Microsporidia are unicellular fungi that are obligate endoparasites. Although nematodes are one of the most abundant and diverse animal groups, the only confirmed report of microsporidian infection was that of the “nematode killer” (Nematocida parisii). N. parisii was isolated from a wild Caenorhabditis sp. and causes an acute and lethal intestinal infection in a lab strain of Caenorhabditis elegans. We set out to characterize a microsporidian infection in a wild nematode to determine whether the infection pattern of N. parisii in the lab is typical of microsporidian infections in nematodes. We describe a novel microsporidian species named Sporanauta perivermis (marine spore of roundworms) and characterize its infection in its natural host, the free-living marine nematode Odontophora rectangula. S. perivermis is not closely related to N. parisii and differs strikingly in all aspects of infection. Examination by transmission electron microscopy (TEM) revealed that the infection was localized in the hypodermal and muscle tissues only and did not involve the intestines. Fluorescent in situ hybridization (FISH) confirmed infection in the muscle and hypodermis, and surprisingly, it also revealed that the parasite infects O. rectangula eggs, suggesting a vertical mode of transmission. Our observations highlight the importance of studying parasites in their natural hosts and indicate that not all nematode-infecting microsporidia are “nematode killers”; instead, microsporidiosis can be more versatile and chronic in the wild.

Microsporidia are obligate intracellular parasites whose hosts include diverse animals and one protist group (20, 26). Microsporidia are also unicellular fungi with over 1,300 recognized species (7, 14, 20). Their life cycles vary considerably for different species, but they all have at least one extracellular (spore) stage and one intracellular (merogonial/sporogonial) stage (12). Microsporidian spores are distinguished by the presence of an internal tube known as the polar filament (34). Fixed at the apex of the spore by the anchoring disk, the polar filament extends to the posterior of the spore where it is usually found coiled around one or two nuclei. The number of coils and their angle of inclination can often distinguish species. Microsporidian spores are also characterized by having a thick, protective chitinous wall around the cell membrane that allows them to survive outside their hosts. The spore wall thins near the anchoring disk; this is thought to facilitate the exit of the polar filament through the anchoring disk. During infection, the polar filament everts and, upon contacting a host’s cell membrane, transfers the contents of the spore (sporoplasm) into the host cell. The sporoplasm differentiates into the proliferative (meront) stage that goes on to differentiate into spores (sporogony). Mature spores may then infect adjacent host cells or be released into the environment.

Microsporidian infections (microsporidiosis) are highly variable and cause a wide range of symptoms depending on the host and the microsporidium involved. Infections are usually acquired by ingestion of spores (horizontal transmission), but vertical transmission (from mother to offspring) has been observed (mainly in insects) (1). Microsporidian infections may be acute or chronic, affect one or multiple tissue types, cause mild or lethal symptoms, and even change the host’s sex (18, 26).

Nematodes, commonly known as roundworms, are ubiquitous in the environment. In marine sediments, nematodes account for more than 90% of individual animals and over 50% of the total biomass (16), yet reports of microsporidian infections in this animal group are limited and unclear. Indeed, most reports of microsporidian infections in nematodes, including marine nematodes, failed to clearly demonstrate morphologically (presence of polar filament) and genetically that the infecting agent was a microsporidium (17, 21, 28, 29). The only exception is the microsporidian infection described 4 years ago in a lab strain (N2) of the soil nematode Caenorhabditis elegans (33). In this strain, ingestion of microsporidian spores (Nematocida parisii) causes an acute and extremely virulent (100% lethal) infection that affects only the intestinal tissues. N. parisii was originally isolated from a wild Caenorhabditis sp. and transferred to the lab strain; effects of the parasite on the native host are unclear. Given that nematodes are arguably the most abundant and diverse animal phylum (15), the dearth of knowledge regarding their microsporidian parasites is surprising. Here we present the first morphological and molecular description of a microsporidian infection in a marine nematode. We describe Sporanauta perivermis, a novel microsporidian species infecting the free-living marine nematode, Odontophora rectangula (family Axonolaimida). This is, to our knowledge, the first confirmed case of an interaction between a fungal group and nematodes in marine environments (3, 4). Moreover, the characteristics of S. perivermis infection challenge our current understanding of how microsporidia and nematodes interact in the wild by providing a completely different infection pattern from that observed in the lab with N. parisii. Unlike infection with N. parisii, infection with S. perivermis is mild and not severe, chronic and not acute, and it affects the hypodermal and muscles tissues and not the intestines. S. perivermis even differs in its mode of transmission, which appears to be vertical (infection of the eggs) and not exclusively horizontal (ingestion of spores) as it is in N. parisii. Indeed,...
by comparison, *S. perivermis* infection represents the opposing end of the spectrum of microsporidian infection variability, suggesting that host-parasite relationships between nematodes and microsporidia are considerably more complex and diverse than previously considered.

### MATERIALS AND METHODS

**Marine nematode sampling.** Marine nematodes were collected in July and August 2010 and 2011 from Boundary Bay beach (49.013N, 123.036W), Tsawwassen (British Columbia, Canada). Surface sand and sediment samples (1 to 10 cm deep) were collected at low tide using sterile 50-ml falcon tubes (filled 3/4 with sand and 1/4 with seawater). All samples were kept on ice until they were stored permanently at 4°C in the dark and with loosened caps. For each tube, 1 to 2 g of sand was transferred to a sterile petri dish and flooded with cold (4°C) sterile seawater. Live nematodes were observed using a dissecting microscope and transferred individually to a sterile petri dish containing cold sterile seawater. Marine nematodes were then used for DNA extraction, fluorescence in situ hybridization (FISH), and transmission electron microscopy (TEM).

**Microsporidian and nematode rDNA extraction and sequencing.** Microsporidian and marine nematode rDNA were initially extracted from 0.5 to 1 g of sand using the Mobio UltraClean soil DNA isolation kit (Bio/Can Scientific). Beads (included in this kit) and a beadbeater (Mini-Beadbeater-1, BioSpec Products Inc.) were used to disrupt the tissues during the lysis step. This step was modified from the manufacturer’s protocol by adding a 15-min incubation period at 70°C with a 20-s bead beating (2,500 rpm) treatment every 5 min. Microsporidian small-subunit (SSU) ribosomal DNA (rDNA) sequences were amplified using the microsporidian-specific forward primer 18s (35) and a novel microsporidian-specific primer Micro33R (5′-TAGAGACCCTGTGTCTTGGGG-3′) (this study). This microsporidian primer set amplifies fragments of 550 to 650 bp long from the 5′ end of the microsporidian SSU rDNA. PCR amplicons were separated on agarose gels (2%), purified with the UltraClean 15 Mbio DNA purification kit (Bio/Can Scientific), and cloned using the TOPO TA cloning kit with pCR 2.1 vector (Invitrogen). At least three independent clones were sequenced for each DNA extraction. The same procedures were performed on individual live nematodes to determine the host and prevalence of microsporidian infections. Marine nematodes were barcoded using 18s rDNA marine nematode-specific primers (M18F 5′-AGGCGGTAATGTCGGAG3′ and M18R 5′-TCTCGCTC GTTATGCGAAT-3′) (2) and an *S. perivermis*-specific primer set (5′-CG AGATGTCGTAATGTCGGAG-3′) designed from previously amplified sequences. These primers were also used in combination with 530 and 1,492 microsporidian primers (35) to generate a full-length (1,385 bp) *S. perivermis* SSU rDNA sequence (GenBank accession no. JN195782).

**Phylogenetic analysis.** The small-subunit rDNA sequence of *S. perivermis* was examined phylogenetically by comparing it with that of 43 other microsporidian species representing the five major microsporidian groups (36). We also included an environmental microsporidian sequence (accession no. FJ756061.1) identified from the nucleotide BLAST analysis. The fungal species *Basidiobolus ranarum* and *Conidiobolus corona* tus were included as outgroups (36). All sequences were obtained from GenBank.

All sequences were viewed and aligned using the molecular sequence analysis multplatform SeaView version 4. The alignment was adjusted automatically (trmAL; Phylemon) and by eye. The final alignment was used in three phylogenetic analyses: Bayesian inference, maximum likelihood (ML), and approximate likelihood ratio test based on Shimodaira-Hasegawa-like procedure (aLRT-SH).

To perform Bayesian inference, we used the program MrBayes version 3.2. One million generations were run using the general time reversible (GTR) model with two separate runs of four chains each. Sampling was performed every 1,000 trees of which the initial 25% (approximately 25 thousand trees) were discarded before the generation of the best-supported consensus tree. To perform ML and aLRT-SH tests, we used Phyml version 3.0.

**Transmission electron microscopy.** Live *O. rectangula* nematodes were transferred into a sterile polyethylene embedding capsule (size 3; BEEM Inc.) containing 30 μl of cold (4°C) sterile seawater. Nematodes were centrifuged at 3,000 × g for 30 s, and cuticles were nicked with a razor blade. Fixation proceeded immediately by capping the capsules with a piece of Whatman filter paper soaked in a fixative solution containing 1% (wt/vol) osmium tetroxide in 0.2 M sodium cacodylate buffer (pH 7.2) and left on ice for 30 min. Fixative solution was then added directly to each sample and kept on ice for 2 h. This was followed by three 5-min washes with 0.2 M sodium cacodylate buffer (pH 7.2). The samples were dehydrated through an ethanol series (25%, 50%, 75%, 90%, 95%, and 100%) of 20 min at each step. Ethanol was substituted with an ethanol and acetone mixture (1:1) twice (20 min each) and then repeated twice with pure acetone. Acetone was removed, and a mixture (1:1) of acetone and Spurr’s resin was added and left at room temperature for 24 h. Spurr’s resin was washed twice (24 h each) before polymerizing overnight (12 h) at 70°C. Sectioning of each resin was performed using a Leica EM UC6 ultramicrotome (Leica Microsystems) and then stained twice with 2% (wt/vol) uranyl acetate and lead citrate (Reynolds 1963). Each ultrathin section was examined on a Hitachi H7600 TEM.

**Fluorescent in situ hybridization.** Staining of *S. perivermis* was modified from methods described previously (33). A specific *S. perivermis* probe was purified by high-performance liquid chromatography (HPLC) and synthesized with Quasar 570 (Cy3) at the 5′ end (Biosearch Technologies, Inc.). Live *O. rectangula* nematodes were placed in a 1.5-ml Epipendorf tube containing 30 μl of sterile seawater and aggregated into a pellet by centrifugation (3,000 × g for 30 s). Excess seawater was removed, and cuticles were nicked with a razor blade before a 2-h fixation with 4% paraformaldehyde. The samples were then washed twice with 250 μl of phosphate-buffered saline (PBS) plus 0.1% Tween 20 and hybridized at 46°C for 12 h with 60 μl of hybridization buffer (900 mM NaCl, 20 mM Tris [pH 7.5], 0.01% SDS, and 5 ng/μl probe). Following this, samples were washed with 500 μl of wash buffer (900 mM NaCl, 20 mM Tris [pH 7.5], 0.01% SDS, 5 mM EDTA) and placed at 48°C for 1 h. Samples were then stained and mounted with 4′,6-diamidino-2-phenylindole (DAPI) Vectashield mounting medium (Vector Laboratories) and examined using an Olympus CV10 confocal microscope (Olympus Inc.). Marine nematodes collected from the same sand samples as *O. rectangula* were collected and stained alongside as negative controls.

**DNA sequence.** The full-length sequence of the SSU rDNA gene is deposited as GenBank accession no. JN195782.

### RESULTS

The free-living marine nematode *Odontophora rectangula* is infected with a microsporidian parasite. Live marine nematodes were collected at low tide from Boundary Bay beach (Tsawwassen, British Columbia, Canada). DNA was extracted from the nematodes, and microsporidian infection was assessed by PCR amplifying the microsporidian small-subunit (SSU) rRNA gene. An identical 668-bp fragment was amplified, and this sequence was most similar (88% similarity by BLAST) to an undescribed microsporidian sp. (accession no. FJ756061.1), suggesting the presence of a novel and single microsporidian infection in local nematodes.

Given that the initial isolations were done with a pooled, mixed marine nematode sample, we performed DNA extractions on individual marine nematodes to determine the host. Using a primer set specific for a fragment of the SSU sequence described above, individual nematodes were screened for both their infection status with the novel microsporidian and with a set of nematode barcoding primers. In all cases, microsporidian infections were observed only in a single nematode species, predicted to be *Odontophora*
Overall, the majority of *O. rectangula* nematodes (84%; *n* = 27) were infected, and the proportion of infected individuals in each sex was almost the same (83% males and 84% females). Morphologically, these nematodes match the predicted barcoding identification; *O. rectangula* nematodes are distinguished by their body size and tail shape and for having a distinctive set of large teeth (25, 27).

**Phylogenetic position of the novel microsporidian, Sporanauta perivermis.** To establish the phylogenetic placement of the novel microsporidian SSU rDNA sequence, we compared it with 43 microsporidian species that span the known diversity of the group. Overall, recovered trees reflected our current understanding of microsporidian relationships, with the five major microsporidian clades strongly supported by all methods (Fig. 1) (36). Surprisingly, the new species did not show an affiliation with the only other known nematode-infecting microsporidian (*N. parisii*). Instead, all trees strongly support placing the microsporidian infecting *O. rectangula* within clade IV (Fig. 1). We have named this species *Sporanauta perivermis* (for marine spores of roundworms).

The subclade containing *S. perivermis* includes four other microsporidian lineages: (i) the bark louse-infecting microsporidian *Mockfordia xanthocaeciliae* (32), (ii) the daphnia-infecting microsporidia *Ordospora colligata* (30), (iii) an undescribed microsporidian species (top BLAST hit), and (iv) the genus *Encephalitozoon* (five species) that infect a variety of animal hosts (Fig. 1).
The position of *S. perivermis* relative to the rest of its subclade could not be fully resolved, even when restricting the analysis to members of this group and outgroup representatives within clade IV (data not shown).

**Microscopic characterization of *S. perivermis*.** *O. rectangula* nematodes were examined using standard light microscopy techniques for any behavioral changes (i.e., less active) or any morphological abnormalities such as enlargement of specific tissues that could be signs of microsporidian infection. However, no external signs of infection were evident. Therefore, to examine the parasite’s effect on the nematode and confirm morphologically that *S. perivermis* is a microsporidian parasite, we examined *O. rectangula* nematodes by transmission electron microscopy (TEM). All nematode digestive tissues (pharynx, intestine, and lumen) appeared normal. However, dense oval structures were located within the hypodermal and muscle tissues (Fig. 2A and B). These structures were morphologically consistent with microsporidian spores by their size (2.0 to 2.5 μm by 1.1 to 1.5 μm) and ovoid shape. Conclusive evidence that these structures are indeed microsporidian spores includes the presence of a polar filament (3 or 4 coils), an anchoring disk surrounded by polaroplast membranes, and a thin electron-dense exospore over a thick electron-transparent endospore surrounding the cell membrane (Fig. 2C and D).

Spores were not observed to be in direct contact with the host cytoplasm. Instead, each microsporidian spore appeared to be enclosed within a vesicle that was often observed to be connected to adjacent vesicles (Fig. 2). This resulted in a large “cluster” of interconnected vesicles containing *S. perivermis* spores. Proliferative microsporidian life cycle stages (i.e., meronts) were not observed in the tissues examined.
Fluorescent in situ hybridization reveals *S. perivermis* infection of *O. rectangula* eggs. To further assess the extent of *S. perivermis* infection in *O. rectangula*, we performed fluorescent in situ hybridization (FISH) using a fluorescent probe targeting the *S. perivermis* SSU rDNA sequence. Surprisingly, FISH indicated infection differences in male and female nematodes. In males, FISH confirmed our previous TEM observations in which *S. perivermis* infection was localized to the hypodermal and muscular tissues, surrounding the intestine but never within it (Fig. 3). Within these tissues, spores were observed in clusters (few to hundreds of spores) which appeared to be distributed along the length of the body but were never observed in the head. In addition, spore “clusters” appeared enclosed within large vesicles. We interpret these as vesicles of microsporidian origin due to the hybridization of the microsporidian tag. *S. perivermis* spores were oval and consistent in size (≈2 μm by 1 μm) with those observed by TEM.

In females, spores were also observed in clusters within the hypodermal and muscle tissues; however, the spores appeared to be markedly less abundant. Surprisingly, FISH revealed that in female nematodes the microsporidian infection was localized to *O. rectangula* eggs (Fig. 4). In all infected females examined, the eggs showed a very strong signal for *S. perivermis*, but the parasite did not appear to be in the spore stage, as spores were not observed within the eggs (Fig. 4). Aside from the eggs, there appeared to be no other infection of female reproductive tissues.

A small number of juveniles were observed to be infected. Generally, the infection pattern was the same as in adults, but in some cases, the reproductive tissues were labeled with the FISH probe. The sex of most nematodes, including *O. rectangula*, cannot be resolved in juveniles, so it is unclear how this labeling pattern correlates with the gender differences observed in adults.

**TAXONOMY**

*Sporanauta* n. gen. Ardila-Garcia & Fast 2012. This is a novel microsporidian lineage that belongs to microsporidian clade IV (36) based on SSU rDNA phylogenetic analyses. The closest relatives to *Sporanauta* are the microsporidian genera *Mockfordia*, *Encephalitozoon*, and *Ordospora*. The type species is *Sporanauta perivermis* Ardila-Garcia & Fast 2012. The Latin “sporo” (spore)
and the Latin “nauta” (mariner) refer to microsporidian spores to the habitat (marine).

**Sporanauta perivermis** n. sp. Ardila-Garcia & Fast 2012. The spores are ovoid and measured 2.0 to 2.5 μm by 1.1 to 1.5 μm. The polar filament has 3 or 4 coils. Spores are found in vesicles that prevent them from being in direct contact with the host cells. Vesicles may contain a few spores to hundreds of spores. Infection is localized to the hypodermal and muscle tissues in adults, mature eggs in adult females, and the reproductive tissues of juveniles. Infection does not affect the intestinal tissues. Infection is chronic and appears to cause minor symptoms (if any) on the host. Transmission appears to be vertical (transovary). The type host is the free-living marine nematode *Odontophora rectangula* (family Axonolaimidae). The Latin “peri” (round) and the Latin “vermis” refer to roundworms, the common name for nematodes.

**DISCUSSION**

Fungi and nematodes are highly abundant and diverse in marine ecosystems (19, 22), but the nature of their interactions remains largely unknown (3, 4). The present study is, to our knowledge, the first to provide morphological and genetic evidence to show that fungi (Microsporidia) can parasitize marine nematodes. We describe the infection of a novel microsporidian species, *S. perivermis*, in its wild host, the free-living marine nematode *O. rectangula*. The characteristics of *S. perivermis* infection challenge the current view of the nature of microsporidian infections in nematodes. The only other described microsporidian infection in nematodes involves inoculation of *N. parisii* from wild nematodes to a *C. elegans* lab strain (8, 13, 33). Here the exposure to a microsporidian parasite was lethal, but it raises questions as to whether this particular parasite or any other nematode-infecting microsporidian is as damaging and virulent in its natural host. Our observations on *S. perivermis* infection in its wild host indicate that microsporidian infections in the wild can vary considerably in almost every aspect compared to that observed with *N. parisi*.

*S. perivermis* represents a novel microsporidian lineage that grouped with eight other species in a subclade of microsporidian clade IV. Its closest relatives were isolated from a diversity of hosts, including mammals (9–11, 37), lizards (6), crustaceans (30), and insects (23, 32). Despite their differences in hosts, all members of this subclade are morphologically similar in that they are considerably small (spore size, 2 to 4 μm) by microsporidian standards (spore size range, 2 to 40 μm). In addition, their spores are always

**FIG 4** *S. perivermis* infection in *O. rectangula* eggs by FISH. Each column shows a sequence of images taken on the same plane to show the localization of *S. perivermis* inside eggs. Each row shows a different light filter: the nuclear stain DAPI (shown in blue), the *S. perivermis* fluorescent probe (Cy3 [shown in red]), a combination of the two dyes, and the view of the image under phase contrast. Bars = 10 μm.
found within vesicles that prevent them from being in direct contact with the host cytoplasm. Other members of the *S. pervermis* subclade, *O. colligata* and the *Encephalitozoon* group, also develop in isolation from their hosts' tissues. However, their separation is achieved by means of a membrane-bound parasitophorous vacuole (host origin) instead of a vesicle (24, 31). The host-parasite interface varies considerably for other members of clade IV, as some (i.e., *Endoreticulatus* species) develop in parasitophorous vacuoles, while others (i.e., *Nosemi*) develop in direct contact with the host tissues (5). Even though *N. parisiis* spores were also observed inside vesicles (33), *N. parisiis* and the undescribed *Nematocida* sp. strain 1247 did not group in clade IV with *S. pervermis*. Instead, *Nematocida* falls within microsporidian clade II with their closest relative being the beetle-infecting microsporidian *Ovavesicula papillae* (a position consistent with reference 33).

*S. pervermis* and *N. parisiis* also have stark differences in how they interact with their respective hosts. *S. pervermis* causes minor lesions (if any) in the hypodermal and muscle tissues and does not infect the intestinal tissues of *S. perivermis* juveniles, suggesting that *S. perivermis* is perhaps a “nematode killer” of *N. parisiis* (24). On the other hand, *N. parisiis* infection is typical of unbalanced, opportunistic, acute infections which are usually asymptomatic and cause minor effects on the host. Indeed, one would expect microsporidian-nematode relationships to encompass the full spectrum of infection (9) once additional species are described and their interactions with their hosts are understood.

Transmission of microsporidian parasites in their host is generally horizontal (ingestion of spores) as was reported for *N. parisiis*, while microsporidian vertical transmission in this animal group remains unknown. Vertical transmission of microsporidia is common in insects, in which it is usually transovarial and involves the transfer of the parasite within the female’s eggs (1). In the present study, we provided evidence that microsporidian parasites may be transmitted in this manner in nematodes. *S. perivermis* infects the eggs of adult *O. rectangula* females and the reproductive tissues of *S. perifermin* juveniles, suggesting that *S. pervermis* is transmitted from mother to offspring via the eggs.

Our observations of the infection of *S. perivermis* in the free-living marine nematode *O. rectangula* provide the first description of a fungal parasite of nematodes in marine environments. They provide the first indications that the interactions between microsporidia and nematodes in the wild can be highly intricate and involve versatile adaptations such as microsporidian vertical transmission. Our understanding of microsporidia in nematodes is no longer limited to “nematode killers” but has broadened to include interactions more indicative of coexistence.

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