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SYMPOSIUM

Evolution of Feeding Structures in the Marine Nematode Order Enoplida

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Synopsis Marine nematodes of the order Enoplida may represent the earliest lineage of nematodes and have a variety of fixed and movable feeding structures in their stomas. This study used an 18S ribosomal RNA phylogeny of the orders Enoplida and Triplonchida (subclass Enoplia) to explore the evolution of these feeding structures in light of previous hypotheses based solely on morphology. The Enoplida and Triplonchida were found to be paraphyletic, as several taxa currently classified as Triplonchida, such as *Rhabdodemania*, were found to be part of the Enoplida clade. The position of *Rhabdodemania* within Enoplida was unclear, but a close relation to Enoplidae and Thoracostomopsidae was not supported, making it unlikely that its movable odontia are homologous with the mandibles of these families. A member of Anticomidae was well-supported as the base of the clade containing Phanodermatidae, Enoplidae, and Thoracostomopsidae, suggesting that taxa with buccal rods and mandibles evolved from nematodes with unarmed stomas. The Phanodermatidae were shown to be more closely related to the Enoplidae and Thoracostomopsidae than were the Leptosomatidae, suggesting that the buccal rods of the phanoderms (rather than the mandibular ridge/odontia complex of the Leptosomatidae), may be the origin of the mandibles.

Introduction

The phylum Nematoda occurs in a wide variety of habitats, including deep oceanic sediments, beaches, lakes and streams, arid deserts, rain forests, polar regions, agricultural systems, and as parasites inside virtually every metazoan taxon. Nematodes are arguably the most abundant and diverse metazoans on the planet (Lambshead 1993; Baldwin et al. 2000). While estimates of the total number of species of nematodes vary radically, from 500,000 to 100,000,000, approximately 30,000 species have been described (Baldwin et al. 2000; Hugot et al. 2001). Research on nematodes has been strongly biased toward the minority of taxa that parasitize plants and animals, with relatively little attention given to the vastly more abundant and diverse free-living species. Especially neglected have been nematodes from the aquatic environment, where the majority of nematode species occur (Lambshead 1993). Diversity of nematodes in the depths of the sea has been found to be comparable to that of the polychaetes, previously considered to be the most diverse macrofaunal taxon there (Lambshead and Boucher 2003). Approximately 6900 species of free-living marine nematodes have been described, and an estimated 50,000 species remain undescribed (Appeltans et al. 2012). Free-living nematodes are commonly the most abundant microinvertebrate of marine-estuarine sediment (Warwick and Rice 1979) and deep-sea sediment (Lambshead and Schalk 2001; Danovaro et al. 2010). Marine nematodes also are recognized for their important ecological roles in sediments and water (Aller and Aller 1992), and as a major source of food for higher trophic levels (Coull 1990). They are sensitive to a variety of environmental factors, and their ecological significance has led to the examination of nematodes as potential bioindicators for climatic change and ecological disturbance (Lambshead et al. 2003; Bert et al. 2009).

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Of particular evolutionary importance within marine nematodes is the primarily aquatic subclass Enoplia, as previous molecular phylogenetic evidence from 18S ribosomal RNA (rRNA) suggests that the root of Nematoda belongs somewhere between this lineage and two others, the Chromadoria and Dorylaimia (De Ley and Blaxter 2002; Holterman et al. 2006; Smythe et al. 2006; Meldal et al. 2007). More recently, Blaxter et al. (2015) found Enoplia (specifically *Enoplus brevis*) to represent the base of the Nematoda in a phylogenetic analysis of 181 protein-coding genes, although they cautioned that its placement could have been based on phylogenetic artifacts. Enoplia have several unique features thought to be ancestral in nematodes, including highly indeterminate development (Hope 2002) and retention of the nuclear envelope in mature spermatozoa (unlike all other nematode groups thus far investigated, which show a loss of this structure upon maturation) (Baccetti et al. 1983; Justine 2002). Members of Enoplia also lack an asymmetrically dividing germline and bilateral symmetry of the early embryo, both seen in the development of other nematodes (Malakhov 1994; Voronov et al. 1998; Schierenberg 2005). These features, presumed ancestral within Nematoda (Schierenberg 2005), make Enoplia strong candidates for the base of the nematode tree (Maggenti 1963), as suggested by phylum-wide molecular phylogenies (Holterman et al. 2006; Van Megen et al. 2009).

Members of Enoplia are most diverse in marine habitats but several lineages can be found in freshwater and in moist soils (De Ley 2005). Most Enoplia are recognized by the cyathiform (pocket-like or pouch-like) shape of the external aperture of the amphids (Maggenti 1981; Hope 2007), paired organs believed to be chemosensory and found laterally in the head region of all nematodes (Fig. 1A). Lorenzen (1994, 1981b) suggested that the monophyly of Enoplia was supported by a synapomorphy, the presence of metanemes: lateral, filamentous structures that may be stretch receptors (Lorenzen 1978, 1981a) or a form of proprioceptor (Hope and Gardiner 1982). While metanemes have not been described in nematodes outside of Enoplia, few efforts have been made to find them and their absence in many groups may represent a secondary loss (Decraemer et al. 2014). Lorenzen (1981b, 1994) used morphology to distinguish two orders within Enoplia: Triplonchida and Enoplida. Triplonchida includes primarily free-living, soil-dwelling nematodes, but also several plant parasites such as *Trichodorus* Cobb 1913. Enoplida are primarily marine and often are characterized by complex feeding structures of the head and the stomal region, including onchia (usually fixed teeth present in the posterior portion of the buccal capsule or esophagostome) (Figs. 1B, 2A), odontia (fixed or, rarely, movable teeth present in the anterior portion of the buccal capsule or cheilostome) (Figs. 1A, 2B, 2C), and movable mandibles (dense specializations of the cuticular lining on all three walls of the stoma) (Inglis 1964) (Figs. 1C, 2D, 2E). The diversity of stomal morphology is reflected in the diversity of feeding habits within Enoplida, including taxa that are exclusively predators, as well as omnivores and microbivores feeding on algae, diatoms, bacteria, protists, and fungi (Wieser 1953a; Jensen 1987; Moens and Vincx 1997). De Ley and Blaxter (2002, 2004) used a framework from their 18S rRNA phylogeny (Blaxter et al. 1998) as well as morphological considerations to construct the most recent classification of Enoplia; they retained Lorenzen's (1981b, 1994) orders Enoplida and Triplonchida. De Ley and Blaxter (2002, 2004) recognized seven suborders within Enoplida (Enoplina, Trefusiina, Oncholaimina, Ironina, Tripyloidina, Campydorina, and Alaimina) and three suborders within Triplonchida (Diphtherophorina, Tobrilina, and Tripylina). De Ley and Blaxter (2002) supported Lorenzen's (1981b, 1994) contention that Enoplia are monophyletic on the basis of the presence of metanemes, although these structures have been lost (or perhaps only visible with transmission electron microscopy) in several groups (De Ley and Blaxter 2002). Although some members of Triplonchida traditionally have been placed within the order Dorylaimida (Lorenzen 1981b, 1994), De Ley and Blaxter (2002, 2004) placed them in Enoplia with the possible synapomorphy of a capsule-like structure containing the retractor muscles of the spicule (a copulatory structure).

Members of Enoplida are among the largest free-living nematodes (Maggenti 1963), some reaching a length of 4 cm (Hope 1967, 1974), making them particularly amenable to morphological examination. Leptosomatidae (suborder Ironina according to De Ley and Blaxter [2002, 2004]) have been considered by several authors to be the most morphologically "primitive" members of Enoplida (Filipjev 1918, 1934; Maggenti 1963; Platonova 1964, 1976). This idea is based on taxa such as *Leptosomatum* Bastian 1865 (Figs. 1D, 2F) and *Leptosomatides* Filipjev 1918, both with a reduced, nearly absent stoma lacking onchia or odontia (Bongers 1984). Despite this characterization as simple or "primitive", many leptosomatids have what are believed to be unique features of the stoma, including specialized oblique radial

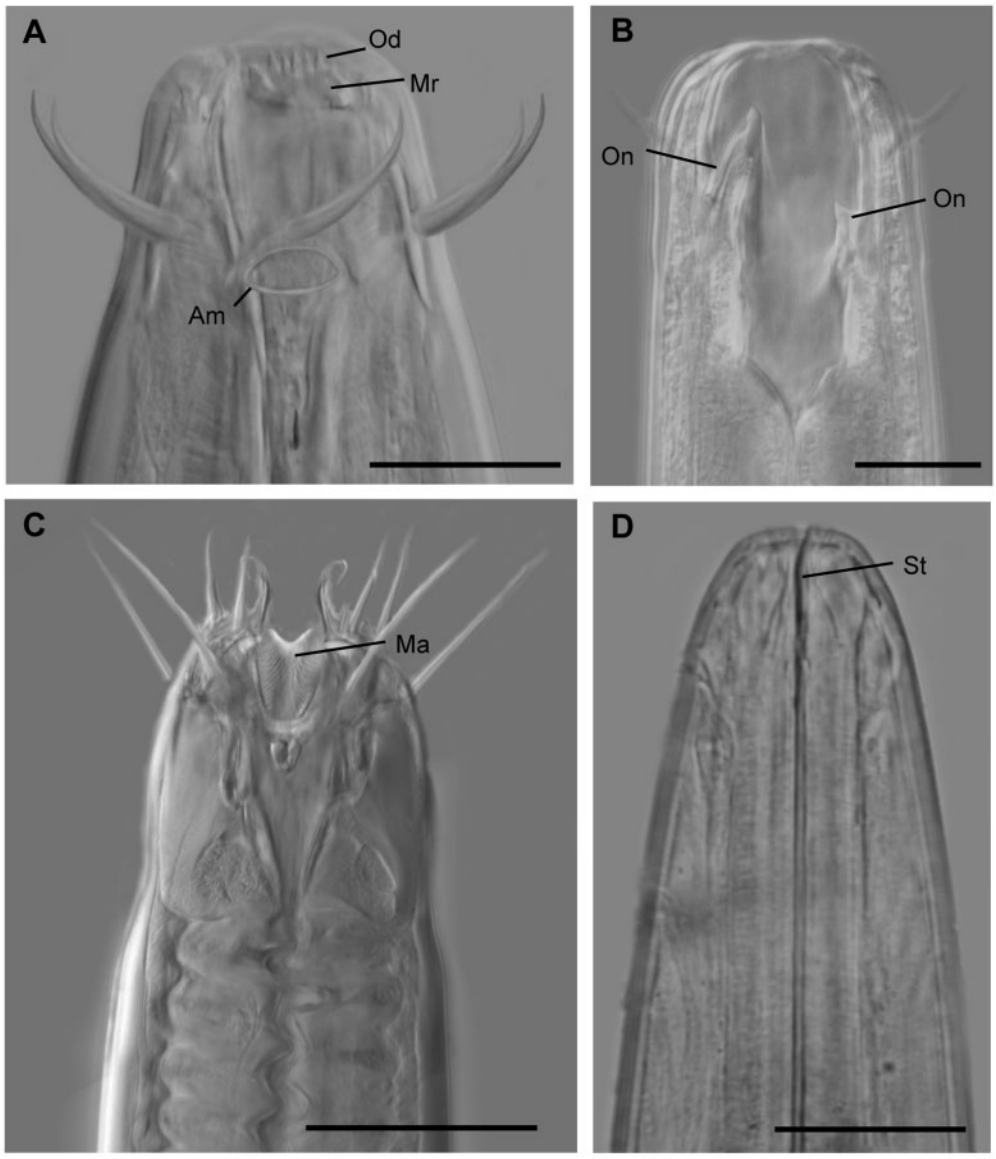

Fig. 1 Anterior region of representative members of Enoplida (composites of multifocal light microscopy images, all in lateral view). (**A**) *Cylicolaimus* sp. (Leptosomatidae). Scale bar = 25 µm. (**B**) *Metoncholaimus* sp. (Oncholaimidae). Scale bar = 15 µm. (**C**) *Enoploides* sp. (Thoracostomopsidae). Scale bar = 50 µm. (**D**) *Leptosomatum* sp. (Leptosomatidae). Scale bar = 30 µm. Am, amphid; Od, odontium; On, onchium; Ma, mandible; Mr, mandibular ridge; St, unarmed stoma.

muscles and labial apodemes that serve to open a deep cheilostome, as well as a microlabium and mandibular ridge on each of the three walls of the triangular stoma (Hope 1982) (Figs. 1A, 2C). Odontia may exist independent of the mandibular ridge or be fused with it to form, at least on the dorsal wall of the stoma, a moveable structure (as evidenced by attached musculature) (Wieser 1956; Inglis 1964) that may be involved in a specialized mode of feeding (Hope 1982). The degrees to which the odontia are incorporated into the mandibular ridge among species of *Deontostoma* Filipjev 1916 represent a transformation series that may be important in analyzing the phylogenetic relationships among species of that large, cosmopolitan genus (Hope 1982).

Members of the enoplid family Oncholaimidae have fixed onchia (usually three) in a spacious stoma (Figs. 1B, 2A) while members of the Enchelidiidae have three onchia, one of which can be protrusible (Smol et al. 2014). Other members of Enoplida have movable mandibles, such as the mandibles of Enoplidae (Fig. 2D) and mandibles, often with associated onchia, of the Thoracostomopsidae (Figs. 1C, 2E) (both families of the suborder Enoplina). Mandibles typically bear a pair of teeth or a transverse rod at the anterior end (Hope 1982)

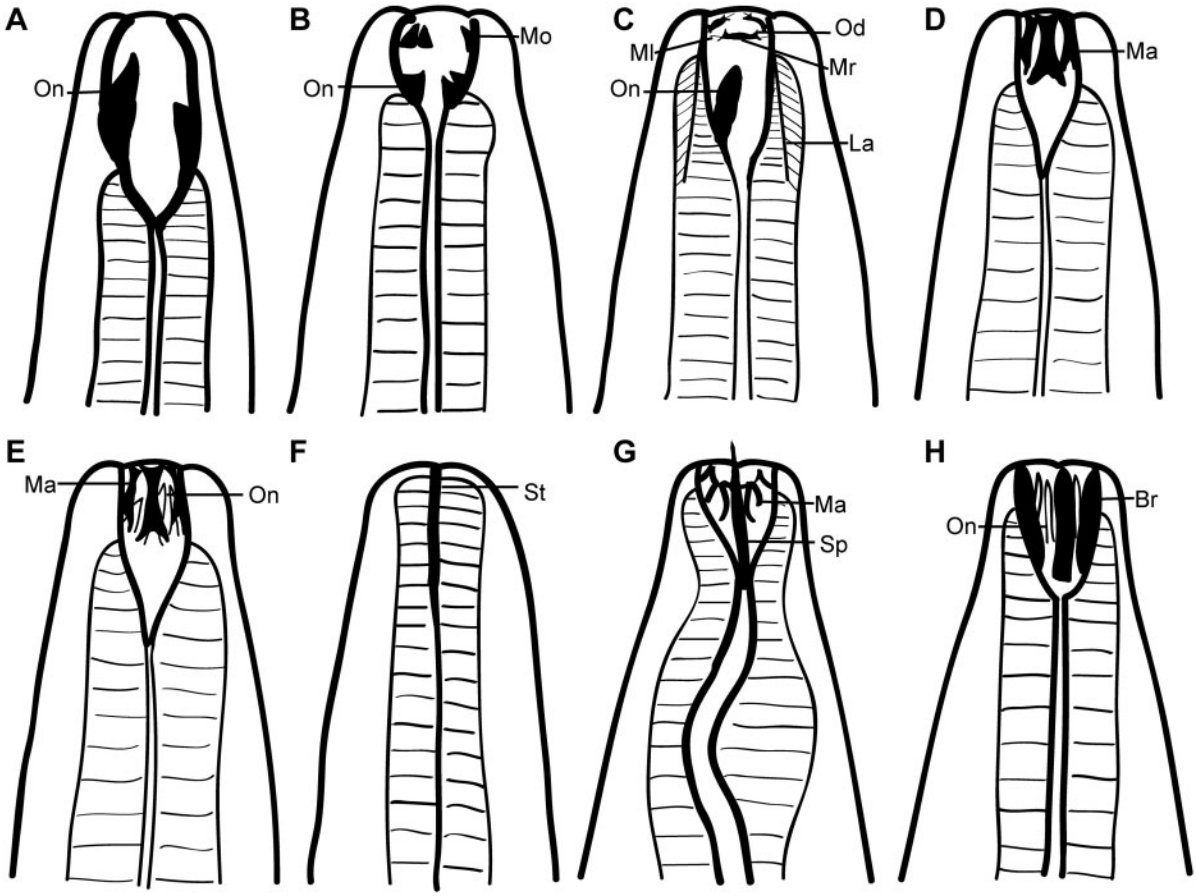

Fig. 2 Drawings of generalized anterior regions of representative members of Enoplida, emphasizing feeding structures of the stoma (amphids, sensilla, and other cuticular surface features not shown). (**A**) *Metoncholaimus* sp. (Oncholaimidae). (**B**) *Rhabdodemania* sp. (Rhabdodemaniidae). (**C**) *Deontostoma* sp. (Leptosomatidae). (**D**) *Enoplus* sp. (Enoplidae). (**E**) *Enoploides* sp. (Thoracostomopsidae). (**F**) *Leptosomatum* sp. (Leptosomatidae). (**G**) *Thoracostomopsis* sp. (Thoracostomopsidae). (**H**) *Phanoderma* sp. (Phanodermatidae). Br, buccal rod; La, labial apodeme; Od, odontium; On, onchium; Ma, mandible; Ml, microlabium; Mo, movable odontium; Mr, mandibular ridge; Sp, spear; St, unarmed stoma.

and move axially, not protruding or everting from the stoma (Inglis 1964). Members of one genus, however, *Thoracostomopsis* (Fig. 2G) have reduced mandibles but three elongate onchia that form a protrusible spear (Smol et al. 2014). Tahseen (2012) suggested that these taxa are predators, and that the movable mandibles are used for grasping and swallowing whole prey. Inglis (1964) proposed that the mandibles of Enoplidae (and taxa later transferred to Thoracostomopsidae) evolved from fixed buccal rods present in many members of the Phanodermatidae (Enoplida) (Fig. 2H). These buccal rods were proposed to be structural and supportive, primarily for the nerves of the labial sensilla (Inglis 1964). As the depth of the stoma increased, further support was needed, which led to cuticularized plates on each of the three walls of the stoma (Inglis 1964; Hope 1982). These plates eventually fused with the buccal rods to form mandibles used in feeding rather than as structural support (Inglis 1964). Hope (1982) proposed that Inglis's (1964) hypothesis was based on a misunderstanding of the cuticle of the head of enoplid nematodes, which Inglis (1964) considered to be a fluid-filled space. Hope (1982), in a detailed ultrastructural analysis of *Deontostoma* (Leptosomatidae) showed that there was no fluid-filled space in need of support, but rather cuticle throughout. Hope (1982) instead proposed that the ancestor of the Enoplidae and Thoracostomopsidae had a microlabium, mandibular ridge, and a pair of odontia on each stomal wall as seen in many species of *Deontostoma*. The mandibular ridge and odontia may have fused, and the resulting mandibular ridge–odontia complex may have moved posteriorly in the stoma to become the mandibles seen in Enoplidae and Thoracostomopsidae, with a transverse bar representing a remaining portion of the mandibular ridge (Hope 1982).

Members of *Rhabdodemania* Baylis and Daubney 1926 (Fig. 2B), the only genus in the Rhabdodemaniidae, have unique feeding structures that have made their taxonomic placement challenging. The 24 species of *Rhabdodemania* have three or rarely two onchia and three pairs of movable odontia (Hope 1988). The homologies of these buccal structures have been difficult to interpret, leading taxonomists to place *Rhabdodemania* in a variety of families and even orders (Hope 1988 and references therein). The most frequent placement has been with the Enoplidae and Thoracostomopsidae, under the assumption that the moveable odontia of *Rhabdodemania* were homologous with the movable mandibles of those taxa (Wieser 1959; Inglis 1964; Platonova 1974). The structure of the amphid, usually indicative of higher-level (ordinal) classification, is also unusual in *Rhabdodemania*, contributing to confusion regarding its broad taxonomic placement. In the amphid of *Rhabdodemania*, the external aperture is reduced to a small pore (invisible with light microscopy) and the fusus (the posterior part of the amphid that bears the dendrites) is extremely long and sinuous (Hope 2007). Hope (1988) suggested that *Pandolaimus latilaimus* (Allgen 1929) Stekhoven 1935, the only genus and species in Pandolaimidae, is likely to be closely related to *Rhabdodemania* due to similarities in the structure of the amphid. De Ley and Blaxter (2002, 2004) placed the Rhabdodemaniidae in the order Triplonchida, suborder Tobrilina, without justification.

Although recent molecular phylogenies have included increasing representation of members of Enoplida (Van Megen et al. 2009; Bik et al. 2010a, 2010b), no attempt to examine enoplid feeding structures in a molecular phylogenetic context has been made. The present study aimed to expand the taxonomic sampling for Enoplida and to explore prior hypotheses of the evolution of morphological features of the stoma, particularly the evolution of mandibles in the context of an 18S rRNA molecular phylogenetic analysis. This study also includes the first nearly full-length sequence from *Rhabdodemania*, affording a chance to examine its phylogenetic affinities and to contribute to an understanding of the evolution of its movable odontia.

Materials and methods

Collection of specimens

Sediment (sand or mud) was collected by hand on beaches and in estuaries, by SCUBA on coral reefs and by dredge in other off-shore habitats of the Atlantic and Pacific oceans and of the Caribbean Sea (Table 1). Although the study lacked specimens from deep-sea sediments, Bik et al. (2010b) showed that there were no major lineages of Enoplida exclusively from that habitat, thereby suggesting that representatives from all major lineages can be collected from shallower habitats. Living nematodes were extracted from sediment by a sieving and decantation method whereby sediment was suspended in a volume of seawater three to four times greater than the volume of sediment. The sediment was allowed to settle for 20–30 s before the supernatant was poured through a mesh sieve of approximately 50 μm. Living nematodes were identified with taxonomic keys (Platt and Warwick 1983; Keppner and Tarjan 1989; Smol and Coomans 2006; Hope 2007), usually to at least genus, before being stored in dimethyl sulphoxide, disodium EDTA, and saturated NaCl (DESS, Yoder et al. 2006) at $-20\,^{\circ}$C.

Molecular and phylogenetic methods

DNA extractions were performed using the DNeasy Blood and Tissue Kit (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. For most specimens, the middle portion of the body was used for extraction of DNA but the head and tail were processed to permanent slides and saved as morphological vouchers to be deposited in the Smithsonian Institution's National Museum of Natural History (voucher information available from first author). Nearly full-length, approximately 1600 base pair (bp) 18S rRNA gene sequences were amplified using forward primer G18S4 and reverse primer 18P (Blaxter et al. 1998). PCR reactions (25 μl total reaction volume) were performed using 2–3 μl of DNA template, 0.5–1 unit of Finnzyme DyNAzyme EXT proofreading polymerase (MJ Research, Waltham, MA), and final concentrations of 0.8 mM deoxynucleoside triphosphates (dNTPs), 1.5 mM $MgCl_2$, and 0.5 μM of each primer. The PCR thermocycling parameters included denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 46–48 °C for 1 min, and 72 °C for 1 min. A final extension period of 5 min at 72 °C concluded the amplification. Prior to direct sequencing, PCR products were enzymatically treated with exonuclease I and shrimp alkaline phosphatase (Pre-Sequencing Kit, USB Corporation, Cleveland, OH) to remove excess primers and dNTPs. Sequencing reactions were conducted by GENEWIZ, Inc. (South Plainfield, NJ) with the original PCR primers in addition to the following internal primers in order to achieve double-stranded sequence coverage: 647 (Nadler et al. 2000), 24F1, and 2FX (Meldal et al. 2007). Twenty-five sequences

Table 1 Georeferences for 25 new nematode sequences generated as part of this study.

Taxon	Locality	Depth	GPS	GenBank accession number
Cylicolaimus sp.	Carrie Bow Cay, Belize	27 m	16°48′07.6″N, 88°04′45.2″W	KR265030
Deontostoma washingtonense	Dillon Beach, Bodega, CA	Intertidal	38°15′17.6″N, 122°58′12.4″W	KR265031
Dolicholaimus sp.	Shell Beach, Bodega, CA	Intertidal	38°25′01.0″N, 123°06′19.9″W	KR265032
Enoploides sp.	Capron Shoals, Ft. Pierce, FL	9 m	27°26′36.7″N, 80°13′58.3″W	KR265033
Enoplolaimus sp.	Tom's Cove, Chincoteague Island, VA	Intertidal	37°53′07.4″N, 75°20′36.9″W	KR265034
Enoplus sp. 1	Seto Marine Lab, Shirahama, Japan	Intertidal	33°41′40.2″N, 135°20′12.7″E	KR265035
Enoplus sp. 2	Weekapaug Point, Westerly, RI	Intertidal	41°19′32.6″N, 71°45′10.6″W	KR265036
Epacanthion sp.	Weekapaug Point, Westerly, RI	Intertidal	41°19′32.6″N, 71°45′10.6″W	KR265037
Eurystomina sp.	Seto Marine Lab, Shirahama, Japan	Intertidal	33°41′40.2″N, 135°20′13.0″E	KR265038
Mesacanthion sp.	Capron Shoals, Ft. Pierce, FL	9 m	27°26′36.7″N, 80°13′58.3″W	KR265039
Mesacanthoides sp.	Carrie Bow Cay, Belize	30 m	16°48′07.6″N, 88°04′36.4″W	KR265040
Metenoploides sp.	Goat Island, Tobago	22 m	11°17′57.0″N, 60°31′20.2″W	KR265041
Meyersia sp.	Carrie Bow Cay, Belize	5 m	16°48′08.9″N, 88°04′47.1″W	KR265042
Oncholaimellinae sp.	Bathtub Beach, Fort Pierce, FL	Intertidal	27°11′08.8″N, 80°09′35.8″W	KR265043
Oncholaimus sp.	Seto Marine Lab, Shirahama, Japan	Intertidal	33°41′40.2″N, 135°20′12.7″E	KR265044
Oxystomininae sp.	Capron Shoals, Ft. Pierce, FL	8 m	27°26′43.2″N, 80°13′51.6″W	KR265045
Phanoderma sp.	Seto Marine Lab, Shirahama, Japan	Intertidal	33°41′40.2″N, 135°20′13.0″E	KR265046
Proplatycoma fleurdelis	Carrie Bow Cay, Belize	Intertidal	16°48′09.1″N, 88°04′55.6″W	KR265047
Pseudoncholaimus sp.	Sebastian Inlet, Vero Beach, FL	Intertidal	27°51′45.4″N, 80°26′48.8″W	KR265048
Rhabdodemania sp.	Higgins 5 mile station, Ft. Pierce, FL	15 m	27°30′08.3″N, 80°12′44.2″W	KR265049
Symplocostoma sp.	Crawl Cay, Bocas del Toro, Panama	2 m	9°15′06.4″N, 82°07′42.9″W	KR265050
Thoracostomopsis sp.	Carrie Bow Cay, Belize	42 m	16°48′07.6″N, 88°4′36.4″W	KR265051
Tobrilus pellucidus	Roosevelt Island, Potomac River, Washington, DC	Intertidal	38°53′58.4″N, 77°03′58.0″W	KR265052
Tripyloides sp.	Capron Shoals, Ft. Pierce, FL	7 m	27°26′39.6″N, 80°13′46.2″W	KR265053
Tylolaimophorus cylindricum	Plummers Island, Potomac River, Maryland	Humus	38°58′15.7″N, 77°10′20.6″W	KR265054

produced for this study (Table 1) were combined with 59 sequences available in GenBank. Previously published taxa were chosen to represent additional diversity from Enoplida, Triplonchida, and several taxa from other orders that other studies have suggested belong in Enoplida: *Trefusia* sp. HM564478 (Trefusiida), *Rhabdolaimus terrestris* KJ636366 (Chromadorida), *Campydora* sp. FJ969118 (Dorylaimida). Two members of Dorylaimida were chosen as outgroup taxa: *Longidorus* sp. EU503145, *Xiphinema americanum* AY283170. Sequences were aligned using ClustalW 1.82 (Thompson et al. 1997) using default parameters. jModelTest2 (Guindon and Gascuel 2003; Darriba et al. 2012) was used to choose GTR (Tavare 1986) plus G (gamma-shaped rate variation among sites) and I (a proportion of invariable sites) as the best-fitting model of nucleotide substitution by the Bayesian Information Criterion (Schwarz 1978). RAxML v. 8.1.11 (Stamatakis 2014) was used to conduct a maximum likelihood (ML) and bootstrap analysis (1000 replications) using the GTR plus G model (the RAxML manual recommends against including I). MrBayes v. 3.2.3 (Ronquist et al. 2012) was used for a Bayesian inference (BI) analysis to approximate posterior probabilities (PPs) using the 4×4 DNA model, the GTR (Nst = 6 command) substitution model with the Invgamma rates command, default priors, four chains for 5×10^6 generations, discarding the first 25% of samples as ''burn in'', and sampling Monte Carlo Markov chains every 1000 generations. All phylogenetic analyses were conducted on the CIPRES Gateway (Miller et al. 2010). FigTree v. 1.4.2 (Rambaut 2014) was used to visualize phylogenetic trees after analysis.

Results

The BI phylogeny revealed two well-supported primary clades, one including taxa currently placed in

Triplonchida (Bayesian PP, BPP = 1; bootstrap value percent, BV = 100) and one including predominantly members of Enoplida (BPP = 0.93; BV = 78; Fig. 3). Several taxa currently assigned to Triplonchida (*Trischistoma* sp., *Tripylina tamaki*, and *Rhabdodemania* sp.) were placed in the large clade with members of Enoplida. *Rhabdodemania* sp. was placed as part of an unresolved and poorly supported (BPP = 0.56; BV = 20) clade with members of the Ironidae (*Ironus* spp. and *Dolicholaimus* sp.), *Halalaimus* spp. (Oxystominidae), and a well-supported subclade (BPP = 1; BV = 100) including members of the Oncholaimidae and Enchelidiidae. The Oncholaimidae were shown to be paraphyletic although the Enchelidiidae were shown to be well-supported (BPP = 1; BV = 81) as a monophyletic group nested within the oncholaims.

Trefusia sp. (Trefusiina) was also placed within the Enoplida, as sister to the clade uniting *T. tamaki*, *Trischistoma* sp. and *Trefusia* sp. *Rhabdolaimus terrestris* (Chromadorida) and *Campydora* sp. (Dorylaimida) were also placed within the Enoplida in a well-supported clade with *Syringolaimus* sp. (Ironidae). A moderately-supported clade (BPP = 0.93; BS = 57) united the other members of Oxystominidae beyond *Halalaimus* spp., rendering that family paraphyletic. The larger clade within Enoplida uniting those members of Oxystominidae as well as Ironidae, Chromadorida, Dorylaimida, Trefusiina, and Triplonchida was very poorly supported (BPP = 0.54; BV = 4).

A moderately well-supported clade (BPP = 0.98; BV = 61) within the Enoplida included two clades of the (paraphyletic) Anoplostomatidae at its base, as well as Leptosomatidae, Anticomidae, and Phanodermatidae as sister to the Enoplidae and the Thoracostomopsidae. Members of the Leptosomatidae were shown to be strongly supported as a monophyletic group (BPP = 1; BV = 99), but all taxa beside *Leptosomatides*, placed at the base of the clade, formed a weakly-supported sub-clade (BPP = 0.66) with little resolution of the rest of the members of the family. The Thoracostomopsidae was well supported as a monophyletic group (BPP = 1; BV = 100) with two species of *Enoploides* as the earliest branching lineage and sister to two other clades, one well-supported with *Thoracostomopsis*, *Mesacanthoides*, and *Epacanthion* and the other poorly-supported (BPP = 0.62; BS = 40) containing *Metenoploides*, *Mesacanthion*, and two species of *Enoplolaimus*. The ML phylogeny (not shown) was congruent with the BI phylogeny in all major clades and relevant taxa.

Discussion

The BI and ML phylogenetic analyses suggest that the subclass Enoplia requires taxonomic revision with respect to the orders Enoplida and Triplonchida, which were shown to be paraphyletic. Three taxa placed in Triplonchida by De Ley and Blaxter (2002, 2004) were shown to be part of the well-supported clade of Enoplida: *Trischistoma* sp., *T. tamaki* (Tripylidae), and *Rhabdodemania* sp. (Rhabdodemaniidae). Other recent 18S rRNA phylogenies have also shown these members of Tripylidae to be placed within Enoplida rather than with other members of Triplonchida (Holterman et al. 2006; Meldal et al. 2007; van Megen et al. 2009; Zhao and Buckley 2009). Bik et al. (2010a, 2010b) found the same result and indicated that they had found Triplonchida to be monophyletic, considering *Tripylina* and *Trischistoma* to be members of Enoplida, contrary to the currently accepted classification of De Ley and Blaxter (2002, 2004). Enoplida was also shown to be paraphyletic with respect to several other orders with taxon representatives that were placed in Enoplida, including the Trefusiina (*Trefusia* sp.), Dorylaimida (*Campydora* sp.), and Chromadorida (*R. terrestris*). A strongly supported clade including *Rhabdolaimus*, *Campydora*, and *Syringolaimus* was also reported by Bik et al. (2010a, 2010b), and Meldal et al. (2007) showed *Campydora* as sister to *Syringolaimus* within Enoplida but did not include *Rhabdolaimus* in their study.

While the Leptosomatidae have been considered morphologically ''primitive'' within Enoplida by several authors (Filipjev 1918, 1934; Maggenti 1963; Platonova 1964, 1976), the present analysis suggests they may be derived and not near the base of Enoplida. As the relationships among the major lineages of Enoplida remain unclear in the present analysis, it is not possible to determine the features of the ancestral or ''primitive'' enoplid, but the placement of the Leptosomatidae as well-nested within a clade containing members of Anoplostomatidae and other families suggests that leptosomatids do not represent the earliest enoplid nematodes. Within the Leptosomatidae, *Leptosomatides*, with a reduced, unarmed, stoma lacking onchia, odontia, microlabia, or mandibular ridges, was well-supported as the earliest lineage in the family. This placement supports the idea that although the Leptosomatidae are not an early branch of the Enoplida, the members of Leptosomatidae with simple, reduced stomas are early diverging members of that family. The present phylogenetic results also suggest that *Leptosomatides*

does belong in the Leptosomatidae, contrary to Bongers (1984), who suggested that it might belong with the Thoracostomopsidae. Relationships among the rest of the members of Leptosomatidae included in the analysis were unresolved or poorly supported using the 18S rRNA locus. Additional analyses using other loci such as the 28S rRNA may provide further resolution as it has for other nematode families or genera (e.g., Stock et al. 2001; Smythe and Nadler 2006; Subbotin et al. 2008).

The present analysis provides the first full-length 18S rRNA sequence data for *Rhabdodemania*, allowing for its inclusion in broader phylogenetic analyses and evaluation of the evolution of its unique feeding structures, the movable odontia. The first sequence data from *Rhabdodemania* was provided by Litvaitis et al. (2000), who used 28S rRNA in their phylogenetic analysis of 49 marine and terrestrial nematodes. Litvaitis et al. (2000) found *Rhabdodemania* to form a poorly supported clade with some members of the Enoplidae and Thoracostomopsidae (Enoplida), although they did not find most Enoplida to be monophyletic, as subsequent studies have. Pereira et al. (2010), in an analysis limited only to Enoplida, found moderate support with 28S rRNA for the placement of *Rhabdodemania* in Enoplida, part of a clade with *Bathylaimus* (Tripyloididae) as sister to the Thoracostomopsidae. Using a small (350 bp) fragment of 18S rRNA, Pereira et al. (2010) also found moderate support for the placement of *Rhabdodemania* with members of the Oncholaimidae, taxa with fixed onchia.

The current analysis suggests that *Rhabdodemania* belongs within the Enoplida, and not with Triplonchida, where De Ley and Blaxter (2002, 2004) placed it. Its close relatives within Enoplida, however, are still uncertain. Although its placement in a clade with members of Ironidae and Oncholaimidae is poorly supported, a possible close relationship with Ironidae provides an interesting suggestion regarding the origin of the movable odontia. While members of Ironidae have movable teeth (usually three but sometimes four or five), these teeth are likely unique in Enoplida (Lorenzen 1981b, 1994; Hope 2007) because in juveniles, replacement teeth are formed in pharyngeal pouches posterior to the functional ones (Smol et al. 2014). The teeth of members of Ironidae also move and protrude longitudinally (Van der Heiden 1974), unlike the mandibles of Enoplidae and Thoracostomopsidae that move axially and do not protrude. As juveniles mature, the replacement teeth move forward to become functional with each molt. While these primary teeth of ironids are unlikely to be homologous with the movable odontia of *Rhabdodemania* (Lorenzen 1981b, 1994; Hope 2007), some ironids also have small denticles that may be movable given the complex musculature of their stoma (Van der Heiden 1974). Further detailed investigation of the denticles of ironids, such as *Ironella* Cobb 1920, is warranted, given a possible close relationship with *Rhabdodemania*. The current analysis provides no evidence that *Rhabdodemania* is closely related to Enoplidae and Thoracostomopsidae, as those families are part of a well-supported clade that does not include *Rhabdodemania*. Thus, the movable odontia of *Rhabdodemania* are unlikely to be homologous with the mandibles of the Enoplidae and Thoracostomopsidae as previously suggested (Wieser 1959; Inglis 1964; Platonova 1974).

Inglis (1964) proposed that the movable mandibles of Enoplidae and Thoracostomopsidae evolved from fixed buccal rods present in many members of the Phanodermatidae, while Hope (1988) suggested instead that the mandibles evolved from the microlabium and mandibular ridge/odontia complex seen in the Leptosomatidae. All of these families likely evolved from nematodes lacking teeth entirely, as the current analysis places them as nested within a well-supported clade with members of Anoplostomatidae at its base, although Anoplostomatidae is not shown to be monophyletic. *Chaetonema* and *Anoplostoma*, representative members of Anoplostomatidae in this study, have a wide, sclerotized but unarmed (toothless) stoma. Other studies have shown a similar placement of the Anoplostomatidae in relation to these families (Pegova et al. 2004; Bik et al. 2010a, 2010b). The Anoplostomatidae are placed at the base of two clades, the monophyletic Leptosomatidae and a clade containing one representative of the Anticomidae (*Anticoma* sp.), the Phanodermatidae, Enoplidae, and the Thoracostomopsidae. While the Leptosomatidae are not distantly related to the taxa with mandibles, the Enoplidae and Thoracostomopsidae, in the present analysis *Anticoma* sp., which has a small, unarmed stoma, is the sister to the lineages containing taxa with mandibles, suggesting that the mandibular ridge and odontia of the Leptosomatidae may not be the origin of the mandibles. Instead, a toothless ancestor may have given rise to a lineage with buccal rods (Phanodermatidae) and the mandibles both of Enoplidae and of Thoracostomopsidae. Improved taxon sampling of the Anticomidae may shed light on the origin of the buccal rods and mandibles, as at least one genus not yet sequenced for any loci, *Odontanticoma*, possesses odontia. The sister relationship of the Enoplidae to the Phanodermatidae is not strongly supported, although other studies have shown strong support for this relationship (Bik et al. 2010a,

2010b). In addition to buccal rods, members of Phanodermatidae have one small dorsal and two larger ventrosublateral teeth (Smol et al. 2014). This close relationship of the Phanodermatidae to both families of taxa with movable mandibles suggests that the buccal rods and/or teeth of the phanoderms, rather than the microlabium and mandibular ridge/odontia complex of the Leptosomatidae, may indeed be the origin of the mandibles (Inglis 1964). This suggests that further morphological examination of the Phanodermatidae, the stoma of which have never been explored in detail, is warranted, although Hope (1982) noted that there is no obvious evidence of microlabia, odontia, or mandibular ridges in this family. Hope (1982) did note that he had unpublished SEM evidence of microlabia in *Enoplus* sp. (Enoplidae), suggesting that perhaps the microlabia and mandibles are independent structures and that the microlabia have not been incorporated into the mandibles.

The Thoracostomopsidae as currently organized contains three subfamilies: the Thoracostomopsinae (with only *Thoracostomopsis* spp.), the Trileptiinae (with only *Trileptium* spp.), and the Enoplolaiminae (with all the remaining genera) (Smol et al. 2014). Smol et al. (2014) suggested that the Enoplolaiminae needs to be revised as the complex nature of the stoma has been misunderstood by many taxonomists, leading to poorly described species. Pereira et al. (2010) included in their analysis three species of *Trileptium*, which have weakly developed mandibles but strongly developed onchia. Pereira et al. (2010) found that both 28S and 18S rRNA showed a number of genera in Thoracostomopsidae to be paraphyletic, including *Trileptium* and *Mesacanthion*, with one species of *Trileptium* forming a well-supported clade with two species of *Mesacanthion*. The current analysis included only members of Thoracostomopsinae and Enoplolaiminae and showed *Thoracostomopsis*, unique within the family with its reduced mandibles and protrusible spear, to be nested within a well-supported clade of members of Enoplolaiminae. The current analysis did not show any genera of Thoracostomopsidae to be paraphyletic, but only *Enoploides* and *Enoplolaimus* were represented by two species and thus available for testing monophyly. Consistent with the 18S rRNA results of Pereira et al. (2010) and Bik et al. (2010b), the current analysis placed *Enoploides* spp. at the base of the Thoracostomopsidae. As the name suggests, *Enoploides* is similar to *Enoplus*, with solid mandibles with teeth at the anterior end, but having moderately well-developed onchia while *Enoplus* lacks onchia (Smol et al. 2014). The placement of *Enoploides* at the base of the Thoracostomopsidae suggests that perhaps the earliest members of the family had robust mandibles and that mandibular variations and reductions such as the long, slender mandibles of *Metenoploides* or the delicate, arch-shaped mandibles with an anterior transverse rod of *Enoplolaimus* are derived. Filipjev (1927) believed that the powerful mandibles of *Enoplus* represented the ancestral condition in the Enoplidae and Thoracostomopsidae, a similar evolutionary scenario as the current analysis suggests. Other authors have suggested that more robust, simple mandibles are derived and that taxa with small onchia and less robust mandibles like *Enoplolaimus* represent the ancestral form (Wieser 1953b), an opposite scenario to that suggested by the present analysis. Platonova (1984) proposed *Parenoplus* to represent the earliest member of the Enoplidae/Thoracostomopsidae, with arch-shaped mandibles and onchia only visible in juveniles, suggesting that the oral armature in this taxon was used only for structural support and not for feeding. The 28S results of Pereira et al. (2010) placed *Oxyonchus*, with arch-shaped, toothed mandibles (bearing additional denticles) with an anterior rod, at the base of the Thoracostomopsidae, suggesting yet another evolutionary scenario. *Parenoplus* and *Oxyonchus* are rarely collected, and neither have been sequenced for full-length 18S, thereby highlighting the challenge of including all known morphological diversity in modern phylogenetic hypotheses.

The present study represents a first attempt to explore and understand the evolution of feeding structures in the Enoplida using a molecular phylogenetic framework. Any effort to understand morphological diversity should include as much of the known diversity as possible, a significant obstacle in taxa such as marine free-living nematodes, with many species that are rarely found and difficult to collect. For example, this analysis included only 7 of the 19 currently recognized genera of the Thoracostomopsidae and 7 of the 32 genera of the Leptosomatidae (Smol et al. 2014), suggesting that interpretations of morphological evolution are premature. Preliminary discussions of the evolution of these fascinating structures can, however, inform, guide, and spur further research. Putative relatives of taxa of interest can be proposed for further examination, perhaps allowing the discovery of homologous structures not previously identified. With regard to the evolution of feeding structures in Enoplida, taxa of particular interest that have received relatively little morphological attention include the Phanodermatidae, Ironidae, and the Anoplostomatidae. Future research should not only

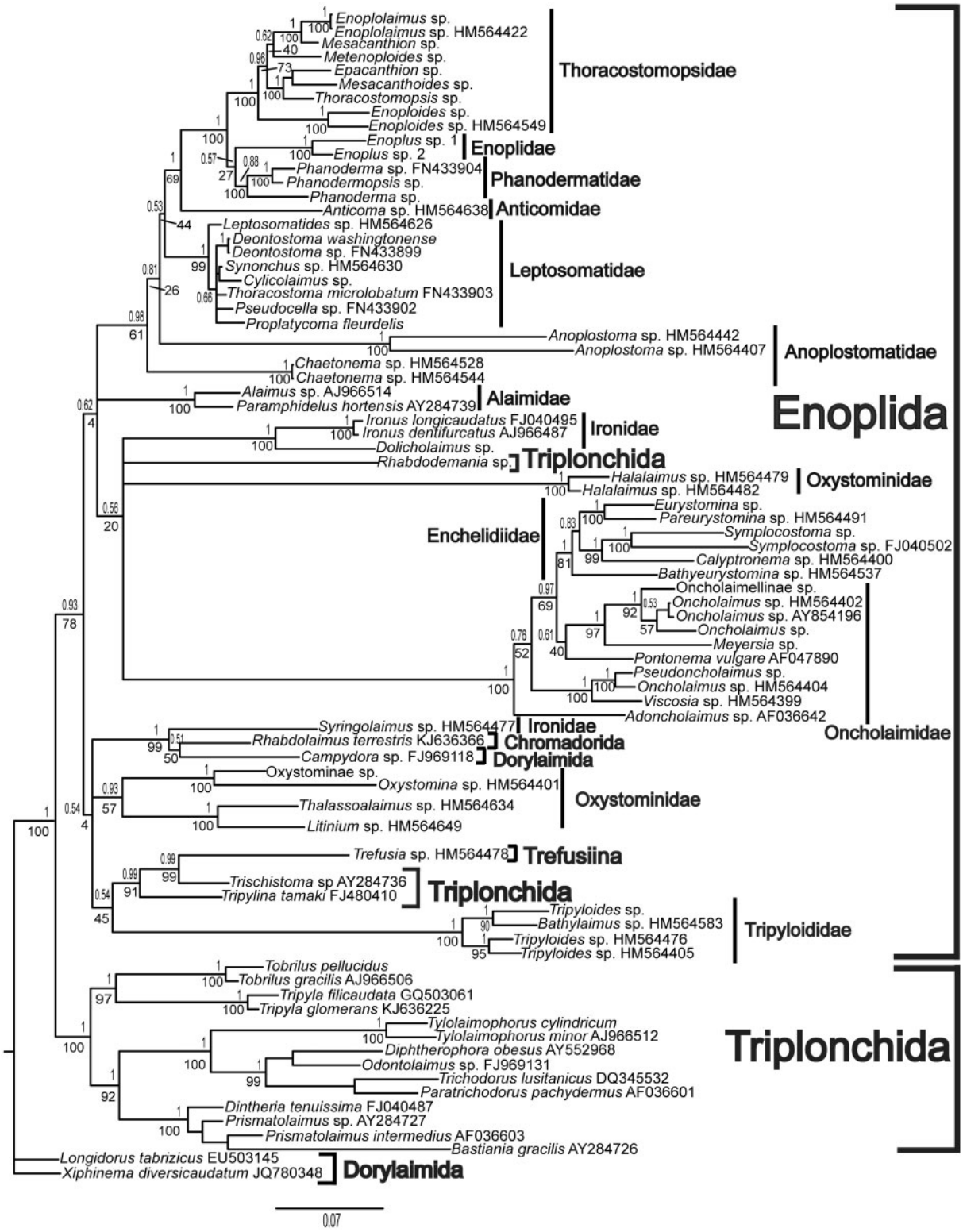

Fig. 3 BI tree of 84 18S rDNA sequences from nematodes in the subclass Enoplia. Taxonomic names followed by GenBank accession numbers represent previously published sequences from other studies. Two plant parasitic nematodes, *Xiphinema diversicaudatum* JQ780348 and *Longidorus tabrizicus* EU503145, members of the order Dorylaimida, were used as outgroups to root the tree. Numbers above nodes represent BPPs, numbers below nodes represent bootstrap support values for the same clades found in the ML analysis, and the scale-bar represents the number of substitutions per site. Bold labels indicate orders to which species are currently assigned according to De Ley and Blaxter (2002, 2004).

attempt to include additional taxa and clarify poorly-supported relationships but also incorporate morphological visualization techniques that have yet to be applied to marine nematodes, such as serial thin-section transmission electron microscopy and three-dimensional reconstruction (e.g., Bumbarger et al. 2009) and confocal imaging and functional analysis of musculature (e.g., Hochberg et al. 2010; Oliveira and Mayer 2013). Inglis (1964) provided detailed descriptions and functional morphological hypotheses of the musculature associated with the feeding structures of members of Enoplida using light microscopy. Modern examinations of the musculature of the enoplid stoma would allow interpretations of the functional morphology of movable feeding structures, comparisons to previous hypotheses, and a greater understanding of the evolution of the morphological and functional diversity in nematodes.

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