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The nematode tapeworm: rediscovery of the bizarre parasite of nematodes, *Spirogyromyces vermicola* Tzean & Barron (Fungi, incertae sedis) in northeastern Mexico

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Abstract. We report the second world observation of the bizarre nematode-parasitic fungus-like organism, *Spirogyromyces vermicola*, from forest soil at Saltillo, Mexico. It is a benign parasite of nematodes that fills their intestine. Its phylogenetic position remains a mystery, but its morphology and development are reminiscent of Harpellales and Orphellales in the Kickxellomycotina. *Spirogyromyces* was cultivated *in vivo* in the original host (*Rhabditis*) and in *Caenorhabditis elegans*. *Spirogyromyces* proliferated in both hosts, and it did not appear to affect significantly health, reproduction, or numbers of hosts. The rediscovery of *Spirogyromyces* will highlight its potential in the study of parasitic systems in nematodes, including *Caenorhabditis* research.

Keywords. *Caenorhabditis*, commensal, parasite, *Rhabditis*, intestine

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Introduction

Nematodes are organisms of paramount ecological importance in natural ecosystems. They also affect human welfare as parasites of plants, animals, and humans. The knowledge of organisms antagonistic to or parasitic on nematodes is of great relevance from basic and applied standpoints (Barron 1977). Most fungi–nematode interactions result in the killing of the nematode host or prey by the fungus; that is the case for the Orbiliales and their anamorphs, common specialized nematode hunters (Li et al. 2005). The fungal parasites of nematode eggs, interspersed among several phylogenetic branches (Olpidiomycota, Zoopagomycota, and Ascomycota, among others: Drechsler 1943; Barron and Szuarto 1986; Wijayawardene et al. 2018) also kill their hosts. In 1981, during surveys of nematophagous fungi, the prominent mycologist George C. Barron and his sabbatical guest, Shean-Shong Tzean, discovered and described an unusual fungus-like organism, *Spirogyromyces vermicola* Tzean and Barron, 1981, parasitizing rhabditid nematodes in soil in the tropical greenhouse of the University of Guelph, Ontario, Canada (Tzean and Barron 1981). These authors described in great detail the morphology and development of this organism. *Spirogyromyces* appears to fill the ecological role of a tapeworm (Cestoda) inside nematodes. The sporulating, spiral fertile thallus eventually fills to a large extent the nematodes's intestine; however, infected nematode females are able to grow and reproduce in an apparent normal way (Tzean and Barron 1981). This organism has not been detected or reported again since Tzean and Barron's report, and its phylogenetic position remains unknown.

In the present work, we describe the rediscovery of *Spirogyromyces vermicola*, from forests in the Sierra

Madre Oriental mountains of northeastern Mexico, during a survey of nematophagous fungi from soil for biological control of plant-parasitic nematodes. We also describe the *in vivo* maintenance of the organism on the nematodes *Rhabditis* Dujardin, 1845 sp. and *Caenorhabditis elegans* Maupas, 1900, as well as a basic experiment on its effects on host populations in the laboratory.

Methods

Soil sampling. Soil was collected from an oak forest (*Quercus laeta* Liebm. and *Quercus saltillensis* Trel.) near Saltillo, state of Coahuila, Mexico (Fig. 1), on the western (rain-shadow) slope of the Sierra Madre Oriental. Other vegetation elements there are Weeping Juniper, *Juniperus flaccida* Schltdl., and Texas Madrone, *Arbutus xalapensis* Kunth. The soil is a very shallow, limestone (calcareous)-derived litosol with a 3–5 cm overlaying layer of leaf litter. Yearly precipitation at this site is around 400 mm. Thirty samples were randomly collected, separated by 10 m. Each sample consisted of 3 g of soil and leaf litter in sterile plastic bags, taken to a depth of 10 cm. Soil samples were baited with nematodes following Barron (1977).

Bait nematode rearing. A colony of uninfected *Rhabditis* sp. (Rhabditida, Rhabditidae) was maintained in the laboratory, as follows: 2–4 freshly killed, macerated, laboratory-reared Mealworm (*Tenebrio molitor* Linnaeus, 1758) larvae were placed in moist chambers (100 mm diameter Petri dishes lined with washed, moist, sterile, brown-bag paper. Bacteria were used as the nematode food source; therefore, when bacterial growth was obvious as viscid growth on insects, 10–20 *Rhabditis* sp. of both sexes (juveniles and adults) were added to the chambers and maintained at room temperature (23–26 °C). Every two weeks, nematodes from these colonies were subsequently transferred *en masse* (100–200 nematodes) to moist chambers with 2–4 killed mealworms colonized by bacteria. Under these conditions, each Petri dish produced approximately 30,000–50,000 *Rhabditis* sp. in two weeks.

Nematophagous fungus detection, increase, and *in vivo* culture of *Spirogyromyces*. Each 3 g soil sample were added to moist chambers, based on the general methods and concepts described by Barron (1977). Each soil sample and about 300 *Rhabditis* nematodes of different ages (juveniles and adults) were added to individual moist chambers set as described. Moist chambers were incubated and examined after two and three weeks. In each date, samples (small masses) of nematodes were collected and picked up with bent pins under a stereomicroscope. Temporary slides of live nematodes were made in cotton blue stain or distilled water and observed under bright field (cotton blue stain) or phase contrast microscopy in an Olympus CKX41 microscope fitted with a IX2-SLP phase-contrast slider (Olympus, Tokyo, Japan). Vouchers of infected nematodes are at the insect and invertebrate collection of the Laboratory of Insect Ecology and Biological Control at Universidad Autónoma Agraria Antonio Narro, Saltillo, Coahuila, as specimens UAN-2015-67. Attempts to amplify fungal-diagnostic genomic regions (ITS-4) for phylogenetic placement of this organism were unsuccessful

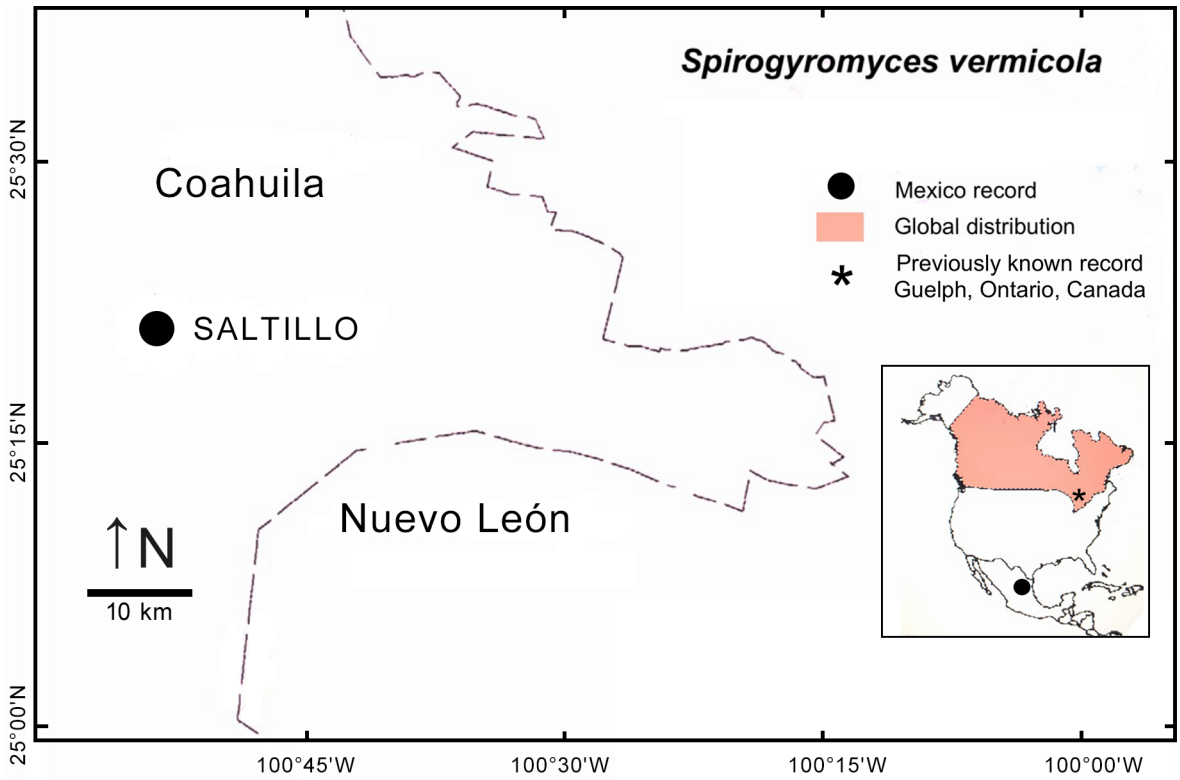

Figure 1. Sampled area (Saltillo), historical global distribution and new Mexican record of *Spirogyromyces vermicola*.

(K. Seifert pers. comm.). The reasons for these negative results are unknown; processed nematode samples yielded amplified sequences from saprophytic fungi present in these nematode cultures.

For *in vivo* culture of *Spirogyromyces*, groups of 10 nematodes infected with the fungus were transferred to Petri dishes with macerated insects and uninfected nematodes produced as in the section on bait nematode rearing, above. To verify infection status, every five days five samples of nematodes from inoculated dishes were taken with a pin, and 40–50 nematodes were inspected under the microscope searching for fungal infections. Dishes where the fungus of interest was observed were used for serial transfers and maintenance of fungal cultures.

Effect of *Spirogyromyces* on population increase of *Rhabditis* sp., and infection of *Caenorhabditis elegans* in the laboratory. Five to 10 *Spirogyromyces*-infected nematodes were transferred to moist chambers as described, containing 10 disinfected, macerated mealworm larvae and 100 uninfected *Rhabditis* nematodes of different ages and sexes per dish. Uninoculated dishes were prepared the same way, but without adding infected nematodes. There were 10 replicates (Petri dishes) per treatment (inoculated and uninoculated nematodes). All dishes were incubated at room temperature. After nine days, whole paper disks from each dish (with the nematode colonies on them) were transferred separately to glass bottles with 200 ml of distilled water and vigorously shaken to suspend nematodes for counts and population estimation, using a Neubauer chamber under a compound microscope. Therefore, numbers of nematodes (i.e. population growth) were compared in *Spirogyromyces*-inoculated and non-inoculated moist chambers.

With identical methods, a colony of *Caenorhabditis elegans* (N2 strain) was started on Petri dishes and exposed to *Spirogyromyces* from 10 infected *Rhabditis* hosts, to induce infections in this host. Groups of 10 *Rhabditis* nematodes infected with the fungus were transferred to Petri dishes with colonies of thousands of uninfected *C. elegans* nematodes. These were produced as in the section on *Rhabditis* nematode rearing, above. To verify infection status, every five days five samples of nematodes from inoculated dishes were taken with a pin, and 40–50 nematodes were inspected under the microscope searching for fungal infections.

Results

***Spirogyromyces vermicola* Tzean & Barron.** (Tzean and Barron 1981)

Figure 2

Materials examined. MEXICO – **Coahuila** • Municipality of Saltillo; 25°21′42.5″N, 100°58′19.7″W; 1700 m elev.; 15.V.2015; S. R. Sanchez-Peña, leg; soil and litter samples in oak (*Quercus taeda* and *Q. saltillensis*) forest; UAN-2015-67.

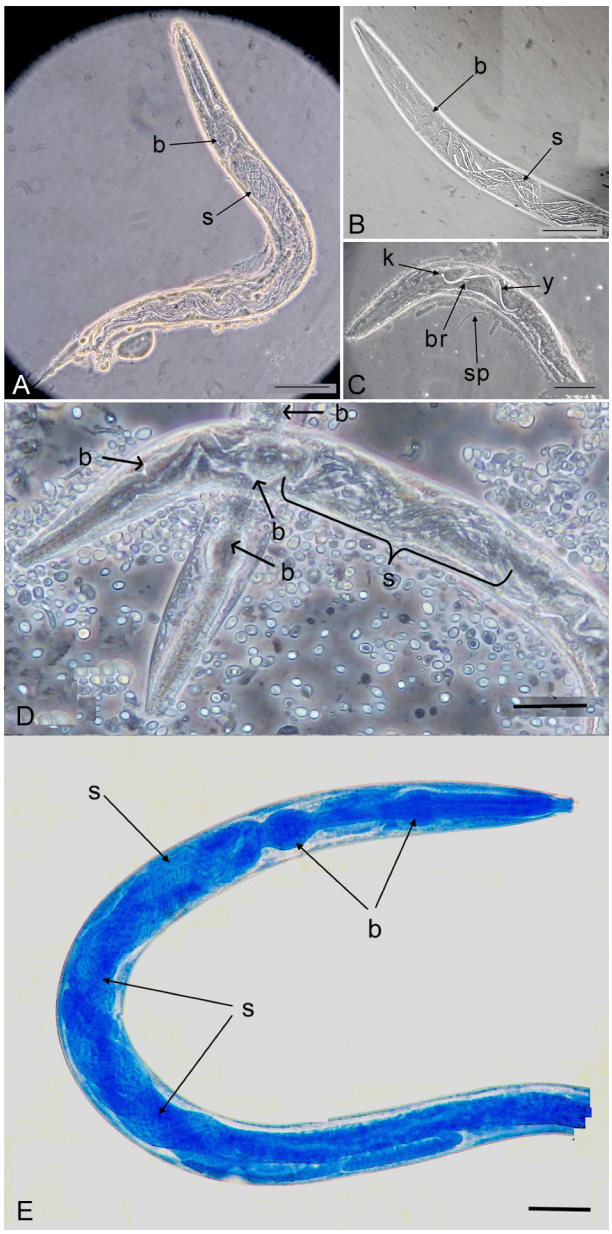

Figure 2. *Spirogyromyces vermicola*. **A.** General habitus of *S. vermicola* in the intestine of *Rhabditis* sp. nematode host. Profuse helicoidal growth of vegetative thalli (s) is visible in the intestine posterior to the esophageal bulb (b) of worm. **B.** General habitus of *S. vermicola* in the intestine of *Caenorhabditis elegans* nematode host. Profuse helicoidal growth of vegetative thalli (s) is visible in the space posterior to the esophageal bulb (b) of worm. **C.** Young thallus (y) of *S. vermicola* in intestine of rhabditid nematode host. Characteristic knob (k) on tip of vegetative thallus; b, one branch arising at septum of vegetative thallus; spores originate after elongation of these branches. Mature spore (sp) floating outside nematode host. **D.** Helicoidal growth (s) in *Caenorhabditis elegans*; two pharyngeal swellings (metacorpus and terminal bulb) (b) that differentiate *C. elegans* from *Rhabditis*; oval bodies around the worms are saprophytic yeasts from culture. **E.** Helicoidal growth in *C. elegans*; two pharyngeal swellings (b). A–D: phase contrast. E: cotton blue stain in bright field. Scale bars: A, B = 100 µm; C, E = 40 µm; D = 50 µm.

One soil sample yielded one colony of nematodes infected by *Spirogyromyces*. Our observations closely match the description and first record of the organism

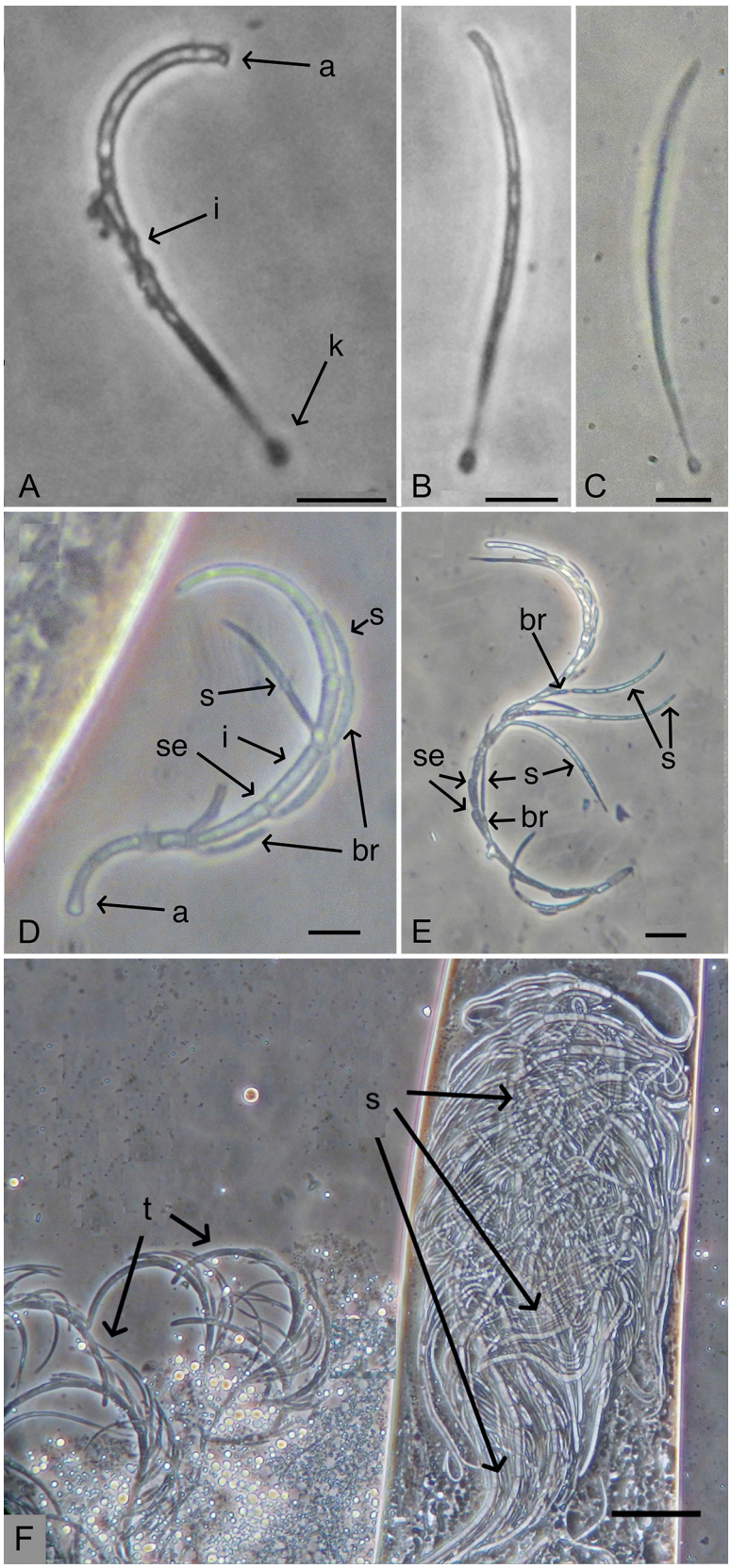

Figure 3. *Spirogyromyces vermicola.* **A–C.** Mature spores; apical "knob" (k) on cytoplasm-empty end of spore, distal regarding attachment to conidiophore; (a), attachment point of spore to gut lining proposed by Tzean and Barron (1981); this point has a barely visible attachment "beak"; (i), intercalary growth zone in spore. Equivalent to (i) in **D. D.** Young branching thallus; (a), point of attachment of spore to gut lining; (br), branches producing and bearing still immature spores (s) apically; (i), zone of intercalary elongation and division of the spore; (se), septum. **E.** Older branching thallus; (br), branches producing and bearing more mature, septate spores (s) apically; (se), septa and constrictions on main filament. **F.** Densely packed *S. vermicola* thalli (s) from ruptured intestine of rhabditid host. The helicoidal arrangement of the thalli is lost due to slide preparation and disruption of body wall and intestine. Several thalli (t) expelled from the worm. All phase contrast. Scale bars: A –C, E = 10 µm; D = 15 µm; F = 40 µm.

from a greenhouse at Guelph, Ontario, Canada (Tzean and Barron 1981). Specimens are preserved in formaldehyde and absolute ethanol. Preserved specimens were also confirmed as *S. vermicola* by Dr. Keith Seifert, Biodiversity Section (Mycology and Microbiology) Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada.

Identification of *Spirogyromyces*. Mature thalli of *S. vermicola* resemble the green alga *Spyrogyra* (Link) Nees (hence the name), and they fill to a considerable extent the intestine of infected nematodes, posterior to the pharynx (Fig. 2). Figure 3C–E shows developing thalli released from crushed infested hosts. Mature thalli produce abundant filiform, slightly bent (question mark-shaped) spores (conidia) (Fig. 3A, B). There are also nearly straight spores (Fig. 3C). Spores can usually be circumscribed in a rectangle measuring 40.7 (30.0–55.0) × 21.25 (11–24.5) µm (n = 20). They show a swollen area (a "knob") distal to their attachment point on the conidiophore (Fig. 3A–C). This knob is 1.5–2.5 µm in diameter; below the knob there is a neck or narrowing of the spore (0.7 µm in diameter). Spores are transversally septate (3–6 septa), divided in several compartments. Two or three compartments next to the swelling or knob are devoid of cytoplasm, and the remaining compartments contain live cytoplasm. Spores are released or voided into the environment most likely with the host's feces, where they are ingested by other nematodes. The blunt end of the spore is the attachment point to the gut lining (Fig. 3A, "a") proposed by Tzean and Barron (1981). This point has a barely visible attachment (the "beak"). After attachment, the ingested spore elongates. The zone of intercalary elongation and division of the spore is the (proximal) cytoplasm-containing part of the spore adjacent to the cytoplasm-empty section. Eventually the spores become an elongate, undulating filament (thallus) bearing multiple branches where spores are produced apically (Fig. 3D, E). Multiple fully developed thalli inside a host give the appearance of a multiple helix or braid (Fig. 1).

***In vivo* cultures.** We have maintained *Spirogyromyces in vivo* for more than four years in Petri dishes with *Rhabditis* sp. nematodes and bacteria proliferating on killed Mealworms as the nematode food source. By transferring a few live infected individuals to cultures of uninfected hosts, these non-axenic cultures have been kept with minimal saprophytic fungal growth. Our stocks of *Spirogyromyces*-infected *Rhabditis* were maintained without apparent abnormalities.

Effect of *Spirogyromyces* on population increase of *Rhabditis* sp., and infection of *Caenorhabditis elegans* in the laboratory. There were no significant differences in nematode population increase among the *Spirogyromyces*-inoculated and uninoculated dishes (mean = 37,000 and 47,000 nematodes/dish, respectively) (t-test, df = 19, p = 0.3511).

We successfully established *Spirogyromyces* infections in *C. elegans* (Figs. 2B, D, E) with inoculum originating from *Rhabditis* sp. This fungus-like organism was able to colonize the gut of this nematode. We did not observe any obvious differences in the morphology or growth pattern of *S. vermicola* in this host. Development of the nematode host was apparently normal. Infected *C. elegans* females were able to reproduce, since gravid females were observed, as in *Rhabditis*. However, infection appeared to be unstable in *C. elegans*, as infected nematodes were not observed before 13 days in *C. elegans* colonies. In contrast, generalized infections are observed after seven days in *Rhabditis* colonies. Furthermore, *C. elegans* populations in individual Petri dishes, infected by transfer of infected *Rhabditis* hosts, yielded infections by *S. vermicola* only for 15–20 days. Samples taken after this period were free of infected live nematodes. Only spores were present on the paper substrates of the colonies. Therefore, it appears that *S. vermicola* infections might not be self-sustaining in populations of this host. In comparison, infected colonies of *Rhabditis* sp. were maintained for at least four years, by serial transfer of infected hosts onto uninfected colonies.

Discussion

This is the second observation worldwide of *Spirogyromyces*, extending its known geographical distribution by more than 2000 km from Guelph, Ontario, Canada to Saltillo, Mexico (Fig. 1). The dissimilarity of the sites where it was collected—a tropical greenhouse in Ontario (Tzean and Barron 1981) and a xeric oak forest in Mexico—suggests that this organism may be widespread in soils with abundant leaf litter.

Tzean and Barron (1981) reported the fungal development in detail and described primary, secondary, and tertiary growth of the organism. Spores are ingested by nematodes and attach to the inner wall of the intestine. Tzean and Barron proposed that attachment occurs at the proximal end of the spore (see description). Primary growth consists of elongation of the ingested spores at their mid-region (elongation is not due to apical growth). This new growth produces septa, forming an undulating, septate hyphae. Primary growth follows and reflects the curving of the spore. The spore thus becomes a young multiseptated thallus. Secondary growth is the formation of short lateral branches next to septa. Each branch in turn produces one slightly or markedly curved, somewhat question mark-shaped spores (tertiary growth).

However, we suggest that the bulbous tip of spores appears to be involved in attachment to the intestine, as shown in Figure 1C and in the general thallus positioning in the nematode. It appears contradictory that the attachment point would be caudad and thallus elongation would then proceed cephalad. The opposite (cephalad attachment and caudad thallus elongation and spore release) seems more plausible, particularly

considering that spore release is posterior, through the anus, into the environment. Additional observations are required.

Similar to Tzean and Barron (1981), we observed that nematode females heavily colonized by *Spirogyromyces* (their intestine completely filled with thalli) are still able to reproduce in an apparently normal fashion. From our observations, *Spirogyromyces* is a mild parasite, a commensal or a non-essential symbiont, and rhabditid nematodes are its hosts in nature; *Rhabditis* sp. and two additional unidentified species of Rhabditida were readily infected in the laboratory (Tzean and Barron 1981). In the present work, we induced infections in the rhabditid *C. elegans*. Therefore, the organism seems to be non-specific, at least within the Rhabditidae.

Under the microscope the organism is reminescent of the arthropod-commensalistic Harpellales (e.g. *Smittium*) and Orphellales ("Trichomycetes"; now Kickxellomycotina in part) (Wijayawardene et al. 2018; Davis et al. 2019). Tzean and Barron (1981) also compared *Spirogyromyces* to the Trichomycetes, which are now considered an invalid polyphyletic assemblage of true fungi and protist-like organisms (Reynolds et al. 2017; Wijayawardene et al. 2018).

Similarities between the true fungal Harpellales and Orphellales, and *Spirogyromyces* are the habitat, which is the intestine of ecdysozoan invertebrates (Phillipe et al. 2005); its apparent innocuity towards hosts; the attachment of the fungal thallus to the gut lining; and the release of spores from the host gut into the environment. On the other hand, the host (nematodes vs. arthropods), the lack of spores with filaments (trichospores), and zygospores separate *Spirogyromyces* from the Harpellales and Orphellales (Tzean and Barron 1981). We think that it probably is a highly derived and specialized, true member of the Fungi, probably in the Kickxellomycotina. But it is also possible that *Spirogyromyces* is a non-fungal eukaryote, like the filamentous "Pseudofungi" (Heterokonta) (Cavalier-Smith and Chao 2006). Functionally, *Spirogyromyces* is similar to metazoan parasites of vertebrates like *Taenia* tapeworms parasitic on mammals, which release very large numbers of infective propagules into the environment (Garcia et al. 2003).

Infections in Rhabditidae, and particularly in *C. elegans*, introduce a completely new and unusual host–parasite system in model organism research. Additional surveys are required to better understand the geographical distribution of this organism, and to obtain additional isolates for experimental research on its host range and particularly, on its host–parasite interactions. These should unveil unique, novel mechanisms and interactions at the genetic, molecular, cellular and organismic levels. Also, phylogenetic molecular analysis of *Spirogyromyces* is required to elucidate its systematic and evolutionary position in the tree of life.

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Author Contributions

Conceptualization: SRSP, MSS. Formal analysis: MSS, SRSP, MFV. Funding acquisition: SRSP. Investigation: SRSP, MSS, MFV. Methodology: MSS, SRSP. Resources: ABC. Supervision: SRSP. Visualization: MSS, MFV. Writing – original draft: MSS, SRSP. Writing – review and editing: MSS, SRSP, MFV.

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