Parasitology International 68 (2019) 24–30

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Molecular phylogeography and genetic diversity of *Angiostrongylus cantonensis* and *A. malaysiensis* (Nematoda: Angiostrongylidae) based on 66-kDa protein gene

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ARTICLE INFO

Keywords:
Angiostrongylus nematodes
Molecular phylogeography
Haplotype diversity
Genetic divergence
Thailand

ABSTRACT

Angiostrongylus cantonensis is the main causative agent of human angiostrongyliasis. A sibling species, *A. malaysiensis* has not been unequivocally incriminated to be involved in human infections. To date, there is only a single report on the application of the partial 66-kDa protein gene sequence for molecular differentiation and phylogeny of *Angiostrongylus* species. Nucleotide sequences of the 66-kDa protein gene of *A. cantonensis* and *A. malaysiensis* from Thailand, as well as those of the laboratory strains of *A. cantonensis* from Thailand and Hawaii, *A. cantonensis* from Japan and China, *A. malaysiensis* from Malaysia, and *A. costaricensis* from Costa Rica, were used for the reconstruction of phylogenetic tree by the maximum likelihood (ML) method and the haplotypes by the median joining (MJ) network. The ML phylogenetic tree contained two major clades with a full support bootstrap value – (1) *A. cantonensis* and *A. malaysiensis*, and (2) *A. costaricensis*. *A. costaricensis* was basal to *A. cantonensis* and *A. malaysiensis*. The genetic distance between *A. cantonensis* and *A. malaysiensis* ranged from $p = .82\%$ to $p = 3.27\%$, that between *A. cantonensis* and *A. costaricensis* from $p = 4.90\%$ to $p = 5.31\%$, and that between *A. malaysiensis* and *A. costaricensis* was $p = 4.49\%$ to $p = 5.71\%$. Both *A. cantonensis* and *A. malaysiensis* possess high 66-kDa haplotype diversity. There was no clear separation of the conspecific taxa of *A. cantonensis* and *A. malaysiensis* from different geographical regions. A more intensive and extensive sampling with larger sample size may reveal greater haplotype diversity and a better resolved phylogeographical structure of *A. cantonensis* and *A. malaysiensis*.

1. Introduction

The rat lungworm *Angiostrongylus cantonensis* (Chen, 1935) is the main causative agent of human angiostrongyliasis, a disease characterized by eosinophilic meningitis or eosinophilic meningoencephalitis [1,2]. To date, more than 2800 cases of human angiostrongyliasis have been recorded worldwide [3]. A sibling species, *A. malaysiensis* Bhaibulaya and Cross, 1971, reported in Malaysia, Thailand, Indonesia, Japan, Laos and Myanmar [4–6], has a similar life cycle but has not been unequivocally incriminated to be involved in human infections [2,4,7]. The distributions of these two *Angiostrongylus* species broadly overlap in Laos, Cambodia, Myanmar, and Thailand [5,6]. *A. cantonensis* does not occur in Malaysia.

Rodents are normally the definitive hosts of *A. cantonensis* [8–11], while snails and slugs are the intermediate hosts [2,12]. Humans, as an accidental host, are infected by consumption of raw or poorly cooked snail meat and a variety of paratenic hosts which harbor the third-stage infective larvae [4,13]. This zoonotic parasite appears to have spread from its native areas in recent years, with cases of *A. cantonensis* infection reported throughout the Southeast Asia, Pacific islands, Africa, Australia, North, Central and South America and the Caribbean islands [1–3]. It is of increasing public health importance as globalization contributes to the geographical spread and more international travelers encounter the disease. The rapid global spread of the disease has posed

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https://doi.org/10.1016/j.parint.2018.09.006
Received 7 August 2018; Received in revised form 30 August 2018; Accepted 25 September 2018

challenges in its molecular epidemiology studies.

Both mitochondrial and nuclear gene sequences have been used for molecular differentiation and phylogenetic analyses of *Angiostrongylus* species. The nuclear genes include the 66-kDa protein gene [14], ribosomal transcribed spacer (ITS) regions [15,16], and the small subunit (SSU) ribosomal RNA (18S rRNA) gene [15,17,18]. The mitochondrial genes include cytochrome *c* oxidase subunit I (COXI) [5,19–24], and cytochrome *b* (CYTB) [6,25]. In addition, the complete mitochondrial genome has also been used for species differentiation and phylogenetic analysis [26–29].

As far as we are aware, there is only a single report on the application of the partial 66-kDa protein gene sequence for molecular differentiation and phylogeny of *Angiostrongylus* species [14]. However, a 232-bp fragment of this 66-kDa protein gene was used to identify the serum samples of three patients with histopathological diagnosis of abdominal angiostrongyliasis [30]. There is no report on the phylogeography of *Angiostrongylus* worms based on this genetic marker.

Based on cytochrome *c* oxidase subunit I (COI) gene [4], distinct haplotypes for *A. cantonensis* were identified in seven regions of Thailand – AC10 in Phitsanulok (northern region), AC11 in Nakhon Phanom (northeastern region), AC15 in Trat (eastern region), AC16 in Chantaburi (eastern region), AC4 in Samut Prakan (central region), AC14 in Kanchanaburi (western region), and AC13 in Ranong (southern region). Phylogenetic analysis revealed that these haplotypes formed distinct lineages. On the other hand, nine COI haplotypes were identified for *A. malaysiensis* [20]. In general, the COI sequences did not differentiate unambiguously the various geographical isolates of *A. cantonensis* as well as *A. malaysiensis*. Based on cytochrome *b* (CYTB) gene [6], a total of 15 CYTB haplotypes was found in 37 sequences of *A. cantonensis* from 14 geographical localities (covering north, west, east, central and south regions) in Thailand. Some CYTB haplotypes appeared to be confined to specific geographical localities.

The present study on the molecular phylogeography and genetic diversity of *A. cantonensis* and *A. malaysiensis*, from different regions of Thailand and other countries, based on the partial sequence of the 66-kDa protein gene was carried out to add to our knowledge on the findings based on COI and CYTB genes.

2. Materials and methods

2.1. Collection of Angiostrongylus specimens

Adult male and female *Angiostrongylus* worms were collected from the pulmonary arteries and heart of wild caught rodents in different provinces around Thailand [31]. The worms were examined microscopically and identified based on the morphological characteristics according to existing keys and descriptions of the *Angiostrongylus* species [2,32,33]. After morphological identification, the worms were preserved in absolute alcohol and kept at –70° C until DNA extraction. The locality and rodent host of the *Angiostrongylus* specimens used in the present study are summarized in Table 1.

All procedures involving animals were conducted under animal use protocols approved by the Animal Ethical Committee of the Ministry of Public Health, Thailand (approval no. FWA00013622).

2.2. DNA extraction, amplification and nucleotide sequencing

Genomic DNA was extracted from individual adult female and male *Angiostrongylus* worm by using the fast technology for analysis of nucleic acid (FTA) classic card method (Whatman BioScience, Newton Center, Massachusetts, USA) and according to the instructions of the manufacturer [14,19]. Captured nucleic acids on the FTA cards were purified and polymerase chain reaction (PCR) master mix was added directly to the DNA punch in a PCR tube, followed by amplification [14,19].

The sequencing procedure follows that previously described by Eamsobhana et al. [14]. DNA amplification was conducted using the previously published primers AC1 5′–CTCGGCTTAATCTTTGCGAC–3′ and AC2 5′–AACGAGCGGCAGTAGAAAAA–3′ [14,30]. The PCR mixture was denatured at 94 °C for 3 min, followed by 35-cycles at 94 °C for 2 min, 58 °C for 3 min and 72 °C for 3 min [14]. The PCR products, after electrophoresis in agarose gel and ethidium bromide staining, were visualized under ultraviolet (UV) light.

Nucleotide sequences of the 66-kDa protein gene of adult male and female *A. cantonensis* from Thailand and Hawaii lab strains (from experimentally infected albino rats), *A. cantonensis* adult worms from Japan and China (from wild-caught rodents), *A. malaysiensis* adult worms (from the pulmonary arteries of wild-caught *Rattus tiomanicus* in Malaysia), and *A. costaricensis* adult specimens from Costa Rica (from experimentally infected cotton rats) were included in this study for phylogenetic analysis. The locality and rodent host of these *Angiostrongylus* taxa are shown in Table 1.

2.3. Sequence alignment and phylogenetic analysis

ChromasPro v.1.5 (Technelysium Pty Ltd., Australia) software was used to edit and assemble the 66-kDa sequences. Multiple sequence alignment was conducted with ClustalX [34] and the resulting alignment was trimmed using BioEdit v.7.0.5.3 [35]. The best-fit nucleotide substitution model (HKY + Gamma) for maximum likelihood (ML) was selected using the corrected Akaike Information Criterion [36] and determined by Kakusan v.3 [37]. The phylogenetic tree was reconstructed using TreeFinder [38] prior to the annotations of bootstrap values (BP) generated via 1000 ML bootstrap replicates. Two independent runs of 2×10^6 generations with four chains were performed, with trees sampled every 200th generation. Likelihood values for all post-analysis trees and parameters were evaluated for convergence. The first 200 trees from each run were discarded as burn-in (where the likelihood values were stabilized prior to the burn-in), and the remaining trees were used for the construction of a 50% majority-rule consensus tree. The final phylogenetic tree was edited using FigTree v.1.4 [39].

2.4. Haplotype network reconstruction

The genealogical relationships of the haplotypes were reconstructed by the median joining (MJ) network [40]. The MJ network was calculated using NETWORK v5.0.0.3 (http://www.fluxus-engineering.com).

2.5. Haplotype and nucleotide diversity

Genetic differentiation among populations was estimated by analysis of molecular variance (AMOVA) using Arlequin v.3.5.2.1 [41].

2.6. Genetic divergence

Uncorrected (*p*) pairwise genetic distances were estimated using PAUP* 4.0b10 software [42] to assess the level of genetic variation.

3. Results

3.1. Phylogenetic relationships

The 66 aligned sequences (39 *A. cantonensis*, 23 *A. malaysiensis*, and 4 *A. costaricensis*) of the 66-kDa gene consisted of 245 sites, of which 222 sites were invariable (monomorphic), and 22 sites (with 24 mutations) were variable (polymorphic) consisting of 6 singleton variable sites and 16 parsimony informative sites.

The lnL likelihood for ML tree was −529.40. The ML tree contained two major clades with a full support bootstrap value – (1) *A. cantonensis* and *A. malaysiensis*, and (2) *A. costaricensis* (Fig. 1). *A. costaricensis* was

Table 1
Specimens of *Angiostrongylus* lungworms used for sequencing 66-kDa gene marker.

Species	Code	Haplotype	Location/Origin	Rodent host	GenBank accession number
A. cantonensis	AcF-1Cm	Ac66–1	N Thailand: Chiang Mai	*Bandicota indica*	MH562054
	AcM-1Cm	Ac66–1	N Thailand: Chiang Mai	*Bandicota indica*	MH562055
	AcM-24Rn	Ac66–1	S Thailand: Ranong	*Rattus norvegicus*	MH562063
	AcM-1Rn	Ac66–1	S Thailand: Ranong	*Rattus rattus*	MH562072
	AcF-2Sr	Ac66–1	S Thailand: Surat Thani	*Bandicota indica*	MH562074
	AcF-13Bn	Ac66–1	C Thailand: Bangkok	*Rattus norvegicus*	MH562084
	AcM-14Lb	Ac66–1	C Thailand: Lopburi	*Rattus norvegicus*	MH562085
	AcM-2Psl	Ac66–1	C Thailand: Phitsanulok	*Rattus rattus*	MH562088
	AcF-3H	Ac66–1	Hawaii: lab strain	*Rattus norvegicus*	MH562089
	AcM-3H	Ac66–1	Hawaii: lab strain	*Rattus norvegicus*	MH562090
	AcF-3 T	Ac66–1	Thailand: lab strain	*Rattus norvegicus*	MH562091
	AcF-1 J	Ac66–1	Japan: Okinawa	*Rattus* sp.	MH562092
	AcM-2 J	Ac66–1	Japan: Okinawa	*Rattus* sp.	MH562093
	AcM-1 J	Ac66–5	Japan: Okinawa	*Rattus* sp.	MH562097
	AcM-3 T	Ac66–6	Thailand: lab strain	*Rattus norvegicus*	MH562094
	AcF-1 T	Ac66–7	Thailand: lab strain	*Rattus norvegicus*	MH562095
	AcF-10Sk	Ac66–4	S Thailand: Song Khla	*Rattus rattus*	MH562068
	AcM-10Sk	Ac66–4	S Thailand: Song Khla	*Rattus rattus*	MH562069
	AcM-1Kk	Ac66–4	NE Thailand: Khon Kaen	*Rattus rattus*	MH562086
	AcF-2 T	Ac66–4	Thailand: lab strain	*Rattus norvegicus*	MH562102
	AcM-2 T	Ac-664	Thailand: lab strain	*Rattus norvegicus*	MH562103
	AcM-1H	Ac66–9	Hawaii: lab strain	*Rattus norvegicus*	MH562096
	AcF-2Psl	Ac66–3	C Thailand: Phitsanulok	*Rattus rattus*	MH562087
	AcF-1Sr	Ac66–2	S Thailand: Surat Thani	*Bandicota indica*	MH562073
	AcM-4Sp	Ac66–2	C Thailand: Samut Prakan	*Rattus rattus*	MH562081
	AcF-1Sp	Ac66–2	C Thailand: Samut Prakan	*Rattus rattus*	MH562082
	AcM-10Bn	Ac66–2	C Thailand: Bangkok	*Rattus norvegicus*	MH562083
	AcF-2 J	Ac66–2	Japan: Okinawa	*Rattus* sp.	MH562099
	AcF-1H	Ac66–8	Hawaii: lab strain	*Rattus norvegicus*	MH562098
	AcF-1C	Ac66–10	China: Guanhxi	*Rattus* sp.	MH562101
	AcF-3C	Ac-66-10	China: Guangxi	*Rattus* sp.	MH562104
	AcM-2C	Ac66–10	China: Guangxi	*Rattus* sp.	MH562105
	AcM-2H	Ac66–10	Hawaii: lab strain	*Rattus norvegicus*	MH562108
	AcF-2H	Ac66–11	Hawaii: lab strain	*Rattus norvegicus*	MH562109
	AcF-6Pk	Ac66–12	W Thailand: Prachuap Khiri Khan	*Rattus rattus*	MH562064
	AcM-6Pk	Ac66–12	W Thailand: Prachuap Khiri Khan	*Rattus rattus*	MH562065
	AcF-2C	Ac66–12	China: Guangxi	*Rattus* sp.	MH562100
	AcM-3C	Ac66–12	China: Guangxi	*Rattus* sp.	MH562106
	AcM-1C	Ac66–13	China: Guangxi	*Rattus* sp.	MH562107
A. malaysiensis	AmF1-5Bkk	Am66–1	C Thailand: Bangkok	*Rattus exulans*	MH562052
	AmF-1Cr	Am66–1	N Thailand: Chiang Rai	*Bandicota indica*	MH562056
	AmM-1Cr	Am66–1	N Thailand: Chiang Rai	*Bandicota indica*	MH562057
	AmM-2Kb	Am66–1	W Thailand: Kanchanaburi	*Rattus norvegicus*	MH562062
	AmM-1 Tak	Am66–1	W Thailand: Tak	*Bandicota bengalensis*	MH562077
	AmF-1Mh	Am66–1	N Thailand: Mae Hong Son	*Rattus losea*	MH562080
	AmF-1Mal	Am66–1	Malaysia: Pahang	*Rattus tiomanicus*	MH562110
	AmM-1Mal	Am66–1	Malaysia: Pahang	*Rattus tiomanicus*	MH562111
	AmF-2Mal	Am66–1	Malaysia: Pahang	*Rattus tiomanicus*	MH562112
	AmM-2Mal	Am66–1	Malaysia: Pahang	*Rattus tiomanicus*	MH562113
	AmF-10Nk	Am66–2	NE Thailand: Nong Khai	*Rattus norvegicus*	MH562060
	AmM-10Nk	Am66–3	NE Thailand: Nong Khai	*Rattus norvegicus*	MH562061
	AmF-1Nk	Am66–4	NE Thailand: Nong Khai	*Rattus norvegicus*	MH562058
	AmM-38St	Am66–4	S Thailand: Satun	*Rattus rattus*	MH562071
	AmF-1 Tak	Am66–4	W Thailand: Tak	*Bandicota bengalensis*	MH562078
	AmM-2Mh	Am66–4	N Thailand: Mae Hong Son	*Rattus losea*	MH562079
	AmM-1Png	Am66–5	S Thailand: Phang Nga	*Rattus rattus*	MH562075
	AmM-2Png	Am66–5	S Thailand: Phang Nga	*Rattus rattus*	MH562076
	AmF2-5Bkk	Am66–6	C Thailand: Bangkok	*Rattus exulans*	MH562053
	AmF-1 Tr	Am66–6	S Thailand: Trang	*Rattus rattus*	MH562066
	AmF-11Tr	Am66–6	S Thailand: Trang	*Rattus rattus*	MH562067
	AmF-38St	Am66–7	S Thailand: Satun	*Rattus rattus*	MH562070
	AmM-1Nk	Am66–8	NE Thailand: Nong Khai	*Rattus norvegicus*	MH562059

basal to *A. cantonensis* and *A. malaysiensis*. *A. cantonensis* and *A. malaysiensis* were clearly separated but had low bootstrap support value of 56% and 57% respectively. The lineages within them had poor or no bootstrap support value. There was no clear distinction between the *A. cantonensis* as well as the *A. malaysiensis* samples from different geographical localities.

3.2. Haplotype diversity and nucleotide diversity

Analysis based on Arlequin suite of *A. cantonensis* (39 specimens) and *A. malaysiensis* (23 specimens) indicates that the number of usable loci with less than 5.00% missing data was 245. There were 8 polymorphic sites for *A. cantonensis*, 5 for *A. malaysiensis*, and 13 for *A. cantonensis* + *A. malaysiensis*. The gene diversity for *A. cantonensis* was 0.85 ± 0.04, and that for *A. malaysiensis* was 0.78 ± 0.07. The mean number of pairwise differences was 1.59 ± 0.97 for *A. cantonensis*,

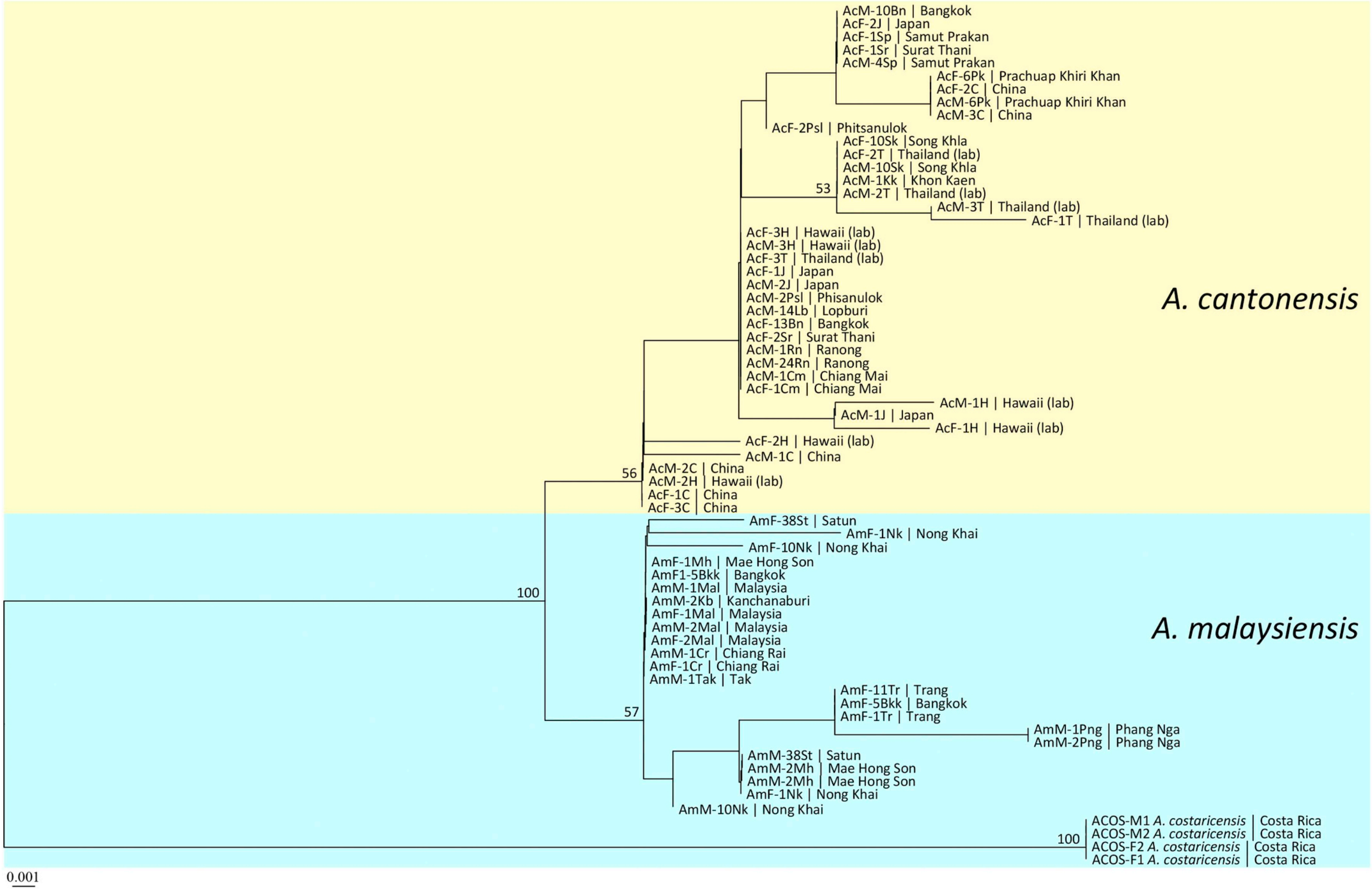


Fig. 1. Maximum-likelihood (ML) tree of *Angiostrongylus cantonensis* (Ac), *A. malaysiensis* (Am), and *A. costaricensis* (ACOS), based on partial sequences of 66-kDa protein gene. F, female; M, male. The bootstrap values are shown above the branches.

1.39 ± 0.88 for *A. malaysiensis*, 2.74 ± 1.47 for *A. cantonensis* + *A. malaysiensis*, and 3.83 ± 1.95 for *A. cantonensis* + *A. malaysiensis* + *A. costaricensis*. The nucleotide diversity (average over loci) was 0.007 ± 0.004 for *A. cantonensis*, 0.006 ± 0.004 for *A. malaysiensis*, 0.011 ± 0.007 for *A. cantonensis* + *A. malaysiensis*, and 0.016 ± 0.009 for *A. cantonensis* + *A. malaysiensis* + *A. costaricensis*.

The 39 specimens of *A. cantonensis* were represented by 13 haplotypes, Ac66–1 to Ac66–13 (Tables 1, 2; Fig. 2). Haplotype Ac66–1 was the commonest haplotype present in 13/39 (33.33%) specimens – 9 for Thailand from 6 localities and a lab strain (2 from Chiang Mai, North Thailand; 2 from Ranong and 1 from Surat Thani, South Thailand; 1 each from Bangkok, Lopburi, and Phisanulok, Central Thailand; and 1 from a laboratory strain originated from Khon Kaen, Northeast Thailand), 2 for Hawaii (lab strain), and 2 for Japan (Okinawa). Seven Ac66 haplotypes were represented in the 23 specimens from 10 geographical regions of Thailand (Table 2). The samples from Bangkok and Phitsanulok in Central Thailand, Surat Thani in South Thailand, Guangxi in China, Okinawa in Japan, the Thailand lab strain (originated from Khon Kaen in Northeast Thailand), and the Hawaii lab strain were variable (Tables 1, 2; Fig. 2). Eight haplotypes were each confined to a single locality – Ac66–3 in Phisanulok (Central Thailand); Ac66–6 and Ac66–7 in Thailand lab strain; Ac66–13 in Guangxi (China); Ac66–5 in Okinawa (Japan); and Ac66–8, Ac66–9 and Ac66–11 in Hawaii lab strain (Table 2).

Eight haplotypes were represented in the 23 specimens of *A. malaysiensis* (Tables 1, 2; Fig. 2), with the commonest haplotype Am66–1 present in 10/23 (43.48%) specimens – 6 for Thailand from 5 localities (2 from Chiang Rai and 1 from Mae Hong Son in North Thailand; 1 each from Tak and Kanchanaburi in West Thailand; and 1 from Bangkok in Central Thailand). All the 4 specimens from Malaysia had haplotype Am66–1. Eight Am66 haplotypes were represented in the 19 specimens from 9 regions in Thailand (Table 2). The specimens from Mae Hong Son in North Thailand, Nong Khai in Northeast Thailand, Tak in West Thailand, Bangkok in Central Thailand, and Satun in South Thailand were variable. Five haplotypes were each confined to a single locality – Am66–2, Am66–3 and Am66–8 in Nong Khai (Northeast Thailand); Am66–7 in Satun (South Thailand); and Am66–5 in Phang Nga (South Thailand).

3.3. Genetic divergence

The uncorrected *p*-distance values for *A. canronensis* ranged from $p = 0\%$ to $p = 2.04\%$ and that for *A. malaysiensis* ranged from $p = 0\%$ to $p = 1.63\%$ (Supplementary file 1: Table S1). There was no variation for the four *A. costaricensis* specimens. The genetic distance between *A. cantonensis* and *A. malaysiensis* ranged from $p = .82\%$ to $p = 3.27\%$, that between *A. cantonensis* and *A. costaricensis* from $p = 4.40\%$ to $p = 5.31\%$, and that between *A. malaysiensis* and *A. costaricensis* was $p = 4.49\%$ to $p = 5.71\%$.

4. Discussion

The 66-kDa protein was first reported to be present only in *A. cantonensis* adult female worms in the muscle cell membranes adjacent to the pseudocoelom [43]. A 232-bp fragment of this 66-kDa protein gene was used to identify the serum samples of three patients with histopathological diagnosis of abdominal angiostrongyliasis [30]. This gene marker was also able to detect *A. cantonensis* DNA in the cerebrospinal fluid (CSF) samples from four out of 10 patients who were serologically positive for infection [44]. It was employed for the

Table 2
Diversity of 66-kDa haplotype in *Angiostrongylus cantonensis* and *A. malaysiensis* lungworms from different geographical regions.

Species	Locality	No. specimens	No. haplotype
A. cantonensis	Chiang Mai, N Thailand	2	1 (Ac66–1)
	Lopburi, C Thailand	1	1 (Ac66–1)
	Khon Kaen, NE Thailand	1	1 (Ac66–4)
	Prachuap Kiri Khan, W Thailand	2	1 (Ac66–12)
	Bangkok, C Thailand	2	2 (Ac66–1, Ac66–2)
	Phitsanulok, C Thailand	2	2 (Ac66–1, Ac66–3)
	Samut Prakan, C Thailand	2	1 (Ac66–2)
	Ranong, S Thailand	2	1 (Ac66–1)
	Surat Thani, S Thailand	2	2 (Ac66–1, Ac66–2)
	Song Khla, S Thailand	2	1 (Ac66–4)
	Guangxi, China	6	3 (Ac66–10, Ac66–12, Ac66–13)
	Okinawa, Japan	4	3 (Ac66–1, Ac66–2, Ac66–5)
	Thailand lab strain	5	4 (Ac66–1, Ac66–4, Ac66–6, Ac66–7)
	Hawaii lab strain	6	5 (Ac66–1, Ac66–8, Ac66–9, Ac66–10, Ac66–11)
A. malaysiensis	Chinag Rai, N Thailand	2	1 (Am66–1)
	Mae Hong Song, N Thailand	2	2 (Am66–1, Am66–4)
	Nong Khai, NE Thailand	4	4 (Am66–2, Am66–3, Am66–4, Am66–8)
	Tak, W Thailand	2	2 (Am66–1, Am66–4)
	Kanchanaburi, W Thailand	1	1 (Am66–1)
	Bangkok, C Thailand	2	2 (Am66–1, Am66–6)
	Satun, S Thailand	2	2 (Am66–4, Am66–7)
	Phang Nga, S Thailand	2	1 (Am66–5)
	Trang, S Thailand	2	1 (Am66–6)
	Pahang, Malaysia	4	1 (Am66–1)

molecular differentiation and determination of the phylogenetic relationships of three *Angiostrongylus* species (*A. cantonensis*, *A. malaysiensis*, and *A. costaricensis*) [14].

Phylogenetic analysis of the present study confirms earlier finding [14] that *A. costaricensis* is genetically well separated from the clade of *A. cantonensis* and *A. malaysiensis* (Fig. 1). The sister group relationship of *A. cantonensis* and *A malaysiensis* agrees with earlier findings based on 66-kDa protein gene [14], small subunit (SSU) ribosomal RNA (18S rRNA) gene [17], cytochrome *c* oxidase subunit I gene [4,19,20], cytochrome *b* (CYTB) gene [6], and the complete mitochondrial genome [26–29].

In our earlier study, only two specimens each of *A. cantonensis* from Thailand (lab strain), Hawaii (lab strain), China, and Japan as well as the nucleotide sequence of *A. cantonensis* from Taiwan (GenBank Accession No. U17585) were used for 66-kDa protein gene analysis [14]. In the present study, we investigated a total of 39 *A. cantonensis* specimens – 18 specimens from 10 geographical regions of Thailand, 5 specimens of the Thailand lab strain, 6 specimens of the Hawaii lab strain, 6 specimens from China, and 4 specimens from Japan (Tables1 and 2). The nucleotide sequence of *A. cantonensis* from Taiwan (GenBank Accession No. U17585) was not included as our earlier study showed it to be very distant from other *A. cantonensis* samples, with uncorrected *p*-distance values ranging from 26.87% to 29.92% [14].

Thirteen haplotypes (Ac66–1 to Ac66–13) were present in the 39 specimens of *A. cantonensis* in the present study (Fig. 2). The samples did not cluster unequivocally according to their geographical origin (Thailand, China, Japan and Hawaii). The commonest haplotype (Ac66–1) was present in 13/39 (33.33%) specimens – 9 for Thailand from 6 localities and a lab strain (originated from Khon Kaen, Northeast Thailand), 2 for Hawaii (lab strain), and 2 for Japan (Okinawa).

It is noteworthy that the Thailand and Hawaii lab strains of *A. cantonensis*, despite having been maintained for many generations, possessed a high diversity – 4 haplotypes out of 5 specimens of the Thailand lab strain and 5 haplotypes out of 6 specimens of the Hawaii lab strain; with haplotype Ac66–10 being common to both strains (Table 2, Fig. 2). Haplotype diversity was also present in the *A. cantonensis* specimens from Bangkok and Phitsanulok in Central Thailand, Surat Thani in South Thailand, Guangxi in China, and Okinawa in Japan (Table 2, Fig. 2).

The single Khon Kaen specimen of *A. cantonensis* from the house rat *Rattus rattus* shared the same haplotype Ac66–4 with two specimens of the Thailand lab strain (originated from Khon Kaen). More field specimens from Khon Kaen are needed to determine the extent of Ac66 diversity and if Ac66–4 is the commonest haplotype in this geographical region.

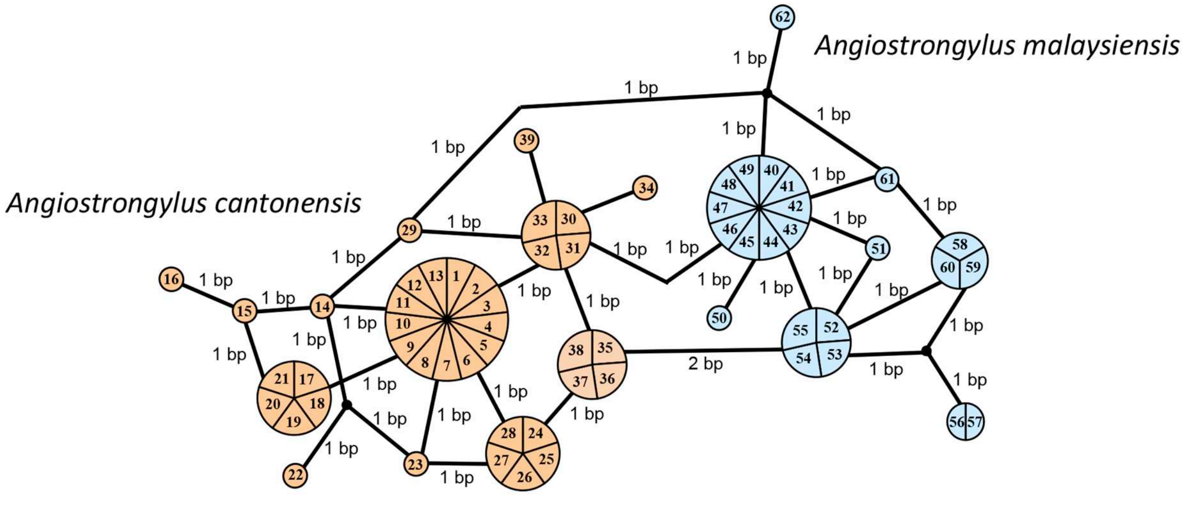
In the present study, the Am66 haplotype diversity of *A malaysiensis* (8 haplotypes for 19 specimens from 9 geographical regions in Thailand) is comparable to the Ac66 haplotype diversity of *A. cantonensis* with 7 haplotypes for 23 specimens from 10 geographical regions in Thailand (Table 2). The commonest Am66–1 haplotype was present in 5 specimens from 4 geographical regions of Thailand (representing North, West, Central and South Thailand) as well as 4 specimens from Malaysia.

High haplotype diversity of other genetic markers has been reported for *A. cantonensis* and *A. malaysiensis*. Fifteen cytochrome *b* (CYTB) haplotypes were observed in 38 sequences from 15 geographical localities in Thailand [6]. For the cytochrome *c* oxidase subunit I (COXI) gene, 16 haplotypes have been reported for *A. cantonensis* worldwide [4], and 9 haplotypes for *A. malaysiensis* from Thailand, Malaysia, Laos and Myanmar [20].

The present and earlier findings of high haplotype/genetic diversity in *A. cantonensis* and *A. malaysiensis* indicate the occurrence of an elevated mutation rate in both the nuclear and mitochondrial genes. A more intensive and extensive sampling with larger sample size of these parasites is needed to determine the extent of haplotype diversity and phylogeographical structure. Studies are also needed on convergent and parallel mutations, genetic drift and gene flow among populations.

In summary, phylogenetic analysis of the present study confirms that *A. costaricensis* is basal to the clade of *A. cantonensis* and *A. malaysiensis*. Both *A. cantonensis* and *A. malaysiensis* from various geographical localities possess high 66-kDa haplotype diversity. However the sample size of the conspecific taxa from each locality was too small for conclusive inference of distinct phylogeographic patterns. As in other nuclear and mitochondrial genetic markers, such as cytochrome *c* oxidase subunit I and cytochrome *b*, the 66-kDa protein gene could be useful for species identification and delimitation as well as for studying genetic diversity, population structure and phylogeography.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2018.09.006.



Angiostrongylus cantonensis

1. AcF-1Cm | Chiang Mai (Ac66-1)
2. AcM-1Cm | Chiang Mai (Ac66-1)
3. AcM-24Rn | Ranong (Ac66-1)
4. AcM-1Rn | Ranong (Ac66-1)
5. AcF-2Sr | Surat Thani (Ac66-1)
6. AcF-13Bn | Bangkok (Ac66-1)
7. AcM-14Lb | Lopburi (Ac66-1)
8. AcM-2Psl | Phisanulok (Ac66-1)
9. AcF-3H | Hawaii – lab (Ac66-1)
10. AcM-3H | Hawaii – lab (Ac66-1)
11. AcF-3T | Thailand – lab (Ac66-1)
12. AcF-1J | Japan (Ac66-1)
13. AcM-2J | Japan (Ac66-1)
14. AcM-1J | Japan (Ac66-5)
15. AcM-3T | Thailand – lab (Ac66-6)
16. AcF-1T | Thailand – lab (Ac66-7)
17. AcF-10Sk | Song Khla (Ac66-4)
18. AcM-10Sk | Song Khla (Ac66-4)
19. AcM-1Kk | Khon Kaen (Ac66-4)
20. AcF-2T | Thailand – lab (Ac66-4)
21. AcM-2T | Thailand – lab (Ac66-4)
22. AcM-1H | Hawaii – lab (Ac66-9)
23. AcF-2Psl | Phitsanulok (Ac66-3)
24. AcF-1Sr | Surat Thani (Ac66-2)
25. AcM-4Sp | Samut Prakan (Ac66-2)
26. AcF-1Sp | Samut Prakan (Ac66-2)
27. AcM-10Bn | Bangkok (Ac66-2)
28. AcF-2J | Japan (Ac66-2)
29. AcF-1H | Hawaii – lab (Ac66-8)
30. AcF-1C | China (Ac66-10)
31. AcF-3C | China (Ac66-10)
32. AcM-2C | China (Ac66-10)
33. AcM-2H | Hawaii – lab (Ac66-10)
34. AcF-2H | Hawaii – lab (Ac66-11)
35. AcF-6Pk | Prachuap Khiri Khan (Ac66-12)
36. AcM-6Pk | Prachuap Khiri Khan (Ac66-12)
37. AcF-2C | China (Ac66-12)
38. AcM-3C | China (Ac66-12)
39. AcM-1C | China (Ac66-13)

Angiostrongylus malaysiensis

40. AmF1-5Bkk | Bangkok (Am66-1)
41. AmF-1Cr | Chiang Rai (Am66-1)
42. AmM-1Cr | Chiang Rai (Am66-1)
43. AmM-2Kb | Kanchanaburi (Am66-1)
44. AmM-1Tak | Tak (Am66-1)
45. AmF-1Mh | Mae Hong Son (Am66-1)
46. AmF-1Mal | Malaysia (Am66-1)
47. AmM-1Mal | Malaysia (Am66-1)
48. AmF-2Mal | Malaysia (Am66-1)
49. AmM-2Mal | Malaysia (Am66-1)
50. AmF-10Nk | Nong Khai (Am66-2)
51. AmM-10Nk | Nong Khai (Am66-3)
52. AmF-1Nk | Nong Khai (Am66-4)
53. AmM-38St | Satun (Am66-4)
54. AmF-1Tak | Tak (Am66-4)
55. AmM-2Mh | Mae Hong Son (Am66-4)
56. AmM-1Png | Phang Nga (Am66-5)
57. AmM-2Png | Phang Nga (Am66-5)
58. AmF-5Bkk | Bangkok (Am66-6)
59. AmF-1Tr | Trang (Am66-6)
60. AmF-11Tr | Trang (Am66-6)
61. AmF-38St | Satun (Am66-7)
62. AmM-1Nk | Nong Khai (Am66-8)

Fig. 2. Haplotype network of *Angiostrongylus cantonensis* and *A. malaysiensis* based on 66-kDa protein gene sequences generated by NETWORK software. Circles represent haplotypes and numbers represent individuals sharing the specific haplotype. Haplotype in brackets.

Acknowledgements

We thank our institutions for providing various research facilities and other support. We also thank Dr. Hong-Man Zhang (CDC, Guangxi), Dr. Ichiro Miyagi and Dr. Takako Toma (University of the Ryukyus), Dr. Akira Ishih (Hamamatsu University), Dr. Elizabeth Abrahams-Sandi and Dr. Gabriela Solano (University of Costa Rica) for the gifts of *Angiostrongylus* materials, and Prof. Yukifumi Nawa (the editor) and the two anonymous reviewers whose comments led to a greatly improved manuscript.

Funding

This work was funded in part by the Department of Disease Control, Ministry of Public Health, Thailand, and the University of Malaya (H-5620009 to HSY).

Competing interests

The authors declare that they have no competing interests.

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