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Mitochondrial genome of *Angiostrongylus vasorum*: comparison with congeners and implications for studying the population genetics and epidemiology of this parasite

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ABSTRACT

Angiostrongylus vasorum is a strongylid nematode of major clinical relevance in canids, causing angiostrongylosis. In spite of its increasing importance, the genetics, epidemiology and biology of this parasite are not entirely understood. Mitochondrial (mt) DNA provides useful markers for studies of these areas, but genetic data are scant for *A. vasorum* and its congeners. Here, the mitochondrial genome was amplified by long-range polymerase chain reaction (long-PCR) from a portion of a single male adult of *A. vasorum*, sequenced using 454 technology and annotated employing a semi-automated bioinformatic pipeline. This circular mitochondrial genome is 13,422 bp and contains 12 protein-encoding, 22 transfer RNA, and 2 ribosomal RNA genes, consistent with its congeners and other secernentean nematodes. This mt genome represents a rich source of markers for future investigations of the population genetics and epidemiology of *A. vasorum*. Molecular tools, employing such mt markers, should be useful for explorations into host specificity and for prospecting for cryptic species, and might also underpin the diagnosis of canine angiostrongylosis.

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1. Introduction

Angiostrongylus vasorum (Baillet, 1866) is a metastrongyloid nematode of dogs and other canids, including various species of fox, wolf, coyote and jackal (definitive hosts) (reviewed by Koch and Willeesen, 2009). This parasite causes angiostrongylosis, a disease that typically manifests itself in respiratory distress, coughing, dyspnoea, exercise intolerance, weight loss, vomiting, diarrhoea and/or neurological signs, coagulopathies or sudden death (Koch and Willeesen, 2009; Schnyder et al., 2010). *A. vasorum* has emerged as an important pathogen of canids in various countries in the western Palaearctic and eastern Nearctic ecozones (Bwangamoi, 1972; Bolt et al., 1992; Lima et al., 1994; Conboy, 2004; Morgan et al., 2008; Taubert et al., 2008; Jefferies et al., 2010; Di Cesare et al., 2011).

Angiostrongylus vasorum has an indirect life cycle. The dioecious adults of *A. vasorum* live in the heart and the pulmonary arteries of the definitive canid host; here, the oviparous females produce eggs, from which first-stage larvae (L1s) hatch within lung tissue to penetrate into the alveoli. L1s then migrate 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) within ~16 days (Guilhon and Bressou, 1960; Guilhon, 1963). Frogs can act as paratenic hosts, following the ingestion of infected snails or slugs (Bolt et al., 1993). L3s within an infected intermediate or paratenic hosts are then ingested by canids, penetrate the gut wall and then migrate to the abdominal lymph nodes. Here, they moult to fourth-stage (L4), enter the portal circulation, migrate through the liver parenchyma and eventually reach the right ventricle and pulmonary arteries, where they develop to adult worms. The prepatent period is reported to range from 28-108 days (see Koch and Willeesen, 2009).

With an apparent spread and increasing clinical relevance of canine angiostrongylosis, particularly in Europe and the Americas (Morgan et al., 2007; Conboy, 2011), there has been a focus on enhanced treatment and control. An emphasis has been placed on targeted treatment of clinical cases with anthelmintics, such as fenbendazole, milbemycin oxime and moxidectin (Schnyder et al., 2009; Willeesen et al., 2007). However, in spite of the clinical significance of canine angiostrongylosis in dogs, little is known about the genetics and epidemiology of *A. vasorum*, which is critical for the sustained control of this disease.

Molecular tools using suitable genetic markers can readily underpin such fundamental studies. Mitochondrial (mt) genomes provide suitable markers for genetic and epidemiological investigations of species of strongylid nematodes, with a perspective on discovering population variants and cryptic species, and exploring transmission patterns linked to particular genotypes of a parasite (cf. Hu and Gasser, 2006; Jex et al., 2010). Recent advances in high throughput sequencing and computational techniques now provide a solid footing for the rapid determination of mt genomes from parasitic nematodes as a source of genetic markers for such studies. In the present study, we employed a massively parallel sequencing method and semi-automated pipeline for the characterisation of the mt genome of *A. vasorum*, which we compared directly with those of two congeners, *Angiostrongylus cantonensis* and *A. costaricensis* (see Lv et al., 2012), for which mt genome data already exist. We also studied the genetic relationships of these species of *Angiostrongylus* and representatives of different superfamilies within the Strongylida, and identified regions in the genome of *A. vasorum* likely to serve well as markers for future molecular epidemiological studies of this species in different countries.

2. Materials and methods

2.1. Collection of parasite and isolation of genomic DNA

Adult specimens (males and females) of *A. vasorum* were collected following reverse perfusion of the lungs from monospecifically infected dogs (see Schnyder et al., 2009, 2010), washed extensively in physiological saline and then stored at -80 °C. Upon thawing, the anterior and posterior ends of a single male worm were excised and cleared in lactophenol for microscopic identification. The mid-body section was used for the extraction of genomic DNA 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 automated sequencing (BigDye chemistry v.3.1) (cf. Gasser et al., 2006).

2.2. Sequencing and assembly of the mt genome

From the genomic DNA from the mid-body section of 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 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 and quantified spectro-photometrically (ND-1000 UV-VIS spectrophotometer v.3.2.1; NanoDrop Technologies). Following an electrophoretic analysis of quality, 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. JX268542) was then assembled using the program CAP3 (Huang et al., 1999) from individual reads (of ~300 bp).

2.3. Annotation and analyses of sequence data

Following assembly, the mt genome of *A. vasorum* was annotated using a semi-automated annotation pipeline (Jex et al., 2010). In brief, each protein encoding mt gene was identified by local alignment comparison (performed in all six reading frames) using amino acid sequences conceptually translated from corresponding genes from the mt genomes of two congeners (e.g., *A. cantonensis* and *A. costaricensis*; accession nos. GQ398121 and GQ398122, respectively; Lv et al., 2012). The large and small subunits of the mt ribosomal RNA genes (*rns* and *rnl*, respectively) were identified by local alignment (i.e., using nucleotide sequence data). All transfer RNA (tRNA) genes were identified using a multi-step approach. Initially, 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 clustered into groups, based on 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 representing each amino acid group were ranked based on structural “strength” (as inferred by the number of nt mismatches in each stem), and the 100 best-scoring structures for each group 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 of each mt genome were then identified and annotated based on having the highest sequence identity to known nematode tRNAs. Annotated sequence data were imported using the program SEQUIN (available via <http://www.ncbi.nlm.nih.gov/Sequin/>) for the final verification of the mt genome organisation and subsequent submission to the GenBank database.

Sliding window analysis was performed on the aligned, complete mt genome sequences of the three *Angiostrongylus* species using the program DnaSP v.5 (Rozas et al. 2003). The alignment of these sequences was achieved using MUSCLE v. 3.8 (Edgar, 2004), as implemented in SeaView v.4 (Gouy et al., 2010). The only ambiguously alignable regions were within the control regions; all other genes aligned easily. A sliding window of 300 bp (steps of 10 bp) was used to estimate nucleotide diversity (π) over the entire alignment; indels were excluded using DnaSP. Nucleotide diversity for the entire alignments was plotted against midpoint positions of each window, and gene boundaries were defined. Similar, pairwise analyses were performed among *Angiostrongylus* species to demonstrate regions with different magnitudes of nucleotide diversity.

2.4. Alignment and phylogenetic analysis of concatenated amino acid sequence data

The amino acid sequences conceptually translated from individual genes of the mt genome of *A. vasorum* were concatenated, as were those of *A. cantonensis* (accession. no. GQ398121) and *A. costaricensis* (GQ398122). Also used for comparative purposes were concatenated amino acid sequences predicted from published mt genomes from nematodes selected to represent individual superfamilies of the order Strongyloidea, including *Haemonchus contortus* (NC_010383; Trichostrongyloidea), *Metastrongylus pudendotectus*, *Metastrongylus salmi* (GQ888714, GQ888715; Metastrongyloidea), *Ancylostoma caninum* (FJ483518; Ancylostomatoidea) and *Oesophagostomum dentatum* (GQ888716; Strongyloidea) (cf. Jex et al., 2010). All amino acid sequences (considering all homologous characters) were aligned using MUSCLE (Edgar, 2004) and then subjected to phylogenetic analysis using Bayesian inference (BI) (as described by Jex et al., 2010) employing the software package MrBayes v.3.1.2 (<http://mrbayes.csit.fsu.edu/index.php>) run on a four dual-core Opteron-based Unix cluster (<http://pug.nhm.ac.uk>).

3. Results

3.1. Features of the mt genome of *A. vasorum*

The circular mt genome sequence of *A. vasorum* is 13,422 bp in size (Fig. 1), similar to those of *A. cantonensis* (13,497 bp) and *A. costaricensis* (13,585 bp) (Lv et al., 2012). The nucleotide composition of the mt genome of *A. vasorum* is 21.5% for A; 24.7% for G; 6.1% for C and 47.6% for T (Table 1). As expected for strongylid nematodes, the genome is AT-rich, with T being the most favoured nucleotide and C the least favoured. The mt genome of *A. vasorum* contained genes encoding 12 proteins (COX1-3, NAD1-6, NAD4L, ATP6 and CYTB), two ribosomal and 22 tRNA genes, but lacked an *atp8* gene. All of the 36 genes are transcribed in the same direction (5'→3') (Fig. 1). Also the arrangement of the mt genome of *A. vasorum* was consistent with GA2 representing all other nematodes of the order Strongyloidea investigated to date (see Hu and Gasser, 2006; Jex et al., 2010). The longest non-coding (AT-rich) region between the *trnA* and *trnP* was 219 bp in length (see Fig. 1); its AT-content was 79.0%, significantly greater than for the rest of the genome (Table 1).

3.2. Protein genes and codon usage

Predicted initiation and termination codons for the protein encoding genes of *A. vasorum* were compared with those of the two other congeners (Table 2). The commonest start codon for *A. vasorum* was TTG (for 5 of 12 proteins), followed by ATT (4 genes), ATA (2 genes) and ATG (1 gene). Ten mt protein genes of *A. vasorum* were predicted to have a TAA or TAG translation termination codon. The other two protein genes ended in an abbreviated stop codon, such as T or TA (Table 2). For *A. vasorum*, the 3'-end of these genes was immediately adjacent to a downstream tRNA gene (Fig. 1 and Table 3), which is consistent with the arrangement in the two other species of *Angiostrongylus* (cf. Lv et al., 2012).

The codon usage for the 12 protein genes of *A. vasorum* was also compared with that of the two other congeners (Table 3); 61 of the 64 possible codons were used. Neither codons CTC (Leu), CGC (Arg) and ACC (Thr) were used in the *A. vasorum* mt genome, nor was CGC. The preferred nucleotide usage at the third codon position of mt protein genes of *A. vasorum* 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. vasorum*, the codons ending in G have higher frequencies than the codons ending in A, which, interestingly, is similar to, for example, *Ascaris suum* and *Onchocerca volvulus*, but distinct from other members of the order Strongylida and *Caenorhabditis elegans*. As the usage of synonymous codons is hypothesized to be preferred in gene regions of functional significance, codon bias is thought to be linked mainly to selection at silent sites and to maximize translation efficiency (Sharp and Matassi, 1994; Durent and Mouchiroud, 1999).

AT bias in the nucleotide composition also reflected a bias in the amino acid composition of 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. vasorum*, 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, 13.1%; TTC, 0.2%), Ile (ATT, 4.4%; ATC, 0.1%), Tyr (TAT, 5.5%; TAC, 0.1%) and Asn (AAT, 2.6%; AAC, 0.1%), the frequency was always less if the third position was a C.

3.3. Transfer RNA genes

Twenty-two tRNA gene sequences were identified in the mt genome of *A. vasorum*. These sequences ranged from 52-61 nt in length. The tRNA structures had a 7 bp amino-acyl arm, a 3-4 bp DHU arm, a 4-5 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 *trnS* genes) have a predicted secondary structure with a 3-4 bp DHU stem and a DHU loop of 5-8 bases, in which the variable T ψ C arm and loop are replaced by a "TV-replacement loop" of 6-12 bases, in accordance with other nematodes (Hu and Gasser, 2006). The mt *trnS* for *A. vasorum* has a secondary structure consisting of a DHU replacement loop of 4-6 bases, 3 bp T ψ C arm, T ψ C loop of 6-9 bases and a variable loop of 4 bases, consistent with other members of the Secernentea (Okimoto et al., 1992; Keddie et al., 1998), but distinct from *T. spiralis* (Adenophorea) (see Lavrov and Brown,

2001). An overlap of one nucleotide is found between the *trnS* (UCN) and *trnN* genes in the mt genome of *A. vasorum*. Another overlap was detected between *trnE* and *rrnS*.

3.4. Ribosomal RNA genes

The *rrnS* and *rrnL* genes of *A. vasorum* were identified by sequence comparison with *A. cantonesis* and *A. costaricensis*. The *rrnS* gene was located between *trnE* and *trnS* (UCN), and *rrnL* was between *trnH* and *nad3*. The two genes were separated from one another by the protein genes *nad3*, *nad5*, *nad6* and *nad4L* (Fig. 1). The size of the *rrnS* gene of *A. vasorum* was 696 bp. The size of the *rrnL* gene of *A. vasorum* was 961 bp. The lengths of these two genes were similar to those of other nematodes (for *rrnS*, 697 bp in *C. elegans*, 700 bp in *As. suum* and 684 bp in *O. volvulus*; for *rrnL*, 953 bp in *C. elegans*, 960 bp in *As. suum* and 987 bp in *O. volvulus*), and were amongst the shortest for any metazoan organism (cf. Lavrov and Brown, 2001). The AT content of the *rrn* genes was 72.3% (Table 1). The overall identities in the *rrnS* sequence between *A. vasorum* and *A. cantonesis*, and between *A. vasorum* and *A. costaricensis* were 81.1% and 78.1%, respectively, whereas for the *rrnL*, the identities were 75.2% and 73.9%, respectively.

3.5. Nucleotide variation across mt genomes of *Angiostrongylus* species

Nucleotide diversities across the mt genomes between or among *A. vasorum*, *A. cantonesis* and *A. costaricensis*, achieved by sliding window analysis, are shown in Fig. 2. Pairwise analyses between species largely mirrored the overall pattern of diversity across all three species. Also indicated on the graph are the regions of *cox1* (~700 bp; Folmer et al., 1994) and *rrnL_nad3* (~445 bp; Jefferies et al., 2010) used previously to determine phylogeographic relationships of and genetic diversity within *A. vasorum* populations (Jefferies et al., 2010). Greatest nucleotide diversity was detected in the D-loop region, which was expected, given the sequence variability within and the difficulty in aligning across this region. Gene-by-gene nucleotide diversity was highly variable. The most variable genes included *rrnL*, *nad6*, *atp6* and the 5'-ends of *nad4* and *nad5*; the least variable genes included *rrnS*, *cox1* and *cox2*.

3.6. Amino acid sequence comparisons and genetic relationships of *A. vasorum* with two congeners and other strongylids

The amino acid sequences predicted from individual protein encoding mt genes of *A. vasorum* were compared with those of *A. cantonesis* and *A. costaricensis* (Table 4). Pairwise comparisons of the concatenated sequences revealed identities of 65.7-81.5% among the three *Angiostrongylus* species. Based on identity, COX1 was the most conserved protein, whereas NAD2 and NAD6 were the least conserved. For all mt proteins, the amino acid sequence identity was greater between *A. vasorum* and *A. cantonesis* than between *A. vasorum* and *A. costaricensis*, except for COX3, NAD2, NAD3 and NAD4L (see Table 4). Phylogenetic analysis of the concatenated amino acid sequence data for the 12 mt proteins showed that *A. vasorum* was more closely related to *A. cantonesis* than to *A. costaricensis* (pp = 1.00), to the exclusion of *Metastrongylus* species (Metastrongyloidea), *H. contortus* (Trichostrongyloidea), *Ancylostoma caninum* (Ancylostomatoidea) and *Oesophagostomum dentatum* (Strongyloidea) (Fig. 3).

4. Discussion

There is major significance in the use of mt DNA markers for investigating the genetic composition of species of *Angiostrongylus* (Nematoda: Strongylida), given that there are no morphological features that allow the unequivocal specific identification of some developmental stages (e.g., larvae) (Anderson, 2000) and given that cryptic species have been detected within the Strongylida (Gasser, 2006; Gasser et al., 2008). In nematodes, mt DNA is predicted to be maternally inherited and is usually more variable in sequence within a species than nuclear ribosomal DNA (Gasser, 2006), making mt gene regions well-suited for studying the population genetics of parasitic nematodes (Blouin, 2002; Anderson et al., 1998; Hu et al., 2004). The characterization of the complete mt genome sequence of *A. vasorum* herein also provides a foundation for addressing ecological or epidemiological questions regarding this and related species (including *A. andersoni*, *A. mackerrasae*, *A. malayensis* and *A. siamesensis*) (Anderson, 2000). This is particularly pertinent, given that significant population variation has already been detected within *A. vasorum* (see Jefferies et al., 2009, 2010) and that nothing is known about the affiliations of particular genetic variants to various molluscan, canine and/or other hosts.

Sliding window analysis revealed patterns of nucleotide diversity among the mt genomes of *Angiostrongylus* species. These patterns of high and low variability are of interest to researchers wishing to identify new mt markers for molecular ecological, biogeographical and population genetic investigations. Regions of high variability offer an opportunity to provide greater resolution in differentiating populations. Jefferies et al. (2010), relying on limited primer sets, which were available at the time, elected to sequence fragments of *cox1* and a region incorporating the 3'-end of *rrnL* and the complete *nad3* gene (*rrnL_nad3*) (see Fig. 3). Although these genes provided adequate resolution for their study, particularly when combined, the sliding window analysis conducted here suggests that these regions were amongst the most conserved, a feature that enables primer design, but can miss the very signal pursued. Many more regions in the mt genome are now revealed as potentially suitable for molecular ecological studies. Additionally, the comparison between and among entire mt genomes provides an opportunity to develop genus- or species-specific oligonucleotide primers to capture regions of variability best suited to the application (e.g., PCR-coupled mutation scanning and sequencing) or resolution required.

Conserved oligonucleotide primers can now be rationally and selectively designed to relatively conserved regions flanking "variable sequence tracts" in the mt genome considered as most informative, based on sequencing from a small number of individuals from particular *A. vasorum* populations, or genetic variants detected using nuclear ribosomal markers (cf. Gasser et al., 2006). Using such primers, PCR-based single-strand conformation polymorphism (SSCP) analysis (Gasser et al., 2006) could be applied to pre-screen large numbers of individual specimens representing different populations, in order to establish levels of haplotypic variability (at any life cycle stage). Such an approach has been shown to be effective, for example, to explore the genetic variation in populations of hookworms (Hu et al., 2002a) and *Dictyocaulus viviparus* of cattle in Sweden (Hu et al., 2002b). The results of the latter study, for instance, revealed that both the mt DNA diversity within populations and the gene flow among populations of *D. viviparus* were low, which was similar to findings for some parasitic nematodes of insects and plants, but, interestingly, distinct from results achieved for gastrointestinal trichostrongyloid nematodes (e.g., *Haemonchus placei* and *Ostertagia ostertagi*) of domesticated ruminants, considered to have relatively high levels of diversity and gene flow (see Blouin et al., 1992, 1995, 1997). Such differences were interpreted to relate mainly to differences in host movement as well as parasite biology, population sizes and transmission patterns, and should thus be of epidemiological relevance.

Clearly, these aspects are worthy of study in *A. vasorum* populations in different countries and distinct hosts.

It would be interesting to explore whether specific genotypes/haplotypes of *A. vasorum* have a particular affinity to different canid hosts and/or predilection sites in particular organs, cause different clinical forms/manifestations of angiostrongylosis (reviewed by Willesen and Koch, 2007) and/or whether particular subpopulations of *A. vasorum* might undergo hypobiosis (arrested development) in tissues. Using molecular tools (based on mt markers), in combination with traditional parasitological and serological methods, it might be possible to assess disease caused by particular strains (haplotypes) in the canine system (cf. Schnyder et al., 2009). Furthermore, it would also be interesting to extend mt genomic sequencing to a range of *Angiostrongylus* species of rodents, examine their genetic/phylogenetic relationships and utilize mt markers to explore species associated with human cases of angiostrongylosis globally. Although human angiostrongylosis is currently recognised to relate mainly to *A. cantonensis* or *A. costaricensis* (see Punyagupta, 1975; Jaroonvesama, 1985; Lv et al., 2009), there has been limited study of the genetic identity and pathogenicity of the parasites affecting humans. Clearly, mt markers would be useful for investigating transmission patterns by genetically matching the identity of larvae in intermediate molluscan to adults in mammalian hosts, and also predicting the zoonotic risk linked to particular species of infected intermediate hosts.

In conclusion, the present study emphasizes the relevance of the mt genome of *A. vasorum* defined here, as a basis for future systematic, population genetic, epidemiological, ecological and biological studies. In particular, studying members of this genus provides a stimulus to re-examine the phylogenetic relationships of members of the Metastrongyloidea with other superfamilies in the order Strongylida. The PCR-coupled sequencing and bioinformatic platform established for the direct sequencing of mt genomes from single worms (Jex et al., 2010) provides exciting prospects for large-scale, high throughput population genetic and mt genomic studies of parasitic nematodes.

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Table 1

Nucleotide composition (%) of the entire mt genome of *Angiostrongylus vasorum* and of protein and RNA genes within this genome, and comparison with the congeners *A. cantonensis* and *A. costaricensis* (see Lv et al., 2012).

Species		Length (bp)	A	C	T	G	A+T
<i>A. vasorum</i>	Entire sequence	13422	21.5	6.1	47.6	24.7	69.1
	Protein genes	10398	18.8	6.2	49.0	26.0	67.8
	RNA genes	1659	30.5	6.3	43.0	20.1	73.5
<i>A. cantonensis</i>	Entire sequence	13497	24.5	6.2	48.7	20.6	73.2
	Protein genes	10461	22.0	6.3	50.2	21.5	72.2
	RNA genes	1659	32.3	6.2	44.3	17.2	76.7
<i>A. costaricensis</i>	Entire sequence	13585	25.4	6.6	47.8	20.2	73.2
	Protein genes	10335	22.8	6.8	49.2	21.2	72.1
	RNA genes	1663	32.9	6.5	43.0	17.6	75.9

Table 2

Locations and lengths of protein encoding genes in the mt genome of *Angiostrongylus vasorum* as well as their initiation and termination codons as well as the lengths of the predicted proteins, and comparisons with the congeners *A. cantonensis* and *A. costaricensis* (see Lv et al., 2012).

Gene	Positions and nt sequence lengths (bp)			Initiation-termination codons and amino acid sequence lengths		
	<i>A. vasorum</i>	<i>A. cantonensis</i>	<i>A. costaricensis</i>	<i>A. vasorum</i>	<i>A. cantonensis</i>	<i>A. costaricensis</i>
<i>cox1</i>	1 – 1573	1-1579	1-1579	ATA-TAA (523)	ATT-TAG (525)	ATT-TAA (525)
<i>tmC</i>	1577 – 1634	1578-1634	1579-1634			
<i>tmM</i>	1637 – 1695	1637-1693	1635-1693			
<i>tmD</i>	1699 – 1755	1702-1754	1695-1748			
<i>tmG</i>	1755 – 1808	1755-1811	1752-1809			
<i>cox2</i>	1808 – 2504	1812-2505	1810-2503	ATT-TAG (231)	TTG-TAG (230)	TTG-TAA (230)
<i>tmH</i>	2505 – 2560	2503-2557	2509-2564			
<i>rrnL</i>	2557 – 3518	2558-3519	2565-3531			
<i>nad3</i>	3521 – 3854	3517-3853	3531-3867	TTG-TAG (111)	TTG-TAG (111)	TTG-TAG (111)
<i>nad5</i>	3886 – 5453	3855-5437	3880-5461	ATA-T (544)	ATA-T (526)	ATA-T (526)
<i>tmA</i>	5453 – 5507	5437-5491	5462-5516			
<i>tmP</i>	5726 – 5783	5723-5777	5782-5835			
<i>tmV</i>	5787 – 5840	5782-5835	5838-5892			
<i>nad6</i>	5850 – 6276	5845-6271	5901-6333	ATG-TAG (141)	ATG-TAG (141)	ATG-TAG (143)
<i>nad4L</i>	6279 – 6511	6271-6503	6333-6564	ATT-T (76)	ATT-T (76)	ATT-T (76)
<i>tmW</i>	6511 – 6568	6503-6559	6565-6623			
<i>tmE</i>	6569 – 6623	6561-6615	6631-6689			
<i>rrnS</i>	6622 – 7318	6616-7312	6690-7385			
<i>tmS</i> (UCN)	7319 – 7375	7311-7366	7385-7439			
<i>tmN</i>	7374 – 7428	7365-7420	7438-7497			
<i>tmY</i>	7433 – 7487	7425-7484	7502-7556			
<i>nad1</i>	7485 – 8361	7485-8361	7557-8430	TTG-TAG (291)	TTG-TAG (292)	TTG-TAG (290)
<i>atp6</i>	8366 – 8963	8363-8963	8445-9045	ATT-TAA (198)	ATT-TAG (200)	ATT-TAG (200)
<i>tmK</i>	8966 – 9026	8964-9024	9046-9105			
<i>tmL</i> (UUR)	9029 – 9083	9025-9080	9107-9162			
<i>tmS</i> (AGN)	9084 – 9136	9080-9132	9163-9215			
<i>nad2</i>	9136 – 9979	9131-9980	9215-10064	TTG-TAG (281)	TTG-TAG (282)	TTG-TAA (282)
<i>tmI</i>	9988 – 10045	9991-10047	10071-10124			
<i>tmR</i>	10045 – 10097	10048-10102	10125-10176			
<i>tmQ</i>	10098 – 10151	10102-10157	10178-10232			
<i>tmF</i>	10155 – 10211	10158-10213	10235-10291			
<i>cob</i>	10212 – 11319	10214-11324	10300-11401	TTG-TAG (369)	TTG-TAA (369)	ATG-TAG (366)
<i>tmL</i> (CUN)	11326 – 11385	11323-11378	11401-11456			
<i>cox3</i>	11377 – 12145	11379-12145	11457-12225	ATT-TAA (256)	TTG-T (255)	TTG-T (254)
<i>tmT</i>	12143 – 12199	12145-12202	12223-12280			
<i>nad4</i>	12199 – 13420	12203-13433	12281-13511	TTG-TAG (406)	TTG-TAG (409)	TTG-TAA (409)

Table 3

Codon usages (%) in mt protein genes of *Angiostrongylus vasorum* compared with those of *A. cantonensis* and *A. costaricensis* (see Lv et al., 2012).

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

Table 4

Pairwise comparisons (in %) of the amino acid sequences predicted from the three mt genomes of *Angiostrongylus vasorum* (this study) as well as *A. cantonensis* and *A. costaricensis* (cf. Lv et al., 2012).

Predicted protein	<i>A. vasorum</i> vs. <i>A. cantonensis</i>	<i>A. vasorum</i> vs. <i>A. costaricensis</i>	<i>A. cantonensis</i> vs. <i>A. costaricensis</i>
ATP6	85	77	77.5
COB	85.4	84.2	85.8
COX1	92.8	91.8	93.3
COX2	84	81.4	87.4
COX3	82.3	83.5	83.5
NAD1	86.3	84.2	70.5
NAD2	67.8	70	69.3
NAD3	77.9	79.5	74.1
NAD4	80.6	80.4	80.2
NAD4L	70.1	75.9	77
NAD5	74	70.6	77.6
NAD6	77.5	69.9	71.8
All genes	73	65.7	81.5

Fig. 1. Schematic representation of the circular mt genome of *Angiostrongylus vasorum*. 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 *Angiostrongylus vasorum* with two congeners (*A. cantonensis* and *A. costaricensis*) and selected species representing different superfamilies of the order Strongylida, including *Metastrongylus pudendotectus*, *M. salmi* (Metastrongyloidea), *Haemonchus contortus* (Trichostrongyloidea), *Oesophagostomum dentatum* (Strongyloidea) and *Ancylostoma caninum* (Ancylostomatoidea), 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.

0.05

Fig. 3. Sliding window analysis of complete mitochondrial genome sequences of three *Angiostrongylus* species. The black line indicates nucleotide diversity in a window size of 300 bp, with step size of 10 bp. Individual pairwise results are shown by symbols (see key). Gene boundaries indicated with shaded columns of gene regions used previously by Jefferies et al. (2010).

Angiostrongylus vasorum is a strongylid nematode of major clinical relevance in canids, causing angiostrongylosis. In spite of its increasing importance, the genetics, epidemiology and biology of this parasite are not entirely understood. Mitochondrial (mt) DNA provides useful markers for studies of these areas, but genetic data are scant for *A. vasorum* and its congeners. Here, the mitochondrial genome of this parasite was amplified by long-range polymerase chain reaction (long-PCR) and sequenced using 454 technology and annotated using a semi-automated bioinformatic pipeline. This circular mitochondrial genome is 13,422 bp and contains 12 protein-encoding, 22 transfer RNA, and 2 ribosomal RNA genes, consistent with its congeners and other secernentean nematodes. This mt genome represents a rich source of markers for investigating the population genetics and epidemiology of this parasite and its congeners. Molecular tools, employing such mt markers, will be useful for explorations into host specificity and for prospecting for cryptic species, and should also underpin the diagnosis and control of canine angiostrongylosis.

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