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Characterization of the complete mitochondrial genome of *Ortleppascaris sinensis* (Nematoda: Heterocheilidae) and comparative mitogenomic analysis of eighteen Ascaridida nematodes

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Abstract

Ortleppascaris sinensis (Nematoda: Ascaridida) is a dominant intestinal nematode of the captive Chinese alligator. However, the epidemiology, molecular ecology and population genetics of this parasite remain largely unexplored. In this study, the complete mitochondrial (mt) genome sequence of *O. sinensis* was first determined using a polymerase chain reaction (PCR)-based primer-walking strategy, and this is also the first sequencing of the complete mitochondrial genome of a member of the genus *Ortleppascaris*. The circular mitochondrial genome (13,828 bp) of *O. sinensis* contained 12 protein-coding, 22 transfer RNA and 2 ribosomal RNA genes, but lacked the ATP synthetase subunit 8 gene. Finally, phylogenetic analysis of mtDNAs indicated that the genus *Ortleppascaris* should be attributed to the family Heterocheilidae. It is necessary to sequence more mtNDAs of *Ortleppascaris* nematodes in the future to test and confirm our conclusion. The complete mitochondrial genome sequence of *O. sinensis* reported here should contribute to molecular diagnosis, epidemiological investigations and ecological studies of *O. sinensis* and other related Ascaridida nematodes.

Introduction

Ortleppascaris sinensis, a new nematode species, is described from specimens found in the stomach and intestine of the captive Chinese alligator *Alligator sinensis* Fauvel 1879 (Crocodilian: Alligatoridae) in China (Zhao *et al.*, 2015, 2016). Recently, mitochondrial (mt) genomics have been widely used for species-specific identification and differentiation of many zoonotic nematodes (Xie *et al.*, 2011b). Moreover, mitochondrial genomes contain useful genetic markers for studying the genetic structure and systematics of nematodes, as well as clinical diagnosis and epidemiology of parasitic disease, due to mutation rates that are proposed to be more rapid than those of nuclear genes (Hu *et al.*, 2004). To date, over 100 complete mitochondrial genome sequences have been published for class Chromadorea nematodes and 20 for class Enoplea, although about 15,000 species of nematodes have been recorded (https://www.ncbi.nlm.nih.gov/genomes/OrganelleResource.cgi?opt=organelle&taxid=6231). However, complete information on the mt genome of nematodes in the genus *Ortleppascaris* was not previously available.

Here, we report the complete nucleotide sequence of the mt genome from *O. sinensis* for the first time, and compare

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the sequence and genome organization with the sequences from related nematodes in the same order, Ascaridida. Additionally, the phylogenetic relationships of the genus *Ortleppascaris* within the order Ascaridida were investigated by the construction of phylogenetic trees (Bayesian inference (BI), maximum parsimony (MP) and maximum likelihood (ML)) using the sequence of protein-coding genes (PCG) dataset. More mitogenomes from related species will further enhance our understanding of molecular evolution and phylogenetic relationships within Ascaridida.

Materials and methods

Parasite collection and DNA extraction

Adult nematodes were obtained from the stomach and intestine of the Chinese alligator *A. sinensis* in the National Nature Reserve of China Alligator (Chinese Crocodile Lake) in Anhui Province, China. Nematodes were washed in physiological saline, identified to species *O. sinensis* based on morphological and molecular characterization (Zhao *et al.*, 2016), fixed in 75% ethanol and stored at −20°C until use. Total genomic DNA was isolated from individual nematodes using a TIANamp Genomic DNA Kit (Tiangen, Beijing, China) according to the manufacturer's protocol.

Primer design, PCR amplification and sequencing

To sequence the complete mitogenome of *O. sinensis*, short segments of *cox*1, *cox*1–*rrn*L and *rrn*L–*nad*3 were amplified by polymerase chain reaction (PCR) using a known specific degenerate primer (Hu *et al.*, 2002). After obtaining the mitogenome segments, we designed species-specific primers for the remaining parts, based on the conserved sequences in Ascaridida nematodes (see supplementary table S1).

PCR amplification reactions were performed in 50-µl volumes: 25 µl Premix Taq (LA Taq Version 2.0; Takara, Japan), 1.0 µl of each primer (forward and reverse; 20 µM), 2.0 µl template DNA and 21 µl sterile double-distilled water (ddH_2O). PCRs were performed with the following cycling parameters: 1 min at 94°C; followed by 35 cycles of 94°C for 30 s and 55–60°C for 5.0 min; and a final extension at 72°C for 10 min. PCR products were electrophoresed, excised and purified with a column DNA gel extraction kit (Sangon Biotech, Shanghai, China), and cloned into the pMD™19-T vector (Takara). The positive clones were sequenced directionally (ABI 3730; Applied Biosystems, Foster City, California, USA). To verify the number of variable-length AT-repeats in the largest non-coding region, DNA extracted from ten individual nematodes was used as a template for PCR.

Sequence analysis and annotation

The nucleotide sequences obtained were compared using BLAST against known sequences in GenBank, and then assembled with SeqMan software (DNAStar version 5.0; http://www.dnastar.com/). Protein-coding genes (PCGs) were determined by DNAStar version 5.0, implemented at the National Center for Biotechnology Information (NCBI) website with the invertebrate mitochondrial genetic code, as well as aligning their similarity against the published mitochondrial sequences of Ascaridida nematodes using Clustal X 2.0 and MEGA 6.0 (Tamura *et al.*, 2013). The majority of tRNA genes and their secondary structures were identified using tRNAscan-SE software V1.21 (Mohandas *et al.*, 2014). Two large non-protein-coding regions were candidates for the rRNA genes (*rrn*L and *rrn*S) by comparison with sequence similarity to genes in Ascaridida nematodes and previously reported conserved areas (Hu *et al.*, 2002; Xie *et al.*, 2011b). The secondary structure of non-coding regions (containing a putative control region) was folded using Mfold Server (Zuker, 2003). When multiple secondary structures were possible, the most stable one (lowest free energy (−ΔG)) was preferred. The base composition, codon usage, relative synonymous codon usage (RSCU) values and nucleotide substitution were analysed with Mega 6.0. Nucleotide composition skewness was calculated as AT skew = (A − T)/(A + T) and GC skew = (G − C)/(G + C), respectively.

Phylogenetic analyses

For the phylogenetic analysis, 17 nematode mitochondrial genome sequences of Ascaridida available from GenBank were used, in addition to the complete mtDNA sequences of *O. sinensis* determined in this study (see supplementary table S2); the classification status of the nematodes listed in supplementary table S2 was according to the GenBank. To infer the phylogenetic relationships of genus *Ortleppascaris* in the Ascaridida, we constructed phylogenetic trees based on the 12 protein-coding genes. Phylogenetic analyses were conducted using BI, MP and ML methods using *Wuchereria bancrofti* (NC_016186) as the outgroup (McNulty *et al.*, 2012). The GTR + G model was selected as the most appropriate model by MODELTEST v.3.7 (Abascal *et al.*, 2005) based on the Akaike information criterion (AIC). MP trees were implemented with a gap option as 'missing data' using PAUP* 4.0b10 (Swofford, 2002). The ML tree was reconstructed using PhyML 3.1 software (Guindon *et al.*, 2010) to assess the amount of phylogenetic signal in the dataset. As for BI analysis, the MrBayes 3.2 software was used to construct the tree (Ronquist *et al.*, 2012). The first 25% of the trees were discarded and the remaining trees were used to calculate Bayesian posterior probabilities (PP). Statistical tests for the alternative tree topology based on previously proposed hypotheses were performed using the likelihood-based Shimodaira–Hasegawa test implemented in BI, MP and ML trees.

Results and discussion

Genome structure, organization and composition

The complete mitogenome of *O. sinensis* was 13,828 bp in length, and its sequence has been deposited in GenBank under the accession number KU950438. The mitogenome encodes 12 PCGs (*cox*1–3, *nad*1–6, *nad*4L, *atp*6, *cytb*), 2 ribosomal RNAs (*rrn*S and *rrn*L), 22 tRNAs (*trn*A, *trn*R, *trn*N, *trn*D, *trn*C, *trn*Q, *trn*E, *trn*G, *trn*H, *trn*I, *trn*L1 CUN, *trn*L2 UUR, *trn*K, *trn*M, *trn*F, *trn*P,

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*trn*T, *trn*W, *trn*Y, *trn*V, *trn*S1 AGN and *trn*S2 UCN) , a non-coding region (NCR), an AT-rich region and lacks *atp*8. These genes are all located on the J-strand and are transcribed in the same direction, like the genomes of other nematodes of Chromadorea (fig. 1) (Lavrov & Brown 2001; Waku *et al*., 2016). The mitochondrial arrangement of *O. sinensis* was fully in accordance with the mode of GA7 (gene arrangement, GA) described by Yatawara *et al*. (2010). Eleven intergenic spacers totalling 50 bp, and five overlapping regions totalling 5 bp were scattered throughout the whole genome (table 1). Compared to the mitochondrial arrangement of Ascaridida nematodes, that of *O. sinensis* was similar to those of the Ascaridae, Toxocaridae and Anisakidae, but different from those of Cucullanidae and Ascaridiidae (fig. 2) (He *et al*., 2005; Li *et al*., 2008; Xie *et al*., 2011a). Compared to the mitochondrial arrangement of *Ascaridia columbae* and *Ascaridia galli* in the Ascaridiidae, there were large differences: *cox*1 and *rrn*S were translocated, and tRNAs, such as *trn*D, *trn*E, *trn*S2, *trn*F and *trn*C, were severely rearranged, translocated and/or inverted. Moreover, the position of the AT-rich region of *O. sinensis* was between the genes *trn*S2 and *trn*N, and the position of the NCR of *O. sinensis* was between *nad*4 and *cox*1. This is the same as reported for the nematodes of the families Ascaridae, Toxocaridae and Anisakidae (Li *et al*., 2008; Xie *et al*., 2011b), whereas the AT-rich region was located between genes *trn*A and *trn*P in hookworms (*Ancyclostoma caninum*, *A. duodenale*, *Bunostomum phlebotomum* and *Necator americanus*). Such differences and similarities in the mitogenome of different nematodes suggests that structure and composition of the mitochondrial genome can be used to analyse phyletic evolution and population genetics for nematode species (J.K. Park *et al*., 2011; Mohandas *et al*., 2014).

Base content

Like other Ascaridida nematodes, the nucleotide composition for the *O. sinensis* mitochondrial DNA was also biased toward A and T (A = 26.1%, T = 48.0%, C = 8.4%, and G = 17.6%) (see supplementary table S3). We can see from the table that the AT content of *O. sinensis* (74.0%) was the highest among the 18 Ascaridida nematodes, though its mtDNA length was the shortest apart from that of *Contracaecum osculatum*. In each Ascaridida nematode, the nucleotide composition of these genes (PCGs, rRNA, tRNA and AT-rich region) were biased toward A

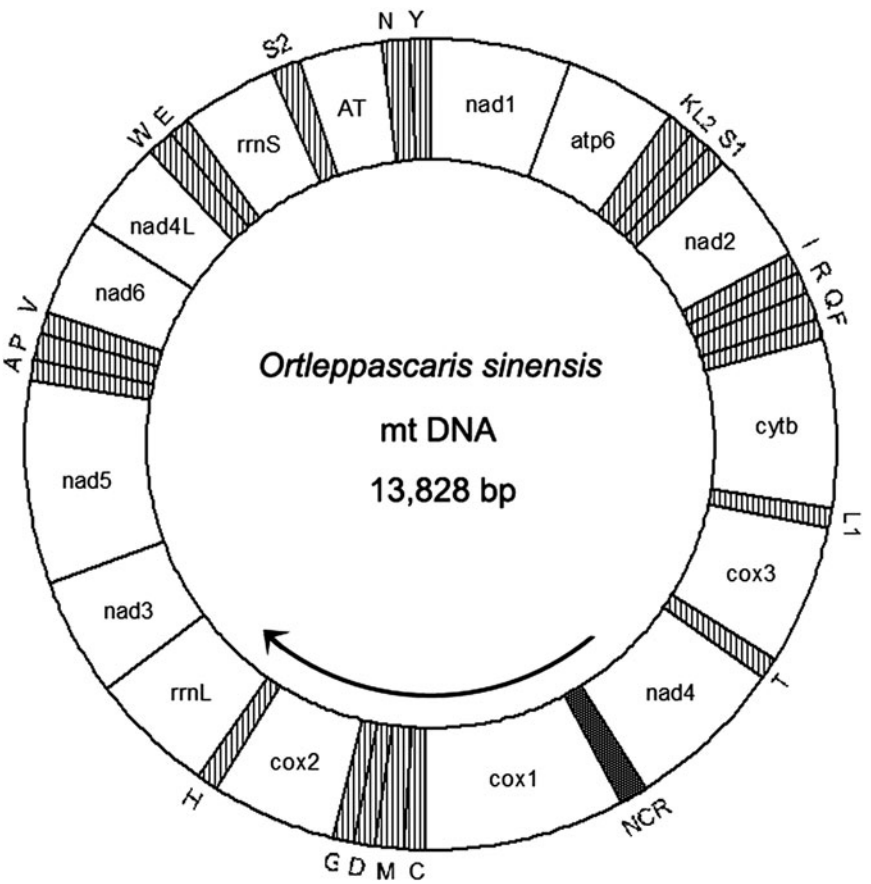

Fig. 1. Circular representation of the mitochondrial genome of *O. sinensis*. Protein-coding and ribosomal genes are shown with standard abbreviations. Genes for tRNAs are abbreviated by the single-letter amino acid code, with S1 = AGN, S2 = UCN, L1 = CUN, and L2 = UUR. AT: AT-rich region; NCR: non-coding region. Genes are not to scale and the tRNA genes are indicated by hatched areas.

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Table 1. Organization of the *O. sinensis* mitochondrial genome.

Gene	Position	Size	Spacer(+) Overlap(−)	Start codon	Stop codon	AA	Anticodon	AT-skew	GC-skew
*nad*1	1–873	873	1(−)	ATT	TAA	290		−0.38	0.40
*atp*6	873–1470	598	0	ATA	T	199		−0.38	0.43
*trn*K	1471–1533	63	1(+)				TTT		
*trn*L2 UUR	1535–1589	55	0				TAA		
*trn*S1 AGN	1590–1644	55	0				TCT		
*nad*2	1645–2488	844	0	GTA	T	281		−0.36	0.37
*trn*I	2489–2551	63	15(+)				GAT		
*trn*R	2567–2622	56	1(−)				ACG		
*trn*Q	2622–2676	55	7(+)				TTG		
*trn*F	2684–2742	59	0				GAA		
*cyt*b	2743–3847	1105	0	ATA	T	368		−0.38	0.32
*trn*L1 CUN	3848–3902	55	0				TAG		
*cox*3	3903–4670	768	1(−)	ATT	TAG	255		−0.41	0.27
*trn*T	4670–4727	58	1(+)				TGT		
*nad*4	4729–5958	1230	0	ATG	TAA	409			
NCR	5959–6049	91	0						
*cox*1	6050–7627	1578	1(−)	TTG	TAG	525		−0.36	0.34
*trn*C	7627–7645	56	0				GCA		
*trn*M	7646–7743	61	0				CAT		
*trn*D	7744–7799	56	0				GTC		
*trn*G	7800–7855	56	0				TCC		
*cox*2	7856–8593	738	2(+)	ATT	TAA	245		−0.29	0.39
*trn*H	8596–8652	57	3(+)				GTG		
*rrn*L	8656–9616	961	0						
*nad*3	9617–9952	336	1(−)	TTG	TAA	111		−0.39	0.65
*nad*5	9952–11533	1582	0	ATA	T	527		−0.36	0.39
*trn*A	11534–11591	56	1(+)				TGC		
*trn*P	11593–11645	55	0				TGG		
*trn*V	11646–11700	55	0				TAC		
*nad*6	11701–12135	435	0	TTG	TAG	144		−0.46	0.38
*nad*4L	12136–12369	234	6(+)	ATT	TAG	77		−0.36	0.56
*trn*W	12376–12434	59	1(+)				TCA		
*trn*E	12436–12496	61	2(+)				TTC		
*rrn*S	12499–13197	699	11(+)						
*trn*S2 UCN	13209–13262	54	0				TGA		
AT	13263–13714	452	0						
*trn*N	13715–13771	57	0				GTT		
*trn*Y	13772–13828	57	0				GTA		

AT: AT-rich region. NCR: Non-coding region.

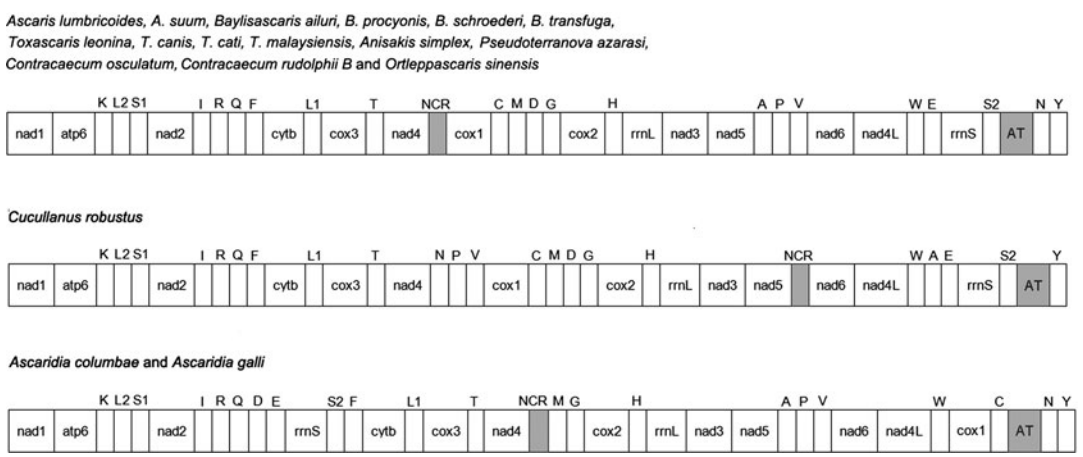

Fig. 2. Mitochondrial genome organization of 18 Ascaridida nematodes. Protein-coding and ribosomal genes are shown with standard abbreviations. Genes for tRNAs are abbreviated by the single-letter amino acid code, with S1 = AGN, S2 = UCN, L1 = CUN, and L2 = UUR. AT: AT-rich region; NCR: non-coding region. Genes are not to scale.

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and T; the AT content of the AT-rich region was the highest, followed by that of rRNA; and the AT content was lowest in PCGs compared to the other genes. In general, Ascaridida nematodes have an AT-specific bias and rejection of C in nucleotide composition (Kim *et al.*, 2006; Li *et al.*, 2008).

A comparative analysis of AT% vs. AT-skew and GC% vs. GC-skew across complete mitogenomes of 18 Ascaridida nematodes is shown in fig. 3. The average AT-skew of the mitogenome of Ascaridida nematodes was −0.36, ranging from −0.28 in *A. columbae* to −0.41 in *A. galli*, whereas the AT-skew in the mitogenome of *O. sinensis* was −0.30. The average GC-skew of the mitogenome of Ascaridida nematodes was 0.41, ranging from 0.32 in *Anisakis simplex* to 0.49 in *A. galli*, whereas the GC-skew in the *O. sinensis* mitogenome was 0.35. The mitochondrial genomes of Ascaridida nematodes usually have negative AT-skews and positive GC-skews, like the mitochondrial genomes of metazoans (Lavrov & Brown 2001). Although the exact mechanism causing the AT

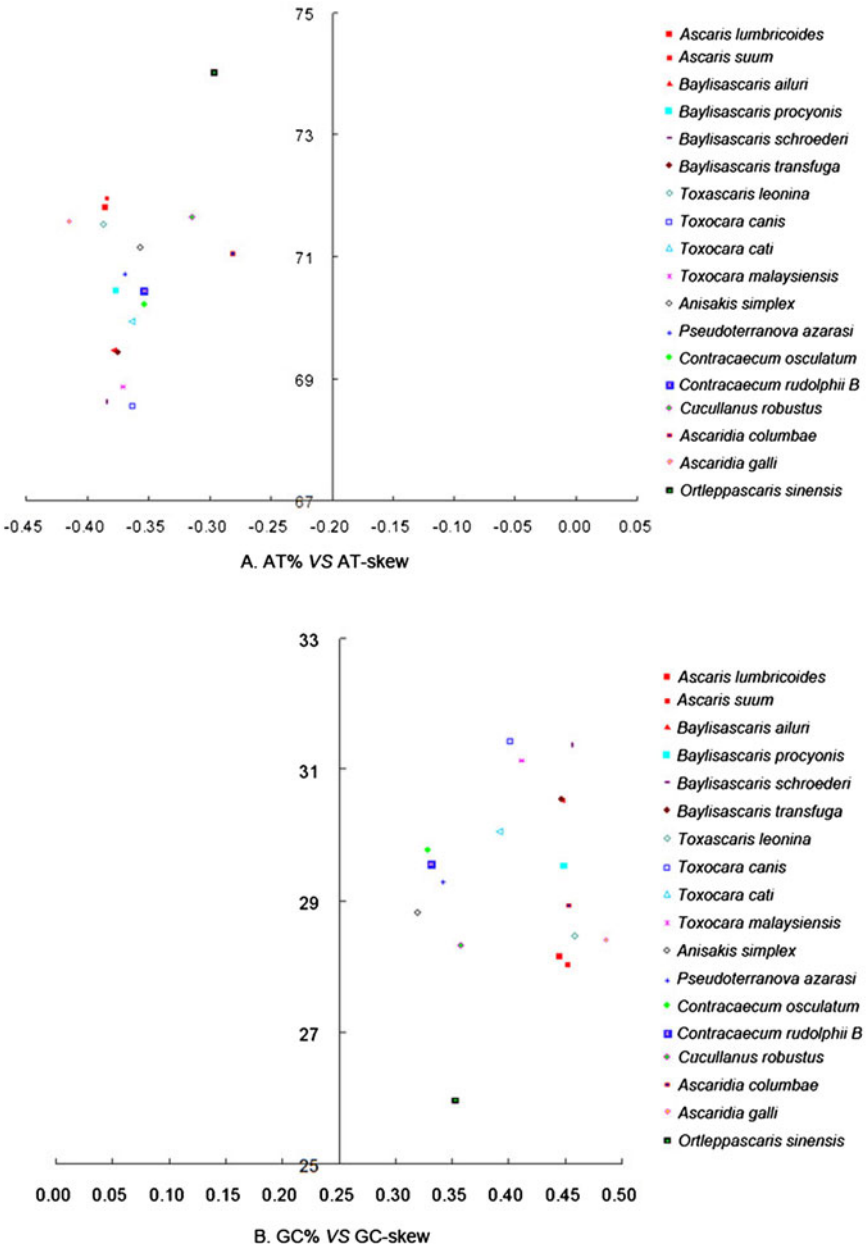

Fig. 3. AT% vs. AT-skew and GC% vs. GC-skew of the mitochondrial genomes of 18 Ascaridida nematodes. Values are calculated for full-length mitochondrial genomes. The *x*-axis shows the level of nucleotide skew and the *y*-axis indicates the nucleotide percentages.

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bias in mtDNA remains unknown, such a situation could be the result of inversions of the AT-rich regions and replication origin (Oliveira *et al.*, 2015).

Protein-coding genes and codon usage

The overall length of the 12 PCGs in the *O. sinensis* mitogenome was 10,319 bp, accounting for 74.62% of the total length of the mitogenome, encoding 3419 amino acids. Among the 12 PCGs of *O. sinensis*, *nad*5 is the longest (1582 bp) and *nad*4L is the shortest (234 bp). However, among the 12 PCGs of the 18 Ascaridida nematodes, *nad*3 (333 bp or 336 bp) and *nad*6 (1230 bp or 1236 bp) are the most conserved genes, and *cytb* (1101 bp, 1102 bp, 1104 bp, 1105 bp, 1107 bp, 1095 bp, 1098 bp or 1099 bp) is the most variable gene in length; the AT content *of nad*4L and *nad*6 is relatively high, but *cox*1, *cox*2 and *cytb* kept a relatively low level (see supplementary table S4). This nucleotide bias is bound to have an effect on the codon use of mtDNAs and the kinds of amino acids.

In *O. sinensis* all the PCGs utilized typical ATN, GTA and TTG start codons (see supplementary table S5). In detail, four genes (*nad*1, *cox*3, *cox*2 and *nad*4L) started with codon ATT, three genes (*atp*6, *cytb* and *nad*5) with ATA, three genes (*cox*1, *nad*3 and *nad*6) with TTG , one gene (*nad*4) with ATG and one gene (*nad*2) with GTA. Although almost all the PCGs utilized the conventional stop codons TAA (four times) or TAG (four times), the remaining genes (*atp*6, *cytb*, *nad*5 and *nad*2) used incomplete stop codons, such as a single T. In Ascaridida nematodes, the most commonly used start codons were TTG and ATT, but GTA, GTT, GTG, ATA, ATG and TTT were also used, of which TTT was only used as a start codon by *nad*6 genes in *A. galli*. The stop codons most commonly used were the complete termination codons TAA or TAG, but incomplete termination codons (T or TA) were also found. The presence of an incomplete termination codon is commonly observed in metazoan mitochondrial genomes and these incomplete termination codons would likely be completed via post-transcriptional polyadenylation (Kim *et al.*, 2006; Li *et al.*, 2008; Oliveira *et al.*, 2015; Grosemans *et al.*, 2016).

The codons of the 12 PCGs of *O. sinensis* are analysed in supplementary table S6, including the frequency of each codon and relative synonymous codon usage (RSCU). We found that three codon positions possess different nucleotide biases – mean U in the second codon position and U, A or G in the third codon position. Among them, UUU was the most common (468), CUC, CAC and CCG were used only once, yet CGC (R) and CCG (P) did not exist in the codons of the 12 PCGs for *O. sinensis*. There was also codon bias (a tendency to use codons with A, U and G) in the other nematodes of Ascaridida. The codons with higher frequency of use were UUU (F), AUU (I), UUA (L), UUG (L), GUU (V), UAU (Y), UCU (S), GGU (G), AAU (N) and AGU (S). In addition, codons with a lower frequency of use, and even a lack of some codons, were all seen in some nematodes of Ascaridida. For example, the codons CUC (L), CCC (P) and CGC (R) were not present in *Ascaris lumbricoides*, and the codons CUC (L), CCC (P), UGC (C) and CGC (R) were not present in *Ascaris suum*. The RSCU for the mitochondrial proteins in *O. sinensis* are shown in supplementary fig. S1. The highest frequency of RSCU was CGU (3.64), and the lowest frequency was CUC (0.01). The frequency of RSCU is significantly reduced if the third base position is replaced by C; for example, the frequency of the UUU codon encoding phenylalanine (F) for *O. sinensis* mtDNA is 13.69%, while that of the synonymous codon UUC was just only 0.38%. This phenomenon could be explained by synonymous codon usage bias. Generally codon bias was proposed to be highest in gene regions of functional significance and is believed to be important for maximizing translation efficiency (Duret & Mouchiroud, 1999).

Excluding the start and stop codons, the amino acid frequencies are similar among the 18 nematodes of Ascaridida (see supplementary table S7). The most overused amino acids were Leu (145.92–167.60‰), Phe (129.95–151.04‰), Ser (102.76–113.20‰) and Val (89.21–117.73‰), and the amino acid with lowest use was Arg (9.102–10.93‰). The mitogenome of *O. sinensis* has a relatively lower value in the proportion of the amino acids Leu (147.70‰, average 151.25‰), Phe (140.68‰, average 143.15‰) and Val (89.21‰, average 103.54‰). Usually, proteins encoded by mitochondrial genomes are mostly membrane proteins, and Phe and Leu occur in hydrophobic side chains of such proteins (Li *et al.*, 2008; Xie *et al.*, 2011b).

Transfer RNA genes

Twenty-two tRNA genes were identified in the mitochondrial genome of *O. sinensis* (table 1). The tRNA sequences ranged from 55 to 63 bases in length, and have non-canonical secondary structures (see supplementary fig. S2). The tRNA structures shared some features with those of other metazoans, including a 7-bp amino-acyl arm, a 4-bp dihydrouridine (DHU) arm, a 5-bp anticodon stem, a 7-base anticodon loop, a T always preceding an anticodon, as well as a purine always following an anticodon. Twenty tRNAs (*trn*A, *trn*R, *trn*N, *trn*D, *trn*C, *trn*Q, *trn*E, *trn*G, *trn*H, *trn*I, *trn*L1 CUN, *trn*L2 UUR, *trn*K, *trn*M, *trn*F, *trn*P, *trn*T, *trn*W, *trn*Y and *trn*V) have a 4-bp DHU stem and a DHU loop of 4–11 bases, but lack the TΨC arm and instead have a 'TV-replacement loop' of 7–13 bases. The tRNA genes without TΨC arm for *A. suum* also had normal biological function (Okimoto *et al.*, 1992). The *trn*S for *O. sinensis* possesses a secondary structure consisting of a DHU replacement loop of 7 bases (*trn*S1 AGN) or 4 bases (*trn*S2 UCN), a 3-bp TΨC arm, a TΨC loop of 6 bases (*trn*S1 AGN) or 7 bases (*trn*S2 UCN) and a variable loop of 4 bases (*trn*S1 AGN) or 5 bases (*trn*S2 UCN). Their tRNA secondary structures were similar to those of all other Secernentea nematodes studied to date (Kim *et al.*, 2006; Li *et al.*, 2008), but were distinct from those of *Trichinella spiralis* (Lavrov & Brown, 2001). This unusual feature has been described not only in Ascaridida nematodes but also in other invertebrates, such as *Pupa strigosa* (opisthobranch gastropod), *Laqueus rubellus* (articulate brachiopod) and *Katharina tunicata* (chiton) (Kurabayashi & Ueshima, 2000). The secondary structure and anticodon of tRNA genes in the mitochondrial genome of metazoans were relatively conservative, which can be used for the classification of the families or genera for analysis of system evolution.

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There were also a certain number of base mismatches in the amino-acyl arm, DHU arm, anticodon arm and TΨC arm, and give priority to G·U weak pairing. This phenomenon of base mismatch can be corrected by RNA editing (Fujishima & Kanai, 2014).

Ribosomal RNA genes

The *rrn*S and *rrn*L genes of *O. sinensis* were identified by sequence comparison with Ascaridida nematodes. The *rrn*S gene was located between *trn*E and *trn*S2 UCN, and *rrn*L was located between *trn*H and *nad*3. The two genes were separated from one another by the protein genes *nad*3, *nad*5, *nad*6 and *nad*4L (fig. 2). The size of the *rrn*S gene for *O. sinensis* was 699 bp, and the size of the *rrn*L gene was 961 bp. The sizes of these two genes for *O. sinensis* were similar to those of other nematodes (for *rrnS*, 677–703 bp; for *rrn*L, 955–961 bp) (see supplementary table S3) (Kim *et al*., 2006; Li *et al*., 2008). The AT contents of the *rrn*S (73.8%) and *rrn*L (77.9%) for *O. sinensis* were higher when compared with other Ascaridida nematodes (for *rrn*S, 66.4–73.9%; for *rrn*L, 70.1–78.9%), and only slightly lower than those of *Cucullanus robustus* (supplementary table S3).

Non-coding regions

In the *O. sinensis* mitochondrial genome, the longest non-coding region (AT-rich region) was located between genes *trn*S UCN and *trn*N, as in the mitochondrial genomes of nematodes *A. lumbricoides*, *Baylisascaris ailuri*, *Toxascaris leonina*, *A. simplex*, *Pseudoterranova azarasi* and *C. osculatum*, but differing from *C. robustus* and *A. columbae* (J.K. Park *et al*., 2011; Y.C. Park *et al*., 2011; Liu *et al*., 2013, 2014; Mohandas *et al*., 2014; Liu *et al*., 2015; Xie *et al*., 2011b) (fig. 2). The length of the AT-rich region of *O. sinensis* was 452 bp, which was the shortest, apart from *C. osculatum*, compared with other Ascardida nematodes (239–1516 bp). The AT-rich region is the most variable portion of the genome, both in terms of length and nucleotide sequence, among the mitochondrial genomes of 18 Ascardida nematodes (see supplementary table S8). The AT content of the AT-rich region for *O. sinensis* was 88.9%, significantly greater than that of other Ascaridida nematodes (68.3–89.1%) apart from *Contracaecum rudolphii B*. Additionally, the AT-rich region of the *O. sinensis* mtDNA contained four regions with varying numbers of the dinucleotide TA repeats ($n = 8$, 10, 26, 33) within a total of 154 bp. Similar multiple TA repeats have been described in the AT-rich region of the mitochondrial genomes of other Ascaridida and Strongylida species (Li *et al*., 2008). The AT-rich region has been known to serve as an essential element involved in the initiation of replication and transcription of the mitogenome. Thus, it possibly functions as a control region (Janssen *et al*., 2016).

The second-longest non-coding region (NCR) of *O. sinensis* mtDNA was located between genes *nad*4 and *cox*1, and is relatively shorter (91 bp, average 113 bp) compared with the other 17 Ascaridida nematodes; the NCR of *A. galli* (157 bp) is the longest in length, and *A. suum* (70 bp) is the shortest. However, the NCR of *O. sinensis* has a relatively higher value of AT content (76.9%, average 75.1%) (supplementary table S8). The NCR may function as splicing recognition sites during processing of the transcripts (He *et al*., 2005).

Phylogenetic analysis

The phylogenetic relationships were constructed using BI, MP and ML analyses based on a concatenated mitochondrial sequence dataset of the 12 PCGs of *O. sinensis* and all the other 18 Ascaridida nematodes available

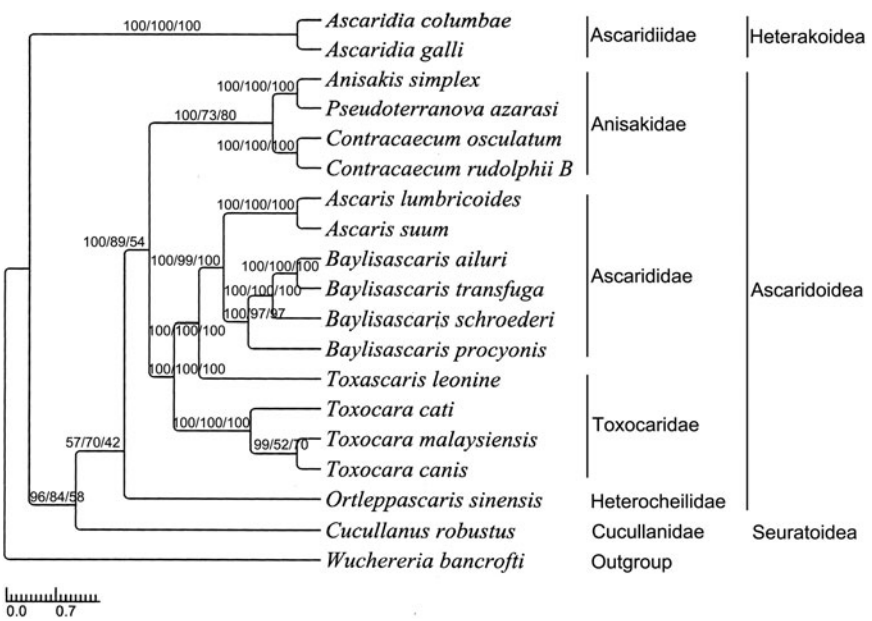

Fig. 4. Phylogenetic tree of 18 Ascaridida nematodes based on the data of 12 protein-coding genes (BI/MP/ML).

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(supplementary table S2). Three phylogenetic trees inferred by BI, MP and ML analyses had identical topologies (fig. 4). Almost all nodes were well supported by high bootstrap values and high Bayesian posterior probabilities, except for some node confidence values. All the nematodes of the order Ascaridida belong to three superfamilies (Ascaridoidea, Seuratoidea and Heterakoidea), six families (Anisakidae, Ascarididae, Toxocaridae, Heterocheilidae, Cucullanidae and Ascaridiidae) and nine genera (*Anisakis*, *Pseudoterranova*, *Contracaecum*, *Baylisascaris*, *Toxocara*, *Ortleppascaris*, *Cucullanus* and *Ascaridia*). Heterocheilidae and Anisakidae/Toxocaridae/Ascarididae have a closer bond than Cucullanidae and Ascaridiidae within Ascaridida. In addition, our analyses showed that *Baylisascaris* (four nematodes) and *Ascaris* (two nematodes) had a close affinity, and then clustered as a taxonomic group with *Toxocara* (four nematodes) within the family Ascarididae; *Anisakis* (one nematode) and *Pseudoterranova* (one nematode) had a close affinity, and then made a sister taxon with *Contracaecum* (two nematodes). The results are similar to both morphological classification and phylogenetic analysis based on molecular evidence (Xie *et al.*, 2011b).

The genus *Ortleppascaris* was removed from the genus *Dujardinascaris* by Sprent (1978) and placed in a new genus *Ortleppascaris* according to morphological characteristics (Sprent, 1978). Recently, *Dujardinascaris* has been attributed to the Heterocheilidae (Lakshmi & Sudha, 2000; Masova *et al.*, 2014) or Anisakidae (Moravec & Jirku, 2014) when some species of this genus were reported, and *Ortleppascaris* was attributed to the Ascarididae (Silva *et al.*, 2013) or just Ascarididea (Sprent, 1977, 1978; Zhao *et al.*, 2016) when some species were reported. So, it is not very clear to which family the genera *Otleppascaris* and *Dujardinascaris* should be attributed. In the trees inferred by the BI, MP and ML methods, the newly sequenced *O. sinensis* clustered distinctly as a single clade. In addition, this clade consistently clustered with the Anisakidae group with high BI, MP and ML bootstrap values (100, 89 and 54, respectively). Our analyses indicate that *O. sinensis* cannot be allocated to the families Anisakidae and Ascarididae, and may be attributed to another family – Heterocheilidae – which had been supported by some morphological and molecular data (Lakshmi & Sudha, 2000; Masova *et al.*, 2014). Although our results have indicated that *O. sinensis* belongs to the family Heterocheilidae, we should be cautious because no other mtDNA sequences of Heterocheilidae are available to date. So it is necessary to sequence more mtDNAs of *Ortleppascaris* nematodes in the future, to test and confirm our conclusion. Furthermore, given that, in the present study, the phylogenetic analysis was based only on mtDNA sequences of 12 PCGs, it is still necessary to employ more molecular datasets of the Ascaridida nematodes, or to expand taxon sampling from the lineage, to examine the phylogenetics and genome evolution of the Ascaridida.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X17000542

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Conflict of interest

None.

References

Abascal, F., Zardoya, R. & Posada, D. (2005) ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* **21**, 2104–2105.

Duret, L. & Mouchiroud, D. (1999) Expression pattern and, surprisingly, gene length shape codon usage in *Caenorhabditis*, *Drosophila*, and *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **96**, 4482–4487.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**, 294–299.

Fujishima, K. & Kanai, A. (2014) tRNA gene diversity in the three domains of life. *Frontiers in Genetics* **5**, 142. doi: 10.3389/fgene.2014.00142.

Grosemans, T., Morris, K., Thomas, W.K., Rigaux, A., Moens, T. & Derycke, S. (2016) Mitogenomics reveals high synteny and long evolutionary histories of sympatric cryptic nematode species. *Ecology and Evolution* **6**, 1854–1870.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* **59**, 307–321.

He, Y., Jones, J., Armstrong, M., Lamberti, F. & Moens, M. (2005) The mitochondrial genome of *Xiphinema americanum sensu stricto* (Nematoda: Enoplea): considerable economization in the length and structural features of encoded genes. *Journal of Molecular Evolution* **61**, 819–833.

Hu, M., Chilton, N.B. & Gasser, R.B. (2002) The mitochondrial genomes of the human hookworms, *Ancylostoma duodenale* and *Necator americanus* (Nematoda: Secernentea). *International Journal for Parasitology* **32**, 145–158.

Hu, M., Chilton, N.B. & Gasser, R.B. (2004) The mitochondrial genomics of parasitic nematodes of socio-economic importance: recent progress, and implications for population genetics and systematics. *Advances in Parasitology* **56**, 133–212.

Janssen, T., Karssen, G., Verhaeven, M., Coyne, D. & Bert, W. (2016) Mitochondrial coding genome analysis of tropical root-knot nematodes (*Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution. *Scientific Reports* **6**, 22591. doi: 10.1038/srep22591.

Kim, K.H., Eom, K.S. & Park, J.K. (2006) The complete mitochondrial genome of *Anisakis simplex* (Ascaridida: Nematoda) and phylogenetic implications. *International Journal for Parasitology* **36**, 319–328.

Kurabayashi, A. & Ueshima, R. (2000) Complete sequence of the mitochondrial DNA of the primitive opisthobranch gastropod *Pupa strigosa*: systematic implication of the genome organization. *Molecular Biology and Evolution* **17**, 266–277.

Lakshmi, B.B. & Sudha, M. (2000) Note on *Dujardinascaris cybii* Arya and Johnson, 1978 (Nematoda: Heterocheilidae) from new host, *Mugil cephalus* (Linnaeus). *Boletin Chileno de Parasitologia* **55**, 45–46.

Lavrov, D.V. & Brown, W.M. (2001) *Trichinella spiralis* mtDNA: a nematode mitochondrial genome that encodes a putative ATP8 and normally structured tRNAs and has a gene arrangement relatable to those of coelomate metazoans. *Genetics* **157**, 621–637.

Li, M.W., Lin, R.Q., Song, H.Q., Wu, X.Y. & Zhu, X.Q. (2008) The complete mitochondrial genomes for three *Toxocara* species of human and animal health significance. *BMC Genomics* **9**, 224. doi: 10.1186/1471-2164-9-224.

Lin, R.Q., Liu, G.H., Zhang, Y., D'Amelio, S., Zhou, D. H., Yuan, Z.G., Zou, F.C., Song, H.Q. & Zhu, X.Q. (2012) *Contracaecum rudolphii* B: gene content, arrangement and composition of its complete mitochondrial genome compared with *Anisakis simplex* s.l. *Experimental Parasitology* **130**, 135–140.

Liu, G.H., Shao, R., Li, J.Y., Zhou, D.H., Li, H. & Zhu, X. Q. (2013) The complete mitochondrial genomes of three parasitic nematodes of birds: a unique gene order and insights into nematode phylogeny. *BMC Genomics* **14**, 414. doi: 10.1186/1471-2164-14-414.

Liu, G.H., Zhou, D.H., Zhao, L., Xiong, R.C., Liang, J.Y. & Zhu, X.Q. (2014) The complete mitochondrial genome of *Toxascaris leonina*: comparison with other closely related species and phylogenetic implications. *Infection, Genetics and Evolution* **21**, 329–333.

Liu, S.S., Liu, G.H., Zhu, X.Q. & Weng, Y.B. (2015) The complete mitochondrial genome of *Pseudoterranova azarasi* and comparative analysis with other anisakid nematodes. *Infection, Genetics and Evolution* **33**, 293–298.

Masova, S., Barus, V., Seifertova, M., Malala, J. & Jirku, M. (2014) Redescription and molecular characterisation of *Dujardinascaris madagascariensis* and a note on *D. dujardini* (Nematoda: Heterocheilidae), parasites of *Crocodylus niloticus*, with a key to *Dujardinascaris* spp. in crocodilians. *Zootaxa* **3893**, 261–276.

McNulty, S.N., Mullin, A.S., Vaughan, J.A., Tkach, V.V., Weil, G.J. & Fischer, P.U. (2012) Comparing the mitochondrial genomes of Wolbachia-dependent and independent filarial nematode species. *BMC Genomics* **13**, 145. doi: 10.1186/1471-2164-13-145.

Mohandas, N., Jabbar, A., Podolska, M., Zhu, X.Q., Littlewood, D.T., Jex, A.R. & Gasser, R.B. (2014) Mitochondrial genomes of *Anisakis simplex* and *Contracaecum osculatum* (sensu stricto) – comparisons with selected nematodes. *Infection, Genetics and Evolution* **21**, 452–462.

Moravec, F. & Jirku, M. (2014) *Dujardinascaris mormyropsis* n. sp. (Nematoda: Anisakidae) from the osteoglossiform fish *Mormyrops anguilloides* (Linnaeus) (Mormyridae) in Central Africa. *Systematic Parasitology* **88**, 55–62.

Okimoto, R., Macfarlane, J.L., Clary, D.O. & Wolstenholme, D.R. (1992) The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum*. *Genetics* **130**, 471–498.

Oliveira, M.T., Haukka, J. & Kaguni, L.S. (2015) Evolution of the metazoan mitochondrial replicase. *Genome Biology and Evolution* **7**, 943–959.

Park, J.K., Sultana, T., Lee, S.H., Kang, S., Kim, H.K., Min, G.S., Eom, K.S. & Nadler, S.A. (2011) Monophyly of clade III nematodes is not supported by phylogenetic analysis of complete mitochondrial genome sequences. *BMC Genomics* **12**, 392.

Park, Y.C., Kim, W. & Park, J.K. (2011) The complete mitochondrial genome of human parasitic roundworm, *Ascaris lumbricoides*. *Mitochondrial DNA* **22**, 91–93.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M.A. & Huelsenbeck, J.P. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**, 539–542.

Silva, J.P., da Silva, D.C., Melo, F.T., Giese, E.G., Furtado, A.P. & Santos, J.N. (2013) Liver histopathology in the cane toad, *Rhinella marina* (Amphibia: Bufonidae), induced by *Ortleppascaris* sp. larvae (Nematoda: Ascarididae). *Journal of Parasitology* **99**, 250–256.

Sprent, J.F. (1977) Ascaridoid nematodes of amphibians and reptiles: *Dujardinascaris*. *Journal of Helminthology* **51**, 253–287.

Sprent, J.F. (1978) Ascaridoid nematodes of amphibians and reptiles: *Gedoelstascaris* n.g. and *Ortleppascaris* n.g. *Journal of Helminthology* **52**, 261–282.

Swofford, D.L. (2002) *PAUP*:Phylogenetic Analysis Using Parsimony (and other methods), version 4.0b10*. Sunderland, Sinauer.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725–2729.

Waku, D., Segawa, T., Yonezawa, T., Akiyoshi, A., Ishige, T., Ueda, M., Ogawa, H., Sasaki, H., Ando, M., Kohno, N. & Sasaki, T. (2016) Evaluating the phylogenetic status of the extinct Japanese otter on the basis of mitochondrial genome analysis. *PLoS One* **11**, e0149341. doi: 10.1371/journal.pone.0149341.

Wolstenholme, D.R., Okimoto, R. & Macfarlane, J.L. (1994) Nucleotide correlations that suggest tertiary interactions in the TV-replacement loop-containing mitochondrial tRNAs of the nematodes, *Caenorhabditis elegans* and *Ascaris suum*. *Nucleic Acids Research* **22**, 4300–4306.

Xie, Y., Zhang, Z., Niu, L., Wang, Q., Wang, C., Lan, J., Deng, J., Fu, Y., Nie, H., Yan, N., Yang, D., Hao, G., Gu, X., Wang, S., Peng, X. & Yang, G. (2011a) The mitochondrial genome of *Baylisascaris procyonis*. *PLoS One*, **6**, e27066. doi: 10.1371/journal.pone.0027066.

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. (2011b) Complete mitochondrial genomes of *Baylisascaris schroederi, Baylisascaris ailuri* and *Baylisascaris transfuga* from giant panda, red panda and polar bear. *Gene* **482**, 59–67.

Yatawara, L., Wickramasinghe, S., Rajapakse, R.P. & Agatsuma, T. (2010) The complete mitochondrial genome of *Setaria digitata* (Nematoda: Filarioidea): mitochondrial gene content, arrangement and composition compared with other nematodes. *Molecular and Biochemical Parasitology* **173**, 32–38.

Zhao, J., Wang, S., Tu, G., Zhou, Y., Wu, X. & Li, C. (2015) Histopathology of gastric wall in chinese alligator *Alligator sinensis* infected with *Ortleppascaris sinensis* (Nematoda: Ascaridoidea). *Nutricion Hospitalaria* **32**, 1180–1183.

Zhao, J.H., Wang, S.S., Tu, G.J., Zhou, Y.K. & Wu, X.B. (2016) Morphological and molecular characterization of *Ortleppascaris sinensis* sp. nov. (Nematoda: Ascaridoidea) from the Chinese alligator *Alligator sinensis*. *Journal of Helminthology* **90**, 303–311.

Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research* **31**, 3406–3415.