CHAPTER 6

Genetic Characterization of Zoonotic *Ancylostoma Ceylanicum* from Humans, Dogs and Cats in Rural communities in Peninsular Malaysia targeting the Second Internal Transcribed Spacer (ITS-2) Ribosomal RNA and Partial Mitochondrial Cytochrome Oxidase C Subunit I (*Cox* 1) Genes

6.1 INTRODUCTION

Besides anthroponotic hookworms, humans may also harbor zoonotic infections with canine and feline hookworm species. For example, cutaneous larva migrans (CLM) or 'creeping eruptions' is a hypersensitivity reaction caused by migrating nematode larvae, of which *Ancylostoma braziliense* is the most frequently implicated aetiological agent in humans (Chaudhry et al, 1989; Malgor et al, 1996; Bouchaud et al, 2000; Manning et al, 2006; Bowman et al, 2010; Feldmeier & Shuster, 2012). Hookworm-related CLM cases have also been reported from Malaysian patients (Hanjeet et al, 1988; Robson & Othman, 2008; Yap, 2010; 2011; Hamat et al, 2010) and in tourists who have visited Malaysia (Bouchaud et al, 2000; Lederman et al, 2008; Bowman et al, 2010). Another canine hookworm, *Ancylostoma caninum* is the leading cause of human eosinophilic enteritis (EE) with many cases reported in Australia (Loukas et al, 1992; Croese et al, 1994a; Landmann & Prociv, 2003). Cases have also been reported sporadically in the United States (Khoshoo et al, 1995), Egypt (Bahgat et al, 1999), the Philippines, South America and Israel (Croese et al, 1994b).

Ancylostoma ceylanicum however, is the only species of animal hookworm known to produce patent infections in humans. This has been demonstrated both experimentally (Wijers & Smit, 1966; Carroll & Grove, 1986) and naturally. Natural

infections with *A. ceylanicum* have been first reported in Dutch servicemen returning from West New Guinea, who suffered heavy infection with concurrent anemia (Anten & Zuidema, 1964). Zoonotic ancylostomiasis caused by *A. ceylanicum* has been reported in many regions of Asia and Southeast Asia including Philippines (Velasquez & Cabrera, 1968), Taiwan (Yoshida et al, 1968) and India (Chowdhury & Schad, 1972; Traub et al, 2007). Recently, there was an influx of studies focusing on the investigation of prevalence of this parasitic zoonosis using molecular tools. These advanced tools are mainly used to detect and characterize infection directly from eggs in human and animal feces targeting the internal transcribed spacer 2 (ITS-2) of nuclear rDNA as the genetic marker for hookworm species identification (Traub et al, 2007; Traub et al, 2008; Sato et al, 2010; Jiraanankul et al, 2011; Conlan et al, 2012).

To date, a range of various target regions such as nuclear ribosomal DNA particularly the internal transcribe spacer 1 (ITS-1) and ITS-2 rDNA gene have successfully been employed for strongylid nematode including hookworm species or strain identification at the molecular level (Gasser et al, 2006a). This locus has a lower mutation rate, is repetitive due to less sequence variation among or between populations, which make them suitable used as species-specific markers (Elder & Turner, 1995; Hoste et al, 1995; Stevenson, 1995; Romstad et al, 1998). Although the ITS region provides a highly sensitive tool for the detection and differentiation of hookworm DNA at the species level (Traub et al, 2004), it lacks the genetic resolution and no intraspecific variation in order to provide sufficient level of sequence variation within the subspecies level (Hoste et al, 1995; Stevenson, 1995; Romstad et al, 1998). In contrast, mitochondrial (mt) DNA genes have more intraspecific sequence variation because of their maternal inheritance and relatively high evolutionary rates (Avise et al, 1987) than nuclear rDNA, makes them suitable for population genetic studies (Blouin,

2002; Hu et al, 2002; Hu & Gasser, 2006). The use of other markers such as the cytochrome c oxidase subunit 1 (*cox1*), a protein coding in mitochondrial (mt) DNA that shows more intraspecific sequence variation (Blouin, 2002; Hu & Gasser, 2006) has successfully been used to explore and investigate the genetic structure of many strongylid nematodes including hookworm (Hawdon et al, 2001; Zhan et al, 2001; Hu et al, 2002; Li et al, 2004; Hu et al, 2008).

Our previous study among communities living in hookworm endemic areas indicated that almost a quarter (11/58; 19.0%) of hookworm-positive individuals carrying infections with *A. ceylanicum* (i.e., those studied and mentioned in **Chapter 5**). Likewise, our earlier study to determine the prevalence of intestinal helminths infections in dogs and cats in these communities demonstrated that hookworm infections (61.9%; 65/105) were significantly high in both dogs and cats compared to other parasites species (i.e., those studied and mentioned in **Chapter 4**). However, as the species identification of hookworm in human was genetically characterized on the basis of its ITS-2 of nuclear rDNA in the latter, this nuclear locus does not provide sufficient genetic resolution to infer within-species differences (Hoste et al, 1995; Stevenson, 1995; Romstad et al, 1998). Further genetic discrimination at a more variable locus will allow further evidence to support the zoonotic exchange of this hookworm species between humans and animals. With this in mind, we aimed to further investigate the zoonotic potential of *A. ceylanicum* isolates recovered from the feces of humans, dogs and cats living in the same communities in rural Malaysia by targeting the *cox1* gene as complementary to the ITS-2 gene.

6.1.1 Objectives of the study

General objective

To further investigate the zoonotic potential of *A. ceylanicum* isolates recovered from the feces of humans, dogs and cats living in the same rural communities in Peninsular Malaysia.

Specific objectives

1. To determine the species of hookworm in dogs and cats using conventional PCR by targeting the ITS-2 gene.

2. To further explore the zoonotic potential of *A. ceylanicum* isolates recovered from the feces of humans, dogs and cats living in the same communities by targeting the *cox1* gene.

3. To compare the sequence diversity within *cox 1* sequences of *A. ceylanicum* isolates recovered from humans, dogs and cats.

4. To investigate the intraspecies and interspecies sequence variation within *cox 1* sequences of *A. ceylanicum* isolates.

6.1.2 Research hypotheses

1. The common species of hookworm in dogs and cats include *A. ceylanicum*, *A. caninum* and *A. brazilienze*.

2. There will be a considerable level of genetic variation within the *cox 1* sequences of *A. ceylanicum* isolated from humans, dogs and cats.

3. Some of the *A. ceylanicum* strains from the same geographical location and host are expected to be clustered together within the same group.

4. There will be high number of nucleotide substitution (e.g., transversions and transitions) within the *Ancylostoma* genus. These mismatches of nucleotide will allow differentiation between *Ancylostoma* species.

6.1.3 Significance of the study

Parasitic zoonoses pose a continuing public health problem, especially in endemic developing countries where the majority of populations live in poor, overcrowded conditions, lack of education, practice poor standards of hygiene and sanitary behavior. Close contact with domestic animals such as dogs and cats coupled with a poor veterinary care and sanitation expose these communities to high risk of acquiring zoonotic infections. The use of molecular tools as reported here allows us to identify zoonotic hookworm species causing infections to humans and animals, which cannot be achieved by conventional microscopic examination of feces alone. In addition, the use

of more than one genetic marker such as *cox 1* gene as complementary to ITS-2 gene, which has been proven to be more suitable for genetic diversity study, will provides additional evidence to further support the zoonotic exchange of hookworm species between humans and animals, particularly *A. ceylanicum*.

6.2 MATERIALS AND METHODS

6.2.1 Background

Details of the consent, sample collection, sampling scheme, population sampled and microscopic examination of fecal samples prior to this genotyping study have been described previously (**Chapters 3 and 4)**.

6.2.2 DNA amplification of hookworm species in dogs and cats fecal samples targeting the ITS-2 gene using conventional PCR

Specific species identification of hookworm in human has been previously described and discussed in **Chapter 5**. As for dogs and cats, specific species identification of hookworms was carried out using direct conventional PCR assay from microscopically hookworm-positive fecal samples of dogs and cats. In brief, a forward primer NC1 (5'-ACG TCT GGT TCA GGG TTC TT-3') and reverse primer NC2 (5'-TTA GTT TCT TTT CCT CCG CT-3') (Gasser et al, 1993) were used to amplify an approximately 420 bp regions of internal transcribed spacer-2 (ITS-2) and 28S ribosomal RNA region *Ancylostoma* spp following similar protocols as previously described in **Chapter 5.2.7 (Conventional semi-nested PCR)**. In brief, the PCR was carried out in a 50 µl final

mixture containing 10x PCR buffer, 2.5mM dNTPs), 25 mM $MgCl_2$, 10 pmol of each primer, 5U of *Taq* polymerase and 6 µl of DNA template (i.e., hookworm-positive samples). Control samples without DNA were included in each PCR. The sample was heated to 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds (denaturing), 55°C for 30 seconds (annealing), 72°C for 30 seconds (extension) and a final extension at 72°C for 7 minutes.

6.2.3 DNA amplification of *Ancylostoma ceylanicum* targeting cytochrome oxidase c subunit 1 (*cox 1*) gene using conventional PCR

In order to further investigate the zoonotic ancylostomiasis caused by *A. ceylanicum*, human, dog and cat fecal samples that were positive for *A. ceylanicum* based on PCR targeting the internal transcribed spacer-2 (ITS-2) ribosomal gene were then subjected to another PCR assay targeting the mitochondrial cytochrome oxidase c subunit I (*cox 1*) gene. A region of 450 bp in the *cox1* gene was amplified from the genomic DNA using a forward primer JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') and a reverse primer JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG-3') (Bowles et al, 1992), verified to be sufficiently conserved for hookworm by sequencing (Hu et al, 2002). The PCR was conducted in a final mixture of 20 µL containing 10 µL of ExPrime Taq™ Premix (GENET BIO, South Korea), 10 pmole of each primers and 2 µL of DNA template. DNA blank (DNase/RNase free water, Sigma Cat. no. W4502, St. Louis, MO) was also included in each PCR as negative control. The sample was then heated to 94 °C for 5 min (initial denaturation), followed by 30 cycles of 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing), 72 °C for 30 s (extension) and a final extension cycle at 72 °C for 5 min.

6.2.4 PCR product analysis

All-positive amplicon generated from both DNA amplifications for each sample were then purified and sequenced. Sequence chromatograms were manually aligned and the consensus sequence was created for each sample. Homology searches were carried out using Basic Local Alignment Search Tool (BLAST) hosted by National Centre for Biotechnology Information (NCBI) (Bethesda, MD) reference sequences. Details on the PCR product analysis have been previously described in **Chapter 5.2.7(Conventional semi-nested PCR).**

6.2.5 Phylogenetic analysis of *cox1* gene

As the complete mitochondria genome of *A. ceylanicum* is currently not available in the GenBank data, each of the nucleotide sequences was then aligned together with the full mitochondria genome of *A. caninum* (GenBank accession number NC012309 and FJ483518) and *A. duodenale* (GenBank accession number NC003415 and AJ417718) which served as reference sequences and out groups to the sequences generated in the present study using CLUSTAL-W analysis (Thompson et al, 1994). Neighbor-Joining (NJ) algorithm with Kimura 2 parameter analysis and Maximum Parsimony (MP) phylogenetic analysis were conducted using MEGA4 program (Tamura & Kumar, 2002). The tree was drawn to scale, with branch lengths in the same unit as those of the evolutionary distances used to infer the phylogenetic tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test values (1000 replicates) is shown next to the branches. All sequences generated were deposited in NCBI GenBank (Appendix H).

6.3 RESULTS

6.3.1 Identification of hookworm species in dogs and cats targeting the ITS-2 gene

Details on the specific species of hookworm infections in humans were provided in **Chapter 5**. In brief, *N. americanus* 87.2% (41/47) were the most predominant species infecting human followed by *A. ceylanicum* (11/47; 23.4%). No *A. duodenale* were detected in humans. As for dogs and cats, a total of 65 microscopically hookworm-positive fecal samples were subjected to PCR and sequence analysis. The PCR amplicons were successfully amplified and sequenced from 51 (78.5%) microscopic positive samples (Table 6.1). Sequence comparison using BLAST hosted by NCBI showed that 51.0% (26/51), 47.1% (24/51) and 2.0% (1/51) of these fecal samples were infected with *A. caninum, A. ceylanicum* and *A. braziliense*, respectively (Table 6.2). Of this, *A. ceylanicum* were detected in both dogs (79.2%; 19/24) and cats (20.8%; 5/24) whilst *A. caninum* were only found in dogs (100%; 26/26). Likewise, a single *A. braziliense* infection was isolated from cat. High prevalence of *A. caninum* were recorded in both animals in Kuala Pangsun village (25.5%; 13/51), followed by Gurney (9.8%; 5/51), Sungai Bumbun and Kemensah (5.9%; 3/51 each) and the lowest was reported in Pos Iskandar and Sungai Miak (2.0%; 1/55 each). As for *A. ceylanicum*, higher prevalence was reported in Gurney and Kuala Pangsun village (9.8%; 5/51). Pos Iskandar and Sungai Bumbun had the same prevalence of *A. ceylanium* (7.8%; 4/51 each). Similarly, the same prevalence of *A. celanicum* in dogs and cats was reported in Sungai Miak and Kemensah village (5.9%; 3/51). The only *A. braziliense* infection was isolated from cat in Kuala Pangsun.

6.3.2 Genetic characterization of the partial mitochondrial cytochrome oxidase c subunit I (*cox 1*) gene of the *Ancylostoma ceylanicum* from humans, dogs and cats

6.3.2.1 Phylogenetic analysis of *cox 1* gene

A total of 36 genomic DNA fecal samples extracted from humans, dogs and cats in which *A. ceylanicum* infection was previously confirmed were amplified by optimized PCR targeting the *cox1* gene. Of the 36 samples, only 22 samples (i.e., 12 dogs, 8 humans and 2 cats) were successfully sequenced at the *cox1* gene. All sequences from this study representing humans, dogs and cats together with four reference sequences obtained from GenBank database were used to examine the relationship between the *A. ceylanicum* isolates using Neighbor-Joining (NJ) (Figure 6.1) and Maximum Parsimony (MP) phylogenetic analysis (Figure 6.2). The trees were distinctly separated into 3 clusters with the *A. ceylanicum* isolates grouped together, and genetically distinct from *A. caninum* isolates (GenBank accession numbers NC012309 and FJ483518) and *A. duodenale* isolates (GenBank accession numbers NC003415 and AJ417718). Within *A. ceylanicum*, the cluster was divided into two clades, one consisting of 3 human isolates, the other comprising of 19 isolates sourced from both humans and animals origin from different geographical locations (Figure 6.1 and 6.2). This separation was strongly supported by bootstrap analysis (99% for both Neighbor-Joining (NJ) and Maximum Parsimony (MP) methods). The two groups of *A. ceylanicum* could be distinguished from each other by five fixed nucleotide differences at locations 891, 966, 1008, 1077 and 1083 with the highest number of nucleotide variations being noted at location 1083 involving adenine, thymine and guanine bases (Table 6.3). Figure 6.3 shows the multiple sequences alignment of the *A. ceylanicum* isolates from this study representing

humans, dogs and cats together with reference sequences obtained from GenBank database.

Of these, four fixed nucleotide differences at locations 966, 1008, 1077 and 1083 were recorded in human strains, could be distinguished from each other based on their geographical locations. Similarly, the *A. ceylaniucm* strains of dogs isolated from different geographical locations could also be distinguished from one another by four fixed nucleotide differences at locations 891, 1008, 1077 and 1083. In cats, the two *A. ceylanicum* strains from different geographical locations (i.e., Gurney and Pangsun) could be distinguished from one another by two fixed nucleotide differences at locations 891 and 1088. Strains of *A. ceylanicum* from the same geographical location (i.e., Gurney) isolated from different hosts (i.e., humans and dogs) clustered together within the same group providing evidence to show that strains of *A. ceylanicum* are being circulated between human and animal hosts within endemic foci of hookworm infection. However, given the small number of *A. ceylanicum* isolates in the present study, no statistical significance was observed when comparing each strain based on their geographical locations.

6.3.2.2 Intraspecies and interspecies genetic diversity of the *A. ceylanicum*

All 22 *cox1* sequences in this study were multiple aligned against the complete mitochondria genome of *A. caninum* (GenBank accession number NC012309 and FJ483518) and *A. duodenale* (GenBank accession number NC003415 and AJ417718) to investigate the intraspecies and interspecies genetic diversity of the *A. ceylanicum*. Altogether 59 different mismatches were recorded within the genus represented either by transversions (Tv), transitions (Ts) or multiple substitution events (Table 6.3). Within each *A. ceylanicum*, *A. caninum* and *A. duodenale*, the percentage of

transversions (62.7%; 37/59) was higher than transitions (55.9%; 33/59). However, the Tv:Ts ratio was not statistically significant as assessed by Pearson's chi-square test (X^2 test) ($p>0.05$). In each sequence or region of the *A. ceylanicum*, *A. caninum* and *A. duodenale*, A and T transversions were most common (28 transversions), reflecting the A and T biasness in this data.

Other mismatches including transversions were also observed though rare by comparison, for example G and T (9 transversions) and one transversion of A and C. Other nucleotide variations such as transitions were also present including 25 transitions of A and G and 4 of C and T. These mismatches of nucleotide at different locations allowed the discrimination between *Ancylostoma* species. For example, mismatches at positions 858, 939, 966, 996, 999 and 1083 separated *A. ceylanicum*, *A. caninum* and *A. duodenale* among each other. Mismatches at locations 729, 738, 819, 822, 837, 858, 867, 909, 912, 918, 924, 945, 948, 969, 975, 981, 987, 1018, 1026, 1038 and 1062 allowed differentiation of *A. ceylanicum* with *A. caninum* and *A. duodenale*.

Table 6.1: Hookworm infections detected by microscopy and conventional PCR assay [a] of dogs and cats fecal samples in Peninsular Malaysia

Location	Dogs (N=77)					Cats (N=28)				
	No. Examined	Microscopy		PCR assay		No. Examined	Microscopy		PCR assay	
		n	%	n	% [b]		n	%	n	% [b]
Gurney	12	11	91.6	9	81.8	2	2	100	1	50.0
Pos Iskandar	12	5	41.7	5	100	0	0	0	0	0
Kuala Pangsun	27	20	77.8	17	80.9	17	2	11.8	2	100
Bukit Serok	0	0	0	0	0	0	0	0	0	0
Sungai Bumbun	8	7	87.5	5	71.4	8	6	75.0	2	33.3
Sungai Layau	0	0	0	0	0	0	0	0	0	0
Sungai Miak	8	5	62.5	4	80.0	0	0	0	0	0
Kemensah	10	6	60.0	5	83.3	1	1	100	1	100
Total	**77**	**54**	**70.1**	**45**	**83.3**	**28**	**11**	**39.3**	**6**	**54.5**

[a] Species identification was carried out using conventional PCR targeting the **ITS-2 gene**

[b] Frequency was calculated based on number of PCR-positive sample divided by number of microscopy-positive sample for respective location

Table 6.2: Hookworm species in dogs and cats fecal sample detected by conventional PCR assay [a] according to locations in Peninsular Malaysia

Location	Dogs							Cats					
	PCR	*A. ceylanicum*		*A. caninum*		*A. braziliense*		PCR	*A. ceylanicum*		*A. caninum*		*A. braziliense*	
	Positive	n	%	n	%	n	%	Positive	n	%	n	%	n	%
Gurney	9	4	44.4	5	55.5	0	0	1	1	100	0	0	0	0
Pos Iskandar	5	4	80.0	1	20.0	0	0	0	0	0	0	0	0	0
Kuala Pangsun	17	4	23.5	13	76.5	0	0	2	1	50.0	0	0	1	50.0
Bukit Serok	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sungai Bumbun	5	2	40.0	3	60.0	0	0	2	2	100	0	0	0	0
Sungai Layau	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sungai Miak	4	3	75.0	1	25.0	0	0	0	0	0	0	0	0	0
Kemensah	5	2	40.0	3	60.0	0	0	1	1	100	0	0	0	0
Total	**45**	**19**	**42.2**	**26**	**57.8**	**0**	**0**	**6**	**5**	**83.3**	**0**	**0**	**1**	**16.7**

[a] Species identification was carried out using conventional PCR targeting the **ITS-2 gene**

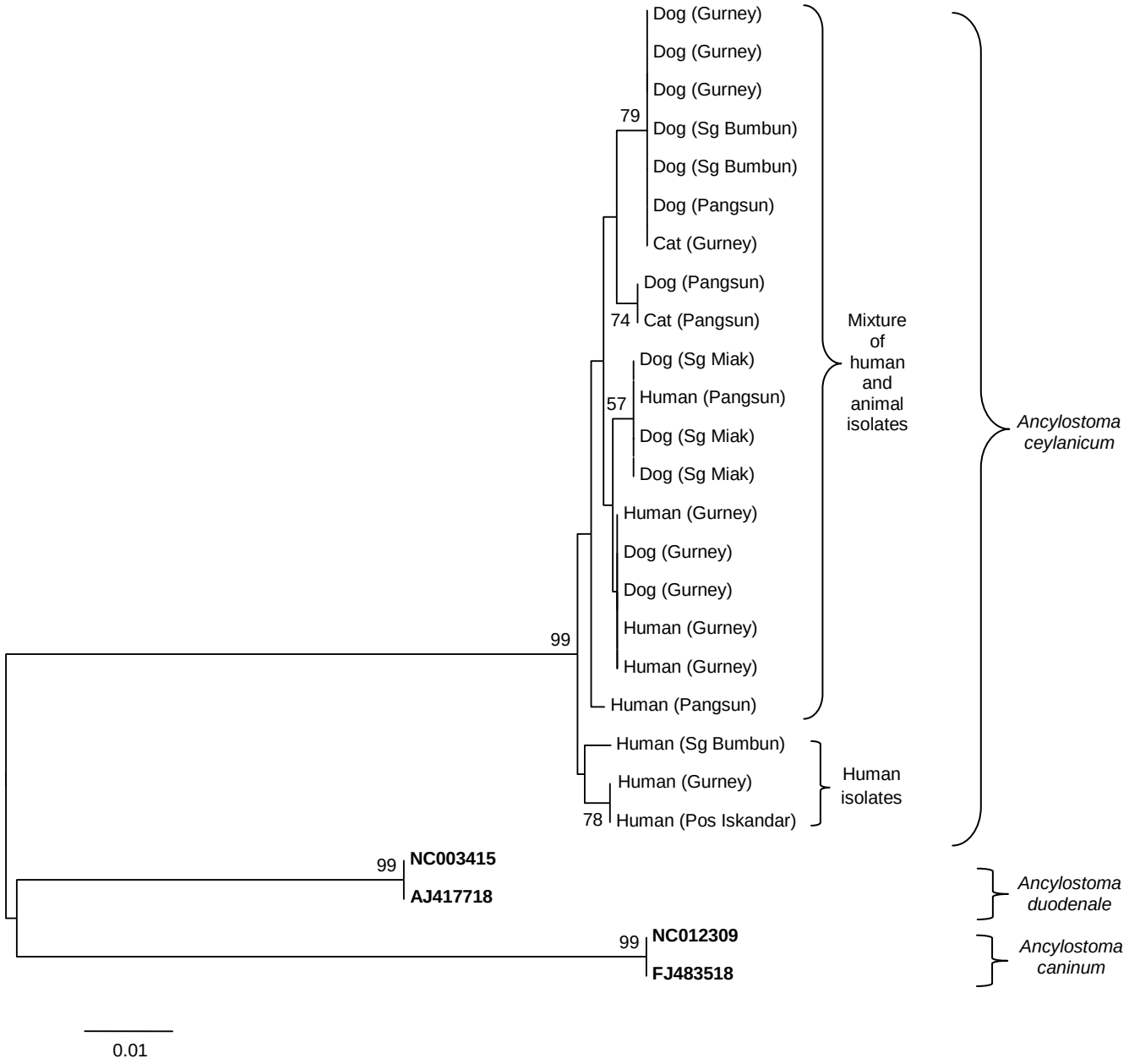

Figure 6.1: Phenogram of the *A. ceylanicum* sequences of ***cox 1* gene** constructed using Neighbor-Joining (NJ). Sequences from the present study and reference sequences from GenBank are indicated. Figure at nodes represent the degree of bootstrap support based on 1000 bootstrapped trees. Only bootstrap values >50% are shown

* The isolates were labeled according to the host and geographical location

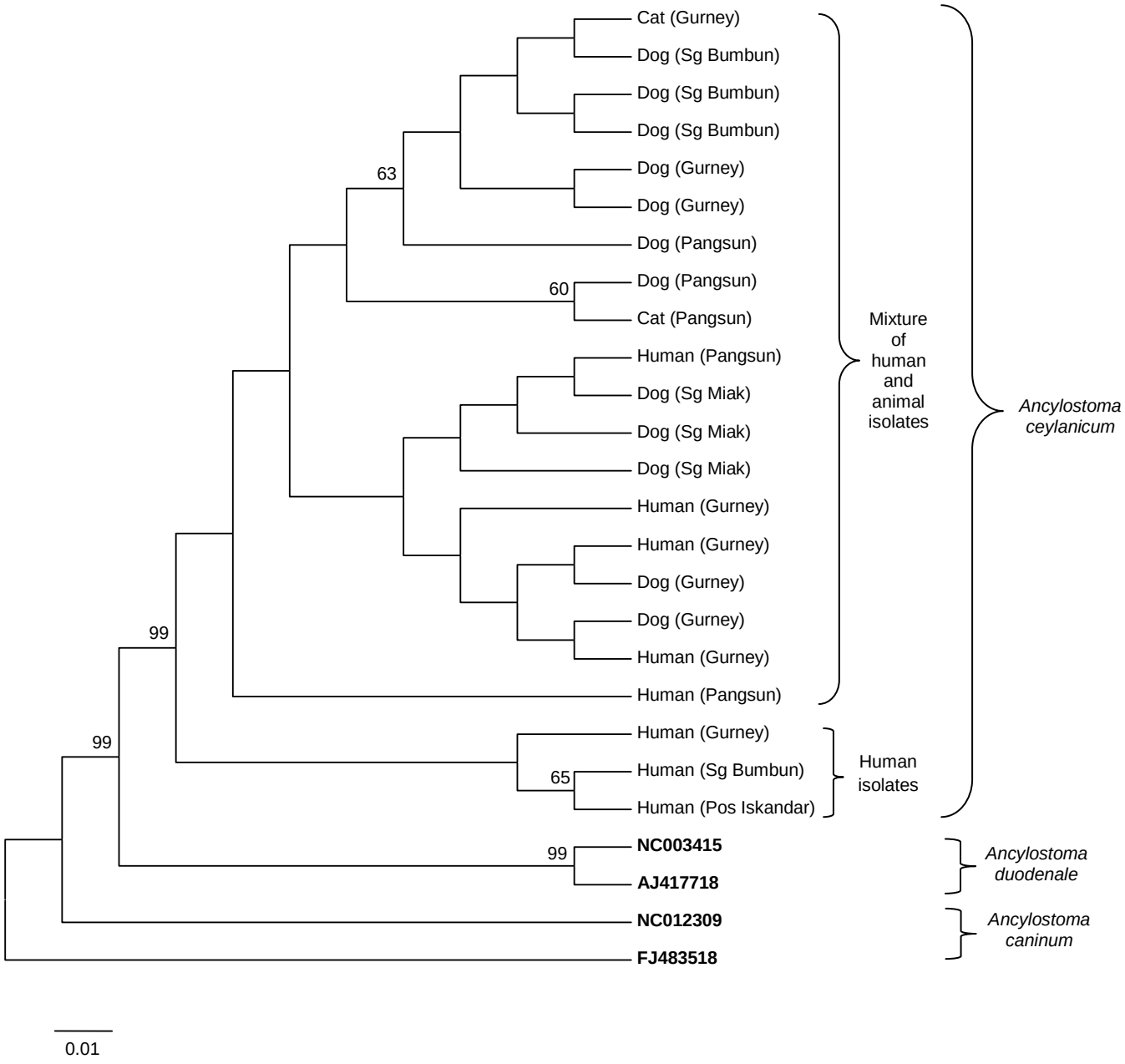

Figure 6.2: Phenogram of the *A. ceylanicum* sequences of ***cox 1*** **gene** constructed using Maximum Parsimony (MP). Sequences from the present study and reference sequences from GenBank are indicated. Figure at nodes represent the degree of bootstrap support. Only bootstrap values >50% are shown

* The isolates were labeled according to the host and geographical location

Table 6.3: Multiple alignments of ***cox 1* gene** of *A. ceylanicum* sequences from this study with the reference sequences obtained from GenBank, representing *A. caninum* and *A. duodenale*

Isolates	GenBank Accession Number	Nucleotide position from the start of the gene																																												
		729	738	741	759	768	783	786	807	819	822	828	834	837	843	846	849	858	867	879	882	885	888	891	903	906	909	912	918	924	927	939	942	945	948	951	954	960	963	966	969	975	978	981	987	990	996
A. caninum	**FJ483518**	**A**	**T**	**C**	**A**	**T**	**T**	**A**	**T**	**A**	**A**	**T**	**A**	**T**	**A**	**C**	**T**	**A**	**G**	**G**	**T**	**G**	**A**	**T**	**G**	**G**	**T**	**G**	**G**	**T**	**G**	**T**	**A**	**A**	**T**	**A**	**A**	**A**	**A**	**T**	**T**	**A**	**G**	**T**	**G**	**A**	**A**
A. duodenale	**NC003415**	.	.	**T**	**G**	**A**	.	**T**	**A**	.	.	.	**G**	.	**T**	**T**	**A**	**T**	.	**A**	**G**	**A**	**T**	**C**	**T**	**A**	.	.	.	.	**T**	**G**	**T**	.	.	**G**	**G**	**T**	**T**	**A**	.	.	**A**	.	.	**G**	**C**
Dog (Pangsun)	KC247727	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	**C**	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Dog (Pangsun)	KC247742	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Dog (Sg Bumbun)	KC247729	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	**C**	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Dog (Sg Bumbun)	KC247730	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	**C**	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Dog (Sg Bumbun)	KC247731	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	**C**	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Dog (Gurney)	KC247732	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	**C**	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Dog (Gurney)	KC247733	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	**C**	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Dog (Gurney)	KC247735	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Dog (Gurney)	KC247736	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Dog (Sg Miak)	KC247739	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Dog (Sg Miak)	KC247741	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Dog (Sg Miak)	Resubmitted	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Cat (Gurney)	KC247728	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	**C**	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Cat (Pangsun)	KC247743	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Human (Gurney)	KC247737	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Human (Gurney)	KC247745	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	.
Human (Gurney)	KC247734	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Human (Gurney)	KC247738	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Human (Pangsun)	KC247740	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	**T**
Human (Pangsun)	KC247744	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	.
Human (Sg Bumbun)	Resubmitted	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	.
Human (Pos Iskandar)	Resubmitted	**T**	**G**	**T**	.	.	**A**	.	**A**	**G**	**G**	**A**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	.	**A**	**T**	.	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**A**	**T**	**T**	**A**	.	.	**T**	**T**	**G**	**A**	**T**	.	**A**	**A**	.	.

Isolates	GenBank Accession Number	Nucleotide position from the start of the gene												
		999	1002	1008	1018	1019	1026	1035	1038	1041	1047	1062	1077	1083
A. caninum	**FJ483518**	**G**	**G**	**A**	**A**	**A**	**A**	**G**	**G**	**A**	**A**	**G**	**A**	**A**
A. duodenale	**NC003415**	**A**	**A**	**T**	**.**	**T**	**.**	**A**	**.**	**G**	**G**	**.**	**T**	**T**
Dog (Pangsun)	KC247727	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**G**
Dog (Pangsun)	KC247742	**T**	**.**	**.**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**G**
Dog (Sg Bumbun)	KC247729	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**G**
Dog (Sg Bumbun)	KC247730	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**G**
Dog (Sg Bumbun)	KC247731	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**G**
Dog (Gurney)	KC247732	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**G**
Dog (Gurney)	KC247733	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**G**
Dog (Gurney)	KC247735	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**.**
Dog (Gurney)	KC247736	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**.**
Dog (Sg Miak)	KC247739	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**.**	**.**
Dog (Sg Miak)	KC247741	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**.**	**.**
Dog (Sg Miak)	Resubmitted	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**.**	**.**
Cat (Gurney)	KC247728	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**G**
Cat (Pangsun)	KC247743	**T**	**.**	**.**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**G**
Human (Gurney)	KC247737	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**.**
Human (Gurney)	KC247745	**T**	**.**	**.**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**.**	**.**
Human (Gurney)	KC247734	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**.**
Human (Gurney)	KC247738	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**.**
Human (Pangsun)	KC247740	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**.**	**.**
Human (Pangsun)	KC247744	**T**	**.**	**T**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**.**
Human (Sg Bumbun)	Resubmitted	**T**	**.**	**.**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**T**
Human (Pos Iskandar)	Resubmitted	**T**	**.**	**.**	**G**	**T**	**G**	**A**	**A**	**G**	**.**	**A**	**T**	**T**

Bold alphabets represent nucleotide substitutions from the start of the gene. Dots (.) represent nucleotide identities

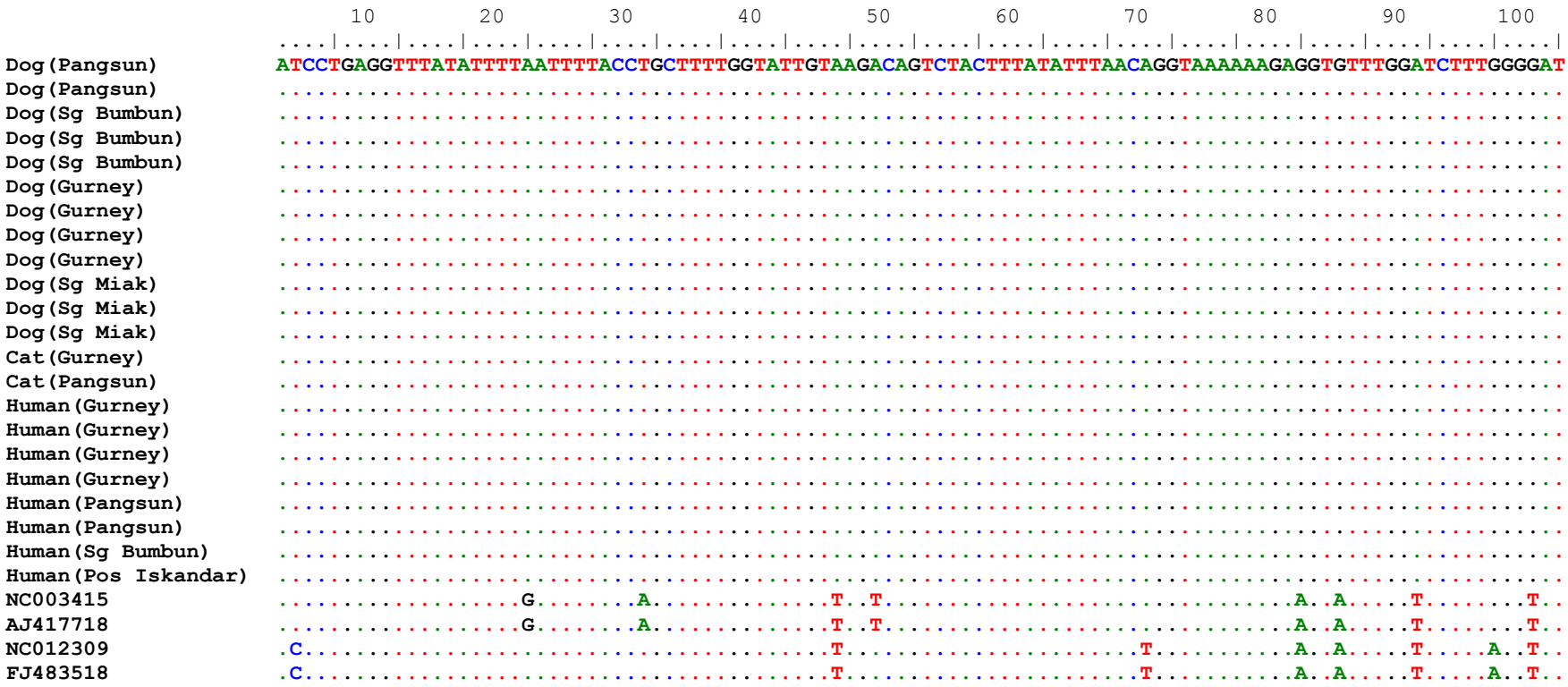

Figure 6.3: Multiple sequences alignment of ***cox 1 gene*** for *A. ceylanicum* isolates from this study representing humans, dogs and cats together with reference sequences obtained from GenBank database. Dots (.) represent nucleotide identities

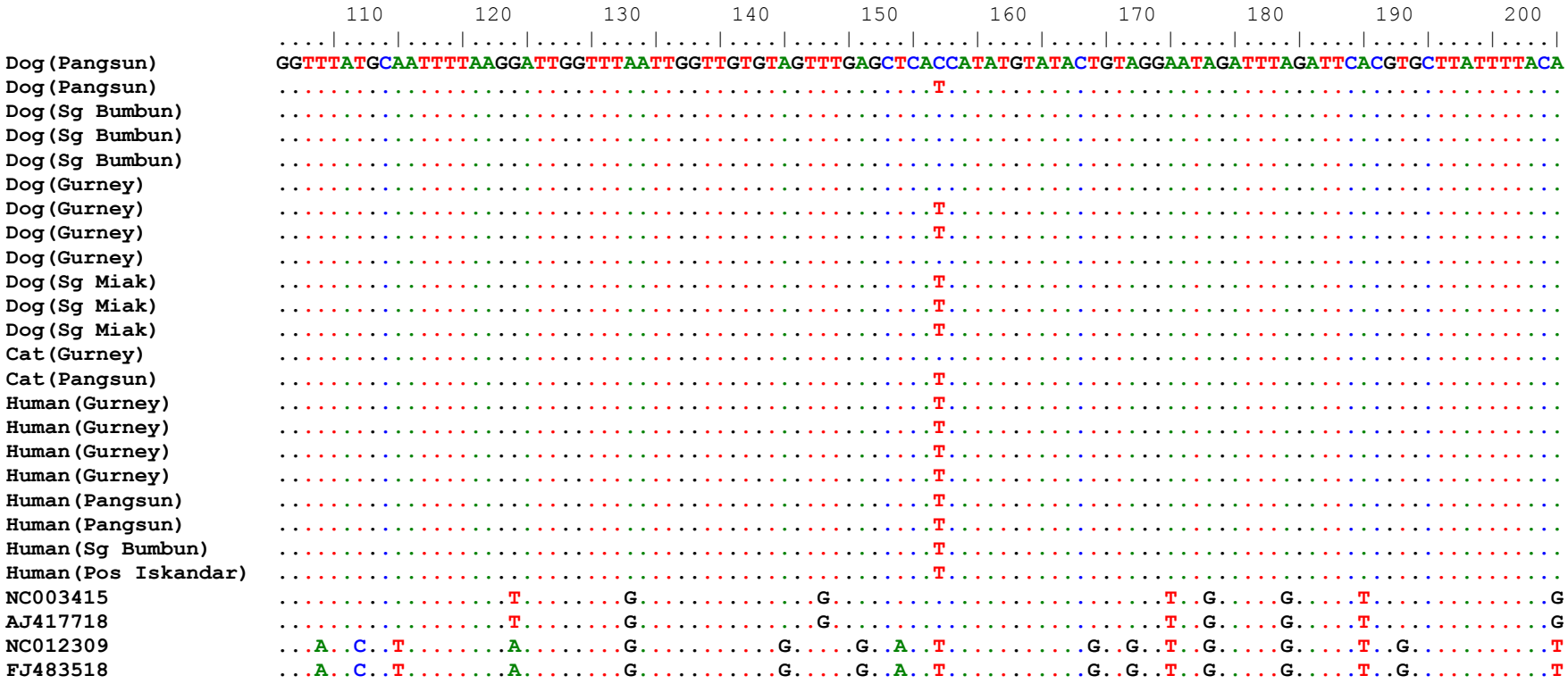

Figure 6.3 (Continued)

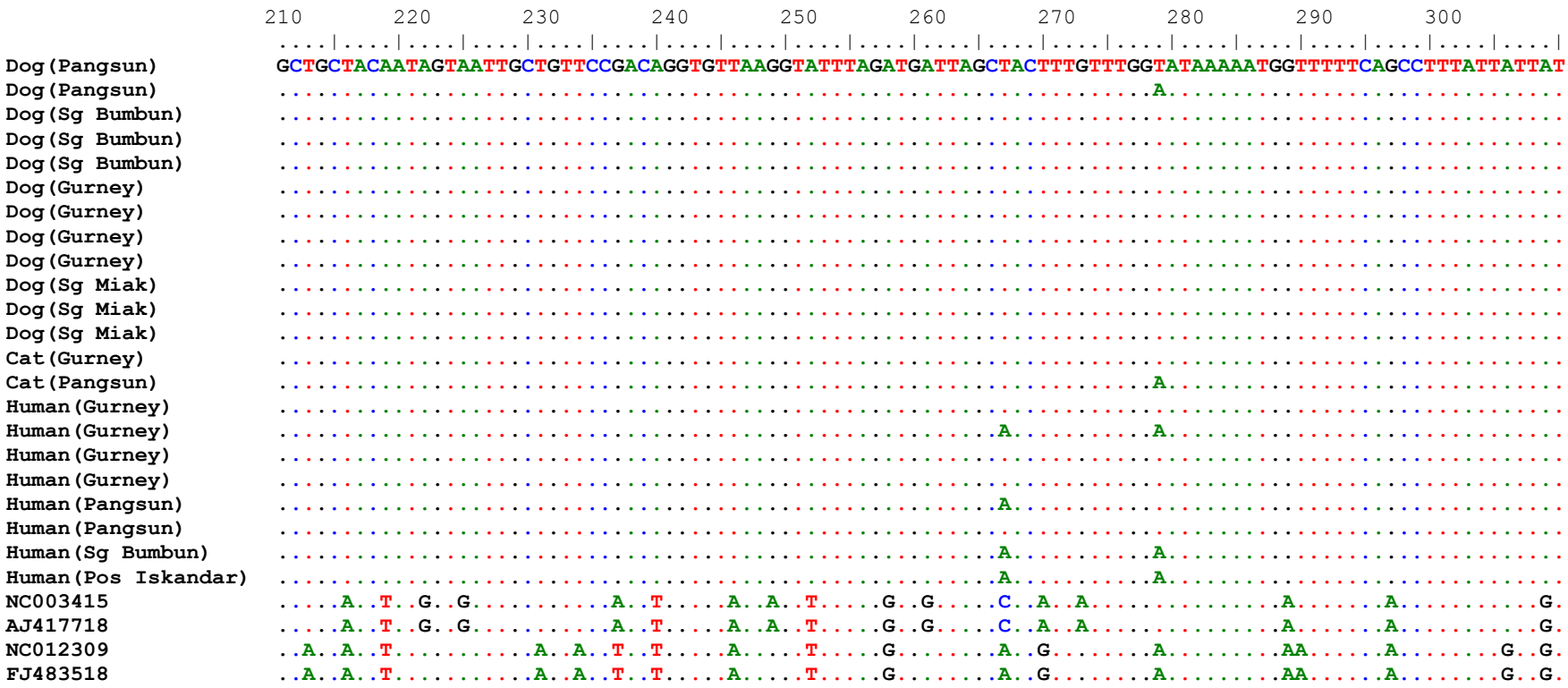

Figure 6.3 (Continued)

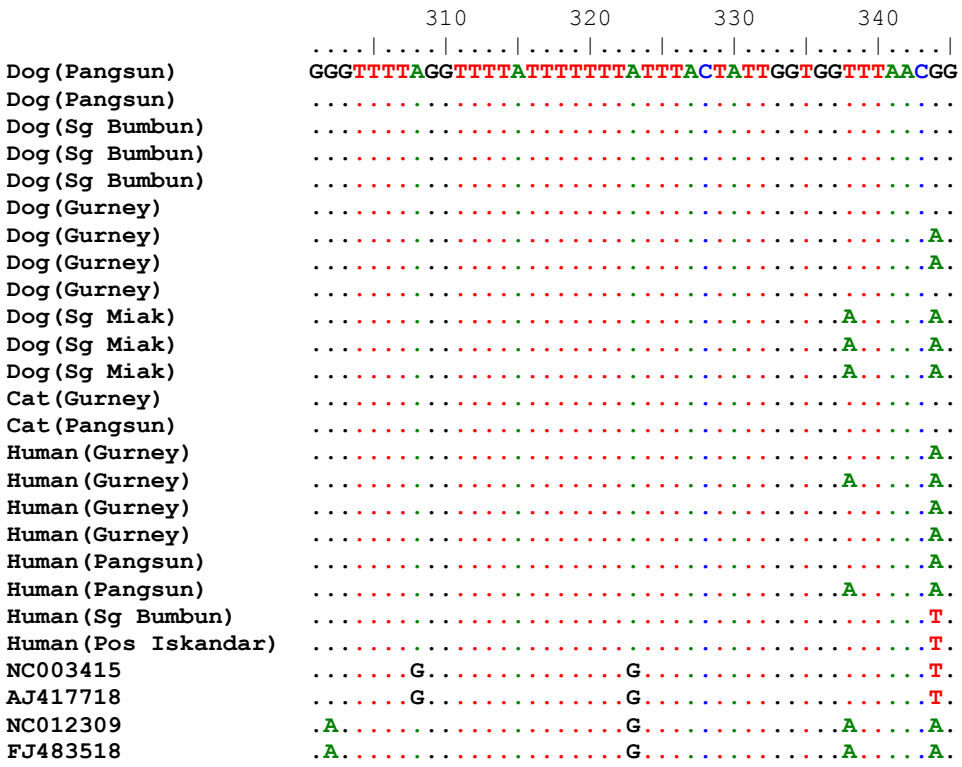

Figure 6.3 (Continued)

6.4 DISCUSSION

This study demonstrates that *A. ceylanicum* and *A. caninum* are highly endemic hookworm species among dogs and cats residing in Orang Asli communities in rural areas of Peninsular Malaysia. In our study, *A. ceylanicum* infections were also found in human (i.e., Orang Asli communities), indicating that dogs and cats may act as possible sources of infection to humans. This highlights that zoonotic transmission of *A. ceylanicum* to humans is still a largely overlooked public health problem. The result was further strengthened by our earlier epidemiological analysis (i.e., those studied and mentioned in **Chapter 3**) which revealed that close contact with dogs and cats were significantly associated with hookworm infections in these rural communities. As dogs and cats were the most common domestic animals in the studied communities and they roam freely and defecate indiscriminately around the neighborhood. Such human-animal cohabitation puts humans at risk for an array of zoonotic parasitic diseases. Likewise, the indiscriminate defecation habit particularly among children around their houses without parental supervision may also facilitate transmission of parasites from humans to humans, humans to animals or vice versa.

Although emerging zoonotic case reporting is increasing worldwide, these parasites are still regarded as rare and abnormal hookworms of humans and largely overlooked in human parasite surveys. The epidemiological and clinical significance of *A. ceylanicum* remains largely unresolved due to the limited availability of published research data. Clinical signs reported ranged from asymptomatic, light to heavy infections with anemia, lethargy and excessive hunger (Anten & Zuidema, 1964). In an experimental infection with *A. ceylanicum* in human (Wijers & Smit, 1966; Carroll & Grove, 1986), subjects developed clinical signs similar to those described from

experimental infection with *N. americanus* and *A. duodenale* (Brumpt, 1952; Wright & Bickle, 2005). However, in the present study, all eleven infected individuals with *A. ceylanicum* were asymptomatic. Unfortunately, the clinical observation of creeping eruptions, and eosinophilic enteritis (EE) and iron deficiency anemia were not investigated in the present study. In contrast, study on zoonotic ancylostomiasis caused by *A. ceylanicum* in Thailand reported that infected individuals reported to suffer from poor health and abdominal pain (Traub et al, 2008). As the clinical impact of *A. ceylanicum* in humans remains to be investigated, further studies investigating the epidemiology, transmission dynamics and clinical significance of *A. ceylanicum* in a community endemic for hookworm disease will be beneficial in unravelling the true significance of this zoonosis in humans.

Besides *A. ceylanicum,* more than half of the infected dogs were also harboring *A. caninum*. Reports from other countries have highlighted that *A. caninum* is ubiquitously found in dogs and cats in tropical and sub-tropical climates including Thailand (Traub et al, 2008), India (Traub et al, 2002; 2005) and Australia (Traub et al, 2004; 2007; Palmer et al, 2007). However, it is difficult to compare the current status of hookworm infection among animals especially dogs and cats in Malaysia since there is limited prior documented data. Even if data was available, the incrimination of a specific species is not possible as molecular tools were not employed in these previous studies. The only prevalence data on hookworm species available was among stray dogs in Kuala Lumpur and Sarawak (East Malaysia) (Yoshida et al, 1973; Choo et al, 2000), where more than 95% of dogs were infected with *A. ceylanicum* based on necropsy examination. To date, there is no reported data on *A. caninum* among dogs in Malaysia. None of the cats in this study were infected with *A. caninum*. In contrast, *A.caninum* infections have been previously reported among cat populations in Australia, although

this species is regarded as an uncommon parasite of cats (Stewart, 1994). In addition, our results also indicated that one case of *A. braziliense* was reported in cat. Malaysia is one of the few countries besides Australia to report on *A. braziliense* infections in the Asia-Pacific region (Stewart, 1994).

Animal hookworms such as *A. caninum* and *A. braziliense* can establish pathogenic human infections such as cutaneous larva migrans (CLM) or human eosinophilic enteritis (EE) dependent on the migration of L3 to the ectopic site in the human host (Bowman et al, 2010; Feldmeier & Schuster, 2012). To date, few cases of CLM have been reported in Malaysia (Hanjeet et al, 1988; Robson & Othman, 2008; Hamat et al, 2010; Yap, 2010). A recent report indicated that 31 patients with CLM were referred to Kuala Lumpur general hospital (Yap, 2011). Few cases have also been reported sporadically among travelers returning from Malaysia (Lederman et al, 2008). However, it is unclear which *Ancylostoma* species caused these CLM cases. Hookworm-related CLM or EE is usually caused by *A. braziliense, A. caninum, A. tubaeforme* and *Uncinaria stenocephala* (Bowman et al, 2010; Feldmeier & Schuster, 2012). However, no studies of *A. tubaeforme* and *U. stenocephala* have been reported in Malaysia and only one case of *A. braziliense* was reported to date, it is assumed that *A. caninum* might be the potential species causing the hookworm-related CLM in Malaysia.

In the current study, sequences of the cytochrome c oxidase subunit 1 (*cox 1*) were successfully amplified and sequenced for isolates of *A. ceylanicum* representing strains from different geographical locations and hosts within Malaysia. Of the 36 *A. ceylanicum* isolates subjected to PCR at the *cox 1* gene, only 22 samples were successfully sequenced. Failure of PCR amplification on the 14 samples could be caused by low concentration of parasite DNA due to the low intensity of infection for

the respective samples. In addition, it was also thought to be associated with the low sensitivity of the PCR assay. In this study, we used the existing primer set developed by Bowles et al. (1992) and Hu et al. (2002). The reported works did not state the sensitivity of their PCR assays. Thus, it is difficult to estimate the lowest detectable concentration in which parasite DNA can be detected for our study.

Sequence analysis showed that the *A. ceylanicum* strains could be divided into two distinct groups, one consisting of human isolates whilst the other consisting of a mixture of human and animal isolates from different geographical locations. These two groups of *A. ceylanicum* had differences at five fixed nucleotide differences in the *cox 1* sequence at locations 891, 966, 1008, 1077 and 1083, which could be distinguished from one another. This might suggest that the two groups within *A. ceylanicum* represent genetically distinct haplotypes rather than cryptic species. However, this hypothesis requires further statistical investigation given the small number of *A. ceylanicum* isolates in the current work. Similarly, two distinct groups were detected within *A. duodenale* from humans from Zhejiang Province in China, which suggested that the two groups within *A. duodenale* represent genetically distinct subpopulation, rather than cryptic species (Hu et al, 2002). Likewise, study conducted in dogs in Townsville, Australia revealed that two genetically distinct groups were detected within *A. caninum* from these dogs (Provic & Croese, 1996). Previous morphological and clinical studies in Australia have indicated that *A. caninum* is not only specific to dogs, but also capable of infecting other hosts including cats and humans causing eosinophilic enteritis (Loukas et al, 1992; Croese et al, 1994a).

In addition, some of the *A. ceylanicum* strains from both human and animal host within the same geographical location (e.g., Gurney) clustered together within the same group. This provides evidence to show that dogs and humans share genetically similar

genotypes of *A. ceylanicum* within the same geographical location. These results add weightage to confirm our findings in the previous study that demonstrated individuals who had close contact with dogs and cats were eleven times more likely to be infected with *A. ceylanicum* (i.e., those studied and mentioned in **Chapter 3**). Additionally, zoonotic ancylostomiasis caused by *A. ceylanicum* in humans has also been reported in other South East Asia countries including Thailand (Traub et al, 2008; Jiraanankul et al, 2011) and Laos PDR (Sato et al., 2010; Conlan et al, 2012). Therefore, it is tempting to postulate that certain genetically distinct groups within *A. ceylanicum* can selectively infect non canine host such as humans. However, further statistical investigation is required to prove this hypothesis since our sample numbers were limited. Perhaps such pattern of variability within *A. ceylanicum* may have arisen due to secondary contact between allopatrically evolved populations or subpopulations in different or non overlapping geographical areas (Avise et al, 1987), which may have arisen due to host movement from other geographical locations where this species are endemic.

High numbers of nucleotide variations were also observed in the present study, a finding that was consistent with other observations when mtDNA was used as the genetic marker (Blouin, 2002; Hu et al, 2002; Hu et al, 2008). In each of our *cox1* sequence, more than 80% of the nucleotide compositions contained adenine (A) and thymine (T). Few studies have also reported that the nematode mitochondrial genome is highly in favor of A and T nucleotides, reflecting the extreme A and T biasness (Okimoto et al, 1992; Blouin et al, 1998). McDonnell et al. (2000) also reported a similar finding in their mtND4 gene analysis of Cyathostominae and Strongylianes. As more distantly related species were compared, both transversions (A and T) and transitions appeared to be more saturated. When the species were compared within a genus, the number of A and T transversions had outpaced transitions, as reported in the

present study. This result was also in agreement with those findings by Thomas & Wilson (1991) and McDonnell et al. (2000) when sharp decrease in transition and transversion ratios were recorded from intraspecific to interspecific comparisons in nematode worms. However, Blouin et al. (1998) reported that transitions were 5-6 times more common than transversions of A and T in intraspecific comparisons.

The detection of considerable level of mitochondrial genetic variation within *cox 1* sequence of *A. ceylanicum* might suggest different biological, epidemiological (e.g., transmission) and disease characteristic (i.e., pathogenic effects) linked to different genotypes. However, there is presently no evidence to support this hypothesis, warranting further investigation using other loci and further isolates as genetic markers. In addition, further studies investigating the epidemiology, transmission dynamics and clinical significance of *A. ceylanicum* in endemic communities with hookworm disease will be beneficial in unravelling the true significance of this zoonosis in humans. As all the *A. ceylanicum* strains in human were isolated from asymptomatic individuals, it would also be of particular interest to compare the genetic make-up of *A. ceylanicum* in symptomatic human infected with this zoonotic species with those from dogs or cats from the same geographical areas. In conclusion, the detection of genetically distinct groups and considerable level of genetic variation within the *cox 1* sequence of *A. ceylanicum* as reported in the present study might suggest potential haplotype-linked differences in zoonotic, epidemiological and pathobiological characteristics, a hypothesis that still need further investigation. Addressing these questions should offer valuable insight into aspects of *A. ceylanicum* or other hookworm transmission, thus having implication on how disease control measures can be instituted.

6.5 CONCLUSIONS

The present study provides the first documented data on species specific hookworm in humans, dogs and cats inhabiting the same geographical locations in Peninsular Malaysia. In addition, it allows us to further explore and discuss the possible role of dogs and cats as zoonotic transmission agents of the hookworm infections in these parasite-endemic communities in Peninsular Malaysia.

The following conclusions are a synopsis of the analysis undertaken through this study in which they were discussed:

1. Out of 65 microscopically positive dogs and cats fecal samples, 51 (78.5%) were successfully amplified and genetically characterized on the basis of their DNA sequences of the ITS-2 region of the ribosomal RNA gene.

2. Of these, *A. caninum* (51.0%; 26/51) was the most common hookworm species detected in both dogs and cats, followed by *A. ceylanicum* (47.1%; 24/51) and *A. braziliense* (2.0%; 1/51).

3. *A. ceylanicum* were detected in both dogs (79.2%; 19/24) and cats (20.8%; 5/24). In contrast, only dogs were infected with *A. caninum* (100%; 26/26). Likewise, a single *A. braziliense* infection was isolated from cat.

4. The phylogenetic analysis showed that *A. ceylanicum* isolates were grouped together and genetically distinct from *A. caninum* isolates and *A. duodenale* isolates.

5. Within *A. ceylanicum*, the cluster was divided into two clades, one consisting of 3 human isolates, the other comprising of 19 isolates sourced from both humans and animals origin from different geographical locations. This separation was strongly supported by bootstrap analysis (99% bootstrap value).

6. The two groups of *A. ceylanicum* could be distinguished from each other by five fixed nucleotide differences at locations 891, 966, 1008, 1077 and 1083.

7. It was also noted that some *A. ceylanicum* strains from the same geographical location and host clustered together within the same group, thus providing some degree of evidence that the same strains of *A. ceylanicum* might be circulating between human and animal hosts in the same geographical location.

8. Multiple alignment comparison of the *cox1* sequences in this study against the mitochondria genome of *A. caninum* and *A. duodenale* showed that 59 different mismatches were recorded within *Ancylostoma* genus.

9. Within *A. ceylanicum*, *A. caninum* and *A. duodenale* sequences, the percentage of transversions (62.7%; 37/59) was higher than transitions (55.9%; 33/59); with A and T transversions being the most common (28 transversions).

10. The mismatches of nucleotide at different locations allowed the discrimination between *Ancylostoma* species. For example, separation of *A. ceylanicum, A. caninum* and *A. duodenale* by mismatches at positions 858, 939, 966, 996, 999 and 1083.