Research article

Toxocara canis (Ascaridida: Nematoda): Mitochondrial gene content, arrangement and composition compared with other *Toxocara* species

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Abstract

Introduction: The complete mitochondrial (mt) genomes of nematodes sequenced thus far are circular and small in length, containing 37 genes, including 12–13 protein-coding genes: namely, *cytochrome oxidase subunits* 1–3 (*cox*1–3), *nicotinamide adenine dinucleotide dehydrogenase subunits* 1–6 and 4L (*nad*1–6 and *nad*4L), *ATP synthase subunit* 6 and 8 (*atp*6 and 8), *cytochrome* b (*cyt*b), small subunit ribosomal RNA gene (*rrn*S), large subunit ribosomal RNA. The aims of the present study are to determine the gene order, composition, codon usage pattern, and translation initiation/termination codons of the mt genome of *Toxocara cani*s found in Sri Lanka and to compare those findings with other *Toxocara* species.

Methodology: *Toxocara canis* worms used in the present study were obtained from Sri Lanka. The total genomic DNA was extracted using a part of a single worm using an Easy-DNATM Kit. PCR reactions were performed as described previously by Wickramasinghe et al., (2009). Nucleotide sequences were determined with an ABI PRISM 3100 Avant DNA sequencer using a Big Dye Terminator v3.1 Cycle Sequencing Kit. The open-reading frames and codon usage profiles of protein-coding genes were analyzed using the program DNAsis and GENETYXMAC (ver. 6.0).

Results: The partial mt genome of *T. canis* is 9611 bp in length. This partial mt genome contains 9 protein-coding genes (*cox*1-3, *nad*2-5, *atp*6 and *cyt*b), 14 transfer RNA (tRNA) genes and one ribosomal RNA gene. All genes are transcribed in the same direction as found in other members of the genus *Toxocara.* The nucleotide compositions of the obtained partial mtDNA sequences for *T. canis* is biased toward A and T, with T being the most favored nucleotide and C the least favored, in accordance with mt genomes of other nematodes. The content of A+T is 67.48% for *T. canis* (19.9% A, 47.5% T, 23.3% G and 9.3% C). The start codons we inferred in *T. canis* were ATT, GTG, ATA, GTT, TTG and ATG. Stop codons were TAG, TA and T. Six of the 8 protein genes used TAG as a translation termination codon and the other two genes (*atp6 and nad2*) used TA and T respectively. The length of 14 tRNAs is ranging from 52 bp to 68 bp and 8

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more tRNAs remain to be sequenced and identified. The *rrnL* gene of *T. canis* was identified by sequence comparison with other *Toxocara* species. The *rrnL* is located between tRNA-His and *nad*3. The *rrnL* gene is 958 bp in length. A non-coding region is located between *nad4* and *cox1*. The length of this region is 112 bp in *T. canis.*

Conclusion: The present study determined the partial mt genome sequence of *T. canis* obtained from a dog in Sri Lanka. This study provides molecular markers for studying the systematics, population genetics and ecology of the nematodes of socioeconomic importance. Furthermore, we observed a significant difference in nucleotide length of the mitochondrial protein-coding genes, tRNAs, *rrnL* and non-coding regions of *T. canis* from three different geographical locations, Sri Lanka, China and Australia. These variations should be further investigated in future studies.

Note: Nucleotide data reported in this paper is available in the GenBank™, EMBL and DDBJ databases under the accession number **JN593098**.

Key words: Toxocara canis, mitochondrial genome, tRNA, ribosomal RNA, protein-coding genes, gene arrangement

Introduction

Mitochondria are sub-cellular organelles involved in most of the energy production of the cell. In addition, they play other important roles such as iron homeostasis, intermediary metabolism and apoptosis.[1] The proto-mitochondrion entered the primitive eukaryotic cell between two and three billion years ago. Initially, the bacterial genome encoded all the genes necessary for a free-living organism. However, as the symbiosis matured, many bacterial genes were transferred to extra bacterial plasmids (chromosomes), such that today, the maternally inherited mitochondrial DNA (mtDNA) retains only the genes for the 12S and 16S rRNAs and the 22 tRNAs required for mitochondrial protein synthesis plus 12-13 polypeptides of the mitochondrial energy generating process, oxidative phosphorylation. The remaining ~1500 genes of the mitochondrial genome are now scattered throughout the chromosomal DNA.[2]

Within these organelles, most metazoan species possess a compact, circular mitochondrial genome (mt genome), which varies in size from 14 to 20 kb.[1,3] The nematode mt genome contains 12–13 protein-coding genes, 22 transfer RNAs, and small subunit and large subunit ribosomal RNAs.[1,4] All mitochondrial genes sequenced thus far in *Toxocara* species are transcribed in the same direction and are rich in A and T with no introns within genes. Furthermore, mt genome sequences can be used as powerful molecular markers to discriminate not only *Toxocara* species but also other nematodes.[5-7]

Toxocara canis is the most common round worm found in dogs (especially in puppies). Toxocariasis is an important zoonotic disease caused by accidental ingestion of embryonated eggs of *T. canis* and *T. cati*. There are several clinical manifestations in humans such as ocular larva migrans (OLM), visceral larva migrans (VLM), and spinal or neurological forms of disease.[8-13]

The objectives of the present study were to determine the structure, organization and sequence of

the mt genome of *T. canis* of medical and veterinary significance. Furthermore, as mt genome sequences provide useful molecular markers to discriminate closely related species, genomics data obtained in this study can be used in comparative genomics and phylogeographical research.

Materials and methods

Parasite and DNA extraction

Toxocara canis worms used in the present study were obtained from Sri Lanka. Adult worms were fixed in 70% (v/v) ethanol and stored at -20°C until use. The total genomic DNA was extracted using a part of a single worm using an Easy-DNA™ Kit (Invitrogen Corporation, Carlsbad, CA). Briefly, a piece of worm was vacuum dried to evaporate ethanol, then the sample was incubated with TE buffer (320 µl), Solution A (20 µl), Solution B (10 µl) and proteinase K (5 mg/ml) at 60°C overnight. The next day, Solution A (300 µl), Solution B (120 µl) and chloroform (750 µl) were added. DNA in the final aqueous solution was precipitated using ethanol (100%). Finally, the DNA pellet was re-suspended in TE buffer (49µl) and 1 µl of 2mg/ml Rnase was added. The specific identity of *T. canis* DNA was verified from other *Toxocara* species using the sequence of the nuclear ribosomal internal transcribed spacers 2 (ITS-2) and small subunit ribosomal RNA (*rrnS*).[14]

PCR amplification

Primers used to sequence the mt genome of *T. canis* are the same as used for the amplification of *T. vitulorum* mt genome,[15] names and primer sequences of which are given in Table 1.

All the polymerase chain reactions (PCRs) were carried out in a final reaction volume of 25 µl. Amplifications were performed with 1µl of genomic DNA extract using 1 U Ex *Taq* DNA Polymerase (TaKaRa, Japan), 10X Ex *Taq* buffer, 0.2 mM each dNTPs and 10 pm of each primer. PCR conditions used were 3 min denaturation at 94°C; 30-35 cycles of 30 s at 94°C; 30 s at 50°C and 1 min at 72°C; followed by a final 5 min polymerization at 72°C. All the PCR reactions were carried out in a MyCycler™ Thermal cycler (BioRad, USA). Amplicons were detected on 1% agarose gels stained with ethidium-bromide. Amplified PCR products were purified in agarose gel using GENECLEAN II Kit.

Table 1
Primers used in this study to obtain 9611 bp mitochondrial sequence of *T. canis*

Primer name	Primer sequence (5'-3')
NemamitoND1F	TTAGATTGTAAATCTAAAGA
NemamitoND1R	TAAAATAACCHCCAAAACAACA
NemaATPase6F	TWYCCWCGTTWTCGTTATGA
NemaATPase6R	CTTAAAACAAATRCAYTTMT
NemamitoND2F	TTCAAAGTTATATTTTYTC
NemamitoND2R	GAACTTTRCCTTATCAAGA
NemamitoCytbF	ATTATRTCGTCTTGATAAGG
NemamitoCytbR	TTATTWGGAATAGCACGCAAAAT
NemamitoCOIIIF	GGTGATTATGATAARGTTTG
NemamitoCOIIIR	ATGATGATTATAATTAAAATG
NemamitoND4F	TTATTTATCTACNGGTTTTCA
NemamitoND4R	AGAHTCATGATAAAATTCMCC
NemamitoCOIF	GCTTATTCTTCWRTTACTCATA
NemamitoCOIR	ATTACGATCAGTTAACAACAT
NemamitoCOIIF	AATTTGCGTAGTAGTTCTATTTC
NemamitoCOIIR	AGGGTATGAACCTHACAGACT
TcND3F	AAGCGGGTATGTGTTTGGTC
TcND3R	TGCTACCTTAATGTCCTCACGC
TcND5F	TAAATGGCAGTCTTAGCGTGAGG
TcND5R	CAACCTCTAAAAGGAAACTGAGG

Sub-cloning

Purified PCR products were sub-cloned in pGEM^R T-vector system (Promega, USA). The ligation mixture contained 2X ligation buffer 5µl, T-vector 1µl, T_4 DNA ligase 1µl and purified DNA 3µl. Ligation mixtures were incubated at 4°C overnight. Ligated products were transformed into *E. coli* JM109 cells. Briefly, 2 µl of ligation mixture was added to a sterile 1.5 ml tube containing 20 µl of *E. coli* JM109 cells. The tubes were gently flicked and incubated on ice for 30 min. Then, heat shock was done in a water bath at exactly 42°C for 45-50 seconds and tubes immediately transferred into ice for 2 min. SOC or LB solution (180µl) were then added to the tubes and incubated at 37°C for 90 min with shaking (~150 rpm). Transformation culture was plated gently onto duplicate LB/ampicillin/IPTG/X-gal plates. Positive clones were obtained and plasmid DNA extraction was performed using the alkaline SDS method.

Sequence analysis and gene identification

Nucleotide sequences were determined with an ABI PRISM 3100 Avant DNA sequencer using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, CA, USA). Sequences were annotated manually and/or using BioEdit/ClustalW/ApE-plasmid Editor programmes and aligned against the complete/partial mt genome sequence of *Ascaris suum, T. canis, T. cati, T. vitulorum* and *T. malaysiensis* to identify the genes, translation initiation and translation termination codons and gene boundaries[16-18, 21]. The open-reading frames and codon usage profiles of protein-coding genes were analyzed using the program DNAsis and GENETYXMAC (ver. 6.0). Ribosomal RNA genes and tRNAs were identified by aligning sequences with known *Toxocara* species.[16-18]

Results and discussion

General features of the mt genome of *Toxocara canis*

The partial mt genome of *T. canis* was determined. The length of the partial mt genome was 9611 bp. This partial mt genome contains 9 protein-coding genes (*cox*1- *cox*3, *nad*2-*nad*5, *atp*6 and *cyt*b), 14 transfer RNA genes (Lys, LeuUUR, Ser, Ile, Arg, Gln, Phe, tRNA-LeuCUN, tRNA-Thr, tRNA-Cys, tRNA-Met, tRNA-Asp, tRNA-Gly, and tRNA-His) and one ribosomal RNA gene (*rrnL*). This partial genome has a high T content (23.3%) and a low C content (9.3%). The content of A+T was 67.48% and G content was 23.3%. A schematic presentation of the genomic organization of mt genome of *T. canis* characterized in the present study is given in Figure 1. Mitochondrial genomes are circular in nematodes sequenced thus far. The gene arrangement and content of *T. canis* are similar to other *Toxocara* mitochondrial genomes (*T. canis*, *T. cati* and *T. malaysiensis* from China[17], *T. canis* from Australia[18] and *T. vitulorum* from Sri Lanka[14]) and *Ancylostoma duodenale*, *Necator americanus*, *Cooperia oncophora* and *Caenorhabditis elegans*.[19-22] However, *Trichinella spiralis* has *atp*8 gene in its mt genome. This gene is not present in *Toxocara* species.[17] Mitochondrial genes of *T. canis* are transcribed in the same direction similar to other *Toxocara* species.[17-21]

Non-coding region (NCR) found (112 bp) in *T. canis* was between *nad*4 and *cox*1. There was another NCR reported between *nad*3 and *nad*5 in *A. duodenale* and *N. americanus*. [20] However,

it was not found in *T. canis*. The genome arrangement of *T. canis* is the same as *T. cati*, *T. malaysiensis*, *T. vitulorum* but significantly differ from those of *Setaria digitata*, *Onchocerca volvulus*, *Dirofiliria immitis*, *Strongyloides stercoralis, N. americanus* and *T. spiralis*.[22-25]

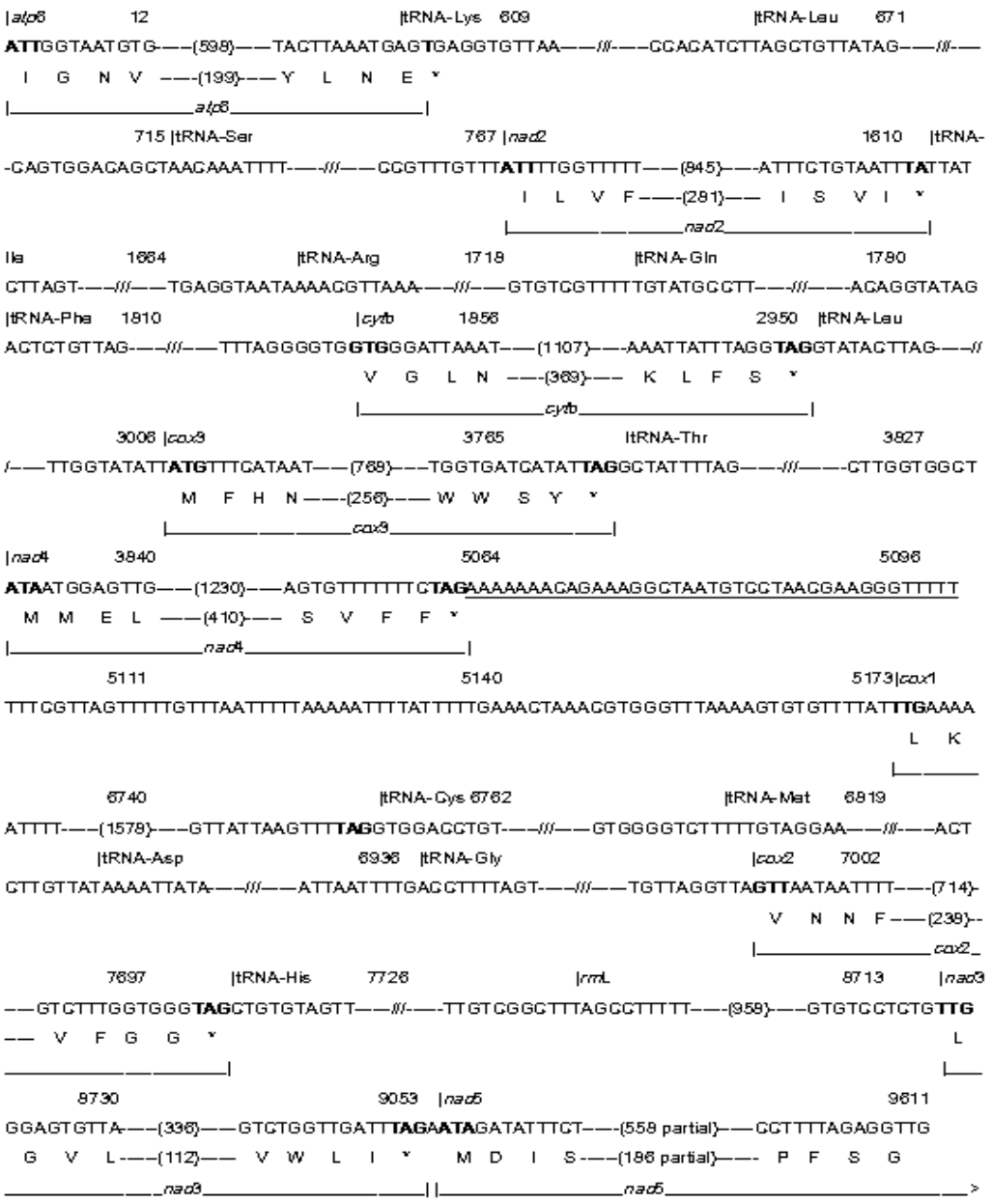

Figure 1 Schematic representation of the genomic organization of a large portion of the *T. canis* mt genome. This region contains 9 protein-encoding genes (*atp6, cytb, cox3, nad2, nad4, cox1, cox2, nad3, nad5*), 14 tRNAs, a non-coding region (underlined) and the *rrnL* gene. The symbol | above the sequence indicates the beginning of each gene. The start and stop codons are shown in bold. * indicates a stop codon

The nucleotide compositions of the obtained partial mtDNA sequences for *T. canis* is biased toward A and T, with T being the most favored nucleotide and C is the least favored, in accordance with mt genomes of other nematodes.

Protein-coding genes, tRNAs, rrnL and non-coding regions

Nucleotide and amino acid length for protein-coding genes are shown in Table 2. Our results indicated that the length of the nucleotide sequences of some protein-coding genes and tRNAs are different from the mitochondrial genomes of *T. canis* from Australia[17] and China[18]. However, cox*3* and *nad4* genes are similar in length (768 bp and1230 bp) from three different geographical locations whereas the nucleotide length of *atp6* (598 bp), *cytb* (1107 bp), *cox1* (1578 bp) and *nad3* (336 bp) of *T. canis* are similar in Sri Lanka and China. However, the length of these genes is varied in Australian isolates (599 bp, 1101 bp, 1575 bp and 330 bp respectively). *cox2* gene is 714 bp and 711 bp in length in Sri Lankan and Chinese isolates and 698 bp in length in an Australian isolate. Interestingly, *T. canis* was collected from a dog in Sri Lanka and China[17] and from a fox in Australia.[17,18] This might be a one reason for sequences length variation. However, this should be further investigated.

Table 2 Positions and characteristics of mitochondrial genes and non-coding sequences of mt genome of *Toxocara canis* and comparison with *T. vitulorum*, *T. cati* and *T. malaysiensis*.

Genes	Positions (5'-3')	Nucleotide (bp)	No. of amino acid/Initiation and temination codons							
	T. canis	*T. canis*	*T. canis*		*T. vitulorum*		*T. cati*		*T. malaysiensis*	
atp6	1-598	598	200	ATT/T	200	ATT/T	200	TTG/TAA	200	ATT/T
tRNA-Lys	599-660	62								
tRNA-Leu	661-715	55								
tRNA-Ser	716-767	52								
nad2	768-1612	845	282	ATT/TA	282	ATT/T	282	GTT/T	282	ATT/T
tRNA-Ile	1613-1674	62								
tRNA-Arg	1675-1728	54								
tRNA-Gln	1729-1785	57								
tRNA-Phe	1786-1843	58								
cytb	1844-2950	1107	369	GTG/TAG	369	TTG/TAA	369	ATG/TAA	369	GTG/TAG
tRNA-Leu	2951-3006	56								
cox3	3007-3774	768	256	ATG/TAG	256	TTG/TAG	256	ATG/TAA	256	GTG/TAG
tRNA-Thr	3775-3831	57								
nad4	3832-5061	1230	410	ATA/TAG	410	GTG/TAG	410	ATA/TAG	410	ATA/TAG
non-coding region	5062-5173	112								
cox1	5174-6751	1578	526	TTG/TAG	527	TTG/TAG	526	TTG/TAG	527	TTG/TAG
tRNA-Cys	6752-6808	57								
tRNA-Met	6809-6876	68								
tRNA-Asp	6877-6936	60								
tRNA-Gly	6937-6991	55								
cox2	6992-7705	714	238	GTT/TAG	238	GTT/TAG	237	GTG/TAA	237	GTA/TAG
tRNA-His	7706-7759	54								
rrnL	7760-8717	958								
nad3	8718-9053	336	112	TTG/TAG	112	TTG/TAA	112	TTG/TAG	112	TTG/TAG
nad5	9054-9611	558 (partial)	186 (partial)	ATA/?	184 (partial)	ATG/?	528	ATA/T	528	ATG/T

As shown in Table 2, the predicted initiation and termination codons for the protein-coding genes of *T. canis* were compared with those of other *Toxocara* species. The start codons we inferred in *T. canis* were ATT, GTG, ATA, GTT, TTG and ATG. Stop codons were TAG, TA and T. Six of the 8 protein genes used TAG as a translation termination codon and the other two genes (*atp6 and nad2*) used TA and T respectively. Three *Toxocara* species used similar start codons in *nad1, atp6, cox1,* and *nad3* (Table 2). Twenty-two transfer RNA genes have been identified in the mt genomes of the nematode species.[17-25] In our study, we have determined 14 of these, ranging

from 52 bp to 68 bp in length (Table 2) and 8 more remain to be sequenced and identified. The length of tRNAs are varied among *T. canis* from three geographical locations.[17,18] The *rrnL* gene of *T. canis* was identified by sequence comparison with other *Toxocara* species. The *rrnL* is located between tRNA-His and *nad*3. The *rrnL* gene is 958 bp in length. The second non-coding region is located between *nad4* and *cox1*. The length of this region is 112 bp for *T. canis.*

Conclusion

The present study determined the partial mt genome sequence of *T. canis* of human and animal health significance. The arrangement, composition and content of the mitochondrial genes of *T. canis* are similar to other *Toxocara* species. However, sequence length of the mitochondrial protein-coding genes, tRNAs, *rrnL* and non-coding regions of *T. canis* are significantly different in three different geographical locations. This may be due to genetic diversity among different populations and/or errors in sequencing or annotation. These variations should be further investigated in future studies.

Conflicts of interest statement

The authors have no conflicts of interest concerning the work reported in this paper.

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