

The complete mitochondrial genome of the rodent intra-arterial nematodes *Angiostrongylus cantonensis* and *Angiostrongylus costaricensis*

Shan Lv · Yi Zhang · Ling Zhang · Qin Liu ·
He-Xiang Liu · Ling Hu · Fu-Rong Wei ·
Peter Steinmann · Carlos Graeff-Teixeira ·
Xiao-Nong Zhou · Jürg Utzinger

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Abstract The two rodent intra-arterial nematodes, *Angiostrongylus cantonensis* and *Angiostrongylus costaricensis*, can cause human ill-health. The present study aimed to characterize and compare the mitochondrial (mt) genomes of these two species, and clarify their phylogenetic relationship and the position in the phylum Nematoda. The complete mt genomes of *A. cantonensis* and *A. costaricensis* are 13,497 and 13,585 bp in length, respectively. Hence, they are the smallest in the class of Chromadorea characterized

thus far. Like many nematode species in the class of Chromadorea, they encode 12 proteins, 22 transfer RNAs, and two ribosomal RNAs. All genes are located on the same strand. Nucleotide identity of the two mt genomes is 81.6%, ranging from 77.7% to 87.1% in individual gene pairs. Our mt genome-wide analysis identified three major gene arrangement patterns (II-1, II-2, and II-3) from 48 nematode mt genomes. Both patterns II-1 and II-2 are distinct from pattern II-3, which covers the Spirurida, supporting a closer

S. Lv (✉) · Y. Zhang · L. Zhang · Q. Liu · H.-X. Liu · L. Hu ·
F.-R. Wei · X.-N. Zhou
National Institute of Parasitic Diseases,
Chinese Center for Disease Control and Prevention,
Shanghai 200025, People's Republic of China
e-mail: lvshan000@eyou.com

Y. Zhang
e-mail: zhang1972003@yahoo.com.cn

L. Zhang
e-mail: zhangling2613884@163.com

Q. Liu
e-mail: liuqin0901@sohu.com

H.-X. Liu
e-mail: hxliu17@hotmail.com

L. Hu
e-mail: linghu_99@yahoo.com

F.-R. Wei
e-mail: nettle520@163.com

X.-N. Zhou
e-mail: xiaonongzhou1962@gmail.com

S. Lv · P. Steinmann · J. Utzinger
Department of Epidemiology and Public Health,
Swiss Tropical and Public Health Institute,
CH-4002 Basel, Switzerland

P. Steinmann
e-mail: peter.steinmann@unibas.ch

J. Utzinger
e-mail: Juerg.Utzinger@unibas.ch

S. Lv · P. Steinmann · J. Utzinger
University of Basel,
CH-4003 Basel, Switzerland

L. Zhang
Xinjiang Provincial Center for Disease Control and Prevention,
Urumqi 830001, People's Republic of China

C. Graeff-Teixeira
Laboratório de Biologia Parasitária da Faculdade de Biociências da
PUCRS e Laboratório de Parasitologia Molecular do Instituto de
Pesquisas Biomédicas da PUCRS,
91619-900, Porto Alegre, Brazil

C. Graeff-Teixeira
e-mail: graeff.teixeira@gmail.com

relationship between Ascaridida and Strongylida rather than Spirurida. Thymine (T) was highly concentrated on coding strands in Chromadorea, but balanced between the two strands in Enoplea, probably due to the gene arrangement pattern. Interestingly, the gene arrangement pattern of mt genomes and phylogenetic analysis based on concatenated amino acids indicated a closer relationship between the order Ascaridida and Rhabditida rather than Spirurida as indicated in previous studies. These discrepancies call for new research, reassessing the position of the order of Ascaridida in the phylogenetic tree. Once consolidated, the findings are important for population genetic studies and target identification.

Introduction

Rodent *Angiostrongylus* belong to the superfamily Metastrongyloidea of the phylum Nematoda. They parasitize rodents and are located in the bronchioles (e.g., *Angiostrongylus andersoni*), pulmonary arteries (e.g., *Angiostrongylus cantonensis*, *Angiostrongylus mackerrasae*, and *Angiostrongylus malaysiensis*), or mesenteric arteries (e.g., *Angiostrongylus costaricensis* and *Angiostrongylus siamensis*) (Anderson 2000). As an exception in the bursate group, *Angiostrongylus* spp. require intermediate hosts to complete their life cycle (Anderson 2000). Terrestrial mollusks, such as snails and slugs, normally play this role, but some freshwater snails have been found to be particularly important for the transmission of angiostrongyliasis (Yousif and Lammler 1975; Morley 2006; Lv et al. 2008, 2009a). Most of them are highly specific with regard to their definitive rodent host species and even intermediate mollusk hosts, although cross infections have been observed (Lv et al. 2008). Hence, angiostrongyliasis is locally endemic due to the biogeography of definitive and intermediate host species. With regard to *A. cantonensis*, it is commonly believed that this species originated in Southeast Asia from where it subsequently spread over tropical regions following the biological invasion of their suitable definitive hosts *Rattus norvegicus* and *Rattus rattus* (Kliks and Palumbo 1992; Prociv et al. 2000).

Among the many species of rodent intra-arterial nematodes described thus far, only *A. cantonensis* and *A. costaricensis* are known to cause human ill-health. The former species is implicated in eosinophilic meningitis, whereas the latter can cause granulomatous inflammation of the intestinal wall (Kramer et al. 1998; Wang et al. 2008; Lv et al. 2010). An infection in humans is acquired primarily via consumption of undercooked snails or foodstuff contaminated with the third-stage infective larvae (Lv et al. 2010). Several studies reported that *A. cantonensis* not only infects humans but also wildlife, such as primates, flying foxes, and birds (Barrett et al. 2002; Kim et al. 2002; Duffy et al. 2004; Monks et al. 2005; Gelis et al. 2011). Hence, *A. cantonensis*

is a potential threat to wildlife. Regarding *A. costaricensis*, this species can also parasitize primates (Miller et al. 2006). Interestingly, *A. mackerrasae* (endemic in Australia) and *A. malaysiensis* (endemic in Malaysia) virtually have the same life cycles like *A. cantonensis*, whereas *A. siamensis* in Thailand shares a very similar life cycle with *A. costaricensis*. However, neither *A. mackerrasae*, nor *A. malaysiensis*, nor *A. siamensis* have been reported to be involved in human and wildlife infections. At present, the diagnosis of an *Angiostrongylus* infection in humans and animals is most often based on morphological characteristics of adult worms or larvae (Lv et al. 2009b) and immunological tests (Geiger et al. 1999; Intapan et al. 2003). However, several *Angiostrongylus* species show similar morphology and migration routes in the hosts, and immunological diagnosis demonstrates low specificity. Therefore, differential diagnosis is a challenge. Indeed, there is a need for a more accurate diagnosis and molecular approaches might play a role to readily distinguish closely related species and different isolates.

Genetic markers derived from mitochondrial (mt) DNA hold promise for molecular diagnosis. The rapid mutation rate of mt DNA compared to nuclear DNA renders the former a promising genetic marker to distinguish various clades or species at a lower taxonomic level, which might explain their frequent use in population genetic and diagnostic studies (Blouin et al. 1998; Gissi et al. 2008). An mt genome-wide analysis between different *Angiostrongylus* species is therefore needed not only for the identification of suitable genetic markers for population genetics and diagnosis, but also for clarifying their phylogenetic relationship from a molecular point of view.

The aims of the current study are (1) to characterize the mt genomes of *A. cantonensis* and *A. costaricensis* and (2) to compare these mt genomes with other species of the phylum Nematoda.

Materials and methods

Parasites and DNA extraction

A. cantonensis was obtained from Lijiang, Fujian province in the People's Republic of China. The nematode was maintained in the laboratory of the National Institute of Parasitic Diseases (Shanghai, People's Republic of China). Adult worms were recovered from the pulmonary arteries of an infected Sprague–Dawley rat. *A. costaricensis*, obtained from Santa Rosa, Rio Grande do Sul in Brazil, was maintained in the laboratory of the Instituto de Pesquisas Biomédicas da PUCRS (Porto Alegre, Brazil). Adult worms were recovered from mesenteric arteries of rodents and kept in 70% ethanol.

From both species, a single female worm was used. Specimens were washed several times with physiological

saline. Total genomic DNA was extracted from nematodes using sodium dodecyl-sulfate/proteinase K treatment (Gasser et al. 1993). The individual worm was put into a 2.5-ml tube with 500 μ l extraction buffer and homogenized with a polytron. The tubes were incubated at 37°C overnight. The suspension was then centrifuged for 60 s at 10,000 \times g and the supernatant transferred to a clean tube and extracted with phenol/chloroform/isoamyl alcohol (v/v/v=25:24:1). The aqueous phase was precipitated with a double volume of absolute ethanol and centrifuged for 3 min at 10,000 \times g. The DNA pellet was suspended in 50 μ l H₂O and kept at -20°C.

PCR amplification and sequencing

The primers were designed according to the conversed sequences of currently available mt genomes, i.e., those of *Ancylostoma duodenale* [NC_003415], *Necator americanus* [NC_003416], and *Cooperia oncophora* [NC_004806], which are close relatives of *Angiostrongylus* spp. according to conventional classification. Some of the primers used for *A. costaricensis* were designed based on the sequenced mt genome of *A. cantonensis*. All adjacent fragments overlapped. PCR cycling conditions used were 94°C for 10 min, and then 35 cycles with 94°C for 60 s, 48°C for 90 s, and 72°C for 90 s, followed by 72°C for 10 min for the final extension. Each PCR reaction yielded a single band detected in a 1% agarose gel upon ethidium bromide staining. PCR products were recovered from the gel using Mini-Spin Columns (Axygen). Purified PCR products were ligated into pGEM®-T Easy vectors with the LigaFast ligation system (Promega). The plasmid vector with the target fragment was transformed into JM109 or DH5 α *Escherichia coli*, following the manufacturer's instructions. The positive clones were sequenced with the dideoxynucleotide termination method.

Sequence analyses

The sequences were assembled and edited using the Vector NTI package (version 9.1, Invitrogen). The proteins encoding genes were identified using ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) set for the invertebrate mt genetic codes. The initiation and termination codons were determined by comparison with the corresponding sequences of *A. duodenale*, *N. americanus*, and *C. oncophora*. Two ribosomal RNA (*rnr*) genes were identified by comparison to other nematode mt genomes. Transfer RNA (*trn*) genes in the mt genome of *A. cantonensis* were identified using the tRNA scan program (Lowe and Eddy 1997), whereas two *trnS* genes were recognized by their potential to be folded into *trn*-like secondary structures and by their anticodon sequences. Secondary structures of tRNAs and rRNAs were edited using RNAviz (De Rijk and De Wachter 1997). The stem-loop structures of non-coding mt regions were inferred

using the web Mfold program (Zuker 2003). The adenine-thymine (AT)-rich region was determined using the “Tandem Repeats Finder” program (Benson 1999).

The arrangement of genes in the nematode mt genome was compared among all nematodes whose mt genome sequences have been determined and made publicly available. Adenine plus thymine (A+T) contents were compared among all nematodes whose mt genomes are available.

Phylogenetic analysis

For the phylogenetic analysis, 46 nematode mt genomes available from GenBank were used, in addition to the complete mtDNA sequences of *A. cantonensis* and *A. costaricensis* determined in this study. These mtDNA sequences were: *Agamermis* spp. BH-2006, *Ancylostoma caninum*, *A. duodenale*, *Anisakis simplex*, *Ascaris suum*, *Brugia malayi*, *Bunostomum phlebotomum*, *Caenorhabditis briggsae*, *Caenorhabditis elegans*, *Chabertia ovina*, *Chandlerella quiscalis*, *Contraecum rudolphii*, *C. oncophora*, *Cylicocyclus insignis*, *Dirofilaria immitis*, *Enterobius vermicularis*, *Haemonchus contortus*, *Heterorhabditis bacteriophora*, *Hexameris agrotis*, *Mecistocirrus digitatus*, *Metastrongylus pudendotectus*, *Metastrongylus salmi*, *N. americanus*, *Oesophagostomum dentatum*, *Oesophagostomum quadripinulatum*, *Onchocerca volvulus*, *Pristionchus pacificus*, *Radopholus similis*, *Romanomermis culicivora*, *Romanomermis iyengari*, *Romanomermis nielsenii*, *Setaria digitata*, *Steinernema carpocapsae*, *Strelkovimermis spiculatus*, *Strongylus vulgaris*, *Strongyloides stercoralis*, *Syngamus trachea*, *Teladorsagia circumcincta*, *Thaumamermis cosgrovei*, *Toxocara canis*, *Toxocara cati*, *Toxocara malaysiensis*, *Trichinella spiralis*, *Trichostrongylus axei*, *Trichostrongylus vitrinus*, and *Xiphinema americanum*.

The amino acid sequences encoded by 12 protein coding genes from each species were individually concatenated and subjected to alignment using ClustalX. Conversed blocks were selected for phylogenetic analysis using the G-blocks website service (Castresana 2000). The molecular phylogeny was reconstructed based on Bayesian inference using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). The posterior probabilities were calculated using Metropolis-coupled Markov chain Monte Carlo simulations. The consensus tree was drawn in TreeView version 1.6.6.

Results and discussion

Mitochondrial genome of *A. cantonensis* and *A. costaricensis*

The complete mt genome of *A. cantonensis* (GQ398121) and *A. costaricensis* (GQ398122) were sequenced based on genetic material isolated from single female worms. Both mt

genomes are circular with 13,497 and 13,585 bp, respectively. These two mt genomes are the smallest thus far characterized in the class of Chromadorea. Indeed, the average size of the mt genome of the 39 Chromadorea nematode species for which sequence data are currently available is 14.16 ± 0.55 kb. The respective size is 18.80 ± 3.23 kb for the nine Enoplea nematodes. Unlike *X. americanum*, which lacks several tRNA genes (He et al. 2005), *A. cantonensis* and *A. costaricensis* possess the same mt gene content as other nematodes, except for *T. spiralis*, which has a unique *atp8* gene (Lavrov and Brown 2001). Specifically, the mt genomes of the two *Angiostrongylus* species sequenced here contain 12 protein-coding genes (*atp6*, *cox1-3*, *cytb*, *nad1-6*, and *nad4L*), two ribosomal RNA genes (*rrnS* and *rrnL*), and 22 tRNA genes. Moreover, the mt genomes have the same gene arrangement pattern and all genes are transcribed in the same direction (Fig. 1). There are two major non-coding regions (NCR) in both mt genomes. The shorter NCR is located between *nad4* and *cox1*, while the longer NCR (A+T-rich region or putative control region) is located between tRNA-Ala and tRNA-Pro genes.

The overall identity between the mt genomes of *A. cantonensis* and *A. costaricensis* was 81.6%, with a considerable range (from 77.7% for *nad6* to 87.1% for *rrnS*) observed for protein-coding and rRNA genes (Table 1).

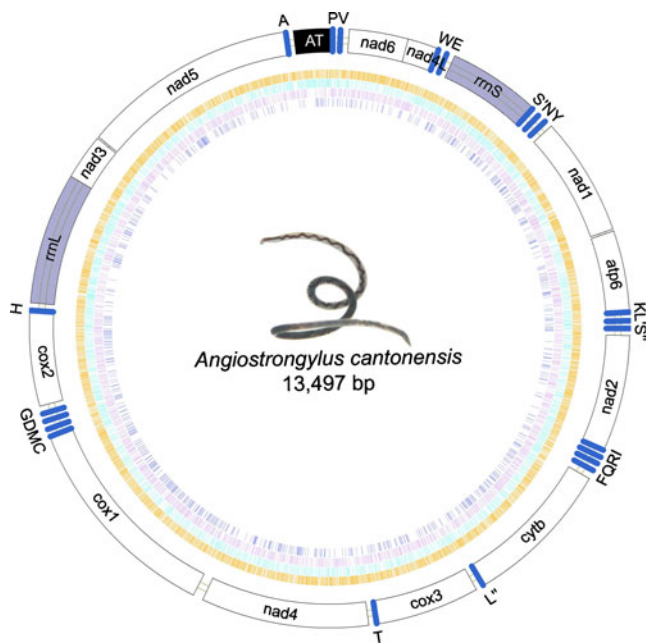


Fig. 1 The consensus structure of the mitochondrial (mt) genome based on that of *A. cantonensis*. Each tRNA gene was indicated by the amino acid code according to the International Union of Pure and Applied Chemistry (IUPAC). The two leucine genes were annotated by L'(UAA) and L''(UAG), and the two serine genes by S'(UGA) and S''(UCU). AT with black background denotes A+T-rich region. All the genes are located on the same strand and transcribed in the same direction (clockwise). Four barcode-like circles indicate different bases and their distribution in the genome (from outer to inner circle: T, A, G, C)

Table 1 Comparison of protein-coding genes and ribosomal RNA genes of *A. cantonensis* and *A. costaricensis*

Gene	<i>A. cantonensis</i>		<i>A. costaricensis</i>		Identity
	Ini/Ter	Length	Ini/Ter	Length	
<i>atp6</i>	ATT/TAG	600	ATT/TAG	600	0.80
<i>cox1</i>	ATT/TAG	1,578	ATT/TAA	1,578	0.86
<i>cox2</i>	TTG/TAG	693	TTG/TAA	693	0.86
<i>cox3</i>	TTG/T	766	TTG/T	766	0.84
<i>cytb</i>	TTG/TAA	1,110	ATG/TAG	1,101	0.81
<i>nad1</i>	TTG/TAG	876	TTG/TAG	873	0.82
<i>nad2</i>	TTG/TAG	849	TTG/TAA	849	0.79
<i>nad3</i>	TTG/TAG	336	TTG/TAG	336	0.80
<i>nad4</i>	TTG/TAG	1,230	TTG/TAA	1,230	0.79
<i>nad4L</i>	ATT/T	232	ATT/T	232	0.84
<i>nad5</i>	ATA/T	1,582	ATA/T	1,582	0.81
<i>nad6</i>	ATG/TAG	426	ATG/TAG	432	0.78
<i>rrnL</i>		961		966	0.82
<i>rrnS</i>		696		695	0.87

Ini and Ter denote initiation and termination codon, respectively

Consistent with previous studies (Hu et al. 2002; Li et al. 2008; Jex et al. 2009), our findings showed a similar identity pattern in protein-coding and rRNA genes. For example, the genes *cox1-3* and *rrnS* showed higher identity among family members, whereas *nad2*, *nad4*, and *nad6* showed higher variation. Hence, the genes *nad2*, *nad4*, and *nad6* could be used as markers in population genetic studies of *A. cantonensis*. However, it should be noted that the comparison of the mt genome of different isolates of the hookworm species *N. americanus* indicated a different pattern at intraspecific level: *nad1* and *nad3* showed higher difference than other protein-coding genes (Hu et al. 2003a, b). It follows that the final candidate marker for population genetics of *A. cantonensis* should ideally be determined based on the comparison of mt genomes of different strains or isolates. Nonetheless, the analysis of the two close *Angiostrongylus* relatives, in our view, provides helpful information for the identification of candidate biomarkers.

The length of each mt gene pair of *A. cantonensis* and *A. costaricensis* was similar; a small difference could be identified in *cytb*, *nad1*, *nad6*, as well as the two rRNA genes. Initiation codons were the same for each gene pair except for *cytb*, for which the initiation codon was TTG for *A. cantonensis* but ATG for *A. costaricensis*. However, termination codon usage showed considerable variation; a difference was found in *cox1*, *cox2*, *nad2*, *nad4*, and *cytb*. For the first four genes, *A. cantonensis* employed TAG as termination codon, while *A. costaricensis* utilized TAA. A reversed termination codon usage occurred in the *cytb* gene. As has been observed for other nematodes (Hu et al. 2003a, b; Li et al. 2008), *A. cantonensis* and *A. costaricensis*

utilized truncated termination codons, like those observed in *cox3*, *nad4L*, and *nad5*.

A considerable bias of codon usage (frequency and relative synonymous codon usage) in protein-coding genes in the mt genome of *A. cantonensis* and *A. costaricensis* was identified (Fig. 2). Overall, UUU (Phe), UUG (Leu), UUA (Leu), GUU (Val), UAU (Tyr), and AUU (Ile) are dominant

codons, which is similar to other nematode species (Lavrov and Brown 2001; Hu et al. 2002, 2003a, b; Montiel et al. 2006; Kang et al. 2009). However, the proportion of these codons showed a marked variation in different genes. For example, UUU (Phe) exceeded 20% in *nad4L* in both mt genomes, but was below 10% in *cox1* and *cox2*. Pairwise comparison of specific genes between these two genomes

Codon	Amino acid	average		<i>atp6</i>		<i>cox1</i>		<i>cox2</i>		<i>cox3</i>		<i>cytb</i>		<i>nad1</i>		<i>nad2</i>		<i>nad3</i>		<i>nad4</i>		<i>nad4L</i>		<i>nad5</i>		<i>nad6</i>	
		a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
UUU	Phe	13.8	13.6	12.5	13.0	9.1	9.1	8.7	7.8	14.1	14.5	13.0	12.8	14.7	14.8	16.6	16.7	17.9	16.1	13.7	13.9	22.1	20.8	15.7	15.4	20.4	16.0
UUG	Leu	10.1	8.3	8.0	6.5	9.1	7.4	10.0	7.8	9.4	4.7	9.5	8.4	13.0	11.7	11.7	11.0	13.4	14.3	8.3	6.3	5.2	9.1	12.0	8.3	9.9	9.0
UUA	Leu	6.6	7.6	8.5	7.5	5.3	5.9	4.3	6.1	8.2	10.6	6.8	6.8	5.8	7.2	8.8	7.4	5.4	7.1	8.3	8.8	3.9	2.6	6.1	9.9	6.3	6.9
GUU	Val	5.8	4.4	7.5	4.0	5.3	4.8	3.5	3.9	4.7	3.5	7.9	6.0	4.5	4.8	6.0	7.1	8.9	6.3	6.1	2.4	10.4	6.5	4.7	3.0	7.0	4.2
UAU	Tyr	5.6	5.5	7.0	6.5	5.7	5.5	5.7	6.9	5.9	5.1	5.4	6.3	6.5	5.5	4.6	3.5	1.8	2.7	6.6	6.1	2.6	2.6	4.9	4.7	7.7	8.3
AUU	Ile	5.3	5.7	5.5	8.0	5.3	6.3	7.0	7.8	3.9	5.1	4.6	5.4	5.5	3.8	6.7	5.7	5.4	8.0	5.1	5.6	9.1	11.7	4.9	3.6	3.5	4.9
AUG	Met	4.1	3.3	5.5	3.5	3.2	3.2	2.2	2.2	1.2	0.0	2.7	3.0	5.5	3.1	4.9	4.2	5.4	2.7	4.4	4.6	9.1	6.5	5.1	3.4	4.9	4.9
GGU	Gly	3.8	3.5	4.0	1.5	5.9	4.6	3.9	3.9	3.9	3.9	3.8	4.4	2.7	4.1	3.9	2.8	2.7	4.5	2.4	2.7	3.9	2.6	3.8	2.8	2.1	2.8
AAU	Asn	3.2	2.7	3.0	1.5	3.2	3.0	5.2	4.3	3.1	2.7	3.3	3.0	0.7	0.7	3.2	3.9	3.6	0.9	2.9	2.7	1.3	1.3	4.4	3.2	2.8	2.1
UCU	Ser	3.1	3.1	3.5	4.5	3.6	3.4	3.0	3.0	3.5	3.1	1.9	1.6	4.5	3.8	1.8	1.4	2.7	4.5	3.9	3.9	2.6	2.6	2.5	2.5	3.5	4.9
AGU	Ser	3.0	3.3	2.0	4.0	3.4	3.2	2.2	2.2	2.7	2.7	3.5	2.7	2.4	2.7	1.8	3.2	2.7	3.6	2.7	1.5	1.3	3.9	4.0	5.1	5.6	6.3
AUA	Met	2.5	3.3	2.0	1.5	2.1	2.3	1.7	1.7	0.8	2.4	2.7	4.4	1.4	2.4	3.5	4.9	1.8	2.7	4.6	4.1	0.0	2.6	3.2	4.4	0.7	2.8
AGA	Ser	2.2	2.1	1.0	2.5	1.9	1.1	2.2	2.2	3.5	2.7	2.2	2.2	2.4	2.7	2.5	1.4	1.8	1.8	2.2	1.7	2.6	2.6	2.7	3.0	1.4	1.4
GUG	Val	2.0	2.1	2.5	2.0	2.1	1.7	0.9	0.9	2.4	3.1	1.6	2.2	1.7	2.4	2.5	3.2	3.6	0.9	2.0	3.2	3.9	0.0	1.5	1.1	3.5	2.8
GAU	Asp	1.9	1.9	1.5	1.0	2.7	2.7	4.8	3.5	2.0	2.0	1.4	2.2	1.4	1.4	0.7	0.4	1.8	2.7	0.7	1.2	1.3	1.3	2.3	2.3	0.7	2.1
GCU	Ala	1.8	1.7	1.5	1.0	2.7	3.0	2.2	1.7	2.4	1.2	2.4	2.2	2.1	2.7	0.4	0.0	0.0	0.9	2.0	2.2	0.0	0.0	1.7	1.3	0.7	0.7
AAG	Lys	1.8	1.5	0.5	2.0	1.7	0.8	2.6	2.2	1.6	0.8	2.2	1.4	2.1	1.4	3.5	3.2	1.8	2.7	1.0	1.2	3.9	2.6	1.3	0.9	0.0	1.4
ACU	Thr	1.7	1.7	2.5	2.5	3.4	3.2	0.9	1.7	2.4	2.4	1.6	1.6	0.7	0.7	0.7	1.1	0.9	0.9	1.0	0.2	0.0	0.0	1.7	2.1	1.4	1.4
CCU	Pro	1.6	1.4	0.5	0.0	3.2	2.5	2.2	2.6	1.2	1.2	1.9	2.2	1.4	2.1	1.8	1.1	0.0	0.0	1.7	1.0	0.0	0.0	0.9	0.9	0.7	0.7
CAU	His	1.5	1.4	0.5	0.5	2.9	2.7	1.7	1.7	4.3	4.3	1.9	1.9	0.3	0.3	0.7	0.7	0.9	0.9	1.0	1.0	1.3	1.3	0.8	0.6	0.0	0.0
GGG	Gly	1.4	1.8	0.5	3.0	2.1	3.0	1.3	3.0	2.7	2.4	0.3	0.8	1.0	0.7	0.4	1.8	1.8	0.9	2.0	1.5	2.6	2.6	0.8	0.9	2.8	2.1
GUA	Val	1.3	2.4	1.5	4.5	0.2	1.1	0.9	1.3	2.4	2.4	1.6	1.4	1.0	3.8	2.5	2.5	2.7	4.5	1.0	2.2	1.3	3.9	1.7	2.5	0.7	3.5
UGU	Cys	1.3	1.3	2.0	2.0	1.1	1.3	3.5	3.9	1.6	1.2	0.8	1.1	0.3	0.7	0.4	0.4	0.9	0.0	1.2	1.7	1.3	1.3	1.5	1.1	2.1	1.4
AGG	Ser	1.3	1.5	1.0	2.0	1.1	2.1	1.3	1.3	0.8	1.6	1.1	1.6	1.4	1.0	1.1	0.4	0.9	0.9	2.0	2.2	2.6	3.9	1.5	1.3	1.4	0.7
GAG	Glu	1.2	1.4	3.0	3.0	0.8	1.1	2.6	3.9	1.6	1.2	0.8	0.5	2.1	3.1	0.7	0.7	1.8	0.9	1.2	1.2	1.3	1.3	0.4	0.8	0.7	0.0
GAA	Glu	1.2	0.8	1.0	1.5	0.8	0.4	2.2	1.7	0.8	1.2	0.5	0.8	2.4	1.0	1.4	0.7	1.8	2.7	0.7	0.5	1.3	0.0	1.3	0.6	1.4	0.7
AAA	Lys	1.1	1.4	2.0	0.5	0.6	1.5	0.0	0.4	0.4	1.2	1.1	1.4	1.0	1.7	1.4	1.4	1.8	0.9	1.7	1.7	0.0	1.3	1.5	2.3	0.7	0.0
UGA	Trp	1.0	0.9	0.5	1.0	1.5	0.8	1.3	0.4	1.6	2.0	2.2	1.1	0.7	0.0	1.1	1.1	0.9	0.0	0.7	0.7	0.0	1.3	0.0	0.9	1.4	1.4
GGA	Gly	1.0	0.8	1.5	0.0	0.8	1.0	2.6	0.9	1.6	2.0	1.1	0.5	1.7	0.0	0.0	0.4	0.0	0.9	0.5	1.0	0.0	0.0	0.8	1.3	0.7	0.0
UGG	Trp	0.9	1.1	0.5	0.0	1.0	1.7	0.9	1.7	1.6	1.2	1.4	2.2	0.0	0.7	1.4	1.4	0.9	1.8	0.5	0.5	1.3	0.0	1.1	0.6	0.0	0.7
CAA	Gln	0.6	0.4	1.0	0.0	0.6	0.6	1.7	0.9	0.0	0.0	0.5	0.3	0.7	0.7	0.7	0.4	0.0	0.0	0.5	0.2	0.0	0.0	0.8	0.8	0.0	0.0
CGU	Arg	0.6	0.7	1.5	1.0	1.3	1.3	1.3	0.9	0.4	0.4	0.0	0.3	1.4	1.0	0.0	0.0	0.0	0.0	0.0	0.5	1.3	1.3	0.6	0.6	0.0	0.7
CAG	Gln	0.6	0.8	0.5	1.5	0.6	0.6	0.9	1.7	0.4	0.4	1.1	1.1	1.0	1.0	0.4	0.7	0.9	0.9	0.0	0.5	0.0	0.0	0.6	0.6	0.0	0.0
UCA	Ser	0.5	0.6	1.0	1.0	0.8	0.6	0.4	1.3	0.8	0.4	0.3	0.5	0.0	0.0	0.4	0.0	0.0	0.0	1.2	1.0	0.0	1.3	0.2	0.2	0.7	0.7
UCG	Ser	0.5	0.5	0.0	0.0	0.6	0.6	0.0	0.0	0.0	0.0	0.5	0.3	0.3	1.0	0.0	0.4	0.9	0.0	1.0	0.7	2.6	0.0	0.4	0.8	0.7	0.0
ACG	Thr	0.4	0.3	0.5	0.5	1.1	0.6	0.4	0.0	0.0	0.0	0.5	0.0	0.0	0.3	0.0	0.4	0.0	0.0	0.2	0.5	0.0	0.0	0.6	0.2	0.0	0.0
CUU	Leu	0.4	0.4	0.0	0.0	0.4	0.6	1.3	0.4	0.4	0.0	0.0	0.3	0.0	0.3	0.4	0.0	0.0	0.0	0.5	0.7	0.0	0.0	0.2	0.6	1.4	0.7
CCA	Pro	0.4	0.4	0.0	0.0	0.2	0.8	0.4	0.0	0.4	0.4	0.5	0.0	1.0	0.3	0.0	0.4	0.0	0.0	0.2	0.7	0.0	0.0	0.4	0.2	0.7	0.7
ACA	Thr	0.3	0.5	0.5	1.0	0.2	0.4	0.9	0.4	0.0	0.0	0.3	1.1	0.0	0.0	0.4	0.0	0.0	0.0	0.5	1.0	0.0	0.0	0.6	0.8	0.0	0.0
GCG	Ala	0.3	0.4	0.0	0.5	0.6	0.6	0.0	0.0	0.0	0.4	0.8	0.8	0.7	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.6	0.0	0.0
CUG	Leu	0.2	0.3	0.5	0.0	0.2	0.2	0.0	0.0	0.0	0.0	1.6	0.5	0.3	0.3	0.0	0.4	0.9	0.9	0.0	0.2	0.0	1.3	0.2	0.0	0.0	0.0
CUA	Leu	0.2	0.2	0.0	0.0	0.0	0.2	0.4	0.0	0.4	0.4	0.5	0.3	0.3	0.0	0.4	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.4	0.0	0.0
AUC	Ile	0.2	0.1	0.0	0.5	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.3	1.0	0.0	0.0	0.0	0.0	0.0	0.2	0.5	0.0	0.0	0.0	0.2	0.0	0.0
CCG	Pro	0.2	0.3	0.0	0.5	0.4	0.6	0.9	0.4	0.0	0.0	0.0	0.8	0.3	0.0	0.0	0.4	0.0	0.0	0.2	0.5	0.0	0.0	0.0	0.0	0.0	0.0
CGA	Arg	0.2	0.1	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.3	0.5	0.7	1.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.7	0.0
CGG	Arg	0.1	0.1	0.0	0.5	0.2	0.0	0.0	0.4	0.0	0.0	0.5	0.0	0.3	0.3	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
UUC	Phe	0.1	0.3	0.0	1.0	0.0	0.2	0.0	0.0	0.4	0.8	0.0	0.3	0.0	0.0	0.4	0.4	0.0	0.0	0.5	0.2	0.0	0.0	0.0	0.2	0.0	0.0
GUC	Val	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.3	0.0	0.0	0.3	0.0	0.4	0.0	0.0	0.2	0.2	0.0	0.0	0.0	0.4	0.0	0.7
GCC	Ala	0.1	0.2	0.5	0.0	0.2	0.0	0.0	0.0	0.0	0.4	0.0	0.3	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.2	0.4	0.0	0.0
GCA	Ala	0.1	0.4	0.0	0.0	0.2	0.4	0.0	0.0	0.0	0.8	0.0	0.3	0.0	0.0	0.4	0.0	0.0	0.0	0.7	1.2	0.0	0.0	0.0	0.4	0.0	0.0
UAC	Tyr	0.1	0.3	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.8	0.5	0.0	0.0	0.7	0.0	0.0	0.0									

showed a similar codon usage, whereas distinctive differences were observed in some gene pairs. For instance, *A. cantonensis* uses the codon GUU to encode valine, whereas *A. costaricensis* uses codons GUU and GUA, at the same proportion to encode valine.

Twenty and 18 tRNA genes were identified by the scan program in the mt genome of *A. cantonensis* and *A. costaricensis*, respectively (Table 2). The tRNA-Arg genes as well as two tRNA-Ser genes in both mt genomes failed to be identified. For *A. costaricensis* one more tRNA gene (tRNA-Val) was identified by eye. Remarkably, one additional tRNA-Ile gene was found in the AT-rich region in the mt genome of *A. cantonensis* by the tRNA scan program. However, the tRNA lacks a typical secondary structure, the anticodon loop, i.e., one more base located in the loop. Furthermore, it possesses a high proportion of A+T (57/60). This pseudogene was similar to the tRNA-Ile identified in *M. pudendotectus* (Jex et al. 2010).

Table 2 tRNA anticodon and length of *A. cantonensis* and *A. costaricensis*

tRNA	<i>A. cantonensis</i>		<i>A. costaricensis</i>	
	Anticodon	Length	Anticodon	Length
Pro	UGG	55	UGG	54
Val	UAC	54	UAC ^a	54
Trp	UCA	57	UCA	59
Glu	UUC	55	UUC	59
Ser	UGA ^a	53	UGA ^a	52
Asn	GUU	56	GUU	60
Tyr	GUA	60	GUA	55
Lys	UUU	61	UUU	60
Leu	UAA	56	UAA	56
Ser	UCU ^a	50	UCU ^a	52
Ile	GAU	57	GAU	54
Arg	ACG ^a	54	ACG ^a	51
Gln	UUG	56	UUG	55
Phe	GAA	56	GAA	57
Leu	UAG	56	UAG	56
Thr	UGU	58	UGU	58
Cys	GCA	57	GCA	56
Met	CAU	57	CAU	59
Asp	GUC	53	GUC	54
Gly	UCC	57	UCC	58
His	GUG	55	GUG	56
Ala	UGC	55	UGC	55
Ile	GAU ^b	60	–	–

^a tRNAs failed to be identified by tRNAscan-SE program; identified according to other complete mt genomes

^b tRNA identified by tRNAscan-SE program, but lacked typical structure of anticodon loop

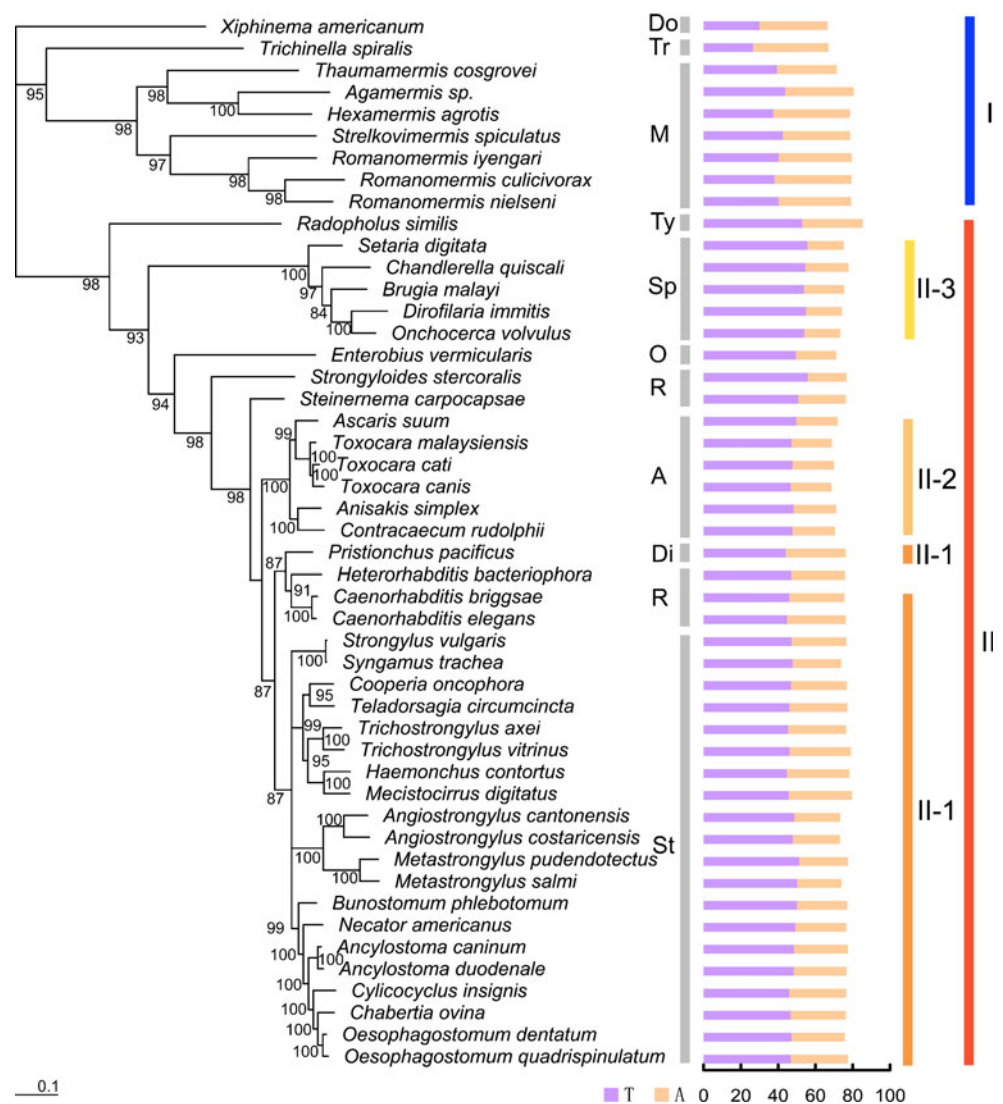
Comparison with other nematode mt genomes

Analysis of a suite of 48 nematode mt genomes revealed a consistently high A+T content, yet variation between species was found to be considerable (Fig. 3). *R. similis*, commonly known as banana root nematode, possesses the highest A+T content (85.4%). On the other band of the spectrum is *X. americanum* (American dagger nematode), which shows the lowest A+T content (66.5%). The variation in A+T content was observed across orders; it ranges from 73.2% (*A. costaricensis*) to 79.7% (*M. digitatus*) in the order Strongylida, from 75.6% (*C. briggsae*) to 76.7% (*S. stercoralis*) in the order Rhabditida, from 68.6% (*T. canis*) to 72.0% (*A. suum*) in the order Ascaridida, from 73.3% (*O. volvulus*) to 77.7% (*C. quisquali*) in the order Spirurida, and from 71.4% (*T. cosgrovei*) to 80.5% (*Agamermis* spp.) in the order Mermithida. The single member *E. vermicularis* (pinworm) in the order Oxyurida and *T. spiralis* in the order Trichocephalida have relatively lower A+T contents, i.e., 71.2% and 67.0%, respectively. Indeed, a strong mutational bias towards A and T has been observed in nematode mt genes (Blouin et al. 1998).

In contrast to the positive skewing in A+T content in each nematode order, heterogeneity was observed in the ratio of A/T. Nematodes in the class Chromadorea, including the orders Strongylida, Diplogasterida, Ascaridida, Rhabditida, Oxyurida, Spirurida, and Tylenchida, without exception, had higher T than A. The percentage (T/(A+T)) ranges from 57.2% (*H. contortus*) to 74.2% (*S. digitata*) with a median of 64.7%. The highest percentages were found in the orders Ascaridida and Spirurida. Indeed, previous studies showed that substitution tended to be T in nematode mt genomes (Blouin et al. 1998; Nadler and Hudspeth 2000). However, in the class Enoplea, consisting of the orders Dorylaimida, Trichocephalida, and Mermithida, the percentage approaches 50% and ranges between 39.6% (*T. spiralis*) and 55.1% (*T. cosgrovei*) with a median of 50.6%. This phenomenon could be explained by the transverse translocation of genes between two DNA strands in Enoplea, which might balance the proportion of A/T on both strands.

The 39 nematode species belonging to the Chromadorea possess a compact mt genome containing 36 genes without repeats. The genes were located on a single strand with the same transcriptional direction. With a few exceptions, e.g., *H. bacteriophora* and *R. similis*, Chromadorea nematodes rarely have long repeated or NCRs except the AT-rich regions. In contrast to the constant gene content, the arrangement of genes showed a variation across orders. Three distinct patterns of gene arrangement (including major non-coding locality) were identified in these nematode mt genomes (Fig. 3; II-1, II-2, and II-3). All nematodes in the order Strongylida fell into group II-1 and shared the same

Fig. 3 Phylogenetic tree of nematode mitochondrial (mt) amino acid sequences based on Bayesian inference. The *gray bars with capital letter* indicate different order of nematode (*Do* Dorylaimida, *Tr* Trichocephalida, *M* Mermithida, *Ty* Tylenchida, *Sp* Spirurida, *O* Oxyurida, *R* Rhabditida, *A* Ascaridida, *Di* Diplogasterida, *St* Strongylida). The *transverse bars* denote the proportion of adenine (*A*) and thymine (*T*) in each nematode mt genome. The *vertical color bars* indicate the arrangement of mt genes (I, gene located on both light and heavy strands; II, gene located on heavy strands; II-1, II-2, II-3 indicate the group in which the members share a similar gene arrangement, respectively). The posterior probability (as a percentage) is indicated on branch lines



gene arrangement with the exception of *M. pudendotectus* in which the tRNA-Ile gene moved close to the AT-rich region. Interestingly, in our study one more tRNA-Ile gene was identified in the AT-rich region by the tRNA scan program. It is similar to the tRNA-Ile of *M. pudendotectus*. However, it can be excluded from the tRNA due to its inconsistency with the typical structure of the anticodon loop. Nevertheless, this finding indicates a high similarity between gene arrangement patterns among these members of the superfamily Metastrongyloidea and should be assigned to group II-1.

Additionally, two species (*C. briggsae* and *C. elegans*) in the order Rhabditida and the single species (*P. pacificus*) in the order Diplogasterida also share the pattern II-1. In contrast, three other members in the order Rhabditida, i.e., *S. carpocapsae*, *H. bacteriophora*, and *S. stercoralis*, show distinct arrangement patterns. The gene arrangement of *S. carpocapsae* was a mediate between II-1 and II-2; namely, a location change of the AT-rich region and a tRNA-Asn

gene, which might result in either II-1 or II-2. A few short linkages of genes in pattern II-1 (e.g., fragments between *cox2* and *nad3*, between tRNA-Gln and *nad4*, between tRNA-Val and tRNA-Arg) could be detected in the mt genome of *H. bacteriophora*, while similar gene linkages have not been observed in *S. stercoralis*. In the order Ascaridida, all mt genomes showed the same arrangement pattern (II-2). The only difference between II-2 and II-1 were in the location of the AT-rich region. Unlike patterns II-2, II-3 was distinctively different from pattern II-1; only a few short gene linkages (two to five genes) could be detected in both II-1 and II-3. Within the group II-3, the gene arrangement of *O. volvulus* and *C. quisquali* was slightly different from the other three species in the location of the five adjacent tRNA genes.

In contrast, the genes of the mt genomes belonging to the class Enoplea were allocated to both strands. Among the seven members of Mermithida duplication or repeats of genes were common. Interestingly, no duplication or repeats

were observed in the other two orders (Dorylaimida and Trichocephalida). Instead, *T. spiralis* (Trichocephalida) showed a unique *atp8* gene (Lavrov and Brown 2001), whereas *X. americanum* (Dorylaimida) lacked the tRNA-Asn, tRNA-Cyr, and one of two tRNA-Ser genes when compared to the other nematode species (He et al. 2005). Unlike species belonging to Chromadorea, all Enoplea nematodes, even within a family, had distinct gene arrangement patterns and lacked detectable similarity.

Phylogenetic analysis

Figure 3 shows a phylogenetic tree, which was constructed based on the concatenated amino acid sequences consisting of 2,266 amino acids according to G-block, which effectively distinguished the orders from each other with the exception of *S. carpocapsae* and *S. stercoralis* that conventionally have been classified as belonging to the order Rhabditida, but were far away from other members. Indeed, the phylogeny of Rhabditida is the most complex in the phylum Nematoda. It might be paraphyletic, as indicated by previous studies (Blaxter et al. 1998). In addition to the phylogenetic analysis based on the amino acid sequence, gene arrangement patterns further support this hypothesis. *H. bacteriophora* was placed in the same clade with *C. elegans* and *C. briggsae*, but has a different gene arrangement pattern. In contrast, *P. pacificus*, which is a member of the order Diplogasterida, showed the same pattern as *C. elegans* and *C. briggsae*, although there was a suppressor tRNA located in the D-loop (Molnar et al. 2011). Indeed, our phylogenetic analysis indicated that *P. pacificus* is genetically close to the order Rhabditida. The inconsistent findings from the phylogenetic analysis and gene arrangement patterns highlight that there is a need for further studies pertaining to *H. bacteriophora*. The trophic niche (*H. bacteriophora* is entomopathogen and *Caenorhabditis* spp. is bacteriovore) should be considered when further pursuing this scientific inquiry.

Previous studies based on the nuclear small subunit ribosomal DNA (SSU) sequence indicated a close relationship between Ascaridida and Spirurida (Blaxter et al. 1998; Meldal et al. 2007). However, findings from the present study along with results from several recent investigations (Kim et al. 2006; Jex et al. 2009; Kang et al. 2009) pertaining to nematode mt genome analysis indicate that Ascaridida have a closer relationship with Rhabditida instead. Furthermore, gene arrangement patterns were more similar between Ascaridida and Rhabditida rather than Spirurida. We also employed the method of maximum parsimony used in previous studies to restructure the phylogeny (data not shown) but failed to significantly change the topology based on Bayesian inference. We also note that some studies indeed implied a potential conflict in phylogeny based on nuclear and mitochondrial DNA (Shaw 2002), although most studies had shown a similar

phylogenetic relationship. Nevertheless, few conflicts were noted at higher taxonomic level. Therefore, the position of the order Ascaridida in the phylogenetic tree should be reappraised.

Conclusions

The complete mt genomes of the two rodent intra-arterial nematodes that can cause human (and wildlife) ill-health, *A. cantonensis* and *A. costaricensis*, represent the smallest mt genomes characterized thus far in the class of Chromadorea. The gene content of these two mt genomes, however, is consistent with that of other species in this class. An mt genome-wide comparison revealed that the mt genomes of *Angiostrongylus* showed considerable variation in different genes, which might provide a basis for identifying markers for population genetic studies and targets for development of novel diagnostic assays. The comparison between 48 mt genomes of different nematode species showed different A+T contents and gene arrangements, which, along with phylogenetic analysis using concatenated amino acids, support a closer relationship between Ascaridida and Rhabditida rather than Spirurida, as suggested by previous studies using nuclear genes. This apparent inconsistency calls for a reappraisal pertaining to the phylogenetic relationship of the order Ascaridida.

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