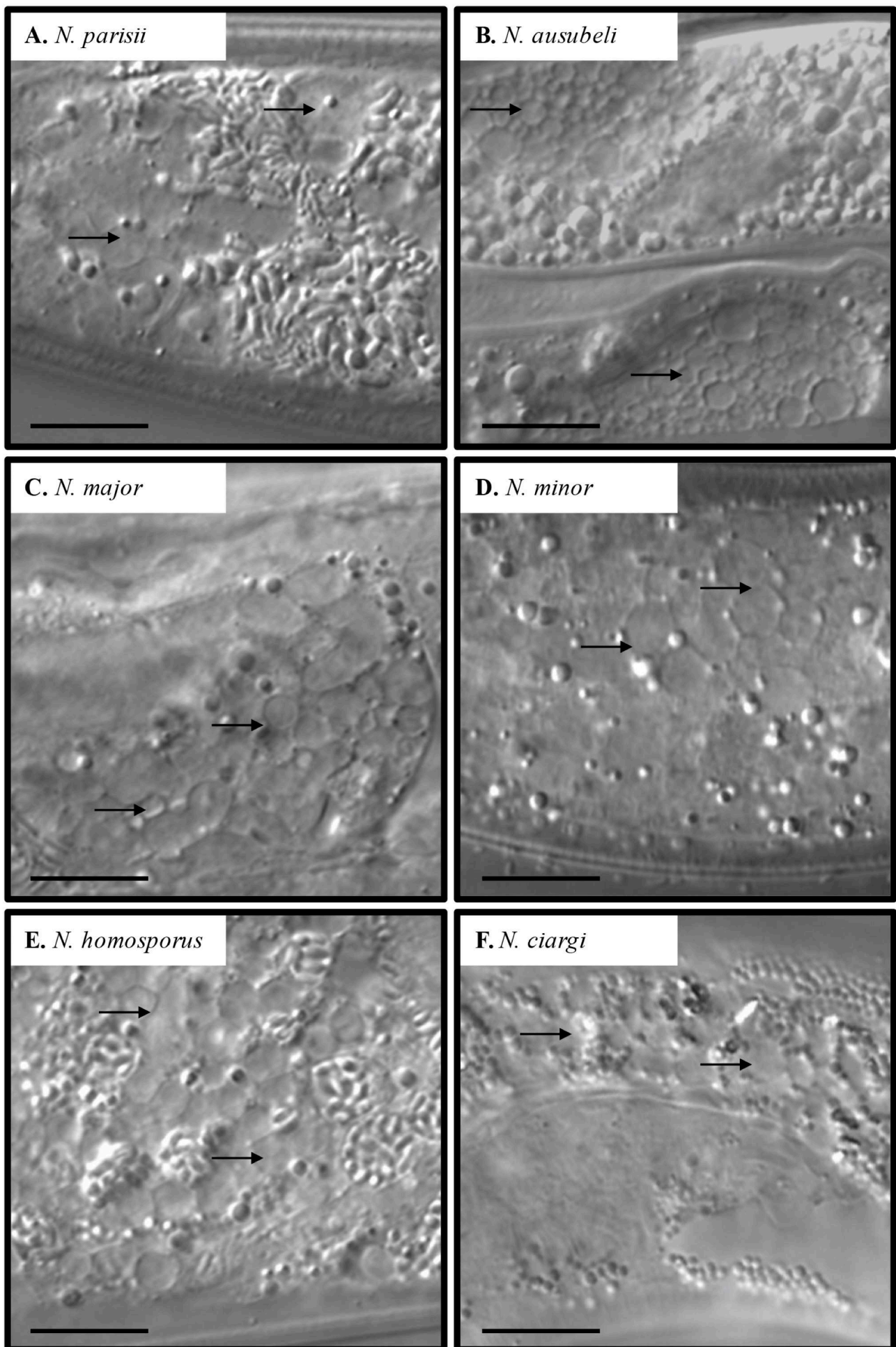


Figure S4

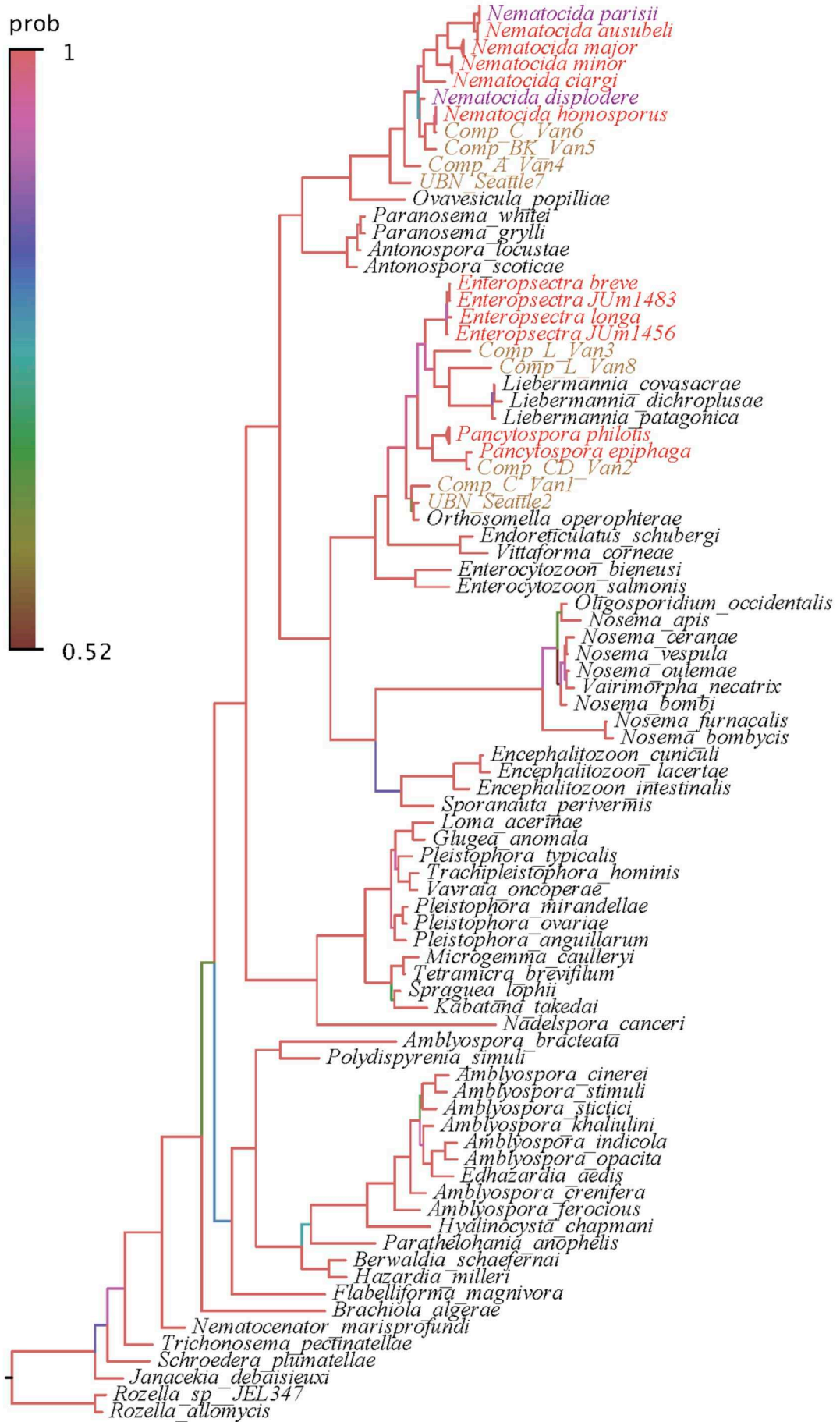


Figure S5

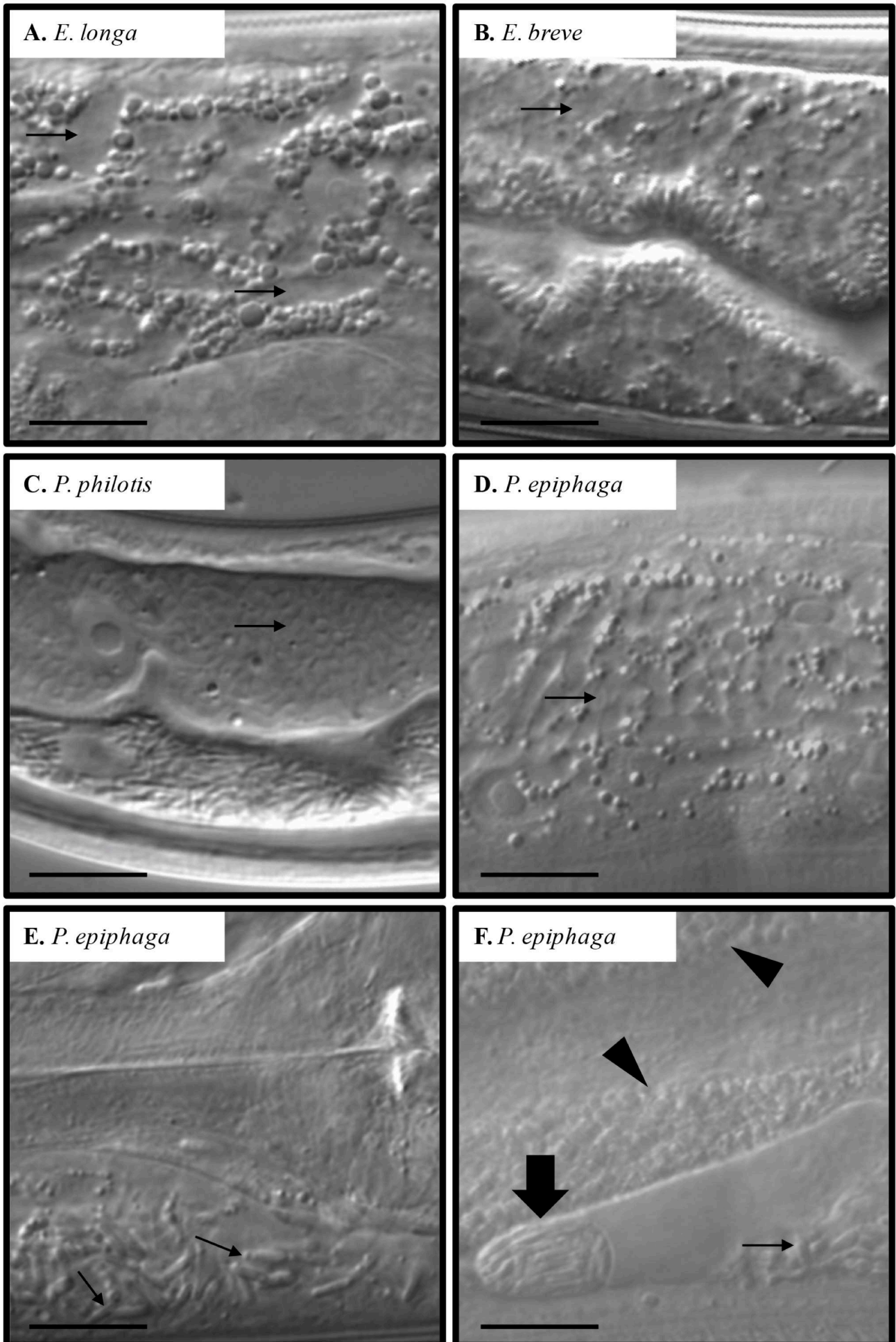


Figure S6

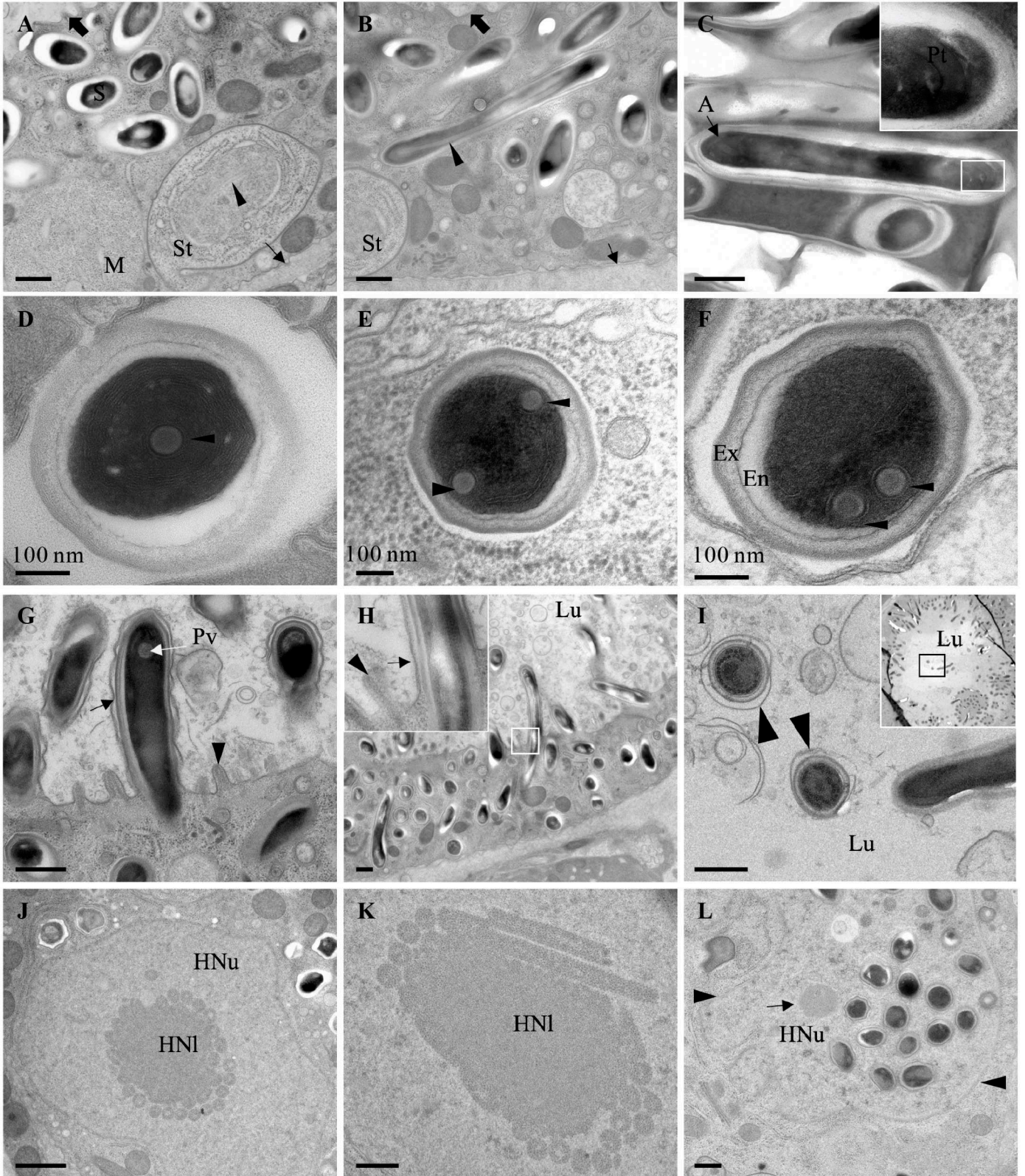
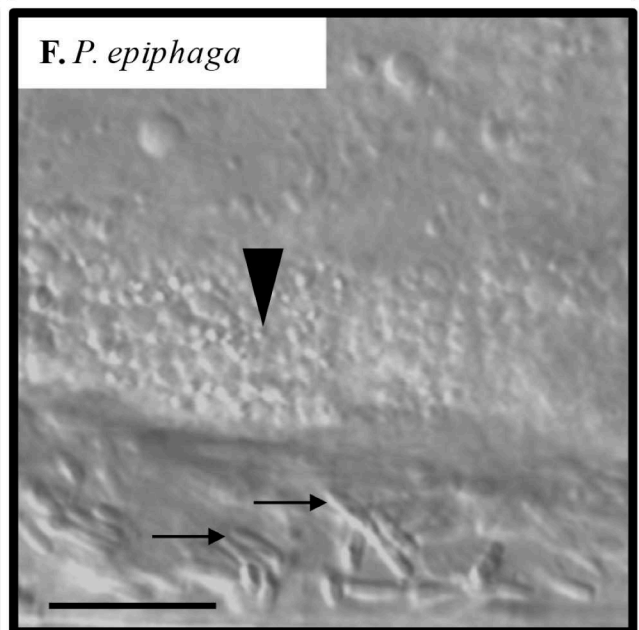
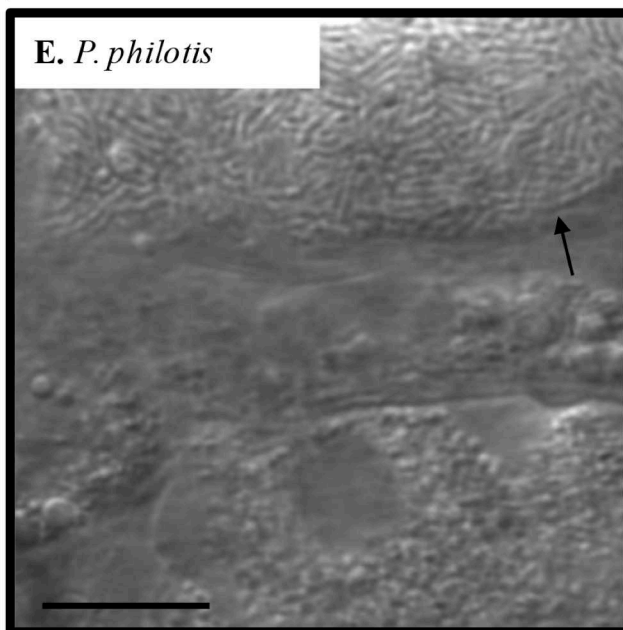
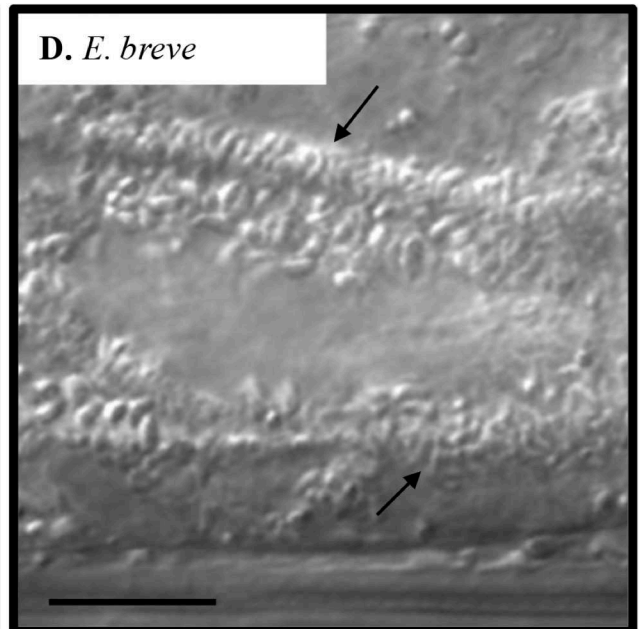
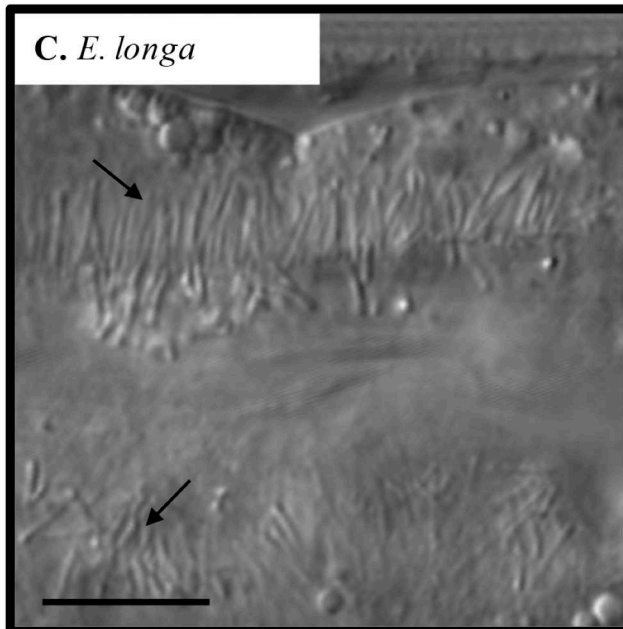
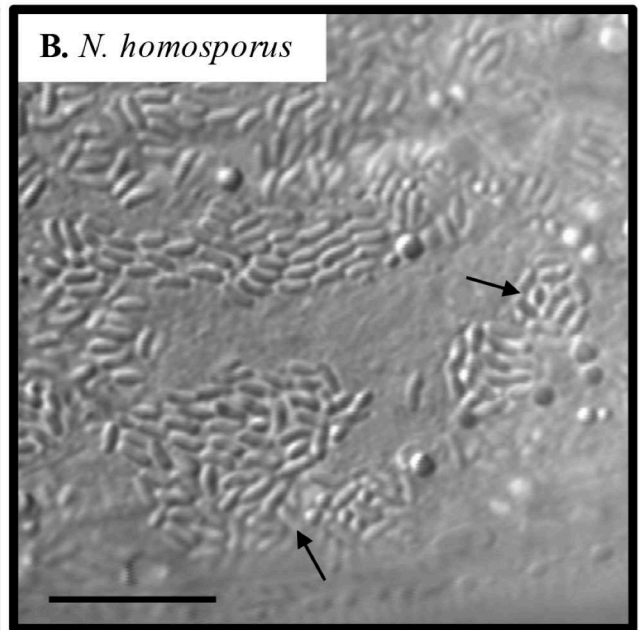
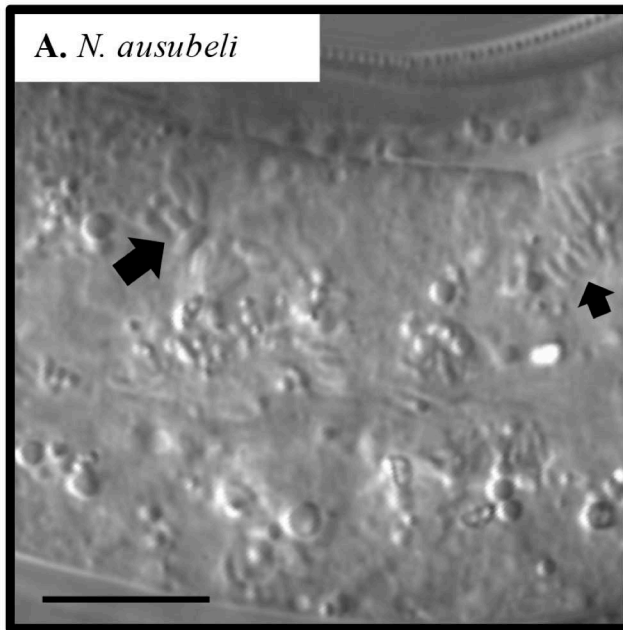


Figure S7

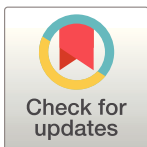


CORRECTION

Correction: A Large Collection of Novel Nematode-Infecting Microsporidia and Their Diverse Interactions with *Caenorhabditis elegans* and Other Related Nematodes

The *PLOS Pathogens* Staff

In [Table 2](#), dividing lines were introduced during typesetting that affect the readability of the table. The publisher apologizes for the error. Please see the corrected [Table 2](#) here.



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Table 2. Collection of other microsporidia species infecting wild nematodes

Microsporidia	Nematode host	Strain	Country / Year	Sample composition	Spore feature
<i>Nematocida major</i> n. sp.	<i>C. briggsae</i>	JUm2507	Thailand / 2013	rotting fruit	2S
	<i>C. briggsae</i>	JUm2747	Guadeloupe / 2014	rotting fruit	2S
	<i>C. tropicalis</i>	JUm2751	Guadeloupe / 2014	rotting fruit	n. d.
<i>Nematocida minor</i> n. sp.	<i>Oscheius tipulae</i>	JUm1510	Czech Republic / 2008	rotting apple	2S
	<i>O. tipulae</i>	JUm2772	Armenia / 2014	rotting fruit	n. d.
<i>Nematocida homosporus</i> n. sp.	<i>O. tipulae</i>	JUm1504	France / 2008	rotting stem	1S
	<i>Rhabditella typhae</i>	NICm516	Portugal / 2013	rotting apple	1S
<i>Nematocida ciargi</i> n. sp.	<i>Procephalobus</i> sp.	JUm2895	Spain / 2015	rotting fruit	1S
<i>Nematocida</i> sp. 7	<i>C. sp. 42</i>	NICm1041	French Guiana / 2014	flower	n. d.
<i>Enteropsectra longa</i> n. sp.	<i>Oscheius</i> sp. 3	JUm408	Iceland / 2002	compost	1S, LT, AP
<i>Enteropsectra breve</i> n. sp.	<i>O. tipulae</i>	JUm2551	France / 2013	rotting apple	1S, SR, AP
	<i>O. tipulae</i>	JUm1483	France / 2008	rotting plum	1S, SR, AP
	<i>O. tipulae</i>	JUm1456	France / 2008	rotting fruit	n. d.
<i>Pancytopora philotis</i> n. sp.	<i>O. tipulae</i>	JUm1505	France / 2008	rotting apple	LT
	<i>O. tipulae</i>	JUm1460	France / 2008	rotting snail	LT
	<i>O. tipulae</i>	JUm1670	France / 2009	rotting apple	LT
	<i>O. tipulae</i>	JUm2552	France / 2013	rotting stem	LT
<i>Pancytopora epiphaga</i> n. sp.	<i>C. brenneri</i>	JUm1396	Colombia / 2008	rotting fruit	LT

The reference strain of each newly found species is in bold. 2S: two distinct sizes of spores; 1S: one size of spores

LT: long, thin rod; SR: small rod (see dimensions in Table 3); AP: form spores first along the apical side of the intestinal cells. n. d.: not determined

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Reference

1. Zhang G, Sachse M, Prevost M-C, Luallen RJ, Troemel ER, Félix M-A (2016) A Large Collection of Novel Nematode-Infecting Microsporidia and Their Diverse Interactions with *Caenorhabditis elegans* and Other Related Nematodes. PLoS Pathog 12(12): e1006093. doi:10.1371/journal.ppat.1006093 PMID: [27942022](https://pubmed.ncbi.nlm.nih.gov/27942022/)

II.

Natural variation of *Caenorhabditis elegans* in sensitivity to microsporidian infection

While studying the diverse microsporidian infections found in the wild nematodes, we also observed natural phenotypic variation in sensitivity of different wild *C. elegans* strains to the same microsporidian infection. In this chapter, I will focus on the comparison of the sensitivity of ten *C. elegans* strains upon the same *N. ausubeli* infection. We chose to focus on the *N. ausubeli* instead of *N. parisii* infection in *C. elegans* with two reasons. First, our previous results showed that *C. elegans* may have a different transcriptional response to the infection by *N. ausubeli* than to the infections by three other *Nematocida* species, including *N. parisii*, *N. major* and *N. homosporus* (see the former chapter). Second, the sensitivity of six *C. elegans* strains to *N. parisii* infection has been studied by Balla et al. (2015). It will be of interest to compare *N. ausubeli* with *N. parisii* in their interactions with *C. elegans*.

Variation in *C. elegans* sensitivity to *N. ausubeli* infection was determined by food consumption tests in the presence or absence of microsporidia. Two *C. elegans* strains, JU1249 and JU2825, displayed the largest sensitivity difference to *N. ausubeli* infection. JU1249 was found to be the most resistant strain, whereas JU2825 was the most sensitive one. Quantitative fluorescence in situ hybridization (FISH) analysis on microsporidian infection in the two strains suggested that the JU1249 and JU2825 strains differ in their tolerance. Competition assays further supported that JU1249 and JU2825 are good candidates for studies on the genetic loci associated with *C. elegans* sensitivity variation to microsporidian infections.

A surprising effect of microsporidia infection on *C. elegans* was also found in this study, which will be described in detail in the next chapter.

My contributions in this study:

I performed all the assays and analyses in this part.

Clément Dubois did his M2 internship in our lab. His work was closely related to this part of my Ph. D. project. I will talk about his results in the Discussion.

II.1. Background

Specificity of infection of *Caenorhabditis* and *Oscheius* species by eight microsporidia species was described in the former chapter. Meanwhile, intraspecific variation of *C. elegans* sensitivity to *N. parisii* and *N. ausubeli* was also observed during the maintenance of wild infected strains. Some infected *C. elegans* strains displayed higher mobility, higher fecundity and greater longevity than other *C. elegans* infected with the same microsporidia. This host phenotypic variation can be host-based or pathogen-based, or dependent on their interaction. Host *C. elegans* strains may have different genetic bases underpinning their microsporidia-defense traits. On the other hand, intraspecific variation for pathogenicity may also exist in microsporidia. Here, we focus on the host *C. elegans*. We were particularly interested in the genetic basis of *C. elegans* sensitivity variation to microsporidia infection, which can be further studied by Quantitative trait locus (QTL) analysis.

Previously, the sensitivity of six *C. elegans* strains to *N. parisii* infection was investigated by Balla et al. (2015). They notably found that one *C. elegans* strain, from Hawaii (HW), has enhanced immunity at the young larval stage (L1), which gives to this strain the ability to clear the *N. parisii* infections. This phenomenon was not observed in the other five strains tested, including N2. The genetic basis of this early-life immunity variation between HW and N2 was further investigated. The results suggested that the special immune ability of the Hawaiian strain was a complex multigenic trait (Balla et al. 2015). Among the six *C. elegans* strains compared in this study, only one strain ERT002 (derived from CPA24) was naturally found infected with *N. parisii* (ERTm1). The other five strains may have never encountered any microsporidia in their natural habitats. To study the evolutionary consequences of microsporidia infection pressures on *C. elegans*, it is relevant to compare *C. elegans* strains that have been exposed to microsporidia in the wild.

The work described in this chapter is the first description of variation among naturally infected *C. elegans* strains in sensitivity to microsporidia infection. While comparing the sensitivity of ten wild *C. elegans* strains to *N. ausubeli* infection, we proved the natural variation for this trait in *C. elegans* species. Interestingly, we found contrasted levels of *C. elegans* tolerance to *N. ausubeli* infections. Indeed, the two *C. elegans* strains, JU1249 and JU2825, showed similar levels of infection but highly contrasted levels of sensitivity (indirect

fitness assay). These two *C. elegans* strains will be further used to identify genetic locus/loci involved in *C. elegans* tolerance to *N. ausubeli* microsporidia.

II.2. Materials and Methods

II.2.1. *C. elegans* and *Microsporidia* strains

In **RESULTS I**, 19 *C. elegans* strains had been isolated with *N. parisii* or *N. ausubeli* infection. Among these *C. elegans* isolates, the strains JU1249, JU1762, JU2009, JU2132, JU2287, JU2526, JU2586, JU2816, JU2825 and the lab reference strain N2 were used. The nine naturally infected strains were subsequently bleached to remove microsporidia infection and any other microbes, and maintained in the lab as described in **RESULTS I**.

The *N. ausubeli* strain used in the first infection assay on all the ten *C. elegans* strains was JUm2009, a *N. ausubeli* strain isolated from the *C. elegans* strain JU2009. Another *N. ausubeli* strain JUm2526, two *N. parisii* strains JUm1249 and JUm2816 were isolated from the *C. elegans* strains JU2526, JU1249 and JU2816, respectively. JUm2009, JUm2526 and JUm1249 were used on the further infection assays on JU1249 and JU2825, to compare their sensitivity variation upon different microsporidia infections. Finally, *N. ausubeli* JUm2009 and *N. parisii* JUm2816 were used to infect the mixed population of JU1249 and JU2825 in the competition assay. Spores were prepared and quantified as described in **RESULTS I**.

II.2.2. Food consumption assays

A. Infection and maintenance

20 uninfected young adults (i.e. prior to first egg formation) were transferred onto a 55mm-diameter NGM plate seeded with *E. coli* OP50. 2 million of microsporidian spores suspended in 100 µl of distilled water were dispensed onto the *E. coli* lawn. The cultures were then incubated at 23 °C. The infection symptoms were monitored by Nomarski optics at 48 hours after inoculation. In order to avoid the possible bias caused by the high or low concentration of spores used in the initial inoculation, and to have a stable infected *C. elegans* population, 20 young adults were transferred to a new plate every two days. Uninfected *C. elegans* populations were also maintained and transferred for a few generations. Three replicates were conducted in parallel for each experiment. Infected and uninfected *C. elegans* populations were maintained for ten days after inoculations. Then the *C. elegans* isolates were used in food consumption assays (the protocols of the food consumption assay and the intensity normalization method were from by Matthew Rockman and Victoria Cattani).

B. Plate seeding, *C. elegans* transfer and fluorescence scan

To study the population growth of different *C. elegans* strains under different conditions, food depletion by each strain was measured in the presence or absence of microsporidia infection.

First, 100 μ L of OP50-GFP (a *E. coli* OP50 strain carrying a GFP marker) were carefully seeded in the center of 6 cm NGM plates. Control 6 cm NGM plates were seeded with 100 μ L of regular OP50, which were required for the normalization of OP50-GFP fluorescence intensity. Seeded plates were incubated at room temperature for 24 hours in the dark. Signal intensity of the seeded plates was then scanned and measured using a Typhoon FLA 9000 laser scanner (GE Healthcare Life Sciences). The fluorescence intensity in this first scan was used as the data at timepoint 1. *C. elegans* individuals from prepared populations were transferred to plates seeded with OP50-GFP soon after the first scan.

From the *C. elegans* populations maintained for ten days, 10 L4s were transferred to a plate seeded with OP50-GFP, with three replicates per experiment. The fluorescence decay in plates was scanned every 24 hours until the depletion of OP50-GFP. Two types of control were also scanned at each timepoint: three plates seeded with OP50-GFP but without *C. elegans* (a control for bacterial growth), and three plates seeded with regular OP50 but without *C. elegans* (a negative control for non-specific fluorescence).

A few plates got contaminated by fungi or other bacteria after several times of scan. These contaminated plates were discarded then.

C. Fluorescence intensity normalization

The mean fluorescence intensity of each plate was corrected by the formula below to obtain the corrected fluorescence intensity (F_c).

$$F_c = \frac{\frac{F_i - F_{backi}}{F_1 - F_{back1}}}{\frac{F_{nowormi} - F_{backi}}{F_{noworm1} - F_{back1}}}$$

F_i = Mean fluorescence intensity of the plate at timepoint i ;

F_{backi} = Mean fluorescence intensity of the plate with regular OP50 without *C. elegans* at timepoint i ;

$F_{nowormi}$ = Mean fluorescence intensity of the plate with OP50-GFP without *C. elegans* at timepoint i .

The food fluorescence intensity on each plate at different timepoints was then analyzed and compared. As the bacteria OP50 could grow in the plate during the experiment, the GFP intensity could initially rise when the bacteria growth rate was higher than the consumption rate of *C. elegans*.

In the food consumption assays focusing on JU1249 and JU2825, infected *C. elegans* populations were harvested after the last scan, and stored in acetone at -20 °C for microsporidia infection measurements by FISH.

II.2.3. Measurements of microsporidia infection by FISH

A. FISH measurements

The infection rate, the pathogen load and the average infected area of *C. elegans* were measured by FISH staining. We used the FISH probe MicroB conjugated to a Cyanine5 (Cy5) dye to stain the small subunit rRNA of *N. parisii* and *N. ausubeli* (Troemel et al. 2008). FISH was performed as previously described (Troemel et al. 2008). Briefly, 5ng/μL of MicroB was used in the hybridization at 46 °C. After overnight hybridization, *C. elegans* populations were washed and incubated at 48 °C for 30 minutes.

Stained *C. elegans* isolates were mounted on glass slides and imaged using the fluorescence microscope under the Cy5 filter with a 10X objective and the Metamorph software. Images were analyzed semi-manually using ImageJ software. The body area, the infected area and the intensity of the infected area in 30 adult *C. elegans* were measured for each replicate. The infection rate, and the average pathogen load were computed and analyzed.

B. Statistical analysis on FISH data

Rstudio (v 0.99.903) and two packages (ggplot2, plyr) were used for the statistical analysis and the Figure 28. The pathogen load was analyzed by a linear model to explain the logarithm of fluorescence intensity (pre-normalized) by the *C. elegans* genotypes. To test whether the pathogen loads of the two *C. elegans* strains significantly differed upon each microsporidia infection, we performed a t-test. To test whether the infection rates of the two *C. elegans* strains significantly differed upon each microsporidia infection, we used a Wilcoxon Rank Sum test.

II.2.4. Competition assays

A. Infection and competition

The *C. elegans* strains JU1249 and JU2825 were competed with or without microsporidia infection. Ten uninfected JU1249 L4 larvae and ten uninfected JU2825 L4 larvae were transferred to a 10 cm NGM plate seeded with *E. coli* OP50. These 20 L4s founded the initial mixed population in the competition assays, with five replicates per experiment. 5 million microsporidian spores in 150 μL distilled water were placed on the *E. coli* lawn. The cultures were then incubated at 23 °C. The infection symptoms were checked by Nomarski optics at 48 hours after inoculation. To avoid starvation, each plate was visually checked twice a day for the amount of OP50 left in the plate; if starvation may occur before the next visual check, a small fraction of the population (about 200 to 400 animals) was transferred to a fresh culture plate. As microsporidia infection delays the growth of *C. elegans* populations, infected populations were transferred fewer times than uninfected populations in the experiment. After

each transfer, animals from old plates were harvested in M9 solution and stored at -80 °C for quantification of the respective proportions of JU1249 and JU2825.

B. Allele quantification

The whole genomes of JU1249 and JU2825 were sequenced using Illumina Hiseq4000 with 20X coverage and paired end 150 bp. For each genome, the raw data were aligned to the reference genome (*C. elegans* WS243) using a combination of softwares - bwa, samtools, Picard and Genome Analysis Toolkit (GATK) (Li and Durbin, 2009; Li et al., 2009; McKenna et al., 2010). From the output files, homozygous single-nucleotide polymorphisms (SNPs) versus the reference genome were called (A pipeline on the genome data analysis was developed in the lab by Fabrice Besnard). A few SNPs between JU1249 and JU2825 were selected manually.

Based on one of the single-nucleotide polymorphisms (SNPs) between JU1249 and JU2825 on chromosome III, pyrosequencing primers III_663310_Forward (5'-GTGACGTACTAGCAACGAGTCGATTTTGGGGATGGA-3'), III_663310_Reverse (5'-CACTAGGCAGGTAGGCATTTTT-3') and III_663310_Sequencing (5'-CCAAACTTTTATAGAGATCA-3') were designed. Allele quantification was performed using pyrosequencing as previously described (Félix and Dubeau, 2012). In brief, harvested *C. elegans* isolates were centrifuged at 3,000 rpm for 2 minutes to generate a nematode pellet. Lysates of 2 µL of the pellet were used as PCR templates, with the primers III_663310_Forward and III_663310_Reverse. The allele frequency of the PCR product was quantified with the pyrosequencer (PyroMark Q96 ID instrument; Biotage), using primer III_663310_Sequencing and a universal biotinylated primer (5'-[Bt_n]TAGCAGGATACGACTATC-3').

The accuracy of this quantification method was estimated by measuring the allele frequencies of PCR products that were amplified using *C. elegans* lysates of known proportions of mixtures of JU1249 and JU2825 individual L4s. On average, 2% of discrepancy existed between expected and observed allele frequencies.

The experiment was continued until all replicates in one treatment reached fixation of either JU1249 or JU2825.

II.3. Results

II.3.1. Natural variation of *C. elegans* population growth, in the presence or absence of *N. ausubeli* infection

In the Felix laboratory collection of wild nematode isolates, 19 *C. elegans* strains were naturally infected with microsporidia (Figure 24). The *N. parisii* infection has been shown to

have different negative effects on *C. elegans*, such as shortening the lifespan, reducing the brood size and increasing the mortality (Troemel et al. 2008; Balla et al. 2015). These negative effects of microsporidia infection may together delay the growth of *C. elegans* populations. As individual-based assays are time-consuming, to assay natural sensitivity to microsporidia infection in the wild *C. elegans* strains, we used a Food Consumption assay to compare the food depletion rate of different strains in the presence and absence of microsporidia infection.

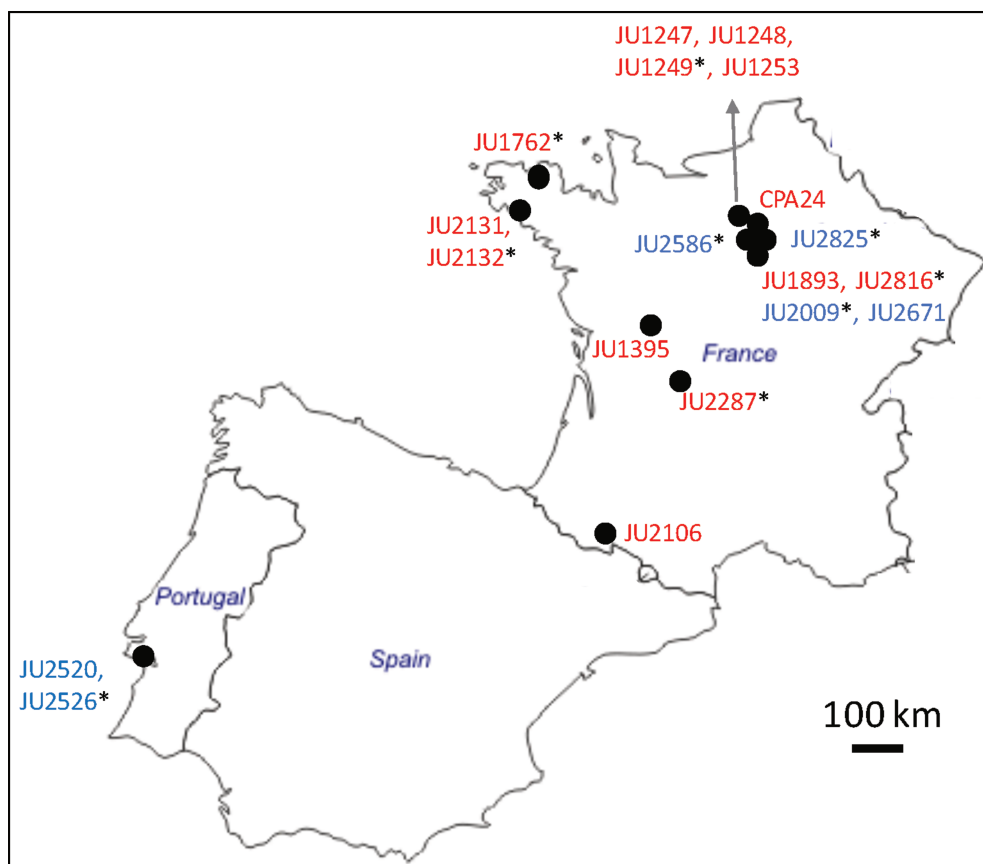


Figure 24. Geographical distribution of known wild *C. elegans* strains with microsporidia infection.

C. elegans strains naturally infected with *N. parisii* (red) or *N. ausubeli* (blue). Strains with “*” were used in the food consumption test to compare their sensitivity to *N. ausubeli* infection. The map is modified from http://www.d-maps.com/carte.php?num_car=4573&lang=en.

The strong assumption of our food consumption assay is that a faster worm population growth causes faster food depletion, i.e. there is a good correlation between fitness and food depletion. Note that a faster food depletion rate is not necessarily fully correlated to the nematode population growth rate. For example, it might also be correlated to the food consumption rate per worm. A small population with a high food consumption rate per worm may consume food faster than a relatively larger population with a lower food consumption rate per worm. However, when considering a single *C. elegans* strain, the impact of microsporidian infection on the host fitness will be reflected by the decay in food consumption caused by the infection. *C. elegans* food consumption rates might be differentially influenced by microsporidian infections, which could indicate variations in the sensitivity of *C. elegans* to microsporidia infection. Although the food consumption assay is a rough proxy, it is a practical and convenient method to quantify *C. elegans* sensitivity to microsporidian infection.

First, we investigated variation in sensitivity among ten *C. elegans* strains, nine of which were naturally found infected with *N. parisii* or *N. ausubeli*, and N2 as a control (Figure 24, marked by “*”). We bleached each strain and infected it with the *N. ausubeli* strain JUm2009. Then these infected populations were maintained for 10 days (about four generations) to a relatively stable state, in which infected *C. elegans* individuals infect other uninfected individuals and *C. elegans* populations continue the infection over generations. From the stable infected population, individual L4s were transferred to plates seeded with *E. coli* OP50-GFP, the fluorescence intensity of which was monitored at different timepoints. Uninfected *C. elegans* isolates were also maintained and tested in parallel.

As expected, we find that for nine *C. elegans* strains (JU2526 will be described in the next chapter), the infected populations consumed food slower than the uninfected populations (Figure 25). This demonstrated that *N. ausubeli* infection negatively affects *C. elegans* food consumption rates. Moreover, this negative impact of *N. ausubeli* infections varies between strains (Figure 25 and Figure 26). For example, with or without infection, JU2287 is the slowest food consumer among the nine strains. However, JU2825, the fastest food consumer when uninfected, only had a medium food consumption rate among the nine strains when they were infected (Figure 26). This demonstrates that the food consumption rates of different *C. elegans* strains were affected differently when they were infected with *N. ausubeli*, which suggested that the natural variation in *C. elegans* response to the *N. ausubeli* infection.

To further understand the genetic basis of *C. elegans* sensitivity variation to microsporidia infection using a quantitative trait locus (QTL) approach, at least two strains showing contrasted phenotypes are required. Among the *C. elegans* strains we tested, JU1249 and JU2825 were chosen as candidates. In the absence of microsporidia infection, JU2825 consumed food faster than JU1249. In contrast, with *N. ausubeli* JUm2009 infection, JU1249 consumed food faster than JU2825 (Figure 25, Figure 26). Thus, *N. ausubeli* JUm2009 infection has a larger negative influence on JU2825 than on JU1249. JU1249 is more resistant to *N. ausubeli* JUm2009 than JU2825. We further investigated whether the host sensitivity variation between JU1249 and JU2825 is specific to *N. ausubeli* JUm2009.

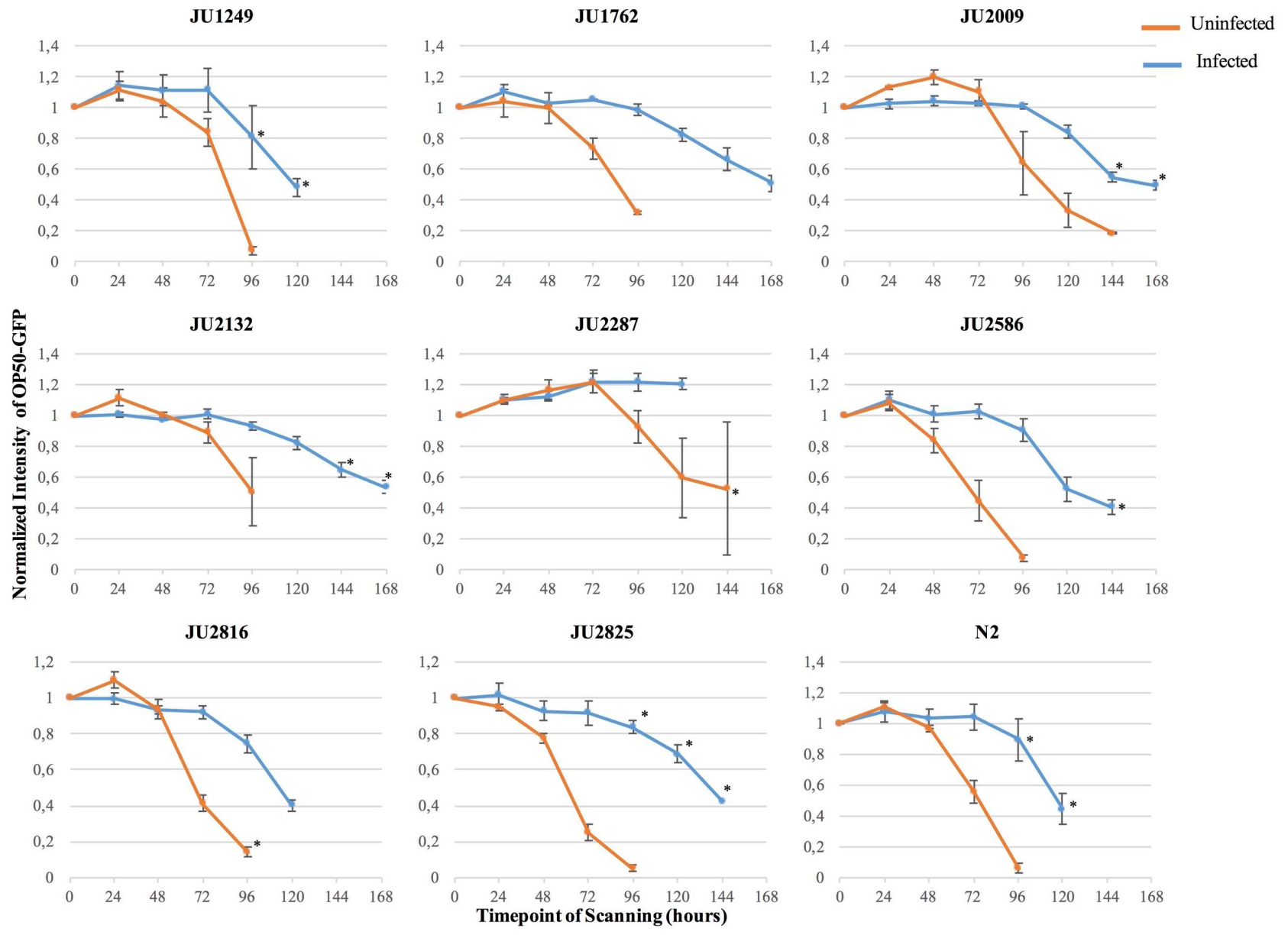


Figure 25. Variation in sensitivity against *N. aeusubeli* infection among nine *C. elegans* wild isolates.

E. coli OP50-GFP intensity over time for nine *C. elegans* strains with or without *N. aeusubeli* JU2009 infection. The Y-axis is the normalized intensity of the fluorescence of OP50-GFP. The X-axis is the scanning time point. Each plate was scanned every 24 hours until full depletion of bacteria on the plate. Data are mean values of three replicates (data marked with “*” are mean values of two replicates), with bars represent standard errors. As the bacteria OP50-GFP could grow on the plate, the intensity increased in the beginning.

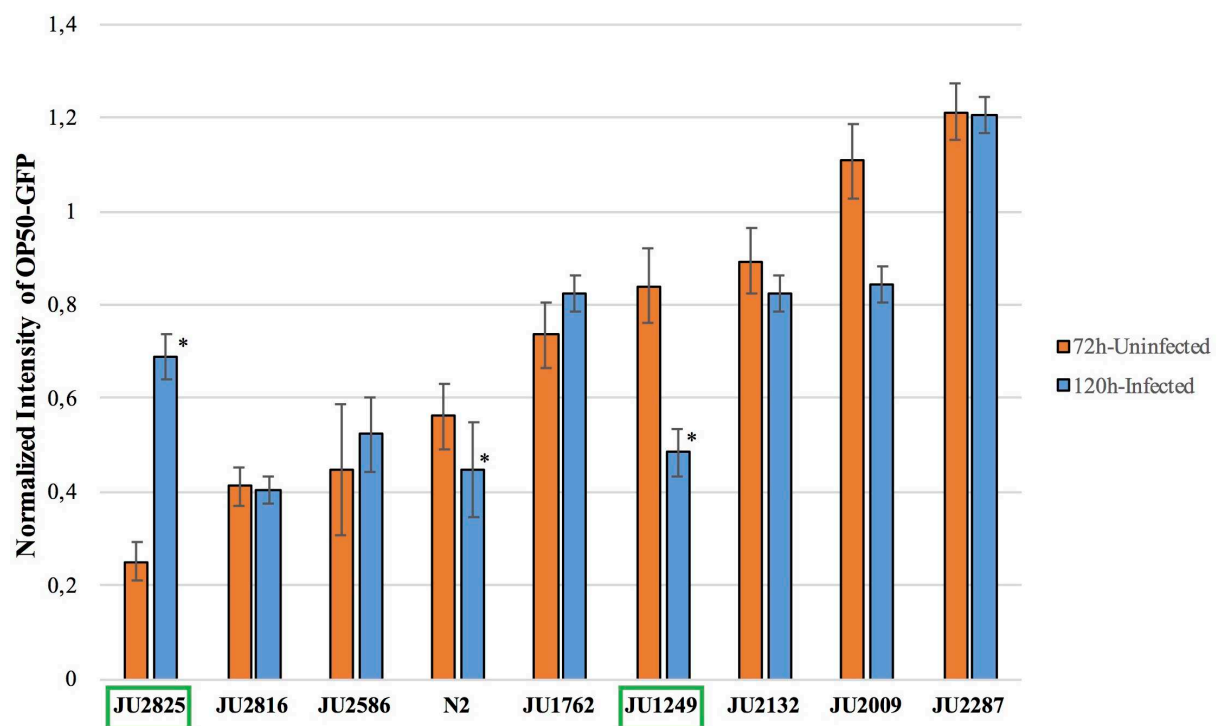


Figure 26. OP50-GFP intensity of nine *C. elegans* strains at two timepoints.

The OP50-GFP intensity of the plates with uninfected *C. elegans* (at 72h) and infected *C. elegans* (at 120h) is shown. Data are mean values of three replicates (data marked with “*” are mean values of two replicates), with bars represent standard errors. The nine strains on the X axis were ordered from left to right by their normalized OP50-GFP intensity at 72 hours. JU2825 and JU1249, which displayed contrasted phenotype, were highlighted with green frames.

II.3.2. Host response of JU1249 and JU2825 to infection by different microsporidia

N. ausubeli JUm2009 has a greater negative impact on JU2825 than on JU1249. We then investigated the impacts of another *N. ausubeli* strain JUm2526 and a *N. parisii* strain JUm1249 on the food consumption rates of JU1249 and JU2825. Populations with JUm2009 infection and uninfected populations were also tested in parallel. As in the previous experiment, without infection, JU1249 consumed food in a similar or a slightly slower rate, compared to that of JU2825. In all the infected populations, JU1249 was faster than JU2825 in the food consumption rate (Figure 27). These results confirmed that JU2825 is more sensitive than JU1249 to microsporidia infection.

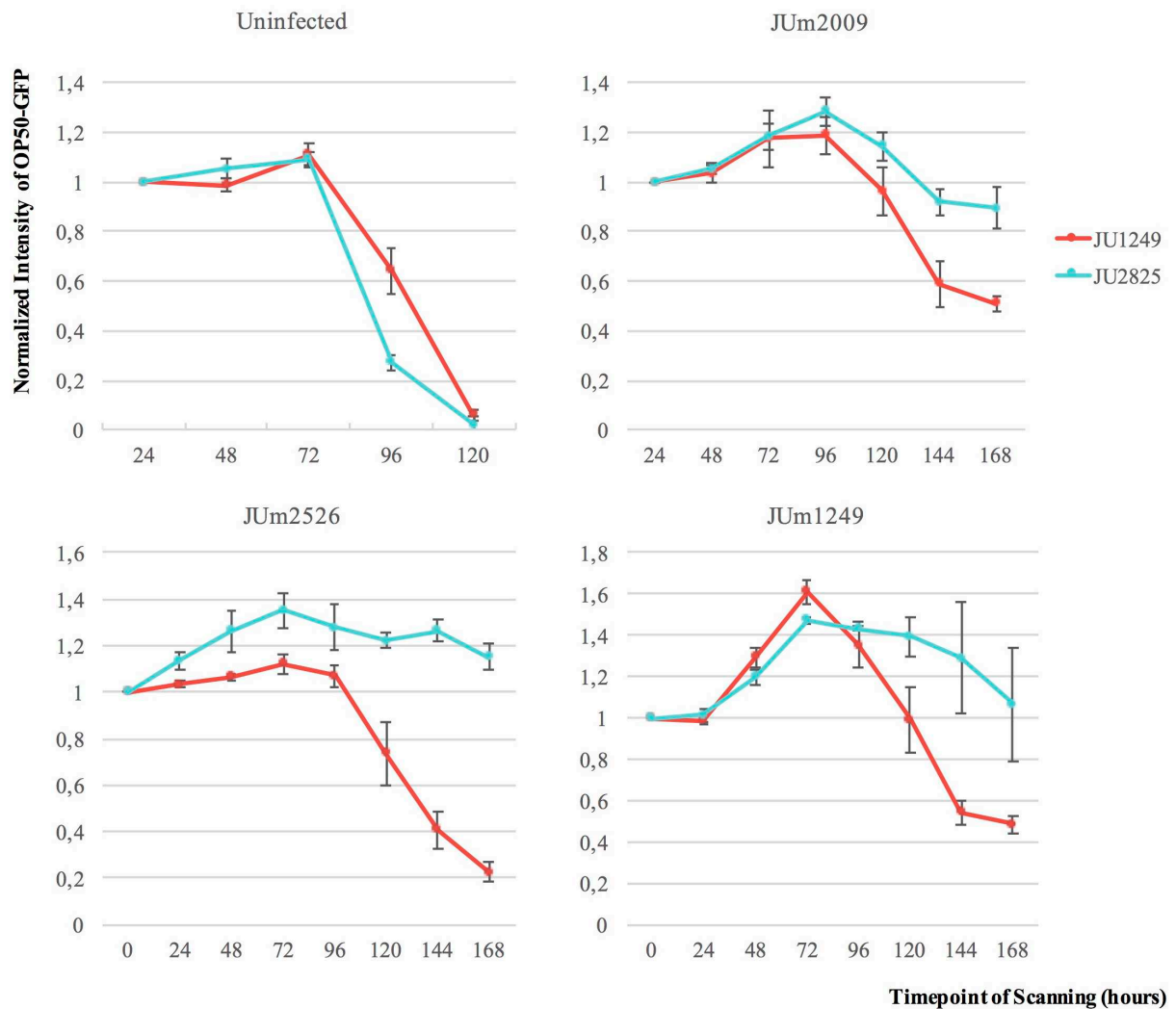


Figure 27. JU2825 is more sensitive than JU1249 to microsporidia infection.

Food consumption of *C. elegans* strains JU1249 and JU2825 was monitored over time in the presence and absence of microsporidia. Two *N. ausubeli* strains (JUm2009 and JUm2526) and one *N. parisii* strain (JU1249) were used in the infection. Data are mean values of three replicates, with bars represent standard errors.

To further understand why JU1249 consumed food faster than JU2825 with different microsporidia infections, we examined the infection rates and the pathogen loads, using a FISH probe targeting *N. parisii* and *N. ausubeli*. We performed this quantitative FISH on the populations used in the Food consumption test after the last scan at 168 hours. When infected with the *N. parisii* JUm1249, JU1249 displayed a significantly higher pathogen load than JU2825 (p-value is 4e-05). With either of the two *N. ausubeli* infections, no significant difference in the pathogen load was observed between JU1249 and JU2825. When they were

infected with any of the three microsporidia, no significant difference in the infection rate was detected between JU1249 and JU2825. These results suggest that JU1249 and JU2825 have different tolerance abilities. Indeed, JU1249 shows a higher food consumption rate than JU2825 with *N. ausubeli* infection, yet it did not show a lower infection rate or a lower pathogen load. Together, FISH tests indicate that a different tolerance of JU1249 and JU2825 affected their performances in the food consumption test.

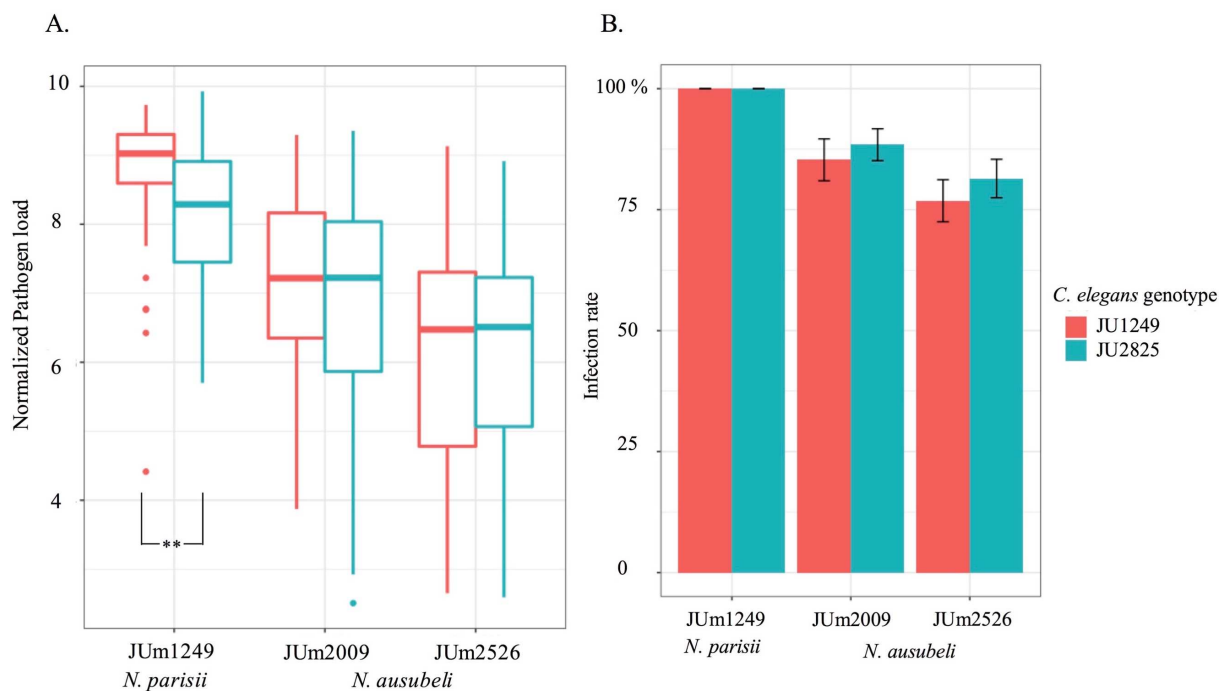


Figure 28. FISH analysis of the pathogen load and the infection rate of JU1249 and JU2825 upon three different microsporidia infections.

C. elegans strains JU1249 and JU2825 were infected with *N. parisii* JUm1249, *N. ausubeli* JUm2009 and *N. ausubeli* JUm2526, respectively. Data are mean values of three replicates, with the standard errors indicated as the error bars. In each replicate, 30 adult *C. elegans* were measured. **A.** Pathogen loads of JU1249 and JU2825 upon different microsporidia infections. A t-test was performed to test whether the pathogen loads of the two *C. elegans* strains significantly differed upon each microsporidia infection. Only upon *N. parisii* JUm1249 infection, JU1249 displayed a significantly higher pathogen load than JU2825 (p-value is $4e-05$, indicated by “***”). No other significant difference between JU1249 and JU2825 was found. **B.** Infection rate of JU1249 and JU2825 upon different microsporidia infections. A Wilcoxon Rank Sum test was performed to test whether the infection rates of the two *C. elegans* strains significantly differed upon each microsporidia infection. No significant difference between JU1249 and JU2825 was found.

II.3.3. JU1249 outcompeted JU2825 when they were infected with microsporidia in laboratory competition assays

Competition assays examine the relative fitness between different co-occurring strains. We performed competition assays between JU1249 and JU2825 populations in the presence and absence of microsporidia infection, to compare their sensitivity to microsporidia infection, and to examine the possible evolutionary consequences of this trait in *C. elegans* and for QTL mapping eventually. In the assay, we bleached and synchronized JU1249 and JU2825, then we mixed the two strains in equal number of individual L4s to seed the populations. *C. elegans* populations with *N. ausubeli* infection, *N. parisii* infection and no infection were assessed, each with five replicate cultures. The populations were maintained by being transferred to fresh plates before starvation. After each transfer, the proportions of JU1249 and JU2825 in the old plates were measured using quantitative pyrosequencing.

Starting from 50% for both strains, in the presence of either microsporidia infection, the resistant strain JU1249 outcompeted the sensitive strain JU2825 (Figure 29). *N. ausubeli* JUm2009 infection had a stronger selective effect on *C. elegans* population than *N. parisii* JUm2816 infection did. Populations infected with JUm2009 reached to fixation of JU1249 at the sixth transfer, while the populations infected with JUm2816 reached to fixation of JU1249 at the eighth transfer. On the other hand, JU2825 outcompeted JU1249 in the uninfected condition. These results confirm that the *C. elegans* strain JU2825 is more sensitive to microsporidia infection than JU1249. The sensitivity variation among different *C. elegans* strains to microsporidia infection may alter the outcomes of intraspecific competition in the wild.

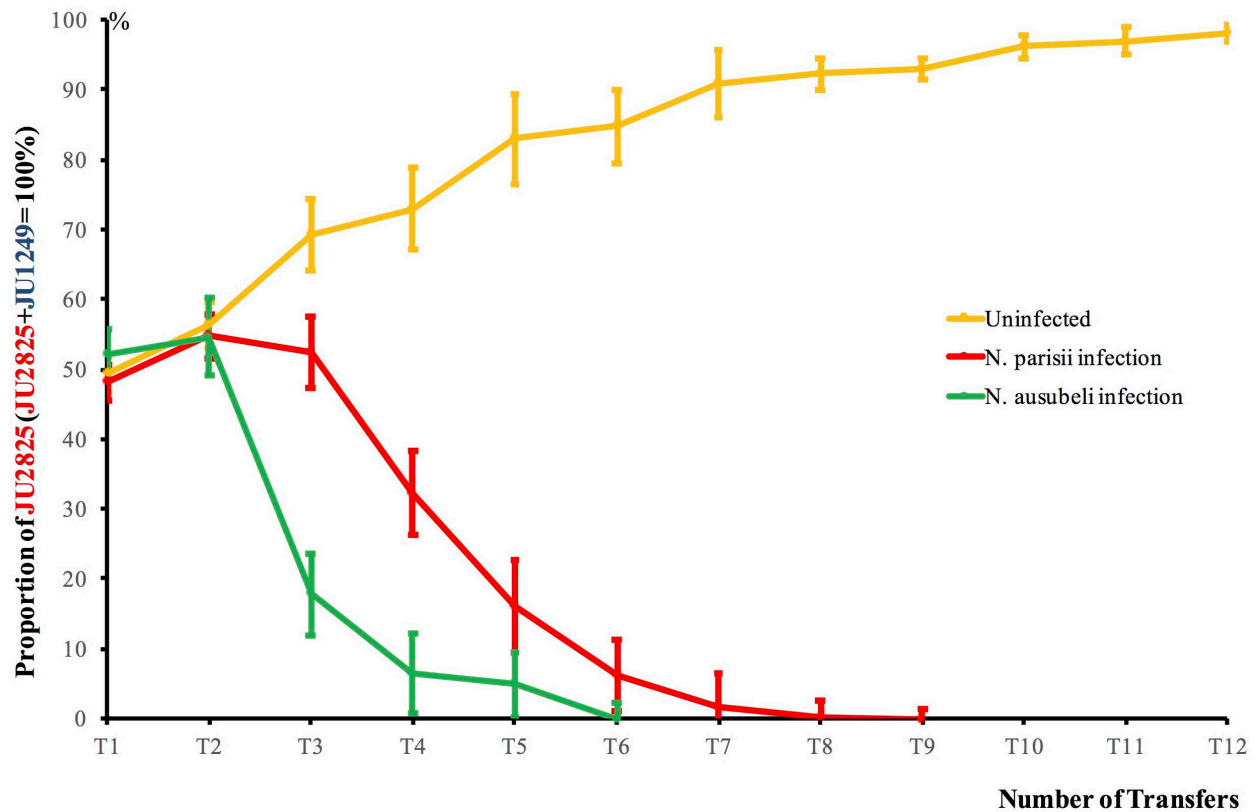


Figure 29. Influence of microsporidian infection in the experimental competition between *C. elegans* JU1249 and JU2825.

The proportion of JU2825 and JU1249 is inferred from the proportion by pyrosequencing of their respective private SNP. The mean proportions of five replicates of each treatment at each transfer were indicated. Error bars are standard errors over replicates. The experiment was continued until all replicates in one treatment reached fixation of either JU1249 or JU2825. In the infected populations, JU1249 outcompeted JU2825, whereas in the absence of microsporidia, JU2825 outcompeted JU1249.

II.4. Conclusion

Overall, we find natural diversity for *C. elegans* sensitivity to *N. ausubeli* or *N. parisii* infections. The *C. elegans* strains JU1249 and JU2825 can be further used to start a QTL analysis on the genetic bases of *C. elegans* sensitivity variation to microsporidian infection.

III.

The effect of microsporidia infection on *Caenorhabditis elegans* with the Mortal germline phenotype

Among the ten *C. elegans* strains used in the food consumption assay, JU2526 showed an intriguing result: the infected JU2526 population consumed food faster than the uninfected JU2526 population. We examined JU2526 and found that it displays a mortal germline phenotype (Mrt). Further studies indicated that microsporidia infection could suppress the Mrt phenotype of some *C. elegans* strains, i.e. extend the number of generations before reaching complete sterility.

Here, I describe my preliminary studies on this serendipitous observation.

III.1. Background

The germ line is an immortal cell lineage, which is passed down indefinitely from generation to generation. In *C. elegans*, the germ line can be passed over generations by self-fertilization. The mortal germline phenotype (Mrt) corresponds to a progressive decline in fertility of the hermaphrodite from one generation to the next, until full sterility. Several *C. elegans* mutants (N2 genetic background), such as *mrt-2*, *hrde-1* and *prg-1*, have been described as Mrt, some of them showing a temperature-dependent phenotype (Ahmed and Hodgkin, 2000; Buckley et al. 2012; Simon et al. 2014). In our lab, we also noticed that several wild *C. elegans* strains exhibited the temperature-sensitive progressive sterility. At 23 °C or 25 °C, some strains with a strong Mrt phenotype become sterile after three to four generations. The molecular mechanism of the progressive sterility among wild *C. elegans* strains remains unknown and is being investigated by Lise Frézal in the lab.

When we investigated the sensitivity variation of different *C. elegans* strains to microsporidia infection, we first compared the food consumption rates of different *C. elegans* strains in the presence and absence of microsporidia infection. All infected strains consumed food slower than when they were uninfected, except JU2526 (Figure 30). Surprisingly, in this food consumption experiment, the uninfected JU2526 populations were only composed of few individuals, whereas the infected JU2526 had a much larger population size than the uninfected JU2526 population. Further tests showed that JU2526 has a Mrt phenotype at 23 °C, the temperature at which we did our food consumption test. As all JU2526 individuals tested in food consumption tests were from the same original population, the larger size of the infected population suggested that microsporidia infection may be able to prevent or attenuate the progressive decrease of population growth in JU2526 when cultured at 23°C.

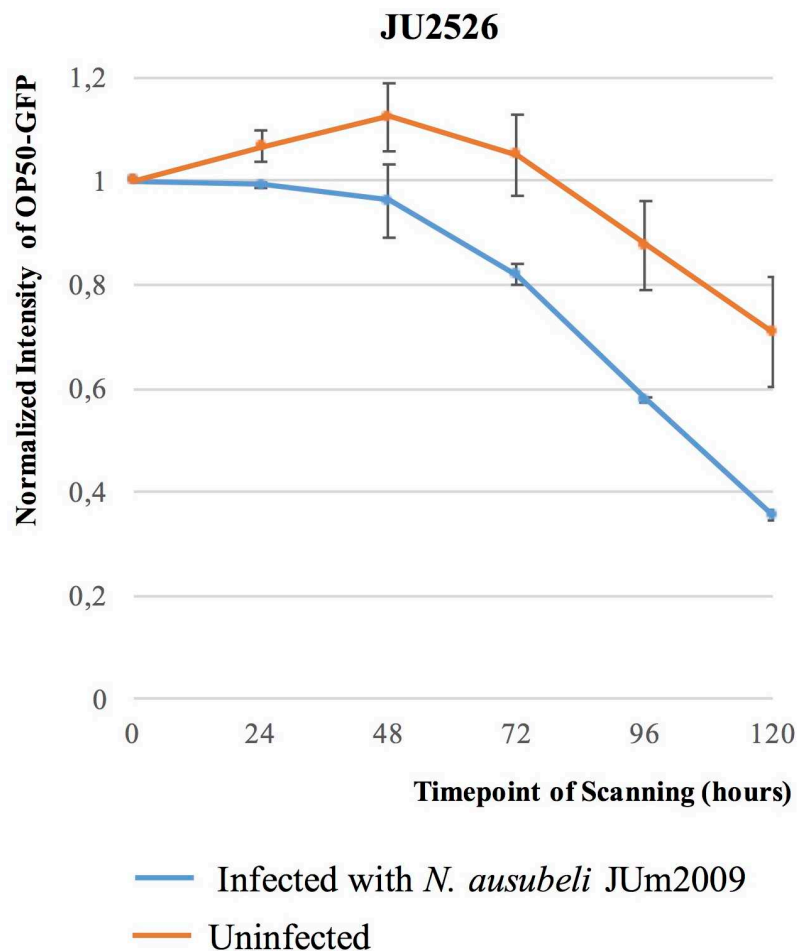


Figure 30. Food consumption of JU2526 with or without microsporidia infection.

This experiment was performed in parallel to those in Figure 25.

We further investigated the microsporidia effect on four *C. elegans* strains with the Mrt phenotype. These strains were tested for the number of generations before becoming completely sterile, in the presence and absence of microsporidia infection. The results demonstrate that both *N. parisii* and *N. aeusubeli* infections could increase the number of generations of JU2526 and JU775 at high temperature.

III.2. Materials and Methods

III.2.1. *C. elegans* and Microsporidia strains

The *C. elegans* strains JU2526, MY10, QX1211 and JU775 were used in this study. All of these four strains have been shown to display the Mrt phenotype. As the Mrt phenotype is temperature sensitive, these four strains were bleached and maintained at 15 °C. The *N. parisii* strain JUm2816 and the *N. ausubeli* strain JUm2009 were used in this study. Spores were prepared and quantified as described in **RESULTS I**.

III.2.2. Mortal germline assay

20 uninfected young adults (i.e. prior to first egg formation) were transferred to a 6 cm NGM plate seeded with *E. coli* OP50. 5 million microsporidian spores in 100 µl distilled water were placed onto the *E. coli* lawn. The cultures were then incubated at 15 °C. The infection symptoms were checked by Nomarski optics at 72 hours after inoculation.

10 L4 larvae from the infected population were transferred to a fresh plate and moved to 23 °C. Uninfected individual L4s were also tested in parallel. The generation moved from 15 °C to 23 °C was called the first generation. Ten L4 animals were transferred onto fresh plates at each generation (2-3 days). The transfer was continued until the sterile generation. i.e. when 10 L4 progenies were not available on the plate. The number of generations before becoming sterile was scored.

Three experiments were performed, each with three treatments: *N. parisii* JUm2816 infection, *N. ausubeli* JUm2009 infection and uninfected control. In the first experiment, we tested *C. elegans* strains JU2526 and MY10, both with three replicates; in the second experiment, we tested JU2526 again with five replicates; in the third experiment, we tested *C. elegans* strains JU775 and QX1211, both with five replicates.

The statistical analysis and the figures in Figure 25 were obtained using Rstudio, as described in **Results II**. To test whether the number of generations before becoming sterile of each strain with different treatments significantly differed, we used a Wilcoxon Rank Sum test on the generation numbers of different treatments within each strain.

III.3. Results

We tested four *C. elegans* strains JU2526, MY10, QX1211 and JU775. MY10, JU775 and QX1211 have been shown to have an extremely strong Mrt phenotype at 25 °C (Marie-Anne Félix's data, not shown). In the experimental framework used in my study, at 23 °C, MY10 and QX1211 become sterile in 3 to 4 generations. JU775 and JU2526 exhibit a milder Mrt

phenotype. In average, JU775 becomes sterile in about 7 generations, and JU2526 in about 9 generations (Figure 31).

With either *N. parisii* JUm2816 infection or *N. ausubeli* JUm2009 infection, JU2526 had a significant increase (p-value is 0.01 for both) in the number of generations before the sterility (Figure 31B). Compared to those with *N. parisii* JUm2816 infection, JU2526 with *N. ausubeli* JUm2009 infection also showed a significant increase (p-value is 0.04) in the generation number. With either microsporidia infection, JU775 had a significant increase (p-value is 0.01 for both) in the number of generations before the sterility (Figure 31C). Microsporidia infections did not seem to rescue the progressive loss of fertility of MY10 or QX1211. These results indicated that microsporidia infections are able to rescue fertility in *C. elegans* strains with a moderate Mrt phenotype.

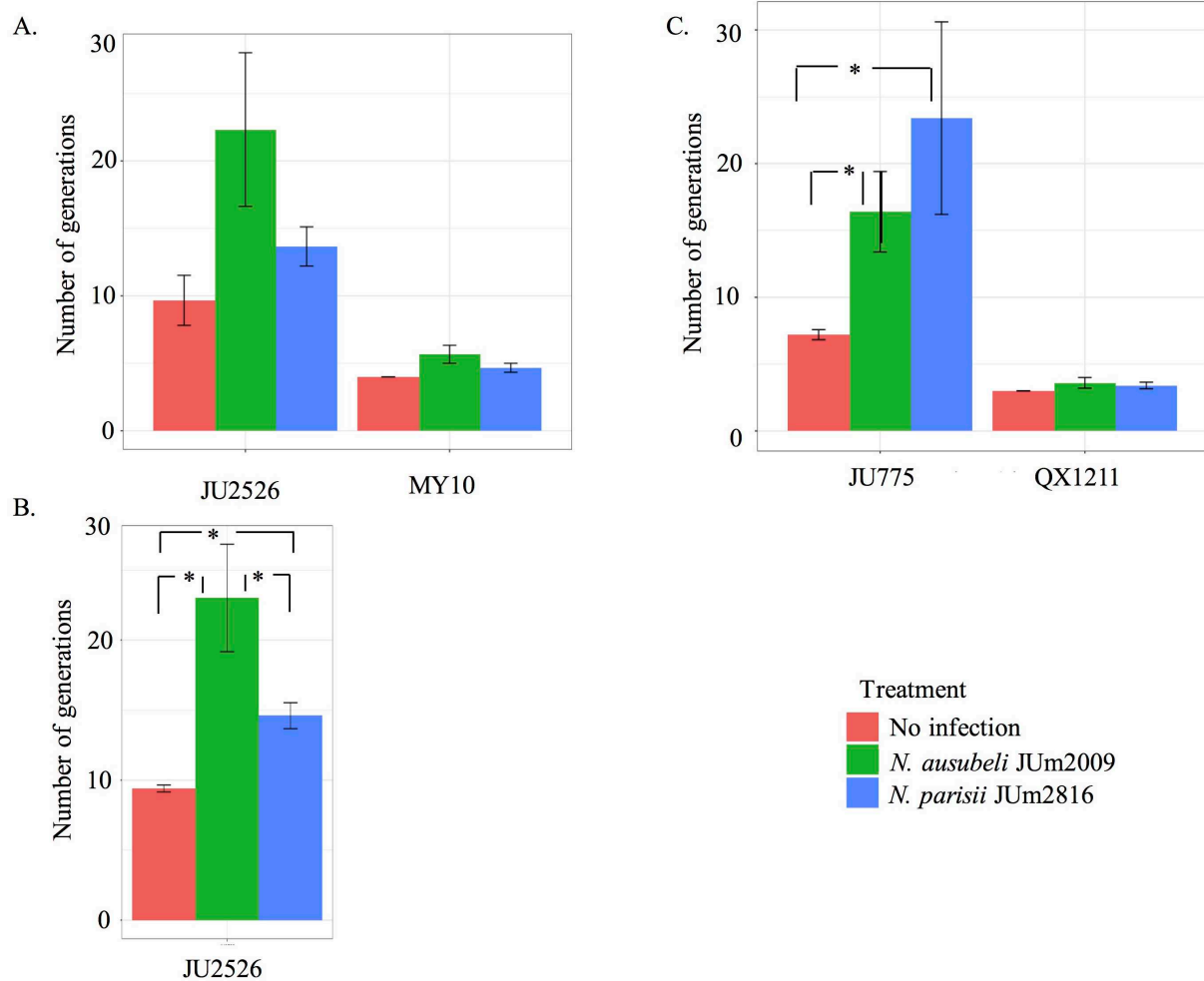


Figure 31. The rescue effect of microsporidian infection on the Mrt phenotype in *C. elegans*.

Data are mean values and standard errors. In each treatment, **A.** Three replicates for JU2526 and for MY10; **B.** Five replicates for JU2526; **C.** Five replicates for JU775 and QX1211. A Wilcoxon Rank Sum test was performed to test whether the number of generations before becoming sterile of each strain with different treatments significantly differed. All the statistical differences were shown, with * for p-value < 0.05. In A, the differences in generations of JU2526 with or without infection are not significant, which may be due to the limited number of replicates.

Discussion

As we have already discussed the results in the paper of **RESULT I**, here I will include again the key points in the discussion of the paper and make further comments on our other results, related studies and some recently reported discoveries.

I. Diverse interactions between rhabditids and microsporidia

I.1. The diversity of microsporidia infections in rhabditids

A great diversity of interactions between microsporidia and rhabditid nematodes have been discovered. In addition to *N. parisii* and *N. ausubeli*, we found five new *Nematocida* species, two *Enteropsectra* species and two *Pancytopora* species; another two new *Nematocida* species, *Nematocida displodere* and *Nematocida ironsii*, were proposed very recently (Troemel et al., 2008, Luallen et al., 2016; Reinke et al., 2017). These 13 microsporidia were isolated from 48 rhabditid nematode strains of six *Caenorhabditis* species, two *Oscheius* species, *Rhabditella typhae* and *Procephalobus* sp. (Troemel et al., 2008, Luallen et al., 2016; Reinke et al., 2017). In addition, two undefined microsporidia species in the genus *Enteropsectra* and some environmental SSU rDNA microsporidia sequences may represent further species in the genera *Nematocida* and *Pancytopora* (paper Figure S2, S4) (Ardila-Garcia et al., 2013). Altogether, these discoveries demonstrate the great diversity of microsporidia infections in wild rhabditid nematodes.

I.2. How were these microsporidia species described?

As it is difficult to do mating test among microsporidia, these nematode-infecting microsporidia species were mainly defined by molecular methods. *N. parisii* was defined to be a new species in a new genus of microsporidia because its SSU rDNA sequence was found to be fairly divergent but most closely related to the SSU rDNA of microsporidia *Ovavesicula popilliae*. *N. ausubeli* was defined to be an independent *Nematocida* species because its SSU rDNA is approximately 95% identical to that of *N. parisii*, which is its most closely related species in the phylogenetic analysis (Troemel et al., 2008).

In our study, the new species *N. major*, *N. minor*, *N. homosporus* and *N. ciargi* were characterized by SSU rDNA and β -tubulin. These four *Nematocida* species all had *N. parisii* or *N. ausubeli* as their top blast hit in GenBank, with similarity between 81%-86% of SSU rDNA and 74%-84% of β -tubulin genes. They formed a single clade with *N. parisii* and *N. ausubeli*, with *O. popilliae* as sister group within Clade II of the microsporidia SSU rDNA phylogeny. The inter-specific genetic distance of SSU rDNA among them is at least 0.06, which is greater than the distance between *N. parisii* and *N. ausubeli*. But their intra-specific distances are all 0.00. Therefore, these four *Nematocida* were defined as new *Nematocida* species. *N. sp. 7* was defined as a new species based on comparison and the phylogeny of β -tubulin genes with other species.

E. longa, *E. breve*, *P. philotis* and *P. epiphaga* were described as new species because their SSU rDNA were closest (88-89% identity) to that of *Orthosomella operophtherae*, which is in the Clade IV of the microsporidia phylogeny. The β -tubulin sequences of the *Enteropsectra* species were closest (75-76% identity) to *Vittaforma corneae*, which is a close relative to *O. operophtherae*. *E. longa*, *E. breve*, *P. philotis* and *P. epiphaga* were placed in two close groups, with *O. operophtherae* as outgroup in the Clade IV of the microsporidia SSU rDNA phylogeny. Among the four strains in the *Enteropsectra* group, we defined *E. longa* (JUm408) and *E. breve* (JUm2551) as two species, with further considerations on their spore morphology and host specificity. We did not define *Enteropsectra* JUm1456 and *Enteropsectra* JUm1483 to a species due to their different placements in the SSU rDNA phylogeny and the β -tubulin phylogeny. The other group, the *Pancytospora* group contains five strains, which were defined as two species based on SSU rDNA, host and tissue specificity.

N. displodere and *N. ironsii* were defined as new *Nematocida* species based on whole genome sequencing and phylogenomic comparison. *N. displodere* was found to be a sister group to *N. parisii* and *N. ausubeli* in their phylogenomic tree. However, *N. parisii* and *N. ausubeli* proteins showed an average amino acid identity of 66% compared to each other, and 48.6% and 48.3% compared to *N. displodere* proteins, respectively. Thus, *N. displodere* was defined as a new *Nematocida* species (Luallen et al., 2016).

N. ironsii was recently separated from *N. parisii* and proposed as an independent species. The type strain of *N. ironsii* is ERTm5, which was previously identified to be a *N. parisii* strain based on rRNA sequence (Balla et al., 2015; Reinke et al., 2017). The genome of ERTm5 was sequenced recently and compared with *N. parisii* ERTm1 and ERTm3. The average genome nucleotide identity between *N. parisii* ERTm1 and ERTm5 is 92.3%, whereas the ERTm1 and ERTm3 strains share 99.8% identical genomes (Cuomo et al., 2012; Reinke et al., 2017). Based on a 95% average genome nucleotide identity standard, ERTm5 was considered as an independent species, named *Nematocida ironsii* (Konstantinidis et al., 2006; Reinke et al., 2017). In genomic phylogeny, *N. ironsii* ERTm5 is the closest species to the clade of *N. parisii* ERTm1 and ERTm3 (Figure 32). Compared to molecular analysis with a few gene regions, genomic comparisons among strains and species may provide a better and more accurate method to distinguish species and study the relationships among species. However, note that the thresholds are artificially set in both cases.

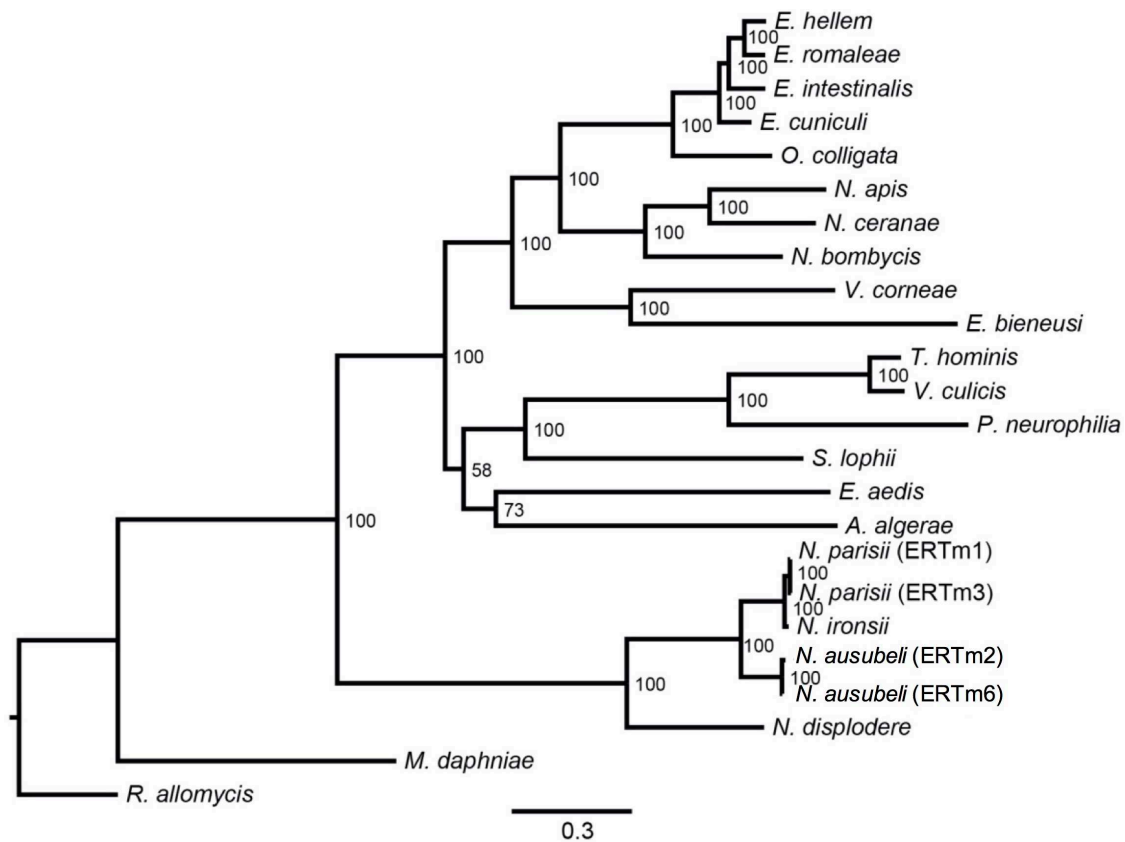


Figure 32. Phylogenomic tree of 21 microsporidia species.

The phylogenomic tree includes six strains of four *Nematocida* species and 17 other microsporidia species. *N. displodere* is in the basal position of the *Nematocida* genus. *N. ironsii* is the closet species to *N. parisii*. Adapted from Reinke et al. (2017).

Interestingly, our phylogenetic analysis based on the β -tubulin gene also showed the same groupings of ERTm1, ERTm3 versus ERTm5, as their genomic phylogeny displayed (Figures 32, 33B). In the β -tubulin phylogeny, the *N. parisii* clade includes two subclades: one subclade includes ERTm1, ERTm3 and six other strains, while the other subclade includes ERTm5 and five other strains (Figure 33B). Thus, the other strains in the same subclade as ERTm5 may also belong to *N. ironsii*. It will be necessary to further perform genomic and phylogenomic analyses on more strains to understand better the relationships and diversity among different “*N. parisii*” strains, as well as among *N. ausubeli* and other nematode-infecting microsporidia.

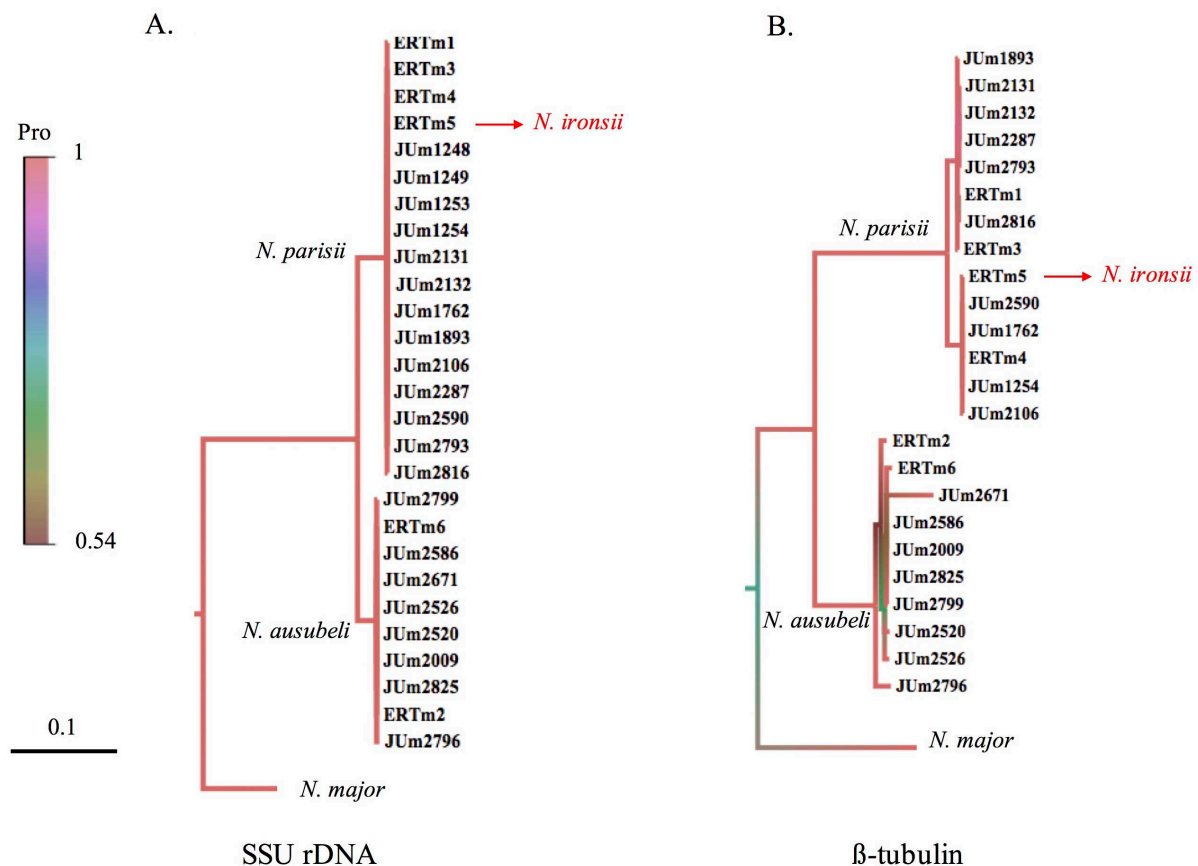


Figure 33. Phylogenies of different *N. parisii*, *N. ausubeli* and *N. ironsii* strains. A. SSU rDNA phylogeny. B. β -tubulin phylogeny. The phylogeny with the β -tubulin gene shows further relationships among different *N. parisii* strains, as well as among different *N.*

ausubeli strains. However, the phylogeny of SSU rDNA does not show any of the “*parisii*” subclades in B. Note that the β -tubulin gene is more divergent than SSU rDNA, which may explain the better resolution of subclades in B. Branch colors show the posterior probability, with the corresponding color code shown on the left. Scale bar indicates expected changes per site.

I.3. Various tissue tropisms of rhabditid-infecting microsporidia

Only horizontal transmission has been observed in the known rhabditid-infecting microsporidia infections, likely via the oral route. Different nematode tissues are infected by the various microsporidia. In the 13 known rhabditid-infecting microsporidia, 11 of them infect the intestinal cells of their nematode hosts, whereas the other two, *N. displodere* and *P. epiphaga*, showed infections in other tissues. *N. displodere* infection has been found in the epidermis, muscles, coelomocytes and neurons of *C. elegans*. *P. epiphaga* infection has been found in the epidermis, muscles and coelomocytes of *C. brenneri* and at least the epidermis of *C. elegans*. The various infection niches show that rhabditid-infecting microsporidia have evolved to use different host tissues to maximize their growth.

N. displodere seemed to have some other special features that are related with its diverse tissue tropisms. For example, with a longer polar tube than that of *N. parisii* (three-fold longer than similar sized spores of the two species), *N. displodere* was suggested to be able to inject the sporoplasms directly into host epidermis and muscles from the intestinal lumen. Unlike the spores of *N. parisii* that are continuously shed through the host defecation process, *N. displodere* spores were observed to accumulate inside the host, mainly in epidermis and muscles, until they are released into the environment through bursting of the host vulva (Luallen et al., 2016). The entry and release mechanisms of *P. epiphaga* remain unknown yet. It would be interesting to further study the infection mechanisms of *N. displodere* and *P. epiphaga*, and compare them with each other, as well as with the intestine-infecting microsporidia.

I.4. Spread of microsporidia within the infected tissue

A cell-to-cell spread mechanism of *Nematocida* was reported recently (Balla et al., 2016). Previously, it was shown that only infected *C. elegans* with *N. parisii* spores inside their intestinal cells are infectious, whereas those with only meronts are not infectious to other *C. elegans* individuals (Troemel et al., 2008). However, within one worm, a single inoculated intestinal cell that only contains *N. parisii* meronts is found to be contagious to its neighboring intestinal cells (Balla et al., 2016). This cell-to-cell transmission of *N. parisii* could proceed throughout up to half of all the intestinal cells without any differentiation of spores, and lead to deformation of cell boundaries and syncytia formation in the worm's intestine. Similar to *N. parisii*, *N. ausubeli* is able to spread across several intestinal cells before forming spores. *N. displodere* could spread across different host muscle and hypodermal cells, and even from the main hypodermal syncytium into the anterior epidermal cells. This cell-to-cell spread mechanism was thus suggested to be a conserved mechanism in the genus *Nematocida* (Balla et al., 2016).

To better determine whether this cell-to-cell spread mechanism is conserved in the genus *Nematocida*, it is necessary to further examine some or all of the five new *Nematocida* species we have found. It would be also interesting to examine if similar mechanisms exist in *Enteropsectra* and *Pancytospora* species, using the single cell inoculation method in Balla et al. (2016).

I.5. Microsporidia exit mechanism from host intestinal cells

To exit from the intestinal cells of *C. elegans*, *N. parisii* first rearranges the host actin (ACT-5) to induce gaps in the terminal web of the host intestinal cells (mentioned in the **Introduction III.1.2.**) (Troemel et al., 2008; Estes et al., 2011). Then the spore-containing compartment (SCC) will be marked with RAB-11, and then traffic to and fuse with the apical membrane of the intestinal cells. After the fusion, spores gain access to the intestinal lumen and are marked by PGP-1 (Figure 22A, Figure 34, left panel (a)) (Szumowski et al., 2014). A very recent study showed an alternative mechanism that spores may have ACT-5 coats in addition to the PGP-1 marker, when they gain the access to the lumen (Figure 34, left panel (b)) (Szumowski et al., 2016). The ACT-5 coat is not necessary for spore exit, but it may promote spore exocytosis. Moreover, later during infection, spore-filled vesicles coated with

CHC-1 (clathrin heavy chain), PGP-1 and ACT-5 were noticed, which was suggested to act as a compensatory endocytosis mechanism for the host cell, due to the massive efflux of intracellular membrane material (Figure 34, right panel) (Szumowski et al., 2016).

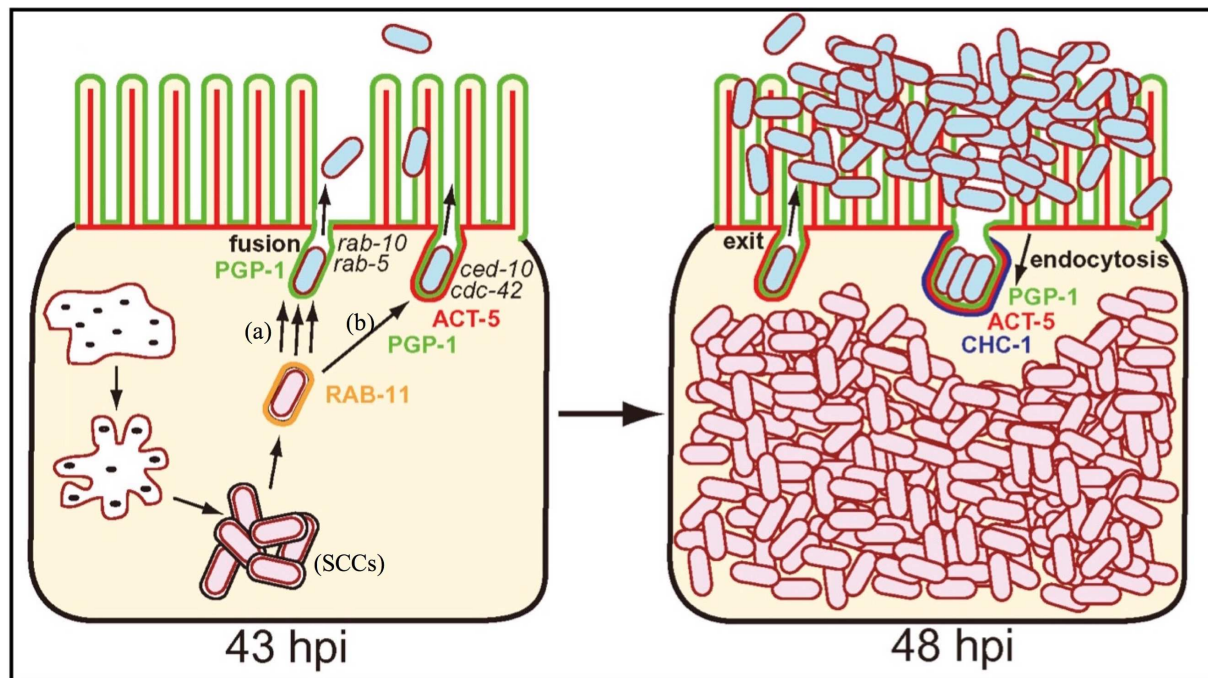


Figure 34. Diagram of *N. parisii* exiting strategy from *C. elegans* intestinal cells.

The model for actin regulation of *N. parisii* spore exocytosis and compensatory endocytosis in *C. elegans* intestinal cells. Left panel: At 43 hpi, the RAB-11 labeled SCCs transfer to and fuse with the apical membrane of the intestinal cells, and acquire (a) a PGP-1 membrane marker or (b) both PGP-1 and ACT-5 markers. Formation of ACT-5 coats is dependent on the Rho GTPases *ced-10* and *cdc-42*, as well as *rab-5* and *rab-11*. Right panel: At 48 hpi, spores has overtaken the host cell, with a lot of exited spores. Endocytic spore-filled vesicles were coated with CHC-1 (clathrin heavy chain), PGP-1 and ACT-5. Actin in the microvilli and the terminal web is represented in bright red; the apical plasma membrane with PGP-1 is in green. Adapted from Szumowski et al. (2016).

In our study, transmission electronic microscopy indicated different exit mechanisms between *Nematocida* and *Enteropsectra* species. *N. ausubeli* probably has a similar exit mode as *N. parisii*: some isolated *N. ausubeli* spores possessed an additional membrane, which likely formed the SCC, and exited from host cells through exocytosis; no additional membranes have been observed around *N. ausubeli* spores in the intestinal lumen (*paper* Figures 5H, K, L, 6A). However, *E. longa* showed a distinct cellular exit mode: no SCCs have been observed

in the *E. longa* infection, precluding exocytosis as an exit mode; *E. longa* spores were seen to protrude on the apical side of the host cell and push out the host cell membrane; some *E. longa* spores appeared to be surrounded with an additional membrane in the intestinal lumen. The exit mode of *E. longa* spores is likely associated to the fact that *E. longa* tends to form its spores first along the apical side of host epithelial cells (*paper* Figures 7A, E, 8L). This polarization may facilitate the exit of *E. longa*, as *E. longa* spores do not need to traffic from other places in the cell to the apical membrane of the intestinal cells, which is probably required for most *N. parisii* and *N. ausubeli* spores. Furthermore, it is likely that because *E. longa* does not exit in the form of SCCs through exocytosis, the host cells will not lose much intracellular membrane material. Thus no compensatory endocytosis mechanism that occurs in *N. parisii* infection is needed for hosts with *E. longa* infection, one proof of which is that no spore-containing vesicles have been observed in the *E. longa* infection. It would be interesting to study the molecular mechanism of the spore exit in *Enteropsectra* species, as well as other new rhabditid-infecting microsporidia.

I.6. The host specificity of various microsporidia

The different microsporidia displayed a range of host specificity. In the genus *Nematocida*, *N. parisii* and *N. major* are relatively specialists as they infect *Caenorhabditis*, but not *Oscheius*. *N. ausubeli*, naturally found in *C. elegans* and *C. briggsae*, is also able to infect *Oscheius* sp. 3. However, *N. ausubeli* does not infect *O. tipulae*, unless at very high dose of spores, which indicates that *O. tipulae* is far less susceptible than *Oscheius* sp. 3 and the two *Caenorhabditis* to *N. ausubeli* infection. *N. homosporus* is a generalist that infects *R. typhae*, two *Caenorhabditis* and two *Oscheius*.

Among the four nematode-infecting microsporidia in Clade IV, except for *P. epiphaga*, the other three were all isolated from *Oscheius* species. *E. breve* is able to infect two *Oscheius* species but not *Caenorhabditis*. *E. longa* and *P. philotis* display an even narrower host specificity: *E. longa* only infects *Oscheius* sp. 3 while *P. philotis* only infects *O. tipulae*.

In the future, it would be interesting to study the specificity of *N. minor*, *N. ciargi* and the two microsporidia with broader tropisms. In the genus *Nematocida*, *N. minor* (both strains) and *N. homosporus* (one of the two strains) were naturally found in *O. tipulae*. Different from *N.*

homosporus, *N. minor* has two sizes of spores, which is a feature shared by *N. parisii*, *N. ausubeli* and *N. major*. Our infection studies showed that when *O. tipulae* or *O. sp. 3* were infected with *N. ausubeli*, which was not known as their natural pathogen, two sizes of spores were observed. It would be interesting to examine the appearance of *N. minor* spores in a *Caenorhabditis* host, if the infection can be established.

N. ciargi, which was placed on the basal position in the phylogeny (based on SSU rDNA and β -tubulin, *paper* Figure 3) of *Nematocida*, was isolated from a *Procephalobus* strain. *Procephalobus* sp. is an outgroup nematode, compared to the other nematode host species described here. The study on host specificity of *N. ciargi* may provide insights on how the host specificity of different *Nematocida* species evolves.

The two nematode-infecting microsporidia with a non-intestinal tropism, *N. displodere* and *P. epiphaga*, were isolated from *C. elegans* and *C. brenneri*, respectively. *P. epiphaga* could also infect *C. elegans* and *C. briggsae*. It is of interest to study the specificity of the two species, for example, to see whether the microsporidia with broader tropisms also have broader host spectra.

On the other hand, many other rhabditid nematode species have not been found with microsporidia infection in the wild or tested for their susceptibility to the known microsporidia in the laboratory. For example, in *Caenorhabditis*, only five species of the *Elegans* group and *C. sp. 42* of the *Drosophilae* group were involved here. It would be interesting to test microsporidia infection in other *Caenorhabditis* species. Furthermore, it would be also interesting to test the susceptibility of non-rhabditid nematodes to microsporidia infections. For example, the diplogastrids *Pristionchus* spp., potential predators of *C. elegans* and other rhabditids, may also be susceptible to some of the known rhabditid-infecting microsporidia.

I.7. Diversification of the nematode-infecting microsporidia

Diversification of rhabditid-infecting microsporidia may take place in three routes. Firstly, diversification and speciation of hosts and pathogens may occur in parallel. The

correspondent positions of *Nematocida* and host nematodes (*Caenorhabditis* and *Oscheius*) on their phylogenies indicates that microsporidia may coevolve with nematodes, and speciation of microsporidia may occur after the divergence of rhabditid species (*paper* Figure 3). The genetically close *Enteropsectra* species and their narrow specificities to *Oscheius* hosts may be also due to the fact that speciation of microsporidia occurred after the divergence of *O. tipulae* and *Oscheius* sp. 3.

Secondly, as rhabditid nematodes share their natural habitats and interact with diverse animals, such as arthropods, mollusks and other nematodes, it is possible that host switch take place among them. For example, the millipede *Diploiuulus londinensis caeruleocinctus* has been found to be infected with *Nosema juli*, the genus of which is in the Clade IV of the microsporidia phylum (Wilson 1971). Snails have been found to be infected with microsporidia, some of which are in the Clade IV of the microsporidia phylum (Cunningham and Daszak, 1998; McClumont et al., 2005). It is possible that microsporidia can switch hosts from millipedes or snails to rhabditids. Another possibility is that microsporidia may shift host among different rhabditids, as pathogens might have a higher chance to establish infection in a new host that is closely related to the original host. After the host shifts, pathogen speciation and host specificity may or may not take place to form new microsporidia with narrow ranges of hosts, or with broad ranges of hosts.

Thirdly, both the first and the second diversification routes may have taken place simultaneously to form the diversity of rhabditid-infecting microsporidia.

The diverse natural nematode-microsporidia pairs, especially those with *C. elegans*, provide many opportunities for studies on the evolution of host shifts and host specificity of pathogens.

I.8. An excellent system to study host-pathogen interactions and coevolution

N. parisii was the first known natural intracellular pathogen of *C. elegans*. *C. elegans* is a successful model organism, with several special features and numerous developed tools. Since the discovery of *N. parisii*, exciting progresses using *N. parisii* and *C. elegans* have

been made on many aspects of host-microsporidia interactions. The simple and transparent *C. elegans* provides a whole-animal host to study microsporidia pathogenesis and transmission *in vivo*, such as how *N. parisii* reconstructs the host intestinal cytoskeleton and takes use of the host cell trafficking pathways to exit the host cells; how *N. parisii* maximizes its growth in the host with the cell-to-cell transmission strategy. On the other hand, host responses and immunity of *C. elegans* have also been further revealed, such as the special clearance ability of L1 larvae of CB4856 to the *N. ironsii* ERTm5 infection.

Our studies further determined that microsporidia are common pathogens to wild rhabditid nematodes, especially *C. elegans*, *C. briggsae* and *O. tipulae*, which indicated that nematodes may have coevolved with microsporidia. It would be interesting to study the counter-adaptation and coevolution between nematodes and microsporidia. Furthermore, as some rhabditids, such as *C. elegans*, *C. briggsae* and *O. tipulae*, are potential competitors in their natural habitats, it would be interesting to study how microsporidia shape the competitions among them. For example, in our collection, the *C. elegans* strains JU1247-9, JU1253 the *C. briggsae* strain JU1254 were isolated from the same apple collected in Santeuil, France. All these five strains were naturally infected with *N. parisii*. The sensitivity of these different *C. elegans* and *C. briggsae* strains to *N. parisii* infection may significantly affect their competitions for food and the expansion of their populations.

The sequenced microsporidia genomes could highly facilitate studies on host-pathogen coevolution. To date, the genome of six *Nematocida* strains has been sequenced: the *N. parisii* ERTm1 and ERTm3, the *N. ausubeli* ERTm2 and ERTm6, the *N. displodere* JUm2807 and the *N. ironsii* ERTm5 (Cuomo et al., 2012; Bakowski et al., 2014c; Luallen et al., 2016; Reinke et al., 2017). Based on the analysis of these genomes, seven *Nematocida* large gene families (NemLGF1-7) were defined. From these *Nematocida* large gene families, 82 host-exposed proteins were identified, which may function in manipulations of host metabolism and defenses. These host-exposed proteins were further found to evolve rapidly, which suggested a common strategy for microsporidia to interact and adapt to the nematode hosts (Reinke et al., 2017). For further studies, it would be necessary to sequence the genomes of all the new rhabditid-infecting microsporidia species, especially *Enteropsectra* and *Pancytospora* species.

II. Natural variation of *Caenorhabditis elegans* in sensitivity to microsporidian infection

II.1. Intraspecific variation of *C. elegans* sensitivity to microsporidia infection

The natural sensitivity variation of *C. elegans* to microsporidia infections has been studied in different sets of *C. elegans* strains to different microsporidia: 1), the sensitivity of six *C. elegans* strains (geographically diverse) to *N. ironsii* ERTm5 infection was investigated by Balla et al. (2015), with the comparison of host survival and pathogen loads; 2), in our study, nine *C. elegans* strains were compared for their sensitivity variations to a same *N. ausubeli* infection (**RESULT II**). All these nine tested strains were naturally found with *N. parisii* or *N. ausubeli* infection (Figure 24); 3), in a related study to **RESULT II**, Clément Dubois further compared nine *C. elegans* strains: JU1249 and JU2825 that were tested in **RESULT II**, the Hawaii strain CB4856 with an enhanced immunity in its L1 larvae (Balla et al. 2015), the other six strains representing genetic and/or geographic diversity. The sensitivity of these nine strains to the infections *N. ausubeli* JUm2009 and *N. parisii* JUm2816 were compared. The studies 2) and 3) used the OP50-GFP food consumption tests to compare the host sensitivity upon microsporidia infection. All the three studies found variation in *C. elegans* sensitivity to microsporidia infection (Figure 23A and B, 25, 35).

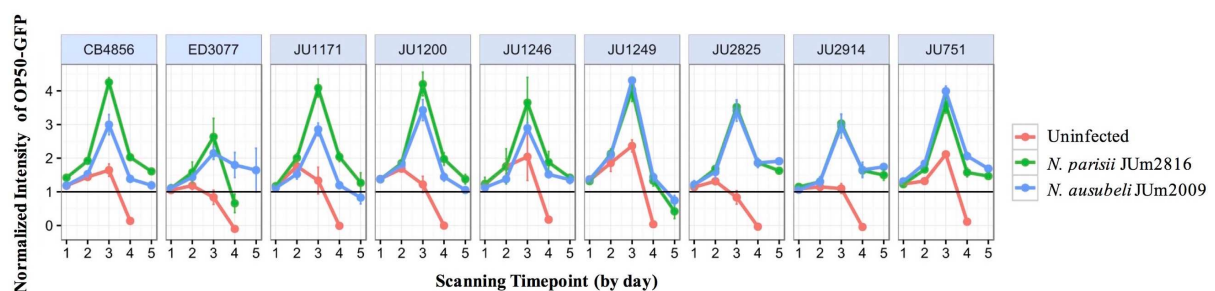


Figure 35. Variation in sensitivity against *N. parisii* and *N. ausubeli* among nine *C. elegans* wild isolates.

The Y-axis is the normalized intensity of the fluorescence of OP50-GFP. The X-axis is the scanning time point. Each plate was scanned every day until full depletion of bacteria on the plate. Data are mean values of three replicates, with bars represent standard errors. The analysis and the figure were performed by Clément Dubois.

II.2. Interesting pairs of *C. elegans* strains for further studies

The enhanced immune ability to control and clear *N. parisii* infection in L1 larvae of CB4856 has been studied with the reference strain N2, which lacks this special immunity (Balla et al. 2015). In our studies, we found that the strains JU1249 and JU2825 showed different sensitivity against both *N. parisii* and *N. ausingtoni* infections (Figure 27). *C. elegans* JU1249 is more resistant than JU2825 to *N. ausingtoni* JUm2009, JUm2526 and *N. parisii* JUm1249 infections. The different sensitivity of JU1249 and JU2825 was reconfirmed in the study by Dubois (Figure 35). Among the nine strains compared by Dubois, JU1249 is the most resistant strain, as the populations of JU1249 consumed the food the fastest upon either of the two microsporidia infections. JU2825, JU2914 and JU751 consumed the food slower compared to other *C. elegans* strains. These results demonstrated that JU1249 and JU2825 have contrasted phenotypes in the sensitivity to microsporidia. These two *C. elegans* can be further used in the quantitative trait locus (QTL) approach for detecting the genetic basis of this phenotype variation.

In the study by Dubois, another pair of *C. elegans* strains, CB4856 and ED3077, showed contrasted variation patterns in sensitivity to different microsporidia infection. With *N. parisii* JUm2816 infection, ED3077 consumed food faster than CB4856, whereas the variation pattern is reversed when the two strains were with *N. ausingtoni* JUm2009 infection (Figure 35, 36). ED3077 seems to be more resistant to *N. parisii* JUm2816 than to *N. ausingtoni* JUm2009, whereas CB4856 appears to be the opposite. These two *C. elegans* strains provide a good pair to study the interactions between *C. elegans* and microsporidia.

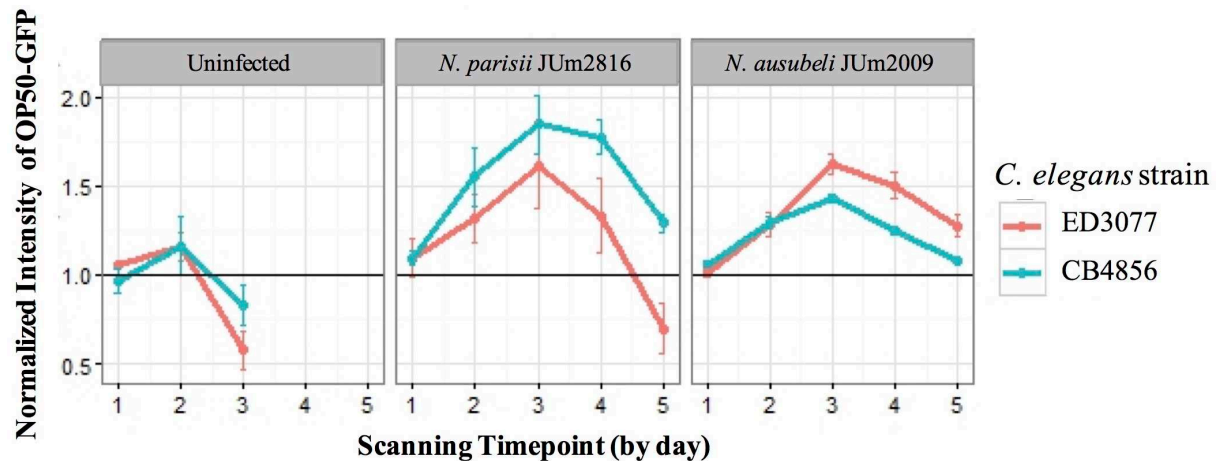


Figure 36. Contrasted sensitivity variation of ED3077 and CB4856 upon different infections.

Food consumption test (a repeated test compared to Figure 35) of ED3077 and CB4856 upon *N. parisii* JUm2816 and *N. ausubeli* JUm2009. Data are mean values of three replicates, with bars represent standard errors. The figure was performed by Clément Dubois.

II.3. The different tolerance of JU1249 and JU2825 to microsporidia infection

Using FISH analyses, we found that though JU1249 consumed food faster than JU2825, populations of JU1249 did not have a lower infection rate nor a lower pathogen load on average than JU2825 populations (Figure 28). In fact, upon *N. parisii* JUm1249 infection, JU1249 had a significantly higher pathogen load than JU2825. These results indicate a different tolerance of JU1249 and JU2825 to microsporidia infection. By contrast, in the study by Balla et al. (2015), the different mortality rates of the six *C. elegans* strains are positively correlated with the differences in pathogen loads. This result indicates variation in host resistance rather than tolerance, which was consistent with their further findings that L1 larvae of *C. elegans* CB4856 displayed a special clearing ability to microsporidia infection. Several reasons may lead to the different results from the two experiments. Firstly, different infection protocols were used in the two experiments, as we measured the fitness of *C. elegans* from a population with stable microsporidia infection (ten days post-inoculation), whereas Balla et al. (2015) measured the mortality rates of infected *C. elegans* immediately (two days post-inoculation). Secondly, we used food consumption tests to compare the sensitivity of different strains. The correlation of the performance of *C. elegans* in the food

consumption test and its mortality is not clear yet. Thirdly, different *C. elegans* strains were compared in the two experiments. Different *C. elegans* strains may display different defensive strategies. Finally, Balla et al., 2015 used *N. ironsii* ERTm5 to apply the inoculation, whereas we used *N. parisii* and *N. ausubeli*. The responses of *C. elegans* to different microsporidia infections may be different.

Further studies on brood size of JU1249 and JU2825 with or without microsporidia infection were performed by Dubois (Figure 37). Three *N. parisii* strains and three *N. ausubeli* strains were used separately to infect JU1249 and JU2825 in this study. First, the infections of the six strains of two *Nematocida* species greatly reduced the brood size of both JU1249 and JU2825, which indicates that neither of the two *C. elegans* strains can clear microsporidia infection like CB4856. Second, the brood sizes of JU1249 and JU2825 were similar when the two strains were uninfected, or infected with any of the three *N. parisii* or *N. ausubeli* JUm2799. With *N. ausubeli* JUm2526, JU1249 had a larger brood size than JU2825 (p-value is 0.004). Together, these results demonstrated that upon *N. ausubeli* JUm2526 infection, *C. elegans* JU1249 has a higher tolerance in the brood size compared to JU2825. This higher tolerance of JU1249 in the brood size may be one factor that leads to the better performances of JU1249 in the food consumption tests and the final winning in the competition assays with JU2825.

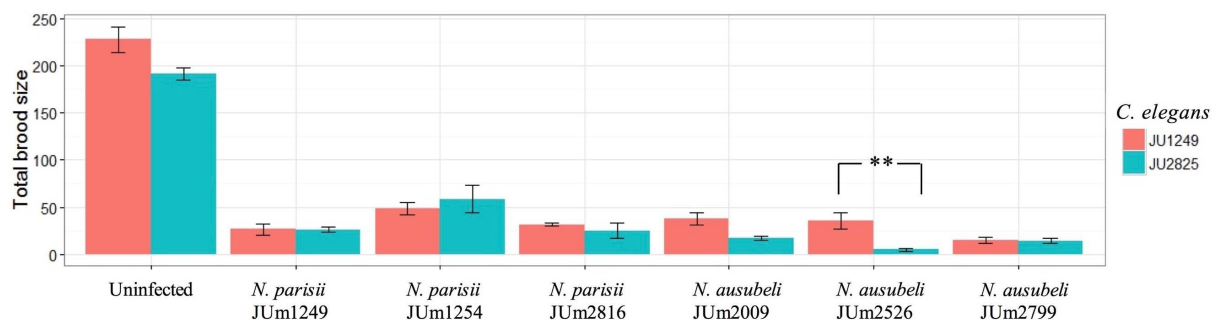


Figure 37. The brood size of *C. elegans* JU1249 and JU2825 with or without microsporidia infection.

In each treatment, 5 L4 stage hermaphrodite *C. elegans* from a stable population (ten days post infection) were transferred to a fresh plate and the number of their progenies were counted. The total number of progenies per hermaphrodite is showed in the figure. Data are mean values of three replicates, with bars represent standard errors. In the pairwise comparisons, JU1249 and JU2825 showed a significant (p-value is 0.004, marked with “**”) brood size difference only upon the infection of *N. ausubeli* JUm2526. The analysis and the figure were performed by Clément Dubois.

II.4. Future studies on the genetic basis of *C. elegans* sensitivity variation to microsporidia infection

With the *C. elegans* strains JU1249 and JU2825 that show contrasted phenotypes in the sensitivity to microsporidia infection, we plan to further study the genetic basis of this natural variation. As proved possible by the competition tests performed between JU1249 and JU2825 in the presence and absence of microsporidia infection, we plan to use Extreme QTL mapping (X-QTL) to detect the underlying genetic loci. X-QTL mapping is a developed bulk segregation technique that employs selection-based phenotyping to large groups of crossed progeny for the trait of interest (Ehrenreich et al., 2010; Chevalier et al., 2014). X-QTL has been used on yeast, schistosome and Arabidopsis, and proven to be a simple and cost-effectively mapping method for traits that are selectable (Ehrenreich et al., 2010; Chevalier et al., 2014; Yuan et al., 2016). It allows a much larger sample size that facilitates detection of multiple loci with small effects.

To assess the genetic basis of *C. elegans* sensitivity variation to microsporidia infection using X-QTL methods, mainly four steps have to be performed. The first step is to cross JU1249 and JU2825 and then generate bulked segregating populations. In the second step (phenotyping), the bulked segregating populations will be treated with or without microsporidia infection and then maintained for several generations. During the maintenance, individuals with microsporidia-resistant (or tolerant) traits are assumed to be selected and accumulated in the population. As indicated by the competition assays between JU1249 and JU2825, the maintenance should be continued at least for 30 days (about 10 to 12 generations). The third step (genotyping) is to sequence each population as a pool. The final step is to analyze the sequenced data of each pool. The frequencies of genetic markers (i.e. SNPs) of each pool will be analyzed and compared. The assumption is that the genome regions containing the microsporidia-resistant (or tolerant) loci will show different frequencies between infected populations and uninfected populations, while other genome regions have equal frequencies between populations with different treatments.

II.5. Other future directions

II.5.1. Screening of other C. elegans strains

Together, 22 *C. elegans* strains were compared for their sensitivity to *Nematocida* infection in three experiments. Though no *C. elegans* strain was found to be fully resistant to the tested *N. parisii* or *N. ausubeli*, the natural variation was determined and three interesting pairs of *C. elegans* (CB4856 versus N2, JU1249 versus JU2825, CB4856 versus ED3077) were found. It would be interesting to compare the sensitivity among more *C. elegans* strains, which may provide many windows to study host-pathogen interactions and coevolution.

II.5.2. Natural variation of C. briggsae in sensitivity to microsporidian infection

In addition to *C. elegans*, natural sensitivity variation can also be studied in *C. briggsae*. *C. briggsae* is one of the most common nematode hosts to microsporidia infections in our collection. Nine *C. briggsae* strains were naturally found infected with four *Nematocida* species. *C. briggsae* was further found susceptible to *N. homosporus*. It will be interesting to study the natural sensitivity variation among *C. briggsae* wild isolates.

II.5.3. Natural variation of Nematocida virulence to C. elegans

The virulence difference between *N. ausubeli* and *N. parisii* was investigated by single-cell inoculation (Balla et al., 2016). The developmental kinetics and symptom severity to the host of *N. ausubeli* infection are different from those after *N. parisii* infection. On average, single *N. ausubeli* infection spreads faster across host intestinal cells, and forms spores earlier than *N. parisii* (Figure 38A). Meanwhile, compared to *N. parisii*, *N. ausubeli* infection leads to a much stronger negative effect on host growth and fecundity (Figure 38B, C) (Balla et al., 2016). Thus, *N. ausubeli* seemed more virulent than *N. parisii* in *C. elegans*.

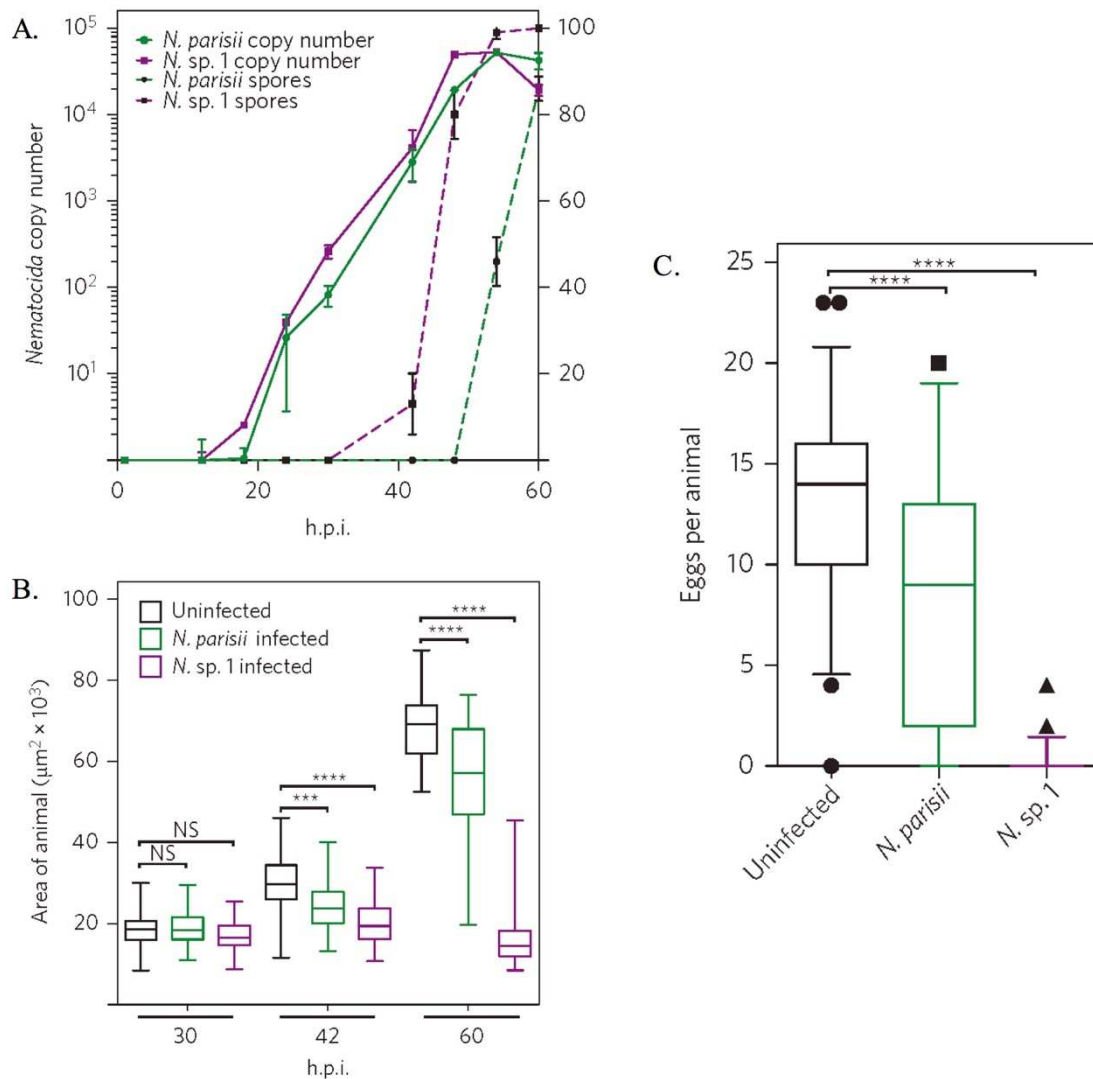


Figure 38. Growth and virulence variations of *N. parisii* and *N. ausubeli* (*N. sp. 1*). Infections were initiated by single-cell inoculation in N2. The *N. parisii* strain ERTm1 and the *N. ausubeli* strain ERTm2 were used here. **A.** Growth kinetics showed by copy number (solid lines) and spore formation (dashed lines). **B.** Sizes of animals over time with different infections and no infection. **C.** Eggs laid by worms with different infections or no infection, at 60 hpi. Adapted from Balla et al. (2016).

Nevertheless, to compare the virulence between *N. parisii* and *N. ausubeli*, it is necessary to test other strains of both species. In the study of Balla et al. (2016), *N. ausubeli* ERTm2 was proven to be more virulent than *N. parisii* ERTm1 to *C. elegans*. However, **RESULT II.3.2** indicated that *N. parisii* JUm1249 is more virulent than *N. ausubeli* JUm2009 and *N. ausubeli* JUm2526 to *C. elegans*, as *N. parisii* JUm1249 induced higher pathogen loads in both *C. elegans* strains than either *N. ausubeli* infection did (Figure 28A). Furthermore, the study

(DISCUSSION II.3, Figure 37) on brood size of JU1249 and JU2825 with three *N. parisii* strains and three *N. ausubeli* strains indicated the variation in virulence of different microsporidia, at both inter- and intraspecific levels. It would be interesting to compare virulence of different microsporidia species and strains using the single cell inoculation method.

In addition, we found that *N. ausubeli* induced a less robust host transcriptional response than *N. parisii* and some other *Nematocida* species in *C. elegans*, by using transgenic *C. elegans* strains with GFP reporter genes that are highly upregulated upon *N. parisii* infection (paper Figure 9). Further studies by Troemel et al. showed that *N. ausubeli* inhibited the induction of host reporter genes by *N. parisii* in co-infection experiments. These results suggested that *N. ausubeli* and *N. parisii* are different in their virulence. It would be interesting to compare virulence of different microsporidia species and strains using transgenic *C. elegans* strains, and further study the competition of *N. ausubeli* and *N. parisii* in the same host.

III. The suppression effect of microsporidia infection on the progressive sterility in *C. elegans*

Our preliminary tests demonstrated that *N. parisii* and *N. ausubeli* infections are able to attenuate the progressive decrease in fertility in *C. elegans* with the mortal germline phenotype (Mrt). In the four *C. elegans* strains we tested, two strains (JU775 and JU2526) with a moderate Mrt phenotype had an increased number of fertile generations upon microsporidia infection. This rescue effect represents an exciting type of host-pathogen interaction and is certainly worth further investigations.

Various negative effects of microsporidia infection on *C. elegans* have been observed and determined. This rescue effect may be a beneficial effect of microsporidia infection on *C. elegans*, which may have great evolutionary and ecological significance. Many interesting questions can be further addressed. For example, is this suppression on *C. elegans* with Mrt phenotype a specific effect of microsporidia infection? Or are other micro-organisms, such as the Orsay virus, bacteria and fungi, also able to display the suppression? Another direction is to identify the molecular mechanisms of this suppression effect, for which reverse, forward and quantitative evolutionary genetic approaches can be further performed.

Conclusion

Our studies revealed the diverse interactions of microsporidia with *C. elegans* and related nematodes. We found that *N. parisii* and *N. ausubeli* are relatively common pathogens of *C. elegans* and *C. briggsae* in the wild. We better described *N. ausubeli* and further found five new *Nematocida* species. We also defined two new genera of nematode-infecting microsporidia, named *Enteropsectra* and *Pancytospora*, which are genetically distinct from *Nematocida*. These microsporidia are diverse in terms of phylogenetic relationships, spore size and shape, host tissue tropism, host cell intracellular localization, cellular exit route, host specificity pattern, virulence and negative effects on hosts, etc. These findings considerably enlarged our knowledge on nematode-infecting microsporidia and on the ecology of *C. elegans* and related nematodes.

To date, 48 strains of 10 nematode species have been found naturally infected with microsporidia. These nematode species displayed interspecific susceptibility and intraspecific sensitivity variations to different microsporidia infections. Here we further determined the intraspecific variation of *C. elegans* in sensitivity to *N. ausubeli* infection. Two *C. elegans* strains, JU1249 and JU2825, displayed the largest sensitivity difference, which may be due to their different tolerance. These two strains are proven to be good candidates for future studies on the genetic loci associated with *C. elegans* sensitivity variation to microsporidian infections. Furthermore, an exciting type of host-pathogen interaction was observed. Microsporidia infection is able to suppress the progressive decrease in fertility in *C. elegans* with the mortal germline phenotype (Mrt). Overall, the diverse interactions of microsporidia with *Caenorhabditis elegans* and related nematodes provide great opportunities to study host-pathogen coevolution.

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Résumé

Les microsporidies sont des pathogènes intracellulaires obligatoires apparentés aux champignons. Elles infectent de nombreux animaux, dont le nématode *Caenorhabditis elegans*. La première microsporidie isolée d'une souche de *C. elegans* sauvage a été nommée *Nematocida parisii*. L'interaction entre *N. parisii* et *C. elegans* est devenue un puissant modèle pour l'étude des interactions hôte-pathogène. Cependant, ce modèle a été récemment découvert et de nombreux détails sur son écologie et sa biologie restaient inconnus. Notamment, nous ignorions l'incidence et la diversité des infections microsporidiennes chez *C. elegans* et autres nématodes dans la nature.

A partir d'une collection de nématodes, de la famille des Rhabditidae, échantillonnés dans le monde entier, j'ai recensé un panel de 47 nématodes présentant des symptômes d'infection par des microsporidies. J'ai caractérisé moléculairement la diversité de ce parasite infectant ces nématodes et déterminé que *N. parisii* (ou *N. ironsii*) est la microsporidie la plus souvent responsable des infections chez *C. elegans* dans la nature. J'ai également décrit et nommé six nouvelles espèces de *Nematocida*. Au cours de mes travaux, j'ai aussi défini deux nouveaux genres de microsporidies génétiquement distincts de *Nematocida*, appelés *Enteropsectra* et *Pancytospora*. Mes travaux ont de plus détaillé la diversité qui existe chez les microsporidies parasites de nématodes. Ces microsporidies présentent des différences en terme de taille et forme de leurs spores, de leur tropismes tissulaire et intracellulaire chez l'hôte, de leur voie de sortie des cellules hôtes mais aussi de spectre d'hôtes. Mes résultats ont démontré que, dans la nature, les infections de *C. elegans* et autres nématodes par les microsporidies sont répandues et diverses.

De plus, j'ai estimé la variation naturelle pour la sensibilité de *C. elegans* à l'infection par *N. ausubeli*. J'ai notamment comparé 10 souches naturelles de *C. elegans* en utilisant des tests de consommation alimentaire. Deux souches de *C. elegans*, JU1249 et JU2825, présentaient des niveaux contrastés de sensibilité, ce que j'ai interprété comme étant une différence de niveau de tolérance aux infections. Ces deux souches se sont révélées être de bons candidats pour une future caractérisation des loci génétiques associés à la variation de sensibilité de *C. elegans* aux infections microsporidiennes. Enfin, j'ai observé un effet surprenant de l'infection de *C. elegans* par les microsporidies. En effet, la présence du pathogène est capable de supprimer le déclin progressif de la fécondité à haute température chez certaines lignées de *C. elegans*.

Mots Clés

Microsporidies, *C. elegans*, diversité, interactions hôte-pathogène, spectre d'hôtes, sensibilité, variation naturelle

Abstract

Microsporidia are fungi-related intracellular pathogens that infect a great variety of animals, including the nematode *Caenorhabditis elegans*. The first microsporidia isolated from wild *C. elegans* was named *Nematocida parisii* in 2008. *C. elegans* and *N. parisii* have been used as a powerful model for the study of host-pathogen interactions. However, it was unclear how widespread and diverse microsporidia infections are in *C. elegans* or other related nematodes in the wild.

By sampling rhabditid nematodes worldwide, we established a collection of 47 nematodes that displayed putative microsporidia infections. We characterized molecularly these infections and determined that *N. parisii* (or *N. ironsii*) is the most common microsporidia infecting *C. elegans* in the wild. We further described and named six new *Nematocida* species. In addition, we defined two new genera of nematode-infecting microsporidia, named *Enteropsectra* and *Pancytospora*, which are genetically distinct from *Nematocida*. Further investigations showed that these microsporidia are diverse in terms of spore size and shape, host tissue tropism, host cell intracellular localization, cellular exit route, host specificity pattern, etc. Overall, these findings illustrate the widespread and diverse microsporidia infections in *C. elegans* and related nematodes in the wild.

We further assayed the natural variation of *C. elegans* in sensitivity to *N. ausubeli* infection, by comparing 10 *C. elegans* strains using food consumption tests. Two *C. elegans* strains, JU1249 and JU2825, displayed the largest sensitivity differences, which were suggested to be a result of the different tolerance between the two strains. These two strains are proven to be good candidates for future studies on the genetic loci associated with *C. elegans* sensitivity variation to microsporidian infections. Furthermore, I observed an exciting effect of host-pathogen interaction. Microsporidia infection is able to suppress the progressive decline in fertility in some *C. elegans* with the mortal germline phenotype (Mrt).

Keywords

Microsporidia, *C. elegans*, diversity, host-pathogen interaction, host specificity, sensitivity, natural variation