Journal of Helminthology

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Characterisation of the mitochondrial genome and phylogenetic analysis of *Toxocara apodemi* (Nematoda: Ascarididae)

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Research Paper

Cite this article: Gao Y, Hu Y, Xu S, Liang H, Lin H, Yin TH and Zhao K (2024). Characterisation of the mitochondrial genome and phylogenetic analysis of *Toxocara apodemi* (Nematoda: Ascarididae). *Journal of Helminthology*, **98**, e33, 1–10
https://doi.org/10.1017/S0022149X24000221.

Received: 12 November 2023
Revised: 04 February 2024
Accepted: 19 March 2024

Keywords:
Toxocara; mitochondrial genome; comparative analyses; phylogenetic analysis

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Abstract

We first sequenced and characterised the complete mitochondrial genome of *Toxocara apodeme,* then studied the evolutionary relationship of the species within Toxocaridae. The complete mitochondrial genome was amplified using PCR with 14 specific primers. The mitogenome length was 14303 bp in size, including 12 PCGs (encoding 3,423 amino acids), 22 tRNAs, 2 rRNAs, and 2 NCRs, with 68.38% A+T contents. The mt genomes of *T. apodemi* had relatively compact structures with 11 intergenic spacers and 5 overlaps. Comparative analyses of the nucleotide sequences of complete mt genomes showed that *T. apodemi* had higher identities with *T. canis* than other congeners. A sliding window analysis of 12 PCGs among 5 *Toxocara* species indicated that *nad*4 had the highest sequence divergence, and *cox*1 was the least variable gene. Relative synonymous codon usage showed that UUG, ACU, CCU, CGU, and UCU most frequently occurred in the complete genomes of *T. apodemi.* The Ka/Ks ratio showed that all *Toxocara* mt genes were subject to purification selection. The largest genetic distance between *T. apodemi* and the other 4 congeneric species was found in *nad*2, and the smallest was found in *cox*2. Phylogenetic analyses based on the concatenated amino acid sequences of 12 PCGs demonstrated that *T. apodemi* formed a distinct branch and was always a sister taxon to other congeneric species. The present study determined the complete mt genome sequences of *T. apodemi*, which provide novel genetic markers for further studies of the taxonomy, population genetics, and systematics of the Toxocaridae nematodes.

Introduction

Toxocaridae contains only two genera, *Porrocaecum* and *Toxocara* (Gu *et al.* 2023). *Toxocara* species can cause toxocariasis and commonly occur in wildlife and domestic animals. The larvae of some *Toxocara* species can also accidentally infect humans; they are therefore of veterinary, medical, and economic significance. Larvae also can migrate into host tissue, leading to tissue damage, inflammatory reactions, visual impairment, and blindness (Zhou *et al.* 2020). *T. canis* can cause detrimental damage to the brain of intermediate or paratenic hosts (Chen *et al.* 2022). Therefore, identifying different *Toxocara* is conducive to preventing and treating nematode infection in humans and animals.

In *Toxocara*, *T. apodemi* and *T. mackerrasae* are non-zoonotic species that are host-specific parasites of Muridae (Olsen 1957; Warren 1972; Asakawa *et al.* 1994). *T. apodemi* mainly infects (*Apodemus. peninsulae*) *A. peninsulae* and *A. agrarius* and has only been reported in China and Korea (Ziegler and Macpherson 2019; Kim *et al.* 2020). The nematode was first reported in *A. peninsulae* from Korea as a new species of the genus Neoascaris and was named *Neoascaris apodemi* by Olsen in 1957 (Olsen 1957). The parasite was then revised as *T. apodemi* after it was reported in striped field mice (*A. agrarius*) in China. Their morphologic characteristics are that external prolongations of the labial pulp are asymmetrical and rounded at the anterior border (Ziegler and Macpherson 2019).

Despite these nematodes' ubiquity and essential roles in diverse ecological systems, the origin and early evolutionary history of these species have long been matters of debate. In recent years, mitochondrial (mt) genomes, as genetic markers, have been widely used to analyse the taxonomy and diversity of certain taxa or specific groups because of their strict maternal inheritance, apparent lack of recombination, rapid evolutionary rate, and comparatively conserved genomic structure (Zhou *et al.* 2020; Gao *et al.* 2020). The study of the complete mitochondrial gene in Toxocaridae will help us to understand its evolutionary relationships. In 1992, the first paper on the complete mt genome of *Ascaris suum* was published, and since then,

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more and more mt genomes of other Ascaridoidea species have been sequenced and annotated (Okimoto *et al.* 1992; Liu *et al.* 2012). The phylogenetic relationships within the superfamily were gradually refined. For example, based on mt genome sequences, *Baylisascaris* species (*B. schroederi*, *B. ailuri*, and *B. transfuga*) are more closely related to *A. suum* than to the 3 *Toxoara* species (*T. canis*, *T. cati*, and *T. malaysiensis*) and *A. simplex* (Xie *et al.* 2011).

Currently, the available molecular information for *T. apodemi* is limited, with only the partial sequence of a small subunit of a ribosomal RNA gene published in GenBank (Kim *et al.* 2020). Thus, the objectives of this study were to describe and determine its complete mt genome sequences, compare its mt genome with those of other *Toxocara* species, and reconstruct its phylogenetic relationships to assess its systematic and phylogenetic position.

Materials and methods

Parasites and species identification

Adult nematodes of *T. apodemi* were obtained from the intestine of a naturally infected wild mouse in Taizhou, Zhejiang Province, China. The parasite was identified at the species level according to previously described morphological features (Asakawa *et al.* 1994; Kim *et al.* 2020; Olsen 1957). The molecular characteristics of 18S and internal transcribed spacers (ITS) were amplified using universal primers NC5 (5′-GTAGGTGAACCTG CGGAAGGATCATT-3′) and NC2 (5′-TTAGTTTCTTTTCCTC CGCT-3′) for ITS sequence (Gasser *et al.* 2008); the genus *Toxocara's* universal primers 18S-F (5′-GCTAATACA TGCACCAAAGC-3′) and 18S-R (5′-GATCACGGAGGATT TTCAAC-3′) were reported previously for 18S rDNA sequence (Kim *et al.* 2020).

Polymerase chain reaction (PCR) amplification of mitochondrial genome and sequencing

In total, 14 specific primers were used to amplify the complete mt genome of *T. apodemi* and were designed based on those of *T. canis* (Accession: NC_010690.1) and *T. cati* (NC_010773.1) published in GenBank (Table S1). PCR reactions were carried out under the following conditions: 94°C for 2 min, then 94°C for 1 min, 40–56°C for 30 s, and 72°C (~1 kb region) for 30 s for 35 cycles, with a final extension at 72°C for 7 min. The positive PCR products were cloned to PMD-18T vectors and then transferred into DH5α cells for positive plasmid sequencing at Sangon Biotech Company (Shanghai, China).

Genome sequence assembly and gene annotation

Sequences were assembled manually and aligned against the mt genome sequences from *Toxocara* species using the program Clustal X (v. 1.83) and the MegAlign procedure within the DNAStar (Burland 2000; Larkin *et al.* 2007). The boundaries of 12 protein-coding gene (PCG) sequences, the 16S ribosomal RNA (*rrn*L) gene, and the 12S ribosomal RNA (*rrn*S) gene were determined using the mt genome sequences of other *Toxocara* nematodes available in GenBank using the software MEGA X (Li *et al.* 2008; Kumar *et al.* 2018; Xie *et al.* 2022). A total of 22 transfer RNA (tRNA) genes were identified using the online tool tRNA scan-SE (http://lowelab.ucsc.edu/tRNAscan-SE) or via visual inspection. Finally, the circular genomic maps of *T. apodemi* were generated using the CGView online server V1.0 (http://stothard.afns.ualberta.ca/cgview_server/).

Comparative mt genome sequences analysis

Comparisons were made among the complete mt genomes available in GenBank for five *Toxocara* nematodes, including gene lengths, A+T contents, nucleotides, and amino acid sequence identities. The A+T content of complete mt genomes, 12 PCGs, and the 1st, 2nd, and 3rd coding positions were computed using DNAStar (v. 12.1) (Burland 2000). The relative synonymous codon usage (RSCU) values of the 12 PCGs of five *Toxocara* species were calculated with MEGA X (Kumar *et al.* 2018). The p-distance model of MEGA X was used for the genetic distance analysis of 12 PCGs among five *Toxocara* species. The rate of nonsynonymous substitutions (Ka) and the rate of synonymous substitutions (Ks) were used to predict evolutionary processes; Ka/Ks ratios were calculated for the nucleotide sequences of all 12 mt PCGs of *T. canis*, *T. cati*, *T. malaysiensis*, *T. vitulorum*, and *T. apodemi* using DnaSP v5 (Librado and Rozas 2009). A sliding window of 300 bp (in 10 bp overlapping steps) was used to estimate nucleotide diversity Pi (π) across the alignment. Nucleotide diversity was plotted against the mid-point positions of each window.

Phylogenetic analysis

Phylogenetic relationships were reconstructed with Bayesian inference (BI) and maximum likelihood (ML) using the concatenated amino acid sequences of 12 PCGs from mt genomes available in GenBank for 35 Ascaridomorpha species, using *Caenorhabditis elegans* as the outgroup (Table S2). The amino acid sequences of 12 PCGs of 36 nematodes were aligned using MAFFT 7.471 and then concatenated into a single alignment (Katoh and Standley 2013). Sites of ambiguous alignment were eliminated using the Gblocks online server (http://www.phylogeny.fr/one_task.cgi?task_type=gblocks). MrBayes 3.1 was used to reconstruct the BI tree and four independent Markov chain runs were performed for 1,000,000 metropolis-coupled MCMC generations, sampling a tree every 100 generations. The mixed model was selected as the best model and was performed using the BI method. The first 25% (2500) of trees were omitted as burn-in, and the remaining trees were used to calculate Bayesian posterior probabilities (Ronquist and Huelsenbeck 2003). The phylograms were drawn using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). An ML tree was inferred by using the JTT matrix-based model and performed using MEGA X (Jones *et al.* 1992; Kumar *et al.* 2018). The tree with the highest log likelihood (–66471.74) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algo-

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Table 1. Mitochondrial genome characteristic of *Toxocara apodemi*

Gene/region	Position (5'–3')	Length (bp)	Initiation and termination codons	Intergenic nucleotides
tRNA–Tyr (Y)	1–57	57		
*nad*1	58–930	873	TTG/TAG	1
*atp*6	932–1529	598	ATT/T	
tRNA–Lys (K)	1530–1592	63		5
tRNA–Leu^{UUR} (L2)	1598–1652	55		
tRNA–Ser^{AGN} (S1)	1653–1705	53		
*nad*2	1706–2551	846	GTT/TAG	1
tRNA–Ile (I)	2553–2612	60		
tRNA–Arg (R)	2613–2668	56		–1
tRNA–Gln (Q)	2668–2722	55		–1
tRNA–Phe (F)	2722–2779	58		
*cyt*b	2780–3886	1107	ATT/TAA	–2
tRNA–Leu^{CUN} (L1)	3885–3939	55		
*cox*3	3940–4707	768	TTG/TAG	1
tRNA–Thr (T)	4709–4764	56		
*nad*4	4765–5994	1230	GTT/TAG	
NCR	5993–6111	117		
*cox*1	6112–7689	1578	TTG/TAA	2
tRNA–Cys (C)	7692–7747	56		
tRNA–Met (M)	7748–7806	59		
tRNA–Asp (D)	7808–7864	57		
tRNA–Gly (G)	7865–7921	57		
*cox*2	7922–8635	714	GTT/TAG	1
tRNA–His (H)	8637–8693	57		
*rrn*L	8694–9648	955		
*nad*3	9649–9984	336	TTG/TAG	
*nad*5	9985–11566	1582	ATG/T	1
tRNA–Ala (A)	11568–11623	56		5
tRNA–Pro (P)	11629–11682	54		
tRNA–Val (V)	11683–11738	56		
*nad*6	11739–12173	435	TTG/TAA	–1
*nad*4L	12173–12404	232	ATT/T	
tRNA–Trp (W)	12405–12463	59		–1
tRNA–Glu (E)	12463–12519	57		
*rrn*S	12520–13216	697		9
tRNA–Ser^{UCN} (S2)	13226–13281	56		
A–T Rich	13282–14247	966		
tRNA–Asn (N)	14248–14303	56		

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rithms to a matrix of pairwise distances estimated using a JTT model, and then the topology with the superior log likelihood value was selected.

Results and Discussion

Molecular and morphological identification of T. apodemi

Adult females measured 68–75 mm in length and 1.8–2.2 mm in width. Eggs were oval and measured 74–78 μm. The tail terminates in a conical, retractible spine-like structure. The surface of the egg has rough dents (Figure S1).

The 18S rRNA sequences of *T. apodemi* were 1352 bp in size with 99.7% identity to the corresponding available sequences in GenBank (Kim *et al.* 2020). The ITS sequences of *T. apodemi* (Accession no. OR231233) were first determined in the present study; it was 957 bp in length and had 80.94% identity to that of *T. canis* in GenBank (Chen *et al.* 2022).

General features of T. apodemi *mitogenome*

The complete mt genome sequences of *T. apodemi* were the first to be sequenced, with a length of 14303 bp (Accession no. OR241493), which was similar to those of *T. canis* (14322 bp) and *T. malaysiensis* (14266 bp), shorter than those of *T. vitulorum* (15045 bp), and longer than those of *T. cati* (14029 bp) (Li *et al.* 2008; Xie *et al.* 2022). The complete mt genome of *T. apodemi* contained 12 PCGs (*cox*1-*cox*3, *cyt*b, *atp*6, *nad*1-*nad*6, and *nad*4L), 22 tRNAs, 2 ribosomal RNAs (rRNAs), and 2 non-coding regions (NCRs) (Table 1, Figure 1). All mt genes of *T. apodemi* were transcribed in the same direction, which was the same as for the other species in *Toxocara* available in GenBank, but different from those of *Ascaridia columbae* and *Ascaridia galli* in superfamily Heterakoidea (Liu *et al.* 2013a; Han *et al.* 2022). A total of 3,423 amino acids were encoded by 12 PCGs in the complete *T. apodemi* mt genome. TTG, ATT, and GTT were used as the start codon of 12 PCGs; TAA and TAG were used as the stop codon of 9 PCGs. "T" as the termination codon also appeared in *atp*6, *nad*5, and *nad*4L,

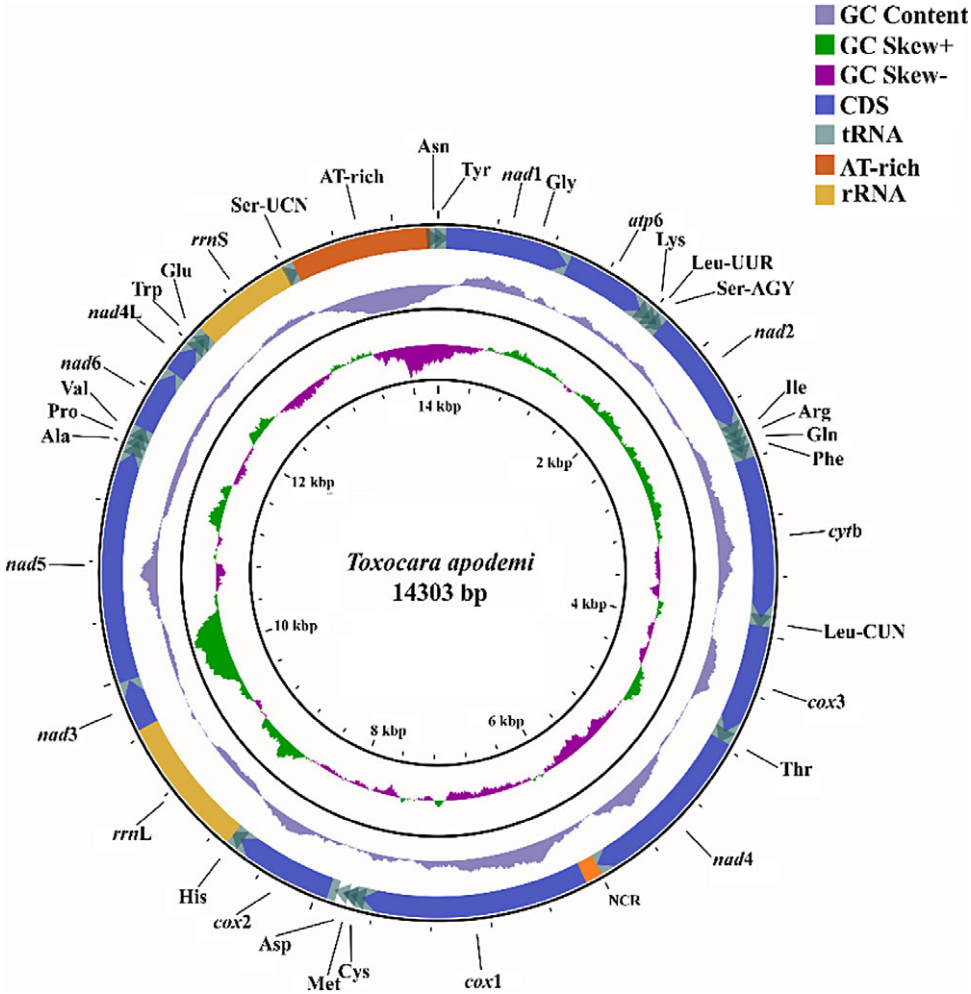

Figure 1. Gene map of *T. apodemi* complete mt genome.

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Table 2. Comparative analysis of mtDNA sequences in genus *Toxocara*

Genes (Regions)	Length/bp (aa)					Identities (%)
	T. apodemi	*T. canis*	*T. cati*	*T. malaysiensis*	*T. vitulorum*	
Complete genome (amino acids)	14303	14322	14029	14266	15045	84.0/82.7/83.2/83.2
	(3423)	(3423)	(3422)	(3422)	(3422)	(90.8/90.9/90.4/89.8)
*nad*1	873	873	873	873	873	86.8/85.6/85.7/86.5
	(290)	(290)	(290)	(290)	(290)	(91.0/93.4/93.1/93.1)
*atp*6	598	598	598	598	598	88.3/88.0/86.3/84.4
	(199)	(199)	(199)	(199)	(199)	(88.4/86.9/84.9/83.4)
*nad*2	846	845	844	844	844	85.5/85.4/83.8/82.6
	(281)	(281)	(281)	(281)	(281)	(84.3/82.9/81.5/79.4)
*cyt*b	1107	1107	1107	1107	1107	84.3/83.2/82.6/84.8
	(368)	(368)	(368)	(368)	(368)	(89.1/89.4/88.0/88/0)
*cox*3	768	768	768	768	768	86.3/86.7/86.5/86.1
	(255)	(255)	(255)	(255)	(255)	(93.7/94.1/94.1/93.3)
*nad*4	1230	1230	1230	1230	1230	82.4/82.3/83.5/83.3
	(409)	(409)	(409)	(409)	(409)	(92.4/92.2/91.7/90.7)
*cox*1	1578	1578	1578	1581	1581	89.1/89.0/89.3/90.1
	(525)	(525)	(525)	(525)	(525)	(96.6/96.4/97.0/96.2)
*cox*2	714	714	711	711	706	87.8/85.8/89.5/88.6
	(237)	(237)	(236)	(236)	(235)	(96.6/97.0/97.5/96.6)
*nad*3	336	336	336	336	336	86.0/87.2/86.3/88.7
	(111)	(111)	(111)	(111)	(111)	(88.3/87.4/87.4/91.0)
*nad*5	1582	1582	1582	1582	1582	84.2/83.6/83.9/85.3
	(527)	(527)	(527)	(527)	(527)	(87.9/87.3/87.1/86.1)
*nad*6	435	434	435	435	435	83.9/85.7/82.9/83.6
	(144)	(144)	(144)	(144)	(144)	(83.3/91.0/88.9/88.2)
*nad*4L	232	233	232	232	232	87.1/85.8/87.9/87/9
	(77)	(77)	(77)	(77)	(77)	(93.5/90.9/92.2/92.2)
*rrn*L	955	958	955	955	954	84.9/85.6/85.8/84.0
*rrn*S	697	697	696	696	688	89.9/91.5/90.6/88/3

Note: Identity: *T. apodemi* vs. *T. canis*, *T. apodemi* vs. *T. cati*, *T. apodemi* vs. *T. malaysiensis*, *T. apodemi* vs. *T. vitulorum*.

which was the same as congeneric species. However, in the *nad*2 gene, TAG was the termination codon in *T. apodemi*, and "TA" or "T" were the termination codons in other species in *Toxocara* (Li *et al.* 2008; Xie *et al.* 2022). The mt genomes of *T. apodemi* had relatively compact structures, with fewer spacer regions and short overlaps between some adjacent genes, in which 11 intergenic spacers fluctuated with 1 to 9 bp, and 5 overlaps were 1–2 bp in length (Table 1). The A+T content of the complete mt genome and the 12 PCGs of *T. apodemi* were 68.38% and 66.54%, respectively. This is in accordance with those of other *Ascaris* species, such as *Toxocara* species, human-type *Ascaris*, pig-type *Ascaris*, and hybrid *Ascaris* (Li *et al.* 2008; Zhou *et al.* 2020; Xie *et al.* 2022), and slightly lower than those of *Parascaris equorum* in Ascaridoidea (70.25%) (Gao *et al.* 2019). The comparison analysis showed that the A+T content of the second coding position of the 12 PCGs was more similar than those of the first and the third coding positions among five *Toxocara* species (Figure 1).

There were 22 tRNAs in the mt genome of *T. apodemi*, ranging from 54 bp (*trn*P) to 63 bp (*trn*K) in size (Table 1). The estimated secondary structures of 22 tRNAs were identical to those of all other chromadorean nematodes investigated so far, except for *T. spiralis* (Gao *et al.* 2022; Xie *et al.* 2022). The lengths of the *rrn*L and *rrn*S of

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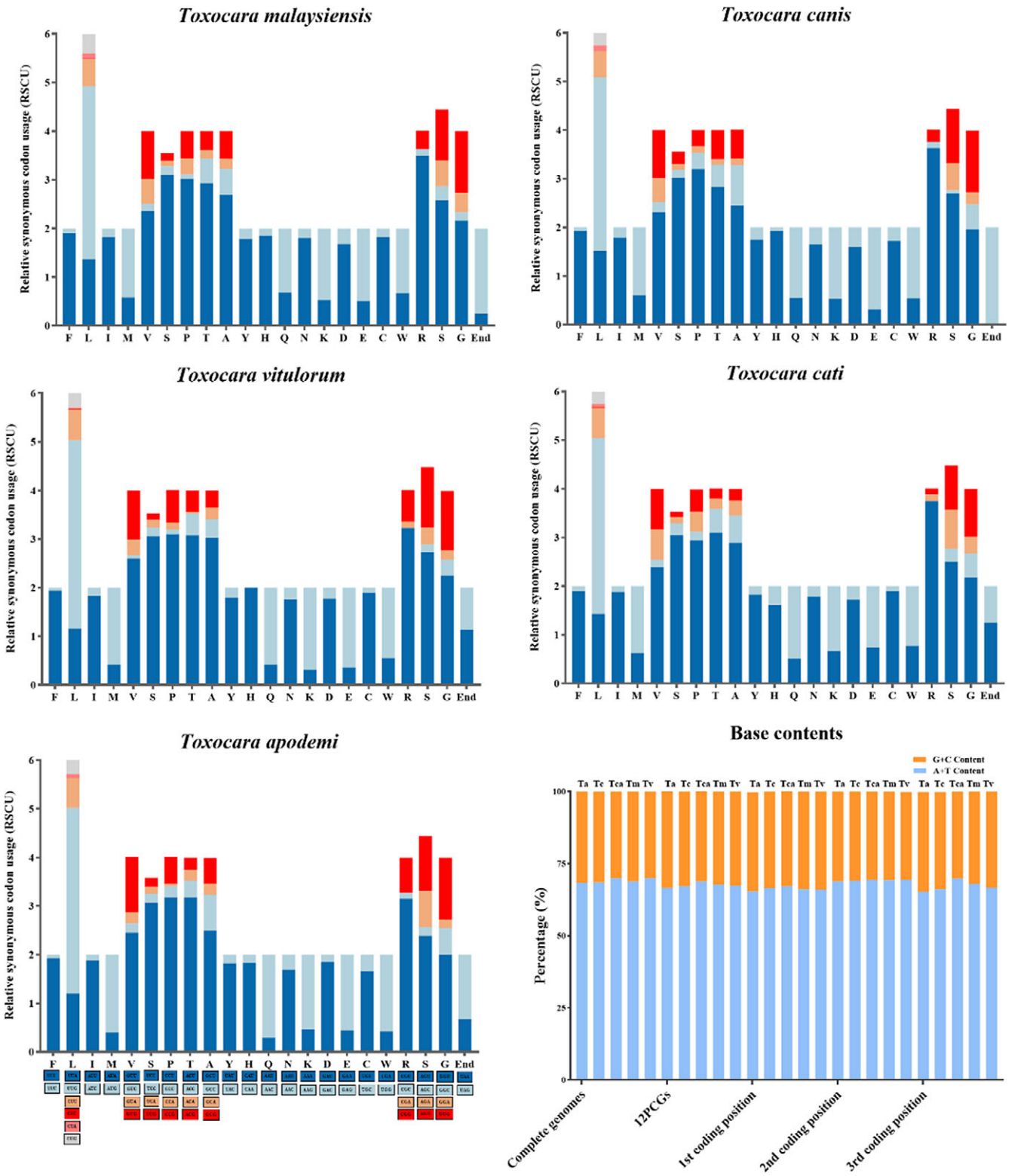

Figure 2. Relative synonymous codon usage (RSCU) of the mitochondrial genomes and A+T contents of complete genomes, 12PCGs, and 1st, 2nd, and 3rd coding position of five *Toxocara* species.
Ta: *T. apodemi*, Tc: *T. canis*, Tca: *T. cati*, Tm: *T. malaysiensis*, Tv: *T. vitulorum*

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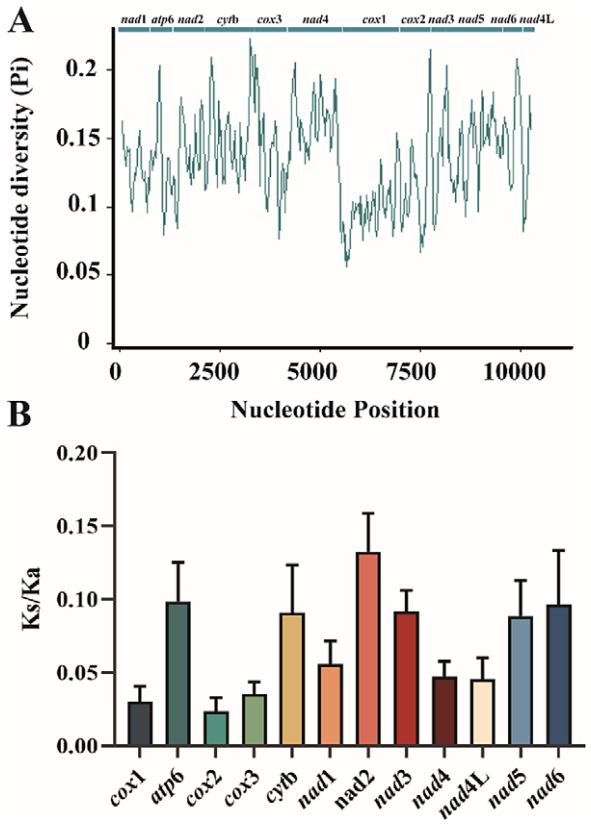

Figure 3. (A) Sliding window analysis of the concatenated alignments of 12 PCGs of five *Toxocara* species. A sliding window of 300 bp (in 10 bp overlapping steps) was used to estimate nucleotide diversity Pi (π) across the alignments. Nucleotide diversity was plotted against the mid-point positions of each window. (B) Evolutionary rates of 12 PCGs among five *Toxocara* species. The ratio of Ka/Ks is calculated for each PCG.

the *T. apodemi* mt genome were 955 bp and 697 bp, respectively, and they were located between *trn*H and *nad*3, and between *trn*E and *trn*S2, respectively. Moreover, there was also a NCR with 117 bp placed in *nad*4 and *cox*1, and an A+T rich with 966 bp placed in *trn*S2 and *trn*N.

Comparative mitogenomics

A comparative analysis of the nucleotide sequences of the complete mt genomes of *T. apodemi* with *T. canis*, *T. cati*, *T. malaysiensis*, and *T. vitulorum* showed that their identities were 84.0%, 82.7%, 83.2%, and 83.2%, respectively (Table 2). *T. apodemi* had higher identities with that of *T. canis* than those of other congeners. This level of mt genome divergence is lower than that between *Ophidascaris wangi* and *Toxascaris leonina* (19.77%), and higher than that of *T. leonina* from cheetah and dog (7.2%) and the *Pseudoterranova decipiens* species complex (3.8–9.4%) (Liu *et al.* 2016; Jin *et al.* 2019; Zhou *et al.* 2021). Nevertheless, the identities of the amino acid sequence of five species in *Toxocara* were similar at 89.8–90.8%. The discrepancy in amino acid sequences was similar to that found between *O. wangi* and *T. leonina* (12.17%) (Zhou *et al.* 2021).

The RSCU reflected the genetic codon usage bias to reveal the relative frequency of synonymous codons (Yang *et al.* 2023). It is a metric commonly used to measure codon usage bias. In Ascaridomorpha, the most commonly used codons are UUG, ACU, CCU, GCU, AGA, and AGU (Han *et al.* 2022). In the complete mt genome of *T. apodemi*, UUG (RSCU = 3.82), ACU (RSCU = 3.18), CCU (RSCU = 3.18), CGU (RSCU = 3.15), and UCU (RSCU = 3.07) were the most frequently used, and CUC (RSCU = 0), CGA (RSCU = 0), CCA (RSCU = 0.05), and UUC (RSCU = 0.07) were the least used (Figure 2). This indicated that *T. apodemi* had similar codon bias in Ascaridomorpha. The RSCU of 5 *Toxocara* species were identical to the most frequently used codon but had a slight difference with the least used codon. For example, the RSCU of AGC was 0.27, 0.29, 0.16, and 0.18 in *T. cati*, *T. malaysiensis*, *T. vitulorum*, and *T. apodemi*, respectively. However, it was 0.06 in *T. canis* (Li *et al.* 2008; Meng *et al.* 2019; Xie *et al.* 2022).

The sliding window analysis of the nucleotide diversity (Pi values) of the 12 aligned PCGs among 5 *Toxocara* species revealed a high degree of nucleotide variation within different genes (Figure 3A). Nucleotide diversity values ranged from 0.09975 (*cox*1) to 0.16268 (*nad*4). *Cox*1 and *cox*2 had relatively low nucleotide diversity values, indicating that they were relatively conserved genes in the 12 PCGs of *Toxocara* species. *Cox*1, as the least variable and most slowly evolving mitogenome gene, was identical to that of *Ascaris* species and Cyathostominae nematodes (Zhou *et al.* 2020; Gao *et al.* 2022). *Nad*4, *cyt*b, *nad*2, and *nad*6 presented higher variability in the five *Toxocara* species. In a previous study, *nad*4 was the least conserved gene between *Parascaris equorum* and *Parascaris univalens* (Gao *et al.* 2019), as was found in the present study. However, *nad*5 had the fewest conservative sites in the mitochondrial genomes for 17 *Ascaris* samples (Zhou *et al.* 2020). These indicate that the least variable and most slowly evolving genes are the same in Ascaridomorpha; however, the least conserved gene is different.

The Ka/Ks ratio is controlled by functionally related sequence contexts, such as encoding amino acids and participating in exon splicing. It is defined as the degree of evolutionary change; when the ratio is greater than 1, positive selection exists, indicating that non-synonymous mutations are more highly favored by Darwinian selection, and they will be retained at a rate greater than that of synonymous mutations (Liu *et al.* 2013b; Xing *et al.* 2022). Our Ka/Ks ratio data showed that all mt genes among *Toxocara* species were subject to purification selection and not a positive selection (Figure 3B). The results were the same as those of the evolutionary rates of PCGs between *T. vitulorum* and 28 other Rhabditida mitogenomes (Xie *et al.* 2022).

Phylogenetic and genetic distance analysis

Complete mitochondrial sequences can provide a great source of species information and can provide new specific molecular markers for species taxonomy, population genetics, and systematics. For some taxonomic groups, genetic distance is the most effective model to quantify sequence divergences among individuals (Chagas *et al.* 2020). In the present study, the average interspecific genetic distances of 12 PCGs among five *Toxocara* species were found to range from 0.1544 (*nad*2) to 0.0259 (*cox*1).

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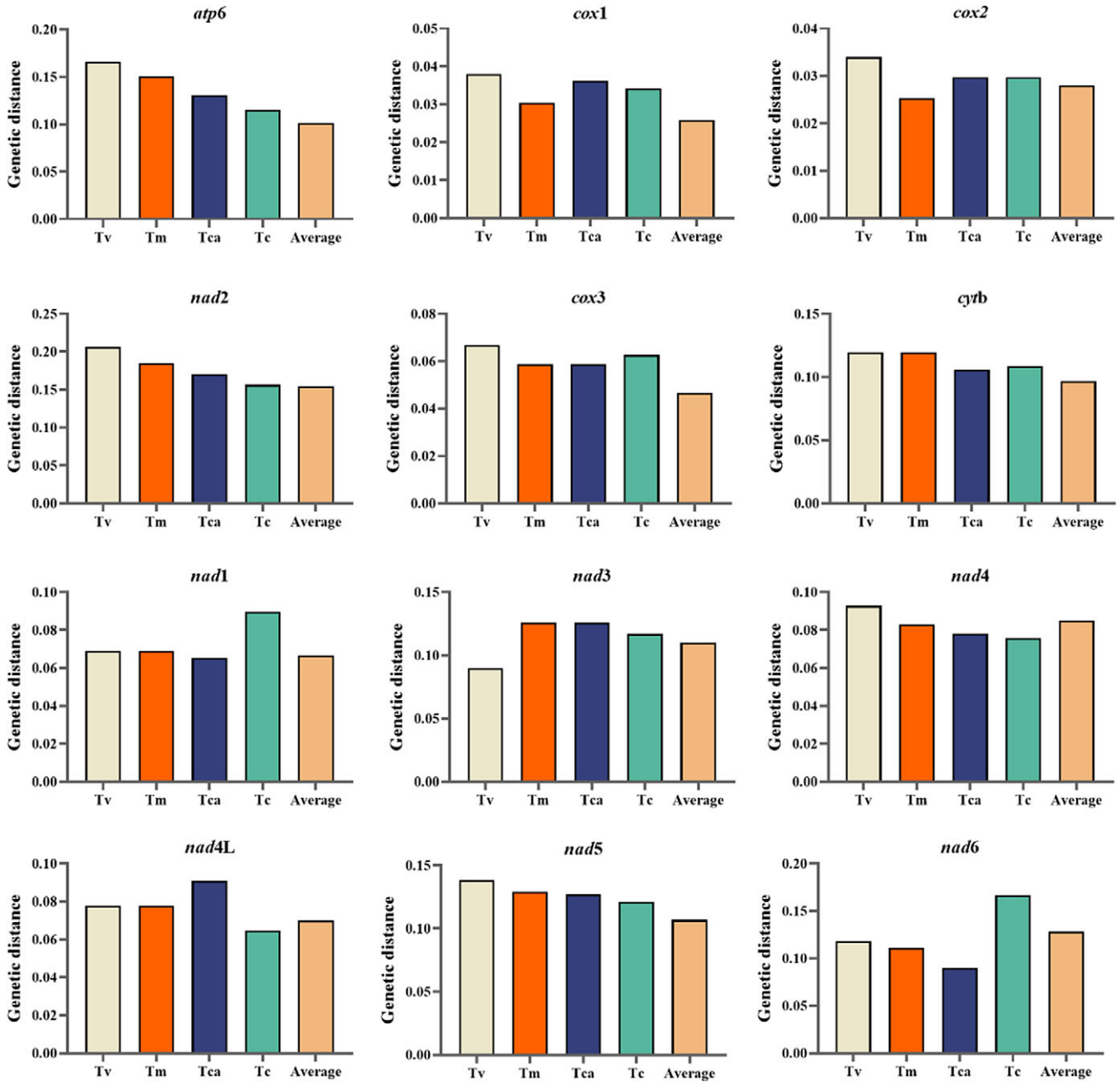

Figure 4. Genetic distance analysis of 12 PCGs among five *Toxocara* species. The genetic distances between *T. apodemi* and the other four *Toxocara* species are shown in the first four columns, and the average interspecific genetic distances of 12 PCGs among five *Toxocara* species are shown in the last column.

In a comparison with *T. apodemi*, the largest genetic distance (0.2064) was observed in the *nad*2 gene with *T. vitulorum*. The smallest genetic distance (0.0169) was found in the *cox*2 gene between *T. apodemi* and *T. malaysiensis* (Figure 4). Genetic distance analysis provided essential insights into the evolutionary mechanisms and patterns of the 12 PCGs in *Toxocara* mitochondrial genomes. The present results were larger than those of the 17 *Ascaris* samples (human-type *Ascaris*, pig-type *Ascaris*, and hybrid *Ascaris*) based on the 12 PCGs and the *cox*1 sequence reported in a previous study (Zhou *et al.* 2020). These findings indicate that the differences in the mtDNA sequence among the sequenced *Ascaris* individuals were smaller than those in *Toxocara*.

Phylogenetic analyses based on the concatenated amino acid sequences of 12 PCGs were used to assess the phylogenetic relationship of order for Ascaridomorpha with BI and ML methods (Figure 5, Figure S2). The two phylograms generated a similar topology, supporting the idea that each genus in the order forms a sister group with high statistical support, which was identical to that reported in a previous study (Xie *et al.* 2022). In the branch of *Toxocara*, *T. apodemi*, *T. canis*, and *T. cati* separately formed a distinct branch and were always sister taxa to other congeneric

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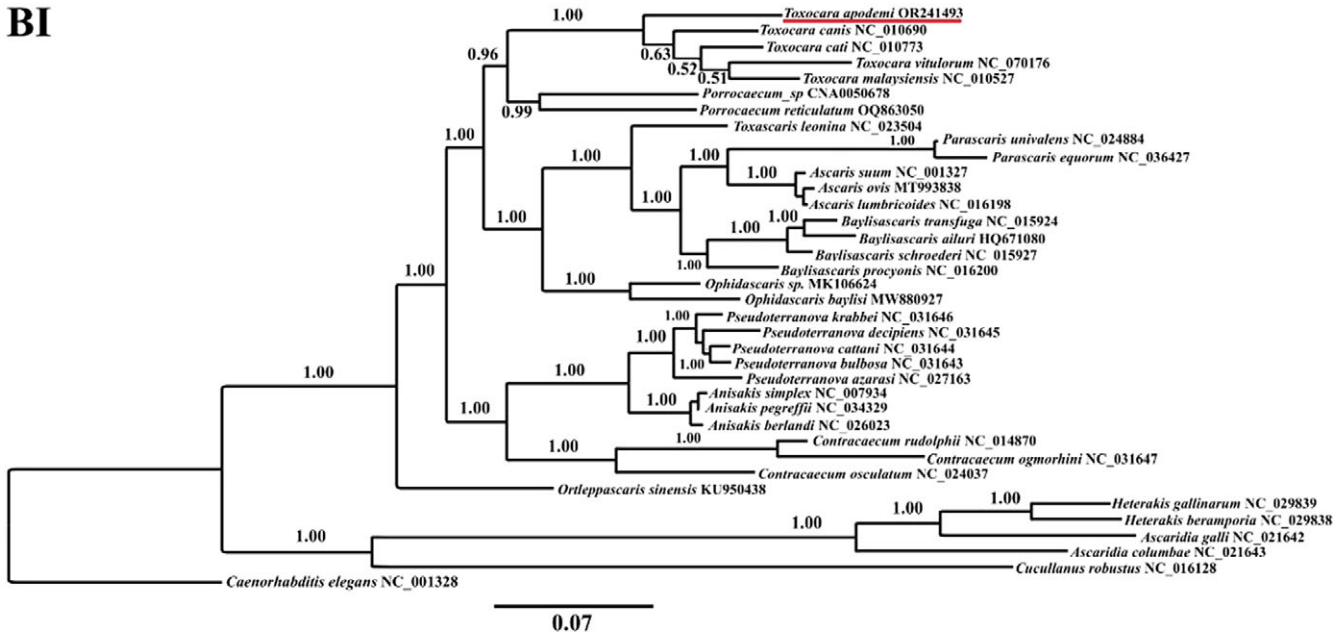

Figure 5. Phylogenetic analyses reconstructed using concatenated nucleotide sequences of 12 PCGs of complete mt genomes in 35 Ascaridomorpha species. The tree was performed by the BI method. *Caenorhabditis elegans* is an outgroup. *T. apodemi* in the current study is underlined.

species. *T. malaysiensis* and *T. vitulorum* clustered together, indicating that the two species were more closely related to each other than to other species in *Toxocara*. These results were inconsistent with findings of previous studies, in which *T. malaysiensis* and *T. cati* formed a sister group, and *T. vitulorum* formed a distinct branch (Li *et al.* 2008; Xie *et al.* 2022; Xing *et al.* 2022). Moreover, the phylogenetic analyses based on 18S showed that *T. apodemi* was more closely related to *T. canis* and *Toxascaris leonina* (Kim *et al.* 2020). In the present study, the mitochondrial genome sequences of *T. apodemi* strongly support it being a member of the *Toxocara* clade.

Conclusions

In the present study, the complete mitogenome sequence of *T. apodemi* was determined and characterised. Comparative genomics suggested that *T. apodemi* was more closely related to *T. canis* in nucleotide and amino acid sequences. Phylogenetic analysis showed that *T. apodemi* was a member of *Toxocara*. These results contribute novel genetic markers for the phylogenetic and evolutionary study of Toxocaridae species.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S0022149X24000221.

Financial support. This work was supported by the National Natural Science Foundation of China (32302897), "Pioneer" and "Leading Goose" R&D Program of Zhejiang (2022C02031), Zhejiang Provincial Key R&D Program of China (2021C02049), Development Science and Technology Program in Taizhou (23sfa02), and Taizhou City Foundation for Talents (TZ2022-2).

Competing interest. None.

Ethical standard. Not applicable.

References

Asakawa M, Li JF, Guo A, Yang X, Huhebateer Liu ZL, Liu YL, Cao X, Chen K (1994). A new host and locality record for *Toxocara apodemi* (Olsen, 1957) (Nematoda:Ascarididae) from striped field mice, *Apodemus agrarius* (pallas) (Rodentia:Murinae) in Changsha, China. *Journal of Rakuno Gakuen University Natural Science* **19**, 193–196.

Burland TG (2000). DNASTAR's Lasergene sequence analysis software. *Methods in Molecular Biology* **132**, 71–91. https://doi.org/10.1385/1-59259-192-2:71

Chagas ATA, Ludwig S, Pimentel JDSM, de Abreu NL, Nunez-Rodriguez DL, Leal HG, Kalapothakis E (2020). Use of complete mitochondrial genome sequences to identify barcoding markers for groups with low genetic distance. *Mitochondrial DNA Part A* **31**, 139–146. https://doi.org/10.1080/24701394.2020.1748609

Chen SY, Qiu QG, Mo HL (2022). Molecular identification and phylogenetic analysis of Ascarids in wild animals. *Frontiers in Veterinary Science* **9**, 891672. https://doi.org/10.3389/fvets.2022.891672

Gao JF, Zhang XX, Wang XX, Li Q, Li Y, Xu WW, Gao Y, Wang CR (2019). According to mitochondrial DNA evidence, *Parascaris equorum* and *Parascaris univalens* may represent the same species. *Journal of Helminthology* **93**, 383–388. https://doi.org/10.1017/S0022149X18000330

Gao Y, Zhang Z, Wang C, Zhao K (2022). The mitochondrial genome of *Cylicocyclus elongatus* (Strongylida: Strongylidae) and its comparative analysis with other *Cylicocyclus* species. *Animals* **12**, 1571. https://doi.org/10.3390/ani12121571

Gasser RB, Bott NJ, Chilton NB, Hunt P, Beveridge I (2008). Toward practical, DNA-based diagnostic methods for parasitic nematodes of livestock–bionomic and biotechnological implications. *Biotechnology Advances* **26**, 325–334. https://doi.org/10.1016/j.biotechadv.2008.03.003

Gu XH, Guo N, Chen HX, Sitko J, Li LW, Guo BQ, Li L (2023). Mitogenomic phylogenies suggest the resurrection of the subfamily Porrocaecinae and provide insights into the systematics of the superfamily Ascaridoidea (Nematoda: Ascaridomorpha), with the description of a new species of *Porrocaecum*. *Parasites & Vectors* **16**, 275. https://doi.org/10.1186/s13071-023-05889-9

Han L, Yang Y, Li H, Zhou X, Zhou M, Liu T, Lu Y, Wang Q, Yang S, Shi M, Li X, Du S, Guan C, Zhang Y, Guo W, Wang J, Chai H, Lan T, Liu H, Liu Q, Sun H, Hou Z (2022). Gene rearrangements in the mitochondrial genome of

ten ascaris species and phylogenetic implications for Ascaridoidea and Heterakoidea families. *International Journal of Biological Macromolecules* **221**, 1394–1403. https://doi.org/10.1016/j.ijbiomac.2022.08.021

Jin YC, **Li XY**, **Liu JH**, **Zhu XQ**, **Liu GH** (2019). Comparative analysis of mitochondrial DNA datasets indicates that *Toxascaris leonina* represents a species complex. *Parasites & Vectors* **12**, 194. https://doi.org/10.1186/s13071-019-3447-2

Jones DT, **Taylor WR**, **Thornton JM** (1992). The rapid generation of mutation data matrices from protein sequences. *Bioinformatics* **8**, 275–282. https://doi.org/10.1093/bioinformatics/8.3.275

Katoh K, **Standley DM** (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* **30**(4), 772–780. https://doi.org/10.1093/molbev/mst010

Kim HC, **Hong EJ**, **Ryu SY**, **Park J**, **Cho JG**, **Yu DH**, **Chae JS**, **Choi KS**, **Park BK** (2020). Morphological and molecular characterization of *Toxocara apodemi* (Nematoda: Ascarididae) from Striped Field Mice, *Apodemus agrarius*, in Korea. *The Korean Journal of Parasitology* **58**, 403–411. https://doi.org/10.3347/kjp.2020.58.4.403

Kumar S, **Stecher G**, **Li M**, **Knyaz C**, **Tamura K** (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* **35**, 1547–1549. https://doi.org/10.1093/molbev/msy096

Li MW, **Lin RQ**, **Song HQ**, **Wu XY**, **Zhu XQ** (2008). The complete mitochondrial genomes for three *Toxocara* species of human and animal health significance. *BMC Genomics* **9**, 224. https://doi.org/10.1186/1471-2164-9-224

Librado P, **Rozas J** (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451–1452. https://doi.org/10.1093/bioinformatics/btp187

Liu GH, **Wu CY**, **Song HQ**, **Wei SJ**, **Xu MJ**, **Lin RQ**, **Zhao GH**, **Huang SY**, **Zhu XQ** (2012). Comparative analyses of the complete mitochondrial genomes of *Ascaris lumbricoides* and *Ascaris suum* from humans and pigs. *Gene* **492**, 110–116. https://doi.org/10.1016/j.gene.2011.10.043

Liu GH, **Shao R**, **Li JY**, **Zhou DH**, **Li H**, **Zhu XQ** (2013a). The complete mitochondrial genomes of three parasitic nematodes of birds: A unique gene order and insights into nematode phylogeny. *BMC Genomics* **14**, 414. https://doi.org/10.1186/1471-2164-14-414

Liu GH, **Gasser RB**, **Otranto D**, **Xu MJ**, **Shen JL**, **Mohandas N**, **Zhou DH**, **Zhu XQ** (2013b). Mitochondrial genome of the eyeworm, *Thelazia callipaeda* (Nematoda: Spirurida), as the first representative from the family Thelaziidae. *PLoS Neglected Tropical Diseases* **7**(1), e2029. https://doi.org/10.1371/journal.pntd.0002029

Liu GH, **Nadler SA**, **Liu SS**, **Podolska M**, **D'Amelio S**, **Shao R**, **Gasser RB**, **Zhu XQ** (2016). Mitochondrial phylogenomics yields strongly supported hypotheses for Ascaridomorph nematodes. *Scientific Reports* **6**, 39248. https://doi.org/10.1038/srep39248

Larkin MA, **Blackshields G**, **Brown NP**, **Chenna R**, **McGettigan PA**, **McWilliam H**, **Valentin F**, **Wallace IM**, **Wilm A**, **Lopez R**, **Thompson JD**, **Gibson TJ**, **Higgins DG** (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947–2948. https://doi.org/10.1093/bioinformatics/btm404

Meng X, **Xie Y**, **Gu X**, **Zheng Y**, **Liu Y**, **Li Y**, **Wang L**, **Zhou X**, **Zuo Z**, **Yang G** (2019). Sequencing and analysis of the complete mitochondrial genome of dog roundworm *Toxocara canis* (Nematoda: Toxocaridae) from USA. *Mitochondrial DNA Part B* **4**, 2999–3001. https://doi.org/10.1080/23802359.2019.1666042

Okimoto R, **Macfarlane JL**, **Clary DO**, **Wolstenholme DR** (1992). The mitochondrial genomes of two nematodes, Caenorhabditis elegans *and* Ascaris suum. Genetics **130**, 471–498. https://doi.org/10.1093/genetics/130.3.471

Olsen LS (1957). A new species of *Neoascaris* (Nematoda) from a Korean wood mouse. *Transactions of the American Microscopical Society* **2**, 205–208.

Ronquist F, **Huelsenbeck JP** (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Warren EG (1972). Two new species of *Toxocara* from viverrid hosts. *Parasitology* **65**, 179–187. https://doi.org/10.1017/s0031182000044978

Xie Y, **Zhang Z**, **Wang C**, **Lan J**, **Li Y**, **Chen Z**, **Fu Y**, **Nie H**, **Yan N**, **Gu X**, **Wang S**, **Peng X**, **Yang G** (2011). Complete mitochondrial genomes of *Baylisascaris schroederi*, *Baylisascaris ailuri* and *Baylisascaris transfuga* from giant panda, red panda and polar bear. *Gene* **482**, 59–67. https://doi.org/10.1016/j.gene.2011.05.004

Xie Y, **Wang L**, **Chen Y**, **Wang Z**, **Zhu P**, **Hu Z**, **Han X**, **Wang Z**, **Zhou X**, **Zuo Z** (2022). The complete mitogenome of *Toxocara vitulorum*: Novel in-sights into the phylogenetics in Toxocaridae. *Animals* **12**, 3546. https://doi.org/10.3390/ani12243546

Xing ZP, **Liang X**, **Wang X**, **Hu HY**, **Huang YX** (2022). Novel gene rearrangement pattern in mitochondrial genome of *Ooencyrtusplautus* Huang & Noyes, 1994: New gene order in Encyrtidae (Hymenoptera, Chalcidoidea). *ZooKeys* **1124**, 1–21. https://doi.org/10.3897/zookeys.1124.83811

Yang J, **Huang X**, **Wang Y**, **Yang H**, **Zhang X**, **Zheng X** (2023). Complete mitogenome of *Nycteribia allotopa Speiser*, 1901 (Diptera, Hippoboscoidea, Nycteribiidae) and comparative analysis of mitochondrial genomes of Nycteribiidae. *Parasitology International* **96**, 102769. https://doi.org/10.1016/j.parint.2023.102769

Zhou C, **Guo T**, **Deng Y**, **He J**, **Ouyang S**, **Wu X** (2020). Mitochondrial phylogenomics of human-type Ascaris, pig-type Ascaris, and hybrid Ascaris populations. *Veterinary Parasitology* **287**, 109256. https://doi.org/10.1016/j.vetpar.2020.109256

Zhou CY, **Ma J**, **Tang QW**, **Zhu XQ**, **Xu QM** (2021). The mitogenome of *Ophidascaris wangi* isolated from snakes in China. *Parasitology Research* **120**, 1677–1686. https://doi.org/10.1007/s00436-021-07069-z

Ziegler MA, **Macpherson CNL** (2019). Toxocara and its species. *CAB Reviews* **14**, 53.

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