

Sequence Analysis of Mitochondrial Genome of *Toxascaris leonina* from a South China Tiger

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Abstract: *Toxascaris leonina* is a common parasitic nematode of wild mammals and has significant impacts on the protection of rare wild animals. To analyze population genetic characteristics of *T. leonina* from South China tiger, its mitochondrial (mt) genome was sequenced. Its complete circular mt genome was 14,277 bp in length, including 12 protein-coding genes, 22 tRNA genes, 2 rRNA genes, and 2 non-coding regions. The nucleotide composition was biased toward A and T. The most common start codon and stop codon were TTG and TAG, and 4 genes ended with an incomplete stop codon. There were 13 intergenic regions ranging 1 to 10 bp in size. Phylogenetically, *T. leonina* from a South China tiger was close to canine *T. leonina*. This study reports for the first time a complete mt genome sequence of *T. leonina* from the South China tiger, and provides a scientific basis for studying the genetic diversity of nematodes between different hosts.

Key words: *Toxascaris leonina*, mitochondrial genome, phylogeny, South China tiger

Toxascaris leonina is a common parasitic nematode of wild mammals, especially tigers. Moreover, it may infect humans, and have the potential to cause a human disease as emerging zoonosis [1,2]. The adult worms live in the gastrointestinal tract of tigers, rob nutrition from the host which can directly affect the nutritional metabolism of tigers, leading to lassitude, emaciation, and developmental delays. It also can cause gastroenteritis, bile duct obstruction, intestinal obstruction, peritonitis, and even lead to death [3]. *T. leonina* belongs to a soil-borne parasite, and its eggs have strong resistance to survive tens of days in the soil. Thus, it can cause repeated infections in a host, and makes it difficult to control effectively. Hence, *T. leonina* has significant impacts on the protection of rare wild animals, such as South China tigers [4].

Since the complete mitochondrial (mt) genome sequence of *Ascaris suum* was first determined [5], a growing number of mt genome sequences of nematodes have been reported. Earlier in 2008, the complete mt genome of *Toxocara* spp., including *Toxocara canis* were sequenced and analyzed based on their mt genetic composition, protein-coding genes, and the loca-

tion of tRNA genes [6]. Partial mt gene sequence of *T. leonina* from Asian lions was isolated, amplified and compared with those of other ascaridoid nematodes [7]. In addition, the complete sequences of mtDNAs of 3 *Baylisascaris* species (*B. schroederi*, *B. ailuri* and *B. transfuga*) were sequenced, and phylogenetically analyzed by maximum likelihood (ML), maximum parsimony (MP), and neighbor-joining (NJ) methods [8]. Recently, partial sequences of *cox1*, *nad1*, and *nad4* genes of *T. leonina* isolated from 4 South China tigers were amplified, sequenced, and analyzed. The DNASTar and MEGA version 5.05 software were used to differentiate the sequence similarities and genetic evolutionary relationship among *T. leonina* strains [4].

This study aimed at amplification of complete mt genome sequence of *T. leonina* from a South China tiger and analysis of its population genetic characteristics. A phylogenetic tree was constructed by MP, ML, and Bayesian inference (BI) methods based on 12 protein-coding genes of the mt genome as the molecular marker, so as to determine the phylogenetic relationships of *T. leonina* from South China tigers with other *Toxascaris* species.

Adult worms were collected from a South China tiger's feces in a zoo of Guangzhou, China and samples were fixed in 75% ethanol and stored at -20°C until use. One sixth of the adult worm was sheared and put in a centrifuge tube, flushed 3-4 times with double distilled water, and cut up again. DNAs

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were extracted using Wizard[®] SV Genomic DNA Purification System according to the manufacturer's instruction (Promega, Wisconsin, USA), and stored at -20°C for use.

Ten pairs of primers F1F (5'-AATATCAAGGTGGGCTTCTGT-3') and F1R (5'-TTTACACCAACAAACATAAGAA-3'); F2F (5'-GTTTTGTTTATGATAAGATGAT-3') and F2R (5'-AAA-CAAGTTCACACAAACAAAT-3'); F3F (5'-TAGTAGTTTTT TTAATTATGT-3') and F3R (5'-CCGGTTGACCCAAAGAACAAG-3'); F4F (5'-CC TTTGTTGAGCCCTGCTTTG-3') and F4R (5'-TAATCAAACITAAAACCCCTTA-3'); F5F (5'-GGGTTTTAAGTTTGAT-TATGGT-3') and F5R (5'-GTACCTAGTATGATAGTACAC-3'); F6F (5'-CTGCATATAAGTATATGTGTGT-3') and F6R (5'-ACT AACATTATAACCACTAACC-3'); F7F (5'-GGGCTAGTAGA-CAGAGTGTGTC-3') and F7R (5'-GAGCCACACCAACTTTCAT-TATCAC-3'); F8F (5'-GGTTCITAT CTAGTGTGGTGAATT-3') and F8R (5'-GTAACAACACCAGCAACAACAAC-3'); F9F (5'-GGTTTACAGTGTCTAGGGCTAC-3') and F9R (5'-TGCACCCCATGAA ACCAGTAGAC-3'); F10F (5'-TTTGCTACTGGTTTTCATGGGG-3') and F10R (5'-AATTTAAATTACAAAATTCAGTCC-3') were designed according to the conserved region of complete mt genome sequences of all relatives published in GenBank and synthesized by Shanghai Sangon Co., China. The PCR mix consisted of 2.5 µl 10× PCR buffer Mg²⁺-free, 3.0 µl of 25 mM

MgCl₂, 2.0 µl of each 2.5 mM dNTPs, 1.0 µl of each primer 25 mM, 1 µl of DNA samples, 0.25 µl of ExTaq polymerase, and 14.25 µl of ddH₂O. PCR was performed as follows: after initial denaturation step at 94°C for 5 min, a set of 35 cycles were run, each consisting of denaturation at 94°C for 30 sec, annealing at 48-52°C for 30, and extension at 72°C for 1.5 min, followed by final extension of 5 min at 72°C. PCR products were deployed in 1% agarose gel by electrophoresis.

The PCR products were cloned and connected with pMD18-T (TaKaRa, Dalian, China), and then transferred into competent cell DH5. Positive clones were screened by bacterial PCR, and plasmids were extracted and sent to Shanghai Sangon Co. for sequencing. To achieve sequence analysis of mtDNA, sequences from 10 amplified fragments were jointed together into complete mt genome via SeqMan in DNASTAR software and manual alignment. After compared with mtDNA of other worms using the MegAlign, location of protein-coding genes and rRNA genes of mt genome of *T. leonina* from South China tiger were determined (Fig. 1). Its genetic composition, fragment size, and arrangement were analyzed. The position of tRNA genes were detected by online software tRNAscan-SE (<http://lowelab.cse.ucsc.edu/tRNAscan-SE>) and manual alignment.

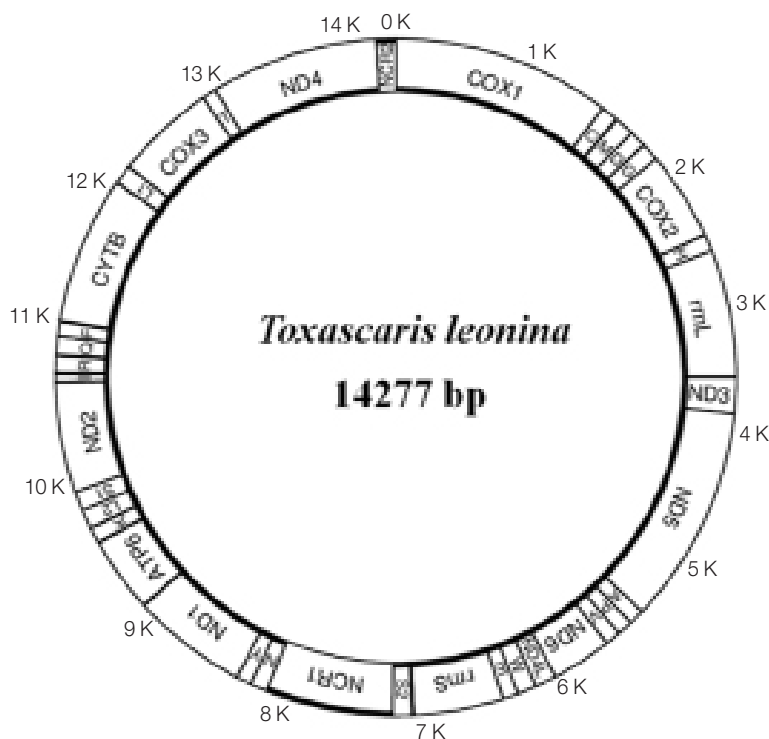


Fig. 1. Arrangement of the mitochondrial genome of *Toxascaris leonina* from South China tiger.

Phylogenetic analyses were performed using the 12 ascaridoid species (Fig. 2) as in-groups, and *Aelurostrongylus abstrusus* serving as an outgroup, based on amino acid sequences of 12 protein-coding genes. The amino acid sequences for each gene were individually aligned using Clustal X, then concatenated into single alignments for phylogenetic analyses. The (JTT+I+G+F) model of amino acid evolution was selected and screened by ProfTest 2.4 software based on Akaike information criterion (AIC). The phylogenetic tree was constructed by MP, ML, and BI methods using *A. abstrusus* (JX519458) as the outside group, drawn, and modified using FigTreev1.4 software.

Adult worms were pale brown, and the anterior end had lancet-shaped cervical alae; the posterior end of males had 2 unequal spicules and no protrusions. The ITS fragment amplified was 907 bp in length, and BLAST analysis indicated 100% similarity with *T. leonina* from the Northeast China tiger (GenBank accession no. JF837175.1).

The complete circular mt genome of *T. leonina* from a South China tiger was 14,277 bp in length, including 12 protein-coding genes, 22 tRNA genes, 2 ribosomal RNA genes, and 2 non-coding regions. All mt genes were located on the same chain (heavy chain) and transcribed in the same direction (Fig. 1). The nucleotide composition of the entire mt genome was: A=21.64%, T=49.39%, G=20.77%, and C=8.19%, and biased toward A and T. There was base overlapping and no introns among some protein-coding genes. The most common start codon for *T. leonina* was TTG, followed by ATT, while GTG

and GTT were the least. The most common stop codon was TAG, and 4 genes were ended with an incomplete stop codon. There were 13 intergenic regions ranging from 1 to 10 bp, the longest one located between tRNA-ser^{icn} and *rrnS*, followed by 9 bp, located between tRNA-Phe and *cytb*. The intergenic regions between NaD1 and Atp6, Atp6 and tRNA-Lys, and tRNA-Lys and tRNA-Leu^{UIR} were 7, 6, and 5 bp, respectively; the remaining genes were separated by 1 bp or none (Table 1).

The phylogenetic trees constructed by ML, BI, and MP methods were basically the same (Fig. 2). Ascaridae and Toxocaridae gathered in Ascaridida and formed a big clade, while Ascaridiidae formed another clade. Among them, *Ascaris*, *Toxascaris*, and *Baylisascaris* were located in Ascaridae branch, the *T. leonina* from South China tiger belonged to *Toxascaris* and had a closest relationship with canine *T. leonina*.

In the mt genome of *T. leonina* from a South China tiger, there are *nad3*, *nad5*, *nad6*, and *nad4* between 2 rRNA genes (*rrnL* and *rrnS*). It can be classified into the GA7 type according to Yatawara et al. [10] based on protein-coding gene number and order between *rrnL* and *rrnS*. Other ascarid nematodes belonging to this genotype are *A. suum*, *B. transfuga*, and others [8,9]. Two non-coding regions (NCR) in the mt genome of *T. leonina* from the South China tiger were NCR1 and NCR2, consisting of abundant multiple copy of fragments with AT-rich region.

Comparing with NCR in the mt genome of *T. leonina* isolated from dogs [11], there were significant distinction in se-

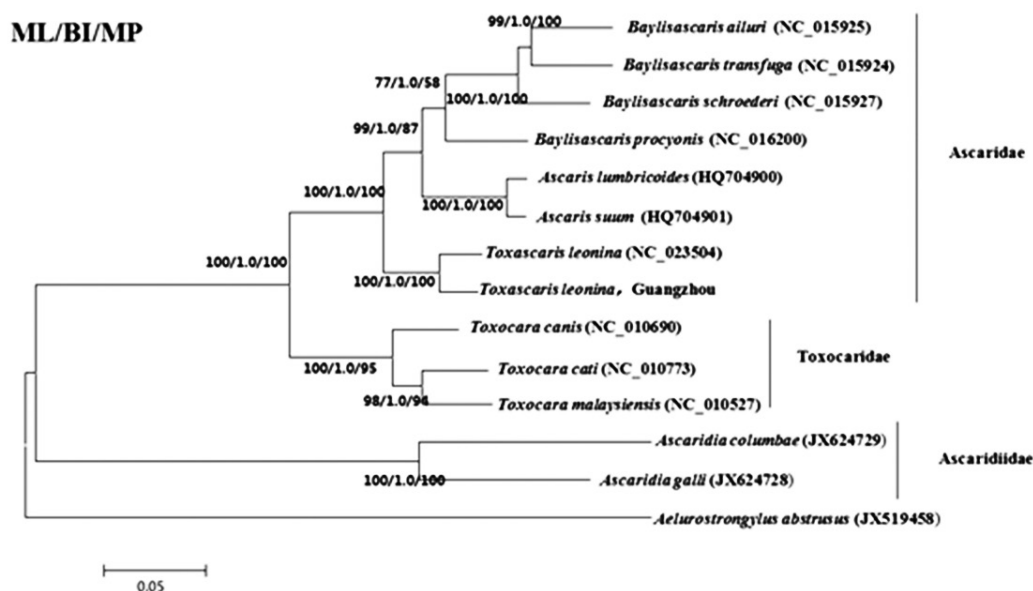


Fig. 2. A phylogenetic tree based on mitochondrial DNA protein-coding genes of *T. leonina* by ML, BI, and MP method.

Table 1. Organization of mitochondrial genome of *Toxascaris leonina* from South China tiger

Genes/regions	Position			Codon		Intergenic nucleotides
	start	end	length	start	stop	
<i>cox1</i>	1	1,578	1,578	TTG	TAG	0
tRNA-Cys	1,578	1,633	56			-1
tRNA-Met	1,640	1,700	61			6
tRNA-Asp	1,701	1,757	57			0
tRNA-Gly	1,761	1,816	56			3
<i>cox2</i>	1,817	2,513	697	TTG	T	0
tRNA-His	2,514	2,572	59			0
<i>rml</i>	2,573	3,529	957			0
<i>nad3</i>	3,531	3,866	336	TTG	TAG	1
<i>nad5</i>	3,867	5,448	1,582	ATT	T	0
tRNA-Ala	5,449	5,504	56			0
tRNA-Pro	5,505	5,559	55			0
tRNA-Val	5,560	5,616	57			0
<i>nad6</i>	5,616	6,050	435	TTG	TAG	-1
<i>nad4L</i>	6,052	6,285	234	ATT	TAG	1
tRNA-Trp	6,286	6,342	57			0
tRNA-Glu	6,342	6,396	55			-1
<i>rrs</i>	6,397	7,096	700			0
tRNA-Ser ^{UCN}	7,107	7,160	54			10
NCR1	7,161	8,088	928			0
tRNA-Asn	8,089	8,146	58			0
tRNA-Tyr	8,147	8,203	57			0
<i>nad1</i>	8,204	9,076	873	TTG	TAG	0
<i>atp6</i>	9,084	9,683	600	ATT	TAG	7
tRNA-Lys	9,690	9,751	62			6
tRNA-Leu ^{UUR}	9,757	9,811	55			5
tRNA-Ser ^{AGN}	9,812	9,863	52			0
<i>nad2</i>	9,864	10,707	844	GTG	T	0
tRNA-Ile	10,708	10,768	61			0
tRNA-Arg	10,769	10,823	55			0
tRNA-Gln	10,825	10,879	55			1
tRNA-Phe	10,883	10,942	60			3
<i>cytb</i>	10,952	12,045	1,094	TTG	TA	9
tRNA-Leu ^{CUN}	12,050	12,107	58			4
<i>cox3</i>	12,108	12,875	768	GTT	TAG	0
tRNA-Thr	12,877	12,932	56			1
<i>nad4</i>	12,933	14,162	1,230	TTG	TAG	0
NCR2	14,163	14,277	115			0

quence size and homology. The start codons of protein-coding genes in the mt genome of *T. leonina* isolated from the South China tiger were TTG, ATT, GTG, and GTT. Except for the stop codon TAG, the incomplete stop codon T and TA frequently occur in protein-coding genes. This phenomenon is widespread in nematode mt genomes, for example, *nad5* uses T as the stop codon in *A. suum* [9] and 5 protein-coding genes use T as the stop codon in *T. canis* [8]. These incomplete codons insert again into AA or A behind TA or T after transcription. This also confirmed the hypothesis that protein-coding genes of mt

genome are the remaining part of un-incorporated nuclear genome [12]. Because of the mt genome of *T. leonina* from the South China tiger prefers AT, the codon usage also preferred to A and T. Also, the higher usage frequency of codon was TTT (13.49%), TTG (9.1%), GTT (7.1%), and ATT (6.2%), respectively. The lower was GGC and CGA. These results were similar to *Toxocara* [6] and *Baylisascaris* [8], indicating that the usage of codon is consistent with the content of mt base composition.

In this study, the results indicated that Ascaridae and Toxocaridae gathered in a same branch, the Ascaridiidae gathered

in another branch; *Ascaris*, *Toxascaris*, and *Baylisascaris* belong to the branch of Ascaridae, the *T. leonina* from the South China tiger is located in the branch of *Toxascaris*. In Toxocaridae branch, the classification of *T. canis*, *T. cati*, and *T. malaysiensis* is conformed to the phylogenetic tree constructed based on the mt genome [6]. In addition, in the branch of *Baylisascaris*, the phylogenetic tree of 3 *Baylisascaris* species (*B. transfuga*, *B. ailuri*, and *B. procyonis*) is the same as the phylogenetic trees constructed using the mt genome as molecular marker [8]. It means that the results of this study conform to other phylogenetic research on *Toxocara* and *Baylisascaris*. In addition, the *T. leonina* from the South China tiger is the most closely related with the canine *T. leonina*, both of them located in the same branch. The phylogenetic analysis of *T. leonina* from the South China tiger was also consistent with the traditional morphological classification and the research result based on *nad1* gene polymorphism [6].

This study obtained for the first time mt genome sequences of *T. leonina* from the South China tiger, enriched mt gene database of *Toxascaris* nematodes, and provided a scientific basis for studying the genetic diversity of nematodes between different hosts.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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