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Genetic characterization of the zoonotic parasite *Ancylostoma caninum* in the central and eastern United States*

T.A. Quintana[1], W.L. Johnson[1], D. Ritchie[1,2], V. Smith[1,2], K.A. Martin[3], K. McMahan[3], M.T. Brewer[3] and J.R.J. Jesudoss Chelladurai[1,2]

[1]Department of Diagnostic Medicine/Pathobiology, Kansas State University College of Veterinary Medicine, Manhattan, KS, USA; [2]Kansas State Veterinary Diagnostic Laboratory, Manhattan, KS, USA and [3]Department of Pathology, Iowa State University College of Veterinary Medicine, Ames, IA, USA

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Corresponding author:
J.R.J. Jesudoss Chelladurai;
E-mail: jebaj@vet.k-state.edu

Abstract

Ancylostoma caninum is the most common nematode parasite of dogs in the United States. The present study aimed to describe the molecular epidemiology of *A. caninum* isolates from the central and eastern states of the United States using the partial mitochondrial cytochrome oxidase (*cox1*) gene and to compare them with those reported globally. We isolated eggs from faecal samples of dogs and characterized each isolate based on *cox1* sequences. A total of 60 samples originating from Kansas, Iowa, New York, Florida and Massachusetts were included. 25 haplotypes were identified in the United States dataset with high haplotype diversity (0.904). Sequence data were compared to sequences from other world regions available in GenBank. Global haplotype analysis demonstrated 35 haplotypes with a haplotype diversity of 0.931. Phylogenetic and network analysis provide evidence for the existence of moderate geographical structuring of *A. caninum* haplotypes. Our results provide an updated summary of *A. caninum* haplotypes and data for neutral genetic markers with utility for tracking hookworm populations. Sequences have been deposited in GenBank (ON980650–ON980674). Further studies of isolates from other regions are essential to understand the genetic diversity of this parasite.

Introduction

Canine hookworms are important nematodes of dogs worldwide and are the causative agents of zoonotic cutaneous larva migrans (CLM) in humans. Of the three canine-adapted hookworm species in the United States, *Ancylostoma caninum*, *Ancylostoma braziliense* and *Uncinaria stenocephala*, *A. caninum* is the most common hookworm in dogs (Bowman *et al.*, 2010). *Ancylostoma ceylanicum*, a zoonotic hookworm endemic in other parts of the world has not been reported in the continental United States.

The prevalence of canine hookworms in the United States between 2017 and 2019, assessed by faecal flotation and antigen testing in dogs that presented to veterinarians for wellness visits, was 4.1%, whereas those that presented for other clinical visits were 4.2% (Sweet *et al.*, 2021). Annual hookworm incidence in dogs across all states increased by 47% between 2015 and 2018 (Drake & Carey, 2019), while some states such as Colorado had an increase of 137% between 2013 to 2017, likely due to dog movement and importation of infected dogs (Drake & Parrish, 2020). Even in large urban areas of the United States where 68.8% of dog owners reported providing parasite control medication to their pets, 7.1% of dogs randomly sampled in dog parks were infected with hookworms (Stafford *et al.*, 2020). Additionally, *A. caninum* isolates resistant to multiple anthelmintics are an emerging problem in United States' greyhounds raised in racing kennels (Jimenez Castro *et al.*, 2019; Kitchen *et al.*, 2019).

The transmission of hookworms begins when infective *A. caninum* third-stage larvae (L_3) penetrate the skin of dogs and humans from dog-faeces contaminated environments. The intensity and life cycle of *A. caninum* infections in dogs depend on host age and immunity. In immunocompetent dogs, infection typically begins by ingestion of L_3 larvae or skin penetration by L_3 larvae. L_3s that penetrate the skin migrate through the bloodstream, to the dogs' lungs and trachea, where they are then coughed up and swallowed and make their way into the intestinal tract. L_3s develop, mature and moult to fourth-stage larvae and later to adult males and females. A proportion of L_3s undergo migration within the body to musculature or fat tissue, where they reside as 'somatic larvae' in an arrested state of development. Upon endocrine stimulation during the last trimester of pregnancy, these arrested larvae reactivate and can pass vertically to nursing puppies through the mammary glands (Burke & Roberson, 1985). Additionally, 'larval leak' may occur, a phenomenon in which reactivated somatic larvae migrate to the lumen of the intestine when existing populations of adult worms in the

intestinal niche are eliminated. Thus, infection may be acquired by dogs from many sources. In dogs, infections result in loss of blood due to the haematophagous nature of the pre-adult and adult stages (Stassens *et al.*, 1996). Clinically, blood loss manifests as a spectrum of signs ranging from asymptomatic infections in adult dogs to peracute disease and death in neonates (Hill, 1946; Miller, 1968). Asymptomatic dogs pose the same level of risk as sources of human infections as symptomatic animals (Savilla *et al.*, 2011).

A few reports of *A. caninum* reaching patency in humans exist, albeit in a minor fraction of infections in tropical regions (George *et al.*, 2016; Furtado *et al.*, 2020). In a larger proportion of people, larval skin penetration by *A. caninum* produces epidermal migration of larvae which manifests as ephemeral papular, pustular, or follicular lesions (Diakou *et al.*, 2019). CLM in humans is often associated with travel and/or close human–animal bonds, especially in areas where anthelmintics are not used in dogs (Heukelbach *et al.*, 2002; Chris & Keystone, 2016). Variability in host and parasite factors have been hypothesized to play a role in the incubation period (Siriez *et al.*, 2010). Parasite factors such as strain variability have been suggested to be related to pathogenicity in the related hookworm *Necator americanus* (Clements & Addis Alene, 2022). Understanding the genetic variability in parasite populations is crucial to elucidating genetic drift in populations, estimating the level of gene flow between and/or within populations, determining if cryptic species exist and explaining phylogeography (Ouborg *et al.*, 2010; Cavallero *et al.*, 2013; Zhao *et al.*, 2022).

The mitochondrial cytochrome oxidase (*cox1*) is a more commonly used barcoding gene to understand population structure than nuclear loci such as internal transcribed spacer 1 and internal transcribed spacer 2 (Gasser *et al.*, 2009). There is relatively little data available on the population structure and molecular epidemiology of *A. caninum* globally. In previous studies, seven haplotypes were identified from 38 adult *A. caninum* from Australia (Hu *et al.*, 2002), 18 haplotypes were identified from 62 United States mid-Atlantic samples from Pennsylvania and North Carolina (Moser *et al.*, 2007) and 30 haplotypes were identified from 160 adult worms from Brazil (Miranda *et al.*, 2008). United States samples from the other states have not been studied. Additionally, sequences from the previous United States study (Moser *et al.*, 2007) are not available in GenBank or other publicly available databases for comparative studies.

The aim of the present study was to understand the haplotype distribution, diversity and phylogenetics of *A. caninum* populations isolated from naturally infected dogs in the United States using the partial mitochondrial *cox1* gene and to compare it with those reported globally. We hypothesized that geographical isolation would drive haplotypic differentiation between *A. caninum* populations in the United States and other global regions. Based on the evidence of association of genetic structuring in parasites with host movement (Blouin *et al.*, 1995), we hypothesized that *A. caninum* isolates within the United States would have population structure.

Materials and methods

Parasites

Faecal samples from naturally infected pet dogs were submitted with owner and veterinarian consent to the Kansas State Veterinary Diagnostic Laboratory between December 2020 and April 2022 for diagnostic parasitology testing. *Ancylostoma caninum* eggs were isolated from faecal samples using a double centrifugal faecal flotation technique. Briefly, approximately 5 g of the faecal sample was mixed with water, strained, sedimented with centrifugation and the supernatant was discarded. The sediment was then mixed with Sheather's sugar solution (Sp. gr. 1.275) and a centrifugal floatation was performed with a coverslip covering the tubes. All parasite stages observed on the coverslip were recorded. If *Ancylostoma* spp. eggs were present, the eggs were collected for inclusion in the study.

Three adult *A. caninum* samples were opportunistically obtained from samples submitted for diagnostic identification to Iowa State University College of Veterinary Medicine by veterinarians. All samples were anonymized for genetic studies, except for the state of origin and reported dog breeds, which were recorded as metadata. Samples for which the dog breed was not provided were coded as 'unknown'. Samples used in the final genetic analysis were obtained from: Kansas (51 samples); New York (four samples); Iowa (three samples); Florida (one sample); and Massachusetts (one sample).

DNA extraction, amplification and sequencing of cox1

Eggs collected from coverslips ($n = 63$ samples) were washed with 1X phosphate-buffered saline and stored at 4°C until DNA extraction. Adults ($n = 3$ samples) were stored in 70% ethanol at room temperature until DNA extraction. DNA was extracted from whole adult worms or eggs suspended in a 200 µl volume using the Qiagen DNeasy Blood and Tissue kit (Valencia, CA) according to the manufacturer's instructions. Total genomic DNA was eluted in 100–200 µl of water and stored at −20°C.

An approximately 400 base pairs (bp) fragment of the *cox1* gene was amplified using primers JB3 (5′-TTTTTTGGGCAT CCTGAGGTTTAT-3′) and JB4.5 (5′-TAAAGAAAGAACATAA TGAAAATG-3′) (Hu *et al.*, 2002). Polymerase chain reaction (PCR) was carried out in a 25 µl volume with 2 µl of DNA, 1 × PCR buffer, 3 mM $MgCl_2$, 200 µM of each dNTP, 200 nM of each primer and 0.3 units of Taq polymerase (GoTaq Flexi, Promega, Madison, WI). PCR conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with the final extension of 72°C for 5 min. PCR reactions were analysed by agarose gel electrophoresis to confirm amplification. Amplicons were enzyme purified (Applied Biosystems, Thermo Fisher Scientific, Vilnius, Lithuania) and sequenced using Sanger sequencing technology (Eurofins Genomics, Louisville, KY). Sequences were analysed and contigs assembled with GeneStudio ver. 2.2.0.

Sequence analysis

Nucleotide Basic Local Alignment Search Tool searches were used to confirm species identity. *Ancylostoma caninum* nucleotide sequences from (Hu *et al.*, 2002) and others from the same portion of the *cox1* gene were obtained from GenBank for inclusion in the present study (table 1). For phylogenetic analyses, *cox1* sequences of *A. tubaeforme*, *A. duodenale* and *A. ceylanicum* were also obtained from GenBank for inclusion. The *cox1* sequences of *A. braziliense* were not available in the nucleotide database of GenBank for inclusion (accessed 19 December 2022).

Multiple sequence alignment was performed with MAFFT (Katoh & Standley, 2013), trimmed to 380 bp in MegaX (Kumar *et al.*, 2018) for haplotypic and network analyses, and

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Table 1. Summary of haplotype analysis of mitochondrial cytochrome oxidase (*cox1*) gene sequences obtained from the present study and sequences derived from GenBank for comparative analysis.

Haplotype designation	Haplotype frequency	Sample code	Location	Breeds	GenBank Accessions	References
US_Hap1	0.28	Samples 5, 8, 11, 13, 14, 15, 17, 21, 22, 32, 34, 35, 38, 41, 42, 50, and 52	United States (USA): Kansas (KS) and New York (NY)	Australian Shepherd, Beagle, Border Collie, German Shepherd, Great Dane, Greyhound, mixed, Shorty Bull and unknown	ON980651	Present study
US_Hap2	0.08	Samples 36, 37, 40, 45 and 53	USA: KS	Great Dane and unknown	ON980663	Present study
US_Hap3	0.08	Samples 43, 44, 56, 64 and 7	USA: KS and Iowa (IA)	Miniature Schnauzer and unknown	ON980664	Present study
US_Hap4	0.05	Samples 28, 30 and 60	USA: KS	Greyhound and mixed	ON980659	Present study
US_Hap5	0.05	Samples 3, 4 and 9	USA: KS	Great Dane and mixed	ON980661	Present study
US_Hap6	0.05	Samples 16, 47 and 65	USA: KS and IA	Greyhound, mixed and unknown	ON980653	Present study
US_Hap7	0.03	Samples 10 and 19	USA: Massachusetts and Florida	Greyhound and mixed	ON980650	Present study
US_Hap8	0.03	Samples 46 and 66	USA: KS and IA	Poodle and unknown	ON980665	Present study
US_Hap9	0.03	Samples 55 and 62	USA: KS	Doberman Pinscher and unknown	ON980670	Present study
US_Hap10	0.03	Samples 12 and 26	USA: KS	Greyhound	ON980652	Present study
US_Hap11	0.03	Samples 27 and 31	USA: NY	Greyhound	ON980658	Present study
US_Hap12	0.02	Sample 6	USA: KS	Shorty Bull	ON980673	Present study
US_Hap13	0.02	Sample 18	USA: KS	American Pitbull Terrier	ON980654	Present study
US_Hap14	0.02	Sample 20	USA: NY	Greyhound	ON980655	Present study
US_Hap15	0.02	Sample 23	USA: KS	Border Collie	ON980656	Present study
US_Hap16	0.02	Sample 25	USA: KS	Greyhound	ON980657	Present study
US_Hap17	0.02	Sample 29	USA: KS	Poodle	ON980660	Present study
US_Hap18	0.02	Sample 33	USA: KS	German Shepherd	ON980662	Present study
US_Hap19	0.02	Sample 48	USA: KS	Mixed	ON980666	Present study
US_Hap20	0.02	Sample 49	USA: KS	Greyhound	ON980667	Present study
US_Hap21	0.02	Sample 51	USA: KS	Unknown	ON980668	Present study
US_Hap22	0.02	Sample 54	USA: KS	Unknown	ON980669	Present study
US_Hap23	0.02	Sample 58	USA: KS	Greyhound	ON980671	Present study
US_Hap24	0.02	Sample 59	USA: KS	Unknown	ON980672	Present study
US_Hap25	0.02	Sample 61	USA: KS	Mixed	ON980674	Present study
Sequences derived from GenBank:						
Aus_Hap1	0.21		Queensland, Australia		AJ407941	Hu *et al.* (2002)
Aus_Hap2	0.05		Queensland, Australia		AJ407962	Hu *et al.* (2002)
Aus_Hap3	0.03		Queensland, Australia		AJ407966	Hu *et al.* (2002)

(*Continued*)

Table 1. (*Continued.*)

Haplotype designation	Haplotype frequency	Sample code	Location	Breeds	GenBank Accessions	References
Aus_Hap4	0.29		Queensland, Australia		AJ407961	Hu *et al.* (2002)
			Australia		FJ483518	Jex *et al.* (2009)
Aus_Hap5	0.13		Queensland, Australia		AJ407964	Hu *et al.* (2002)
Aus_Hap6	0.26		Queensland, Australia		AJ407963	Hu *et al.* (2002)
Aus_Hap7	0.03		Queensland, Australia		AJ407965	Hu *et al.* (2002)
Chi_Hap1					MN215971	Xie *et al.* (2019)
Jap_Hap1					AB751617	direct submission
Jap_Hap2					AP017673	direct submission

with Block Mapping and Gathering with Entropy (Criscuolo & Gribaldo, 2010) for phylogenetic analysis. Haplotype diversity, nucleotide diversity and nucleotide difference analyses were performed in DnaSp ver. 6 (Rozas *et al.*, 2017). Geographical distribution of haplotypes was visualized by mapping using Datawrapper (Datawrapper, 2021) and Microsoft Excel ver. 16.63. A heatmap of breed distribution against haplotypes in the present study was created using plotly (Plotly Technologies, 2015). Median joining networks were generated in Population Analysis with Reticulate Trees (Leigh & Bryant, 2015). Maximum likelihood phylogenetic analysis was performed using PhyML 3.0 (Guindon *et al.*, 2010) after the best substitution model was determined using Bayesian information criterion criteria in Smart Model Selection (Lefort *et al.*, 2017). The tree was visualized with Interactive Tree Of Life ver. 6 (Letunic & Bork, 2021).

Data availability

Unique haplotypic sequences generated in the present study have been deposited in the GenBank nucleotide database under the accession numbers ON980650–ON980674.

Results

Analysis of cox1 sequences reveals high haplotypic variability

An approximately 400 bp region of the *cox1* gene was amplified from 57 of 63 *A. caninum*-positive faecal floats and from three of three *A. caninum* adult worms. The sequences obtained from the 60 samples were found to be distributed across 25 unique haplotypes (table 1, fig. 1a), with haplotype diversity of 0.904 (variance 0.00085 and standard deviation 0.029) and nucleotide diversity of 0.00886 (standard deviation 0.00086). Haplotype designations were provided to the sequences based on frequency of occurrence. The most frequent haplotype (frequency 0.28) was designated US Hap 1, the next most frequent was designated US Hap 2, etc. As shown in fig. 1a, samples from Kansas (n = 51) represented 22 haplotypes (haplotypic diversity: 0.886 ± 0.04). Sequences from Iowa (n = 3) belonged to the same haplotypes as sequences from Kansas (US Hap 3, US Hap 6 and US Hap 8; haplotypic diversity: 1.0 ± 0.27). Three of the sequences from New York represented two unique haplotypes (US Hap 11 and US Hap 14), while the other belonged to US Hap 1 (haplotypic diversity: 0.83 ± 0.22). Two sequences from Massachusetts and Florida (n = 1 each) belonged to a unique haplotype (US Hap 7).

Global haplotype analysis was performed using 60 sequences from the present study, in addition to 11 sequences derived from the GenBank nucleotide database. Sequences from a study in Brazil (Miranda *et al.*, 2008) were derived from a different portion of the *cox1* gene with no overlap with the sequences in Hu *et al.* (2002) and the present study. Sequences from a previous United States study (Moser *et al.*, 2007) were not available in GenBank or any other database for inclusion. Haplotype analysis revealed that the 71 sequences in the final dataset represented 35 haplotypes, with a haplotype diversity of 0.931 (variance: 0.00048 and standard deviation: 0.022) and nucleotide diversity of 0.01624 (standard deviation: 0.00303). Of 380 bp analysed, 59 sites were determined to be polymorphic (variable) with 63 mutations and 43 sites were determined to be parsimony informative. Most of the mutations were silent (synonymous) (fig. 1b), with two

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Fig. 1. (a) Haplotype distribution of mitochondrial cytochrome oxidase (*cox1*) gene sequences from the present study. Haplotype diversity is represented as pie charts with analysis performed according to the state of origin of samples. Hd indicates the haplotypic diversity in each state.(b) Multiple sequence alignment of translated protein coding region of *cox1* haplotypes. Consensus amino acid at each location is shown above the multiple sequence alignment. Each line of the alignment represents a haplotype, shown on the left (with GenBank accession number). Percentage of consensus amino acid at each location is shown at the bottom.

missense (non-synonymous) mutations occurring at amino acid 40 (US Haplotypes 23 and 24) and at 113 (US Haplotype 21).

The occurrence of haplotypes in the different dog breeds included in the present study is shown (table 1). A diverse set of haplotypes were recorded from greyhounds and unknown breeds, which were both overrepresented in the sample set. The most frequently occurring haplotype, US Hap 1, was not restricted to any breed. Unique haplotypes (US Hap 12–25) were overrepresented in greyhounds due to the sampling bias associated with opportunistic sampling.

Haplotype network analysis supports the presence of three clusters

A median joining network of the haplotype dataset ($n = 35$ sequences; each representing one haplotype) was constructed. Haplotypic nomenclature, geographical origin and number of sequences for each haplotype was added from previous studies when available, as summarized in table 1. Three distinct clusters could be visualized, with all haplotypes from the United States and four of the seven haplotypes from Australia forming a cluster

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(cluster A). Three haplotypes from Australia formed a distinct cluster (cluster B), which had an average nucleotide difference of 27.6 from cluster A. Fixation index (Fst) between haplotypes in clusters A and B was 0.80. Fst between all United States haplotypes and all Australian haplotypes (in both clusters A and B) was 0.249. A single GenBank sequence from China was distinct (designated cluster C) with an average nucleotide difference of 16.9 from cluster A. Clusters B and C had an average difference of 32.7 nucleotides. Fst between cluster C and other clusters could not be calculated because cluster C comprised a single sequence. Additionally, there is some evidence for a sub-cluster within cluster A formed by the haplotypes US Hap12, US Hap 18, Aus Hap 7 and Jap Hap1. Fst and average number of nucleotide differences between the sub-cluster population and all the other haplotypes of cluster A is 0.335 and 8.8, respectively. Taken together, these data suggest that there is moderate genetic structuring of global *A. caninum* populations.

Phylogenetic analysis supports the existence of genetic clusters

A maximum likelihood tree of unique haplotypes was constructed using the generalized time reversible GTR model for nucleotide substitutions with discrete gamma model parameters (gamma

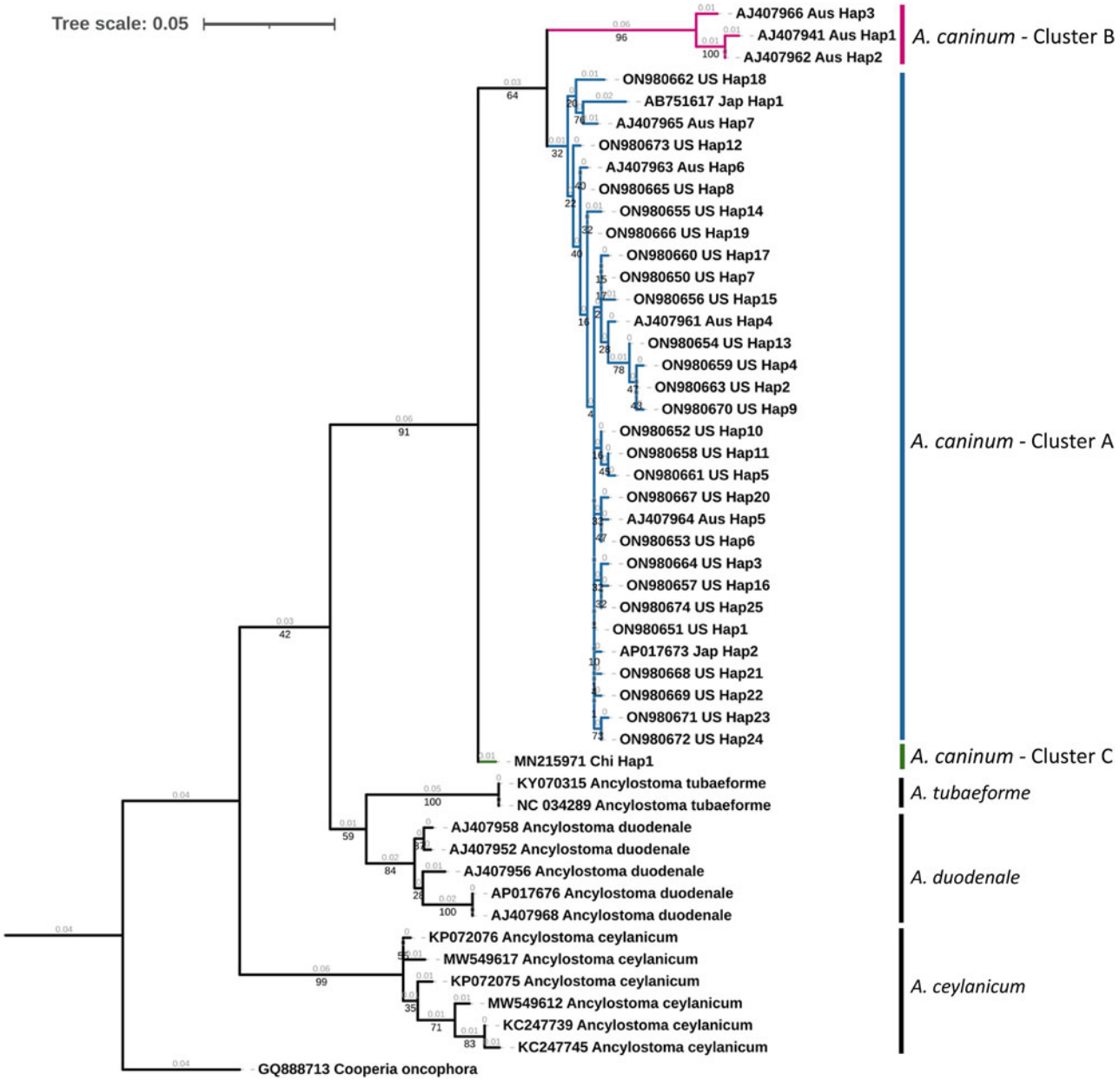

Fig. 2. Phylogenetic tree of *Ancylostoma* spp. A maximum likelihood tree of haplotypes generated from *Ancylostoma caninum* partial mitochondrial cytochrome oxidase (*cox1*) gene sequences in the present study and *Ancylostoma* spp. sequences derived from GenBank. Accession numbers, country code and haplotypic designation are given for each haplotype. *Cooperia oncophora cox1*, trimmed from the complete mitochondrial genome record (Accession number: GQ888713), was used as the outgroup. Cluster designations based on the haplotype network (fig. 3) are provided. Bootstrap values are represented in black under branches. Computed branch lengths are represented in grey above the branches.

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classes: four, shape parameter: 0.057) and bootstrap branch support (fig. 2). The *Cooperia oncophora cox1* sequence (GenBank Accession: GQ888713) was used as the outgroup. All sequences of *A. caninum* formed a monophyletic clade with high statistical support (91%). Sequences from the United States in the study were in cluster A, which appeared monophyletic but with low statistical support (32%). Three haplotypic sequences from Australia formed a distinct cluster (cluster B) with high statistical support (96%), but this cluster was monophyletic with the United States isolates with 64% statistical support. The single *cox1* sequence from China formed a distinct cluster (cluster C) but had low statistical support (<50%). The identity of *A. caninum* sequences from the present study as being distinct from the sequences of other *Ancylostoma* spp. was further highly supported by the monophyly of *A. tubaeforme* (100%), *A. duodenale* (84%) and *A. ceylanicum* (99%).

Discussion

Ancylostoma caninum is one of the most common nematode parasites of dogs in the United States and has the potential to cause significant disease in young dogs. As one of the causative agents of human CLM, its prevalence in dogs, incidence, genetic variation and viability in the environment, among other factors are important from a One Health perspective. Human CLM can be reduced by proper anthelmintic administration to dogs, by removing dog faeces immediately after defecation after donning appropriate personal protective equipment and instituting hygiene practices. Understanding the molecular epidemiology in dogs is a crucial starting point for the control of the infection in humans since prevalence and differences in pathogenicity in CLM at the population level is unknown (Heukelbach *et al.*, 2002). Yet, there is an important need for this information due to the reported increase in the annual incidence of infection in dogs (Drake & Carey, 2019), which increases the risk of human infection. We hypothesized that due to dog movements/importation, *A. caninum* isolates within the United States would have population structure. To test these hypotheses, we examined *A. caninum* in dog isolates from the United States and characterized prevalent *cox1* haplotypes.

Mitochondrial *cox1* has been used as a barcoding marker in multiple parasite studies (Busi *et al.*, 2007; Small *et al.*, 2014; Jesudoss Chelladurai *et al.*, 2017; Zhao *et al.*, 2022). Ideally, molecular epidemiology studies should use published protocols if any exist (such as using the same primer sets) and must deposit sequence data generated in an easily accessible public database. This approach has resulted in a plethora of phylogeographical epidemiological data being generated for important zoonotic parasites such as *Ascaris* spp. and *Echinococcus* spp. (Cavallero *et al.*, 2013; Spotin *et al.*, 2018). In the case of *A. caninum*, however, genetic data are scant due to unavailability of data from previous studies and this is complicated by using different primer sets by different research groups. The complete *cox1* gene is 1577 bp long in the *A. caninum* reference mitochondrial genome (GenBank Accession: NC_012309). The primer set used by Hu *et al.* (2002) flanks nucleotides 754 to 1146 of the *cox1*, while the primer set used by Miranda *et al.* (2008) flanks nucleotides 162 to 628 and the primer set used by Mulinge *et al.* (2021) flanks nucleotides 368 to 830. In the present study, the primers used by (Hu *et al.*, 2002) and (Moser *et al.*, 2007) were used for PCR amplification.

All haplotypes identified in the present study was exclusive to the United States. Our analysis revealed 35 unique *A. caninum*

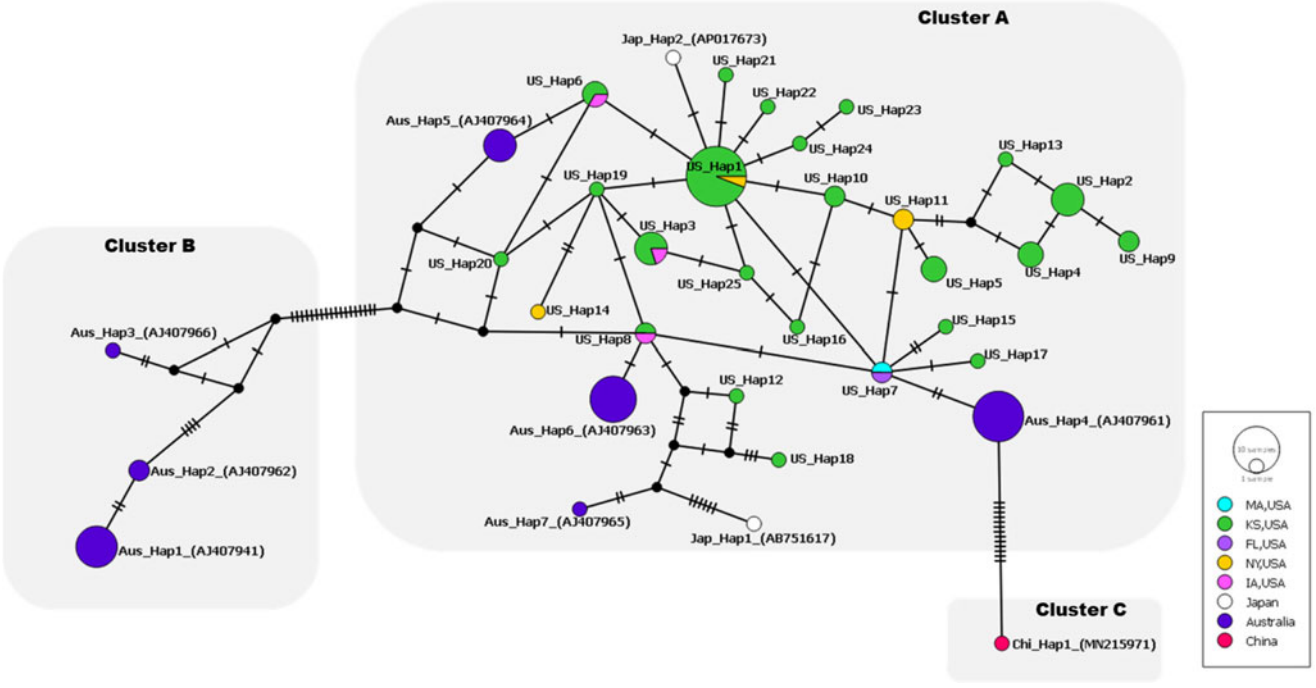

Fig. 3. Median joining network of mitochondrial cytochrome oxidase (*cox1*) gene haplotypes from the present study (designated US Hap 1–25) and GenBank sequences (denoted by country, haplotype designation and accession numbers). Haplotype circles are coloured to represent unique geographical sources of the sequence and are scaled to represent the number of sequences belonging to each haplotype (the present study and Hu *et al.* 2002). Nucleotide differences are denoted by hatch marks across the connecting lines with each mark representing a single nucleotide difference. Unlabelled dark circles represent inferred, unsampled nodes. Three clusters observable in the network are represented by grey boxes.

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haplotypes currently reported globally and 25 unique haplotypes within the United States. To date, there has been a paucity of publicly available molecular data from *A. caninum* populations in the United States. Unique haplotypes were also found in a previous United States study (Moser *et al.*, 2007), but the absence of publicly available data from that study makes direct comparisons impossible. Overall haplotypic diversity among United States samples in the present study (0.931; 60 samples) was higher than diversity at state level – 0.886 in Kansas (51 samples) and 0.833 in New York (four samples; fig. 1a). The higher diversity (1.0) reported from Iowa is attributable to the small random sample obtained for inclusion in the study (three samples). State level haplotype diversities from the present study were comparable to *A. caninum* diversity previously reported from Australia (0.80; 38 samples) (Hu *et al.*, 2002), from North Carolina, United States (0.828; 54 samples) (Moser *et al.*, 2007) and Brazil (0.88; 164 samples) (Miranda *et al.*, 2008). These diversities are significantly higher than the *A. caninum* diversity reported from Massachusetts, United States (0.25; eight samples; (Moser *et al.*, 2007) and diversity of other soil-transmitted zoonotic nematodes such as *Ascaris* from United States (Iowa) populations (0.596; 100 samples; (Jesudoss Chelladurai *et al.*, 2017). Additionally, high numbers of *A. caninum* haplotypes were recorded in greyhounds, a breed overrepresented in reports of anthelmintic resistance (table 1).

Median joining haplotype network analysis demonstrated a complex pattern (fig. 3). *Ancylostoma caninum* occurred in three clusters, which agrees with Moser *et al.* (2007). United States' haplotypes were found only in cluster A, while Australian haplotypes were found in clusters A and B. Cluster B currently only has haplotypes from Australia and cluster C has only one haplotype from China. Combined with the Fst values obtained, it can be concluded that there is moderate population structuring in *A. caninum*. The maximum likelihood tree provided high statistical support for haplotypes in cluster B (fig. 2). Due to the moderately high statistical support for the supercluster containing clusters A and B (64%), it is evident that the sequence in cluster C is distinct from clusters A and B. Given the high divergence of sequences in the clades, an alternate explanation could be that clades B and C are cryptic species.

Haplotype differentiation and spread can be attributed to many factors, such as hybridization, introgression and retention of ancestral polymorphisms (Detwiler & Criscione, 2010). Dog movement and dog importation also play a role in the spread of zoonotic parasites (Wright *et al.*, 2020; von Rentzell *et al.*, 2022). Rates of mutation that led to the formation of novel haplotypes is still relatively unknown in zoonotic helminths (Jesudoss Chelladurai *et al.*, 2017).

Some limitations of the present study include a geographical sampling bias associated with the selection of *A. caninum* positive faecal samples sent to a state veterinary diagnostic laboratory. Since samples were opportunistically collected, clinical history was not considered as a factor in the analysis and often not provided by submitters. The use of Sanger sequencing allowed the capture of only the predominant *cox1* haplotype in each sample. Additionally, the use of *cox1* primers from Hu *et al.* (2002) disallowed us from making comparisons with the dataset from Miranda *et al.* (2008). Furthermore, the lack of *A. caninum* gene sequences from across the world in GenBank and/or other public nucleotide databases led to a low global representation of *A. caninum* populations in the present study. These limitations can be overcome in future studies by prospective selection of dogs with adequate clinical history for inclusion in studies and by using deep amplicon sequencing to detect intra-sample variations in mitochondrial sequences.

Molecular tools can be useful for increasing our understanding of *A. caninum*, and therefore, CLM epidemiology. The usefulness of *cox1* sequences amplified from eggs in describing infection epidemiology has been previously demonstrated with human hookworms (Monteiro *et al.*, 2019). In the present study, we provide molecular characterization of *A. caninum* from dogs in the United States. Sequences from the present study are available in GenBank for future comparative analyses. Our results demonstrated that dogs harbour haplotypes of *A. caninum* unique to the United States. Future studies of *A. caninum* isolates from endemic regions across the world are essential to understand the genetic diversity of this parasite.

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