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Title:
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Date:
2013-03-10

Citation:
Jabbar, A., Jex, A. R., Mohandas, N., Hall, R. S., Littlewood, D. T. J. & Gasser, R. B. (2013). The mitochondrial genome of Aelurostrongylus abstrusus-diagnostic, epidemiological and systematic implications. Gene, 516 (2), pp.294-300. https://doi.org/10.1016/j.gene.2012.10.072.

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PII: S0378-1119(12)01356-X
DOI: doi: 10.1016/j.gene.2012.10.072
Reference: GENE 38034

To appear in: *Gene*

Accepted date: 29 October 2012

Please cite this article as: Jabbar, Abdul, Jexa, Aaron R., Mohandas, Namitha, Hall, Ross S., Littlewood, D. Timothy J., Gasser, Robin B., The mitochondrial genome of *Aelurostrongylus abstrusus* – Diagnostic, epidemiological and systematic implications, *Gene* (2012), doi: 10.1016/j.gene.2012.10.072

GENE-D-12-01040.R1

26th Aug 2012

(~3600 words)

The mitochondrial genome of *Aelurostrongylus abstrusus* – diagnostic, epidemiological and systematic implications

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ABSTRACT

Aelurostrongylus abstrusus (Railliet, 1898) is a metastrongylid nematode of major clinical relevance in felids, causing aelurostrongylosis. In spite of its clinical importance in cats, the genetics, epidemiology and biology of this parasite are not entirely understood. Mitochondrial (mt) DNA can provide markers for studies of these areas, but genetic data are scant for *A. abstrusus* and related lungworms. Here, the mitochondrial genome was amplified by long-range polymerase chain reaction (long-PCR) from a single male adult of *A. abstrusus*, sequenced using 454 technology and annotated using an established bioinformatic pipeline. This circular mitochondrial genome is 13,913 bp and contains two ribosomal RNA, 12 protein-coding and 22 transfer RNA genes, consistent with most other chromadorean nematodes. This mt genome should provide a source of markers for future investigations of the epidemiology and ecology of *A. abstrusus*. Molecular tools, employing such mt markers, are likely to find utility for explorations into the epidemiology, biology and systematics of this parasite, and the diagnosis of feline aelurostrongylosis.

ARTICLE INFO

Keywords:

Aelurostrongylus abstrusus

Aelurostrongylosis

Lungworm

Cat

Mitochondrial genome

1. Introduction

Aelurostrongylus abstrusus (Railliet, 1898) is a metastrongyloid nematode of felids, including domesticated cats (definitive hosts) (Anderson, 2000). This nematode causes aelurostrongylosis, a respiratory disease that typically manifests itself in coughing, tachypnoea, dyspnoea, tachycardia, weight loss and sometimes death (Bowman et al., 2002; Traversa and Guglielmini, 2008; Traversa et al., 2010). The dioecious adults of *A. abstrusus* live in the cardio-respiratory system (terminal bronchioles and alveoli) of the definitive felid host; here, the oviparous females produce eggs, from which first-stage larvae (L1s) hatch within the airways of the lung. L1s then migrate *via* the bronchial/tracheal escalator to the pharynx, are swallowed and then excreted in the faeces. L1s infect a molluscan intermediate host (snail or slug), either by ingestion, while foraging on faeces or by penetration of the epidermis, and then develop, under favourable environmental conditions, into third-stage larvae (L3) (Anderson, 2000). Animals, such as amphibians, reptiles, birds and rodents may act as paratenic host, following the ingestion of infected snails or slugs (Hobmaier and Hobmaier, 1935; Hamilton, 1963; Anderson, 2000). L3s within an infected intermediate or paratenic hosts are then ingested by cats, penetrate the gut wall and then migrate via the lymphatic system or blood stream to the lungs, where they develop to adult worms. The prepatent period is reported to be ~ 1 month (Hamilton and McCaw, 1968).

With an apparent spread and increasing clinical relevance of felid aelurostrongylosis, particularly in Europe, the Americas and Australia (Traversa and Guglielmini, 2008; Lacorcia et al., 2009; Traversa et al., 2010), there has been a focus on improved diagnosis and treatment (Traversa et al., 2010). An emphasis has been placed on targeted treatment of clinical cases with anthelmintics, such as fenbendazole, macrocyclic lactones (off-label) and, more recently, emodepside (Traversa et al., 2009a,b, 2010). However, in spite of the clinical significance of feline aelurostrongylosis, little is known about the epidemiology, ecology and genetics of *A. abstrusus*, which is central to the management of this disease.

Molecular techniques using suitable genetic markers can readily underpin such fundamental studies. Mitochondrial (mt) genomes provide markers suitable for genetic, epidemiological and ecological studies of nematodes, with a perspective on exploring transmission patterns linked to particular genotypes of a parasite and discovering population variants or cryptic species (cf. Hu and Gasser, 2006; Jex et al., 2010a). Recent developments in DNA sequencing and bioinformatics provide a sound foundation for characterizing the mt genomes from parasitic nematodes as a source of genetic markers for fundamental systematic and epidemiological studies. Here, we used a combined massively parallel sequencing-bioinformatics platform (Jex et al., 2010b) for the characterization of the mt genome of *A. abstrusus*, which we compared with those of related metastrongylids, including *Angiostrongylus vasorum*, *An. cantonensis* and *An. costaricensis* (see Gasser et al., 2012; Lv et al., 2012), for which mt genome data already exist. We also studied the genetic relationships among these lungworms and selected representatives of different superfamilies within the order Strongylida, and suggest that some regions in the genome of *A. abstrusus* might serve well as markers for future studies of the molecular epidemiology of this species in various countries around the world.

2. Materials and methods

2.1. Parasite and genomic DNA isolation

Adult worms of *A. abstrusus* were collected from the lung of a fresh cat cadaver (cf. Lacorcia et al., 2009), washed extensively in physiological saline and then stored at -80 °C. Upon thawing, genomic DNA was isolated from a single adult male specimen using a small-scale sodium dodecyl-sulphate (SDS)/proteinase K digestion and column-purification (Wizard DNA Clean-Up kit, Promega, USA) (cf. Gasser et al., 2006). The identity of the specimen was verified by PCR-based amplification of the second internal transcribed spacer

(ITS-2) of nuclear ribosomal DNA using an established method, followed by mini-column purification (Wizard PCR-Preps, Promega) of the amplicon and subsequent sequencing (BigDye chemistry v.3.1) (cf. Gasser et al., 2006).

2.2. Long-PCR, sequencing and mt genome assembly

From the genomic DNA from the single male worm, the complete mt genome was amplified by long-PCR (BD Advantage 2, BD Biosciences) as two overlapping amplicons (~5 kb and ~10 kb), using the protocol described by Hu et al. (2007), with appropriate positive (i.e., *An. vasorum* DNA) and negative (i.e., no template) controls. Amplicons were consistently produced from the positive control samples; in no case was a product detected for the negative controls. Amplicons were then treated with shrimp alkaline phosphatase and exonuclease I (Werle et al., 1994) and quantified spectro-photometrically. Following agarose electrophoretic analysis, the two amplicons (2.5 µg of each) were pooled and subsequently sequenced using the 454 Genome Sequencer FLX (Roche) (Margulies et al., 2005). The mt genome sequence (GenBank accession no. JX519458) was assembled using the program CAP3 (Huang and Madan, 1999) from individual reads (of ~300 bp).

2.3. Annotation and analyses of sequence data

Following assembly, the mt genome of *A. abstrusus* was annotated using an established bioinformatic annotation pipeline (Jex et al., 2010b). Briefly, each protein coding mt gene was identified by local alignment comparison (performed in six reading frames) using amino acid sequences conceptually translated from corresponding genes from the mt genomes of *An. vasorum*, *An. cantonensis* and *An. costaricensis* (accession nos. JX268542, GQ398121 and GQ398122, respectively; see Gasser et al., 2012; Lv et al., 2012). The large and small subunits of the mt ribosomal RNA genes (*rrn*S and *rrn*L, respectively) were identified by

local alignment (i.e., using nucleotide sequence data). The transfer RNA (tRNA) genes were predicted (from both strands) based on their structure, using scalable models based on the standard nematode mt tRNAs (Hu and Gasser, 2006). All predicted tRNA genes were then grouped according to their anti-codon sequence and identified based on the amino acid encoded by this anti-codon. Two separate tRNA gene groups were predicted each for leucine (Leu) (one each for the anticodons CUN and UUR, respectively) and for serine (Ser) (one each for the anticodons AGN and UCN, respectively), as these tRNA genes are duplicated in many invertebrate mt genomes, including those of nematodes (Hu and Gasser, 2006). All predicted tRNAs for each amino acid group were ranked according to "structural strength" (inferred based on minimum nucleotide mismatches in each stem), and, for each group, the 100 best-scoring structures were compared by BLASTn against a database comprising all tRNA genes for each amino acid of all published nematode mt genome sequences (available *via* http://drake.physics.mcmaster.ca/ogre/; Jameson et al., 2003). All tRNA genes were then identified and annotated based on their highest sequence identity to known nematode tRNAs. Annotated sequence data were imported using the program SEQUIN (*via* http://www.ncbi.nlm.nih.gov/Sequin/), the mt genome structure verified and the final sequence submitted to the GenBank database.

2.4. Phylogenetic analysis of concatenated amino acid sequence datasets

The amino acid sequences conceptually translated from individual genes of the mt genome of *A. abstrusus* were concatenated as well as from published mt genomes for related metastrongylids *An. vasorum*, *An. cantonensis*, *An. costaricensis*, *Metastrongylus pudendotectus* and *M. salmi* (GenBank accession nos. GQ398121, GQ398122, JX268542, GQ888714 and GQ888715, respectively; Metastrongyloidea); *Ancylostoma caninum* and *Necator americanus* (FJ483518 and NC_003416, respectively; Anyclostomatoidea); *Haemonchus contortus* and *Trichostrongylus axei* (NC_010383 and GQ888719,

respectively; Trichostrongyloidea); *Oesophagostomum dentatum* and *Strongylus vulgaris* (GQ888716 and GQ888717, respectively; Strongyloidea); and *Strongyloides stercoralis* (AJ558163) (Strongyloidoidea) as an outgroup (Okimoto et al., 1992; Hu et al., 2002, 2003a; Li et al., 2008; Jex et al., 2008, 2009, 2010; Gasser et al., 2012; Lv et al., 2012). All concatenated amino acid sequences of all species were aligned using MUSCLE (Edgar, 2004) and then subjected to phylogenetic analysis using Bayesian inference (BI) (cf. Jex et al., 2010b) employing the software package MrBayes v.3.1.2 (http://mrbayes.csit.fsu.edu/index.php). Analysis was conducted with four independent Markov chain runs for 200,000 metropolis-coupled MCMC generations, sampling a tree every 100 generations in MrBayes v.3.1.2 (http://mrbayes.csit.fsu.edu/index.php). The first 200 trees were omitted as burn-in, and the remaining trees were used to calculate posterior probabilities (pp). Phylograms were drawn using the Tree View program v1.65 (Page, 1996).

3. Results and discussion

3.1. Mt genome features

The circular mt genome sequence of *A. abstrusus* is 13,913 bp in length (Fig. 1). It contains two ribosomal genes, 12 protein-coding (*cox*1-3, *nad*1-6, *nad*4L, *atp*6 and *cyt*B) and 22 tRNA genes. The gene arrangement in the mt genome of *A. abstrusus* was consistent with GA2 representing all other nematodes of the order Strongylida investigated to date (see Hu and Gasser, 2006; Jex et al., 2010b). All of the 36 genes are transcribed in the same direction (5'>3') (Fig. 1). Overall, the genome is AT-rich, as expected for chromadorean nematodes (Hu and Gasser, 2006), with T being the most favoured nucleotide and C the least favoured. The nucleotide contents were 20.9% (A), 6.9% (C), 20.8% (G) and 49.8% (T) (Table 1). The longest non-coding (AT-rich) region, located between the *nad*5 and *trn*A, was 377 bp in

length (see Fig. 1); its AT-content was 76.3%, significantly greater than for all other parts of the mt genome (Table 2).

3.2. Ribosomal RNA genes

The *rrn*S and *rrn*L genes of *A. abstrusus* were identified by sequence comparison with *An. vasorum*. The *rrn*S gene was located between *trn*E and *trn*S (UCN), and *rrn*L was 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. 1). The size of the *rrn*S gene of *A. abstrusus* was 696 bp. The size of the *rrn*L gene of *A. abstrusus* was 961 bp. The lengths of these two genes were similar to those of other Metastrongyloidea for which mt genomes are known (696-699 bp for for *rrn*S, and 958-961 bp for *rrn*L; cf. Jex et al., 2010b; Gasser et al., 2012; Lv et al., 2012; Fig. 1), and amongst the shortest for metazoan organisms (cf. Lavrov and Brown, 2001). The overall identity in the *rrn*S sequence between *A. abstrusus* and *An. vasorum* was 73.3%, whereas for the *rrn*L, the identity was 70.2%.

3.3. Protein-coding genes and codon usage

The prediction of initiation and termination codons for the protein-coding genes of *A. abstrusus* (Table 2) showed that the commonest start codon was ATT (for nine of 12 proteins), followed by TTG (two genes) and GTT (one gene). All mt protein genes were predicted to have a TAA or TAG translation termination codon.

The codon usage for the 12 protein genes of *A. abstrusus* was also compared with that of *An. vasorum* (Table 3); All 64 codons were used. The preferred nucleotide usage at the third codon position of mt protein genes of *A. abstrusus* reflects the overall nucleotide composition of the mt genome. At this position, T is the most frequently, and C the least frequently used. For *A. abstrusus*, the codons ending in G have higher frequencies than the

codons ending in A, which, interestingly, is similar to, for example, *Toxocara cati* and *Dirofilaria immitis* (see Hu et al., 2003b; Li et al., 2008), but distinct from other members of the Strongylida and *Caenorhabditis elegans* (Rhabditida) (see Okimoto et al., 1992; Hu et al., 2002, 2003a). As the usage of synonymous codons is proposed to be preferred in gene regions of functional importance, codon bias appears to be associated with selection at silent sites and to maximize translation efficiency (Sharp and Matassi, 1994; Durent and Mouchiroud, 1999).

The AT bias in the genome is also reflected as a bias in the amino acid composition of predicted proteins. The AT-rich codons represent the amino acids Phe, Ile, Met, Tyr, Asn or Lys, and the GC-rich codons represent Pro, Ala, Arg or Gly. In the mt genome of *A. abstrusus*, the most frequently used codons were TTT (Phe), TTA (Leu), ATT (Ile), TTG (Leu), TAT (Tyr), GGT (Gly) and GTT (Val). Five of these codons are AT-rich, and one of them is GC-rich. Six of the seven codons contained an A or a T in the two positions, except for GGT (Gly), which contained a T only in the third position. None of them had a C at any position. The least frequently used codons were CTA, CTG (Leu), ATC (Ile), GTC (Val), AGC (Ser), CCC (Pro), GCC (Ala), TAC (Tyr), CAC (His), AAC (Asn), CGA (Arg), TCC (Ser) and GGC (Gly). All four GC-rich codons were represented here, and every codon had at least one C. When the frequencies of synonymous codons within the AT-rich group were compared, such as Phe (TTT, 15.5%; TTC, 0.5%), Ile (ATT, 4.7%; ATC, 0.2%), Tyr (TAT, 5.2%; TAC, 0.3%) and Asn (AAT, 2.8%; AAC, 0.03%), the frequency was always less if the third position was a C.

3.4. Transfer RNA genes

Twenty-two tRNA gene sequences were identified in the mt genome of *A. abstrusus*. These sequences ranged from 52-62 nt in length. The tRNA structures had a 7 bp amino-acyl arm, a 4 bp 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 of the 22 tRNA genes (i.e. excluding the two *trn*S genes) have a predicted secondary structure with a 4 bp DHU stem and a DHU loop of 5-10 bases, in which the variable TψC arm and loop are replaced by a "TV-replacement loop" of 4-11 bases, in accordance with most nematodes whose mt genomes are known (Hu and Gasser, 2006). The mt *trn*S for *A. abstrusus* has a secondary structure consisting of a DHU replacement loop of 7 bases, 3 bp TψC arm, TψC loop of 4-8 bases and a variable loop of 3 bases, consistent with other members of the Chromadorea (e.g., Okimoto et al., 1992; Keddie et al., 1998; Jex et al., 2010a; Park et al., 2011), but distinct from *T. spiralis* and *T. murrelli* (Enoplea) (see Lavrov and Brown, 2001; Webb and Rosenthal, 2010, 2011). An overlap of one nucleotide is found between the *trn*S (UCN) and *trn*N genes in the mt genome of *A. abstrusus*. Another overlap was detected between *trn*E and *rrn*S.

3.5. Amino acid sequence comparisons and genetic relationships of A. abstrusus *with other metastrongylids and selected representatives of the Chromadorea*

The amino acid sequences predicted from individual protein encoding mt genes of *A. abstrusus* were compared with those of *An. vasorum, An. cantonensis* and *An. costaricensis* from the dog (Table 4). Pairwise comparisons of the concatenated sequences revealed identities of 55.1-94.3% between these species. Based on identity, COX1 was the most conserved protein, whereas NAD2 and NAD6 were the least conserved. Phylogenetic analysis of the concatenated amino acid sequence data for the 12 mt proteins showed that *A. abstrusus* was more closely related to *An. vasorum, An. cantonensis* and *An. costaricensis* (pp = 1.00) (Metastrongyloidea) than to *M. pudendotectus* and *M. salmi* (Metastrongyloidea) (pp = 1.00), to the exclusion of *H. contortus, T. axei* (Trichostrongyloidea), *Anc. caninum, N. americanus* (Ancylostomatoidea) and *O. dentatum* and *S. vulgaris* (Strongyloidea) (Fig. 2) (pp = 1.00).

3.6. Fundamental and applied implications

The characterisation of the mt genome of *A. abstrusus* paves the way for future genetic, epidemiology/ecology, biology and systematic studies as well as improved diagnosis of *A. abstrusus* infection. Clearly, aelurostrongylosis is a neglected disease of major feline health significance (Traversa et al., 2010). Clinical diagnosis is challenging (Traversa and Gugliemini, 2008), and parasitological diagnosis is often not undertaken in clinical practice; in some cases, misdiagnoses are made mainly due to the similarities in symptoms among a number of respiratory diseases in cats (Bowman, 2000; Bowman et al., 2002). Parasitological diagnosis, which is often based on the detection of L1s in feline faeces, also has its challenges, because the traditional methods, such as the direct faecal smear and flotation (depending on the protocols used) are not sufficiently sensitive for the detection of larvae, and the Baermann technique (Baermann, 1917) is not used routinely in small animal practice, because the overnight incubation to collect/concentrate L1 slows actual diagnosis. Nonetheless, a thorough comparative study (cf. Lacorcia et al., 2009) demonstrated that the Baermann technique was more sensitive than various other traditional approaches, including bronchoalveolar lavage, faecal sedimentation-flotation and necropsy. Recently, molecular methods have been developed to improve detection (reviewed by Traversa and Guglielmini, 2008) and to assist in undertaking prevalence surveys of *A. abstrusus* in cats. In particular, a PCR-based assay, utilizing species-specific genetic markers in the second internal transcribed spacer (ITS-2) region of nuclear rDNA, was evaluated for the diagnosis of *A. abstrusus* infection based on the testing of various types of samples (faeces, flotation supernatant, Baermann sediment and pharyngeal swab) (Traversa et al., 2008). This PCR assay was reported to be specific (100%) and achieved a sensitivity of 96.6% for diagnosis, a practical application being the specific detection of parasite DNA from pharyngeal swabs (Traversa et al., 2008).

Because sequence variation in ITS-2 is usually low within most species of strongylid nematode, mitochondrial DNA is better suited for assessing population variation (see Gasser, 2006). Therefore, having available the mitochondrial genome of *A. abstrusus* sets the scene to develop a combined PCR-based diagnostic and analytical approach, whereby mitochondrial genetic markers, such as *cox*1, *nad*1 and *nad*4 (displaying varying levels of within-species divergence) might be used to explore haplotypic variation in *A. abstrusus* populations in cats (definitive host) and also in molluscan and paratenic hosts. Given that species complexes are commonly encountered in bursate nematodes (Anderson et al., 1998; Blouin, 2002; Gasser, 2006), it would be interesting to prospect for cryptic species, assess whether various haplotypes/genotypes of *A. abstrusus* might have particular host and infection site preferences, and cause different clinical forms/manifestations of aelurostrongylosis (Bowman, 2000; Bowman et al., 2002), and whether particular subpopulations of *A. abstrusus* differ in their immunobiology in their host/s or undergo arrested development (hypobiosis) in tissues to survive for long periods of time in intermediate, paratenic and/or definitive hosts.

PCR-coupled mutation scanning and sequencing of mt gene regions (with differing levels of sequence variation) (cf. Gasser et al., 2006) would be well suited for this purpose, and has been applied previously to the bovine lungworm, *D. viviparus* (see Hu et al., 2004; Höglund et a., 2006). Here, interestingly, both the mt DNA diversity within populations and the gene flow among populations of *D. viviparus* were low, similar to results for selected parasitic nematodes of plants and insects, but, interestingly, different from gastrointestinal trichostrongyloid nematodes of domesticated ruminants, which are considered to have relatively high levels of diversity and gene flow (see Blouin et al., 1992, 1995, 1997). Such differences have been interpreted to relate mainly to parasite biology, transmission patterns, population sizes as well as differences in host movement, and might be of epidemiological relevance. Similar aspects would be interesting to study in *A. abstrusus* in different countries and distinct hosts, particularly given the complexity of the parasite's life cycle, biology and

ecology compared with, for example, *Dictyocaulus* species - which have direct rather than indirect life cycles. In addition, there is a conservation and wildlife management imperative in better identifying and tracking *A. abstrusus*. For example, wild populations of jaguarundi (*Herpailurus yagouaroundi*) in Brazil (Noronha et al., 2002), and recovering, wild populations of Eurasian lynx (*Lynx lynx*) have been found to be infected with this nematode (Schmidt-Posthaus et al., 2002), as have wild and exotic felids held captive in zoos; e.g., cheetah (*Acinonyx jubatus*) (Robert and Walzer, 2009). Planned reintroductions, movement and management of endemic or endangered felids would clearly benefit from improved diagnosis of *A. abstrusus* and other parasitic infections.

Importantly, the findings from the present study also stimulate a reassessment of the evolutionary relationships of lungworms within the order Strongylida using mitochondrial datasets. For decades, there has been considerable debate about the evolutionary relationships of the different groups (e.g., suborders) within this order based on morphological datasets (Skrjabin et al., 1952). For instance, it has been hypothesized that the suborder Metastrongylina (sometimes referred to as "true" lungworms), to which *Aelurostrongylus* and *Angiostrongylus* species belong, came from ancestors in the Trichostrongylina (Schulz, 1951; Durette-Desset et al., 1994) or the Strongylina (Dougherty, 1949, 1951); another proposal was that the Metastrongylina gave rise to the Strongylina (Skrjabin, 1941). A previous study by Chilton et al. (2006) suggested that the Trichostrongylina are basal to the Metastrongylina; their phylogenetic analyses of nuclear ribosomal rDNA sequence data indicated that the Metastrongylina represented a monophyletic assemblage (in agreement with Carreno and Nadler, 2003), and together with the genus *Dictyocaulus*, were a sister group to a clade representing the Trichostrongylina, Ancylostomatina and Strongylina. Given the utility of mt proteomic datasets, high phylogenetic signal and consistent evidence of high nodal support values in recent phylogenetic analyses (Park et al., 2011; Jex et al., 2010b), there is now an opportunity to

test, using such datasets for a broad spectrum of strongylid nematodes, whether the Metastrongylina, including *Dictyocalus* species, represents a monophyletic assemblage and are a sister group to the Trichostrongylina, Ancylostomatina and Strongylina.

Acknowledgements

The Early Career Researcher and Collaborative Research grants (AJ) from The University of Melbourne are gratefully acknowledged. The present study was supported by ARC grant LP100100091 (RBG, ARJ and DTJL). Associated research is also funded by the Australian Research Council (ARC), the National Health and Medical Research Council (NHMRC) and Melbourne Water Corporation.

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Table 1

Nucleotide composition (%) of the entire mt genome of *Aelurostrongylus abstrusus* and of individual protein and RNA genes within this genome.

Nucleotide	Length (bp)	A	C	G	T	A+T
Entire sequence	13913	21.3	7.0	21.1	50.6	71.9
RNA genes	1688	28.3	6.8	19.7	45.2	73.4
Protein genes	10511	18.8	7.3	21.9	52	70.8

Table 2

Positions and nucleotide sequence lengths of individual genes in the mt genome of *Aelurostrongylus abstrusus* as well as codons (start and stop), anticodons and the lengths of predicted proteins.

Gene	Nucleotide positions	Sequence lengths		Codons		Anticodon
		No. of nucleotides	No. of amino acids	Start	Stop	
*cox*1	1 – 1578	1574	524	ATT	TAG	
*trn*C	1577 - 1634	57				GCA
*trn*M	1635 - 1694	59				CAT
*trn*D	1697 - 1751	54				GTC
*trn*G	1759 - 1814	55				TCC
*cox*2	1815 – 2507	690	230	GTT	TAG	
*trn*H	2506 – 2561	55				GTG
*rrn*L	2560 – 3521	961				
*nad*3	3517 – 3855	336	111	ATT	TAG	
*nad*5	3858 – 5459	1599	532	ATT	TAG	
*trn*A	5843 – 5897	55				TGC
*trn*P	6132 – 6190	58				TGG
*trn*V	6200 – 6253	54				TAC
*nad*6	6257 – 6682	423	140	ATT	TAG	
*nad*4L	6744 – 6980	234	77	ATT	TAG	
*trn*W	6986 – 7041	56				TCA
*trn*E	7054 – 7109	56				TTC
*rrn*S	7084 – 7780	696				
*trn*S (UCN)	7779 – 7834	55				GCT
*trn*N	7842 – 7896	54				GTT
*trn*Y	7907 – 7962	56				GTA
*nad*1	7962 – 8828	864	287	ATT	TAG	
*atp*6	8840 – 9439	597	198	ATT	TAG	
*trn*K	9461 – 9522	62				TTT
*trn*L (UUR)	9523 – 9578	55				TAA
*trn*S (AGN)	9577 – 9629	52				TGA
*nad*2	9632 – 10486	852	283	ATT	TAG	
*trn*I	10486 – 10541	56				GAT
*trn*R	10536 – 10589	53				ACG
*trn*Q	10589 – 10646	57				TTG
*trn*F	10661 – 10714	53				GAA
ctyb	10706 – 11809	1101	366	TTG	TAA	

*trn*L (CUN)	11809 – 11866	57				TAG
*cox*3	11859 – 12647	786	261	ATT	TAA	
*trn*T	12631 – 12684	53				TGT
*nad*4	12684 - 13913	1227	408	TTG	TAG	

Table 3

Codon usages (%) in mt protein genes of *Aelurostrongylus abstrusus* compared with those of *Angiostrongylus vasorum* (see Gasser et al., 2012), *An. cantonensis* and *An. costaricensis* (cf. Lv et al., 2012). International Union of Pure and Applied Chemistry (IUPAC) codes (N = A, G, C or T; Y = C or T; R = A or G) were used

Amino acid	Codon	Number of codons and percentage (%) of codon usage			
		A. abstrusus	*An. vasorum*	*An. cantonensis*	*An. costaricensis*
Non-polar					
Alanine	GCN	84 (2.5)	88 (2.5)	75 (1.7)	52 (1.2)
Isoleucine	ATY	242 (7.1)	226 (6.5)	290 (6.4)	306 (6.8)
Leucine	CTN	66 (1.9)	23 (0.7)	646 (14.3)	605 (13.4)
Leucine	TTR	514 (15.0)	566 (16.4)	511 (11.3)	453 (10.1)
Methionine	ATR	103 (3.0)	148 (4.3)	225 (5.0)	191 (4.2)
Phenylalanine	TTY	548 (16.0)	461 (13.3)	614 (13.6)	675 (15.0)
Proline	CCN	75 (2.2)	71 (2.0)	57 (1.3)	35 (0.8)
Tryptophan	TGR	42 (1.2)	58 (1.7)	181 (4.0)	216 (4.8)
Valine	GTN	345 (10.1)	368 (10.6)	370 (8.2)	409 (9.1)
Polar					
Aspargine	AAY	98 (2.9)	92 (2.7)	146 (3.2)	155 (3.4)
Cysteine	TGY	59 (1.7)	77 (2.2)	156 (3.4)	209 (4.6)
Glutamine	CAR	43 (1.3)	38 (1.1)	46 (1.0)	32 (0.7)
Glycine	GGN	222 (6.5)	224 (6.5)	246 (5.4)	237 (5.3)
Serine	AGN	209 (6.1)	245 (7.1)	408 (9.0)	349 (7.8)
Serine	TCN	153 (4.5)	136 (3.9)	111 (2.5)	111 (2.4)
Threonine	ACN	80 (2.3)	77 (2.2)	102 (2.2)	56 (1.2)
Tyrosine	TAY	188 (5.5)	192 (5.5)	288 (6.4)	241 (5.4)
Acidic					
Aspartate	GAY	75 (2.2)	70 (2.0)	122 (2.7)	116 (2.6)
Glutamate	GAR	72 (2.1)	80 (2.3)	105 (2.3)	131 (2.9)
Basic					
Arginine	CGN	100 (2.9)	161 (4.6)	33 (0.7)	34 (0.8)
Histidine	CAY	55 (1.6)	53 (1.5)	48 (1.1)	36 (0.8)
Lysine	AAR	93 (2.7)	93 (2.7)	161 (3.6)	155 (3.4)

Table 4

Pairwise comparisons (in %) of the amino acid sequences predicted from the mt genome of *Aelurostrongylus abstrusus* and those of related lungworms, *Angiostrongylus vasorum* (this study), *An. cantonensis* and *An. costaricensis* (cf. Gasser et al., 2012; Lv et al., 2012).

Predicted protein	*A. abstrusus* vs. *An. vasorum*	*A. abstrusus* vs. *An. cantonensis*	*A. abstrusus* vs. *An. costaricensis*
ATP6	65.5	64	63
COB	76.6	80.0	78.7
COX1	91.2	94.3	92.0
COX2	76.6	82.2	79.6
COX3	77.3	78.8	83.3
NAD1	75.7	74.7	73.3
NAD2	55.1	59.3	59.6
NAD3	61.9	64.6	66.9
NAD4	69.6	72.9	69.5
NAD4L	67.1	78.5	74.7
NAD5	58	64	64
NAD6	60	61	59
All genes	67.8	71.3	69.8

Fig. 1. Schematic representation of the circular mt genome of *Aelurostrongylus abstrusus.* Each transfer RNA gene is identified by a one-letter amino acid code on the outer side of the map. All genes are transcribed in the clockwise direction.

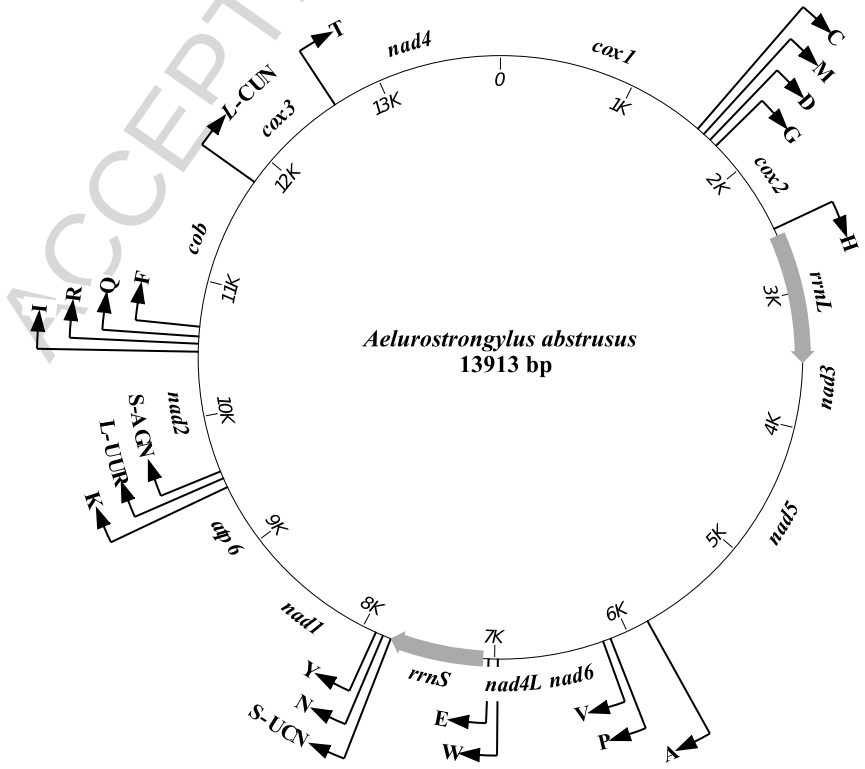
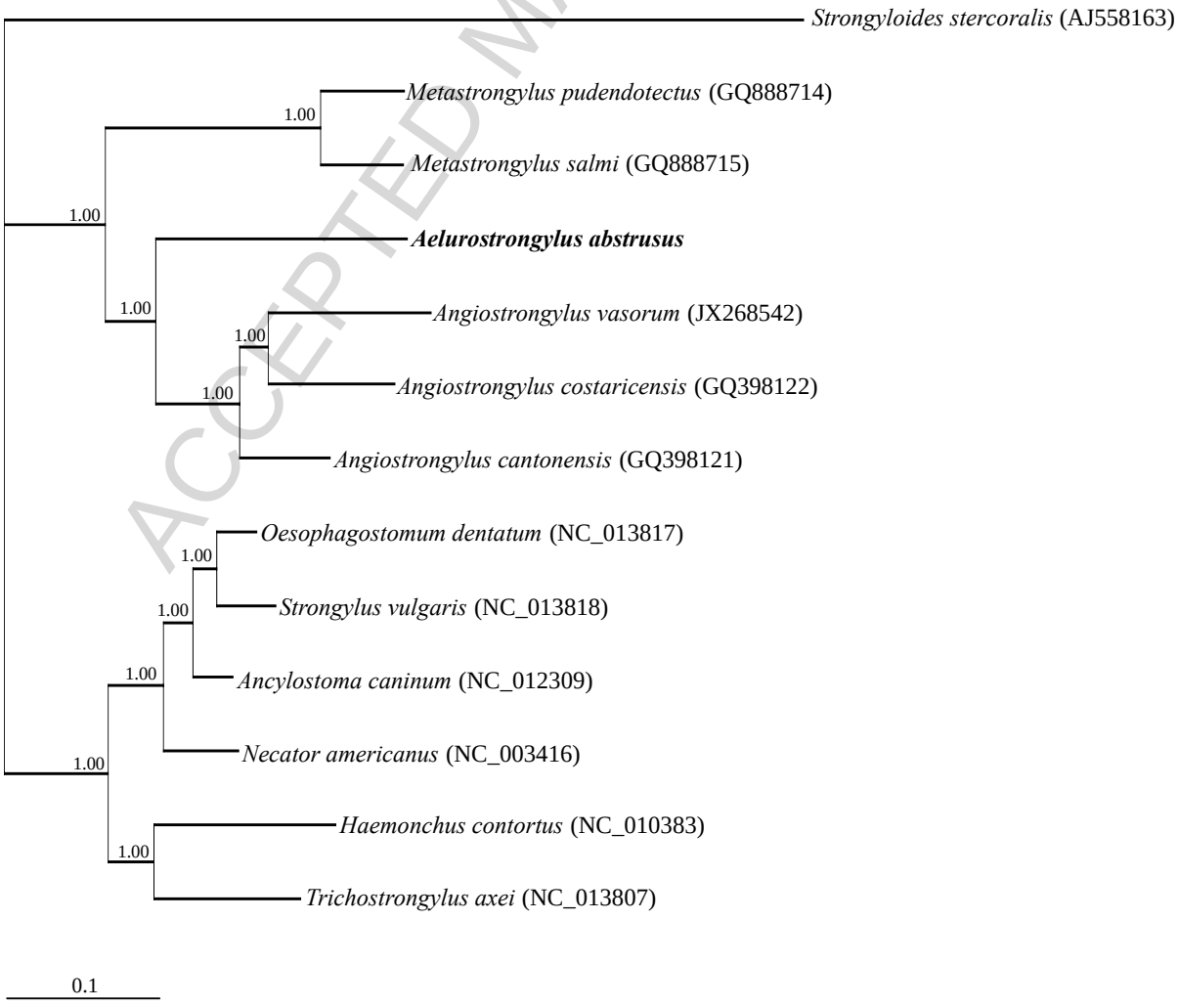


Fig. 2. Relationship of *Aelurostrongylus abstrusus* with the metastrongylids *Angiostrongylus vasorum*, *An. cantonensis*, *An. costaricensis*, *Metastrongylus pudendotectus* and *M. salmi* (Metastrongyloidea), and selected species representing different superfamiles, including *Ancylostoma caninum* and *Necator americanus* (Anyclostomatoidea); *Haemonchus contortus* and *Trichostrongylus axei* (Trichostrongyloidea); *Oesophagostomum dentatum* and *Strongylus vulgaris* (Strongyloidea); and outgroup *Strongyloides stercoralis* (Strongyloidoidea), based on a phylogenetic analysis of concatenated amino acid sequence data for the 12 mt proteins. Absolute support (pp = 1.00) indicated at each node. Accession numbers in round brackets.



Highlights

- *Aelurostrongylus abstrusus* is an important parasitic nematode of felids.
- We characterised the mitochondrial genome of *A. abstrusus* using advanced technologies.
- Markers in this genome should find utility for investigating this parasite.