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Life where you least expect it: Biodiversity, abundance and prevalence of kleptoparasitic nematodes living inside the gastrointestinal tract of North American diplopods

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To the Graduate Council:

I am submitting herewith a dissertation written by Gary Phillips entitled "Life where you least expect it: Biodiversity, abundance and prevalence of kleptoparasitic nematodes living inside the gastrointestinal tract of North American diplopods." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Entomology, Plant Pathology and Nematology.

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Life where you least expect it: Biodiversity, abundance and prevalence of kleptoparasitic nematodes living inside the gastrointestinal tract of North American diplopods

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Dedication

This dissertation is dedicated to all that have supported me:

My family: Sunny and Claire Phillips

My Mom and Dad: James and LaVerne Phillips

My Brothers and their wives: Ronald, Suzette,

Gregory and Sheila Phillips

My Law Enforcement Colleagues:

Jerry and Karen Barnett

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Abstract

Millipede-parasitic nematodes belong to the infraorders Oxyuridomorpha and Rhigonematomorpha. Oxyuridomorpha contains two millipede-parasitic superfamilies (Thelastomatoidea and Coronostomatoidea). Rhigonematomorpha is exclusively parasitic in millipedes and also has two superfamilies (Rhigonematoidea and Ransomnematoidea). An 1853 monograph by Joseph Leidy is still the best reference to these nematodes in North America; currently, only 16 species have been recognized from temperate North American millipede fauna. Most are poorly characterized by today's standards and difficult to place. The primary goal of this research is a comprehensive taxonomic analysis of these nematodes and their specific host-parasite relationships with millipedes. Extensive redescription of nematodes within the millipede digestive tract was conducted utilizing both morphology and molecular analysis. Nematodes were dissected from the intestines of millipedes and studied with several different approaches. Species-level taxa from each millipede were sorted by live microscopic examination of various characters. Some nematodes were fixed in formalin and processed to glycerin for permanent mounts, while others were prepared for SEM and molecular analysis. In dissections undertaken so far, 972 millipedes have yielded 0–1,752 nematodes per specimen. Two families of nematodes appear to favor different regions of the intestine; thelastomatids are often encountered in the posterior gut, while rhigonematids are mostly observed in the midgut. Spirobolid millipedes harbor the greatest abundance and largest nematodes. Rhigonematids typically are more numerous but thelastomatids are more diverse, with at least 20 species found so far. The width of the body is a determining factor for nematode infestation; smaller millipedes, such as some parajulids and platydesmids, are devoid of nematodes. The

intestinal nematode fauna is primarily adult in July, with a rapid shift to almost completely juvenile nematodes by late summer and fall, suggesting these nematodes have one generation per year.

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Chapter 1

Kleptoparasitic and predaceous nematodes in Diplopoda

Abstract

Nematodes inhabit a wide range of ecological niches, including the gastrointestinal tracts of both vertebrates and invertebrates. The most commonly recognized nematodes are those that parasitize vertebrates; however, invertebrates also harbor several species of nematodes that are less recognized by nematologists and parasitologists. Nematodes that live in the intestine of millipedes are known as commensal kleptoparasites. Since the mid-1840s, only a handful of scientists have examined the relationship between millipedes and nematodes. Our current research focuses on the identification, abundance and discovery of new nematodes that live inside the intestines of both native North American and tropically introduced millipedes. Since 2013, 972 millipedes spanning six orders, 15 families and 55 species have been dissected and examined for nematodes and other intestinal and external parasites. Within the orders Oxyurida and Rhabditida, seven superfamilies of nematodes were identified inside the millipede intestine: Rhabditoidea, Diplogasteroidea, Thelastomatoidea, Coronostomatoidea, Rhigonematoidea, Ransomnematoidea and Dorylaimoidea. Nematodes inhabiting the millipede intestine predominantly belong to one of four families: Rhigonematidae (most abundant), Thelastomatidae (most diverse), Hethidae and Coronostomatidae (most restricted). Some millipedes harbored new species of nematodes, including *Coronostoma claireae*, n. sp. (Oxyurida: Coronostomatoidea: Coronostomatidae) from the indigenous and endemic Florida millipede*, Narceus gordanus*. Until recently, only six species of *Coronostoma* spp. have been reported from tropical spirostreptid millipedes and with one from an Australian cockroach. We also have collected at least two additional species of *Coronostoma* from two different orders of millipedes (Spirobolida and Polydesmida) in the southern Appalachian region. At least 20 other

species of nematodes await description or re-description from this research. Rhabditid and diplogasterid nematodes are found in the intestine only as dauer juveniles, but nematode loads ranged from 0–1,752, mostly located in the mid and hind intestine. Certain species of millipedes only have one species of nematode while other millipedes harbored as many as seven species. In general, female nematodes greatly outnumbered males, and during certain times of the year, juvenile nematodes are more abundant than adults. Morphometric analysis suggests that millipede body width may be a limiting factor in successful intestinal colonization.

Introduction

The phylum Nematoda encompasses a diversity of organisms that occupy nearly every environment on Earth (Maggenti, 1981; Bernard, 1991; Mai and Lyon, 1996; Borgonie et al., 2011). Nematodes live on all seven continents (Mai and Lyon, 1996) and free-living forms inhabit the deepest oceans (Nicholas, 1975), the highest mountains (Procter, 1984; Traunspurger et al., 2017) and live in all of our freshwater lakes and ponds (Gaudes et al., 2012). Those that live in terrestrial environments play important roles in nutrient recycling where they release ammonium into the soil, stimulate growth of prey organisms, disperse microbes, are food sources for other organisms (Neher, 2001) and provide nutrient transfer to plants (Perez-Moreno and Read, 2001). They also can have a negative impact on vertebrates and invertebrates in which they act as detrimental parasites, often times causing death to the host (Poinar, 1975; Anderson, 1983). Many nematodes parasitize both plants and animals, their diets consist of bacteria, fungi, plant roots and stems, other nematodes, and various microorganisms, such as bacteria, fungi, and protozoa. Currently, there are approximately 25,000 known nematode species, but total species estimates exceed one million (Nielsen, 2001; Hodda, 2011; Zhang, 2013).

Nematodes are non-segmented, pseudocoelomate, vermiform, bilaterally symmetrical organisms that are diagnosed by two synapomorphies (1) the presence of amphids (chemosensory organisms) and (2) their excretory–secretory system (Pechenik, 2005). Nematodes go through four molts before reaching adulthood, shedding their collagen-based cuticle in order to grow larger. They possess digestive, reproductive, nervous and secretory-excretory systems, but lack respiratory and circulatory systems; respiration takes place or occurs by gas exchange through the cuticle while the

pseudocoelom is responsible for the transportation of nutrients throughout the body. Somatic musculature is composed of only longitudinal muscles. Nematodes range in size from about 100 μm to 8 meters in length and most are sexually dimorphic.

Nematodes have a variety of reproductive strategies: syngamy (sexually - through the union of male and female pronuclei); pseudogamy (eggs develop after penetration of spermatozoa, which does not contribute genetic material to the developing embryo); hermaphroditism (the reproductive system first develops sperm and then switches to produce ova); and parthenogenesis (eggs develop without the assistance of spermatozoa) (Anya 1976). Male reproductive anatomy includes one or two testes, a seminal vesicle, and the vas deferens, which leads to, and opens into, the cloaca. Males are recognized through their modified cuticles called spicules, which function as copulatory vulva dilators when sexual reproduction occurs, as well as forming a passageway for spermatozoa. The female reproductive system consists of one or two ovaries, a seminal receptacle (spermatheca), a uterus, which can be monodelphic (a single genital tract) or didelphic (possessing two genital tracts), an ovijector (distal end of the uterus that forces the egg through the genital pore) and a vulva. Cobb (1929) observed that in many didelphic thelastomatid nematodes, a seminal receptacle was observed on one of two gonads, which he construed as hermaphroditism, thus explaining the lack of males in the family Thelastomatidae. Hermaphroditic nematodes first produce spermatozoa, store it in the seminal vesicle, and then convert to producing ova, which become fertilized. Adamson (1994) hypothesized that a primary characteristic of oxyurid nematodes that parasitize arthropods is haplodiploidy. Haplodiploidy is a condition where males are haploid and develop from unfertilized eggs, whereas females are diploid and develop from eggs that have been

fertilized. Because male nematodes develop from unfertilized eggs, they have half the number of chromosomes than the females.

The study of nematodes that primarily parasitize animals is called helminthology, while the study of nematodes that parasitize plants and other soils and marine organisms is called nematology (Hussey and Bernard 1975). The class Diplopoda (phylum Arthropoda), commonly called "millipedes," has a unique and diverse predatory nematode fauna living kleptoparasitically in their intestinal tracts. Nematodes exclusively living inside the intestines of millipedes belong to the infraorder Rhigonematomorpha while those that live in the intestines of millipedes and other arthropods belong to the infraorder Oxyuridomorpha. In the 1840s and 1850s, Joseph Leidy, a Medical Doctor, examined the flora and fauna of several species of arthropods including such millipedes as *Narceus annularis* Rafinesque, 1820 (order Spirobolida: family Spirobolidae), and he discovered a unique relationship between nematodes, primarily *Rhigonema infecta* Leidy, 1849, and the trichomycete *Enterobryus elegans* Leidy, 1850. Trichomycetes are commensalistic fungi that live inside arthropods intestines (Lichtwardt, 1986). Leidy (1849) observed that both the nematodes and trichomycetes appeared to exist within the millipede's intestinal tract without affecting its health or movement. The effects of oxyuridomorph and rhigonematomorph nematodes and trichomycetes on both millipedes and each other have not been investigated, although the humanly introduced European pest millipede, *Ommatoiulus moreleti* Lucas, 1860 (Julida: Julidae), is being somewhat managed in southern Australia with the parasitic nematode *Rhabditis necronema* Sudhaus and Schulte, 1989 (Bailey, 2016). This nematode, however, is not in the taxa presently under consideration.

Blaxter et al. (1998) identified five clades of nematodes (Table 1.1) (all subsequent tables and figures are located in Appendix 1). Nematodes that primarily parasitize millipedes belong to Clade III. Other phyla that host nematodes include Annelida (Poinar 1978; Spiridonov and Ivanova, 1998), Mollusca (Lopes et al., 2011) and Arthropoda (Basir, 1956; Skrjabin et al., 1951, 1954, 1966; Leibersperger 1960; Kloss, 1965; Poinar, 1975; Adamson and Van Waerbeke, 1992a-c). Nematodes that develop and mature in the intestines of millipedes native to the United States represent the spirurinan infraorders Oxyuridomorpha (ones that parasitize millipedes, insects and scorpions) and Rhigonematomorpha (those that parasitize millipedes exclusively) (De Ley and Blaxter, 2004, 2015) (Table 1.2). The oxyuridomorphs belong to the superfamily Thelastomatoidea, also found in insects and scorpions (Baruš and Koubková, 2002), and are typically nonpathogenic to the hosts. They are characterized by the presence of eight cephalic papillae (labiopapillae), rounded or oval amphids, and males with one, or no spicules. Rhigonematomorphs, obligatory kleptoparasites of millipedes, typically have four cephalic papillae, a basal bulb with a grinding valve, and a muscular esophagus. In females, the vulvae are located in the mid-body or posterior region; males usually have two spicules.

A brief history of the nematodes in arthropods

Karl Hammerschmidt was the first European scientist to clearly illustrate the common nematode, *Hammerschmidtiella diesingi* Hammerschmidt, 1838 (Chitwood, 1932; Carreno, 2014). The first American scientist to describe nematodes inhabiting the hindgut of millipedes was Joseph Leidy (1849, 1853). Leidy (1849) erected the genus *Thelastoma* to accommodate *T. attenuatum* Leidy, 1849 from the spirobolid millipede, *Julus marginatus* (=*Narceus annularis* Rafinesque, 1820) and described two other nematodes from the same

millipede: *Rhigonema infecta,* originally assigned to *Ascaris*, and *Aorurus agile,* originally assigned to *Streptostomum*. A few years later, Leidy (1850) described *Thelastoma labiatum* from the xystodesmid polydesmidan now known as *Apheloria virginiensis* (Drury, 1770) (Shelley et al., 2017).

More oxyurids parasitizing arthropods were described and illustrated in the early 20th century, as detailed by Carreno (2014) in a comprehensive history of Thelastomatoidea. Parasitologists at this time concluded that nematodes used arthropods as definitive hosts instead of being free-living organisms that inhabited them incidentally (Travassos, 1920, 1929; Cobb, 1929; Leibersperger, 1960). Classifying nematodes has proven to be problematic because of disagreement on phylogenetically useful characters, as evidenced by the different groupings and names applied to familial, subfamilial, and generic-level taxa (Travassos, 1920, 1929; Baylis and Daubney, 1926; Poinar, 1977; Adamson and van Waerebeke, 1992a, 1992b, 1992c; Carreno, 2014). The basic separation of oxyurid and rhigonematid nematodes was determined by Artigas (1930), and three decades later, Kloss (1960) proposed the superfamilies Thelastomatoidea and Rhigonematoidea. The former now comprises five families: Hystrignathidae, Protrelloidae, Pseudonymidae, Thelastomatidae and Travassonematidae (Adamson, 1989). Adamson and van Waerebeke (1992a, 1992b, 1992c) accepted this arrangement, which remains the accepted taxonomy for these nematodes.

Most millipede-parasitic nematodes have been described from (sub)tropical diplopods, with M. Adamson, R. A. Carreno, D. J. Hunt, G. R. Kloss, S. V. Malysheva, S. E. Spiridonov, and D. van Waerebeke being prolific authors. Investigations of these nematodes in temperate regions are less numerous and confined largely to North America

and Australia. In North America, Wright (1979) recorded *Rhigonema* sp., *Johnstonia* sp., and *Aorurus* sp. from intestines of *N. annularis*; Adamson (1981) re-described *Rhigonema infecta* from the same; Upton et al. (1983) described *Thelastoma collare* from the anterior hindgut of the desert millipede, *Orthoporus ornatus* Girard, 1853 (Spirostreptida: Spirostreptidae); Poinar (1986) described *Rhabditis myriophila* from the adventive garden millipede *Oxidis gracilis* Koch, 1847 (Polydesmida: Paradoxosomatidae); Carreno (2007) discovered *Thelastoma krausi* in the intestine of *Euryurus leachii* Gray, 1832 (Polydesmida: Euryuridae); and most recently, Phillips and Bernard (2016) described *Coronostoma claireae* from *Narceus gordanus* Chamberlin, 1943 (Spirobolida: Spirobolidae), constituting the first report of *Coronostoma* from North America.

Currently, there are nine genera of kleptoparasitic and predaceous nematodes associated with North American millipedes: *Rhigonema* Cobb, 1898, *Coronostoma* Rao, 1958, *Thelastoma* Leidy, 1849, *Heth* Cobb, 1898, *Carnoya* Gilson, 1898, *Aorurus* Leidy, 1849, *Johnstonia* Basir, 1956, *Aoruroides* Travassos and Kloss, 1958, and *Stauratostoma* Phillips and Bernard, 2017. For the purposes of defining North America, we include Canada, Greenland, Caribbean Islands, Alaska, the continental United States and Mexico. In addition, several species of Rhabditidae, Diplogasteridae and Dorylaimida are sometimes associated with these arthropods, as both phoretics and internal inhabitants.

Problem statement

The internal flora and fauna of North American millipedes have been significantly understudied; approximately 16 nematode species have been described from their intestinal faunas. Joseph Leidy recognized the parasitic relationship between nematodes and millipedes more than 160 years ago, and his comprehensive synthesis (1853) remains

the key publication on these organisms. Since then, fewer than a dozen researchers have published useful accounts of this association in temperate North America. Our ongoing intensive survey of these nematodes demonstrates that this temperate nematode fauna is diverse, with numerous undescribed species and several new genera.

Research questions

1. Which nematodes inhabit millipede digestive tracts in continental North America?
2. Do the same species of nematodes inhabit the digestive tract of allopatric or parapatric congeneric millipede species, e.g. *Choctella cumminsi* Chamberlin, 1918 and *C. hubrichti* Hoffman, 1965?
3. Do nematodes interfere with the health and movement of millipedes?
4. Do all millipedes harbor nematodes, is there a morphometric limit to host nematodes?
5. Do other organisms inhabit millipede digestive tracts?
6. Do nematodes cluster in specific areas of the intestine?
7. Are nematode males, females, and juveniles equally distributed in millipede's intestines?
8. Do commercially reared millipedes or those available from biological supply houses contain nematodes?
9. If millipedes are purchased from such facilities, do they have the potential to harbor undescribed species and can they be used by advanced placement (AP) or honors biology students in a high school setting as potential organisms for general nematode studies?

10. Can exotic nematodes found in purchased exotic millipedes become introduced into native species?
11. What are the phylogenetic relationships between nematodes discovered in millipede intestines?

Objectives

The specific objectives of this research are to (1) determine the biodiversity of nematodes in the intestines of millipedes by conducting a biogeographical survey of millipede-inhabiting nematodes in the United States; (2) determine if different nematode species are found in congeneric millipedes; (3) observe if there are any detrimental effects on the overall health of millipedes when parasitized by nematodes; (4) determine if there is a correlation between millipede size and the presence or absence of nematodes; (5) observe if other organisms parasitize millipedes; (6) document areas within the intestine that nematodes congregate or are found in more, or less, abundance; (7) understand the nematode distribution pattern based on developmental stage while inside the millipede intestine; (8) evaluate commercially available millipedes to determine if millipedes bred and/or maintained in captivity harbor nematodes; (9) develop a teaching curriculum for advanced placement high school students to bring more attention to the biodiversity of both arthropods and nematodes; (10) conduct experiments to determine if exotic nematodes found in purchased exotic millipedes can become introduced into native species; and, (11) analyze kleptoparasitic nematodes molecularly using 28S LSU rDNA.

Materials and methods

Millipedes were collected, identified, and measured prior to dissection. The intestines were removed and examined for nematodes and other parasites. Nematodes

were identified and sorted into morphotaxa, including developmental stages (males, females and juveniles). Representative nematode specimens were killed in hot (60–70˚C) 4% formalin and preserved for microscopic analysis. Brightfield, differential contrast, phase contrast and scanning electron microscopies were used to examine anatomical characters. Molecular analyses were conducted on single nematodes immediately after extraction from the intestine. DNA extraction was accomplished with a Qiagen Blood and Tissue Kit #69506 (Waltham, MA) and PCR products were amplified with 28S LSU rDNA primers designated as LSU391F and LSU501R (Nadler et al., 2006). PCR products were electrophoresed in a 1% agarose gel, bands were excised and cleaned and sent to the University of Tennessee Genomics Core for sequencing. Phylogenetic trees were constructed from a partial sequence of 28S LSU rDNA with maximum parsimony using Paup*(Swofford, 2002). Node support was evaluated using parametric bootstrapping. Statistical analyses of millipede and nematode morphometrics and total nematode loads was conducted with *t*-tests and a randomized block design. Data was subsequently modeled with Poisson regression. Significant effects were identified at $p < 0.05$ and data analyses were conducted using SAS9.4 TS1M3.

Summary results

Between January 2013 and September 2017, 969 millipedes spanning six orders, 17 families and 53 species were collected from 20 states (Table 1.3, Fig. 1.1). A total of 63,203 nematodes from four superfamilies and nine families were extracted from millipede intestines. The distribution of specimens among nematode families were Rhigonematidae (62%), Hethidae (19%), Thelastomatidae (18%), Coronostomatidae (0.2%) and others consisting of unknown juveniles, Dorylaimidae, Diplogasteridae and Rhabditidae (1%) (Fig.

1.2). Approximately 20 new species of nematodes have been recognized and at least three new genera will be described in subsequent reports.

Millipedes with a width or body diameter less than 2 mm rarely have nematodes, while those with diameters of 2 mm or more often harbor them (Fig. 1.3). Of the 969 millipedes dissected, 261 (26.9%) did not have any intestinal nematodes; 559 (57.7%) had 1–100; 94 (9.7%) had 101–250; 27 (2.8%) had 251–500; 17 (1.8%) had 501–1,000; and 12 (1.2%) had 1,001–1,752 (Fig. 1.4). Total nematode loads per millipede ranged from 0–1,752. The highest densities of nematodes occurred in the pyloric area between the mid- and hindguts.

Thelastomatids were most often encountered in the hindgut, while rhigonematids were primarily in the pyloric region of the midgut. In many instances, both families were located in both the mid- and hindguts. No nematodes were observed in the foregut. The largest nematodes, reaching up to 10 mm in length, were *Rhigonema* spp.; while the smallest, males of *Thelastoma* spp. and *Coronostoma* spp., were less than 1 mm in length. Juveniles outnumbered adults of both sexes in Rhigonematidae, Hethidae and Coronostomatidae; female thelastomatids outnumbered juveniles, and females were more prevalent than males in all families. In general, females of all species tended to be larger than males; however, males of most *Thelastoma* spp. were not observed, and males and juveniles of *Coronostoma* spp., *Aorurus* spp. and *Aoruroides* spp. were infrequently encountered. Males were easily detected by their spicules (Fig. 1.15).

The mean number of nematodes per millipede suggested that juveniles consistently outnumbered males and females throughout the year except for a brief period in July. Juvenile populations declined between January and July and increased from July through

December, whereas adult populations of both sexes increased slightly in September and declined in January. These curves, reflecting the sharp numerical increase in juveniles between July and December, suggested that nematode reproduction occurs between July and September (Fig. 1.5).

Kleptoparasitic genera identified in North American millipedes during this study were *Aorurus*, *Aoruroides*, *Coronostoma*, *Heth*, *Oscheius* Andrassy, 1976, *Rhigonema*, *Stauratostoma*, and *Thelastoma*. There were also other Rhabditidae, a new genus of Dorylaimidae related to *Labronemella* Andrassy, 1985, an undescribed species of *Diplogasteroides* de Mann, 1912 (Diplogasteridae), and approximately 10 other unknown taxa. For instance, the spirobolid millipede *N. gordanus* consistently harbored an enigmatic nematode that may be a representative of *Carnoya* (superfamily Ransomnematoidea).

The highest concentrations of nematodes per millipede order were in Spirobolida, followed by Polydesmida, Spirostreptida, and Callipodida; Platydesmida and Julida did not contain any nematodes (Fig. 1.6). Of the Spirobolida, one specimen of *N. gordanus* contained 1,752 nematodes; one of Polydesmida, *Sigmoria* (*Falloria*) *ainsliei* Chamberlin, 1921, had 418; one of Spirostreptida, *O. ornatus*, contained 63; and one specimen of Callipodida, *Abacion magnum* Loomis, 1943, had 61.

During this study, two pairs of congeneric millipede species, apparently separated by distinct geographical barriers, were examined to determine if the nematode fauna was significantly different. *Choctella cumminsi* and *C. hubrichti* are separated by the Tennessee River, the former, living to the north in central Tennessee and northern Alabama, and the latter living to the south and as metropolitan Birmingham. Both harbored primarily one species of *Thelastoma*, but they differed anatomically in the configurations of the heads,

tails, and overall lengths, as well as being genetically different. One individual of each of these millipedes had two species of intestinal nematodes, a juvenile *Rhigonema* sp. in *C. cumminsi* and six adult female *Aorurus* sp. in *C. hubrichti*.

A second congeneric pair of millipedes compared the nematofaunas of *N. gordanus* and *N. americanus*. The former is endemic to xeric scrub environments in peninsular Florida, whereas *N. americanus* occurs in eastern Canada (southern Québec and eastern Ontario), all states east of the Mississippi River, and ten west of the watercourse (Minnesota, Iowa, Missouri, Arkansas, Louisiana, South Dakota, Nebraska, Kansas, Oklahoma, and Texas) (Fig. 1.9) (Shelley et al., 2006; Shelley and Snyder, 2012).

The two *Narceus* spp. were compared by means of *t*-tests to determine significant differences in weight, length and width. Morphometric measurements were recorded for each specimen and the weight, width, and length of each millipede was recorded. *Narceus gordanus* was heavier ($p<0.0001$), wider ($p<0.0001$), and longer ($p<0.0001$) than *N. americanus*. Poisson regression was used to investigate the effects of these millipedes on the abundance of nematodes. Significantly fewer rhigonematids, thelastomatids, *Aorurus* spp., and *Coronostoma* spp. occurred in the intestines of *N. americanus* compared to those of *N. gordanus;* likewise, *N. americanus* contained 16.3% of the total nematode load of *N. gordanus* ($p<0.0001$). Most major groups of nematodes were less abundant in *N. americanus* than in *N. gordanus*. When compared to *N. gordanus*, *N. americanus* had about 37% of Rhigonematidae ($p<0.0001$) loads as *N. gordanus*, 16% of the Thelastomatidae loads ($p<0.0001$), and 5% of *Coronostoma* spp. loads ($p<0.0001$), and 73% of *Aorurus* spp. loads ($p<0.0004$). *Heth* sp. and *Carnoya* sp. were not found in *N. americanus.* Nematode faunas were similar in that both millipedes contained species of *Rhigonema*, *Aorurus,*

Coronostoma, and *Thelastoma;* however, only *N. gordanus* contained representatives of Ransomnematoidea (two spp.), *Heth* sp. and a possible *Carnoya* sp. The *Heth* species is being described (see Chapter 4). The identification of ostensible representatives of *Carnoya* is tentative and requires further anatomical and molecular analyses.

Millipedes purchased from biological supply companies were examined to characterize their nematofauna. Four specimens of *Archispirostreptus gigas* Peters, 1855 and three specimens of *Orthoporus ornatus* were purchased for dissection from Wards Science, Rochester, New York, (permit number P526P-14-03587). One specimen of *A. gigas* yielded 853 nematodes, including two undescribed species of *Coronostoma* and a species of *Brumptaemilius* Dollfus, 1952. Two of the three specimens of *O. ornatus* yielded nematodes, which were identified as *Thelastoma collare* Upton, Crawford and Hoffman, 1983.

Phylogenetic analyses were conducted on a data set including 61 different nematodes, including 22 obtained from GenBank and 39 from this study, the latter extracted exclusively from North American millipedes. Phylogenetic trees were constructed from a portion of 28S LSU rDNA using maximum parsimony as implemented in PAUP* (Swofford, 2002). The data set was comprised of 1,279 aligned characters, 561 of which were parsimony-informative. A heuristic search comprised of one thousand random addition searches using tree bisection and reconnect (TBR) branch swapping was conducted. Node support was evaluated by parametric bootstrapping using 10,000 replicates, each consisting of a single random addition replicate. Twelve most parsimonious trees of 3,013 steps (CI=0.395; RI=0.696; HI=0.605) residing on a single island were found in every search. A majority rule consensus of these MPTs is shown as Fig. 1.10.

Strongyluris Müller, 1894, *Ascaris* Linnaeus, 1758, and *Contracaecum* Railliet and Henry, 1912, all vertebrate parasites, were selected as outgroups. Three described *Rhigonema* spp. were used from the National Center for Biotechnology and Information (NCBI) to compare seven undescribed *Rhigonema* spp. collected during this project. All *Rhigonema* spp. that were extracted during millipede dissections grouped together in a strongly supported monophyletic clade within the superfamily Rhigonematoidea. *Heth* spp., including *H. pivari* Phillips and Bernard, 2017 (see Chapter 4), formed a monophyletic group in the superfamily Ransomnematoidea (Carnoyidae + Hethidae), which consisted of other *Heth* spp., *Carnoya* spp., *Brumptaemilius* spp., *Cattiena* sp., Hunt and Spiridonov, 2001, *Insulanema longispiculatum* Malysheva, Van Luc and Spiridonov, 2012, and *Travossosinema* Rao, 1958. Relationships among other millipede-inhabiting nematodes were less well resolved. *Coronostoma claireae* (Chapter 2) paired with an undescribed *Coronostoma* spp. discovered in North America during this project and this genus formed a monophyletic clade also containing *Aorurus* spp., an unknown juvenile, *Stauratostoma shelleyi*, and several species of *Thelastoma*. Two unique thelastomatid-like nematodes nicknamed “Notch-Head” and “Flat Top” grouped with *Travassosinema* spp. but each likely represent distinct new genera based on morphological characters, but more work is needed to fully resolve their affinities.

Ciliated protists, trichomycetes, bacteria, and gregarines were observed in the intestines, and organisms in the hemocoel were the acanthocephalan *Macracanthorhynchus ingens* von Linstow 1879, hymenopteran eggs and dipteran eggs, probably belonging to Sciomyzidae or Phoridae. Phoretic mites (Acari: Mesostigmata) were routinely observed on millipede venters and male gonopods. Phoretic rhabditid nematodes were encountered on

exoskeletons and between diplosegments. Images of bacteria, trichomycetes, and gregarines are shown in Figs. 1.11–1.14.

Discussion

The kleptoparasitic intestinal nematodes of millipedes are understudied. The distribution of millipede-inhabiting nematode species is poorly known. Nearly every paper published on this interaction has been ad hoc, describing new nematode species as one or more millipede specimens have become fortuitously available. Consequently, there have been many species described but little attempt at synthesis or revision of genera. With between 10,000–12,000 known species of millipedes (Blower, 1985), the magnitude of nematode diversity living inside millipede hosts is surely substantially underestimated. Sixteen extant orders of millipedes exist worldwide today, of which 11 occur in the continental U.S.: Polyxenida, Glomerida, Platydesmida, Polyzoniida, Siphonophorida, Chordeumatida, Callipodida, Polydesmida, Spirostreptida, Spirobolida, and Julida. Our research focused on six of the larger-bodied orders, since preliminary research or experience showed that individuals <2mm wide rarely contain intestinal nematodes. Therefore, the larger Callipodida, Polydesmida, Spirostreptida, and Spirobolida have been the taxa of most interest. We did not examine significant numbers of Polyxenida, Glomerida, Platydesmida, Polyzoniida, Siphonophorida, Chordeumatida, or Julida, most of which are smaller species. Efforts are needed to verify the presence or absence of nematodes in these taxa and to validate the 2-mm-diameter hypothesis. In North America, two *Rhigonema* spp. have been described from millipedes: *Rhigonema infecta* (synonyms *R. nigella* Thomas, 1930, and *R. robusta* Walton, 1928 (Dollfus, 1952; Crites, 1965). Based on both anatomical and molecular assessments, we have discovered at least four additional

new species that await description, indicating a much greater diversity of *Rhigonema* in North American millipedes than the literature shows and one comparable to indigenous faunas in tropical Asia (Hunt, 1998a; Hunt, 1998b; Hunt, 1999; Hunt, 2015.)

Worldwide, seven *Coronostoma* spp. have been described (Chapter 2), mostly from the intestines of spirostreptidan millipedes: *C. singhi* Rao, 1958, from India;, *C. bulbicorpus* Kloss, 1961, from Brazil; *C. diplopodicola* Dollfus, 1964, from the Democratic Republic of the Congo; *C. dentata* van Waerebeke and Adamson, 1986, from Madagascar; *C. gautuni* van Waerebeke and Adamson, 1986, from Burkina-Faso; *C. australiae* Jex, Schneider, and Cribb, 2005 from an Australian cockroach; and *C. claireae* from a Floridian *N. gordanus*. Six of these are from tropical regions of Africa, Brazil, and Australia. Our research has yielded four additional, undescribed species, two from temperate North America (Tennessee and Alabama) and two from a purchased African (Tanzania) specimen of *Archispirostreptus gigas*. These discoveries are significant because they reveal that nematode taxa thought to be restricted to the tropics may be diverse in temperate zones as well. Their discoveries further indicate that the millipede nematofauna in North America has not been deeply investigated and warrants continued effort.

Another focus of this research has been the development of a high-school-level experiential biodiversity-learning module using millipedes and the parasites within them (Chapter 5). Suitable millipedes can be collected in many parts of the country, but in some northern or more arid states they may be difficult to find. Large, common millipedes of several species can be ordered from supply houses at reasonable costs for student use. This approach will allow both rural and urban students to investigate the nature of host-parasite relationships as exemplified in millipedes. For instance, dissection of the

spirostreptidan *A. gigas*, purchased from a biological supply house, yielded 853 nematodes including two undescribed species of *Coronostoma* and a possible new species of *Brumptaemilius*. The overall paucity of knowledge about nematodes in millipedes may even result in new discoveries and range extensions by students investigating parasitism inside their intestines.

Molecular phylogenetic analysis showed Ransomnematoidea as the sister group to Rhigonematoidea plus Thelastomatoidea (Oxyuridomorpha). *Heth pivari* is placed in a lineage with other *Heth* spp., which is nested within sampled Carnoyidae (*Brumptaemilius* spp., *Cattiena fansipanis*, and *Insulanema longispiculum*). North American *Coronostoma* consistently placed near *Stauratostoma shelleyi, Aorurus* spp., and *Thelastoma* spp.

Conclusion

Millipedes are excellent organisms to explore the biodiversity of new life. Nematodes are easily found inside the intestinal tracts and the probability of discovering new species is high. The larger the millipede, the higher the probability will be of discovering kleptoparasitic nematodes. Spirobolid, spirostreptid and polydesmid millipedes offer the best chances of high nematode loads.

In addition to examining the diversity, abundance and prevalence of intestinal nematodes this approach provides a unique opportunity for students, researchers and other professionals to observe other parasitic organisms associated with millipedes. Ciliated protists, gregarines, bacteria, fungi, trichomycetes, and insects can be studied as well. Such study is not restricted to inhabitants of millipedes; students can examine other organisms as potential nematode hosts, especially cockroaches and beetles, to investigate the diversity of their internal nematode faunas.

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Appendix 1

Table 1.1. Nematode clades modified from Blaxter et al. (1998).

Clade	Trophic Group	Taxonomic Rank
I	Bacterivore, Algivore–omnivore–predator	Monochida
I	Invertebrate parasites	Mermithida
I	Algivore–omnivore–predator, Plant parasites	Dorylaimida
I	Vertebrate parasites	Trichocephalida
II	Plant parasites	Triplonchida
II	Bacterivore, Algivore–omnivore–predator	Enoplida
III	Vertebrate parasites	Ascaridida
III	Invertebrate parasites	Rhigonematida
III	Vertebrate parasites	Spirurida
III	Vertebrate, invertebrate parasites	Oxyurida
IV	Fungivore, Plant parasites, Invertebrate parasites	Tylenchida
IV	Fungivore, Plant parasites, Invertebrate parasites	Aphelenchida
IV	Bacterivore	Rhabditida: Cephalobidae
IV	Entomopathogen	Rhabditida: Steinernematidae
IV	Bacterivore	Rhabditida: Panagrolaimidae
IV	Vertebrate parasites	Rhabditida: Strongyloididae
V	Bacterivore, Algivore–omnivore–predator	Diplogasterida
V	Bacterivore, Entomopathogen, Invertebrate parasites	Rhabditida
V	Vertebrate parasites	Strongylida

Table 1.2. Clade III nematodes found in millipedes native to the United States. Phylum Nematoda, Class Chromadorea, Order Rhabditida, Suborder Spirurina (Clade III) (Carreno, 2014; Phillips & Bernard, 2016).

Infraorder	Superfamily	Family
Oxyuridomorpha		
	Oxyuroidea (Vertebrate pinworms)	
		Atractidae
		Heteroxynematidae
		Oxyuridae
		Pharyngonidae
	Thelastomatoidea (Invertebrate pinworms)	
		Hystrignathidae
		Protrelloididae
		Thelastomatidae
		Pseudonymidae
		Travassosinematidae
	Coronostomatoidea	
		Coronostomatidae
		Traklosiidae
Rhigonematomorpha		
	Rhigonematoidea	
		Ichthyocephalidae
		Rhigonematidae
	Ransomnematoidea	
		Carnoyidae
		Hethidae
		Ransomnematidae

Table 1.3. Taxonomic ranks of collected millipedes.

Order	Family	Genus and species
Callipodida	Abacionidae	*Abacion magnum* (Loomis, 1943)
Julida	Blaniulidae	*Blaniulus guttulatus* (Fabricius, 1798)
	Parajulidae	*Oriulus venustus* (Wood, 1864)
		Ptyoiulus impressus (Say, 1821)
	Paeromopodidae	*Californiulus yosemitensis* Chamberlin, 1941
Platydesmida	Andrognathidae	*Brachycybe lecontii* Wood, 1864
Polydesmida	Euryuridae	*Euryurus* sp.
		Euryurus leachii (Gray, 1832)
		Euryurus leachii fraternus Hoffman, 1978
	Nearctodesmidae	*Nearctodesmus* sp.
	Paradoxosomatidae	*Oxidus gracilis* (Koch, 1847)
		Orthomorpha coarctata DeSaussure, 1860
	Polydesmidae	*Pseudopolydesmus canadensis* (Newport, 1844)
		Pseudopolydesmus sp.
	Xystodesmidae	*Apheloria* sp.
		Apheloria montana (Bollman, 1888)
		Apheloria tigana Chamberlin, 1939
		Apheloria virginiensis (Drury, 1770)
		Boraria deturkiana (Causey, 1942)
		Brachoria sp.
		Brachoria initialis Chamberlin, 1939
		Brachoria ochra (Chamberlin, 1918)
		Cherokia georgiana (Bollman, 1889)

Table 1.3. Continued. Taxonomic ranks of collected millipedes.

Order	Family	Genus and species
Polydesmida	Xystodesmidae	*Cherokia georgiana latassa* Hoffman, 1960
		Dicellarius talapoosa (Chamberlin, 1939)
		Harpaphe haydeniana (Wood, 1864)
		Nanaria sp.
		Pachydesmus crassicutis (Wood, 1864)
		Pleuroloma cala (Chamberlin, 1939)
		Pleuroloma flavipes Rafinesque, 1820
		Sigmoria sp.
		Sigmoria (*Falloria*) *aphelorioides* Shelley, 1986
		Sigmoria (*Cheiropus*) *planca* (Loomis, 1944)
		Sigmoria (*Falloria*) *ainsliei* (Chamberlin, 1921)
		Sigmoria (*Falloria*) *mimetica* (Chamberlin, 1918)
Spirobolida	Floridobolidae	*Floridobolus floydi* Shelley, 2014
		Floridobolus penneri Causey, 1957
	Rhinocricidae	*Anadenobolus monilicornis* (Porat, 1876)
	Spirobolidae	*Chicobolus spinigerus* (Wood 1864)
		Narceus americanus (Palisot de Beauvois, 1817)
		Narceus annularis (Rafinesque, 1820)
		Narceus gordanus (Chamberlin, 1943)
		Tylobolus sp.
		Tylobolus uncigerus (Wood, 1864)
	Trigoniulidae	*Trigoniulus corallinus* (Gervais, 1847)
Spirostreptida	Cambalidae	*Cambala annulata* (Say, 1821)
	Choctellidae	*Choctella cumminsi* Chamberlin, 1918
		Choctella hubrichti Hoffman, 1965
	Spirostreptidae	*Archispirostreptus gigas* (Peters, 1855)
		Orthoporus ornatus (Girard, 1853)

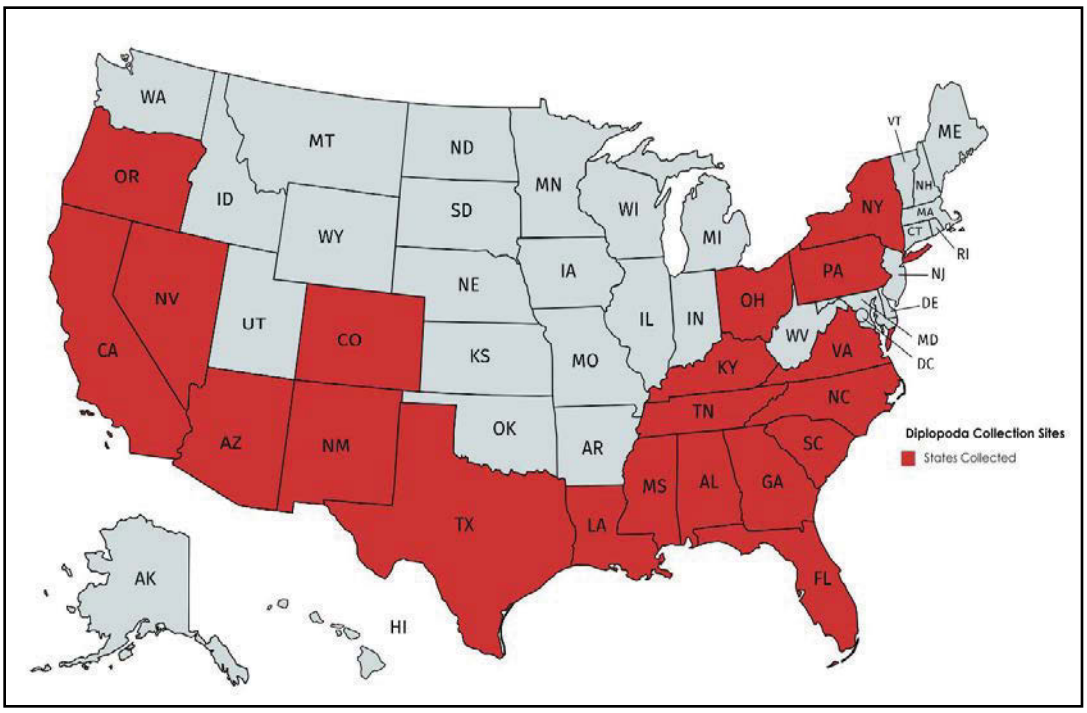

Fig. 1.1. States where millipedes have been sought and collected. Some states did not produce any millipedes, such as Nevada. Other states, such as Texas, New Mexico and Arizona, had relatively few millipedes due to the arid habitats. Colorado millipedes were generally small and did not produce any nematodes.

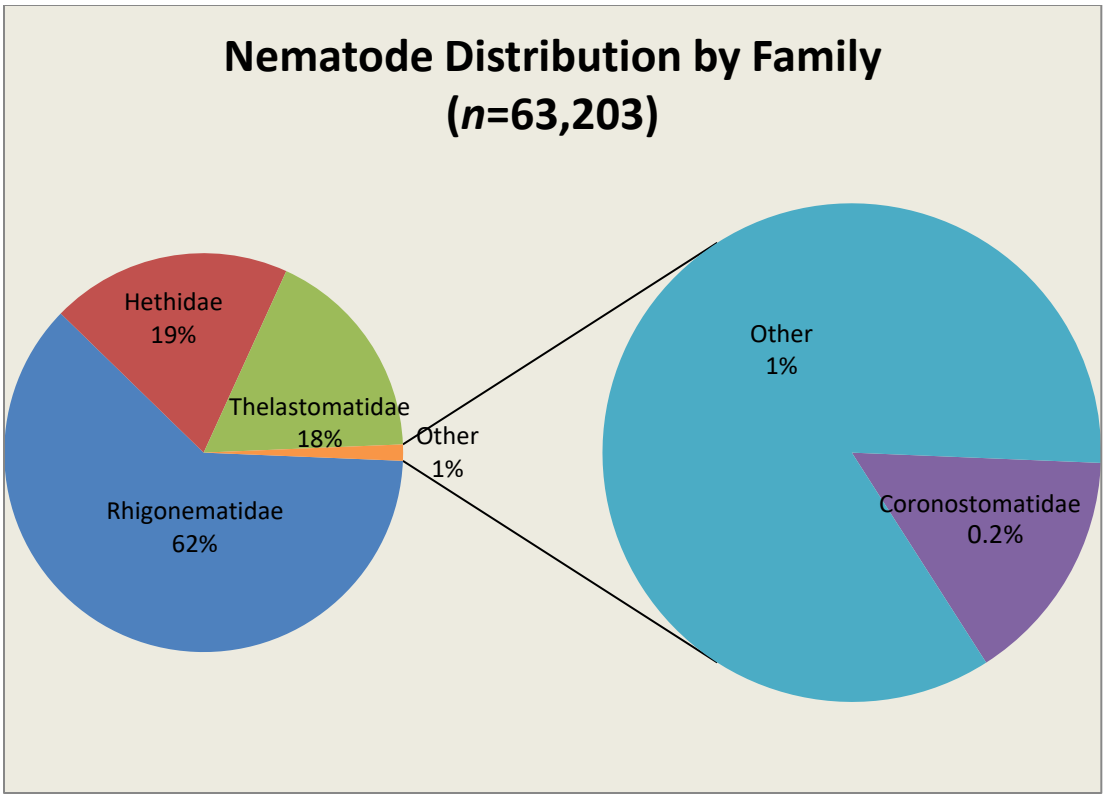

Fig. 1.2. Nematode distribution by Family. Other consisted of unknown juveniles, Rhabditida, Diplogastridae and Dorylaimida. Rhigonematids were most abundant, thelastomatids most diverse, Hethidae was only found in one species of millipede, *Narceus gordanus*, and *Coronostoma* spp. were rarely encountered.

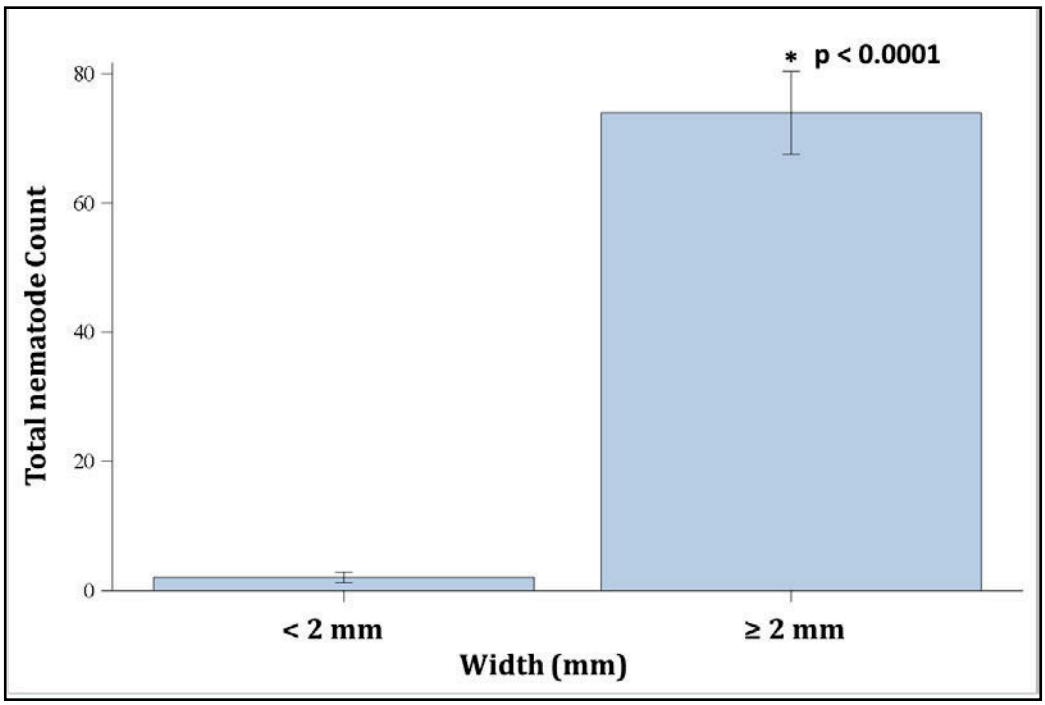

Fig. 1.3. Effect of millipede width on the presence or absence of nematodes. Millipedes < 2mm usually do not contain nematodes, whereas those > 2 mm have a significantly higher probability of containing nematodes.

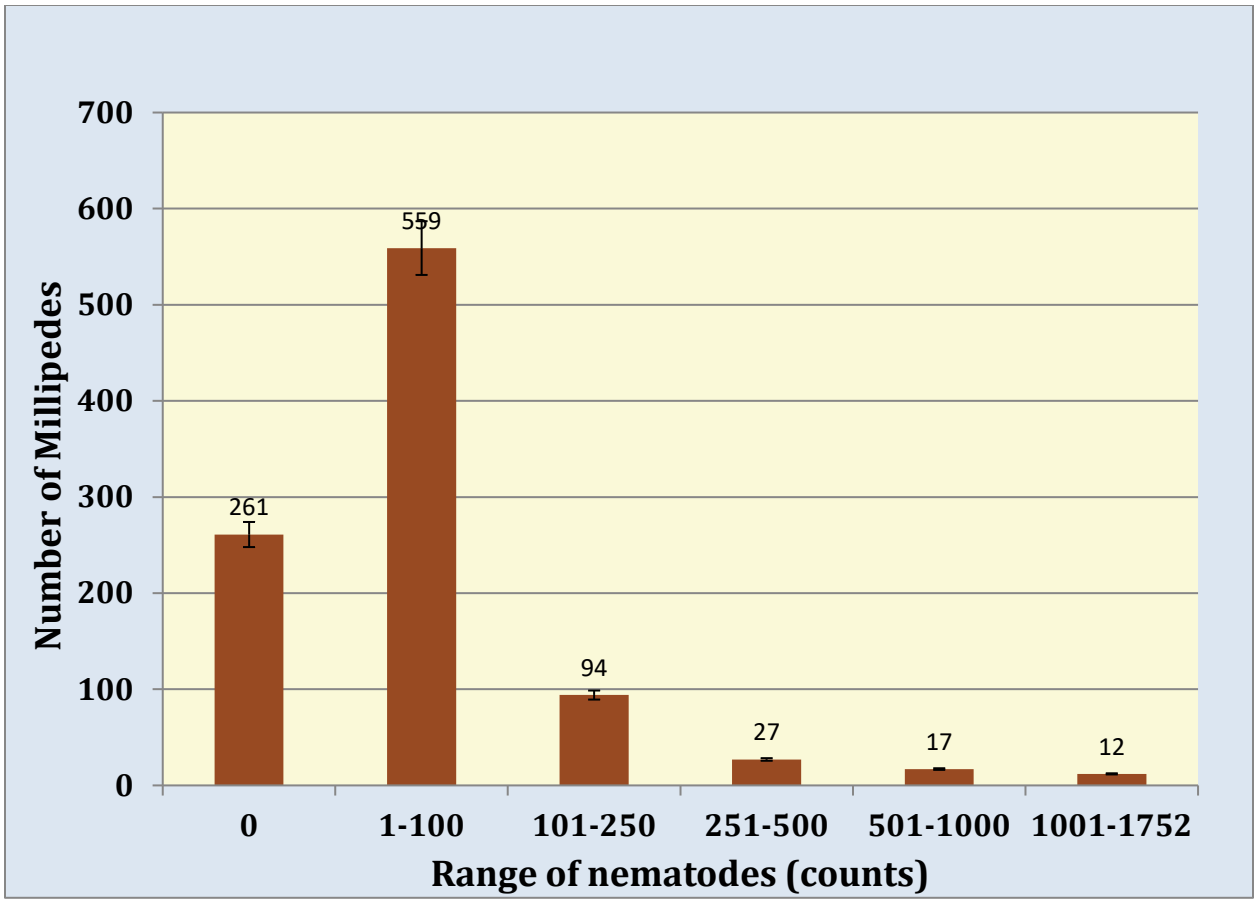

Fig. 1.4. Number of millipedes vs. range of nematodes (*n*=970). Millipedes that did not have any nematodes in the intestines were generally under 2 mm in diameter/width; however, some larger millipedes also did not have nematodes, suggesting that the presence of nematodes in the intestines is a random event. Nearly 58% of millipedes had a range of 0–100 nematodes in the intestine, followed by approximately 10% with 101–250 nematodes.

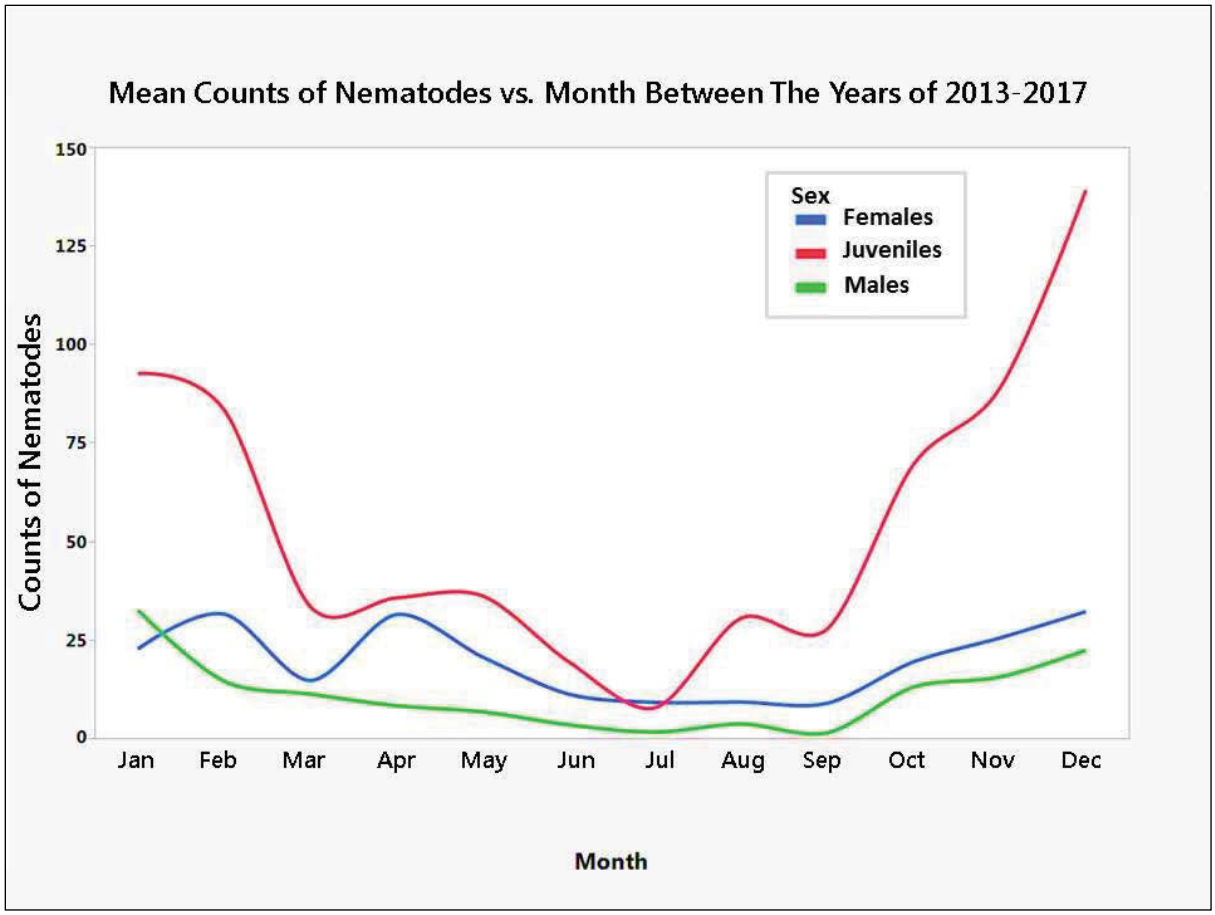

Fig. 1.5. Mean counts of nematodes vs. month (2013-2017). Males were the most infrequently encountered, followed by females and juveniles. Data suggests that juveniles increase their populations after males and females mate in the early summer months. All stages appear to overwinter in late fall and winter. The summer months suggests that nematodes mature and mate primarily in or around July. Many juveniles may not make it to adulthood and die between September and March, since male and female populations stay fairly constant during the second half of the year (July through December).

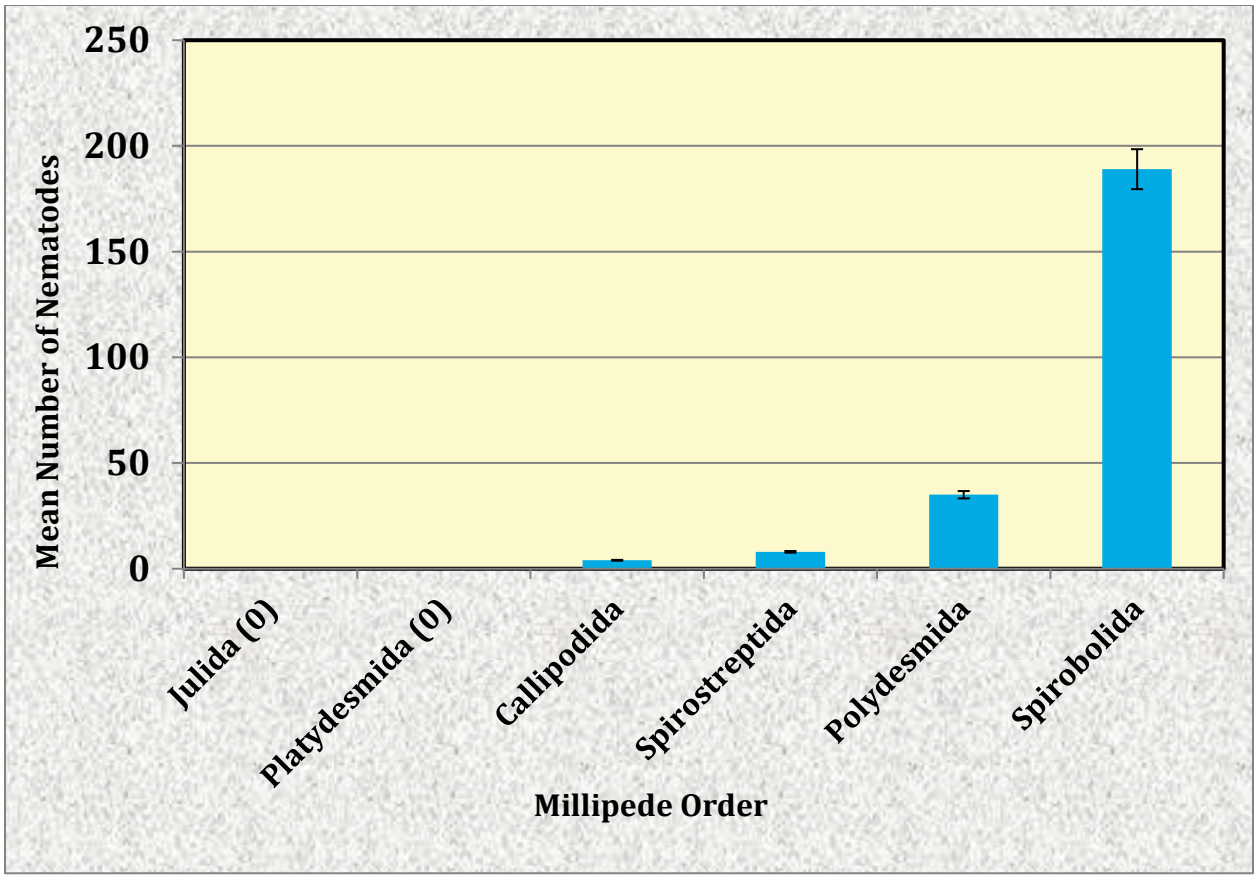

Fig 1.6. Mean number of nematodes per millipede order. Spirobolida had the highest mean number of nematodes followed by Polydesmida, Spirostreptida and Callipodida. Platydesmida and Julida did not yield any nematodes.

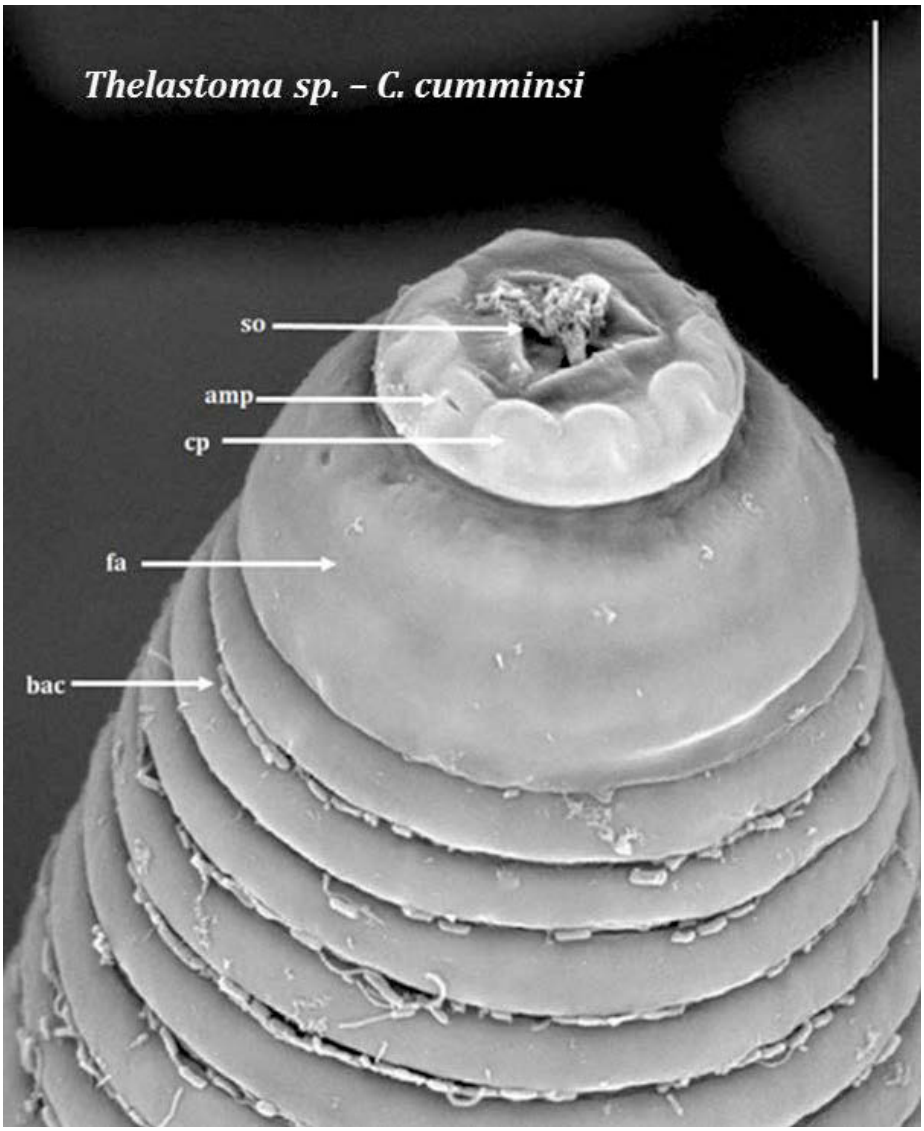

Fig. 1.7. SEM image of undescribed *Thelastoma* sp. from *Choctella cumminsi*. Scale bar 30 µm. Note amphid (amp), cephalic papillae (cp), stomal opening (so), first annule (fa) and bacteria (ba).

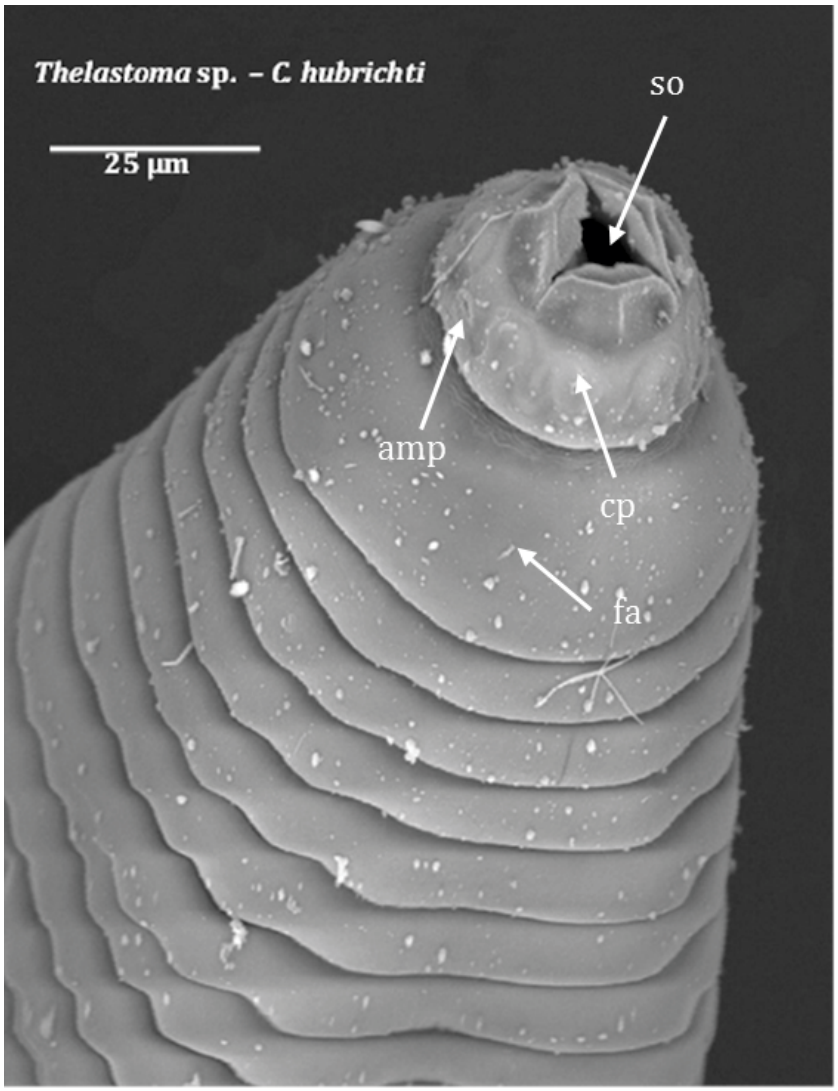

Fig. 1.8. SEM image of female *Thelastoma* sp. from *C. hubrichti.* Amphid (amp), cephalic papillae (cp), stomal opening (so), and first annule (fa).

Fig. 1.9. Projected distribution of *Narceus* spp. Curved line represents outer western boundary of the range for *Narceus americanus*, solid dots represent coastal localities where *N. americanus* has been found. The dot in SW Minnesota is an allopatric population for *N. americanus*. Dashed lines in Florida represent the approximate range for *Narceus gordanus*, within the Florida sand ridges. The stars represent localities for *Narceus woodruffi*. Map and localities from Shelley et al. (2006), Shelley and McAllister (2007) and Shelley and Snyder (2012).

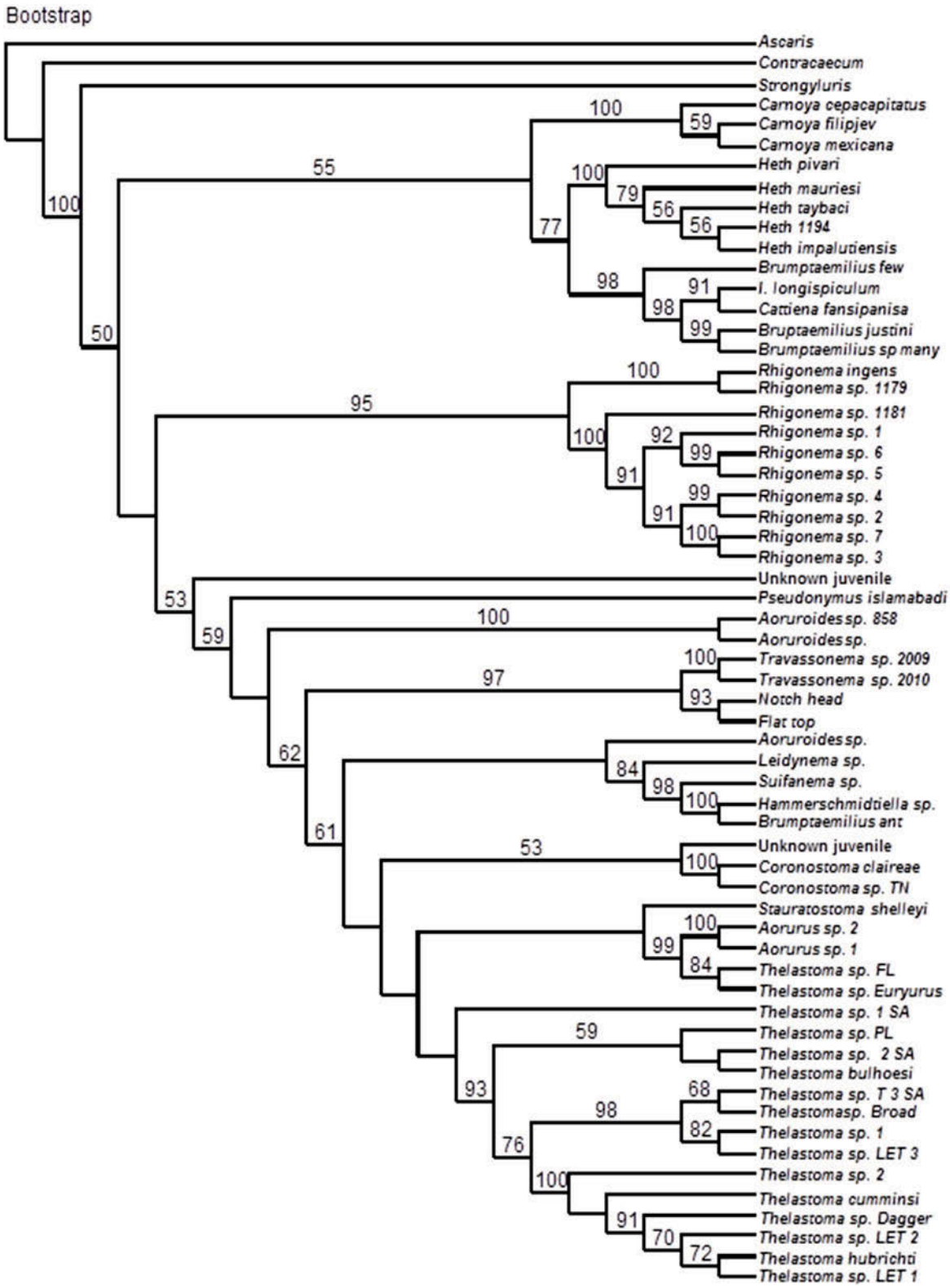

Fig. 1.10. Phylogenetic relationships among studied kleptoparasitic nematodes. Relationships are based on maximum parsimony analyses of partial 28S LSU rDNA. Bootstrap values are given near the branches and nodes.

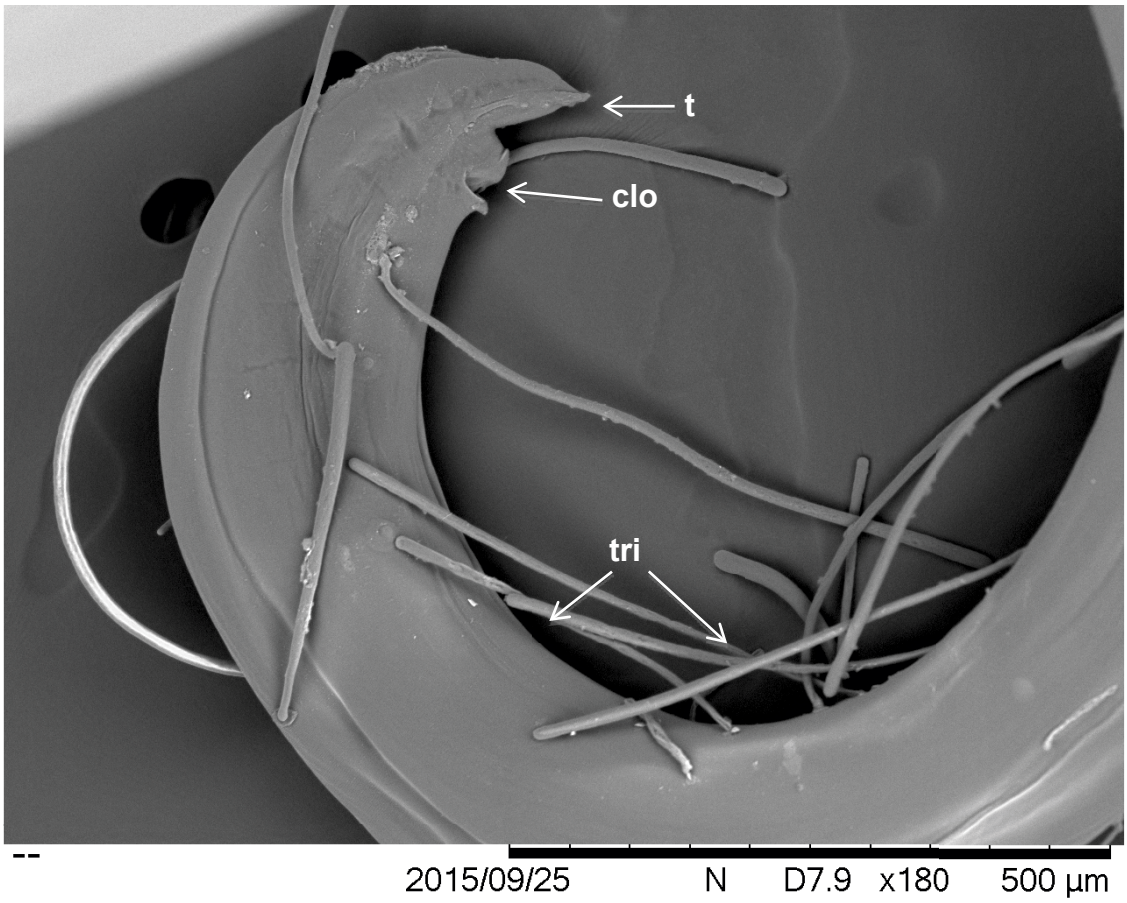

Fig. 1.11. SEM image of male *Rhigonema* sp. Anatomical characters and other organisms are shown showing trichomycetes (tri), cloaca (clo), and tail (t).

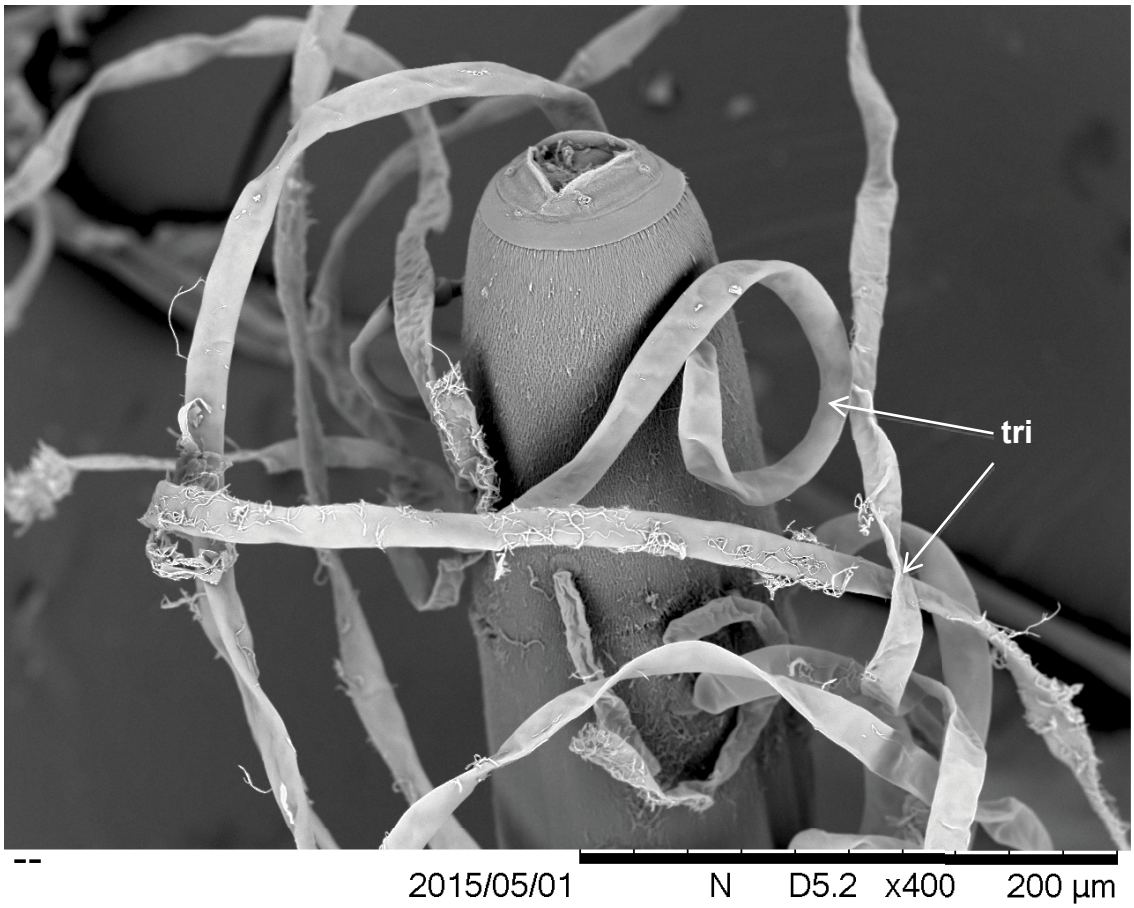

Fig. 1.12. SEM image of female *Rhigonema* sp. Anterior end of nematode is covered in trichomycetes (tri).

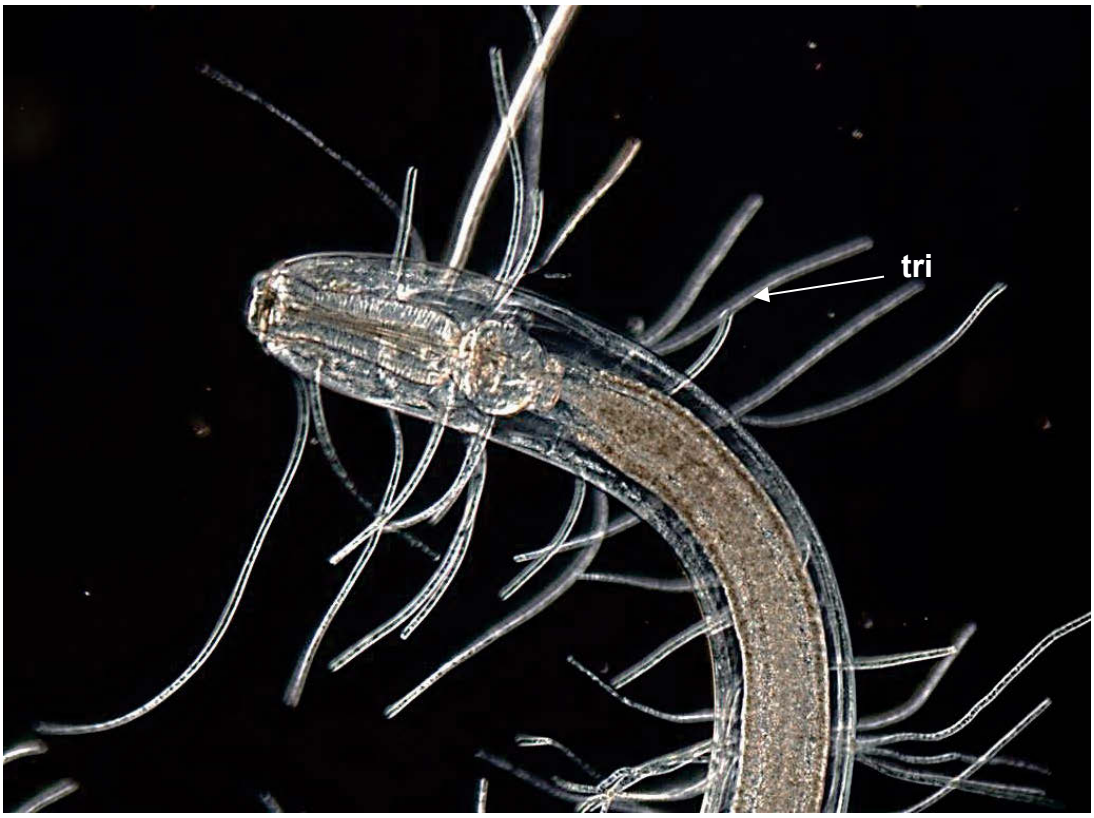

Fig. 1.13. Differential Interference Contrast image of male *Rhigonema* sp. Body covered in trichomycetes (tri).

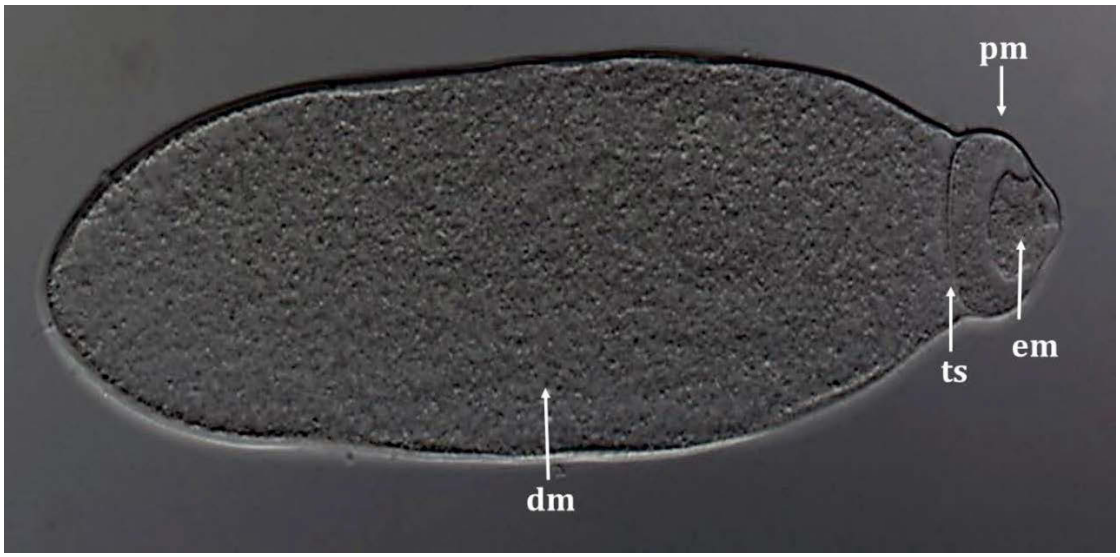

Fig. 1.14. Differential Interference contrast image of gregarine. Gregarine from midgut of a millipede. Commonly found in foregut and midgut of spirobolid, spirostreptid and polydesmid millipedes. Epimerite (em), protomerite (pm), transverse septum (ts), and deuteromerite (dm).

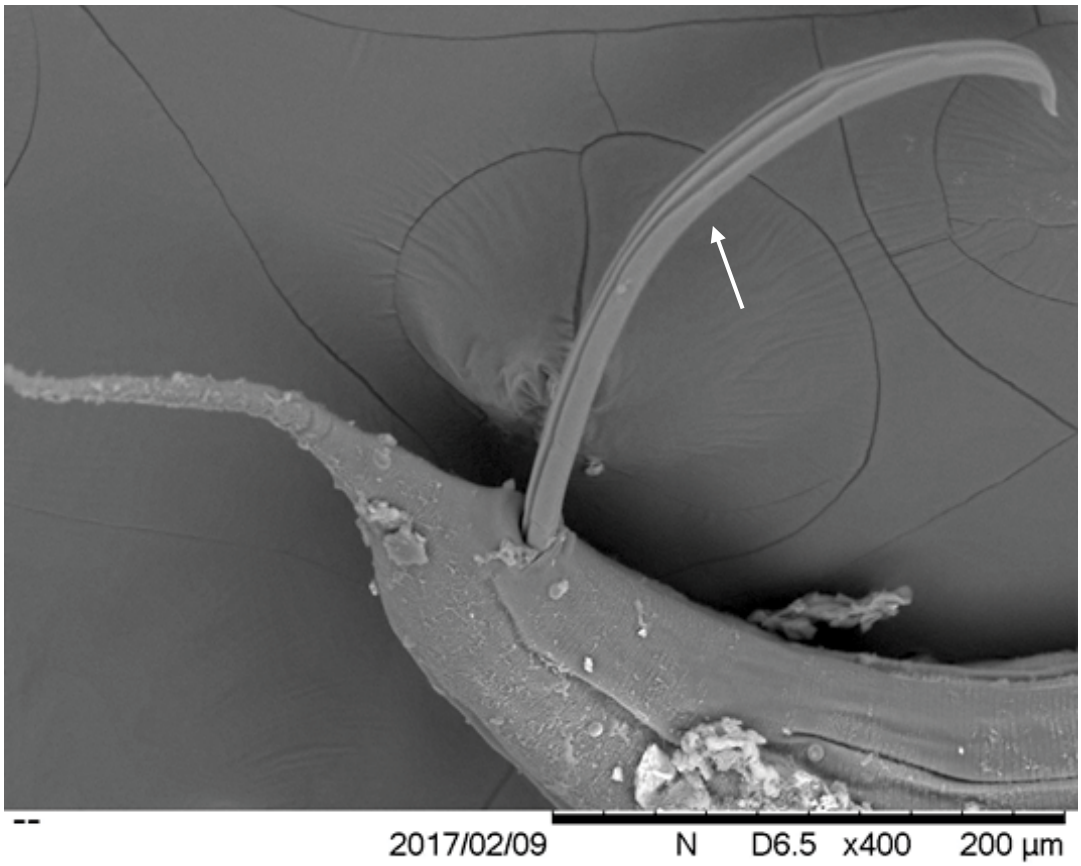

Fig. 1.15. SEM image of male *Brumptaemilius* sp. Everted spicule (arrow) used to dilate the vulva during copulation and to provide a pathway to transfer spermatozoa.

Chapter 2

Coronostoma claireae n. sp. (Nematoda: Rhabditida: Oxyuridomorpha: Coronostomatidae) from the indigenous milliped *Narceus gordanus* (Chamberlin, 1943) (Diplopoda: Spirobolida) in Ocala National Forest, Florida

A version of this chapter was originally published by Gary Phillips et al. (2016) and is cited as follows:

Phillips, G., Bernard, E. C., Pivar, R. J., Moulton, J. K., and Shelley, R. M. 2016. *Coronostoma claireae* n. sp. (Nematoda: Rhabditida: Oxyuridomorpha: Coronostomatidae) from the Indigenous Milliped *Narceus gordanus* (Chamberlain, 1943) (Diplopoda: Spirobolida) in Ocala National Forest, Florida. Journal of Nematology 48(3):159–169.

This chapter is being added to my dissertation as the original article that was submitted to, and published by, the Journal of Nematology. I have received written authorization from Dr. Andrea Skantar, the editor in chief of the Journal of Nematology, that there are no objections in using the published paper as part of my dissertation, and copyright issues are not being violated. I received written authorization from Dr. Skantar on June 27, 2017, stating, "I have reviewed your request and it is fine for you to use your published work in JON as you have indicated. Please include the appropriate citations in your thesis and that is all that we require."

The co-authors and myself conducted the research on *Coronostoma claireae*. I was responsible for the experimental methods, collecting and identifying the organisms, recording and conducting the data analysis, and wrote the chapter. After the chapter/article was written, it was submitted to my committee members and my co-authors before submitting the article to the Journal of Nematology.

Dr. Ernest Bernard is my major professor and was responsible for overseeing this research. He edited the final version, added figures and drawings, and checked for accuracy of the species description. Robert Pivar is a PhD candidate that assisted with

the molecular work and collecting the host organisms that were used during this research. Dr. J. Kevin Moulton is my professor and committee member that oversaw the molecular systematics of *Coronostoma claireae* and aided in editing the chapter. Dr. Rowland M. Shelley is my professor and committee member that oversaw the systematics of the millipede hosts. Dr. Shelley aided in the proper identification of the millipedes and assisted in editing this chapter.

Abstract

Twenty-four individuals of Narceus gordanus (Diplopoda: Spirobolidae) (Chamberlain, 1943) were collected in Ocala National Forest, Florida, between November 2013 and July 2014. Each specimen was dissected to extract the intestine, which was removed and examined for parasitic nematodes. *Coronostoma claireae* n. sp. was collected from the hindgut and midgut of 10 specimens, and its morphology was examined with brightfield, differential interference contrast, phase contrast, and scanning electron microscopy. This species is separated from other *Coronostoma* spp. by the following characteristics: body length less than 3 mm; head sense organs pit-like; first annule long, extending past middle of corpus, width similar to that of second annule; basal bulb pyriform; eggs larger than 60 × 50 μm. This species is the first North American record for the genus *Coronostoma*, which is removed from Thelastomatoidea: Thelastomatidae and reassigned to Coronostomatidae on the basis of presumed apomorphies. A key is provided for known Coronostoma spp. The superfamily Coronostomatoidea is re-established for Coronostomatidae and Traklosiidae.

Introduction

Nematodes that parasitize diplopod intestines are Rhabditida, primarily in the infraorders Rhigonematomorpha and Oxyuridomorpha. The latter infraorder consists of intestinal parasites of both vertebrates and invertebrates. The invertebrate parasites typically are placed in the superfamily Thelastomatoidea, which are well represented in milliped intestines. Both infraorders are most often reported from tropical and sub-tropical regions (Hunt, 1998; Carreno et al., 2013). Their phylogenetic position has been well established within clade III of Nematoda, but they are not considered to be monophyletic (Adamson, 1989; Adamson, 1994; Blaxter et al., 1998; Nadler et al., 2007). Most nematodes that inhabit the gut of diplopods feed on bacteria (Jex et al., 2005); however, species of *Coronostoma* Rao, 1958 are believed to be nematophagous (van Waerebeke, 1986; van Waerebeke and Adamson, 1986; Jex et al., 2005).

Coronostoma previously has been observed exclusively in spirostreptidan millipeds from Brazil (Kloss, 1961), Madagascar (van Waerebeke, 1986; van Waerebeke and Adamson, 1986), Burkina Faso (van Waerebeke and Adamson, 1986), India (Rao, 1958), and from an Australian cockroach (Jex et al., 2005).

Between November 2013 and July 2014, we collected 24 specimens of *Narceus gordanus* (Chamberlin, 1943) (Diplopoda: Spirobolida) from Ocala National Forest, Florida. The intestines of 10 individuals contained a new species of *Coronostoma*, which we name *C. claireae*. This new species is the first record of *Coronostoma* in North America and the first report of a *Coronostoma* sp. from a non-spirostreptidan milliped.

Materials and methods

Specimens of *N. gordanus* (*n* = 24) were collected between December 2013 and July 2014 in Ocala National Forest, Marion County, Florida (29.210833 N, -81.770556 W, elevation 30.4 m). Sex and morphometric data (weight, length, width) were recorded for each milliped. The specimen was then decapitated with a razor blade and the telson was severed. The intestinal tract was removed with the aid of fine-tipped forceps and placed in distilled water, then dissected. Nematodes were separately collected and processed by sequential dissection from the three intestinal regions (Crawford et al. 1983): (i) hindgut – rectum to ileum, (ii) midgut – pyloric region to posterior section of foregut, and (iii) foregut. The intestine was dissected from posterior to anterior and examined for nematodes, gregarines, trichomycetes, acanthocephalans, and ciliated protists with the aid of a Zeiss Stemi 2000 stereo microscope. Collected nematodes were sorted to morphotaxa from each intestinal section, counted, and identified to stage (adult females, adult males, and juveniles). Voucher milliped and nematode specimens are deposited in the Entomology and Plant Pathology Department, University of Tennessee, Knoxville.

Most nematodes were prepared for permanent preservation in glycerin, while others were preserved for molecular analyses or for scanning electron microscopy (SEM). For permanent preservation, nematodes were placed into distilled water, fixed with 4% hot formalin, processed to glycerin by means of a rapid method (Seinhorst 1959), and mounted in anhydrous glycerin on glass slides. All images were made from glycerin-mounted specimens. Most images were produced with a 14-megapixel Q-camera on an Olympus BX-63 DIC microscope system; Figs. 5D and E were obtained with a 17-megapixel DP-73 camera on an Olympus BX-53 phase-contrast microscope. Terminology for stomal

structures follows De Ley et al. (1995). Terminology for arcade-like cells and coelomocytes follows Peregrine (1973), Tahseen (2009) and Weinstein (2006). Measurements given in Table 2.1 were made from glycerin-preserved specimens.

For scanning electron microscopy, formalin-fixed nematodes were washed in distilled water for 20 minutes then dehydrated in a 30-µm microporous specimen capsule (Electron Microscopy Services, Hatfield, PA) using a graded series consisting of 25%, 50%, 75%, 95%, and 100% ethanol, each for 20 minutes. Afterwards, a 1:1 mixture of 100% ethanol and reagent grade hexamethyldisilazane (HMDS) was used in lieu of a critical point dryer. The HMDS series consisted of 25%, 75%, 100% and a second 100% HMDS dehydration, each for 20 minutes. Nematodes were placed on carbon tape affixed to aluminum stubs and sputter-coated with gold for 10 seconds at 20µA in a SPI-Module Sputter Coater (West Chester, PA). Specimens were viewed with a Hitachi TM 3030 electron microscope.

Results

Systematics:

Coronostomatoidea (Kloss, 1961) Poinar, 1977

Coronostomatidae Kloss, 1961

Emended description (based on females)

Obligate inhabitants of the intestine of arthropods. Cuticle without spines. Oral aperture surrounded by 12 equal lobes and an inner ring (*corona radiata*) of numerous setiform or plate-like projections; four inconspicuous to elongated cephalic papillae; amphid aperture near the tip of a projecting conical horn-like structure. Stoma without conspicuous teeth, but subventral stegostomal sectors transverse, plate-like, multi-

denticulate. First annule three or more times wider than other annules. Esophagus massively muscular, composed of a procorpus and basal bulb with or without a short, broad isthmus; grinding valves absent. Secretory-excretory system X-shaped (oxyuroid type) with prominent excretory cell and canals. Reproductive system amphidelphic, vulva transverse and in middle of body, vagina directed anteriorly; each gonad with multiple flexures; zero, one or both spermathecae with sperm, usually posterior gonad with sperm, anterior gonad often lacking sperm. Tail long, tapering, pointed. Egg surface smooth or with small blebs, without filaments.

Male shorter and slenderer than females, esophageal isthmus longer. Anterior end without elaborate *corona radiata* but with amphidial horns. Genital cone projecting with basal and subapical pairs of papilliform supplements; tail with pair of supplements at midpoint. Spicules, gubernaculum and bursa absent. Sperms broadly to narrowly oval.

Sole genus: *Coronostoma* Rao, 1958

Synonym: *Laticorpus* van Waerebeke, 1969 (van Waerebeke, 1986)

Type species: *C. singhi* Rao, 1958

Other species:

Coronostoma australiae Jex, Schneider, Rose and Cribb, 2005

C. bulbicorpus Kloss, 1961

C. claireae n. sp.

C. dentata van Waerebeke and Adamson, 1986

C. diplopodicola (Dollfus, 1964) van Waerebeke, 1986

C. gautuni van Waerebeke and Adamson, 1986

Hosts: Diplopoda of the orders Spirostreptida, Spirobolida and Polydesmida.

Coronostoma was established by Rao (1958) for a new species of nematode (*C. singhi*) inhabiting the intestine of a spirostreptid millipede from Andhra Pradesh, India. He placed this taxon in Aoruridae on the basis of the female esophageal shape and tail morphology of the male. Kloss (1961) erected Coronostomatidae for *Coronostoma*, with a very short diagnosis: female procorpus bulbiform, muscular; female esophageal bulb well-developed but without grinding valves; male lacking spicules, gubernaculum and preanal cup; eggs smooth.

Coronostoma claireae Phillips & Bernard, n. sp.

(Table 2.1, Figs. 2.1–2.5, all subsequent tables and figures are located in Appendix 2)

Description

Female (*n* = 13): Measurements are listed in Table 2.1.

Type locality and habitat: Ocala National Forest, Florida, 29.210833 N, -81.770556 W, elevation 30.4 m, sand pine scrub ecoregion. Dissected from the intestine of *N. gordanus* (Spirobolida: Spirobolidae).

Type designation and deposition: Holotype female and five paratypes deposited in the USDA Nematode Collection, Beltsville, MD. Seven paratypes deposited in the nematode collection in the Entomology & Plant Pathology Department, University of Tennessee, Knoxville, Tennessee.

Description of females: Body cylindrical, stout, head end rounded, tail abruptly narrowing behind anus tapering to a long filiform tip (Fig. 2.1 A). First head annule very large (71–97 μm) reaching to three-fourths of the procorpus (Figs. 2.1 D, 2.1 A, 2.4 A); remaining body annules 9–17 μm wide, each annule with 7–10 fine transverse lines (Figs. 2.1 E, 2.1 H, 2.4 C); annule size and line number becoming less regular near anus, cuticle

anterior to anus with pattern of short lines, forks and whorls (Fig. 2.1 I). Body without lateral alae; lateral field indicated by annule breaks or anastomoses beginning posterior to esophagus (Figs. 2.1 H, 2.4 B). In cross-section, cuticle with short longitudinal lines in each annule (Fig. 2.1 E), lines net-like in tangential view (Fig. 2.5 E). Body musculature in long bands of >200 μm, diagonal muscles not evident. Numerous minute cell-like hypodermal bodies just below cuticle in dorsal and ventral views (Fig. 2.5 E).

Head end with two protuberant amphidal horns, amphidal apertures longitudinal, slit-like (Figs. 2.1 B, 2.2 F, 2.3 C, 2.4 A, 2.4 D). Oral opening surrounded by cheilostom forming a *corona radiata* of 12 entire or bifurcate plates, extending from finely serrated rim (Figs. 2.1 B, 2.1 C, 2.2 B–D, 2.3 A–C, 2.4 A). Between *corona radiata* and amphidal horns, lip region divided into 12 indistinct sectors (Figs. 2.1B, 2.3B). Four minute cephalic pits on anterior end of large head annule (Figs. 2.1 B, 2.4 A). Stoma very shallow, cheilostom and gymnostom fused (Fig. 2.2 D). Subventral stegostomal sectors basally with flattened, enlarged, obliquely oriented plates, covered on anterior surface with numerous minute denticles (Figs. 2.1 C, 2.2 E, 2.2 F, 2.3 D); dorsal metastegostomal plate smaller (Fig. 2.1 D), with one tooth (Fig. 2.3 E).

All parts of esophagus muscular. Procorpus swollen, slightly larger than pyriform basal bulb, isthmus present but short, grinding valve absent, corpus cardiacum prominent (Fig. 2.2 A). Procorpus with longitudinal bands of muscle overlaying transverse muscles (Fig. 2.1 D) anchored at isthmus and almost at level of stegostomal plates (Fig. 2.3 G). Ventral arm of esophageal lumen much wider than subdorsal arms in anterior part of procorpus (Fig. 2.3 F), arms of equal width more posteriorly (Fig. 2.3 G). Nerve ring encircling esophagus at isthmus. Secretory-excretory system distinct, X-shaped, excretory

cell and nucleus large, excretory canal and pore minute, generally just anterior to flexure of anterior gonad (Fig. 2.1 E). Intestinal epithelium composed of discrete polygonal cells. Large arcade-like cells present in pseudocoelom posterior to basal bulb and surrounding anus (Figs. 2.5 A–C); coelomocytes present but not fully catalogued; multilobed giant coelomocyte associated with anterior portion of gonad (Fig. 2.1 F), multivesiculate coelomocyte near anterior arcade-like cells (Fig. 2.5 A).

Vulva at about two-thirds of the head-to-anus length. Vulva transverse, anterior lip not extending as flap over posterior lip. Vagina long, directed anteriorly, with a *vagina vera* composed of two groups of four large cells each (Figs. 2.1 G, 2.4 F). In young females' anterior gonad on right side of body, posterior gonad on left side; in older female's gonads longer and not strictly confined to one side or the other due to flexures. In mature females each gonad reflexed at least once; spermatheca when present occurring in reflexed region. Presence of distinct spermatheca with sperm variable; of 11 females, three without distinct spermatheca with sperm, five with filled posterior spermatheca, three with both spermathecas filled. Sperms narrow-oval, variable in shape, presumed amoeboid (Fig. 2.4 G). Eggs broadly oval, outer shell usually smooth (Fig. 2.6 E), occasionally eggs with blebs on shell.

Phasmid aperture a minute pore posterior to anus, usually with associated small, spherical, subcuticular chamber (Figs. 2.4 E, 2.5 C).

Males not known.

Differential diagnosis:

With the inclusion of the new species, there are now seven described species of *Coronostoma*. Discrimination of these species in earlier papers was partly reliant on

doubtful characters such as position of the nerve ring, length of the gonads, and number of ovarian flexures. Other characters that may be valid will need to be re-evaluated by examination of type material or new specimens. For instance, Rao (1958) and van Waerebeke and Adamson (1986) depicted *C. singhi*, *C. gautuni* and *C. dentata*, respectively, as having four prominent head papillae in addition to the amphidial horns, whereas *C. claireae* n. sp. has minute pits. In the en-face view of *C. australiae*, Jex et al. (2005) placed the amphidial horns and cephalic sense organs within the lip region; this interpretation may have been due to the SEM image of a severely collapsed specimen. The characteristics used in the key are those that are obvious from the relevant illustrations or are measurements that have enough separation to be useful.

Key to species of Coronostoma Rao, 1958

1. Esophagus with short to moderate isthmus, basal bulb pyriform… 2

1’ Esophagus without discernible isthmus, basal bulb subspherical… 6

2. First annule (mega-annule) reaching two thirds or more of corpus… 3

2’ First annule reaches only to middle of corpus …….. ***C. gautuni***

3. Diameter of first annule swollen, wider than succeeding annules … ***C. dentata***

3’ Diameter of first annule width similar to succeeding annules …4

4. Body length at least 4.5 mm ... ***C. singhi***

4’ Body length less than 3 mm ... 5

5. Tail length less than 675 µm, eggs larger than 60 × 50 µm; head sense organs as minute pits… ***C. claireae*** n. sp.

5’ Tail length greater than 700 µm, egg size less than 56 × 41 µm; head sense organs as prominent papillae… ***C. diplopodicola***

6. Basal bulb larger and wider than procorpus … ***C. bulbicorpus***

6’ Basal bulb and procorpus of equal size … ***C. australiae***

Discussion

The 24 *N. gordanus* dissected for this study contained 40–1,750 oxyuridomorph and rhigonematomorph nematodes per milliped in the hind and midgut. Only 10 of these millipeds contained *C. claireae*, with a maximum of nine *C. claireae* in a milliped that contained 1,389 total nematodes. Previous reports of *Coronostoma* spp. similarly list one or a few individuals per milliped. van Waerebeke (1986) observed partly digested nematodes in the intestine of several *C. diplopodicola*, suggesting that this nematode was predacious. During the current study we also found fragmentary remains of a small nematode (stoma, cuticular pieces) in the intestine of a female *C. claireae* n. sp. Therefore, this genus appears to consist of specialized predators of other nematodes inhabiting the milliped’s intestine. However, it seems unlikely that all stages are nematode-predacious. The eggs are of typical nematode size and hatched juveniles would be too small to ingest other nematodes. Rather, small juvenile *Coronostoma* may subsist first on bacteria in the milliped intestine, then switch to predation in the later stages. Similar diet-switching is known in the predacious terrestrial free-living order Mononchida (Yeates, 1987).

How *Coronostoma* spp. actually ingest other nematodes is not completely clear from the stomal analysis of *C. claireae* n. sp. or from shorter descriptions in other papers. The presence of large subventral, multidenticulate stegostomal plates in *C. claireae* n. sp. is an original feature not present in any other terrestrial predacious nematode genus. Another unique feature is the presence of strong longitudinal muscle bands around the periphery of the procorpus. These muscles are anchored to the esophageal isthmus and to the body wall

near the level of the stoma. Thus, they occupy the approximate position of stylet protractor muscles except they extend posteriorly to the isthmus. We hypothesize that contraction of both the transverse and longitudinal procorpus muscles pulls the prey into the stoma while shortening the procorpus, then relaxation of the longitudinal muscles lengthens the procorpus (pulling the prey in farther) and closes the stegostomal plates against the prey to hold it with the denticles. Repetition of the process along with muscular contractions of the entire esophagus could assist in further ingestion.

Coronostoma spp. may be more diverse than their very similar morphologies suggest. The esophagus of *C. claireae* n. sp. at the stomal base is bilaterally symmetrical due to its much-enlarged subventral sectors, assuming a triradiate appearance more posteriorly. The stomal region sketch of *C. diplopodicola* (van Waerebeke 1986) illustrates one small and two large denticulate regions, which suggests an architecture similar to that of *C. claireae* n. sp. On the other hand, *en-face* figures of *C. dentata*, *C. gautuni* (van Waerebeke and Adamson 1986) and *C. australiae* (Jex et al. 2005) show three symmetrical lobes.

Specimens of *C. claireae* were not examined exhaustively for arcade cells and coelomocytes, but very large, conspicuous, nucleated sac-like cells were observed posterior to the esophagus and in the anal-tail region. These cells resemble arcade cells, narrowing and extending anteriorly, but unlike arcade cells were posterior to the esophagus. These arcade-like cells sometimes had distinct protuberances extending into the pseudocoel. In several specimens a vesiculate coelomocyte attached to the hypodermis was observed next to an arcade-like cell. Typical arcade cells are found in the esophageal region (Altun and Hall, 2009; Peregrine, 1973). A cluster of arcade-like cells also occurred around the anal

region, and appeared to have extensions into the tail, superficially resembling the spinneret organ found in Plectida. However, these extensions did not lead to any pore. A single multilobed, giant coelomocyte (Peregrine, 1973) up to 50 μm long was found in the vicinity of the anterior gonad. This particular coelomocyte and its location are known in many nematodes (Peregrine, 1973).

Adamson (1989) provided a list of synapomorphies defining Oxyurida (= Oxyuridomorpha): "…single rather than paired spicule, reduced number of caudal papillae in male, absence of externolateral cephalic papillae, prominent X-shaped excretory system with vesiculate terminal duct, conical spermatozoa, a life cycle involving two molts in ovo with no extraintestinal phase in the host, and haplodiploid reproduction" (p. 176). However, we note that male nematodes in Oxyurida can have two, one, or no spicules. In addition, the eight-celled *vagina vera* seems to be a distinctive feature of female oxyuridomorphs (Chitwood and Chitwood, 1933, 1950). The infraorder is commonly divided into two superfamilies: Oxyuroidea (vertebrate parasites) and Thelastomatoidea (invertebrate parasites). Current classification of Thelastomatoidea generally follows Adamson (1989) and Adamson and van Waerebeke (1992), who recognized five families in Thelastomatoidea, with *Coronostoma* placed in Thelastomatidae.

Coronostomatidae and Robertiidae Travassos and Kloss, 1961 were segregated from Thelastomatidae and placed in a new superfamily, Coronostomatoidea (Poinar, 1977). A separate diagnosis of this superfamily was not presented but it was separated in the accompanying key from other Oxyurida by the lack of a valve in the basal bulb. Adamson and van Waerebeke (1992) placed Coronostomatidae in synonymy with Thelastomatidae without explanation, but they did suggest that Thelastomatidae was paraphyletic and

lacked unifying synapomorphies. Shah et al. (2012) also included *Coronostoma* in their key to genera of Thelastomatidae. Jex et al. (2005) avoided assigning thelastomatoid species to families, and Carreno (2014) did not refer to *Coronostoma* in his review of Thelastomatoidea.

Coronostomatidae differs from Thelastomatidae *s. str.*, and indeed from Thelastomatoidea, in several important characters. The amphid apertures are carried near the tips of horn-like protrusions, while thelastomatids have pore-like amphids directly on the lips. This horn-like amphid structure appears to be unique among Nematoda. Coronostomatids have 12 equal, shallow lobes surrounding the oral aperture; thelastomatid nematodes typically have eight such lobes. The described males of *Coronostoma* lack a spicule, whereas some male Thelastomatidae possess a spicule. Finally, the coronostomatid esophagus is strongly muscled and consists of enlarged procorpus and basal bulb, the latter with little or no isthmus and without grinding valves. In contrast, thelastomatids have a long, slender procorpus and a basal bulb equipped with grinding valves. Coronostomatidae is differentiated from the other thelastomatoid families (Adamson and van Waerebeke, 1992; Shah et al., 2012) by the above characters as well as the midbody location of the vulva (anterior to esophageal base in Protrelloididae); lack of cervical spines (present in Hystrignathidae); and lack of egg filaments (present in Pseudonymidae and Travassosinematidae).

Validity of a separate superfamily Coronostomatoidea is supported morphologically by the 12-lobed lip region, unique amphidial horns and the massively muscled esophagus that lacks the grinding valve present in Thelastomatoidea. Coronostomatoidea and Thelastomatoidea, however, are sister taxa on the basis of the morphology of the male tail.

We did not collect any male *Coronostoma*, but males are known for *C. dentata*, *C. gautuni* and *C. singhi* (Rao, 1958; van Waerebeke and Adamson, 1986). Males of these species are much reduced in size relative to the female and have an arrangement of cloacal and tail papillae similar to that of many thelastomatoid males.

Poinar (1977) included the poorly known beetle and milliped-parasitic family Robertiidae Travassos and Kloss, 1960 (genera *Robertia* Travassos and Kloss, 1961 and *Triumphalisnema* Kloss, 1962; see Bernard and Phillips (2015) for chronology problems with Robertiidae and *Robertia*) in Coronostomatoidea on the basis of an apparently similar esophagus. The family and type genus are now Traklosiidae and *Traklosia* due to homonymy with a fossil synapsid (Bernard and Phillips, 2015). Traklosiidae generally fits the superfamily only on the basis of the muscular esophagus lacking a grinding valve, although an *en-face* SEM of *Triumphalisnema bialulaundatum* Hunt, 1989 shows 11 or 12 irregular lobes surrounding the oral opening (Hunt et al., 1989). *Coronostoma* spp. typically have 12 lobes in the lip region (van Waerebeke and Adamson, 1986; this paper) but Jex et al. (2005) illustrated the head end of *C. australiae* with 11 lobes. The position of Traklosiidae cannot be more precisely determined without additional specimens and analysis, and so the family is retained in Coronostomatoidea.

Conclusion

Most *Coronostoma* spp. have been found in millipeds, with only one species from a cockroach. Millipeds are among the oldest terrestrial arthropods, with molecular clock results and paleobiogeographic reconstructions converging at an origin date of about 524 mya (Pisani, 2009; Shelley and Golovatch, 2011). As herbivores, millipeds likely would have been among the first arthropods ingesting bacterivorous nematodes or nematode eggs

(Adamson, 1994). In the near-neutral fermentative intestine of millipeds (and somewhat later, cockroaches), such nematodes would have been preadapted for feeding on the rich bacterial flora that inhabited the gut. In this environment morphological adaptations for feeding directly on a host would not be necessary. Present-day invertebrate-inhabiting oxyuridomorphs can differ spectacularly in external head morphology (e.g., *Travassosinema* Rao, 1958, see Spiridonov and Cribb, 2012), but in general, morphology and internal anatomy are quite uniform for this large taxon of some 850 species (Adamson, 1994; Spiridonov and Cribb, 2012). Functionally, all oxyuridomorphs of invertebrates are bacterivorous kleptoparasites rather than true parasites, with the exception of the predator *Coronostoma*, which likely represents a relict group of early predacious nematodes within Oxyuridomorpha. The scattered distribution of *Coronostoma* spp. and their evident infrequency and rarity in hosts suggests an early taxon, strengthening the argument that the genus belongs in a separate superfamily coordinate with Thelastomatoidea.

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Appendix 2

Table 2.1. Morphometrics of *Coronostoma claireae* n. sp.

Character	Holotype female	Paratype females (n = 11)	
		Mean	Range
Measurements (μm)			
Length	2359	2201.6	1672–2598
Maximum width	192.2	190.5	129–278
First annule width	87.0	83.5	71–94
Second annule width	13.9	10.1	7–12
Body annule width	13.6	12.9	9–16
Esophagus length	203.4	195	182–213
Corpus length	103.3	102.5	95–112
Bulb length	94.8	96.1	88–104
Excretory pore to anterior end of head	443.8	374.5	322–417
Distance of vulva to anterior end of head	1190	1020.3	797–1250
Distance of anus to anterior end of head	1823	1606.7	1178–2057
Egg length	61.6	70.2	59–80
Egg width	49.7	48.1	45 - 50
Tail length	592.5	603.3	499 - 673
Ratios			
a	12.3	11.9	10.8–13.9
b	11.6	11.3	9.2–13.1
c	4.0	3.6	3.2–4.1
V (%)	50.4	46.8	45–51
V’	0.65	.64	0.59–0.68

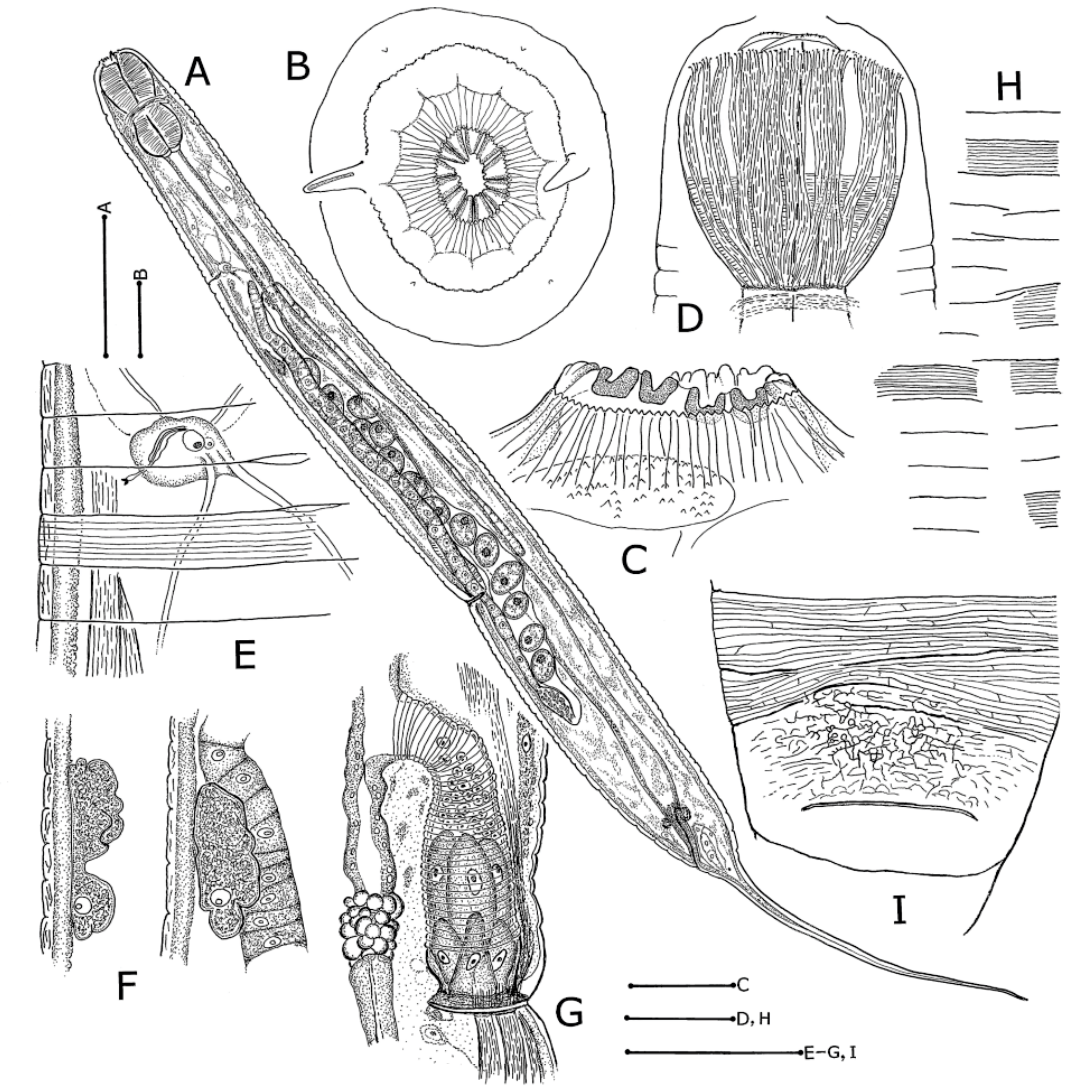

Fig. 2.1. *Coronostoma claireae* n. sp. illustrations. A) Female. B) En–face view. C) Corona radiata. D) Longitudinal muscles surrounding procorpus, procorpal radial muscles partially drawn. E) Secretory–excretory system complex. F) Giant coelomocytes near anterior ovary. G) Base of reproductive system of young female. H) Lateral field posterior to basal bulb. I) Cuticular region around vulva, ventral view. Scales: A, 250 µm; B, C, 10 µm; D–I, 50 µm.

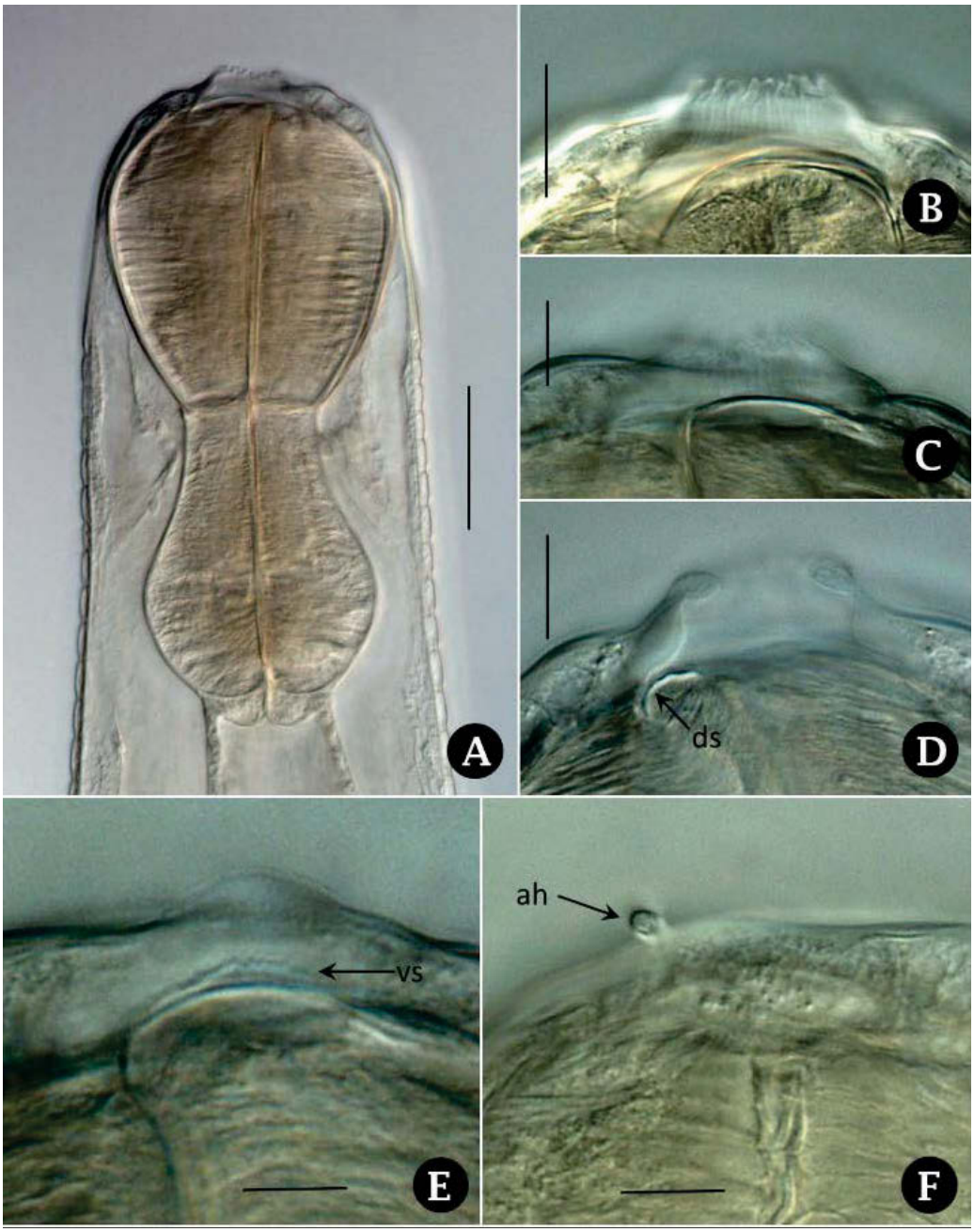

Fig. 2.2. *Coronostoma claireae* n. sp. DIC images. A) Anterior region. B) *Corona radiata* and associated ridges. C) Serrated oral margin. D) Anterior region of stoma with profile of dorsal stegostomal plate (ds). E) Subventral stegostomal plate (vs) showing denticles in profile. F) Subventral stegostomal plate, oblique view, with numerous denticles; ah: amphidial horn. Scales: A, 50 µm; B, 20 µm; C–F, 10 µm.

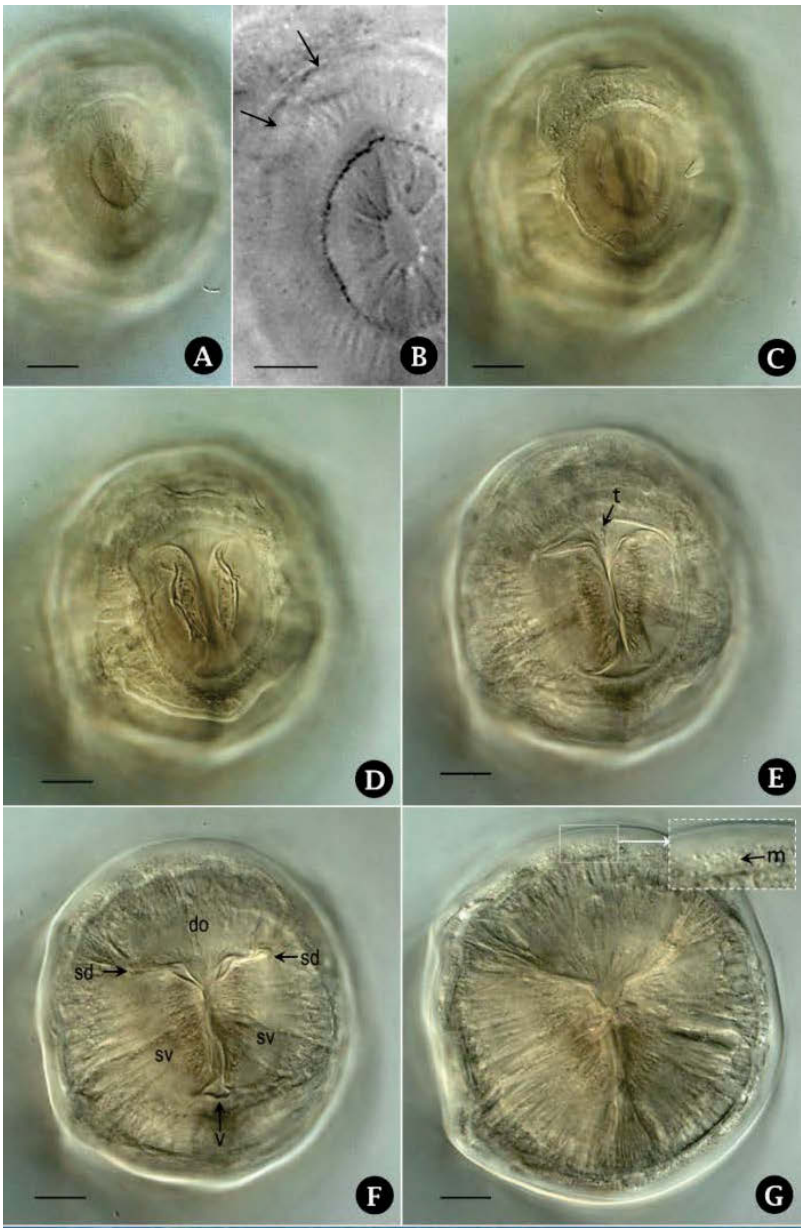

Fig. 2.3. *Coronostoma claireae* n. sp., optical cross–sections. Cross-sections through first 23 µm from anterior end. All images oriented dorsal side up. A) *En–face* view of anterior end. B) Close–up of *en–face* view showing serrated oral margin, fine ridging on oral disc, and weakly lobed edge of oral disc (two of 12 lobes indicated by arrows). C) Cross–section 4 µm below anterior end, interior serrated oral lining and amphidial horns visible. D) Cross–section 6 µm below anterior surface, showing denticulated subventral stegostomal plates. E) Cross–section 12 µm below surface, with small dorsal sector and large subventral sectors; dorsal sector with small tooth (t). F) Cross–section 16 µm below anterior surface, ventral arm (v) of esophageal lumen much longer than subdorsal (sd) arms; dorsal muscular sector (do) much smaller than subventral muscular sectors (sv). G) Cross–section 23 µm below anterior surface, esophageal muscle sectors approximately equal in size. Inset: longitudinal muscle fibers (m) around periphery of esophagus.

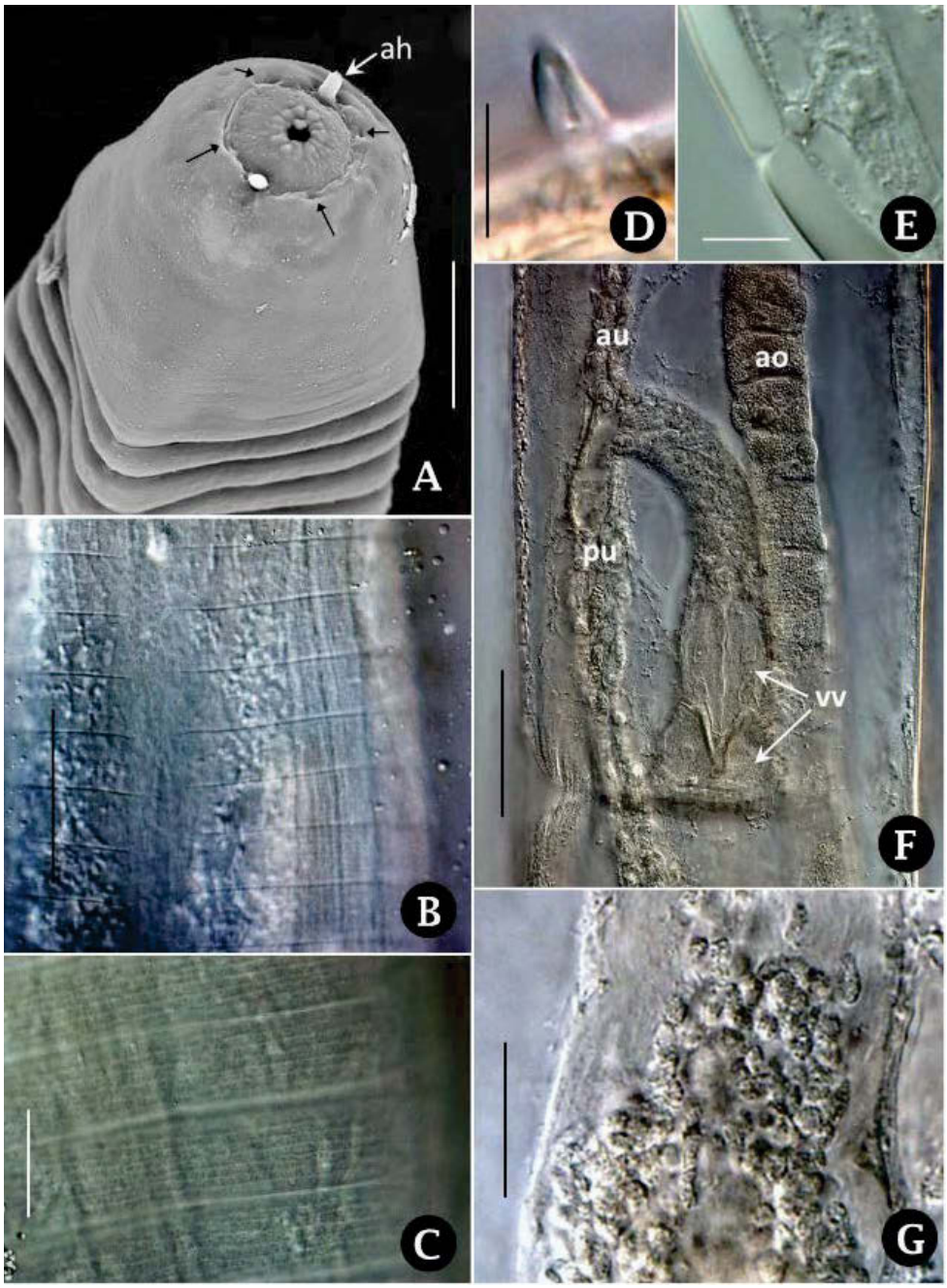

Fig. 2.4. *Coronostoma claireae* n. sp. SEM and DIC images. A) Scanning electron micrograph of anterior end; ah: amphidial horn. Small arrows indicate pore locations. B) Lateral field of interrupted annules. C) Annules posterior to vulva with fine transverse lines. D) Amphidial horn with aperture. E) Phasmid pore and associated subculticular body. F) Basal region of reproductive system, ventral view; vv: cells of *vagina vera*; au: anterior uterus; ao: reflexed anterior ovary; pu: posterior uterus. G) Portion of posterior spermatheca with sperm. Scales: A, B, F, 50 µm; C, G, 20 µm; D, E, 10 µm.

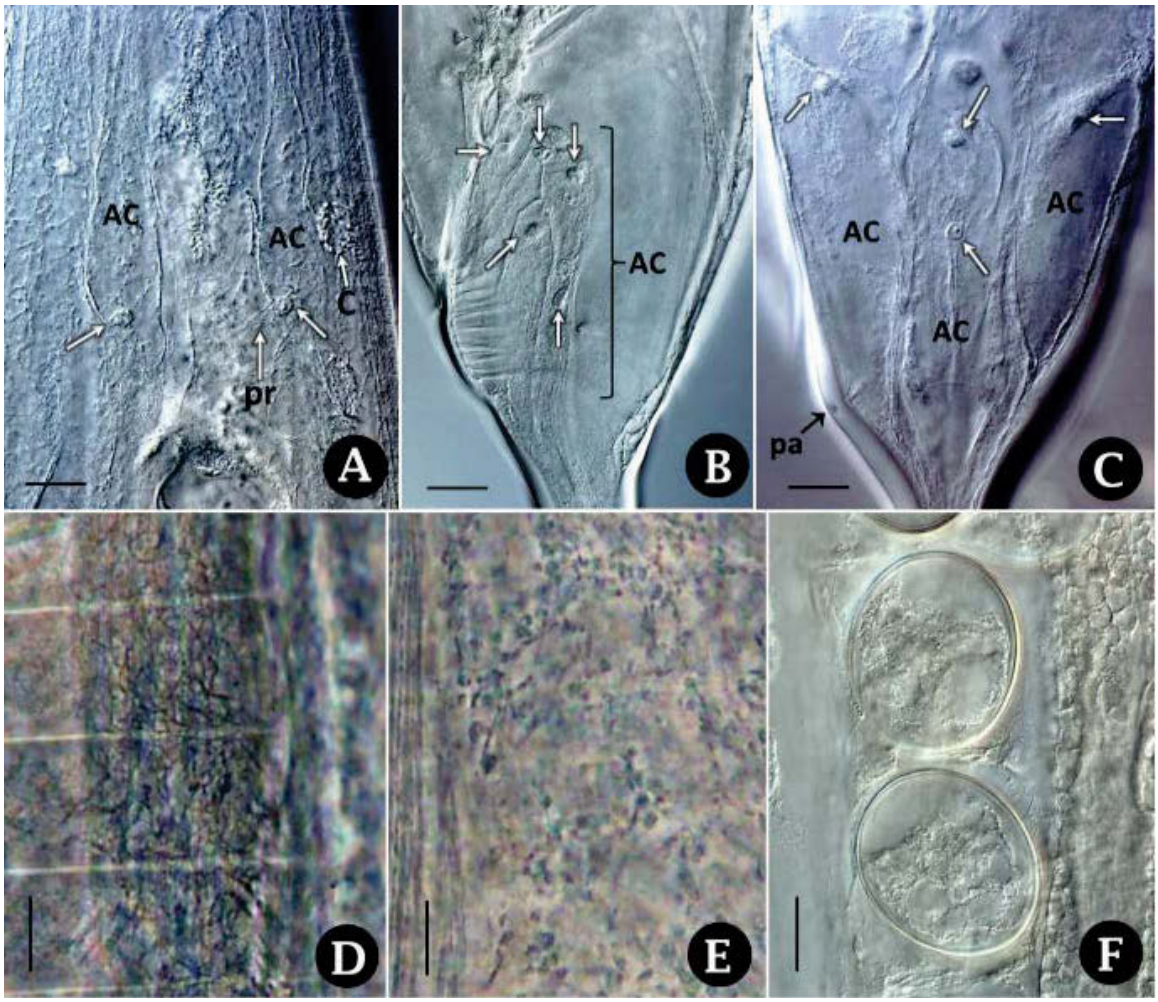

Fig. 2.5. *Coronostoma claireae* n. sp., internal, cuticular and subcuticular features. A) Arcade–like cells and multi–vesiculate coelomocyte between basal bulb and gonad region. B) Posterior arcade–like cells associated with anus and tail, lateral view. C) Posterior arcade–like cells associated with anus and tail, ventral view. D) Net–like structures of presumed medial zone of cuticle, 2 µm below surface. E) Non–muscular dorsal region with numerous irregular bodies, 4 µm below surface. F) Eggs. AC: arcade–like cell; C: multivesiculate coelomocyte; pa: phasmid aperture; pr: process extending from AC toward intestine. Arrows without labels indicate relevant nuclei. Scales: A–C, F, 20 µm; D, E, 10 µm.

Chapter 3

Stauratostoma shelleyi n. gen., n. sp. (Nematoda: Rhabditida: Thelastomatidae) from Appalachian polydesmid millipedes (Polydesmida: Xystodesmidae)

Abstract

Between February 2013 and July 2017, 352 millipedes in the family Xystodesmidae (order Polydesmida) were collected and dissected. Nematodes were removed from the gastrointestinal tract and taxonomically classified. *Stauratostoma shelleyi* n. gen., n. sp. was discovered in the midgut and hindgut from nine different species in this family collected in the southern Appalachian regions of North Carolina, Tennessee and Alabama. Specimens of *S. shelleyi* were morphologically examined using differential interference contrast, phase contrast and scanning electron microscopy. Thirteen specimens from various hosts were sequenced for 28S LSU rDNA and compared to other millipede-inhabiting nematodes. The head of *S. shelleyi* differs from other thelastomatid nematodes in having a head region mushroom-shaped in profile; cruciform stomal opening formed from four flaps; greatly expanded labial disc; and eight labiopapillae compressed into annule-like column supporting the labial disc. *Stauratostoma shelleyi* is the sister group to the few *Thelastoma* spp. that have been molecularly characterized using the D2-D3 expansion segments of the 28S LSU rDNA.

Introduction

The nematode fauna living within the gastrointestinal tract of North American millipedes is not well documented (Carreno, 2007). North American millipedes are understudied hosts of oxyuridomorph and rhigonematomorph nematodes, and records of their distribution in North America are scant. Most studies of nematodes that parasitize diplopods have originated from tropical areas (Carreno et al., 2013). Joseph Leidy, the father of American parasitology, was one of the first researchers to document the existence of nematodes living inside the intestine of millipedes in temperate North America (Leidy, 1849, 1850, 1851, 1853). The first known thelastomatid nematode recorded by Leidy was *Thelastomum* (=*Thelastoma*) *attenuatum* Leidy 1849, from the millipede host *Julus marginatus* (=*Narceus americanus* Beauvois, 1817) (Leidy, 1853). Leidy also described *Thelastomum* (=*Thelastoma*) *labiatum* Leidy 1850 (Leidy, 1853); however, he provided a very superficial anatomical description with only one partial sketch of the *T. labiatum* type specimen. Based on the lack of sound descriptions of some older specimens of *Thelastoma* spp., and a lack of specimens for comparisons, the taxonomic understanding of this genus has been challenging (Carreno, 2007). Re-descriptions of older type materials, when available, and a comprehensive survey of nematode fauna of North American millipedes are needed to understand phylogenetic relationships between *Thelastoma* and other genera of nematodes inhabiting the intestinal tract of millipedes (Carreno, 2007).

Nematodes parasitizing diplopods are taxonomically placed in the infraorders Oxyuridomorpha and Rhigonematomorpha. Their phylogenetic placement has been well established among Clade III nematodes but their sister group has not been entirely

resolved, and it has been established that they are not a monophyletic group (Blaxter et al., 1998; Nadler et al., 2007).

In the course of a recent survey of Appalachian millipedes in Tennessee, North Carolina and Alabama, as well as other areas from the southeastern and western United States, many species of Rhigonematomorpha and Oxyuridomorpha were collected from millipedes. Oxyuridomorph nematodes are kleptoparasites in the intestine of cockroaches, millipedes and scorpions, while rhigonematomorph nematodes are found only in millipedes. Among the thelastomatid and rhigonematid nematode taxa collected in the southern Appalachian region of North America was an unusual species that could not be placed in any described genus. A new genus and species of nematode, *Staurastoma shelleyi* n. gen., n. sp., is described herein.

Materials and metods

Between February 2013 and July 2017, a total of 352 xystodesmid millipedes were collected in many locations in the southeastern U.S., including several Tennessee and Alabama State Parks. Millipedes were transported back to the lab where they were maintained in enclosures with their natural substrate and fed pieces of cucumber fruit. Prior to dissection, morphometric data were recorded for each millipede, including weight, length, width and sex. Millipedes with movement activity typical of their species were considered healthy.

Millipedes were dissected by severing the head and epiproct with a razor blade as described by Phillips et al. (2016). The intestine was pulled intact from the body cavity with fine-tip forceps and placed in a Syracuse watch glass containing distilled water. The

intestine was sectioned into three parts: foregut, midgut and hindgut (Crawford et al. 1983). The intestine was sketched and then dissected with the aid of Zeiss Stemi 2000 or an Olympus SZ51 stereomicroscope. Nematodes were removed from intestinal tissue and sorted. Males, females and juveniles were segregated, counted, and grouped to family or genus-level taxa according to general features. Each dissected millipede was preserved as a voucher specimen in 70% or 95% ethanol.

Specimens were prepared for light microscopy, scanning electron microscopy (SEM) or molecular analysis. Most specimens were killed and fixed in 60–70˚C 4% formalin, then later processed to anhydrous glycerin (Seinhorst,1959) for permanent mounts on glass slides or long-term storage in vials. Slide-mounted specimens were examined with an Olympus BX-63 DIC microscope system and imaged with a 14-megapixel Q-camera. Measurements in Table 3.2 were made from glycerin-preserved specimens. The holotype and paratypes are deposited in the USDA Nematode Collection in Beltsville, Maryland. Remaining specimens are deposited in the collection of the Entomology and Plant Pathology Department, University of Tennessee, Knoxville.

To better visualize the lateral field, head region, and phasmid features, several females were fixed in 95% ethanol for two days and mounted directly on slides into Hoyer's medium. Slides were placed in a 50°C oven for three days to expand the nematodes and harden the mounting medium, then ringed with red insulating varnish. Features were imaged with a 17-megapixel DP73 camera on an Olympus BX-53 phase-contrast microscope.

For scanning electron microscopy, methods used by Phillips et al. (2016) were followed. Formalin-fixed nematodes were washed in distilled water for 20 minutes then placed into a 12-mm × 30-µm microporous specimen capsule (Electron Microscopy Services, Hatfield, PA). Each capsule was placed in a 5-ml glass well and dehydrated with a graded ethanol series consisting of 25%, 50%, 75%, 95%, and 100% ethanol, each for 20 minutes. Following the 100% ethanol dehydration step, a 1:1 mixture of 100% ethanol and reagent grade hexamethyldisilazane (HMDS) was used in place of a critical point dryer. The HMDS series consisted of 75% and two 100% dehydrations, each for 20 minutes. Nematodes were placed on carbon tape affixed to a 45°/90° aluminum stub and sputter-coated with gold for 10 seconds at 20µA in a SPI-Module Sputter Coater (West Chester, Pennsylvania). Specimens were viewed with a Hitachi TM 3030 electron microscope at a voltage of 15kV.

Total genomic DNA was extracted from representative single specimens with the Qiagen DNeasy Blood and Tissue Kit #69506 (Waltham, MA) differing from the manufacturer's instructions only in reduction of the final elution volume to 70 µl (2 x 35 µl) from 400 µl (2 x 200 µl). The resulting gDNA was stored at -20°C. PCR was carried out with TaKaRa Ex Taq Hotstart DNA polymerase (Takara Bio, Shiga, Japan) per the manufacturer's suggested protocol, plus 2µL of DNA template and 3µl of 20µM working stocks of 28S LSU rDNA primers. Several primer pairs were employed, with the greatest success resulting from use of LSU391F: 5'- AGCGGAGGAAAAGAAACTAA- 3' and LSU501R: 5'- TCRGARGGAACCAGCTACTA - 3' (Nadler et al., 2006). Cycling was done using a GenePro (Bioer Technology Co., Hangzhou, China) thermal cycler using the following PCR regime: initial 90s denaturing step at 94˚C, then 4 cycles of 30s at 94˚C, 30s at 56˚C and 75s at 72˚C,

followed by 4 cycles of 30s at 94˚C, 25s at 52˚C and 75s at 72˚C, 9 cycles of 30s at 94˚C, 20s at 48˚C and 75s at 72˚C and finally, 38 cycles of 30s at 94˚C, 20s at 45˚C and 75s at 72˚C.

PCR products were electrophoresed in 1% agarose gels at 110V for 30 minutes. Bands were excised from the gel, cleaned using QiaQuick Gel Extraction Kits and eluted in 37µL of elution buffer. Cleaned PCR products were used as templates for cycle sequencing (Sanger) reactions using 1.5 µl of the PCR primers at a working concentration of 5µM. Sequencing was performed in both directions using BigDye v3.1 terminators (Applied Biosystems, Carlsbad, CA) in a 1/20th reaction using 0.4 µl BigDye terminators and 3–5 µl of homemade 5X sequencing buffer cocktail in a 20 µl reaction volume. Centrisep columns (Princeton Separations, Adelphia, NJ) were used to clean the sequencing reactions, which were then dried in a Centrivap Concentrator (LABCONCO, Kansas City, MO). Dried samples were sent to the University of Tennessee Genomics Core for sequencing. Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI) was used to reconcile and verify opposing strands for accuracy. The 28S rDNA sequences of 11 females and 2 juveniles (n=13) of *S. shelleyi* were obtained, with resulting sequence lengths of 821–1,038 base pairs due to different forward and reverse primers being used to amplify them. The sequence fragments were invariant and therefore were converted into a single consensus sequence submitted to the National Center for Biotechnology Information (NCBI).

In order to ascertain the phylogenetic position of *Stauratostoma shelleyi*, phylogenetic studies were conducted. The consensus sequence of *Stauratostoma shelleyi* was aligned with orthologous sequences of 36 additional nematode taxa either obtained during the course of this research or oxyuridomorph and rhigonematomorph sequences obtained from the National Center for Biotechnology Information (NCBI) (See Fig. 3.5 for

accession numbers). Alignment was completed using Opal (Wheeler and Kececioglu, 2007) via the Opalescent package within Mesquite 3.03 (Maddison and Maddison, 2015). Reconstruction of relationships was carried out using maximum parsimony methods as implemented in PAUP* (Swofford, 2002) and Bayesian inference methods implemented within MrBayes v3.2.2 within the Cyberinfrastructure for Phylogenetic Research (CIPRES) portal v. 3.3 (Miller et al., 2010). The distal outgroup consisted of a single representative of the nematomorphan *Gordionus* spp. Proximal outgroups included the root knot nematode *Melioigyne incognita* as well as two free-living bacteria feeding species, *Distolabrellus veechi* and *Plectus murrayi*. Node support was gauged by nonparametric bootstrap resampling (10,000 reps of a single random addition sequence) as well as Bayesian posterior probabilities.

Parsimony analysis was comprised of a heuristic search employing 1,000 random addition sequence replicates using tree bisection and reconnection (TBR) branch rearrangement. Of 1,279 aligned characters, 610 were parsimony informative. A single most parsimonious tree of 3,179 steps (CI: 0.506; RI: 0.625; HI: 0.494) was recovered in 900 of the 1,000 replicates conducted. Prior to Bayesian analysis, JModeltest v. 2.1.10 (Darriba et al., 2012) was used to determine the most appropriate evolutionary model. The best-fit model chosen was GTR+I+G: (-lnL: 16,894.3004). A Bayesian phylogeny was estimated using Markov Chain Monte Carlo methods implemented within MrBayes 3.2.2 (Ronquist et al., 2012) through the online CIPRES Science Gateway (Miller et al., 2010). No partitions within 28S LSU rDNA were recognized. Nucleotide substitution matrix, rate variation, gamma shape parameter, and base frequencies were estimated (nst = 6; rate = invgamma; unlink statefreq = (all); revmat = (all); tratio = (all); shape = (all); pinvar = (all);

prset applyto = (all) ratepr = variable). Two runs with six chains each were run for a total of 10 million generations. Markov chains were sampled at intervals of 500 generations and the first 35% of trees discarded as burn-in prior to assembling a 50% majority rule consensus tree. Verification that stationarity had been reached was measured by the standard deviation of split frequencies being less than 0.1), the Potential Scale Reduction Factor approaching 1.0, and the MrBayes output overlay plot depicting no directional trend for either run. Resulting phylogenetic trees were modified using Canvas 8.0.5 (Deneba Systems) to produce publication-grade figures.

Millipede and nematode morphometric data were analyzed with mixed model analysis for a nested design, with nematode length as the response variable and millipede species as the categorical independent variable, and millipede length, width and weight as numeric independent variables, respectively. Rank data transformation was applied when the diagnostics analysis showed non-normality and unequal variance on residuals. Significant effects were identified at $p<0.05$. Data analysis was conducted in SAS9.4 TS1M3 (SAS Institute Inc., Cary, NC).

Results

During this research, 972 millipedes spanning six orders, 16 families and 53 species were collected from 20 middle Atlantic, southeastern, southwestern and western states. A total of 66,685 nematodes were extracted and separated into morphotaxa. Of the 972 millipedes dissected, 352 (36.2%) were millipedes from the order Polydesmida. Within the order Polydesmida, only one family (Xystodesmidae) contained *S. shelleyi*.

Specimens in 15 genera of xystodesmid millipedes (Table 3.1, all subsequent tables and figures are in Appendix 4.1) were dissected to determine the presence or absence of intestinal nematodes. Total nematode loads ranged from 0–418 nematodes/specimen, primarily represented by genera in Thelastomatidae, Rhigonematidae, Aoruridae, and Coronostomatidae. Among these 15 genera, nine (60%) contained specimens of *S. shelleyi*. *Stauratostoma shelleyi* was found in 27 of 58 (46.6%) specimens, with 0–32 *S. shelleyi* per millipede. Males attributable to this species were not found and juveniles were rarely encountered. This new species was most often located in the hindgut of the millipede host, although there were a few occasions when we observed them in the pyloric region of the midgut; no nematodes were found in the foregut.

Phylogenetic analysis of 28s rDNA was conducted using two different methods: maximum parsimony and Bayesian Inference. Both analyses yielded largely congruent trees, differing chiefly in the relationships among the basal-most clades, which were weakly supported. The Bayesian inference is presented as Figure 3.5. Both trees recovered *S. shelleyi* as the sister group to *Thelastoma* spp.

The length of each specimen of *S. shelleyi* was measured and compared to the length, width and weight of the most frequently encountered millipede hosts: *Apheloria montana* (Bollman, 1887) and *A. virginiensis* (Drury, 1770) (for taxonomy see Shelley et al., 2017). Other host millipede species were excluded due to insufficient specimen numbers for analysis.

Systematics

Thelastomatidae Travassos, 1929.

Stauratostoma Phillips and Bernard, new genus

Description

Obligatory kleptoparasitic inhabitants of the hindgut and midgut of some xystodesmid millipedes.

Etymology: *Stauratostoma* is developed from the Latin word *staurato-*, cross-shaped or cruciate, combined with –*stoma* (mouth), reflecting the unique appearance of the anterior end.

Females: Body strongly annulated. Tail long, attenuated to fine tip. Anterior end mushroom-shaped in profile. Labial disc greatly expanded, wider than columnar first apparent annule (circumoral annule), with cruciform stomal opening formed from four smooth, rounded, lip-like flaps; labial papillae obscure. First apparent annule cylindrical, divided into eight sectors by longitudinal grooves (Figs. 3.1 A, 3.1 B, 3.2 A-D, 3.3 A, 3.3 B); dorsal and ventral grooves extending entire width of first apparent annule, other six grooves sublateral, not reaching second cephalic annule. Second cephalic annule wider than oral disc (Fig. 3.1 B, 3.2 A-C). Esophagus with long procorpus and distinct basal bulb with grinding valves (Fig. 3.1 A). Secretory-excretory gland massive, transversely oval pore with apparent sub-surface operculum (Figs. 3.1 A, 3.1 C, 3.3 B, 3.3 C). Anterior end of intestine swollen (Fig. 3.1 A). Reproductive system amphidelphic, vulva near midbody; both gonads doubly reflexed, spermatheca present in posterior gonad, sperm oval, absent from anterior gonad; both uteri on right side of body; vagina strongly muscled, directed anteriorly (Figs. 3.1 D-G). Phasmid apertures pore-like, on tail (Fig. 3.1 F). Juveniles similar to adult females except in development of reproductive system. Males not encountered.

Type species: *Stauratostoma shelleyi* Phillips & Bernard, new species

Stauratostoma shelleyi Phillips & Bernard, n. gen., n. sp.

(Table 3.2, Figs. 3.5; 3.6 B-D)

Description

Female (*n* = 31): Measurements are listed in Table 3.2.

Type locality and habitat: Holotype female, 12 paratype females, Tennessee, Anderson County, Powell, Powder House Road, 36.054521, -84.11283889, elevation 324 m, rocky, mixed hardwood and pine ecoregion, collected from the hind and midgut of *Apheloria montana* Bollman, 1887, 31 March 2013 and 18 additional females collected from *A. montana* at same locality throughout the year except in January. Numerous females collected from the gastrointestinal tract of nine species of xystodesmid millipedes from the following counties: in Tennessee, Anderson, Blount, Campbell, Hamblen, Knox, Loudon, White, Wilson, Union; Haywood and Swain Counties, North Carolina; and Shelby County, Alabama (Fig. 3.6 A).

Type designation and deposition: Holotype female and 12 paratype females deposited in the USDA Nematode Collection, Beltsville, MD. Eighteen additional paratype females and many additional *S. shelleyi* specimens collected during this study are deposited in the Entomology & Plant Pathology Department, University of Tennessee, Knoxville, Tennessee.

Etymology: It is our pleasure to name *Stauratostoma shelleyi* n. gen., n. sp. after the renowned millipede expert Dr. Rowland M. Shelley, Adjunct Professor, University of Tennessee, Knoxville, Tennessee.

Description of females: Body cylindrical, white, robust, widening from head to vulva, slightly tapering to anus (Fig. 3.1 A) then attenuating at tail. Cuticle strongly annulated. Body without prominent lateral alae; lateral field originating in the esophageal region as a single incisure, then expanding as a flat field beginning at the basal bulb, interrupting the annules but without incised boundaries, running the length of the body past the anus (Figs. 3.3 E, 3.3 F); tail long, filiform, not annulated (Fig. 3.1 A). Annules prominent, cuticle thicker in esophageal region than on rest of body. First body annule larger and more pronounced than second annule. Oral disc greatly widened, borne on narrower annule-like column divided into eight longitudinally oriented sectors (Figs. 3.2 A-D).

Basal layer of cuticle composed of bands of pebble-like sectors (Figs. 3.4 A, 3.4 C) and more amorphous sectors (Figs. 3.4 B, 3.4 D) in each annule, with pebbly sectors more apparent in muscle-free zones (Figs. 3.4 A, 3.4B). Pseudocoelom in esophageal region with numerous crisscrossed connective fibers anchored at hypodermis and esophageal wall (Fig. 3.4 D); fibers sparse along most of intestinal length, more abundant in posterior intestine-rectal region (Fig. 3.1 F).

Head end with two pairs of flattened, bifurcated flaps forming a cruciate oral opening (Figs. 3.2 A-D). Amphid apertures inconspicuous, small, linear, each with a minute associated guard spine (Figs. 3.2 A-B, 3.2 D). Stoma wide; cheilostom thin, sinuous in profile; gymnostom appearing as a thick, curved rod wider at base than at apex; telostom thick, angled, bearing three pairs of pointed teeth, larger teeth appearing less sclerotized than smaller teeth (Fig. 3.1 B). Esophagus with long cylindrical corpus, short isthmus and pyriform basal bulb containing a grinding valve; terminus of corpus with distinct esophago-intestinal valve. Anterior region of esophagus surrounded by six glands (dorsal, ventral,

four sublateral), each with prominent granules and 1 or 2 nuclei (Fig. 3.1 B). Anterior end of intestine swollen (Figs. 3.1 A).

Nerve ring encircling esophagus at middle of procorpus. Secretory-excretory system consisting of massive ampulla and large oval pore located level with anterior end of basal bulb at about the 34th annule (Figs. 3.1 C, 3.3 B, 3.3 C), canals not seen. Reproductive system amphidelphic, vulva located just anterior to midbody, transverse, anterior lip flap-like; vagina strongly muscular, directed anteriorly; both uteri on right side of body, gonads doubly reflexed in mature females, stretching from anterior intestinal region nearly to anus; anterior gonad without spermatheca, posterior gonad with an axial spermatheca containing small, broadly oval sperm (Figs. 3.1 A, 3.1 D-G). Four ventral coelomocytes present, most anterior coelomocyte associated with anterior flexures of gonads, other three coelomocytes in region of anterior uterus (Figs. 3.1 A, 3.1 D-F). Intestine composed of single layer of polygonal cells (Fig. 3.1 D; rectum about two body annules long, straight, not inflated (Fig. 3.1 F). Anus without prominent flap. Phasmid aperture a minute pore about 80 μm posterior to anus (Fig. 3.1 F).

Males not known.

Juveniles similar to females except for development of reproductive system (Fig. 3.1 E); four coelomocytes distributed as in female: one at anterior-most part of gonads, the other three closer to developing vagina.

Egg (Fig. 3.3 D) broadly oval, shell thin, minutely roughened. Newly formed eggs with cytoplasm distributed uniformly inside egg, older eggs with cytoplasm contracted (Figs. 3.1 F, 3.1 G).

Differential diagnosis: The morphology of the head region, with expanded labial disc with four flaps, cruciate stomal opening and supporting eight-sectored annule-like column, appears to be unique among oxyuridomorph parasites of arthropods, setting this new genus and species apart from the other members of the infraorder. There are now six recognized thelastomatid species known from North American diplopods: *S. shelleyi*, *Thelastoma attenuatum* (Leidy, 1849), *T. collare* Upton, Crawford and Hofmann, 1983, *T. krausi* Carreno, 2007, *T. labiatum* Leidy, 1850 and *T. spicatum* Cobb, 1929. Differentiation of these species in previous papers was based on morphological characters such as the position of the excretory pore and length of the tail (Leibersperger, 1960; Jarry and Jarry, 1968; Carreno, 2007). Due to the lack of type material from older specimens such as *T. labiatum*, coupled with scant drawings and descriptions, additional characters need to be re-evaluated by examination of new specimens.

Discussion

Stauratostoma n. gen. is distinctive in the unique shape of the anterior end, in which the labial disc is greatly expanded. This disc surmounts an apparent columnar first annule, which appears to be longitudinally divided into eight sectors. Those eight sectors probably correspond to the eight circumoral papillae (labiopapillae) often observed in *Thelastoma* spp. This interpretation is based on the location of the amphid apertures, which are near the lateral edges of the disc. In *Thelastoma* spp. the amphid apertures are in the margin between the labial disc and the papillae. Adamson and van Waerebeke (1992) considered *Thelastoma* to have two cephalic annules, with eight labiopapillae on the first annule surrounding the labial disc. We consider the columnar structure to be a pseudoannule formed from the eight labiopapillae found in typical *Thelastoma* spp. The labial disc plus

this apparent or pseudoannule therefore form the first cephalic annule, with the second annule either absent or present as the apparent first body annule. This arrangement differs from that in other thelastomatid nematodes, in which a more conventional smaller labial disc is surrounded by the wider cephalic annule. Thelastomatidae has been considered a paraphyletic group (Adamson and van Waerebeke, 1992) without a unifying synapomorphy, a hypothesis supported by a limited molecular survey of thelastomatid species from a cockroach (Jex et al., 2005). The family as currently circumscribed contains about 30 genera following additions and subtractions (Adamson and van Waerebeke, 1992; Jex et al., 2005; Phillips et al., 2016). None of these genera have the cephalic development seen in *Stauratostoma*, nor do any possess a cruciate stomal opening. The only other thelastomatid species with a similarly constricted head region is *Cranifera cranifera* (Chitwood, 1932) from the gut of the cockroach *Archimandrita tesselata* Rehn, 1903. The lateral view of the *C. cranifera* head region (Chitwood, 1932; Carreno and Tuhela, 2011) resembles that of *S. shelleyi* in being mushroom-shaped, but in *C. cranifera* the labial disc is surrounded by eight lobes as in typical Thelastomatidae. In *S. shelleyi* these lobes are subsumed into a column-like false neck annule. In frontal view, the stomal opening of *C. cranifera* is circular and without flaps or other projections, whereas *S. shelleyi* has four distinct flaps that together form a cruciate stomal opening. The stoma of *C. cranifera* is cylindrical and apparently without teeth, whereas *S. shelleyi* has an array of teeth arising from the telostom.

Thelastoma labiatum Leidy, 1850 was described from a millipede now known as *Apheloria virginiensis*, one of the common hosts of *S. shelleyi*. Leidy (1853) provided a small illustration of the anterior part of *T. labiatum*, which bears some resemblance to *S. shelleyi*

in having the head end set off from the body by a significant constriction. In the brief description Leidy (1850, 1853) stated that the head annule was inflated and had six lobes at the margin. Cobb (1929) described *T. myolabiatum* from the millipede *Fontaria marginata* Say[1], with eight prominent lobes as in most other *Thelastoma* spp. Christie (1938) synonymized *T. myolabiatum* with *T. labiatum*, an action followed by Basir (1956). Van Waerebeke (1987) considered both *T. labiatum* and *T. myolabiatum* to be *species inquirendae*. Regardless of the taxonomic status of these two species, *S. shelleyi* differs from both in the expanded labial disc with four distinct flaps forming a cruciate oral opening, surmounting a narrower column formed of the eight labiopapillae.

One other species shows a possible slight convergence with *S. shelleyi*. *Thelastoma retrovulvaris* Adamson, 1987, described from a large millipede collected in the Seychelles, has a dome-like head end with the eight labiopapillae lying on the lower, wider part of the dome. This character is difficult to assess since the specimens were preserved in ethanol before being studied, and significant shrinkage may have occurred. The two taxa differ further in that the vulva is at midbody in *S. shelleyi* and just anterior to the anus in *T. retrovulvaris*.

Molecular comparisons: Appalachian representatives of Thelastomatidae have several morphological characteristics in common with *S. shelleyi*: a long esophagus, eight labiopapillae, a grinding valve in the basal bulb, thin eggshells, and a long, filiform tail. Molecular analysis supported the inclusion of *S. shelleyi* in the family Thelastomatidae as the sister group to *Thelastoma.* The 28s rDNA BI and MP trees suggested that the *Thelastoma* + *Stauratostoma* + *Coronostoma* clade is the apparent sister group to nematodes inhabiting cockroaches (*Leidynema* spp. + *Cranifera cranifera* + *Suifunema* sp. +

Hammerschmidtiella spp. group). However, the taxonomic ranks of these various groups is uncertain. *Coronostoma* spp. have major morphological differences from all other Oxyuridomorpha, and were returned by Phillips et al. (2016) to their own superfamily, Coronostomatoidea (Poinar and Willmont, 1977).

The length of each specimen of *S. shelleyi* was measured and compared to the length, width and weight of two of the most prominent millipede hosts: *Apheloria montana* and *Apheloria virginiensis* to determine if there was any correlation between nematode length and millipede size (length, width and weight). Adult nematode length was not affected statistically by millipede length, width or weight (Figs. 3.6 B-D). However, the relationship of *S. shelleyi* length to juvenile millipede morphometrics was not determined, and millipede size or developmental stage could have an effect on when infection can first occur. Almost nothing is known about the host-parasite relationships of any oxyuridomorph nematode parasitic in millipedes.

Conclusion

Stauratostoma shelleyi is unique among the thelastomatid nematodes in that the head shape in profile resembles a mushroom. With a flatten labial disc, cruciate stomal opening, and a series of eight pseudoannules, no other thelastomatid resembles *S. shelleyi*. Molecular analyses consistently place *S. shelleyi* in a monophyletic group with *Thelastoma* spp. as its sister-group. Females were well represented while juveniles were rarely encountered, and males were never recovered. *Stauratostoma shelleyi* was only found in one family of millipede, Xystodesmidae and the range extended from Tennessee, North Carolina and Alabama.

Footnote

[1]*Fontaria marginata* Say, 1821 is an untraceable name. All of the species placed in *Fontaria* are now scattered as synonyms among several tribes of Xystodesmidae. Neither Hoffman (1999) nor Marek et al. (2014) list this binomen in their checklists, nor is the name recognized in on-line literature databases such as Web of Science. R. M. Shelley (*in litt.*) suggested this name may have been a *lapsus* on the part of Cobb (1929), who might have unaccountably written *Fontaria* for *Narceus*, a genus in Spirobolidae (Spirobolida). *Narceus marginatus* Say, 1821 is a synonym of *Narceus annularis* Rafinesque, 1820, which is a host for some Thelastomatidae but not for *S. shelleyi*.

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Appendix 3

Table 3.1. Xystodesmid millipedes examined for *Stauratostoma shelleyi* n. gen., n. sp.

Species	Millipedes dissected (*n*)	Millipedes with *S. shelleyi*	Total *S. shelleyi*	Mean	Range
Apheloria montana	77	59	269	3.5	0–19
Apheloria tigana	4	0	0	0	0
Apheloria virginiensis	56	35	276	4.9	0–31
Boraria deturkiana	1	0	0	0	0
Brachoria initialis	6	4	41	6.8	0–21
Brachoria tenebrans	3	2	45	15	0–32
Cherokia georgiana	47	5	41	0.9	0–23
Dicellarius talapoosa	2	0	0	0	0
Harpaphe haydeniana	29	0	0	0	0
Pleuroloma cala	56	0	0	0	0
Pleuroloma flavipes	44	0	0	0	0
Sigmoria ainsliei	2	2	20	10	5–15
Sigmoria aphelorioides	1	0	0	0	0
Sigmoria cheiropus	14	1	6	0.4	0–6
Sigmoria mimetica	10	2	2	0.2	0–1
Total	352	110	700	2.8	0–32

Table 3.2. Morphometrics of female *Staurastoma shelleyi* n. gen., n. sp. Measurements taken from the xystodesmid millipede *Apheloria montana*. The Coefficient of Variation is designated CV.

Measurements (µm)	Holotype female	Paratype females (n=30)			
		Mean	SD	Range	CV
Length	2,522	2,357	249	1,805–2,683	10.6
Maximum width	192	178	33.9	98–237	19.1
First annule width	11.1	10.3	0.89	8.1–12.3	8.3
Second annule width	8.4	7.8	1.2	5.3–9.9	14.9
Esophagus length	508	486	50.0	347–548	10.3
Basal bulb length	116	111	8.6	84–126	7.8
Head to excretory pore	404	389	43.9	274–445	11.3
Vulva to anterior head	991	935	105.3	676–1066	11.3
Anus to anterior head	1,570	1,517	174.1	1,105–1,715	11.5
Egg length	75.2	74.2	3.4	67.2–79	4.5
Egg width	48.4	49.1	2.7	43.9–55.4	5.4
Tail length	952	84.0	90.8	649–973	10.8
Ratios					
a	13.1	13.6	2.2	10.1–18.4	16.5
b	5.0	4.9	0.43	4.3–5.9	8.9
c	2.6	2.8	0.2	2.4–3.3	5.5
V	39.3	39.7	2.4	33.2–44.4	6.1
V’	0.63	0.62	0.03	0.53–0.69	5.1

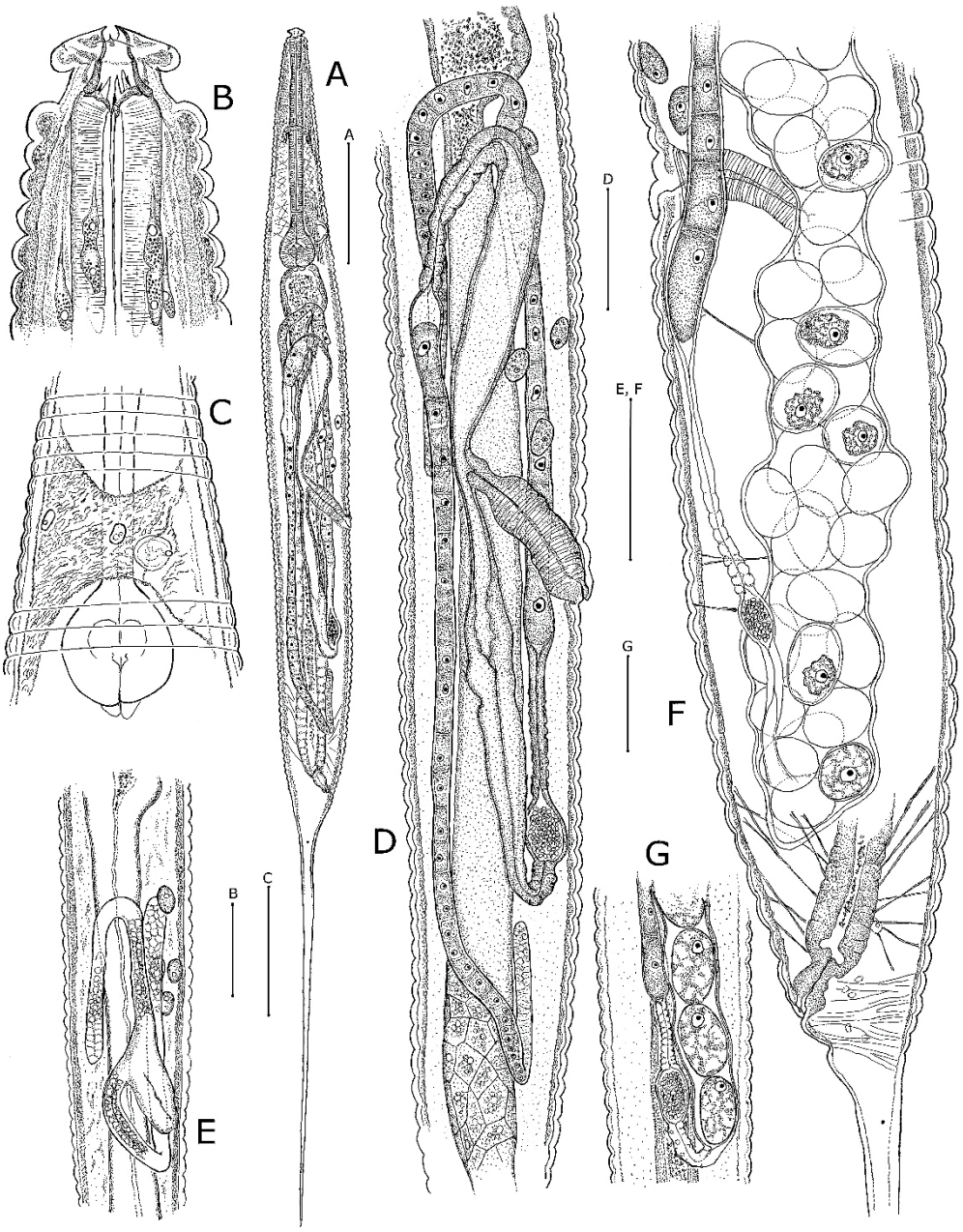

Fig. 3.1. *Stauratostoma shelleyi* n. gen., n. sp. illustrations. A) Habitus, lateral view. B) Anterior end, lateral view. C) Secretory-excretory system region, ventral view. D) Reproductive system of non-gravid female, right-side lateral view; intestinal detail provided only for anterior and posterior parts. E) Developing reproductive system of presumed fourth-stage juvenile. F) Posterior region of reproductive system of older gravid female, left-side lateral view; only the posterior end of the digestive system is illustrated. G) Posterior-most portion of posterior gonad of younger female with eggs in single file. Scales: A = 200 µm; B, E, F = 50 µm; C, D, G = 100 µm.

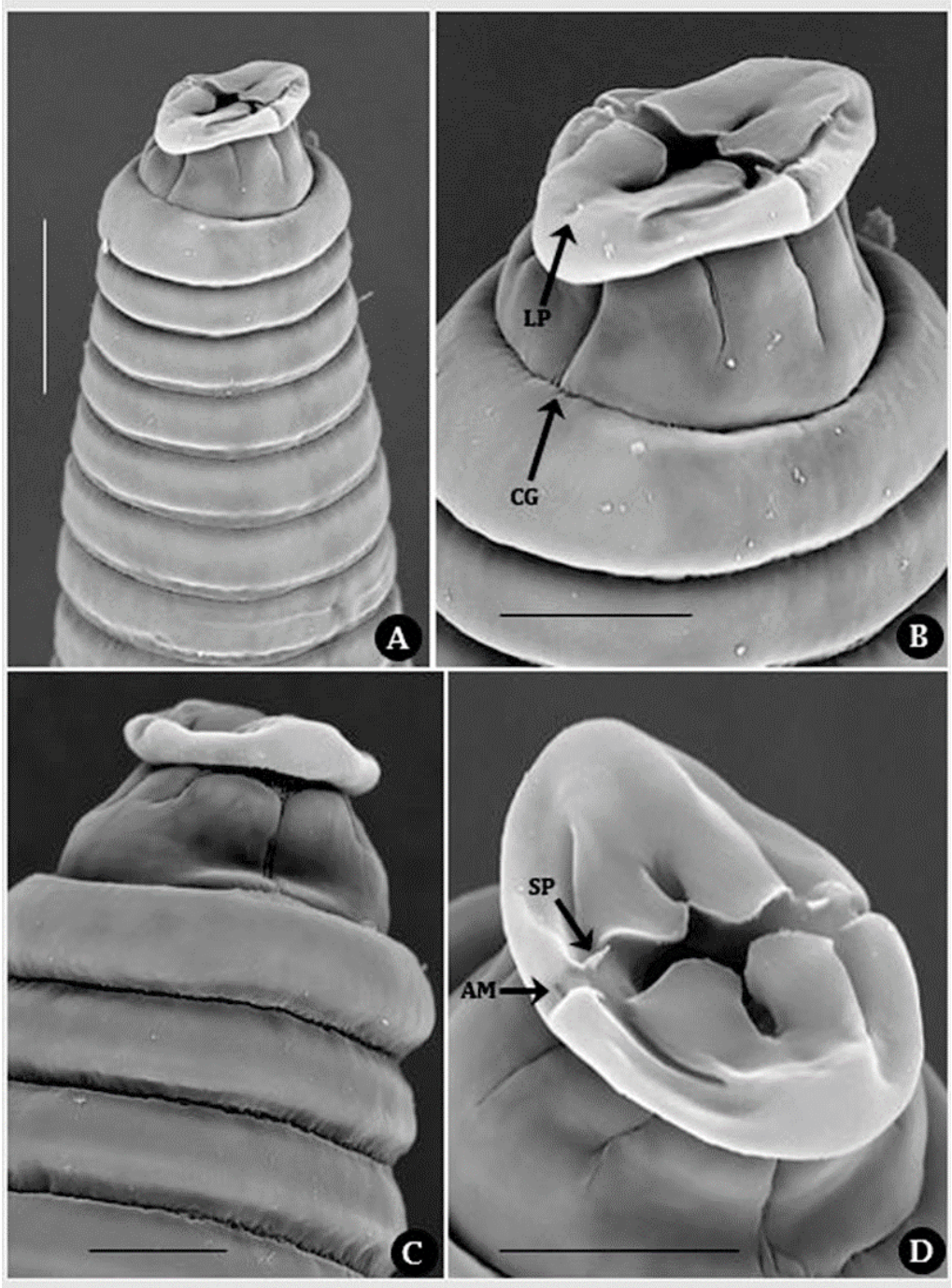

Fig. 3.2. *Stauratostoma shelleyi* n. gen., n. sp., SEMs. A) Anterior region, sublateral view. B) Enlargement of 2A, showing lateral labial pit or depression (LP), complete dorsal or ventral groove (CG) and sublateral and lateral incomplete grooves. C) Dorsal or ventral view showing complete groove and reticulated surface between lobes and labial disc. D) Labial disc with amphid aperture (AM) and associated spine (SP). Scales: A = 20 μm; B–D = 10 μm.

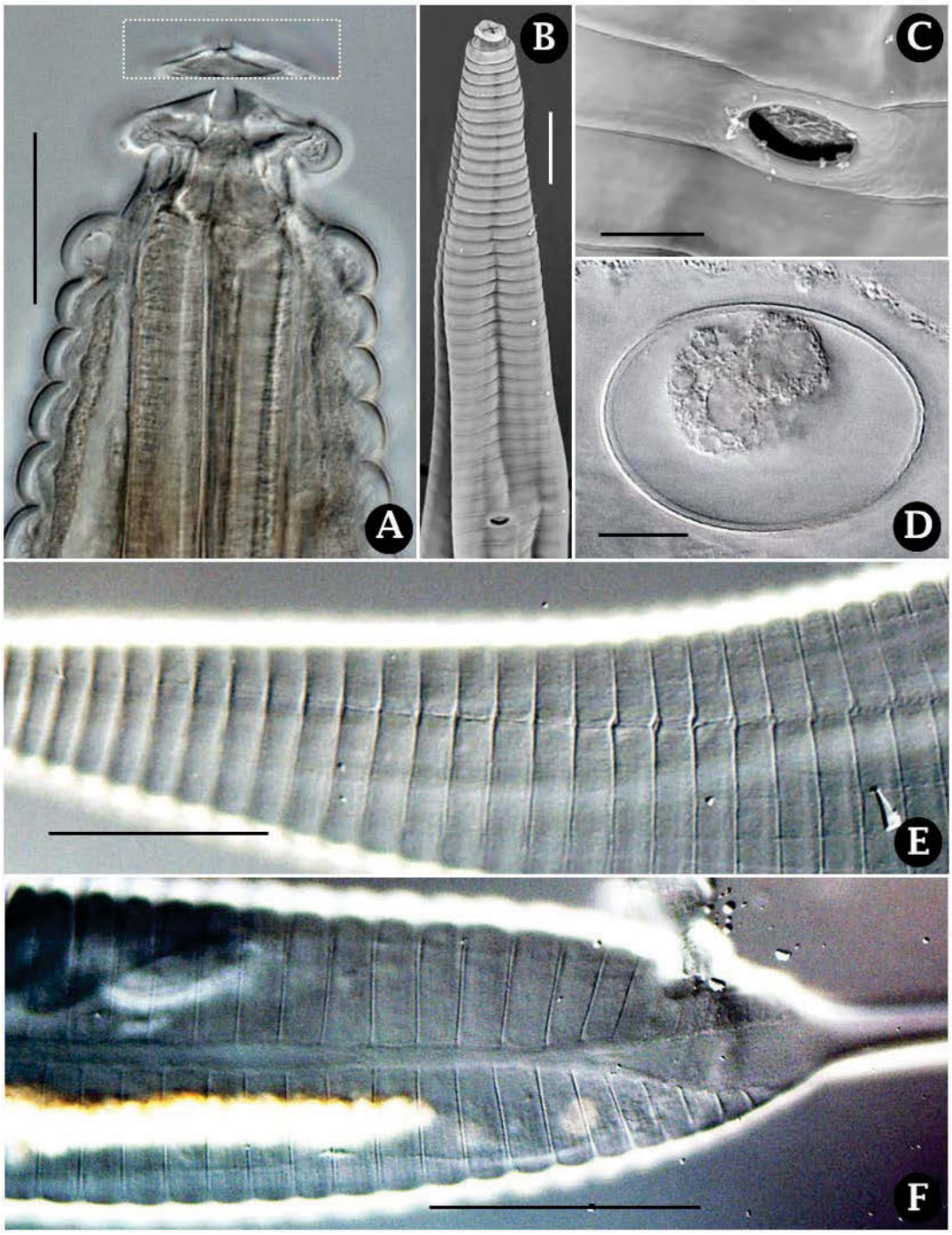

Fig. 3.3. *Stauratostoma shelleyi* n. gen., n. sp., SEMs and DICs. A) Head end, lateral view; inset shows accessory spine near amphid aperture. B) SEM of anterior region showing large S-E pore. C) S-E pore with operculum. D) Typical egg, with slightly roughened outer layer. E) Lateral field, esophageal region. F) Lateral field, posterior region. Figs. 3E and 3F are phase-contrast images. Scales: A, D = 20 µm; B, F = 50 µm; C = 10 µm; E = 100 µm.

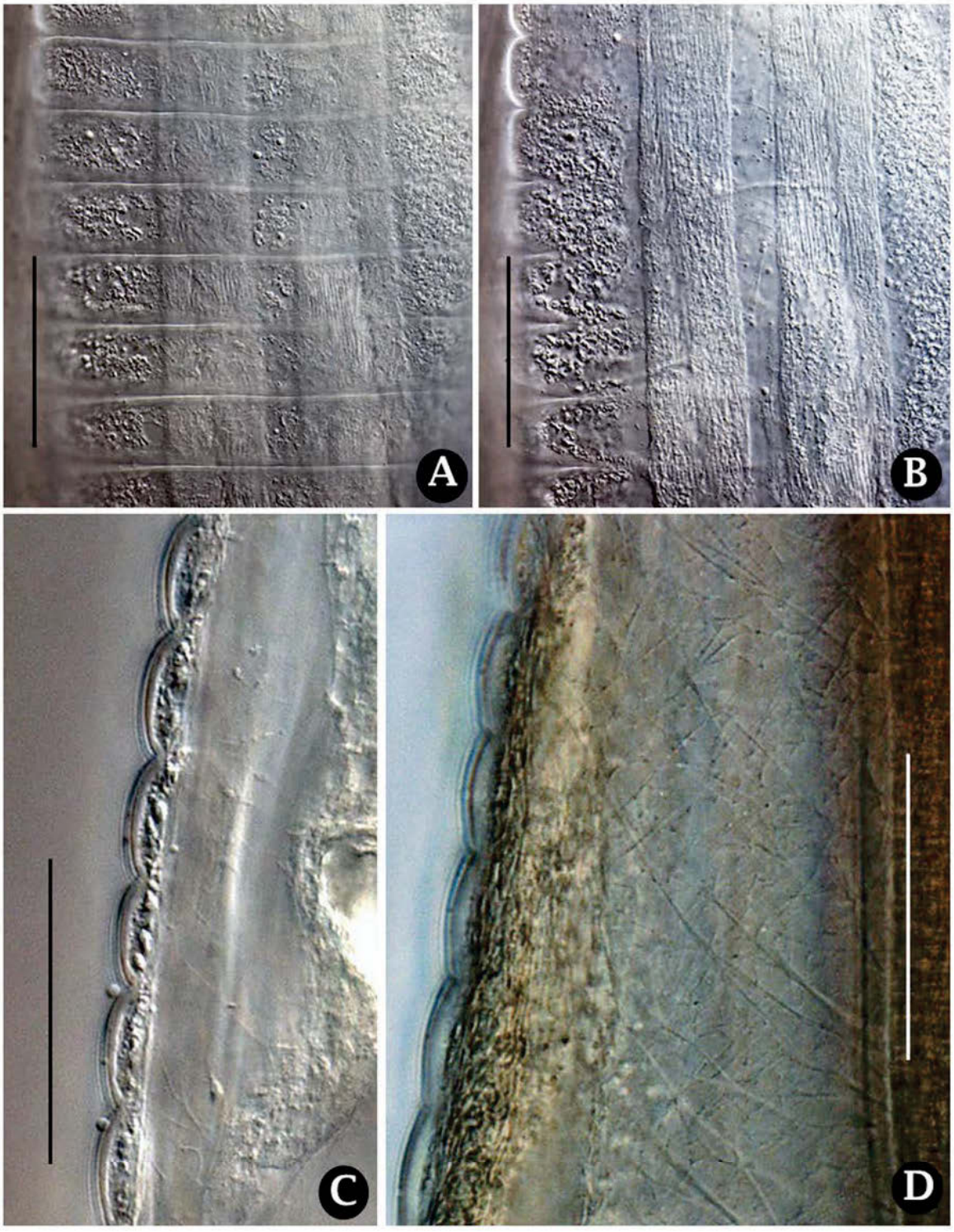

Figure 3.4. *Stauratostoma shelleyi* n. gen., n. sp. DIC of annules and cuticle. A, B) Pebble-like longitudinal subcuticular bands alternating with muscle bands; figures are of same area but imaged at different focus depths. C) Annules in profile with pebbly subcuticle. D) Annules in profile at a muscle zone, and extensive lattice of fibers in pseudocoel. Scales = 50 μm.

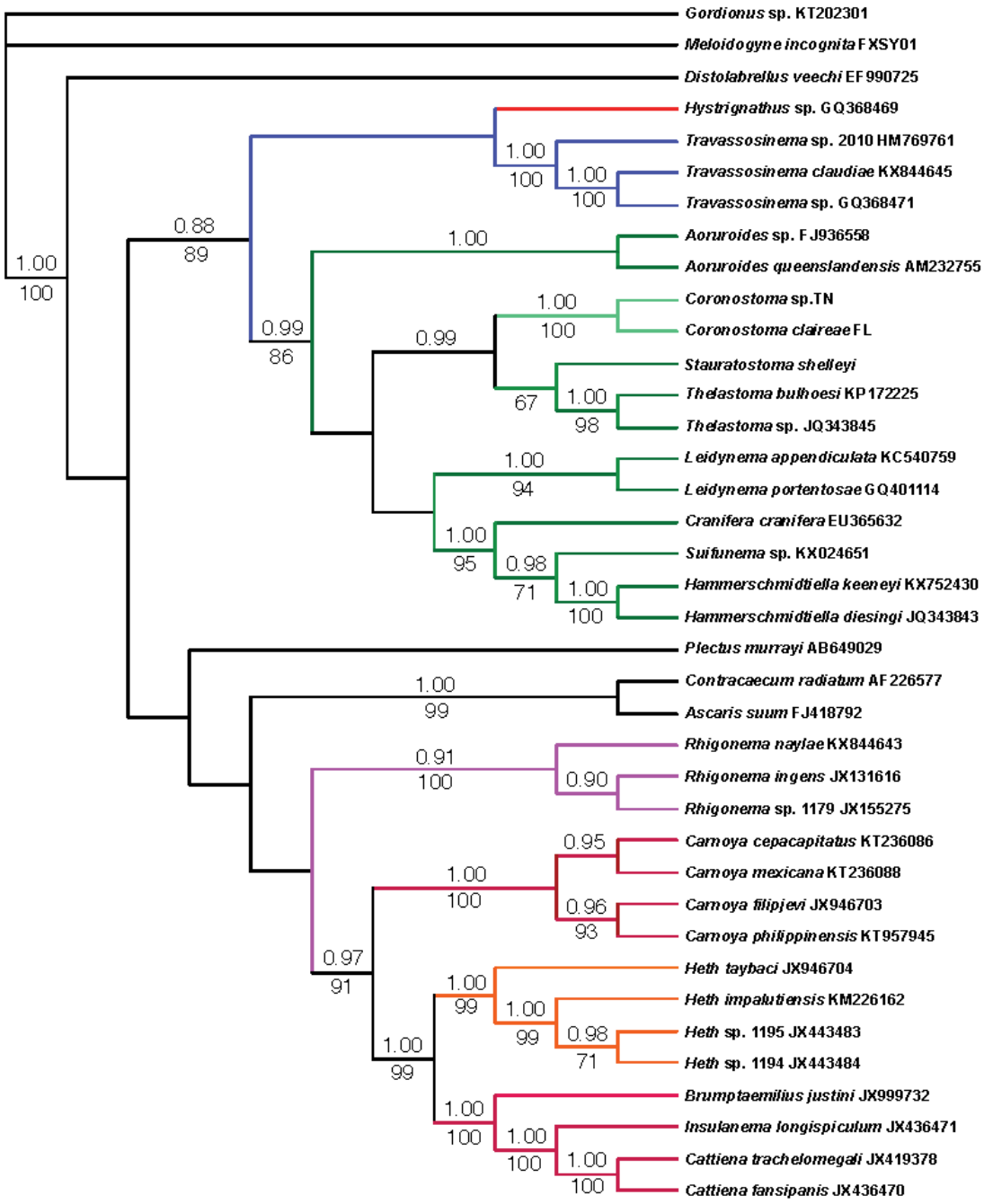

Figure 3.5. Phylogenetic relationships. Oxyuridomorpha and Rhigonematomorpha groups based on partial sequences of the D2-D3 expansion segments of 28S LSU rDNA. Posterior probability values are given on top of the branches and parametric bootstrap values on the bottom. *Stauratostoma shelleyi* is shown as the sister group of *Thelastoma* spp.

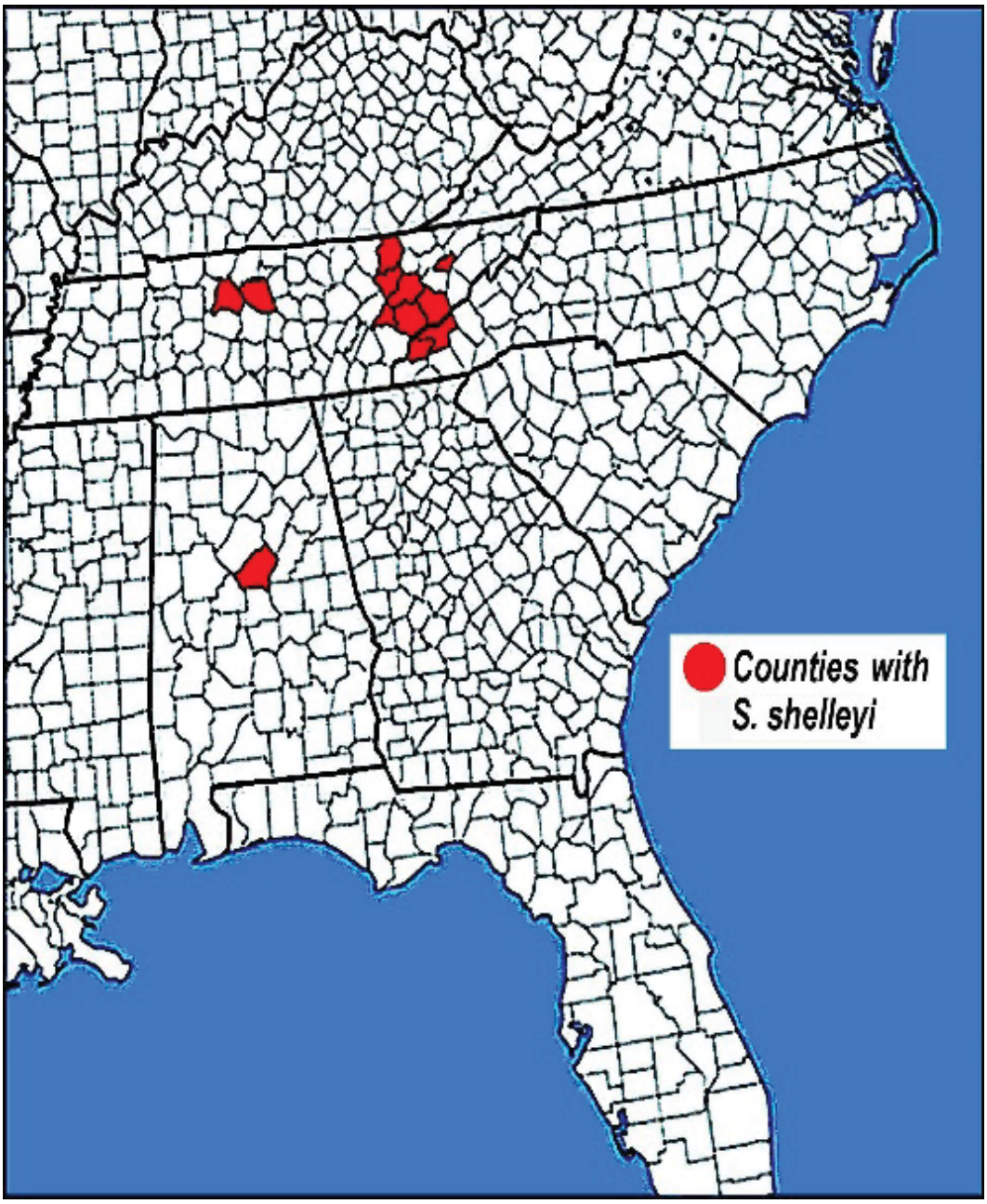

Fig. 3.6 A. Geographic range for *Stauratostoma shelleyi* n. gen., n. sp. Known distribution by county in southeastern United States.

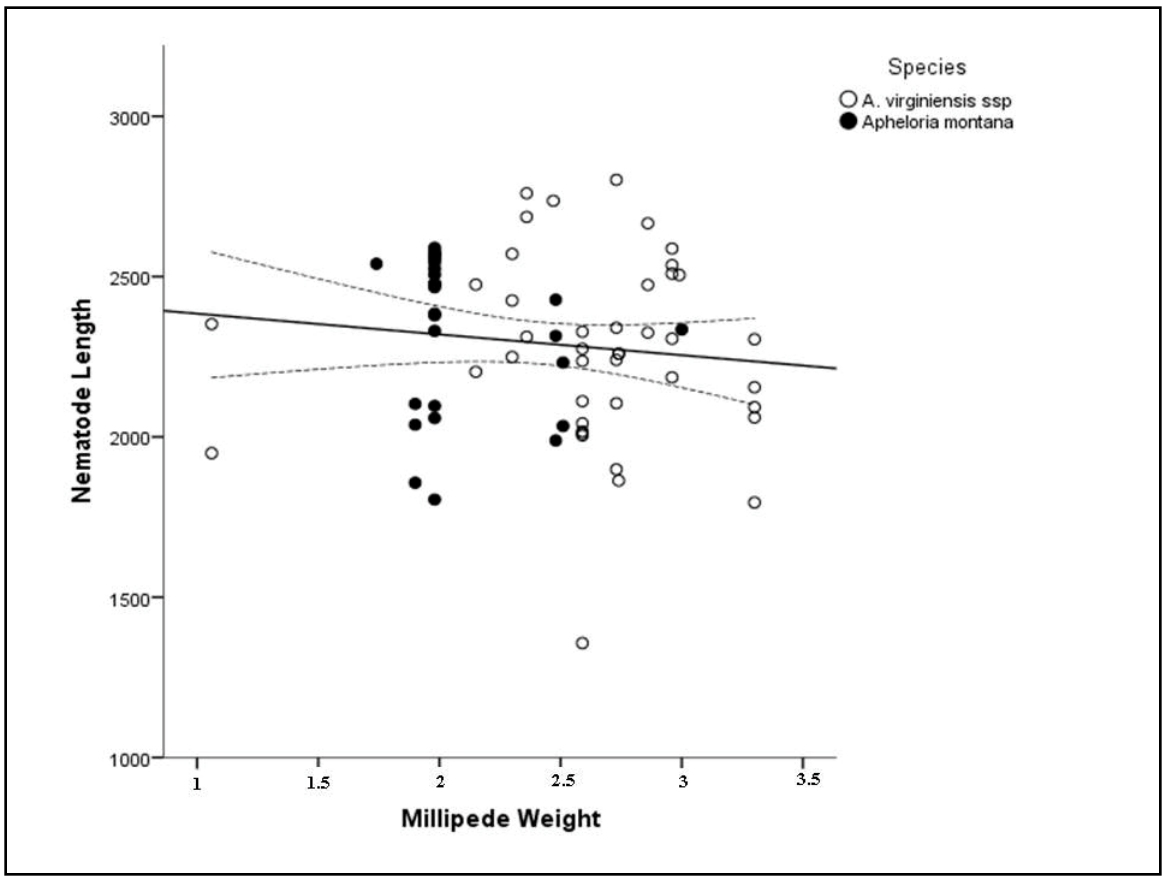

Fig. 3.6 B. Relationship between Millipede weight (g) v. Nematode length (μm). Morphometrics from the millipede species *Apheloria montana* and *Apheloria virginiensis*. The dashed lines define the 95% confidence intervals.

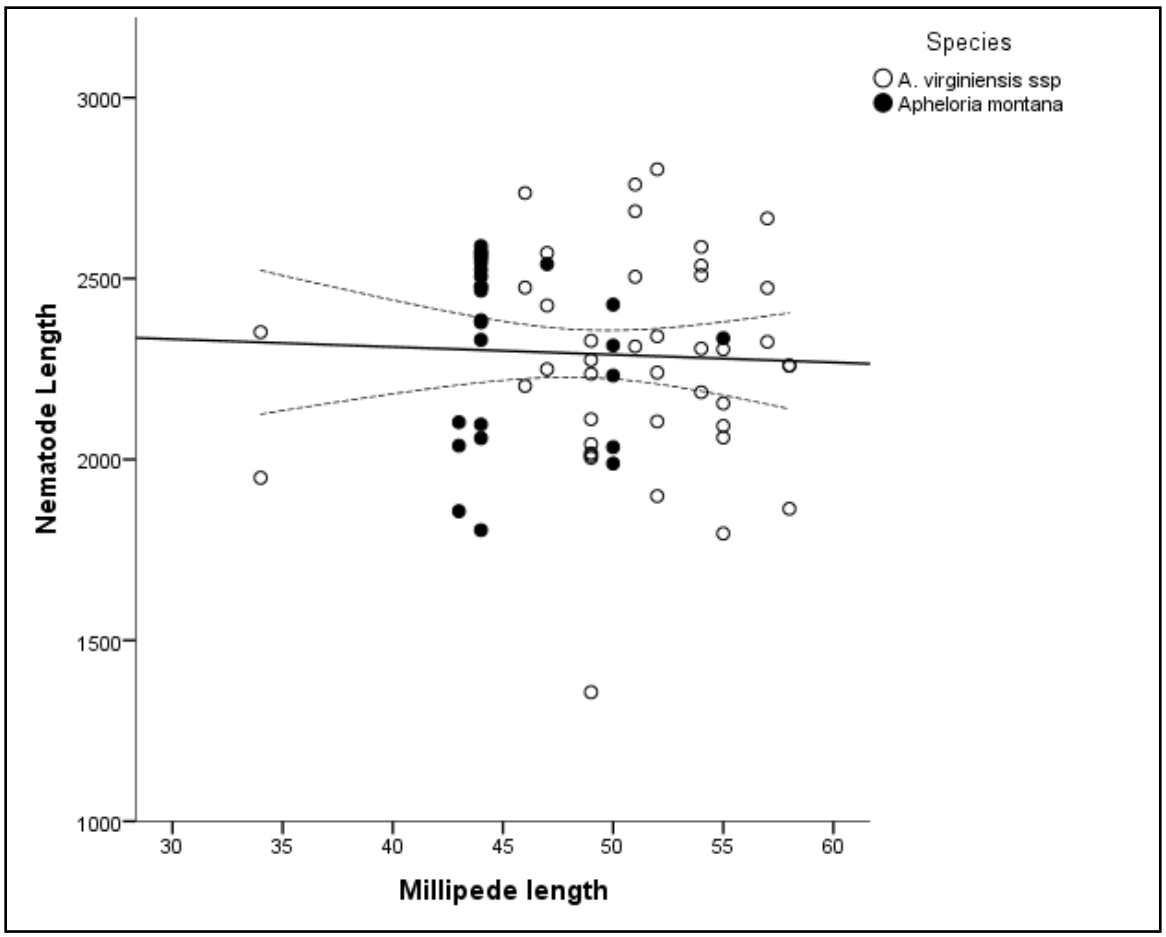

Fig. 3.6 C. Relationship between Millipede length (mm) v. Nematode length (µm). Morphometrics from millipede species *Apheloria montana* and *Apheloria virginiensis*. The dashed lines define the 95% confidence intervals.

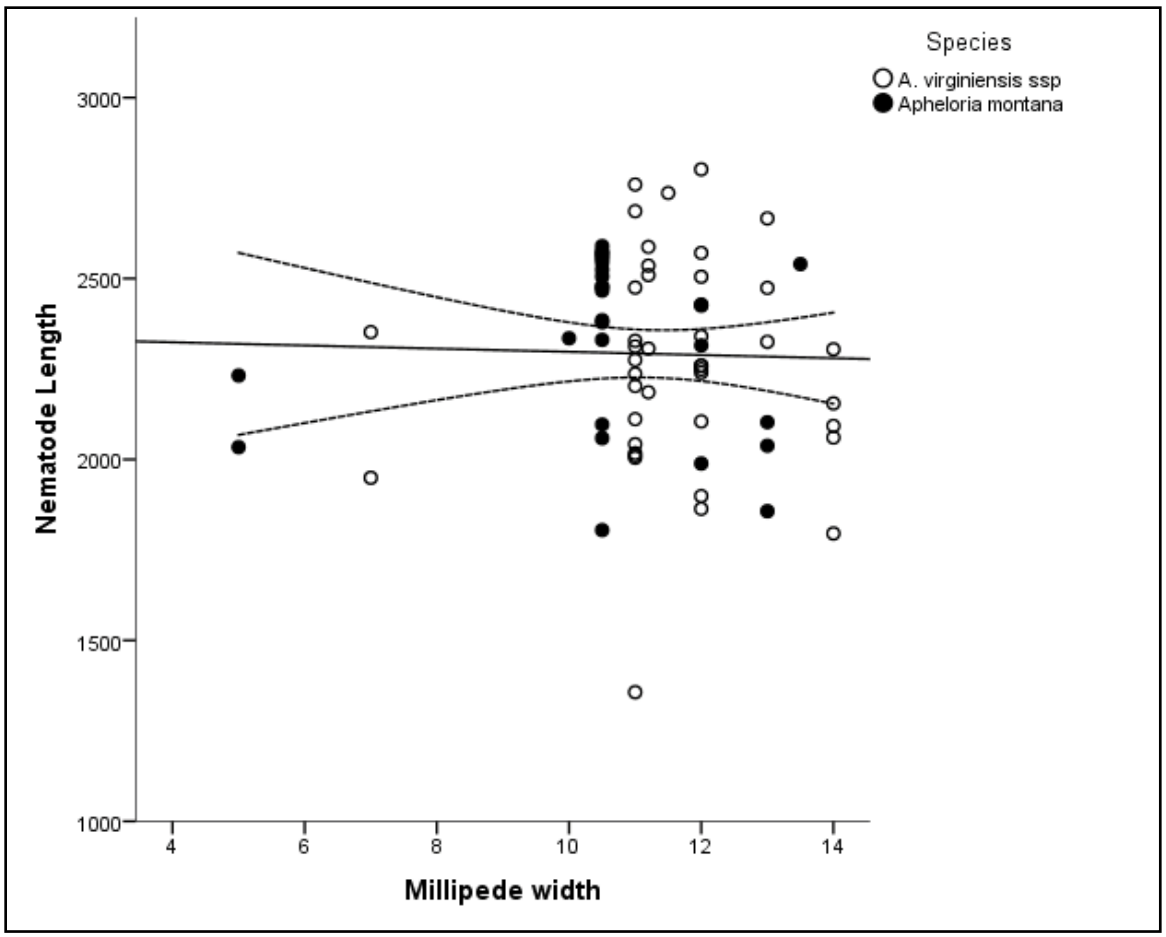

Fig. 3.6 D. Relationship between Millipede width (mm) v. Nematode length (µm). Morphometrics for millipede species *Apheloria montana* and *Apheloria virginiensis*. The dashed lines define the 95% confidence intervals.

Chapter 4

Heth pivari n. sp. (Nematoda: Ransomnematoidea: Hethidae) from the indigenous North American millipede *Narceus gordanus* (Spirobolida: Spirobolidae) from endemic areas in the Florida sand ridges including keys for worldwide *Heth* spp.

Abstract

Between December 2013 and September 2017, 44 specimens of the millipede *Narceus gordanus* (Chamberlin, 1943) (Spirobolida: Spirobolidae) were collected from Alachua, Citrus, Hernando, and Marion counties in peninsular Florida. Morphometric data were recorded for each. Nematodes were dissected from the intestine of each individual and sorted into morphotaxa. *Heth pivari* n. sp. (Oxyurida: Ransomnematoidea: Hethidae) was discovered in 33 (75%) of the millipedes and examined with bright field, differential interference contrast, phase contrast and scanning electron microscopies. LSU rDNA sequences of representative males, females and juveniles of *H. pivari* were analyzed and compared to sequences of 16 nematodes in the infraorder Rhigonematomorpha. *Heth pivari* females differ from those of other *Heth* spp. in having smooth, button-like somatic and cervical papillae and shallow, shield-like cervical collars; males have slit-like, rather than circular, stomal openings. *Heth pivari* is the first species of this genus found in an indigenous, temperate United States millipede. Keys differentiate the 49 known *Heth* spp. based on female cervical ornamentation.

Introduction

Diplopods (Myriapoda: Diplopoda) often harbor a rich diversity of life within their intestines and hemocoels, including gregarine protists, ciliates, bacteria, fungi, trichomycetes, dipterans, nematomorphans, acanthocephalans and nematodes (Leidy 1853; Cloudsley-Thompson 1949; Remy 1950; Crites 1964; Schmidt-Rhaesa et al., 2009; Hash and Brown, 2015). Within the phylum Nematoda, two orders (Oxyurida and Rhabditida) are divided into four superfamilies that contain internal kleptoparasites of temperate North American diplopods: Ransomnematoidea, Rhabditoidea, Rhigonematoidea and Thelastomatoidea. Hitherto, Ransomnematoidea were known only from *Heth mauriesi* (Adamson, 1982), and were described from the invasive bumblebee millipede, *Anadenobolus monilicornis* (von Porat, 1876) (Carreno et al., 2013). *Heth pivari* n. sp., in the indigenous Florida millipede *Narceus gordanus* (Chamberlin, 1943) (Spirobolida: Spirobolidae), represents the first ransomnematoidean in a native North American millipede.

Heth spp. have a generally tropical distribution, occurring in millipedes in Southeast Asia, Australasia, South and Central America, and Caribbean Islands (Kloss 1965; Adamson 1982, 1985; Bowie 1986; Spiridonov 1989; Hunt 1994; Malysheva and Spiridonov 2010; Carreno et al. 2013). Worldwide, there are 49 species of *Heth* from millipedes of the orders Polydesmida, Spirobolida and Spirostreptida. All are obligate kleptoparasites of diplopods (Phillips et al. 2015) that are specialized to feed on bacteria in the latter's intestinal tracts (Spiridonov 1989; Spiridonov and Yushin 2000; Malysheva and Cribb 2012). Identifying species of *Heth* relies primarily on cephalic characters of females (Adamson 1983; Hunt

1994; Spiridonov and Yushin 2000); males do not have ornate heads, usually lack anterior cephalic spines and are unknown for some species.

Narceus gordanus is one of the largest-bodied North American millipedes; it is endemic to peninsular Florida and is abundant in xeric scrub environments in inland sand ridges (Shelley and Bauer 1997; Shelley et al. 2006; Shelley and Floyd, 2014). Its intestines contained thousands of an undescribed species of *Heth* that we name *H. pivari* and describe herein.

Materials and methods

Forty-four specimens of *N. gordanus* were collected between December 2013 and September 2017 in four Florida locations: The Ocala National Forest, Marion County (Co.) (20.257726 N, –81.778702 W); Ridge Manor, Hernando Co. (28.509794 N, –82.186692 W); Citrus Springs, Citrus Co. (29.9786111 N, –82.4327777 W); and Gainesville, Alachua Co. (28.707009 N, –89.395251 W). Initially, they were measured, weighed, sexed and monitored for visible signs of disease based on their natural ambulatory motion. Each was decapitated with a razor blade, the epiproct was severed, and the intestine was pulled out of the hemocoel through the caudal end with fine-tip forceps. The remaining millipede carcasses were placed in 70% or 95% ethanol for long-term storage as vouchers. After removal, the intestinal tract was placed in a watch glass filled with water, partitioned into fore-, mid- and hindguts (Crawford et al. 1983), and dissected with the aid of a Ziess Stemi 2000 or an Olympus SZ51 stereomicroscope. Nematodes were removed from each intestinal section by squirting water into the intestine or they were manipulated with a minuten pin to dislodge them from the intestinal wall, then sorted into morphotaxa.

For light microscopy, nematodes were killed and fixed in an enclosed fume hood and heated to 60–70°C with 4% formalin (Seinhorst, 1959), mounted in anhydrous glycerin on permanent glass slides, and examined with an Olympus BX-63 differential interference contrast (DIC) microscope with a 14-megapixel Q-camera or an Olympus BX53F phase-contrast microscope with an Olympus 17-megapixel DP73 camera. Morphometric measurements of specimens of *H. pivari* in Table 4.1 and 4.2 originated from glycerin-mounted slides (all subsequent tables and figures are in Appendix 4). The holotype and paratypes are deposited in the United States Department of Agriculture Nematode Collection, Beltsville, Maryland; all additional specimens are housed in the Entomology and Plant Pathology Department, University of Tennessee, Knoxville.

For scanning electron microscopy (SEM), specimens were prepared using methods described by Phillips et al. (2016). Fixed nematodes were removed from formalin fixation and placed in 30-μm × 12-mm microporous specimen capsules (Electron Microscopy Services, Hatfield, PA) that were subsequently placed into individual 5-ml glass wells. The contents were cleaned and rinsed with distilled water for 20 minutes and then dehydrated in 20 minute rinses with 25%, 50%, 75%, 95%, and 100% ethanol. Capsules were then immersed in a 1:1 mix (v/v) of hexamethyldisilazane (HMDS) and 100% ethanol, followed by 20 minute dehydrations in 75% HMDS and two in 100% HMDS. Nematodes were stored in individual microporous capsules overnight in a fume hood and allowed to dry then positioned on 45°/90° aluminum stubs affixed with two-sided carbon tape. The stubs with nematodes were gold coated for 10 seconds at 20 μA in an SPI-Module sputter coater (West Chester, PA) and viewed and imaged with a Hitachi TM 3030 scanning electron microscope (Tokyo, Japan) at 15kV.

Molecular analyses of *H. pivari* were conducted on representative specimens of males, females, and juveniles. Total Genomic DNA (gDNA) was extracted from single specimens with a DNeasy Blood and Tissue Kit #69506 (Qiagen, Waltham, MA) according to the manufacturer's protocols. However, we reduced the final elution from the recommended 2 x 200 µl to 2 x 35 µl elution's to obtain more concentrated end products. Total gDNA was stored at –20°C until polymerase chain reaction (PCR) was conducted. Amplification was achieved with a GenePro (Bioer Technology, Hangzhou, China) thermal cycler with TaKaRa Ex Taq Hotstart DNA polymerase (Takara Bio, Shiga, Japan) with 2 µL of DNA template and 3 µL of 20 µM primers. LSU rDNA (28S) was sequenced with the primers designated as LSU391F: 5'– AGCGGAGGAAAAGAAACTAA– 3' and LSU501R: 5'– TCRGARGGAACCAGCTACTA – 3' (Nadler et al., 2006). PCR was initiated with a 90s denaturing step at 94˚C, then 4 cycles of 30s at 94˚C, 30s at 56˚C and 75s at 72˚C, followed by 4 cycles of 30s at 94˚C, 25s at 52˚C and 75s at 72˚C, 9 cycles of 30s at 94˚C, 20s at 48˚C and 75s at 72˚C and finally, 38 cycles of 30s at 94˚C, 20s at 45˚C and 75s at 72˚C.

End products from PCR were electrophoresed in 1% agarose gels for 30 minutes at 110V. Bands were cut, removed, cleaned with QiaQuick Gel Extraction Kits, and eluted with 37 µL of buffer. Cleaned templates were used for sequencing reactions with the same primers from the PCR reactions. Both 5' and 3' directions were sequenced with BigDye v3.1 terminator (Applied Biosystems, Carlsbad, CA) in a $1/20^{th}$ reaction using 0.4 µl BigDye terminators and 5 µl of a proprietary 5X sequencing buffer in a 20 µl reaction. Centrisep columns (Princeton Separations, Adelphia, NJ) were used to clean the sequencing reactions, which were subsequently dried in a Centrivap Concentrator (LABCONCO, Kansas City, MO), and then dried and sequenced by the University of Tennessee Genomics Core. Sequencher

4.7 (Gene Codes Corp., Ann Arbor, MI) was used to reconcile and verify opposing strands for accuracy. The D2-D3 segments of 28S LSU rDNA sequences of 14 females, 2 males and 1 juvenile (n=17) of *H. pivari* were attained, with sequences ranging from 534–1,042 base pairs. The range in base pairs was due to invariant sequence fragments because of the use of different sets of forward and reverse primers used for amplification. Sequence fragments were converted into a consensus and submitted to the National Center for Biotechnology Information (NCBI) in GenBank.

The consensus sequence of *Heth pivari* was aligned with orthologous sequences of 16 nematode taxa obtained from this research as well as the inclusion rhigonematomorph sequences obtained from the National Center for Biotechnology Information (NCBI) (See Fig. 4.1 A for accession numbers). Alignment was completed using Opal (Wheeler and Kececioglu, 2007) via the Opalescent package within Mesquite 3.03 (Maddison and Maddison, 2015). Relationship reconstructions was accomplished with maximum parsimony methods as implemented in PAUP* (Swofford, 2002) and Bayesian inference methods implemented using MrBayes v3.2.2 in the Cyberinfrastructure for Phylogenetic Research (CIPRES) portal v. 3.3 (Miller et al., 2010). The nematomorphan *Gordionus* sp. was used as the distal outgroup. Node support was gauged by nonparametric bootstrap resampling (10,000 reps of a single random addition sequence) as well as Bayesian posterior probabilities. Parsimony analysis was comprised of a heuristic search employing 1,000 random addition sequence replicates using tree bisection-reconnection. The resulting phylogenetic tree was modified using Canvas 8.0.5 (Deneba Systems) to produce a publication-grade figure.

Total kleptoparasitic *Heth pivari* loads inside the intestine were analyzed using a randomized block design with each specimen as the block factor, and the location and sex as the fixed independent effects. Total kleptoparasitic *Heth pivari* loads was modeled as Poisson distribution. Multiple comparisons were conducted with Tukey's adjustment. Significant effects were identified at $p<0.05$. All data analysis was conducted using PROC GLIMMIX in SAS9.4 TS1M3 for Windows 64x (SAS Institute Inc., Cary, NC).

Results

Males, females and juveniles of *H. pivari* primarily inhabited the millipedes' hindguts ($p < 0.0001$). Juveniles outnumbered females and males in both the hind- and midguts; females outnumbered males; and males were least frequently encountered in the mid- and hindguts ($p < 0.0001$). Comparisons of sexes, developmental stages, and locations within the intestine were statistically significant ($p < 0.0229$) except for females found in the hindgut when compared to juveniles in the midgut ($p = 0.1064$) (Fig. 4.1 B). Other nematodes observed in *N. gordanus* intestinal tracts included *Rhigonema* spp., *Coronostoma claireae* (Phillips and Bernard, 2016), *Thelastoma* spp., *Aoruroides* spp., *Carnoya* sp. and *Aorurus* spp.

Systematics

Infraorder Rhigonematomorpha

Superfamily Ransomnematoidea Travassos, 1930

Family Hethidae Travassos and Kloss, 1960

Heth Cobb, 1898

Heth pivari n. sp.

(Tables 4.1, 4.2; Figs. 4.1–4.6, all tables and figures in Appendix 4)

Description

Type locality and habitat: Florida: Marion County, Silver Springs, Ocala National Forest (29.257726 N, –81.778702 W), xeric scrub located near Mount Dora Sand Ridge.

Type designation and deposition: Holotype female and ten male paratypes deposited in the USDA Nematode Collection, Beltsville, Maryland. Additional specimens are deposited in the nematode collection in the Entomology and Plant Pathology Department, University of Tennessee, Knoxville, Tennessee.

Etymology: We are privileged to dedicate this new species to Robert J. Pivar, graduate student in the Entomology, Nematology and Plant Pathology Department, University of Tennessee, Knoxville.

Description of females: Measurements and ratios in Table 4.1. Body cylindrical, white in life, robust, anterior head region broad; maximum diameter near mid-body, then tapering posteriorly and terminating in filiform tail. Differentiated lateral field absent. Annulations 1–1.5 µm wide posterior to cervical collar, each annule from neck to anal region with numerous longitudinal striae; neck region finely annulated but with weak or no transverse striations (Figs. 4.3 A–D, 4.4 F). Buccal cavity elliptical, about 35 µm long by 10–12 µm wide; amphids circular, 2–4 µm in diameter, located on convex lateral aspect of each pseudolabium (Figs. 4.3 A; 4.5 A, B). Pseudolabia rectangular in dorso-lateral view, ornamented with combs and spines; small pectinate combs with 2–3 µm-long bristles on interior lateral, median and cleft margins, and larger combs with 5–7 µm-long bristles continuing along dorso-ventral aspect of each pseudolabium; dorsal and ventral bristles

rounded distally; length of bristles on each margin approximately equal (Fig. 4.2 A). Neck region with four folds anterior to cervical collar, with second, third and fourth folds each containing one smooth, knob-like cervical sensory papilla per quadrant each; papilla about 1–2 µm in diameter (Figs. 4.2 A–C; 4.4 D). Cervical collar with about 72 spines, 5–6 µm long; collar interrupted laterally on each side by lappet shield, adjacent spines often bi- or trifurcate. (Figs. 4.2 A; 4.4 E). Two lateral pairs of large, acute spines in tandem, 10–13 µm long; anterior pair sometimes connected by a shallow ridge, posterior pair less frequently so; (Figs. 4.2 A, B, D; 4.4 C, E). Annulation between each pair of spines irregular and areolated. Knob-like somatic papillae scattered along length of body (Figs. 4.2 A, B). Two-part esophagus consisting of a procorpus and basal bulb with grinding valve (Fig. 4.5 B), anteriorly surrounded by six prominent, uninucleate, amber-colored glands. Excretory pore minute, inconspicuous, 167–234 µm from anterior head (Fig. 4.5 A). Three coelomocytes, one in esophageal region, two in intestinal region. Two gonads, pro–didelphic, reflexed; spermatheca present, filled with large, elongated sperm. Eggs large, eggshells thin, few in number. Vulva located near anus. Phasmids minute pores located just posterior to anus. Tail filiform and attenuated to a fine point.

Description of males: Measurements given in Table 4.2. Body smaller than females, white, head and neck without ornamentation; lateral field absent; cuticle finely annulated, each annule 1–1.5 µm wide, with scattered, smooth papillae (Figs. 4.3 A, F). Stomal opening slit-like, 13–18 µm wide. Lips slightly elevated, surrounded by fine ridges. Amphids inconspicuous, circular, approximately 3 µm in diameter (Figs. 4.3 A, E); four inconspicuous cephalic papillae. Six brown, uninucleated arcade cells surrounding the anterior part of esophagus. Esophagus with pyriform basal bulb containing grinding valve. Intestine dilated

at anterior end, then attenuating to a uniform diameter and narrowing at cloaca. Reproductive system monorchic, reflexed. Sperm comet-fusiform shaped, about 88 µm long × 15 µm at widest point (Figs. 4.5 D, E). Ventral sucker present, 202–330 µm anterior to cloacal opening, flanked by two posterior-lateral papillae (Figs. 4.3 B, D). Anterior, posterior and lateral to the ventral sucker, four 1–2 µm somatic papillae (Fig 4.3 D). Two equal spicules fused distally; each spicule with distinct capitulum. Gubernaculum with proximal end pointed, distal end broad and flattened. Seven pairs of genital papillae and one median ventral papilla present. Genital papillae pattern as follows: one pair of subventral papillae, located posterior and lateral to ventral sucker; one pair about 30 µm anterior to cloaca; two pairs anterior and lateral to cloaca; single, medial papilla on anterior cloacal lip; two subventral pairs posterior to cloaca and one pair dorsal (Figs. 4.3 B–D).

Description of juveniles: Male juveniles similar to adults. Some with more open stomas resembling keyholes, contrasting to slit-like openings of adults (Fig 4.3 D). Juvenile amphids more conspicuous than those of adults (Fig. 4.3 D). Cephalic papillae smaller and more difficult to observe compared to those of adults.

Differential diagnosis

Heth pivari females are characterized by smooth, knob-like cervical and somatic papillae, shallow cuticular shields, continuous cuticular collars with approximately 72 subequal spines, and two pairs of large anterior and posterior lateral spines. Males lack ornate cuticular ornamentations, have slit–like stomal openings and smooth somatic papillae, but otherwise are similar to other described males. *Heth pivari* is distinct from the lone congeneric North American species, *H. mauriesi*, in the adventive millipede, *Anadenobolus monilicornis* (Spirobolida: Rhinocricidae), in that females are longer (2,190–

4,483 µm) versus (1,575–2,000 µm). *Heth pivari* also has smooth cervical and somatic papillae, whereas those of *H. mauriesi* have multi-cusped sensory papillae (Adamson, 1982). *Heth pivari* possesses a trapezoidal shield that interrupts the cervical collar, whereas the shield is replaced by two stout spines in line with the smaller collar spines in *H. mauriesi*. Differentiation of *H. pivari* from other congeners is provided in the accompanying species key.

Heth pivari most closely resembles *H. hamatus* Bowie, 1985 in that both have smooth cervical and sensory papillae and a shallow shield-like continuous collar, but they differ in morphometrics and the number/shape of the lateral spines. Both possess two pairs of anterior and posterior lateral spines and a cuticular collar that dips slightly distad; however, the anterior pair in *H. hamatus* bifurcates, and is broader and fused proximally. The posterior spine of *H. hamatus* is broad and narrows to a single point (Bowie, 1985) whereas in *H. pivari*, both pair of lateral spines are either separated or connected by a thin strip of cuticle and are bifurcated. The posterior lateral spines of the cuticular collar are more pronounced in *H. hamatus* as compared to the slender and smaller lateral ones of *H. pivari*. In males of both species the distal end of the spicule is fusiform, and both have seven pair of papillae. However, *H. pivari* has an additional medial precloacal ventral papilla, whereas Bowie (1985) did not mention such a papilla in the original description of *H. hamatus*.

Females of *H. pivari* resemble those of *H. taybaci* (Malysheva and Spiridonov, 2010) and *H. tuxtlensis* (Mejia-Madrid, 2014) in that the shields are similar shapes. The posterior most lateral spines of the cuticular collar nearly reach the base of the anterior lateral spine; and, all three have smooth somatic sensory papillae. *Heth pivari* has fewer collar spines,

approximately 72, compared to about 88 around the circumference of *H. taybaci* and 100 in *H. tuxtlensis*, respectively. *Heth pivari* also differs in that the anterior and posterior lateral spines are smaller than those of *H. taybaci* and *H. tuxtlensis*; the posterior lateral spines in *H. tuxtlensis* are larger, fused, and broader than those of *H. pivari*. Males of *H. pivari* have smaller spicule arcs (mean 109 µm) than *H. taybaci* (136 µm) and about the same compared to *H. tuxtlensis*. *Heth pivari* males are generally longer (1,897–2,609 µm) compared to *H. hamatus* (1,290–1,960), *H. taybaci* (1,520 µm) and *H. tuxtlensis* (1,400–1,500 µm). Among *Heth* species with a continuous collar that is shield-like, *H. insularis* (Brazil), *H. orthopori* (Paraguay) and *H. tuxtlensis* (Mexico) are located in the same hemisphere as *H. pivari*, while all others are located in Australia, New Zealand, and Asia. *Heth insularis* and *H. orthopori* differ from *H. pivari* by cervical ornamentations.

Discussion

In her key, Chitwood (1935) distinguished five species of *Heth* by the presence or absence of subcephalic collars and whether they had lateral shield-like components. Adamson (1983) split *Heth* into two groups, one with continuous collars, and equivalently-sized spines sharing common bases, and the other without continuous collars and discontinuous spines that do not share common bases. We divide *Heth* into four unnamed major components that do not reflect phylogeny (Table 4.3): Group 1 - 15 species with continuous collars that are not shield-like (Figs. 4.6 A, B; 4.7 A–J; 4.8 K–O); Group 2 - 16 species with continuous collars that are shield-like (Figs. 4.9 A–L; 4.10 M–P); Group 3 - 14 species – discontinuous spines and a lacking shield-like collars (Figs. 4.11 A–H; 4.12 I–N); and, Group 4 - four species that do not conform to the prior categories (Fig. 4.13 A–D).

Terminology for *Heth* varies through all the descriptions, so we apply the following definitions. A continuous collar is one that has cuticular spines with a common root and wraps around the circumference of the neck, with an interruption at the junction of the lateral spines (Figs. 4.2 A; 4.6 A, B). A non-continuous collar is one in which the spines are discontinuous and not connected by a common root around the circumference of the neck and without a shield (Figs. 4.11 A–G; 4.12 H–M). The shield is the lateral interruption in the collar that is expanded into a plate-like structure (Figs. 4.9 A–L; 4.10 M–P). Papillae are small knob-like structures on the cuticle surface that can be smooth or multi-cusped. Anatomical structures, such as spines, pseudolabia, cervical collars, and anterior and posterior spines, are identified in Figs. 4.2–4.13.

Newer methods are required to separate *Heth* spp. Molecular analyses and detailed anatomical descriptions, with accompanying DIC and SEM images and drawings, will aid in comparing and contrasting them. The keys were generated from anatomical descriptions, drawings, photographs and SEM images from past researchers. Many drawings and images do not comply to today's standards and we judged the anatomical descriptions based on observable and described characters. Illustrations, mostly from the original papers, are included and referenced in the couplets.

Worldwide, 49 *Heth* spp. has been described. Taxonomic authorities, groupings, millipede taxonomies, type specimen localities and other references for *Heth* spp. can be found in Table 4.3. These keys have been designed based on female characters only, in particular, the cervical spines and other cuticular ornamentations. Some *Heth* spp. have very similar ornamentations and are hence very analogous, such as *Heth parartigasi* and *H. magnavulvaris*. In order to differentiate those species that are anatomically similar, other

characters, such as the *area rugosa* and the presence of anal flaps, are used in conjunction with cervical ornamentations to separate species.

Worldwide keys to female *Heth* species

Key to Heth species groups

1 … With a continuous collar of cervical spines; having only a minor interruption of larger lateral spines; no shield (Figs. 4.6 A, B; 4.7 A–J; 4.8 K–O) … Group 1 *Heth* spp.

2 … With a continuous cervical collar with only a minor interruption of larger lateral spines; with a shield (Figs. 4.9 A–L; 4.10 M–P) … Group 2 *Heth* spp.

3 … With a discontinuous cervical collar, no shield (Figs. 4.11 A–G; 4.12 H–M) … Group 3 *Heth* spp.

4 … Without a clear cervical collar; no shield: not conforming to any other group description (Fig. 4.13 A–D) … Group 4 *Heth* spp.

Key to group 1 Heth spp.

Continuous cervical collar without a shield

1 ... With one continuous cervical collar of spines around circumference of neck, or with only minor interruption of cervical lateral spines, or collar absent … 2

1'… With two continuous cervical collars around circumference of neck (Fig. 4.7 E); rows of eight similar-sized spines extending from cervical collar to level of esophageal isthmus; lateral alae originating from posterior-most cervical spines and ending at base of tail; female length 1,326–1,440 µm … ***H. coyi***

2 … Cervical collar not interrupted by larger lateral spines; cervical collar spines about the same size … 3

2' … Cervical collar interrupted by larger lateral spines … 7

2'' … Cervical collar with more than 3 rows of lateral spines extending length of esophagus … 12

3 … Tandem, bifurcated pair of lateral spines posterior to cervical collar … 4

3' … With one defined cervical collar; cuticular collar with approximately 32 fine, attenuating spines becoming longer laterally; broad, short, bifurcated anterior lateral spine; single lateral posterior spine (Fig. 4.8 O); length of female 1,550–1,920 µm … ***H. zeuglocantha***

3'' … Cephalic area devoid of spines; numerous multi-cusped sensory papillae anterior and posterior to cervical collar (Fig. 4.7 A); length of female 1,070–1,210 µm … ***H. albertoi***

4 … Anterior and posterior lateral spine pairs broad … 5

4' … Anterior and posterior lateral spine pairs narrow … 6

5 … Cervical collar with about 92 spines; smooth, bare cervical papillae situated between cuticular spines 13 and 14 creating a distinct notch (total of four papillae – two anterior and two posterior between spines 13 and 14); two broad multi-cusped tandem lateral spines on each side of body; anterior lateral spines (8-9 small spines, larger laterals sometimes forked) and posterior lateral spine (2-6 spines, laterals not forked) (Fig. 4.7 F); length of female 2,600–3,200 µm … ***H. gordae***

5' … Cervical collar with about 60 spines; total of eight cervical papillae between cuticular collar spines forming distinctive notch; cervical papillae posterior to collar, two broad, multi-cusped similar spines nearly encircling the body laterally; anterior lateral spine smaller than posterior spine (Fig. 4.8 N); mean female length 1,400–1,700 µm … ***H. xarochae***

6 … Two pair of anterior spines (cervical collar and anterior lateral spines) (Fig. 4.7 G); posterior lateral spines absent; length of female 1,820–2,070 … ***H. hexaspinosum***

6’ … With anterior and posterior series of discontinuous spines around the circumference of the esophageal region (Fig. 4.7 I); length of female 1,731–2,482 µm … ***H. maicuru***

7 … Large multi-cusped papillae (2–4 per side) only present anterior to cervical collar … 8

7’ … Papillae present both anteriorly and posteriorly to cervical collar; with transverse rows of small spines in cervical folds … 9

8 … Cervical, anterior and posterior lateral spines narrow and about the same size (Fig. 4.8 L); length of holotype 2,800 µm … ***H. perarmatum***

8’ … Cervical, anterior and posterior lateral spines progressively larger, broad and overlapping one another (Fig. 4.7 B); lateral alae extend from posterior esophagus to anus; female length 1,753–1,973 µm … ***H. amazonensis***

9 … Collar interrupted by long, slender spines that reach base of anterior lateral spines; tandem pair of long, slender lateral spines posterior of cervical collar (Fig. 4.8 M); female length 1,610–1,990 µm … ***H. spinosum***

9’ … Cervical collar interrupted by larger lateral spines not reaching base of anterior lateral spines … 10

10 … Anterior and posterior spines not sharing a common base; cervical collar with about 60 unequal spines (Figs. 4.6 A, B; 4.7 J); female length 1,750–1,830 µm … ***H. mauriesi***

10’ … Anterior and posterior spines joined by common base … 11

11 … Approximately 15 rows of small, transverse spines on dorsal and ventral cervical region anterior to cervical collar; cervical collar with 53–67 spines interrupted by larger lateral spines; tandem pairs of large, slender spines; about 20 multi-cusped sensory papillae per side (Fig. 4.7 D); length of female 2,960–3,090 µm … ***H. clunyi***

11’ … Cervical collar with approximately 60 similar sized spines; two tandem pair of lateral spines posterior to collar; anterior to cervical collar, 15–20 transverse rows of small spines; >20 multi-cusped sensory papillae posterior to cervical collar (Fig. 4.7 C); length of female 1,880–2,080 µm … ***H. bifidspiculatum***

12 … Prominent spines extending from posterior cervical collar to beginning of lateral alae (Fig. 4.8 K); lateral alae extending from posterior esophagus to tail; length of female 1,155–1,165 µm … ***H. pinnatum***

12’ … Somatic spines extending from cephalic extremity to isthmus with first three spines small and remaining spines long and curved (Fig. 4.7 H); female length between 1,740–2,000 µm … ***H. hispaniolae***

Key to group 2 female Heth spp.

Continuous cervical collar and with a shield that dips posteriorly

1 … Knob–like papillae present near head region … 2

1’ … Knob–like papillae absent near head region … 8

2 … Lateral cervical collar spines reaching or overlapping proximal base of anterior lateral spines … 3

2’ … Lateral cervical spines not reaching anterior lateral spines … 5

3 … Cervical spines unequal in length, increasing in size to lateral sides and forming serrated lappets on outer margins (Fig. 4.9 E); female 2,610–4,225 µm long

… ***H. impalutiensis***

3’ … Cervical spines about equal in length … 4

4 … Shield wider than long, broad (Fig. 4.9 L); female 1,365–2,075 µm long … ***H. taynguyeni***

4’ … Shield long and narrow (Fig. 4.10 O); females 2,550–2,640 µm long … ***H. vietnamensis***

5 … Shield shallow, nearly 3x wide as long; cuticular collar interrupted laterally by about 8–10 µm gap in between the two most posterior lateral cuticular shield spines; some lateral cervical spines of shield forked or tricuspid; anterior and posterior lateral spines 10–13 µm long (Fig. 4.2 A–D); female 2,190–4,483 µm long … ***H. pivari*** n. sp.

5’ … Cervical shield broad with prominent dip distally (Figs. 4.9 A; 4.9 K, 4.10 M) … 6

6 … Shield up to 3× longer than wide (Fig. 4.10 M); females 2,180–2,370 µm long … ***H. tonkinensis***

6’ … Shield width and length similar, rounded or trapezoidal in shape … 7

7 … Shield rounded or ovoid; anterior and posterior spines less than 20 µm long; (Fig. 4.9 A); female 1,105–1,440 µm long … ***H. baudini***

7’ … Shield trapezoidal; lateral spines 28–30 µm long, (Fig. 4.9 K); female 1,905–2,575 µm long … ***H. taybaci***

8 … Lateral cervical collar spines reaching or overlapping proximal base of anterior lateral spines … 9

8’ … Lateral cervical spines not reaching anterior lateral spines … 12

9 … Eight longitudinal ridges extending from base of posterior lateral spines to vulva; cuticle minutely punctate in cervical region; 38–40 cuticular spines; lateral spines 45–67 µm long with separate bases (Fig. 4.9 B); female 2,160–2,600 µm long … ***H. costata***

9’ … Longitudinal ridges absent … 10

10 … Anterior lateral spines fused at base then bifurcated; posterior spine single, not bifurcated; striae posterior to cuticular collar bearing tiny spines (Fig. 4.9 D); females 1,420–2,500 µm long … ***H. hamatus***

10 … Anterior and posterior spines both bifurcated or separated … 11

11’ … Anterior and posterior lateral spines fused, 26–33 µm long; shield about twice as wide as long (Fig. 4.9 H); posterior lateral shield spines longer than preceding cervical spines; females 2,700–3,024 µm long … ***H. orthopori***

11 … Anterior lateral spines widely separated, 50 µm long; posterior lateral spines 55 µm long, bases closer but not touching (Fig. 4.9 J); females 2,010–2,360 µm long … ***H. sutherlandi***

12 … Shield complete, not broken laterally … 13

12’ … Shield incomplete, interrupted by posterior-most lateral spines … 15

13 … Most cervical spines along shield edge larger than preceding cervical spines and serrated laterally on outer margins; some spines forked; anterior lateral spines fused with broad base, 58–67 µm long; posterior lateral spines 82–94 µm long with fused, broad base; (Fig. 4.10 N); female 1,700–2,800 µm long … ***H. tuxtlensis***

13’ … Cervical spines along shield edge about the same size … 14

14 … Cervical collar turning abruptly before lateral lines and proceeding

backwards in a curved direction, forming a "W" shape; shield as long as wide (Fig. 4.9 G) … ***H. juli***

14' … Shield serrate on outer margin; shield well anterior to anterior lateral spine; anterior and posterior lateral spines bifurcate (Fig. 4.9 C) … ***H. dimorphum***

15 … Lateral alae present; each pseudolabial plate with three conspicuous spines; cuticular collar with over 100 spines; shield about twice as long as wide; outer margin of shield serrate; anterior lateral spines 80–95 µm long; posterior lateral spines 84–107 µm long (Fig. 4.10 P); female length 2,400–2,960 µm long … ***H. xaniophora***

15' … Lateral alae absent … 16

16 … Base of anterior lateral spines widely separated; shield more rounded or ovoid (Fig. 4.9 I); females 1,970–2,630 µm long … ***H. ortonwilliamsi***

16' … Base of anterior lateral spines fused; shield trapezoidal (Fig. 4.9 F); female 1,764–1,962 µm long … ***H. insularis***

Key to group 3 female Heth spp.

Discontinuous cervical collar and no shield

1 … Cervical collar with discontinuous spines; lateral spines present … 2

1' … Cervical collar spines absent; cuticle entirely smooth; base of most posterior fold in neck with small, single posteriorly curved spine (Fig. 4.12 J); females 1,140 – 1,189 µm long … ***H. spoilatus***

2 … Sensory papillae near cervical region present … 3

2' … Sensory papillae near cervical region absent … 5

3 … Head about same width as anterior portion of body; head quadrangular in shape; each side of body with about 12 multi-cusped sensory papillae flanking lateral spines; first pair of lateral spines about twice as wide as long; anterior lateral spine with cervical projections terminating on anterior, submedial margin with posteriorly directed spines; medial lateral and posterior lateral spines 30 µm long; lateral alae present (Fig. 4.11 D); females 1,800 – 2,500 µm long … ***H. imias***

3’ … Head narrower than anterior portion of body … 4

4 … Without an observable cervical collar; first pair of lateral spines with broad, connected base and connected; anterior and posterior lateral spines with broad base and bifurcated; (Fig 4.11 B); females 1,640 – 2,720 µm long … ***H. baracoa***

4’ … Cervical collar with four pair of lamellar sets of similar sized spines, not completely wrapping around circumference of cervical region; first pair of lateral spines slender and not connected; anterior and posterior lateral spines slender (Fig. 4.11 C) … ***H. duvidosum***

5 … Head wide, about 1.5× width of anterior portion of body; cervical collar with about 12 small spines of equal size; one lateral spine in same plane as collar; two posterior lateral spines not sharing common base; (Fig 4.11 E); females 1,312 – 1,600 µm long … ***H. macrocephala***

5’ … Head not greatly enlarged, about same width as anterior portion of

body … 6

6 … With one cervical collar … 7

6’ … With two cervical collars; head narrower than anterior portion of body; anterior collar with 6–8 μm long spines; posterior spines equal in size, 3–5 μm long; anterior and posterior collars without larger lateral spines (Fig. 4.12 H); females 2,142–3,004 μm long … ***H. sinediscus***

7 … Pseudolabium with denticles or small spines; four pairs of lateral spines on each side of body, increasing in length from anterior to posterior, approximately 10, 16, 23 and 32 μm long, respectively (Fig. 4.12 K); females 1,685–1,890 μm … ***H. travassosi***

7’ … Psuedolabium without denticles or small spines … 8

8 … Cervical collar with more than 40 non-contiguous spines of similar length around circumference of neck; posterior to cervical collar, two sets of paired anterior and posterior lateral spines (Fig. 4.12 M); females 2,400–3,090 μm long … ***H. tuzetae***

8’ … Cervical collar with fewer than 40 discontinuous spines ... 9

9 … Transverse rows of small cuticular spines encompassing anterior esophageal region; two pairs of lateral spines; anterior and posterior lateral spines separate, 11-12 μm long (Fig. 4.12 I); females 1,831–2,126 μm long … ***H. spinalatum***

9’ … Esophageal region devoid of transverse rows of small spines … 10

10 … Pseudolabial plate spines absent … 11

10’ … Pseudolabial plate spines present … 12

11 … Cervical collar with 8 spines around circumference of neck, interrupted by two lateral spines within the same plane as cervical spines; two posterior lateral spines per side and widely separated (Fig. 4.12 L); females 1,550–1,830 µm long … ***H. travofilhoi***

11’ … Cervical collar formed by 10 large spines around circumference of neck; one pair of lateral spines (Fig. 4.11 F); female length 2,062–2,186 µm … ***H. multiplus***

12 … Cervical collar with 12 spines consisting of four posterior and four anterior spines, flanked by two sublateral spines on each side; one pair of lateral bifurcated spines posterior to collar, broadly fused at base (Fig. 4.11 A); females 1,550–1,830 µm long … ***H. artigasi***

12’ … Posterior lateral spines not broadly fused, meets at a point … 13

13 … Without an anterior vulval flap; *area rugosa* present anterior to vulva (Fig. 4.11 G) … ***H. parartigasi***

13’ … Anterior lip of vulva with overlapping posterior lip; *area rugosa* absent … ***H. magnavulvaris***

Key to group 4 female Heth spp.

Without a collar, no shield, and with or without anterior body covered in spines

1 … Body covered in spines from cervical collar to anus … 2

1’ … Body with spines from cervical collar to isthmus; with multi-cusped sensory papillae posterior to cervical collar; 5–7 lateral spines extending from buccal cavity to nerve ring (Fig. 4.13 D) … ***H. poeyi***

1'' … Body without prominent somatic spines (excluding lateral spines); anterior-most lateral spine bifurcated; posterior lateral spines with broad bases; spines with curved distal tips; lateral alae present from posterior esophagus to anus (Fig. 4.13 C); females 1,332–1,731 µm long … ***H. lamothei***

2 … With three pairs of bifurcated lateral spines, most anterior spines serrated (Fig. 4.13 B); female length 1,983–2,308 µm … ***H. josephinae***

2' … With four pairs of bifurcated lateral spines, somatic spines becoming serrate at about tenth spine (Fig. 4.13 A); female length 1,562–1,702 µm … ***H. adolphi***

Conclusion

With the inclusion of *Heth pivari*, there are now 49 worldwide species of *Heth*. *Heth pivari* is the first of its genus to be discovered in a temperate, indigenous North American millipede, and the second species to be found in the United States. Revision of *Heth* spp. will take years to complete and better efforts need to be taken to molecularly and morphologically characterize many of the *Heth* spp., especially those originating from Cuba and South America. Many type specimens have been lost or destroyed and additional research will be needed to collect neotypes to accurately described those species that are not available for comparison to newly discovered species.

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Appendix 4

Table 4.1. Morphometric measurements of *Heth pivari* n. sp. females.

		Paratype Females (*n* = 71)			
	Holotype female	Mean	SD	Range	CV
Measurements (μm)					
Length	2,800	3,011	406	2,190–4,483	13.5
Maximum width	182	191	52	131–380	27.2
Esophagus length	382	371	17	327–407	4.6
Basal bulb length	140	148	7.1	135–176	4.8
Egg length	210	209.1	11	180–234	5.3
Egg width	111	93.5	10.7	59–116	11.4
Head to excretory pore	167	195	20	167–234	10.3
Head to vulva	2,189	2,337	326	1,664–3,644	13.9
Head to anus	2,394	2,585	370	1,743–3,938	14.3
Tail length	406	426	50.2	318–545	11.8
Ratios					
a	15.4	16.3	2	10.9–21.7	13.5
b	5.4	5.8	0.74	4.4–8	27.2
c	6.9	7.1	0.64	4.8–9.4	4.6
V (%)	78.2	78	1.6	73–83	4.8
V'	0.91	0.91	0.02	0.87–0.96	5.3

Table 4.2. Morphometric measurements of *Heth pivari* n. sp. males.

	Allotype	Paratype Males (*n* = 30)			
		Mean	SD	Range	CV
Measurements (µm)					
Length	2,075	2,253	280	1,897–2,997	12.4
Maximum width	130	137	29	99–198	21.2
Esophagus length	596	622	28	567–681	4.5
Basal bulb length	138	135	3.1	130–144	2.3
Head to excretory pore	250	264	19	236–290	7.2
Ventral sucker length	20	22.5	3	18–29	13.3
Ventral sucker width	15	15.9	2.7	9–22	17
Ventral sucker–cloaca	219	248	32	202–330	12.9
Spicule arc	102	109	6.4	96–124	5.9
Gubernaculum arc	50	66	5.1	53–79	7.7
Cloaca–head	1,916	2,086	271	1,752–2,815	13
Tail length	159	167	15	137–202	9
Ratios					
a	16	16.8	2.5	13–24	14.9
b	3.9	3	0.35	2.6–3.9	11.7
c	13	13.5	1.4	11–17	10.4
c'	92	93	0.73	91–94	0.8

Table 4.3. Worldwide described *Heth* species.

***Heth* Species**	**Group[1]**	**Millipede Order**	**Millipede species**	**Type locality**	**Other localities**	**Other references**
H. adolphi Sanchez and Velasquez, 1979	4	Polydesmida	*Amplinus* sp.	Mexico	None	None
H. albertoi Garcia and Fontenla, 2004	1	Spirobolida	*Anadenobolus arboreus*	Puerto Rico	None	None
H. amazonensis Kloss, 1965	1	Unknown	Unknown	Brazil	None	None
H. artigasi Dollfus, 1952	3	Spirobolida	*Rhinocricus* sp.	Venezuela	Brazil	Kloss, 1965; Adamson, 1983
		Spirobolida	*Rhinocricus flavocinctus, R. albiventris*			
		Spirostreptida	Unknown		Peru	Tantalean & Altmann, 1988
H. baracoa Spiridonov, 1989	3	Spirobolida	*Rhinocricus* sp.	Cuba	None	None
H. baudini Malysheva and Cribb, 2012	2	Spirostreptida	Iulomorphidae	Australia	None	None
H. bifidispiculum Adamson, 1982	1	Spirobolida	*Rhinocricus flavocinctus*	Venezuela	None	None

Table 4.3. Continued. Worldwide described *Heth* species.

***Heth* Species**	**Group[1]**	**Millipede Order**	**Millipede species**	**Type locality**	**Other localities**	**Other references**
H. clunyi Adamson, 1985	1	Spirobolida	*Rhinocricus bernardinensis*	Paraguay	None	None
H. costata Hunt, 1994	2	Spirobolida	Unknown	New Guinea	None	None
H. coyi Garcia, 1997	1	Spirobolida	*Rhinocricus suprenans*	Cuba	None	None
H. dimorphum Chitwood, 1935	2	Spirostreptida	*Spirostreptus* sp.	Sumatra	Philippines	Chitwood, 1935; Kloss, 1965
H. duvidosum Artigas, 1929	3	Unknown	Unknown	Brazil	None	None
H. gordae Mejia-Madrid, 2014	1	Spirobolida	*Anadenobolus putelais*	Mexico	None	None
H. hamatus Bowie, 1985	2	Spirostreptida	*Eumastigonus kaorinus*	New Zealand	None	None
H. hexaspinosum Chitwood, 1935	1	Spirobolida	*Rhinocricus* sp., *R. padbergi*, *R. cachoeirensis*, *Spirobolus* sp.	Panama	Brazil, Argentina	Kloss, 1965

Table 4.3. Continued. Worldwide described *Heth* species.

***Heth* Species**	**Group[1]**	**Millipede Order**	**Millipede species**	**Type locality**	**Other localities**	**Other references**
H. hispaniolae Garcia, Coy, and Ventosa, 2001	1	Spirobolida	Unknown	Dominican Republic	None	None
H. imias Spiridonov, 1989	3	Spirobolida	*Rhinocricus* sp.	Cuba	None	None
H. impalutiensis Malysheva, Mohagan, and Spiridonov, 2015	2	Spirostreptida	Harpagophoridae	Philippines	None	None
H. insularis Kloss, 1965	2	Unknown	Unknown	Brazil	None	None
H. josephinae Sanchez and Velasquez, 1979	4	Polydesmida	*Amplinus* sp.	Mexico	None	None
H. juli Cobb, 1898	2	Julida	*Julus* sp.	Australia	None	Travassos, 1929; Hunt, 2015
H. lamothei Sanchez and Velasquez, 1979	4	Polydesmida	*Amplinus* sp.	Mexico	None	None
H. macrocephala Kloss, 1965	3	Spirobolida	*Rhinocricus padbergi*	Brazil	None	None

Table 4.3. Continued. Worldwide described *Heth* species.

***Heth* Species**	**Group[1]**	**Millipede Order**	**Millipede species**	**Type locality**	**Other localities**	**Other references**
H. magnavulvaris Adamson, 1985	3	Spirobolida	*Rhinocricus bernardinensis*	Paraguay	None	None
H. maicuru Kloss, 1961	1	Spirostreptida	*Scaphiostreptus buffalus*	Brazil	None	Kloss, 1965; Adamson, 1983;
H. mauriesi Adamson, 1982	1	Spirobolida	*Anadenobolus politus*	Martinique	Guadeloupe,	Spiridonov & Yushin, 2000;
			Leptogoniulus naresi, *Trigoniulus lumbricinus, Anadenobolus monilicornis*		Florida (USA)	Carreno et al., 2013
H. multiplus Kloss, 1965	3	Unknown	Unknown	Brazil	None	None
H. orthopori Adamson, 1987	2	Spirostreptida	*Orthoporus americanus*	Paraguay	None	None
H. ortonwilliamsi Hunt, 1994	2	Spirobolida	*Polyconoceras* sp.	New Guinea	None	None
H. parartigasi Adamson, 1985	3	Spirobolida	*Rhinocricus bernardinensi*	Paraguay	None	None

Table 4.3. Continued. Worldwide described *Heth* species.

***Heth* Species**	**Group[1]**	**Millipede Order**	**Millipede species**	**Type locality**	**Other localities**	**Other references**
H. perarmatum Dollfus, 1952	1	Spirobolida	*Rhinocricus cachoeirensis*	Brazil	None	None
H. pinnatum Garcia and Coy, 1995	1	Spirobolida	*Anadenobolus sagittatus*	Cuba	None	None
H. pivari Phillips and Bernard, n. sp.	2	Spirobolida	*Narceus gordanus*	Florida (USA)	None	None
H. poeyi Coy, Garcia and Alvarez, 1993	4	Spirobolida	*Rhinocricus duvernoyi*	Cuba	None	None
H. sinediscus Kloss, 1965	3	Unknown	Unknown	Brazil	None	None
H. spinalatum Kloss, 1965	3	Unknown	Unknown	Brazil	None	None
H. spinosum Artigas, 1929	1	Spirobolida	*Rhinocricus cachoeirensis*	Brazil	None	None
		Spirobolida	*R. padbergi*, *R. albiventris*, *R. nattereri varians*		Brazil, Peru	Adamson, 1983; Tantalean & Altmann, 1988
		Spirostreptida	*Cladostreptus sebastianu*			

Table 4.3. Continued. Worldwide described *Heth* species.

***Heth* Species**	**Group[1]**	**Millipede Order**	**Millipede species**	**Type locality**	**Other localities**	**Other references**
H. spoliatus Garcia and Coy, 1995	3	Spirobolida	*Anadenobolus sagittatus*	Cuba	None	None
H. sutherlandi Hunt, 1994	2	Unknown	Unknown	New Guinea	None	None
H. taybaci Malysheva and Spiridonov, 2010	2	Spirostreptida	Harpagophoridae	Vietnam	None	None
H. taynguyeni Malysheva and Spiridonov, 2010	2	Spirobolida	*Apeuthes* sp.	Vietnam	None	None
H. tonkinensis Malysheva and Spiridonov, 2010	2	Polydesmida	*Platyrhachus* sp.	Vietnam	None	None
H. travassosi Dollfus, 1952	3	Polydesmida	*Leptodesmus jucundus* *L. paulistus*, *L. rubescens*	Brazil	None	Kloss, 1965
H. travfilhoi Dollfus, 1952	3	Spirobolida	*Rhinocricus padbergi*	Brazil	None	Adamson, 1983
H. tuxtlensis Mejia-Madrid 2014	2	Spirobolida	*Anadenobolus putealis*	Mexico	None	None

Table 4.3. Continued. Worldwide described *Heth* species.

***Heth* Species**	**Group[1]**	**Millipede Order**	**Millipede species**	**Type locality**	**Other localities**	**Other references**
H. tuzetae Dollfus, 1952	3	Spirostreptida	*Pseudonannolene tricolor*	Brazil	None	Kloss, 1965
H. vietnamensis Malysheva and Spiridonov, 2010	2	Spirobolida	*Eucarlia* sp.	Vietnam	None	None
H. xaniophora Hunt, 1994	2	Spirobolida	*Polyconoceras* sp.	New Guinea	None	None
H. xarochae Mejia-Madrid, 2014	1	Spirobolida	*Anadenobolous putealis*	Mexico	None	None
H. zeuglocantha Hunt, 1994	1	Unknown	Unknown	New Guinea	None	None

[1] Grouped as defined in the text and keys.

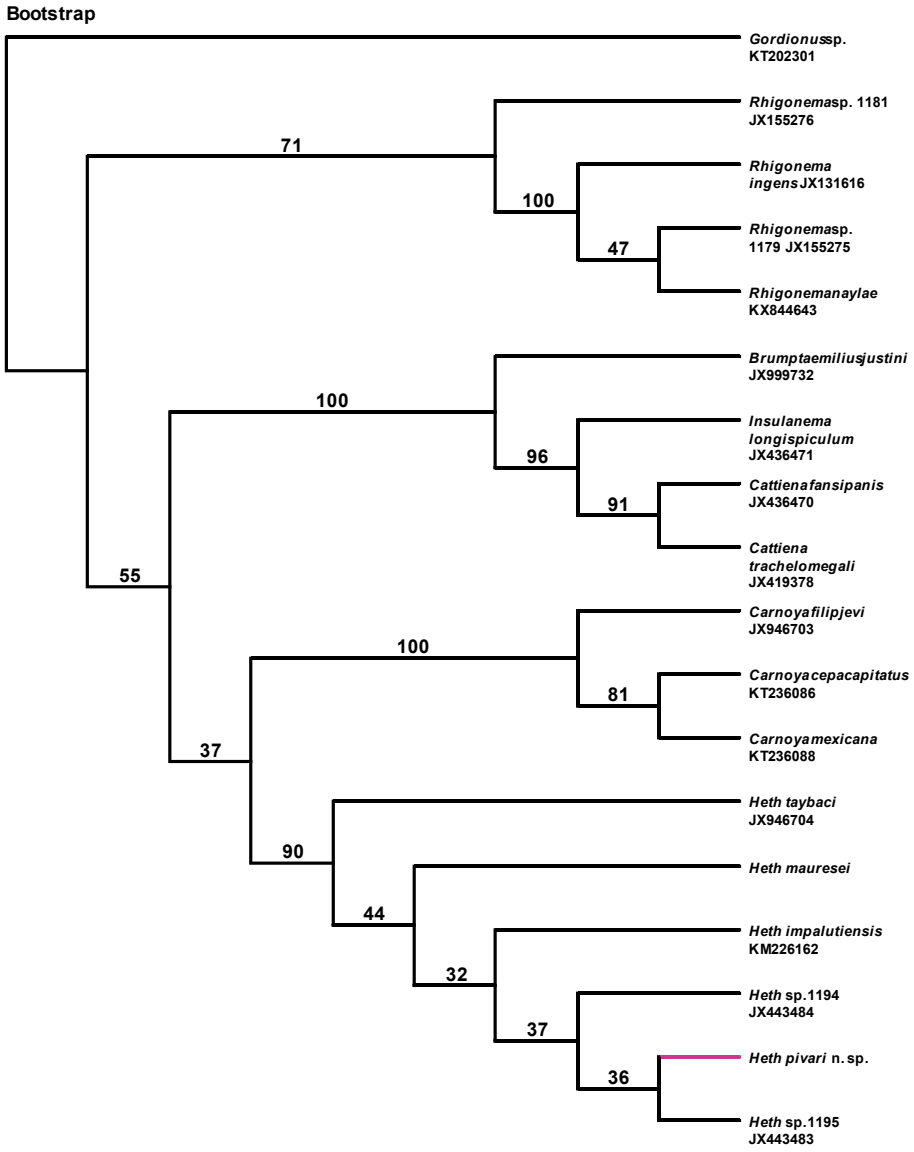

Fig. 4.1 A. Molecular relationships among *Heth pivari* and Rhigonematomorpha. Partial sequences of the D2-D3 expansion segment of 28S LSU rDNA. *Heth pivari* is placed in the family Hethidae and is the sister-group to Carnoyidae.

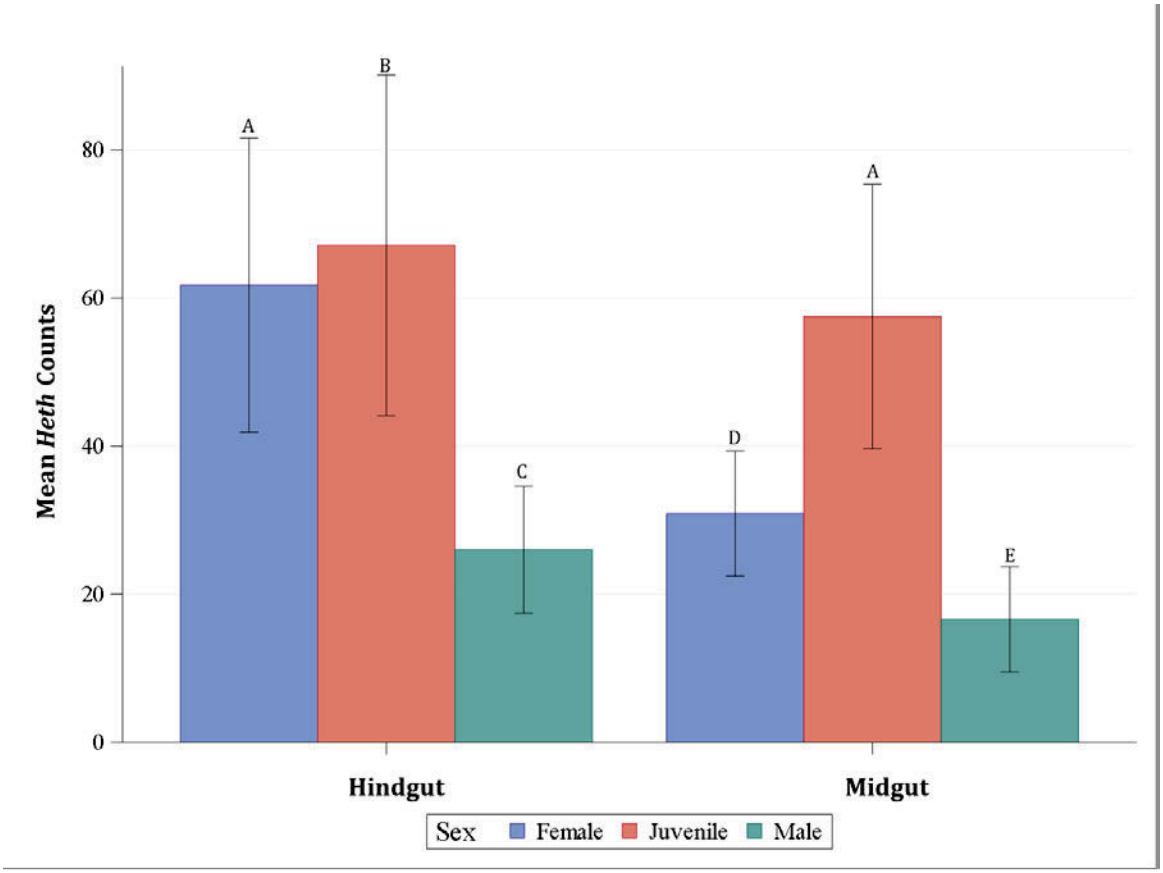

Fig. 4.1 B. Distribution of life stages of *Heth pivari* in the intestine of *Narceus gordanus*. Significant differences observed between all stages ($p<0.0229$) except for females located in hindgut v. juveniles in midgut ($p=0.1064$). Significant *Heth* loads greater in hindgut than midgut ($p<0.0001$).

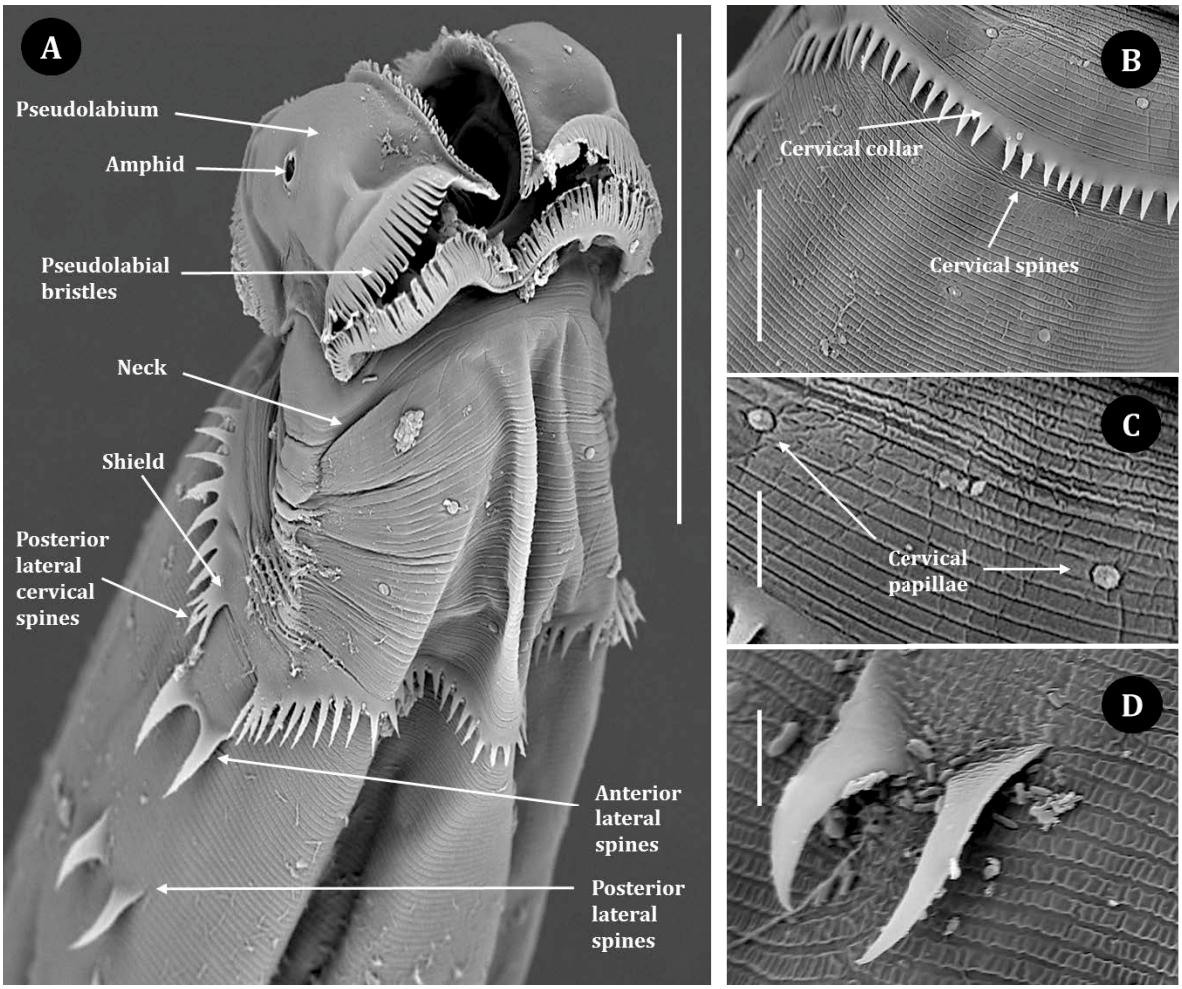

Fig. 4.2. *Heth pivari* n. sp., female SEMs. A) Anterior-lateral view of *H. pivari* showing important anatomical characters, scale bar 50 µm. B) Dorsal-ventral view of cervical collar, scale bar 20 µm. C) Dorsal-ventral view of cervical papillae and cuticle, scale bar 5 µm. D) Subventral lateral spine and cuticle, scale bar 5 µm.

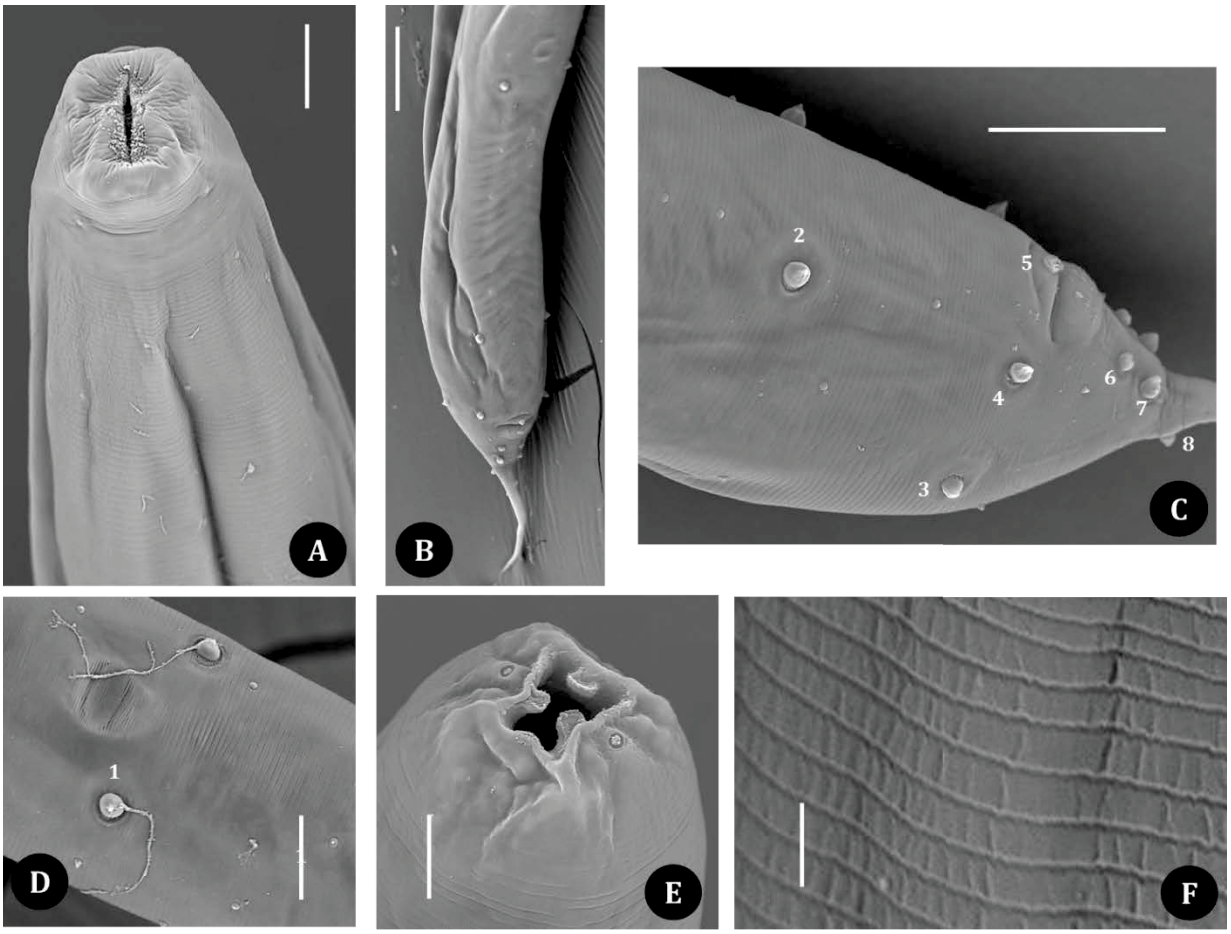

Fig. 4.3. *Heth pivari* n. sp. male SEMs. A) Male anterior end, scale bar 15 µm. B) Posterior showing ventral sucker and genital papillae, scale 70 µm. C) Genital papillae around cloaca and tail, numbers represent pairs of papillae except for number 5 (1 papillae), scale 25 µm. D) Ventral sucker and flanking first pair of papillae, scale 15 µm. E) Juvenile male showing stoma and amphids, scale 10 µm. F) Cuticle and annules around anterior body, scale 3 µm.

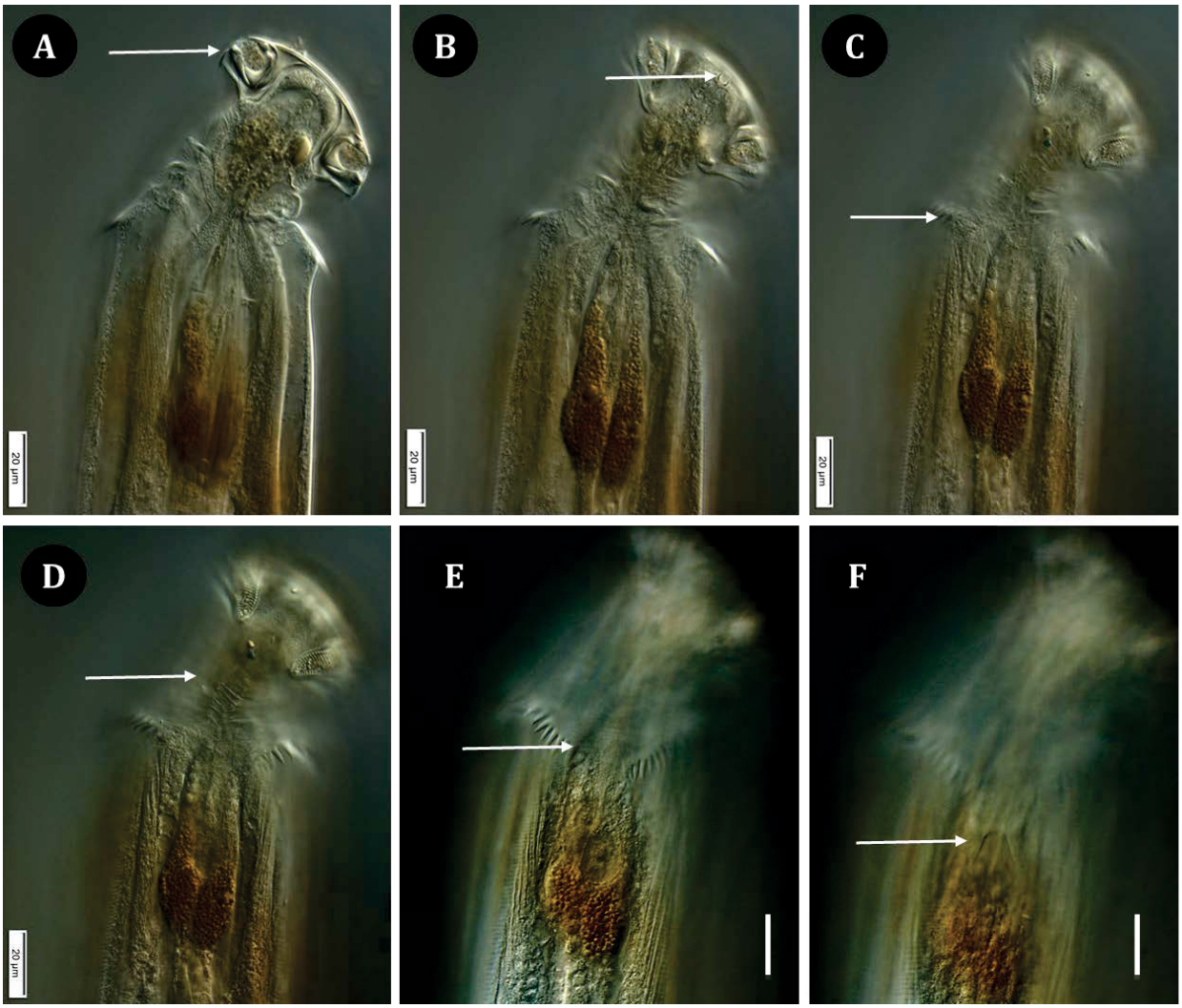

Fig 4.4. *Heth pivari* n. sp., DIC images of anterior head region. A) Psuedolabium. B) Amphid. C) Cervical collar. D) Neck region. E) Cervical lappet shield, scale 20 µm. F) Anterior lateral spine (arrows), scale bar 20 µm.

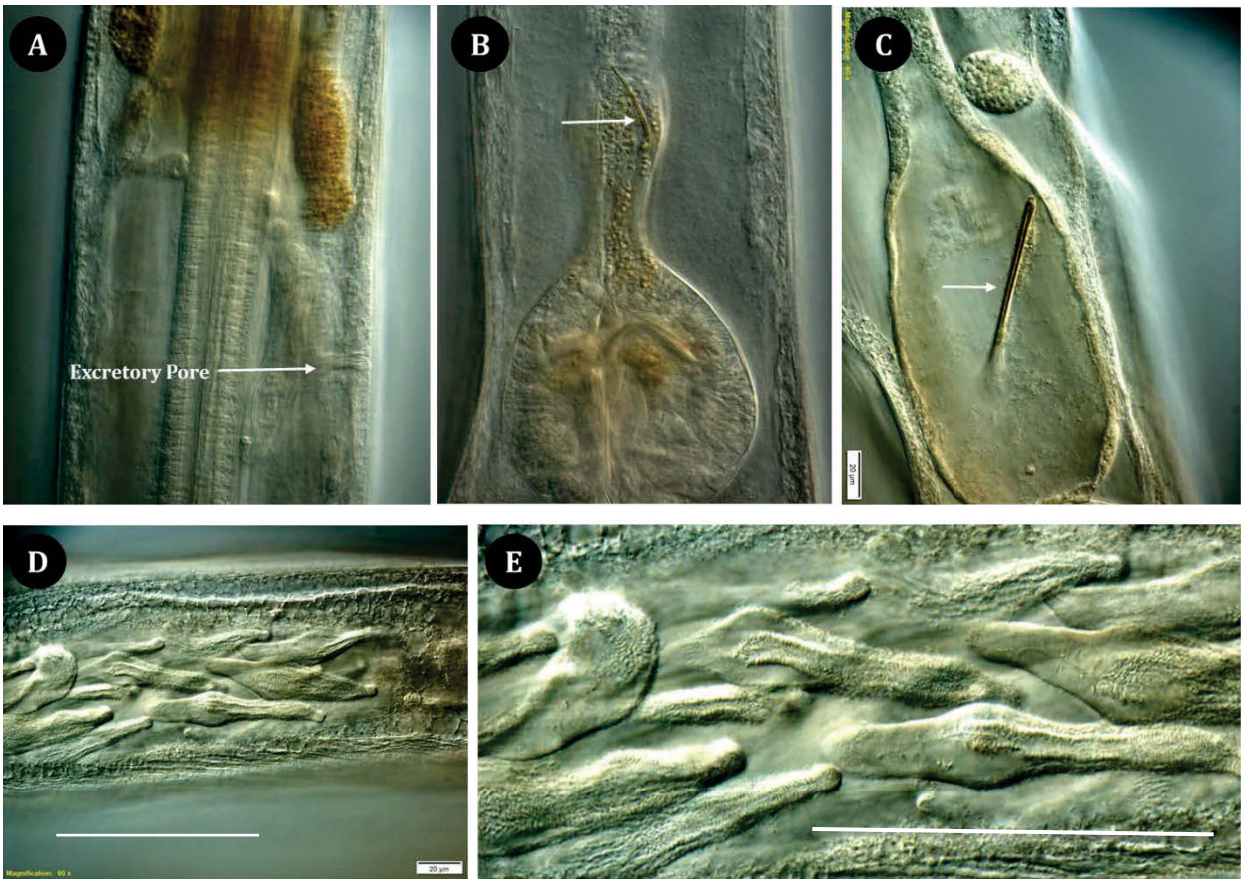

Fig. 4.5. *Heth pivari* n. sp. female and male structures. A) Secretory-excretory pore in female. B) Basal bulb with collembola seta around isthmus (arrow). C) Intestine with collembola seta (arrow). D) Sperm in testis, scale bar 80 µm. E) Fusiform or comet-shaped sperm from *Heth pivari* male, scale bar 80 µm.

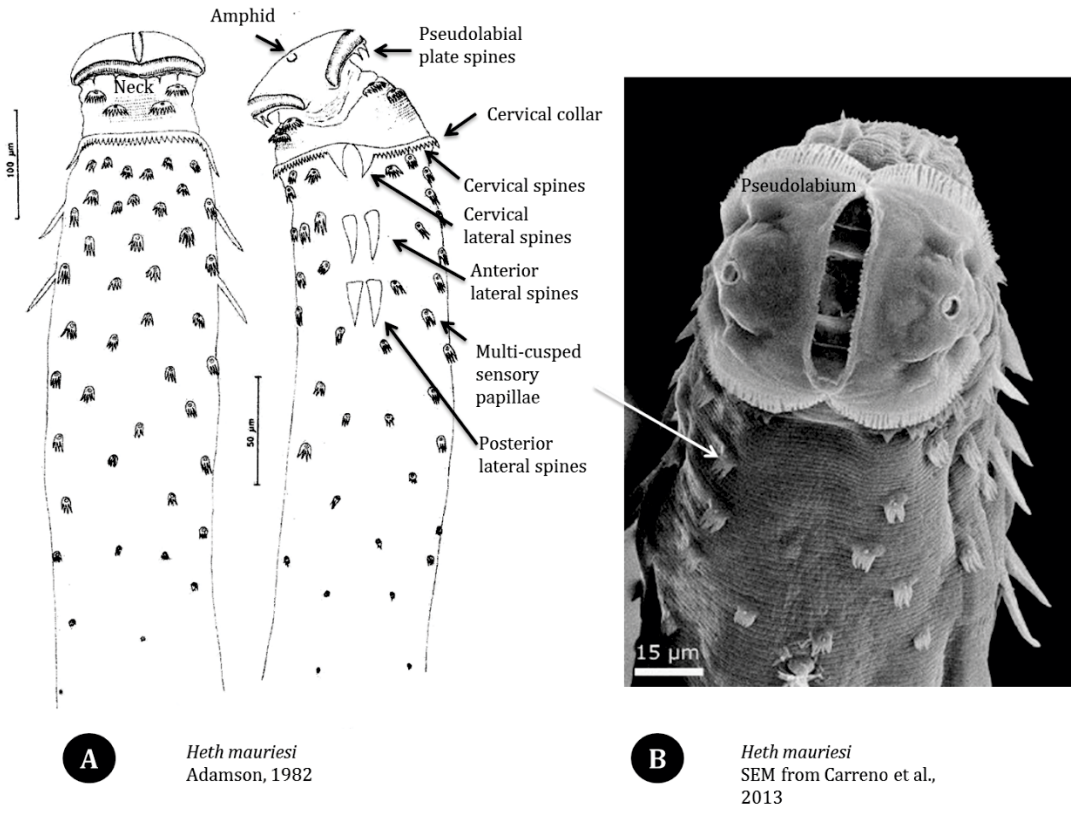

Fig. 4.6. *Heth mauriesi* (Adamson, 1982) illustration and SEM. A) Drawing and B) SEM (Carreno et al., 2013). Common anatomical characters are shown with arrows.

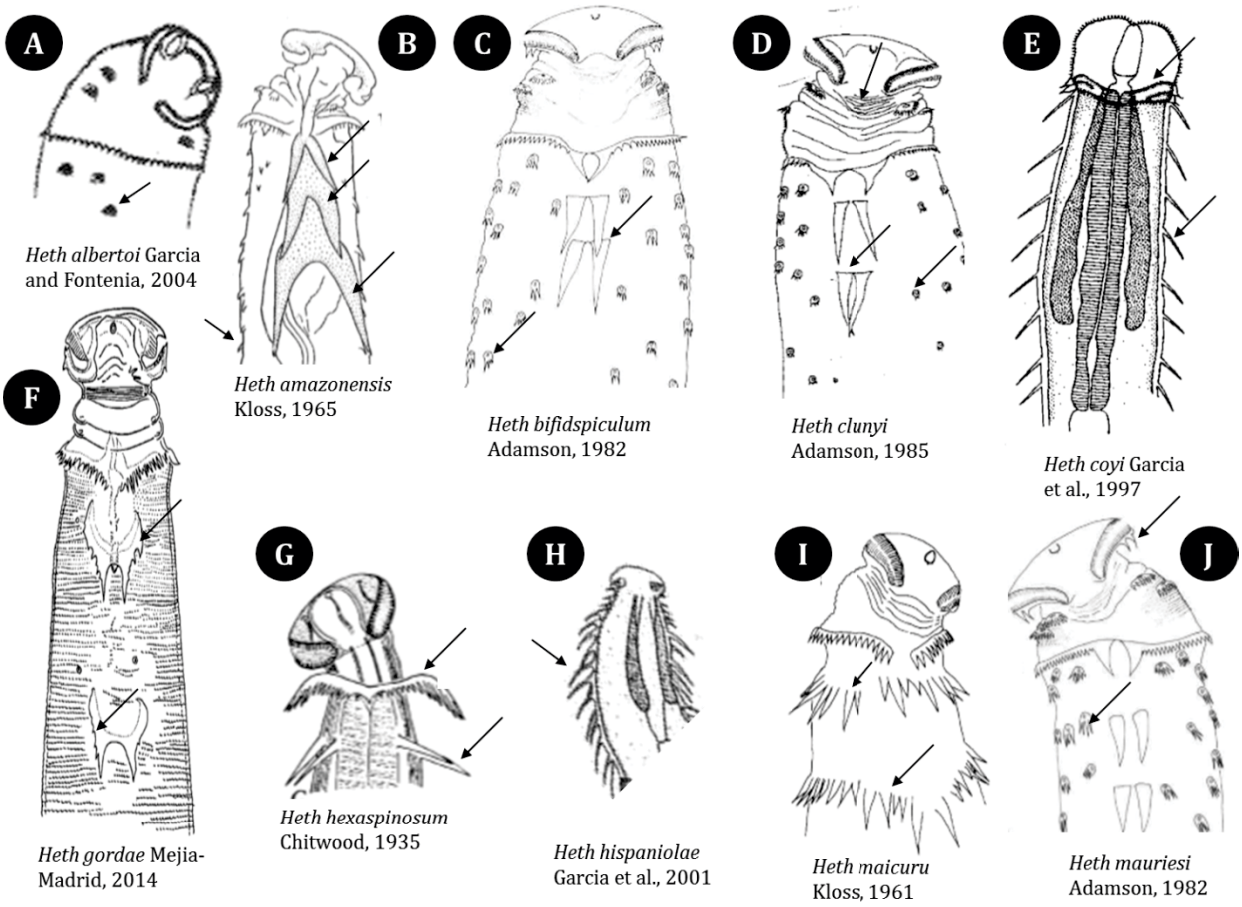

Fig. 4.7. Worldwide *Heth* spp. with continuous collar and no shield (Group 1). A) Cervical and somatic multi-cusped sensory papillae (arrow). B) Increasing sizes of three pair of lateral spines and somatic multi-cusped sensory papillae (arrows). C) Loosely connected lateral spines and somatic multi-cusped sensory papillae (arrows). D) Transverse rows of small spines near cervical folds in neck region and loosely connected anterior and posterior lateral spines and somatic multi-cusped sensory papillae. E) Double row of continuous cervical spines and about 8 rows of somatic spines (arrows). F) Large, broad anterior and posterior lateral spines showing serrated outer shield margins. G) Continuous cervical spines with one set of anterior lateral spines. H) Most anterior somatic spines small and increasing lengths of somatic spines proceeding posteriorly (arrow). I) Two discontinuous cervical collars (anterior and posterior). J) Psuedolabial plate spines and somatic multi-cusped sensory papillae.

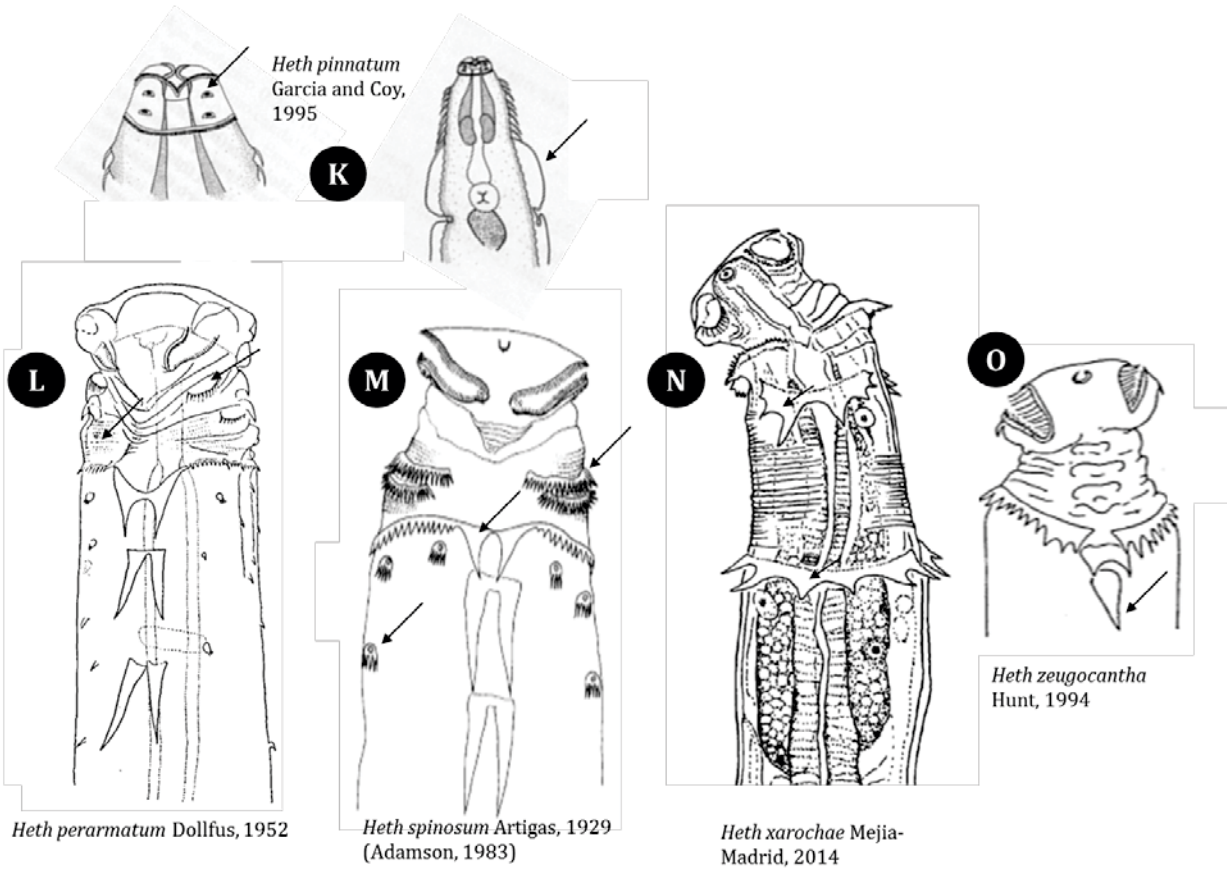

Fig. 4.8. Worldwide *Heth* spp. Group 1 (continued). K) Cervical multi-cusped sensory papillae and lateral alae (arrows). L) Multi-cusped sensory papillae and rows of transverse spines in neck region. M) Four lamellar rows of multi-cusped sensory papillae, large cervical collar lateral spine, and somatic multi-cusped sensory papillae. N) Large multi-cusped, broad anterior and posterior lateral spines. O) Single posterior lateral spine.

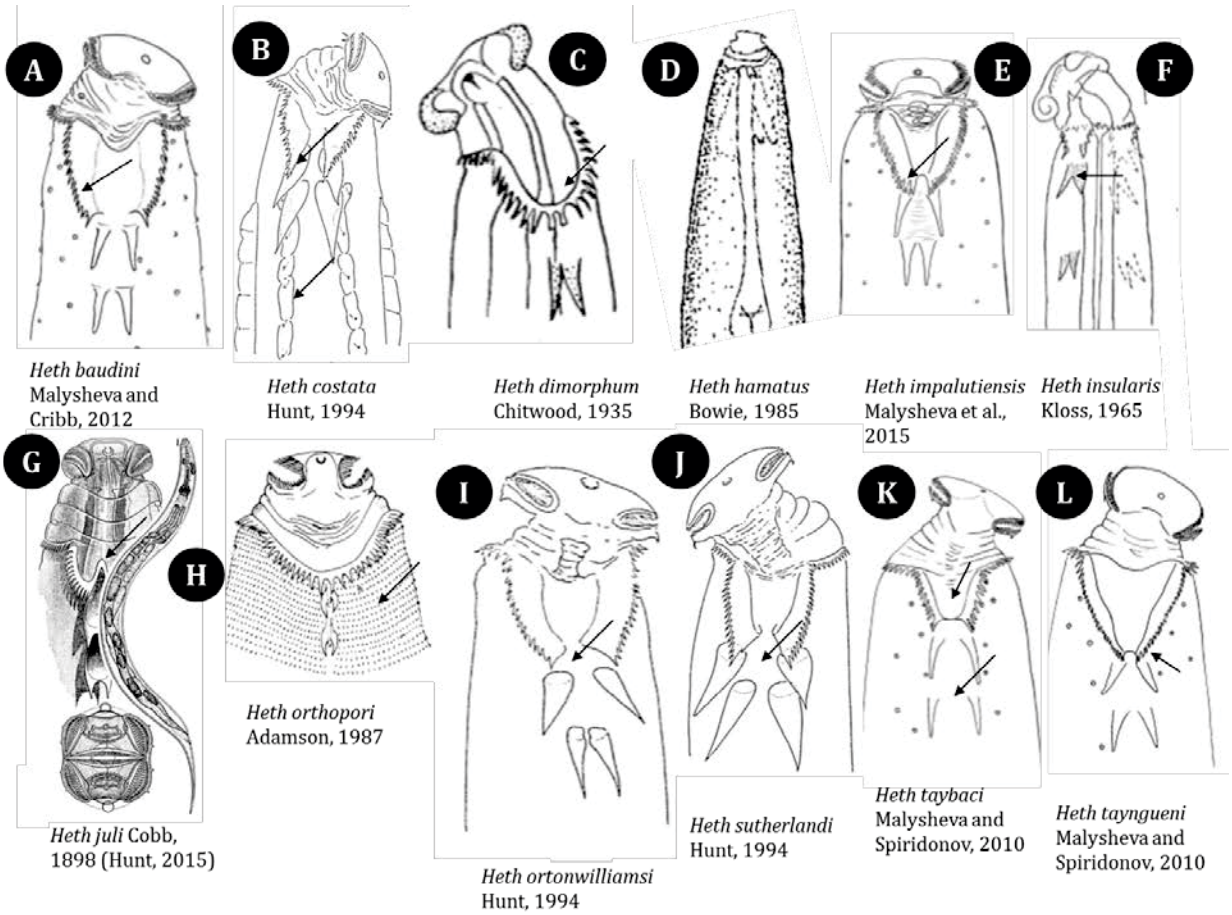

Fig. 4.9. Worldwide *Heth* spp., continuous collar with cervical shield (Group 2). A) Large, broad, rounded shield. B) Posterior lateral cervical spine overlapping anterior lateral spine; somatic ridges. C) "U" shaped shield. D) Female anterior region of *Heth hamatus*. E) Overlapping posterior cervical lateral spines reaching anterior lateral spines. F) Broadly fused, bifurcated anterior lateral spines. G) Type specimen *Heth juli* Cobb, 1898 showing "W" shaped shield. H) Numerous transverse rows of small spines around anterior head region. I) Break in cervical collar and not overlapping anterior lateral spines; widely separated anterior lateral spines and posterior lateral spines nearly or loosely connected. J) Overlapping posterior lateral cervical spines, widely separated anterior lateral spines also overlapping posterior lateral spines. K) Trapezoidal shaped shield; separated anterior and posterior lateral spines. L) Large, triangular shield, cervical spines along posterior lateral aspect bifurcated and overlapping anterior lateral spines.

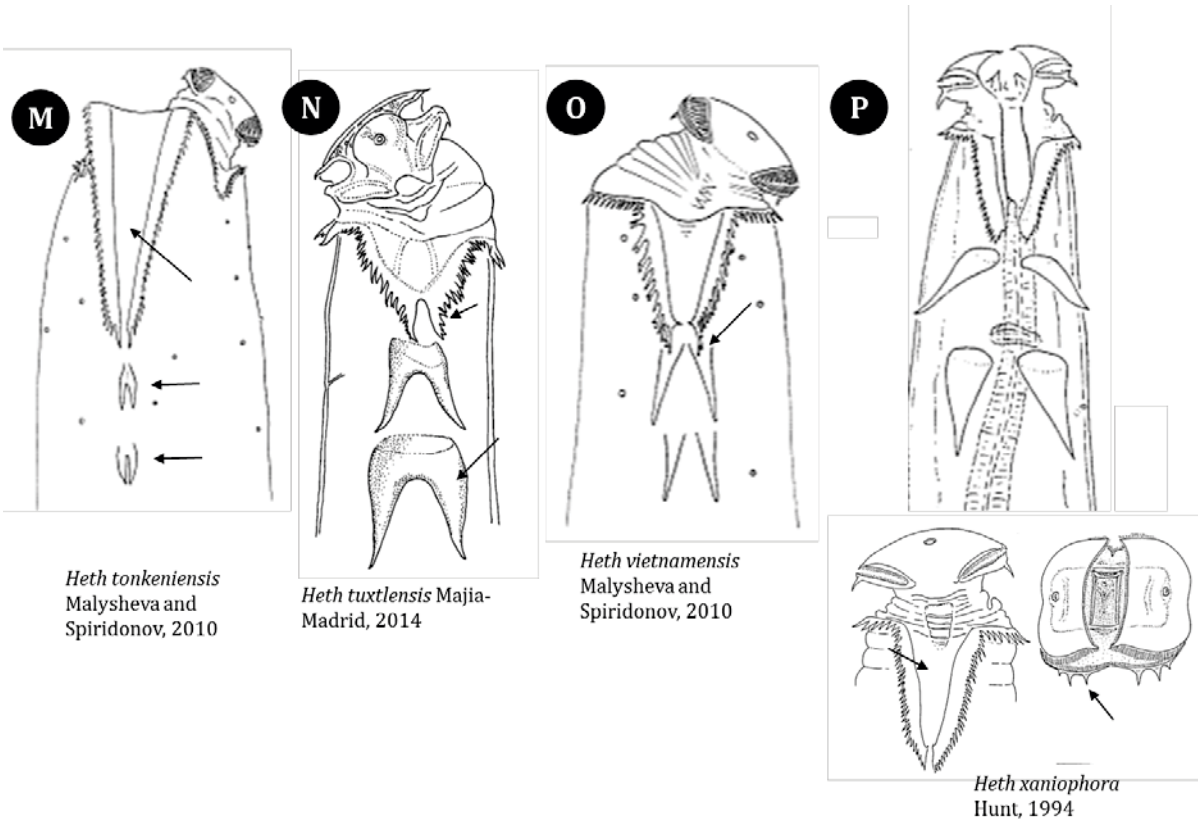

Fig. 4.10. Worldwide *Heth* spp. Group 2 (continued). M) Shield *ca* 3X longer than wide and anterior end extending over neck; fused and bifurcated anterior and posterior lateral spines (arrows); N) Shield with increasingly larger spines, some bifurcated; anterior and posterior lateral spines, broad, fused and large (arrows). O) Posterior lateral cervical spines of shield overlapping anterior lateral spines (arrow). P) Large, robust anterior and posterior lateral spines; shield about twice as long as wide; four sets of three pseudolabial plate spines (arrows).

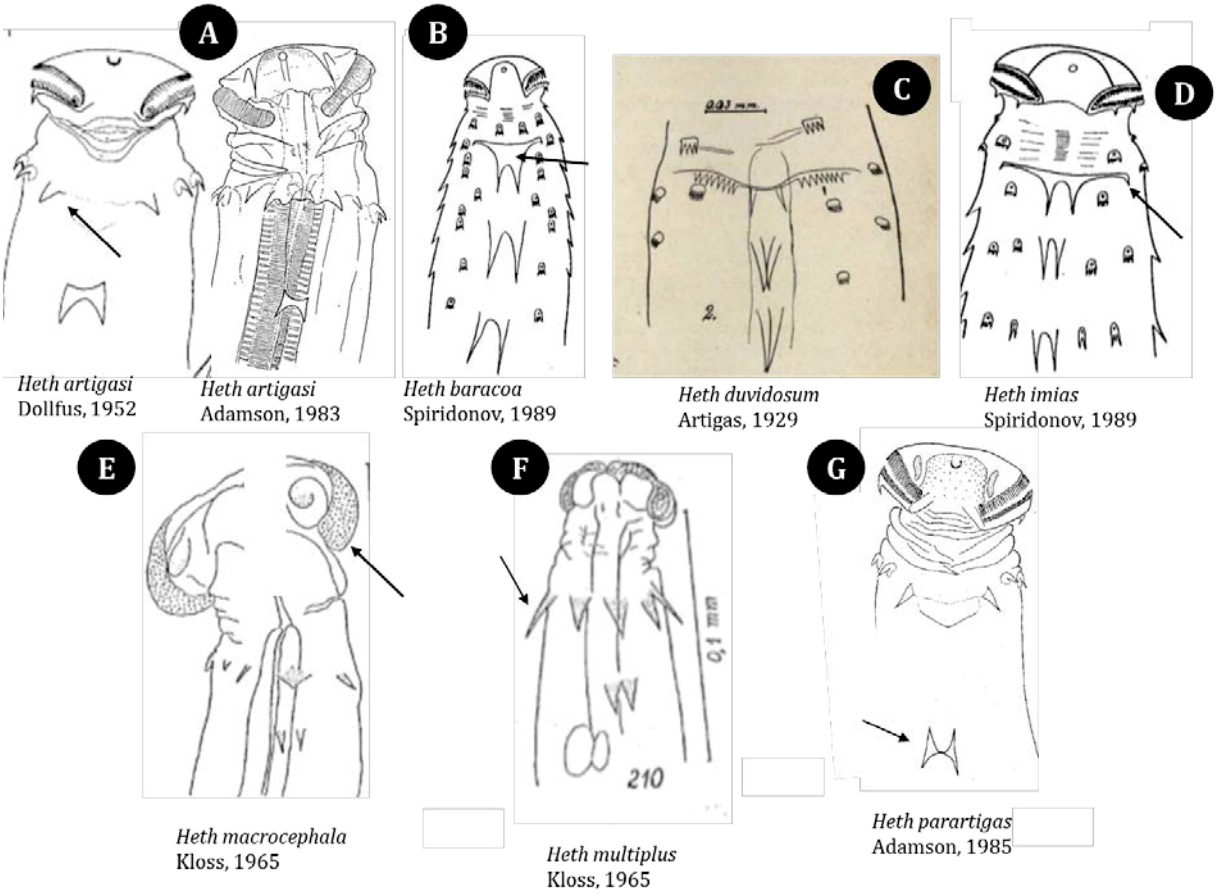

Fig. 4.11. Worldwide *Heth* spp. with discontinuous collar, no shield (Group 3). A) Cervical collar with about 12 spines consisting of four posterior and four anterior spines, flanked by two sublateral spines (arrow); fused posterior lateral spines. B) Head narrower than body; first large lateral spine about same width and length (arrow); approximately 8 cervical spines around circumference and > 20 somatic multi-cusped sensory papillae surrounding lateral spines. C) Two cervical groups of about 8 lamellar spines around circumference of neck; narrow, anterior and posterior lateral spines. D) Head about same with as anterior body; first lateral spine about twice as wide as long with lateral projections directed posteriorly (arrow). E) Large head, about twice as wide as neck region (arrow). F) About 10 discontinuous cervical spines around circumference of neck area (arrow). G) Head region identical to *H. magnavulvaris* Adamson, 1985; female without *area rugosa*.

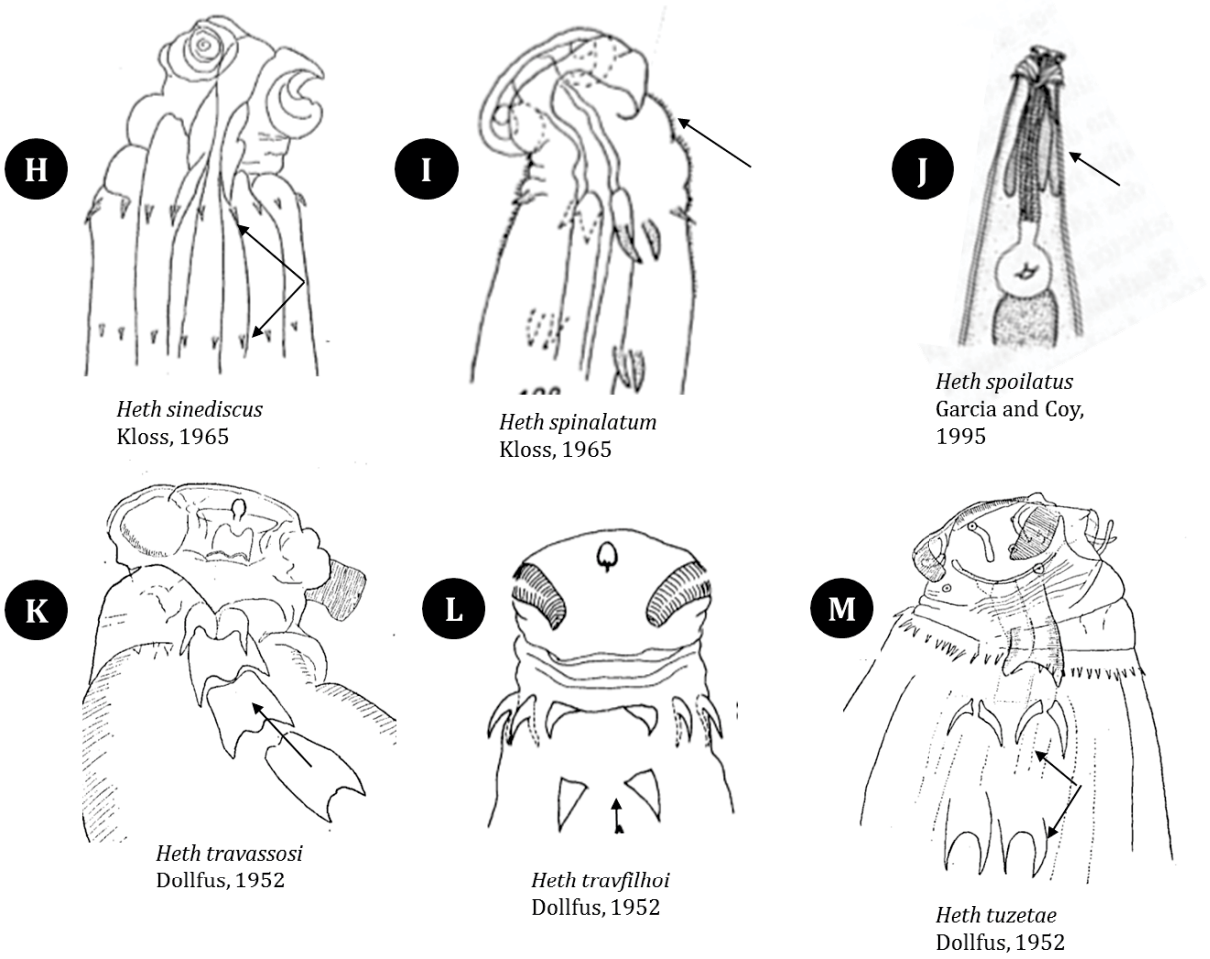

Fig. 4.12. Worldwide *Heth* spp. Group 3 (continued). H) Two conspicuous, discontinuous cervical collars (arrows). I) Small spines encompassing anterior esophageal region (arrow). J) Cervical collar spines absent (arrow). K) Four large, broad lateral spines (arrow). L) Posterior lateral spines widely separated (arrow). M) Two sets of paired anterior and posterior lateral spines.

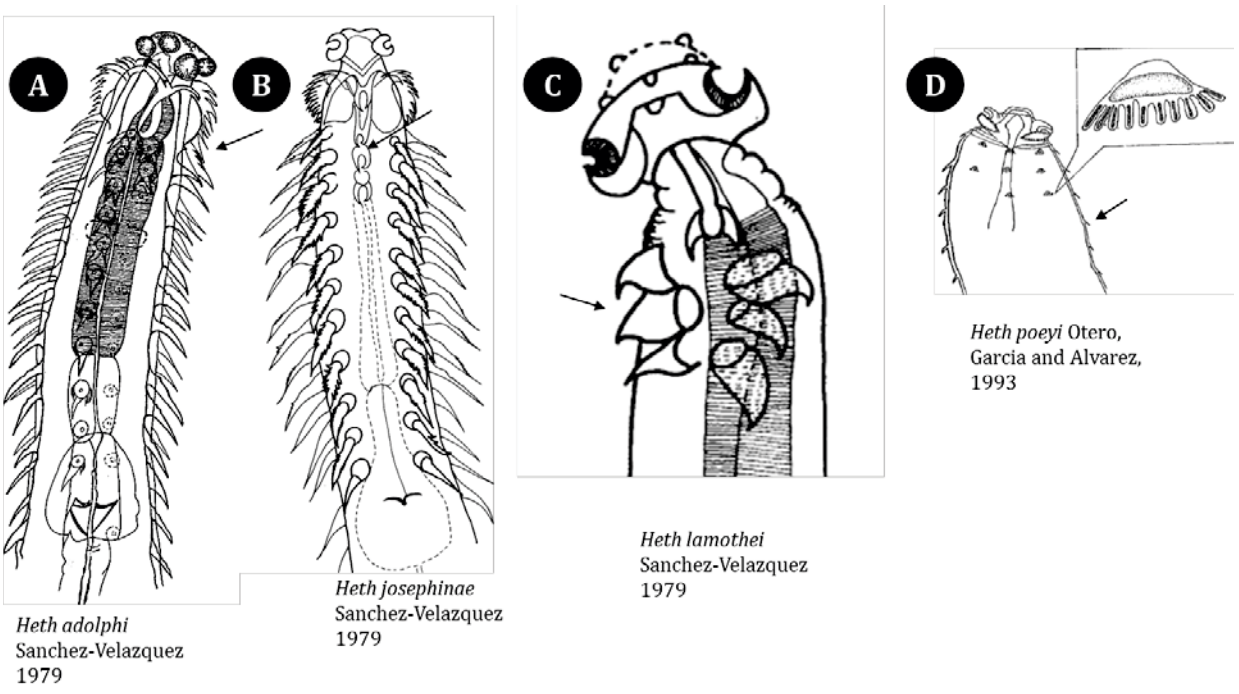

Fig. 4.13. Worldwide *Heth* spp. not conforming to any other group (Group 4). A) Four pair of bifurcated lateral spines; somatic spines begin to be serrate at about tenth spine (arrow). B) With three pair of bifurcated lateral spines (arrow); most anterior somatic spines frequently serrate on both inner and outer margins. C) Body without prominent somatic spines (excluding lateral spines); one bifurcated lateral spine; about 3 pairs of broad based, spines with curved distal tips (arrow). D) Body with somatic spines from cervical collar to isthmus (arrow); with multi-cusped sensory papillae posterior to cervical collar.

Chapter 5

Life in the most unexpected places: Laboratory inquiries to demonstrate commensal relationships and kleptoparasitism in millipedes (Myriapoda: Diplopoda)

Abstract

Parasitism is a fascinating mechanism for an organism to survive and thrive in extreme and harsh environments, such as in the intestine or hemocoel of a millipede. The diversity of life capable of living in such environments is quite remarkable, often times with as many as seven species of nematodes living in the same region of the intestine. The primary goal of this work is to inspire students to gain the requisite skills to discover new life in host organisms that are readily available, accessible, and in most cases, inexpensive or free. We have created a laboratory protocol to study the diversity of life living inside the millipede intestine. This exercise is designed to test a hypothesis, teach students to use keys, dissect a millipede, recover nematodes and other parasites, record data, and formulate a written conclusion. There is a high likelihood that students will discover new species of nematodes during this exercise. The suggested experimental design will catalyze students to investigate the potential of discovering new life in a backyard organism, and simultaneously ignite curiosity and promote a hands-on approach to the application of the scientific method.

Introduction

A parasite is an organism that either lives on or in another organism called the host (Bowman, 2014). An endoparasite lives in the host and an ectoparasite lives on the host. Some parasitic organisms can be commensal, meaning that they benefit from living either inside or on the host while not producing adverse or harmful effects to the host. In true parasitism, the host-parasite relationship is beneficial to the parasite, yet detrimental to the host, often times killing it. Other parasites are kleptoparasitic and obtain food from their hosts' through theft. Kleptoparasitic nematodes obtain food that has already been ingested and digested by certain arthropods, such as millipedes. They feed upon gut bacteria that process food consumed by the millipede. Nematodes that are predators can also be found inside millipede intestines. Adult *Coronostoma claireae* (Phillips and Bernard, 2016) preys on kleptoparasitic nematodes while juveniles probably consume bacteria (Phillips et al., 2016). The relationship between millipedes and nematodes is not truly parasitic, but merely a form of commensalism.

Nematodes belonging to the order Oxyurida, and infraorders Oxyuridomorpha and Rhigonematomorpha, live inside millipede's intestines and are good examples of kleptoparasites. Many kleptoparasites, and some predacious nematodes, can be found in millipedes and other arthropods, and they are easily extracted from their intestines.

Arthropods are invertebrate organisms that are bilaterally symmetrical and have segmented bodies, jointed appendages (legs, antennae, mouth parts), and exoskeletons; examples are insects, arachnids, crustaceans and myriapods (millipedes, centipedes, pauropods and symphylans). Several organisms parasitize arthropods, including bacteria,

protists, fungi, acanthocephalans, insects, and nematodes (Leidy 1853; Cloudsley-Thompson 1949; Remy 1950; Crites 1964).

Millipedes, or diplopods, are among the oldest known terrestrial animals, originating some 524 million years ago in the Cambrian Period, Paleozoic Era (Pisani, 2009; Shelley and Golovatch, 2011). Today, they primarily inhabit moist environments, living on forest floors, in and under decomposing leaf litter, beneath rocks and logs, and below bark of decaying stumps. They are abundant and readily encountered in optimal habitats and are low maintenance organisms in a terrarium. They are macroscopic, non-poisonous, and docile, being unable to bite, pinch, or sting. Millipedes are good organisms to study due to ease of collecting and being free or inexpensive.

Millipede bodies consist of a head and trunk containing at least 11 segments in adults; technically the segments are known as "diplosegments" resulting from embryonic fusion of alternate body somites (Figs. 5.1 A; 5.2 B; all subsequent figures are located in Appendix 5.1). Diplopods are further characterized by a pair of mandibles as the only internal mouthparts, and a gnathochilarium, or a broad, flat, plate-like structure ventral to the mouth that contains separate sclerites (Figs. 5.1 A, 5.2 D, E). Being diplosegments, most of the trunk segments possess two pairs, or four legs, and the body terminates in an apodous (non-leg bearing) segment followed by the terminal end of the body known as the epiproct or telson (Blower, 1985; Hopkin and Read, 1992).

Identifying millipedes to species level usually requires adult males, as the primary diagnostic features are aspects of their gonopods, or copulatory appendages, that replace one or both leg pairs on segment 7 and in some, the anterior pair on segment 8 (Fig. 5.1 A). Other diagnostic features include the shape of the body and head, the relative sizes and

configurations of the gnathochilarial sclerites, the number of trunk segments, the presence or absence of paranota, the arrangement of ocelli, exoskeletal ornamentations, and the presence or absence of a medial sulcus on the frons and labrum.

Excellent examples of published taxonomic keys to Neotropical millipede orders and families are published in Hoffman et al. (1996) and Golovatch et al. (2008). Keys are useful in distinguishing millipedes at any taxonomic level and hence guide students as to which external features to examine for ordinal separation. An abbreviated key adapted from Shelley (1988 and 1999), Kevan and Scudder (1989), Hoffman et al. (1996), Golovatch et al. (2008), and Sierwald (Milli-PEET) (2017) is provided as follows (Figs. 5.1–5.3):

Key to commonly found Appalachian and North American millipede orders

1 Body soft, exoskeleton not calcified, setal tufts present; 11–13 segments (Fig. 5.1 C) … **Order Polyxenida**

1’ Body hard, exoskeleton calcified … 2

2 Adults with 19-21 segments, paranota present at least on anterior-most segments, head and segments about the same width, collum partly overlapping head, large forms often brightly colored (Fig. 5.1 D) … **Order Polydesmida**

2’ Adults with greater than 32 segments … 3

3 Longitudinal groove located on dorsal aspect of tergites, may appear as a single groove or two separate longitudinal lines, strong ridges and crests (arrow); several ocelli present on lateral aspects of head; eyes triangular; adults with 40-60 segments (Fig. 5.2 A) … **Order Callipodida**

3’ Median sulcus present or absent on frons (Fig. 5.2 B, C) … 4

4 Median sulcus extends from apex of labrum to frons (Fig. 5.2 B); body smooth, eyes with numerous ocelli; gonopods concealed (Fig. 5.3 B) … **Order Spirobolida**

4’ Median sulcus does not extend from apex of labrum to frons (Fig. 5.2 C) … 5

5 Medial aspects of gnathochilarium separated and do not meet medially (Figs. 5.2 D; 5.3 A) … **Order Spirostreptida**

5’ Medial aspects of gnathochilarium meet at midline. Fresh specimens have setal fringes along body and in posterior region of body (Figs. 5.2 E; 5.3 C) … **Order Julida**

For the purposes of this laboratory exercise, large-bodied millipedes belonging to the orders Spirobolida, Spirostreptida and Polydesmida are recommended, as their anatomical features are more recognizable. We also recommend that millipedes with a body width of 2 mm or more be used since research has shown that intestinal nematodes are rarely found in more narrow ones (Phillips and Bernard, 2014). Representatives of Spirobolida and Spirostreptida are cylindrical (rounded in cross section), and the frons/labrum of spirobolidans has a medial sulcus, which is absent from spirostreptidans. Commonly called “flat-back” millipedes, polydesmidans lack ocelli, have 19–21 segments, whose sclerites (dorsal tergum, lateral pleura, and ventral sternum) are completely fused into segments lacking suture lines; most also possess lateral expansions of the dorsums called paranota that impart a flattened appearance to them, hence the name “flat-back’ millipedes. It is essential that millipedes be accurately identified so that proper comparisons can be made between their commensal and kleptoparasitic faunas, and perhaps the most common such organisms are oxyurid nematodes.

Nematodes (phylum Nematoda), or "roundworms," are among the most ubiquitous organisms on Earth (Maggenti, 1981). They occupy nearly every biological niche and are considered some of the most dangerous organisms to mankind as parasitic nematodes cause such serious diseases as dracunculiasis (guinea worm infection, primarily African), ascariasis (most common roundworm infection of humans), onchocerciasis (river blindness), and lymphatic filariasis (elephantiasis, primarily African). Given that there are about 25,000 species of identified nematodes, about 3,750 (15%) are parasitic to vertebrates and invertebrates, of which only 60 (0.24%) cause serious health risks to humans; 2,500 (10%) are plant parasites; 6,250 (25%) are free-living terrestrial and freshwater species; and 12,500 (50%) live in a marine environment (del Prado Vera, 2013).

Worldwide, plant parasitic nematodes account for nearly $80 billion dollars in crop loss annually (ARC, 2014). Plant parasitic nematodes play an integral role in vectoring diseases and viruses to crops and other host plants. Severe damage to crops and ornamentals results in low yields, unmarketability, contributes to quarantines, and reduces international trade of essential crops and ornamental plants.

Most nematodes are beneficial to humans and other organisms. They promote nutrient recycling and are important for the overall health of the environment. Some are used to help control insects and other arthropods that destroy plants and crops, which are called entomopathogenic nematodes. One such nematode, *Beddingia (Deladenus) siricidicola*, has been used as a natural biocontrol organism to kill the pine-killing wood wasp, *Sirex noctilio* (Bedding, 2009). When the wasp was accidentally introduced to New Zealand and Australia during the early and mid-20th century, the wasp had the potential to destroy 80% on the pine forests with an estimated loss of $1–4 billion per 30-year pine

rotation. Through the introduction of the biocontrol nematode *B. siricidicola*, much of the pine forest ecoregion in New Zealand and Australia was saved.

Nematodes are incredibly important for medical and genetic research. In the early 1960s, a free-living nematode *Caenorhabditis elegans*, emerged as a model organism that catalyzed the field of molecular biology. This particular nematode is a simple organism to study, with approximately 1,000 somatic cells, it has a simple body plan, is transparent, and easily cultured. Researchers use *C. elegans* as a gateway to understand the individual function of genes and it also offers the potential for unraveling the mysteries of genetic diseases. In the future, nematodes may even aid scientists to understand the aging process, develop cures for cancer, and act as a template to design pharmaceutical drugs.

Given the ecological, environmental and medical importance of nematodes, what exactly are they? Nematodes are bilaterally symmetrical, pseudocoelomate (a fluid filled body cavity located between the endoderm and mesoderm), have a complete digestive tract (mouth, intestinal tract and anus), contain only longitudinal muscles, contain primarily collagen in the cuticle, and are usually sexually dimorphic. Except for lacking respiratory and circulatory systems, nematodes have the same organ systems as vertebrates. Having vulvae and eggs readily identifies females; males are generally smaller and have testes, and frequently have none, one, or two spicules. Nematodes can be as small as 80 μm in length, while the largest, *Placentonema gigantissimia* Gubanov, 1951 can reach a length of 8.4 meters (Gubanov, 1951).

Nematodes living kleptoparasitically in arthropod intestinal tracts are classified as follows: phylum Nematoda, class Chromadorea, subclass Chromadoria, order Rhabditida, suborder Spirurina, and infraorders Oxyuridomorpha and Rhigonematomorpha (De Ley

and Blaxter, 2004; De Ley and Blaxter, 2015). The two infraorders are further divided into four superfamilies – Rhigonematoidea, Thelastomatoidea, Ransomnematoidea and Rhabditoidea.

Kleptoparasitic nematodes are classified using broad anatomical features. Each superfamily encompasses or contains families, and students can differentiate most by the shape of the esophagus, cuticular ornamentation, the number of spicules in the males, and morphometrics.

Millipedes are good host organisms to demonstrate kleptoparasitism. They host a variety of parasites, primarily nematodes that can be readily dissected from their intestines to demonstrate internal biodiversity. Close examination and careful dissections of millipede's intestines allows students to sharpen their observational skills, examine parasitic loads, and foster a grasp of kleptoparasitism. Because millipedes and nematodes are so understudied, students are likely to discover new species when guided and mentored by passionate teachers and advisers. A generalized key that students can use to sharpen their taxonomic skills to identify common nematodes is as follows:

Key to common kleptoparasitic nematodes found in millipedes

1 Esophagus long, basal bulb pyriform, bulb with grinding valve, vulva located near mid-body or posterior body, tail usually long, filiform (Figs. 5.4 A–C, F, G) … 2

1' Esophagus robust, often occupying much of the anterior space, corpus muscular, basal bulb with or without a grinding valve, vulva near mid-body or posterior body (Figs. 5.4 H–J) … 5

2 Procorpus long, straight, without any swelling (Figs. 5.4 A–C, F, G) … 3

2’ Procorpus long, straight, with swellings (Figs. 5.4 F) … 4

3 Head not constricted, often tapered, rounded or flat anteriorly; eggs with thin shell (Figs. 5.4 A; 5.5A, C–E) … ***Thelastoma***

3’ Head constricted, mushroom-like, first annule larger than second annule (Figs. 5.3 B; 5.4 C) … ***Stauratostoma***

3’’ Male with ventral sucker, fused spicules; females highly ornate with cuticular collar spines, pseudolabia present, large circular amphids, with multi-cusped sensory papillae or with smooth, bare sensory papillae (Figs. 5.4 C–E; 5.5 F, G) … ***Heth***

4 Isthmus shorter than basal bulb, vulva at base of tail, eggs with thin shell, tail long, filiform; males, large first annule, straight esophagus with no swelling (Figs. 5.4 F; 5.5 E) … ***Aorurus***

4’ Isthmus longer than basal bulb, vulva usually at midbody (Fig. 5.4 G) … ***Aoruroides***

5 First annule very large, basal bulb without grinding valve, very muscular esophagus, two conical amphids, tail about 1/3 as long as body, males rarely encountered, vulva at about posterior 1/3 of body (Figs. 5.4 H, I; 5.5 H) … ***Coronostoma***

5’ Basal bulb with grinding valve, muscular esophagus, tail generally short, males with two spicules, eggs with thick shell, typically large nematodes up to 10mm in length (Fig. 5.4 J) … ***Rhigonema***

Learning objectives

- Define parasitism, commensalism and kleptoparasitism.
- Explain why millipedes are good organisms to study.
- Identify taxonomic groups of millipedes and nematodes.
- Observe and identify other commensal parasites inside the millipede intestine and hemocoel.
- Prepare a data sheet showing millipede morphometrics, total nematode load, and taxonomic groupings of nematode genera.
- Write a short laboratory report including the following headings: synopsis, results, data analysis and conclusion.

Class schedule to complete project

Two days are needed to complete this laboratory inquiry with each classroom period lasting 90–120 minutes. Approximately two hours will be needed to complete the report, including data analysis, results and conclusions.

National Science and Common Core State Standards

National Science Standard LS4.D for High School students indicates that, "Biodiversity is increased by formations of new species and reduced by extinctions. Humans depend on biodiversity but also have adverse impacts on it. Sustaining biodiversity is essential to supporting life on Earth" (NGSS, 2013). Next Generation Science standards recommend that students have the ability to ask questions, develop models, plan investigations, collate, analyze and interpret data, use mathematics to support scientific claims, articulate explanations, respect critiques, offer clarifications based on scientific

evidence, and effectively communicate findings to peers (NGSS, 2013). This project allows students to fulfill all of these expectations.

Common Core State Standards advises that students should be able to, "Conduct short as well as more sustained research projects to answer a question (including a self-generated question) or solve a problem; narrow or broaden the inquiry when appropriate; synthesize multiple sources on the subject, and demonstrate understanding of the subject under investigation" (CCSS, 2017). Application and implementation of both Next Generation Science and Common Core State Standards can be applied and supported through this laboratory inquiry focusing on millipedes as organisms demonstrating commensal and kleptoparasitic parasitism.

Of certain interest to AP Biology instructors is how the investigation relates to the College Board course requirements for AP Biology. Since the desire for an exposition of the activity's relationship to the course requirements will likely vary between individual readers, the limited discussion of the activity's relationship to the Science Practices and Essential Knowledge for the AP Biology is addressed in Appendix 5.2. Suffice it to say here that the investigation provides for an engaging way to address a number of Science Practices and Essential Knowledge items, as well as provides opportunities for connection to and reinforcement of content addressed by other means during the course. Moreover, it provides an avenue to expose burgeoning future scientists to the field of nematology that, even if pressed for time before the exam, the instructor can utilize in the lab/classroom during the period after the exam yet before the end of the semester.

Experimental design

Hypothesis

Different species of nematodes will be found in millipede intestines, and females and juveniles will be more abundant than males.

Materials

- Gloves
- One large millipede per group of 2-4 students
- Razor blade
- Distilled water, water bottle
- Forceps, minuten pins, dissecting probe, Syracuse watch glass or small plastic petri dish
- Bright field dissecting microscope

Supervised inquiry activity

Activity class, Day 1 (90–120 minutes)

- Divide students into working groups of 2–4.
- Pass out quick keys to identify millipedes and nematodes.
- Discuss general millipede and nematode anatomy.
- Discuss other commensal organisms that may be observed: trichomycetes, gregarines, ciliated protists, bacteria, and acanthocephalans.
- Provide students with live specimens of large (spirostreptid, spirobolid or polydesmid) millipedes. Explain handling techniques and defensive mechanisms of millipedes. Allow students to handle millipedes prior to dissections.

Activity class, Day 2 (90–120 minutes)

- Gather students into groups of 2–4, put on gloves before handling millipedes. Their natural defense mechanism is to release pungent or toxic chemicals (hydrogen cyanide, benzoquinones, alkaloids, phenols, and terpenoids) from their defense gland openings (ozopores) to deter predators; some of these chemicals stain human skin but are not harmful.
- Hold the millipede down on a flat surface, ventral side up, and remove the head with the razor blade, then sever the caudal-most one or two segments (the epiproct and one or two others).
- Hold the millipede between forefinger and thumb, and with fine-tip forceps, grasp the hindgut and gently pull the intestine out of the body. Place the intestine in a Syracuse watch glass or small petri dish filled with distilled water.
- Grasp the millipede carcass, and using a water bottle, forcefully squirt distilled water into the severed anterior or posterior end and flush the hemocoel with water, dislodging any eggs, larvae or acanthocephalans that may be in the body cavity. Direct the flow of water into a separate Syracuse watch glass or Petri dish. Examine and identify any parasites.
- Starting at the hindgut and working from posterior to anterior, take a 30–45˚ angled minuten pin and gently make a longitudinal incision on one side of the intestinal wall, thereby exposing the inner contents. Similarly dissect each section of the intestine to observe which nematode species live in the fore-, mid- and hindgut.
- Gently squirt distilled water on the dissected intestine to dislodge any nematodes.

- Using the minuten pin, separate the different species of nematodes and determine the number of each sex: females have a vulva and eggs, males have testes and spicule(s), and juveniles lack reproductive organs.
- Place the intestine in distilled water, open it, and remove nematodes with probe or a minuten pin.
- Under the dissection microscope, separate nematodes into broad categories: Family: Genera – Coronostomatidae: *Coronostoma*; Hethidae: *Heth*; Rhigonematidae: *Rhigonema*; Thelastomatidae: *Thelastoma*, *Stauratostoma*, *Aorurus* and *Aoruroides*. The shape of the head and esophagus determines the taxonomic family and genus. Record and sketch findings on data sheets.
- Count nematodes, determine the number of males, females and juveniles, record on data sheets.
- Observe and record if other commensal parasites are present, e.g., gregarines, trichomycetes and ciliated protists (Fig. 5.6 A–D).
- Discard the millipede carcass into a plastic bag or place it back into its natural environment for decomposition. Nematodes can be preserved on permanent glass slides using methods designed by Sinehorst (1959), stored in 95% ethyl alcohol, or discarded down the sink.
- Clean workstation, wash hands with warm soapy water, and work on the lab report. Discuss findings within groups, analyze and graph data, and share results with the class upon completion of the lab report.
- Grade the lab report and offer constructive comments.

Data collection

An excel spreadsheet is an easy and effective method for presenting raw data. Excel sheets should be the same for all data entries if they are to be collated and analyzed together. Nematode loads can be compared between different millipede orders, families, genera, sexes, and life stages. Data can be further organized and nematodes can be evaluated based on the proportions of males, females and juveniles in each millipede order and family.

Student assessment

Students can be assessed by direct observations, their interactions, through discussions, and by completed laboratory reports. Demonstration of a solid foundation of the learning objectives and successful completion of basic questions focusing on the laboratory inquiry will provide instructors with information to determine if the student understands the concepts of commensal and kleptoparasitic parasitisms.

Worksheets, taxonomic keys and handouts

The accompanying keys and glossary serve as an instructional guide for students to navigate this laboratory inquiry. Excel spreadsheets enable students to collect and collate raw data, make tables and graphs, and maintain data for future study. Handouts will challenge students to answer questions based on their knowledge and observations of this investigation.

Conclusion

The use of millipedes to demonstrate commensal relationships and kleptoparasitism is a cost-effective and easy way to engage students in the classroom, and at the same time, provide a strong foundation in fulfilling National Science Foundation and

Common Core standards. Millipedes often have five of the six life kingdoms living commensally and kleptoparasitically inside their intestinal tracts, which provide students with a unique opportunity to examine and understand host-parasite relationships. Close examination and attention to detail presents an excellent opportunity for students to identify new species of nematodes and other commensal organisms. The excitement and thrill of being the first to discover a new life form is a monumental experience for any scientist. Students willing to take the time to look for new life, report their findings in a peer-reviewed journal, and establish professional reputations will ultimately propel them to significant academic and personal successes.

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Appendix 5.1

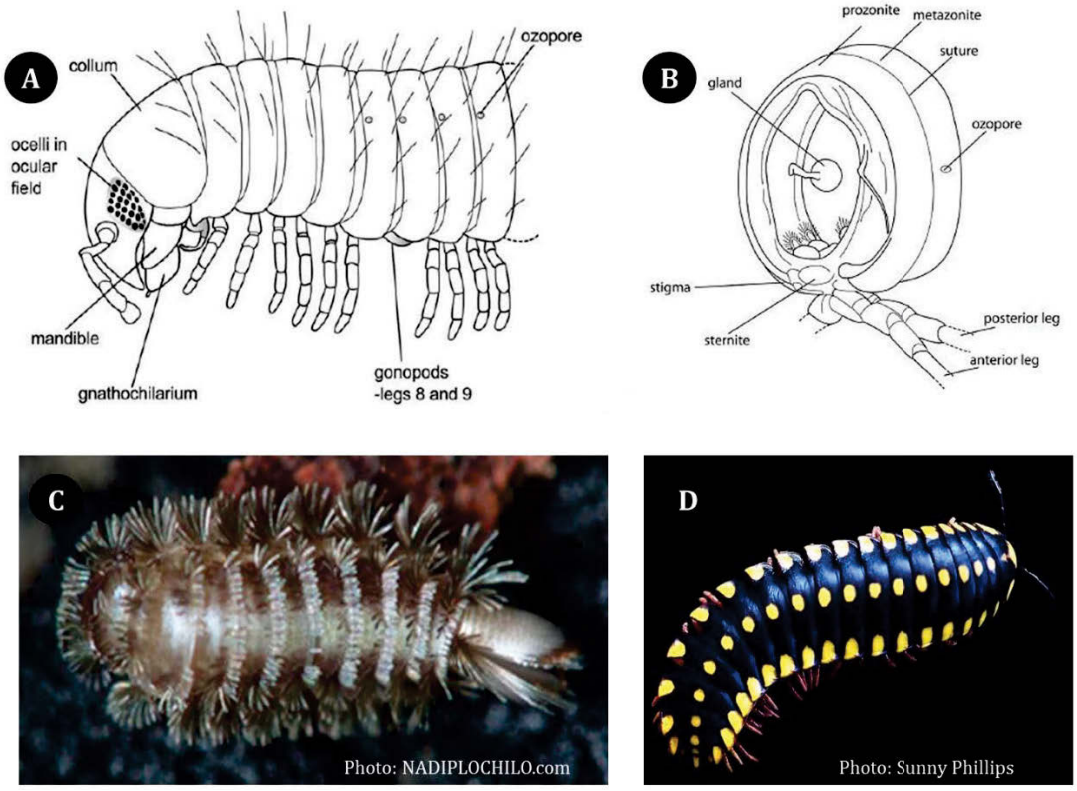
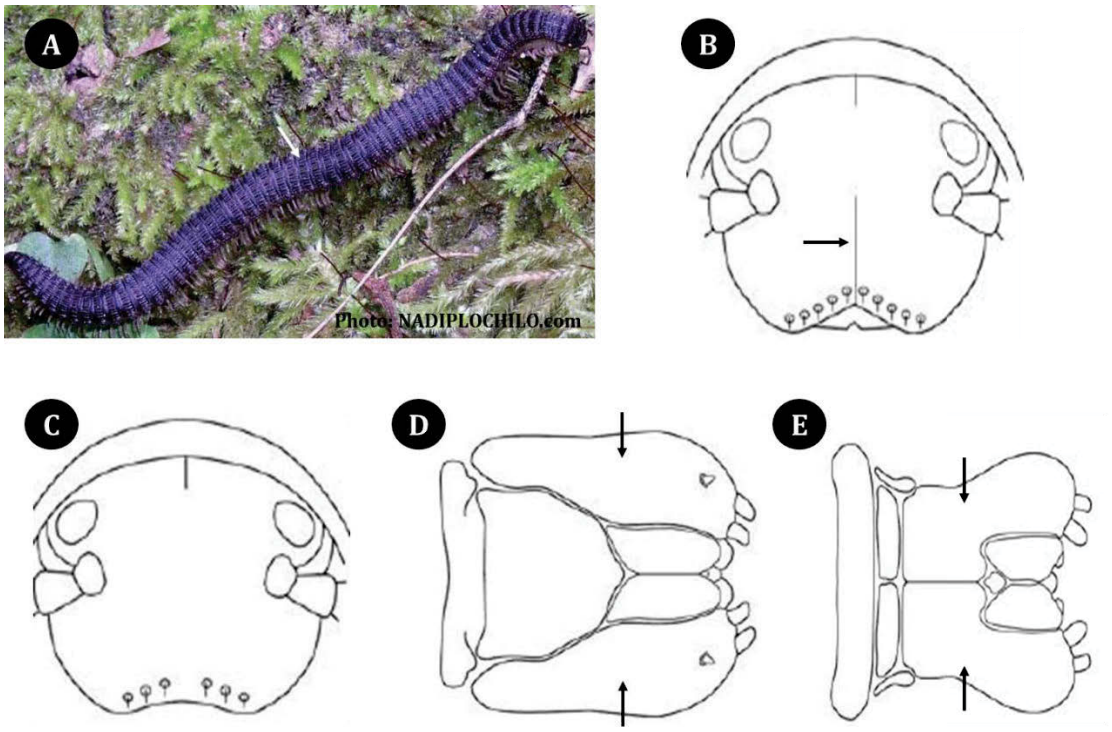


Fig. 5.1. Millipede morphology. A) Anterior end of millipede showing head, diplosegments, gonopods and legs (Blower, 1985). B) Cross section and lateral view of diplosegment (Drawing from Demange, 1981, retrieved from Sierwald, 2017). C) Polyxenida millipede (NADIPLOCHILO.com). D) *Apheloria* sp. millipede.



Drawings from Sierwald (Milli-PEET, 2017) and accessed at https://www.fieldmuseum.org/file/121821

Fig. 5.2. Callipodida and millipede head anatomy. A) Order Callipodida showing crests (arrow). B) Spirobolida, median sulcus extending from labrum to mid frons (arrow). C) Julida, absence of median sulcus from labrum to mid frons. D) Spirostreptida, gnathochilarium are separated (arrows). E) Julida, gnathochilarium are not separated and they meet medially.

Fig. 5.3. Spirostreptid, spirobolid and julid millipedes. A) Spirostreptida*: Archispirostreptus gigas*, scale 25 cm. B) Spirobolida: *Narceus gordanus*. C) Julida: Parajulidae: Aniulini, Photo courtesy of Derek Hennen.

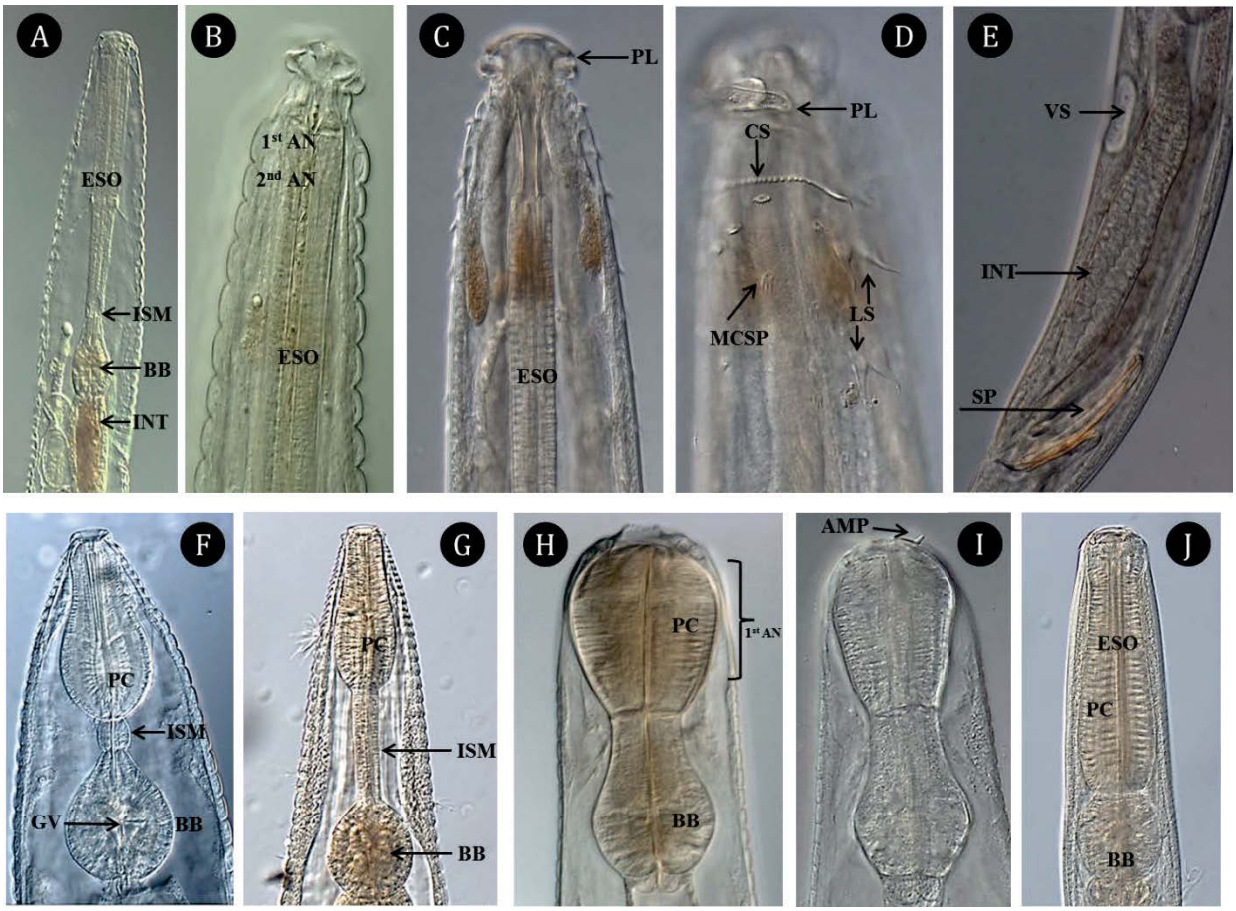


Fig. 5.4. Kleptoparasitic nematodes found in North American millipedes. A) *Thelastoma* sp. with long esophagus (ESO), isthmus (ISM), pear-shaped (pyriform) basal bulb (BB) and intestine (INT). B) *Stauratostoma shelleyi* (Thelastomatidae), first annule (AN) larger than second. C) *Heth* sp., pseudolabium (PL) and esophagus (ESO). D) *Heth mauriesi* (female), cuticular spines (CS), multi-cusped sensory papillae (MCSP), lateral spines (LS). E) *Heth* sp. male, ventral sucker (VS), intestine (INT), and spicules (SP). F) *Aorurus* sp. female, isthmus (ISM) shorter than basal bulb (BB), procorpus (PC), grinding valve (GV). G) *Aoruroides* sp. female, isthmus (ISM) longer than basal bulb (BB). H) *Coronostoma claireae*, female, 1st annule (AN) greatly enlarged. I) *Coronostoma* sp., female, amphid (AMP). J) *Rhigonema* sp. female, esophagus (ESO) takes up most of anterior space, procorpus (PC) and basal bulb (BB).

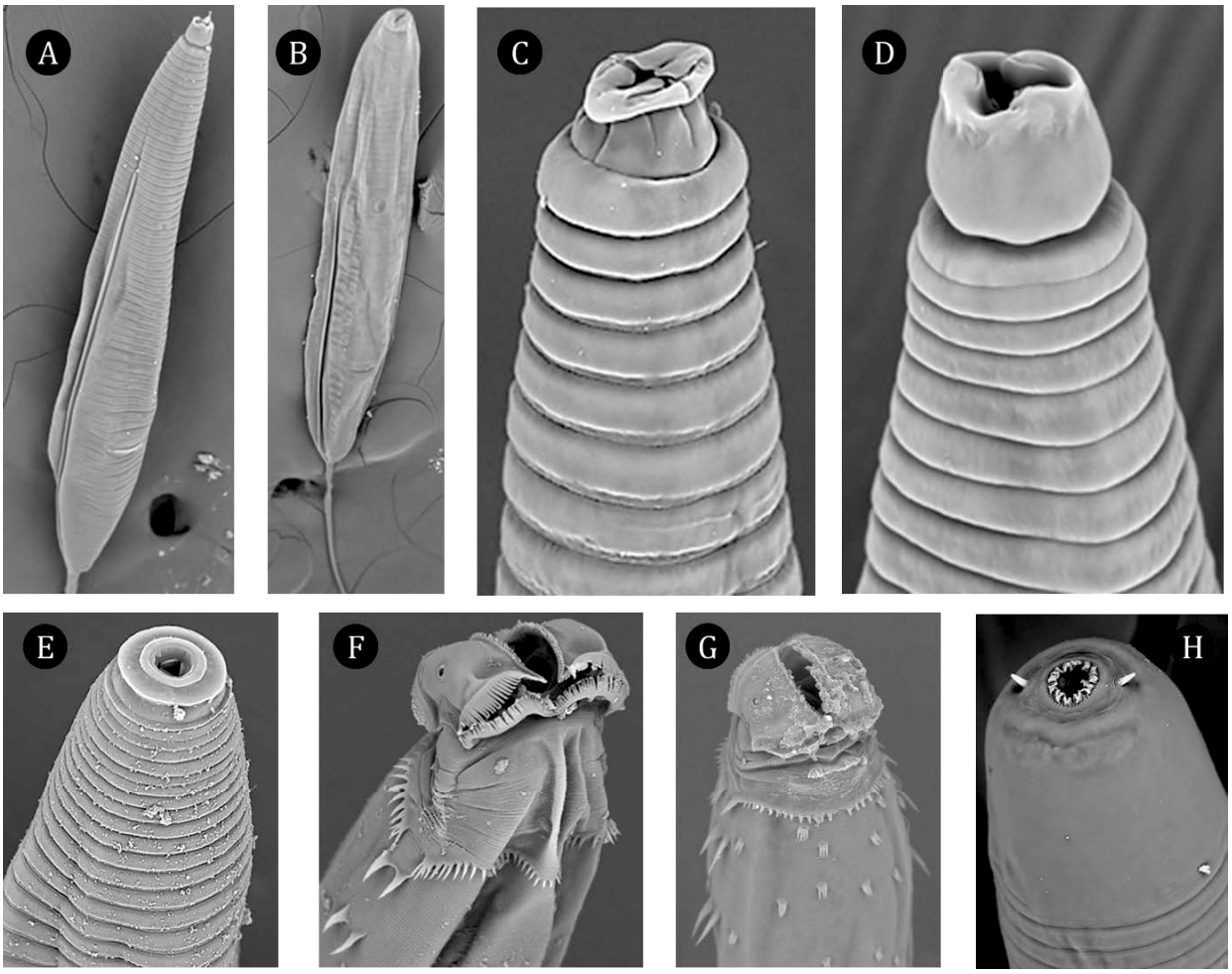


Fig. 5.5. Scanning electron microscope images of kleptoparasitic nematodes. A) *Thelsatoma* sp. female "Notch-Head". B) *Thelastoma* sp. female "Flat-Top". C) *Stauratostoma shelleyi* female. D) *Thelastoma* sp. female "Notch-Head" anterior view. E) *Aorurus* sp. female. F) *Heth pivari* female. G) *Heth mauriesi* female. H) *Coronostoma claireae* female, a nematophagus nematode.

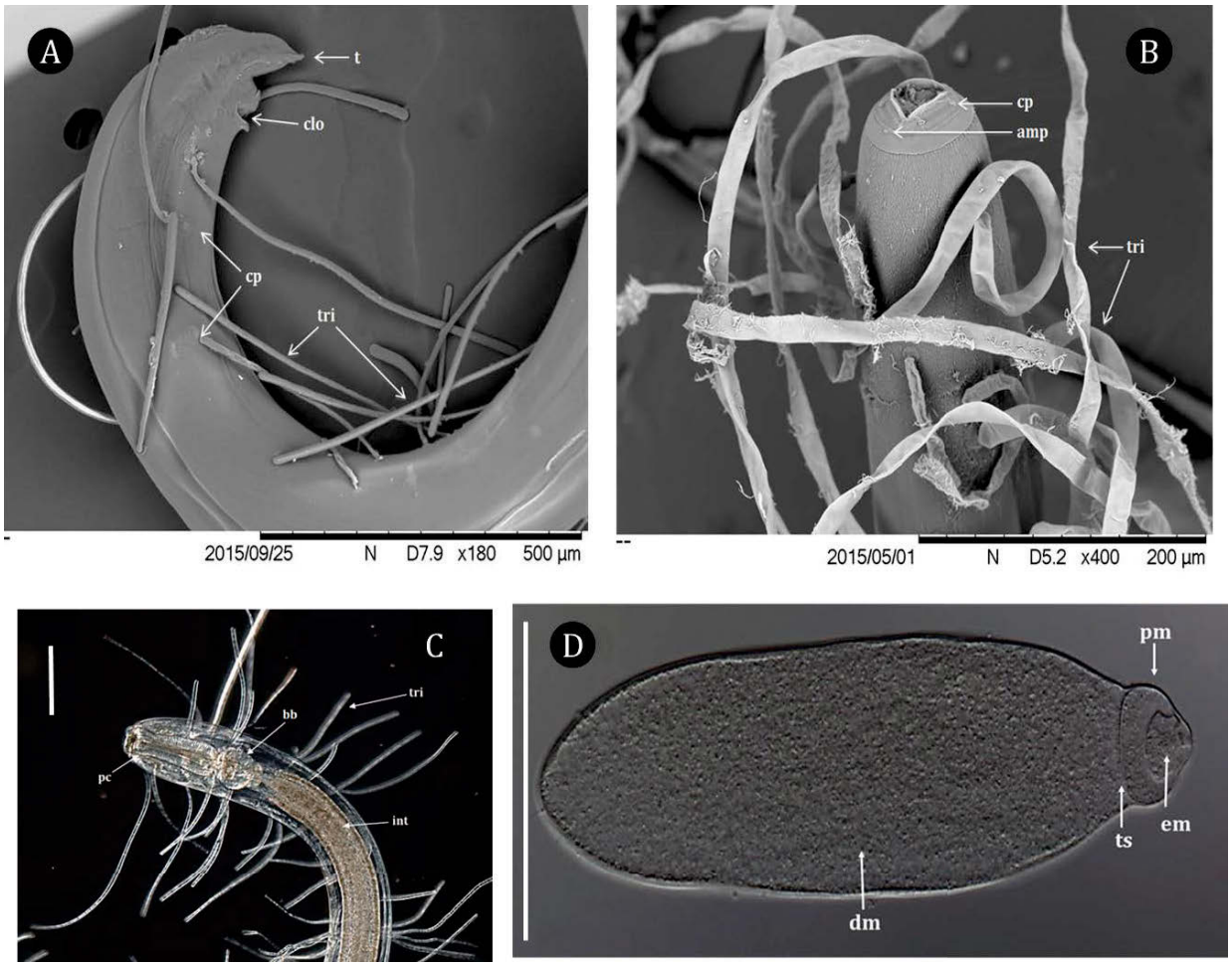


Fig. 5.6. Anatomical characters and organisms found in the millipede intestine. A) SEM image of *Rhigonema* sp. male, Trichomycetes (tri), caudal papillae (cp), cloaca (clo) and tail (t). B) SEM image of *Rhigonema* sp. female, cephalic papillae (cp), amphids (amp), and trichomycetes (tri). C) Differential interference contrast image of *Rhigonema* sp. showing procorpus (pc), basal bulb (bb), trichomycetes (tri), and intestine (int), scale bar 100 µm. D) SEM image of gregarine, epimerite (em), protomerite (pm), transverse septum (ts), and deuteromerite (dm), scale bar 250 µm.

Appendix 5.2

The Advanced Placement Biology College Board Standards (APBCBS, 2017) have identifed several core areas that students should acquire. The APBCBS can serve as a template for designing and implementing lesson plans focusing on kleptoparasitism and commensalism. The nature of the investigation facilitates engaging Science Practices 2, 4, and 5 to varying degrees. The general collection, management, and analysis of the data involved supports Science Practice 2 "The student can use mathematics appropriately" and Science Practice 5 "The student can perform data analysis and evaluation of evidence" quite well. An instructor can engage Science Practice 4 "The student can plan and implement data collection strategies appropriate to a particular scientific question" to varying degrees depending on the instructor's assessment of the student's capacity to select the proper data to record and arrive at the appropriate (general) plan for collecting the data. However, neither a close approximation of nor the precise procedure would likely arise from student construction without a considerable amount of additional time investment from the student and guidance from the instructor.

The topic as it relates to symbiotic relationships between millipedes and the organisms in their digestive systems allows for addressing aspects of Essential Knowledge 2.A.3 "Organisms must exchange matter with the environment to grow, reproduce and maintain organization." Further connection of this investigation to the former and additional Essential Knowledge items (those relating to transport of particles across membranes, for example) might be enhanced through some attention being given to the specific adaptations possessed by the parasitic organisms to facilitate the absorption of nutrients from their host organisms.

The nature of the host-parasite interactions addressed in this investigation also supports Essential Knowledge 2.D.1 “All biological systems from cells and organisms to populations, communities and ecosystems are affected by complex biotic and abiotic interactions involving exchange of matter and free energy” and Essential Knowledge 4.A.5 “Communities are composed of populations of organisms that interact in complex ways”. In addition, depending on the extent it is addressed in the lecture and when addressing other course content, the investigation can further serve as a vehicle for connection with Essential Knowledge 2.D.2 “Homeostatic mechanisms reflect both common ancestry and divergence due to adaptation in different environments, Essential Knowledge 4.A.6 “Interactions among living systems and with their environment result in the movement of matter and energy”, and Essential Knowledge 4.B.3: “Interactions between and within populations influence patters of species distribution and abundance”.

On its own merits, the investigation addresses content of the majority of Science Practices items and a number of Essential Knowledge items. However, its relationship to and connection with the Essential Knowledge items of the Enduring Understandings might be further enhanced by a deliberate effort to reference and use examples relating to entomology when engaging other content throughout the course. Such as focus though potentially highly dependent upon the instructor having (or developing) a deep understanding of entomology including the physiology and biochemistry of insects would be viable and, perhaps, quite powerful for promoting interest in the discipline.

Glossary

- Acanthocephalan: Thorny-headed worms characterized by their eversible and spiny proboscis, which is used to hold onto the intestinal wall of its host.
- Arthropod: Invertebrate organisms that are bilaterally symmetrical, segmented, with multiple and jointed legs, having antennae, with an exoskeleton; examples are insects, spiders, centipedes, shrimp, and crayfish.
- Commensalism: A type of relationship between two organisms where one organism benefits from living either inside or on the host, while not producing adverse or harmful effects to the host.
- Corpus: The musculature surrounding the esophagus.
- Diplopod: A millipede.
- Collagen: A structural protein found in connective tissue.
- Clypeus: A broad plate located under the frons and above the labrum.
- Ectoparasite: A parasite that lives on the host.
- Endoparasite: A parasite that lives in the host.
- Facultative: A condition where an organism can survive with or without another organism.
- Filiform: Long and attenuated, thread-like.
- Frons: The area between the eyes and above the clypeus in arthropods.
- Gonopods: Modeified legs in male millipedes used as copulatory appendages.
- Gnathochilarium: A broad, flat, plate-like structure ventral to the mouth that contains separate sclerites.
- Gregarine: Unicellular, obligate parasites of invertebrates.

- Hemocoel: The body cavity in invertebrates, containing hemolymph.
- Hemolymph: A fluid, similar to blood in vertebrates, occupying the hemocoel in invertebrates.
- Host: The organism that is parastized, which is often times detrimental.
- Hypothesis: An idea, or proposed description or explanation, that is often based upon scant information that leads into a more detailed investigation.
- Kleptoparasite: A relationship between two organisms where the host is unaffected by the theft of food from the second organism.
- Labrum: A broad plate located under the clypeus, also known as the upper lip in arthropods.
- Nematode: Commonly known as "Roundworms," soft bodied organisms that are bilaterally symmetrical, with a complete digestive system, lacking a circulatory and respiratory system, having a cuticle composed of collagen.
- Paranota: Lateral extensions of the terga.
- Parasite: An organism that either lives on or in another organism.
- Pauropod: A relative of millipedes and centipedes, often very small, blind, branching antennae, adults have 9-11 pair of legs, soft-bodied with a distinctive anal plate.
- Pyriform: Shaped like a pear.
- Obligate: A condition in which one organism needs another to survive.
- Ocelli: Simple eyes.
- Oxyuridomorpha: An Infraorder of the Phylum Nematoda, which consist of pinworm nematodes that parasitize both vertebrates and invertebrates.

- Rhigonematomorpha: An Infraorder of the Phylum Nematoda, which consist of pinworm nematodes that only parasitize millipedes.
- Somite: A body division.
- Sclerite: Harden plates forming a section of the arthropod exoskeleton.
- Sulcus: A groove, fissure or furrow.
- Symphylan: Distantly related to the millipedes and centipedes, long, segmented antennae, blind, translucent, adults with 10-12 pair of legs, soft-bodied and typically less than 10 mm in length.
- Terga: A thick plate located on the dorsal side of the exoskeleton of an arthropod.
- Trichomycete: Fungal obligate associates living inside the guts of arthropods.
- Vulva: The external reproductive opening leading into the vagina.

Chapter 6

General conclusions

Conclusion

Kleptoparasitic nematodes and millipedes are severely understudied. With over 12,000 species of millipedes identifed thus far, the kleptoparasitic nematofuana is more widespread than earlier researchers anticipated. Vast areas of the world, in particular, Africa, Asia and North America still need investigation to determine the nematofuana inside the millipedes intestines. Most studies focused on sub(tropical) areas of the world; however, our research has shown that temperate areas also harbor a rich and diverse fauna of nematodes.

Millipedes less than 2 mm in width rarely have nematodes inside the intestines. Those that are greater than 2 mm frequently have nematodes, but some do not have any, suggesting that the ingestion of the nematode egg is a random event during its lifecycle. Among the nematodes living in the millipedes intestines, rhigonematids are most abundant, thelastomatids are most diverse, and coronostomatids are rarely encountered. *Aorurus*, *Aoruroides*, *Carnoya* and many others have also been encountered and await description. We have also discovered nematodes that are not typically observed in the millipede intestine, such as dorylaimids, diplogasterids and certain rhabditids. Millipedes with the most abundant nematodes are spirobolids, followed by polydesmids, spirostreptids and callipodids. The orders Julida and Platydesmida were devoid of nematodes. The remaining five orders of millipedes that are indigenous to the United States still needs investigation to determine if they have nematodes in their intestines.

New species such as *Coronostoma claireae*, *Stauratostoma shelleyi* and *Heth pivari*, are only three of many that have been discovered in the United States during this research. An additional 20 species await further description and some may be re-classified into

higher taxonomic groups, such as “Notch-Head” and “Flat-Top” due to their unique head shapes and other anatomical characters. Congeneric millipedes that are geographically separated, such as *Choctella cumminsi*, *C. hubrichti*, *Narceus gordanus* and *N. americanus*, also have unique and different species of nematodes living in the the millipedes gastrointestinal tracts.

Efforts to revise genera, such as *Heth* and *Thelastoma*, need to be undertaken to resolve the inconsistencies of kleptoparasitic nematode taxonomy. In many instances, type specimens have been destroyed, are missing, or are unavailable for comparison. Several older descriptions, in particular *Heth* and *Thelastoma,* need to be re-evaluated and re-described, both morphologically and moleculary. Among *Heth* spp., only five (10%) and seven *Thelastoma* (10%) have been moleculary analyzed, leaving large gaps in the phylogenetic relationships among both Oxyuridomorpha and Rhigonematomorpha. Much more effort is needed to moleculary characterize clade III nematodes.

Since nematodes and millipedes are so understudied, we have developed a teaching curriculum for advanced placement biology students at the High School level. Our teaching methodologies are to introduce younger generations of students to arthropods, which will allow students to observe the host-parasite interaction in organisms that are free, or readily accessible in rural areas. In situations where millipedes are not available for collection, we have demonstrated that biological supply houses offer millipedes at a low and reasonable costs for teachers to purchase. Millipedes that are purchased offers students the opportunity to discover new speices, such as in *Archispirostreptus gigas* from Tanzania. Our research yielded at least three different undescribed species of nematodes living inside the intestine of *A. gigas*. Giving students the opportunity to discover new life

may catalyze more interest in arthropods and their accompanying parasitic fauna. Millipedes also have other parasites living commensally in their intestines. We have discovered gregarines, trichomycetes, ciliated protists, diptera and hymenoptera eggs, acanthocephalans, and bacteria within the intestines and hemocoels.

With the commercialization and international trade in arthropods, it is unknown if introduced millipedes can transfer their nematode fauna into indigenous North American diplopods. Research needs to be conducted to examine the effects of cross-infection of nematofauna from an introduced species to indiginous species. Millipedes are second to earthworms in nutrient re-cycling and if introduced nematodes are pathogenic to indigenous millipedes, soil dynamics and the soil food-webs may be negatively impacted.

Vita

Gary Phillips was raised in rural Wisconsin and graduated with a B.Sc. degree in Biology from the University of Wisconsin at Whitewater. Upon graduation, he was accepted into federal law enforcment and served 14 years in San Diego, California conducting criminal investigations that focused on narcotics smuggling, money laundering, crimes against children, fraud, weapons and strategic armaments. From San Diego, he was transferred and spent 7 years in SE Asia where he primarily focused on crimes against children, counter-terrorism and other crimes. He retired from federal law enforcment after serving two additional tours in the United States. After retiring, he finished his M.Sc. in Biology with an emphasis on vertebrate decomposition and entomology at the University of Nebraska. Upon graduation, he was accepted into a PhD program at the University of Tennessee at Knoxville under the guidance of Dr. Ernest C. Bernard. In December 2017, he graduated with his PhD in Entomology, Nematology and Plant Pathology. His post-doctoral studies will focus on the completion of numerous taxonomic descriptions of kleptoparasitic nematodes and the initiation of a new field of research in forensic nematology.