Acta Tropica 171 (2017) 141–145

Contents lists available at ScienceDirect

Acta Tropica

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

# Cytochrome *c* oxidase subunit I haplotype diversity of *Angiostrongylus cantonensis* (Nematoda: Angiostrongylidae)

Praphathip Eamsobhana[a,*], Sze-Looi Song[b], Hoi-Sen Yong[c], Anchana Prasartvit[d], Sudarat Boonyong[a], Anchalee Tungtrongchitr[a,*]

[a] Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University – Bangkok 10700, Thailand
[b] Institute of Ocean and Earth Sciences, University of Malaya – 50603, Kuala Lumpur, Malaysia
[c] Institute of Biological Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia
[d] Department of Disease Control, Ministry of Public Health, Nonthaburi, 11000, Thailand

ARTICLE INFO

*Keywords:*
*Angiostrongylus cantonensis*
Angiostrongyliasis
Haplotype
Phylogeography
Rat lungworm
Genetic diversity

ABSTRACT

The rat lungworm *Angiostrongylus cantonensis* is a food-borne zoonotic parasite of public health importance worldwide. It is the primary etiologic agent of eosinophilic meningitis and eosinophilic meningoencephalitis in humans in many countries. It is highly endemic in Thailand especially in the northeast region. In this study, *A. cantonensis* adult worms recovered from the lungs of wild rats in different geographical regions/provinces in Thailand were used to determine their haplotype by means of the mitochondrial partial cytochrome *c* oxidase subunit I (COI) gene sequence. The results revealed three additional COI haplotypes of *A. cantonensis*. The geographical isolates of *A. cantonensis* from Thailand and other countries formed a monophyletic clade distinct from the closely related *A. malaysiensis*. In the present study, distinct haplotypes 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. In general, the COI sequences did not differentiate the worldwide geographical isolates of *A. cantonensis*. This study has further confirmed the presence of COI haplotype diversity in various geographical isolates of *A. cantonensis*. The COI gene sequence will be a suitable marker for studying population structure, phylogeography and genetic diversity of the rat lungworm.

## 1. Introduction

The rat lungworm *Angiostrongylus cantonensis* is a food-borne zoonotic parasite of public health importance worldwide. It has a genome size of about 260 Mb (Yong et al., 2015a). Its life cycle involves mollusk intermediate host and rodent definitive host (Bhaibulaya, 1975). Humans act as an accidental host, acquiring the infection by consuming raw or poorly cooked snail meat which harbors the infective larvae or food contaminated with the third-stage larvae of the parasite (Cross, 1987).

*A. cantonensis* is the primary etiologic agent of eosinophilic meningitis and eosinophilic meningoencephalitis in humans in many countries (Wang et al., 2008, 2012; Eamsobhana, 2014). Angiostrongyliasis cantonensis involving the eyes and lungs have also been reported (Eamsobhana, 2014). In recent years, this neurotrophic nematode has spread from its original endemic areas of Southeast Asia and the Pacific islands to the American continent including the USA, Brazil and Caribbean islands (Wang et al., 2008, 2012). The rapid global spread of the *Angiostrongylus* lungworms and the emerging occurrence of the disease have posed challenges in its molecular epidemiology studies.

Eosinophilic meningitis caused by rat lungworm is highly endemic in Thailand especially in the northeast region of the country where a popular uncooked snail dish “koi-hoi” is often eaten (Eamsobhana, 2014; Eamsobhana et al., 2009). The snail of *Pila* species has an important role of transmitting the infection in Thailand. Although surveys on *A. cantonensis* based on morphological features in a variety of intermediate snail host and definitive rodent host have been continuously performed and reported, there is still limited molecular data on the phylogenetic relationship of this nematode in different regions of Thailand (Vitta et al., 2016). Moreover, apart from the report of Yong et al. (2015b) on molecular phylogeography of *A. cantonensis* and genetic relationships with congeners using the cytochrome *b* gene marker, molecular data on intra-species genetic variations of this neurotrophic lungworm from the definitive rodent host has not been

* Corresponding authors.
*E-mail addresses:* praphathip.eam@mahidol.ac.th (P. Eamsobhana), anchalee.tun@mahidol.ac.th (A. Tungtrongchitr).

http://dx.doi.org/10.1016/j.actatropica.2017.03.020
Received 16 January 2017; Received in revised form 10 March 2017; Accepted 11 March 2017
Available online 27 March 2017
0001-706X/ 

reported from many regions in Thailand.

In our earlier study, the country-wide distribution and incidence of *Angiostrongylus* lungworms and their rodent hosts included 27 provinces around Thailand during the period December 2011 to June 2014 (Eamsobhana et al., 2016). The incidence of infection with *Angiostrongylus* lungworms in positive rats was variable among host species and provinces. In the present study, representative *A. cantonensis* adult worms recovered from the lungs of wild rats in different geographical regions/provinces in Thailand were used to determine their haplotype by means of the mitochondrial partial cytochrome *c* oxidase subunit I (COI) gene sequence. The results revealed distinct COI haplotype of *A. cantonensis* from seven regions in Thailand.

## 2. Materials and methods

### 2.1. Angiostrongylus worms

*A. cantonensis* adult male and female worms were collected from the pulmonary arteries of wild caught rodents in different provinces around Thailand (Eamsobhana et al., 2016). They were identified morphologically under light microscope according to existing keys and descriptions (Bhaibulaya, 1979; Bhaibulaya and Cross, 1971; Eamsobhana, 2014). The worms were preserved in absolute alcohol and kept at −70° C. Table 1 summarizes the locality and rodent host of the eight specimens used for the present study. 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

Genomic DNA extraction from individual adult female and male worms of *A. cantonensis* was carried out using FTA (fast technology for analysis of nucleic acid) classic card method (Whatman BioScience, Newton Center, Massachusetts, USA) as previously described (Eamsobhana et al., 2010a; Eamsobhana et al., 2010b). 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 (Eamsobhana et al., 2010a; Eamsobhana et al., 2010b).

### 2.3. PCR amplification and DNA sequencing

The DNA amplification by polymerase chain reaction was conducted using the previously described primers COIF 5′–TAAAGAAA GAACATAATGAAAATG–3′ and COIR 5′–TTTTTTGGGCATCCTGA GGTTTA–3′ for a partial region of the COI gene (Bowles et al., 1993; Hu et al., 2002; Jefferies et al., 2009; Eamsobhana et al., 2010a). The amplification and sequencing procedure follows that previously described in Eamsobhana et al. (2010a).

**Table 1**
Locality, rodent host, COI haplotype and GenBank accession number of *Angiostrongylus cantonensis* from Thailand. M, male; F, female.

| Specimen | Locality | Rodent host | COI haplotype | Accession number |
|---|---|---|---|---|
| Ac1F | Trat | *Rattus rattus* | AC15 | KY703435 |
| Ac4M | Phitsanulok | *Rattus exulans* | AC10 | KY439004 |
| Ac5F | Phitsanulok | *Rattus exulans* | AC10 | KY439005 |
| Ac7F | Nakhon Phanom | *Rattus rattus* | AC11 | KY703434 |
| Ac8M | Kanchanaburi | *Rattus rattus* | AC14 | KY439007 |
| Ac12F | Samut Prakan | *Rattus rattus* | AC4 | KY439006 |
| Ac13F | Ranong | *Rattus rattus* | AC13 | KY703433 |
| Ac23F | Chantaburi | *Rattus rattus* | AC16 | KY703436 |

### 2.4. Cytochrome c oxidase subunit I nucleotide sequences from GenBank

Representative COI nucleotide sequences of *A. cantonensis* (from Thailand, Myanmar, Cambodia, China, Taiwan, Japan, Hawaii and Brazil) and *A. malaysiensis* were obtained from GenBank for comparison (Fig. 1). *Metastrongylus pudendotectus* and *Metastrongylus salmi* were included as outgroup taxa.

### 2.5. Sequence alignment and phylogenetic analysis

The COI sequences were edited and assembled using ChromasPro v.1.5 (Technelysium Pty Ltd., Australia) software followed by multiple sequence alignment with ClustalX v.1.81 program (Thompson et al., 1997). The resulting alignment was subsequently trimmed using BioEdit v.7.0.5.3 (Hall, 1999). Kakusan v.3 (Tanabe, 2007) was used to determine the best-fit nucleotide substitution models for maximum likelihood (ML) and Bayesian (BI) analyses selected using the corrected Akaike Information Criterion (Akaike, 1973) and the Bayesian Information Criterion (Schwarz, 1978), respectively. Phylograms were reconstructed using TreeFinder (Jobb et al., 2004) prior to the annotations of bootstrap values (BP) generated via 1000 ML bootstrap replicates. Bayesian analyses were conducted using the Markov chain Monte Carlo (MCMC) method via Mr. Bayes v.3.1.2 (Huelsenbeck and Ronquist, 2001). Two independent runs of $2 \times 10^6$ 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 and burn-in using the "sump" command in MrBayes and the computer program Tracer v.1.5 (http://tree.bio.ed.ac.uk/software/tracer/). 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. FigTree v.1.4 (Rambaut, 2012) was used to view and edit the final phylogenetic trees.

### 2.6. Haplotype network reconstruction

The median joining (MJ) network (Bandelt et al., 1999) was used to estimate the genealogical relationships of the haplotypes. The MJ network was calculated using NETWORK v.5.0.0.0 (http://www.fluxus-engineering.com).

### 2.7. Gene/Haplotype and nucleotide diversity

Genetic differentiation among populations was estimated by analysis of molecular variance (AMOVA) using Arlequin v.3.5.2.1 (Excoffier and Lischer, 2010). Statistical significance of AMOVA was tested from 10,000 permutations.

## 3. Results

### 3.1. Phylogenetic relationships

The aligned COI sequences of *A. cantonensis* consisted of 352 sites, of which 261 were invariable and 62 were parsimony informative. The best-fit nucleotide substitution model selected based on AIC was J3 + Gamma, and that selected based on BIC was HKY85 + Gamma. The phylogenetic trees reconstructed using the BI and ML methods had similar topology for the geographical isolates of *A. cantonensis* (Fig. 1). The *A. cantonensis* sequences formed a monophyletic clade distinct from the closely related congener *A. malaysiensis*.

### 3.2. Haplotype diversity and nucleotide diversity

Seven COI haplotypes were revealed in the present eight *A. cantonensis* sequences from seven geographical localities in Thailand (Table 1; Fig. 2). Of these seven haplotypes, AC10 of specimens Ac4M