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Phylogenetic analysis of nematode nuclear 18S rDNA sequences indicates the genus *Tripylina* Brzeski, 1963 (Nematoda: Tripylidae de Man, 1876) should be placed in Enoplida

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Abstract

We have made an extensive study of New Zealand representatives of nematodes from the family Tripylidae de Man, 1876. Based on SSU DNA sequence data and phylogenetic analysis, the genera *Tripylina* Brzeski, 1964 and *Trischistoma* Cobb, 1913 are not closely related to *Tripyla* Bastian, 1865, the type genus of the family Tripylidae de Man 1876. The genus *Tripylina* is sister to *Trischistoma* and *Trefusia* de Man, 1893 and is more closely related to Enoplida than to Triplonchida. Our phylogenetic results indicate that *Tripylina* should be placed in Enoplida.

Key words: *Tripylina*, Tripylidae, Triplonchida, Enoplida, 18s SSU rDNA

Introduction

In the last decade, DNA sequencing and genomics have brought substantial change to nematode taxonomy (Aleshin *et al.* 1998; Blaxter *et al.* 1998; De Ley & Blaxter 2002, 2004.; Holterman *et al.* 2006; Meldal *et al.* 2007). Based on nematode ribosomal RNA small subunit (SSU) phylogenetic trees, Triplonchida and Enoplida are the two sister-orders forming the Enoplia (De Ley & Blaxter 2004; Holterman *et al.* 2006; Meldal *et al.* 2007). However, the relationships of suborders within the two orders remained unresolved. For example, Meldal *et al.* (2007) found that three species that were previously not reliably placed in Enoplia were consistently found in this clade: *Alaimus* sp. (formerly Dorylaimia or Triplonchida), *Campydora demonstrans* (formerly Dorylaimia or Enoplia), and *Trischistoma monhystera* (formerly Triplonchida).

The molecular phylogenetic study of Meldal *et al.* (2007) confirmed that 1) the Triplonchida is an order within Enoplia, consistent with Siddiqi (1983) but contrary to many earlier classifications that were based on morphological data alone and placed part of this group among the Dorylaimia (Thorne 1939; Clark 1961; Siddiqi 1961, 1973; De Coninck 1965; Coomans & Loof 1970); 2) within Triplonchida, the Diphtherophoroidea were well supported as monophyletic; 3) contrary to morphological classifications, *Trischistoma monohystera* appears to be more closely related to Enoplida than to Triplonchida as the latter order forms a well supported clade excluding *T. monohystera*.

The phylogenetic tree of the phylum Nematoda inferred by De Ley & Blaxter (2004) shows that the family Tripylidae de Man, 1876 belongs to the superfamily Tripyloidea, the suborder Triyplina and the order Triplonchida. Nematodes of the family Tripylidae mainly occur in fresh water and soil. The genera *Tripylina* Brzeski, 1963, *Tripyla* Bastian, 1865 (= *Promononchus* Micoletzky, 1923, *Paratripyla* Brzeski, 1963), *Tripylella* Brzeski & Winiszewska-Ślipińska 1993, *Trischistoma* Cobb, 1913 and *Tobrilia* Andrássy, 1967 are included in the family Tripylidae *sensu* Andrássy (2007). To date, there are six valid species in *Tripylina*, twenty four in *Tripyla*, three in *Tripylella*; four in *Trischistoma* and two in *Tobrilia* (Tsalolikhin 1983; Brzeski

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& Winiszewska-Ślipińska 1993; Zullini 2006; Andrássy 2006, 2007). *Tripyla affinis* de Man, 1880 and *Tripylina stramenti* (Yeates, 1972) Tsalolikhin, 1983 have been reported from New Zealand. About 20 species in the family Tripylidae are estimated to occur in New Zealand (Yeates, pers. com.).

Methods

Sampling. Since March 2007, we have been collecting and studying nematodes in the family Tripylidae from the Waitakere Ranges Regional Park, Coromandel Range, Rotorua, Cambridge, Hamilton regions and the Arthurs Pass National Park in New Zealand. A total of 230 mixed soil and litter samples from native forests and conservation parks have been examined. The 0–10 cm topsoil and litter mixtures were collected by trowel from under trees or shrubs. The samples were placed in plastic bags, and transported back to the laboratory and then kept at 10˚C until extraction. Using the Whitehead and Hemming tray method (Southey 1986), nematodes were extracted from sub-samples of 500 g material over 2 days, at room temperature. Using a 20 µm mesh sieve the suspension was reduced to about 5 ml and left to stand for about one hour. The volume was reduced to 3 ml by aspiration of excess fluid. The nematodes were then transferred to a glass block for examination with a dissecting microscope at 8X to 35X magnification (Leica EZ4, Germany).

Morphological identification. The procedure for nematode specimen preparation was similar to the method of Mullin *et al.* (2003). A single tripylid nematode was hand-picked from a living nematode suspension, and mounted in distilled water on temporary glass slides and relaxed using gentle heat. A microscopic attached camera (Nikon Camera Head DS-Fi1) was used to take a series of digital images of key morphological characters of each nematode so as to retain the ability to reevaluate the identity of individual specimens. Series of digital images from individual nematodes are available upon request (from the author, National Nematode Collection New Zealand (NNCNZ)). Nematodes were prepared for PCR as described below in the DNA extraction section. After photographing the specimens, several other nematodes of the same apparent species from the same soil sample collection were put in a tube containing 1M NaCl and stored at –20ºC in a freezer for future DNA extraction. Additionally, many nematodes of the same apparent species from the same collection were processed in glycerol and mounted on glass slides as described by Davies and Giblin-Davis (2004) with nematode extraction numbers for future morphological identification to species level. Nematodes were examined using interference contrast microscope (Nikon ECLIPSE 90i, Japan). Five new species of *Tripylina* from New Zealand are described by Zhao (2009). While the single nematode used as for DNA analysis cannot be preserved physically, permanent conspecific nematode specimens taken from the same sample collection can be compared to the digital vouchers of nematodes to confirm the DNA and the species are correctly matched. Nematodes actively swimming through free water by means of bursts of rapid oscillations of the head, having a narrow stoma, and possessing a pharynx as a muscular tube, were classified as tripylids. In total, based on a more thorough examination of morphological characters (e.g. nematode body length, width; inner, outer labial and cephalic sensillae shape and length; the number of cervical setae and their distance from anterior end; the shape and position of the dorsal tooth; amphid; female vulval position and the structure of reproductive system; tail length, width and shape, etc.), 21 18S rRNA sequences of 19 isolates of Tripylidae, including twelve *Tripyla* spp., three *Tripylina* spp.,one *Tripylella* sp. and three *Trischistoma* spp. were used for molecular phylogenetic analysis.

DNA extraction, Polymerase Chain Reaction (PCR) and DNA sequencing. A modified nematode DNA extraction method of Zheng *et al.* (2002) was used. Total genomic DNA from a single nematode was extracted using worm lysis buffer containing proteinase K (Williams *et al.* 1992). The temporary slides used for morphological identification and photo-documentation were dismantled and individual nematodes removed to an Eppendorf tube which contained 20 µl worm lysis buffer. Tubes containing nematodes were stored at -80ºC at least 30 minutes before DNA extraction taking place. DNA extractions were stored at –4ºC until used as template for PCR amplification. Primers for SSU amplification were forward primer 18S - G18S4 (5’- GCTTGTCTCAAAGATTAAGCC - 3’) and reverse primer 18S - 18P (5’ -

TGATCCWKCYGCAGGTTCAC - 3') (De Ley *et al.* 2002; Dorris *et al.* 2002). The 20 µl PCR reactions contained 10 µl Go Tag® Green Master Mix (Promega Corporation, Madison, WI, USA), 1 µl (0.05 µM) each of forward and reverse primer, and 2 µl of DNA template. The thermal cycling program was as follows: denaturation at 95ºC for 3 min, followed by 30 cycles of denaturation at 94ºC for 60 s, annealing at 55ºC for 45 s, and extension at 72ºC for 45 seconds. A final extension was performed at 72ºC for 10 min. PCR products were purified by Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA). Purified PCR products were sequenced using Big Dye TM Terminator Cycle Sequencing Ready reaction Mix v3.1 kit (Applied Biosystems, USA). Cycle sequencing products were cleaned by 96 well plate ethanol precipitation and analysed on an ABI 3100 Avant genetic analyzer (Applied Biosystems, USA). The quality of each sequence was confirmed by inspection of sequence trace files. The sequences were deposited into the GenBank database and the accession numbers are listed in Table 1.

DNA sequence alignment. Thirty four published sequences from GenBank were included in our phylogenetic analysis (Table 2). Only a few representatives of the suborders of the Enoplia were available on GenBank and so our taxon sampling is limited for some groups. DNA sequences were aligned in Clustal X (Larkin *et al*. 2007) using the multiple alignment method with default parameter values. The resulting alignment was checked by eye and any obviously misaligned bases were corrected.

Phylogenetic inference. We used ModelTest (Posada & Crandall 1998) and PAUP*4.0b10 (Swofford 1998) to select the best AIC model. A Bayesian tree was obtained using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). We ran 4 MCMC chains for 5,000,000 generations under the best-fit model (GTR+I+Γ). Prior distributions were as follows: ratepr = variable, revmatpr = dirichlet (1,2,1,1,2,1), shapepr = exponential (5), brlenspr = unconstrained: exponential (10). We started analysis from a random topology and used a temperature of 0.2, a burnin of 10% and a thinning interval of 1,000. Multiple runs were performed to ensure convergence. We also performed a maximum parsimony analysis in PAUP*4.0b10 (Swofford 1998) with bootstrapping. For the bootstrapping we used 100 replicates with 100 random addition replicates from stepwise addition trees and TBR branch swapping. The trees were rooted using *Monhystera* sp. and *Geomonhystera* sp. from the order Monhysterida, Subclass Chromadoria and Class Chromadorea.

Results

We obtained 21 18S rRNA sequences of New Zealand 19 Tripylidae species and the best-fit model for these sequences and the sequences from GenBank was the GTR+I+Γ model. The Bayesian and maximum parsimony topologies and nodal support measures were very similar. The consensus tree inferred from SSU (Fig. 1) indicated: 1) the Enoplida and Triplonchida were formed with a posterior probability of 100% and a parsimony bootstrap value of 100% respectively; 2) the three suborders of Triplonchida were supported as monophyletic with posterior probabilities and parsimony bootstrap values of 100% , whereas the six suborders of Enoplida formed a paraphyletic grade and relationships among them were poorly resolved; 3) *Tripyla*, the type genus of the family Tripylidae, contained three clades with a posterior probability of 100% and a parsimony bootstrap value of 100% respectively (species with long tails (the de Man's ratio c < 5); species with long cephalic setae (the six long cephalic setae >5 µm) and species with short cephalic setae (the six long cephalic setae <5 µm)); 4) the genera *Tripylina* and *Trischistoma* were closely related with a posterior probability of 100% and a parsimony bootstrap value of 64% respectively, however, they were not in the Triplonchida clade and grouped with genera currently placed in the Enoplida; 5) the genera *Ironus* Bastian, 1865 and *Oxystomina* Filipjev, 1921 were paraphyletic (supposedly they belong to the suborder Ironina); 6) the genera *Tobrilus* Andrássy, 1959 and *Prismatolaimus* de Man, 1880 were paraphyletic (supposedly they belong to the suborder Tobrilina); 7) *Tripyllela* was closer to the suborder Tobrilina than to the suborder Tripylina; 8) *Paratripyla* Brzeski, 1963 (it is no longer a valid genus of Tripylidae (*sensu* Brzeski & Winiszewska-Ślipińska; Zullini; Andrássy) was close to the genus *Tobrilus*.

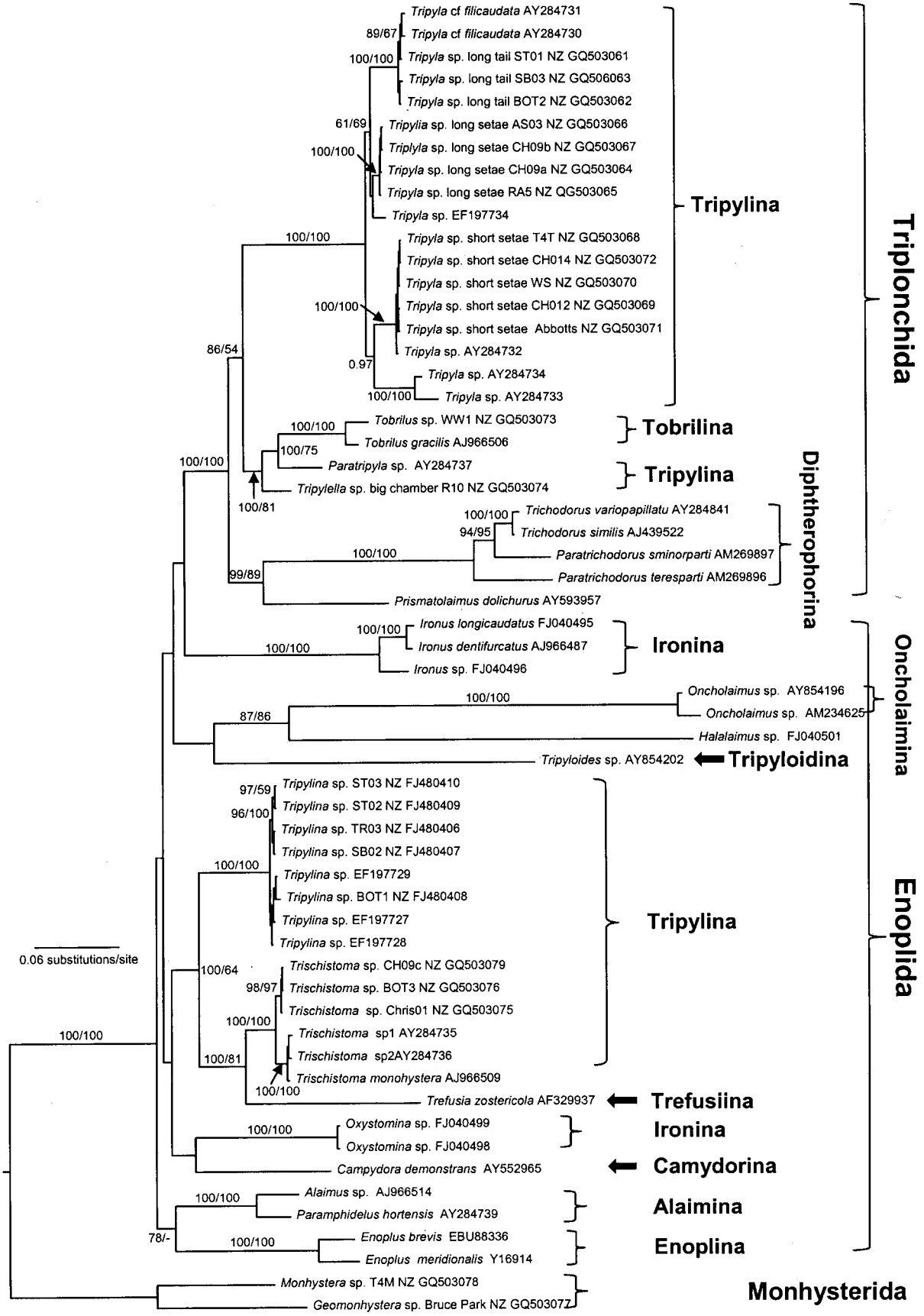

FIGURE 1. Bayesian tree estimated under the GTR+I+Γ model. Branch lengths are drawn proportional to the expected number of substitutions per site. Numbers above branches are posterior probabilities followed by maximum parsimony bootstraps, both expressed as percentages. *Tripylina* sp. ST02 and ST03 = *T. tamaki*; *Tripylina* sp. TR03 and SB03 = *T. tearoha*; *Tripylina* sp. BOT1 = *T. manurewa*.

TABLE 1. Nematode species voucher number, GenBank accession number and collection localities in New Zealand. Area codes follow Crosby *et al.* (1998).

Species	Voucher No.	GenBank Acc.#	Locality information		
			Latitude	Longitude	Locality
Tripyla sp.	NE109	GQ503061	36º52.354 S	174º50.531 E	St Johns Bush, AK
Tripyla sp.	NE164	GQ503062	37º0.657 S	174º54.491 E	Botanical Garden, AK
Tripyla sp.	NE201	GQ503063	36º48.782 S	174º45.026 E	Smith's Bush, AK
Tripyla sp.	NE73a	GQ503064	42º58.099 S	171º34.722 E	Arthurs Pass National Park, NC
Tripyla sp.	NE43	GQ503065	38º0.061 S	175º52.939 E	Rotorua, BP
Tripyla sp.	NE155	GQ503066	41º19.273 S	173º15.237 E	Nelson, NN
Tripyla sp.	NE73b	GQ503067	42º58.099 S	171º34.722 E	Arthurs Pass National Park, NC
Tripyla sp.	NE16	GQ503068	37º8.076 S	175º36.291 E	Thames, CL
Tripyla sp.	NE76	GQ503069	42º 54.441 S	171º33.536 E	Arthurs Pass National Park, NC
Tripyla sp.	NE127	GQ503070	36º51.959 S	174º43.720 E	Western Springs Park, AK
Tripyla sp.	NE128	GQ503071	36º53.093 S	174º49.531 E	Waiatarua Reserve, AK
Tripyla sp.	NE78	GQ503072	43º6.050 S	171º46.574 E	Arthurs Pass National Park, NC
Tobrilus sp.	NE45	GQ503073	36º49.980 S	175º32.840 E	Waiau Falls, CL
Tripylella sp.	NE88	GQ503074	38º34.669 S	174º46.564 E	Rauroa Bush Reserve, WO
Trischistoma sp.	NE106	GQ503075	35º48.610	174º6.074 E	Waiotama, ND
Trischistoma sp.	NE165	GQ503076	37º0.657 S	174º54.491 E	Botanical Garden, AK
Geomonhystera sp.	NE461V	GQ503077	39º56.116 S	175º33.713 E	Bruce Park, Hunterville, RI
Monhystera sp.	NE16	GQ503078	37º8.076 S	175º36.291 E	Thames, CL
Trischistoma sp.	NE73c	GQ503079	42º58.099 S	171º34.722 E	Arthurs Pass National Park, NC
Tripylina tearoha	NNCNZ 2535–2541	FJ480406	37º32.165 S	175º42.911 E	Te Aroha Domain, WO
Tripylina tearoha	NNCNZ 2542–2545	FJ480407	36º48.782 S	174º45.026 E	Smith's Bush, AK
Tripylina manurewa	NNCNZ 2546–2553	FJ480408	37º0.657 S	174º54.491 E	Botanical Garden, AK
Tripylina tamaki	NNCNZ 2546–2553	FJ480409	36º52.354 S	174º50.531 E	St Johns Bush, AK
Tripylina tamaki	NNCNZ 2546–2553	FJ480410	36º52.354 S	174º50.531 E	St Johns Bush, AK

Note: NNCNZ—Nematode National Collection New Zealand; NE—Nematode Extraction.

Discussion

Morphologically, the taxonomy of Tripylidae is still problematic and no firm agreement has been achieved at the generic level based on morphological characters. For example, the latest revision was by Andrássy (2007) and he only partly accepted the revision of Tsalolikhin (1983); Brzeski & Winiszewska-Ślipińska (1993) and Zullini (2006) and listed three subfamilies and five genera in the family Tripylidae. Some taxonomic questions regarding the family are unresolved. For example, *Paratripyla* has not been regarded as a valid genus in the family Trpylidae since 1993 (Brzeski & Winiszewska-Ślipińska 1993; Zullini 2006; Andrássy 2007). However, a SSU sequence of *Paratripyla* (Holterman *et al.* 2006) can be found in the GenBank and our phylogenetic analysis showed that it is closer to the suborder Tobrilina than to Tripylina. In addition, Brzeski & Winiszewska-Ślipińska (1993) removed *Abunema* Khera, 1971 from Tripylidae because it has six lips (instead of three for tripylids) and the shape of the amphids, and this action was accepted by Zullini (2006) and Andrássy (2007). However, *Abunema* was listed as a member of Tripylidae in the framework of Phylum Nematoda (De Ley and Blaxter 2004; De Ley *et al.* 2006).

TABLE 2. Details of the nematode SSU sequences obtained from GenBank.

Nematode Species	Family	Suborder	Order	GenBank Acc. #
Tripyla cf. *filicaudata*				AY 284730
Tripyla cf. *filicaudata*				AY 284731
Tripyla sp.				EF 197734
Tripyla sp.				AY 284732
Tripyla sp.				AY 284733
Tripyla sp.	Tripylidae	Tripylina		AY 284734
Tripylina sp.				EF 197727
Tripylina sp.				EF 197728
Tripylina sp.				EF 197729
Trischistoma sp1				AY 284735
Trischistoma sp2			Triplonchida	AY 284736
T. monohystera				AJ 966509
Paratripyla sp.				AY 284737
Tobrilus gracillis	Tobrilidae	Tobrilina		AJ 966506
Prismatolaimus dolichurus				AY 593957
Trichodorus similis				AJ 439522
T. variopapillatu	Trichodoridae	Diphtherophorina		AY 284841
Paratrichodorus teresparti				AM 269896
P. sminorparti				AM 269897
Ironus longicaudatus				FJ 040495
I. dentifurcatus	Ironidae	Ironina		AJ 966487
Ironus sp.				FJ 040496
Oxystomina sp.				FJ 040498
Oxystomina sp.	Oxystominidae	Ironina		FJ 040499
Halalaimus sp.				
Oncholaimus sp.	Oncholaimidae	Oncholaimina		AY 854196
Oncholaimus sp.				AM 234625
Tripyloides sp.	Tripyloididae	Tripyloidina	Enoplida	AY 854202
Trefusia zostericola	Trefusiidae	Trefusiina		AF 329937
Campydora demonstrans	Campydoridae	Campydorina		AY 552965
Alaimus sp.	Alaimidae	Alaimina		AJ 966514
Paramphidelus hortensis				AY 284739
Enoplus brevis	Enoplidae	Enoplina		EBU 88336
E. meridionalis				Y 16914

Our study is the first extensive molecular phylogenetic study of the family Tripylidae. Our phylogenetic analysis of the New Zealand tripylids and previously published nematode SSU sequences from GenBank indicated (Fig. 1) that 1) the genera *Tripyla* (cuticle annulated, thick; six outer sensillae and four cephalic setae in two well-separated circles; stoma with large dorsal tooth and two subventral denticles; cardiac glands large, composed of three cells; female reproductive system amphidelphic. Male with wide, horn-shaped spicules.), *Tripylina* (cuticle not annulated, thin; body pores numerous; six outer sensillae and four cephalic setae in a single whorl; stoma with large dorsal tooth and two subventral denticles; female reproductive

system prodelphic without postvulval sac; males very rare.) and *Trischistoma* (body much thinner and bent dorsad, mainly in the posterior; cuticle smooth, thin; circles of six outer sensillae and four cephalic setae well separated; buccal denticles minute; without obvious cardiac glands between pharynx and intestine; female genital organ prodelphic, with or without postvulval sac) were well supported as being monophyletic by both morphological and molecular data; 2) the relationships amongst the genera within Tripylidae were not well resolved; 3) the genera *Tripylina*, *Trischistoma* and possibly *Trefusia* belong to a monophyletic group; 4) *Tripylina* and *Trischistoma* are not closely related to *Tripyla*, the type genus of the family Tripylidae; 5) the genus *Tripylina* appears to be monophyletic with respect to *Trischistoma* and to be more closely related to the Enoplida than to the Triplonchida. This finding is consistent with the results of Meldal *et al*. (2007).

To date, only several small-subunit rDNA-based trees have been constructed that covered the entire nematode phylum (Aleshin *et al*. 1998; Blaxter *et al*. 1998; Holterman *et al*. 2006; Meldal *et al*. 2007). Further taxon sampling may yield different relationships among Enoplia lineages. The tripylid nematodes were found to be close to the base of the phylum Nematoda in the embryological and morphological study of Holterman *et al*. (2006). The phylogenetic analysis in our study showed that the Triplonchida is a monophyletic group, and Enoplida is possibly a paraphyletic group (Fig. 1).

In conclusion, nematode molecular phylogenetic studies are still at an early stage in terms of the very limited amounts of DNA sequence data compared with the larger amount of information available from morphological taxonomy. Addition of further taxa or genes to the molecular phylogenetic data set may change the tree topology because currently only relatively limited data are available. This means that a definitive statement about the relationships between Enoplida and Triplonchida, suborders within Enoplida and Triplonchida cannot be made at present. Therefore, more study is needed and as more phylogenetic data becomes available, paraphyletic groups can be exposed, convergent morphological characters can be identified (e.g. cephalic setae, female genital organ) and other characters that are phylogenetically relevant and show true homology will be revealed.

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