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Molecular phylogeography of Angiostrongylus cantonensis (Nematoda: Angiostrongylidae) and genetic relationships with congeners using cytochrome b gene marker

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ARTICLE INFO

Article history:
Received 9 February 2015
Accepted 19 April 2015
Available online xxx

Keywords:
Angiostrongylus cantonensis
Angiostrongylus malaysiensis
Haplotype diversity
Molecular differentiation
Rat lungworms
Systematics
Phylogenetics
Phylogeography
Zoonotic parasites

ABSTRACT

Angiostrongylus cantonensis is an important emerging zoonotic parasite causing human eosinophilic meningitis (or meningoencephalitis) in many parts of the world. To date there is only a single study using mitochondrial cytochrome b (CYTB) gene to determine its genetic structure in eight geographical localities in Thailand. The present study examined the molecular phylogeography of this rat lungworm and its phylogenetic relationship with congeners using CYTB gene marker. A total of 15 CYTB haplotypes was found in 37 sequences from 14 geographical localities (covering north, west, east, central and south regions) in Thailand. These CYTB haplotypes were distinct from those of A. cantonensis for China and Hawaii. In Thailand, some CYTB haplotypes appeared to be confined to specific geographical localities. The partial CYTB DNA nucleotide sequences separated unequivocally the A. cantonensis isolates of Thailand, China and Hawaii as well as the congeners Angiostrongylus malaysiensis, A. costaricensis and Angiostrongylus vasorum, with A. malaysiensis grouped with A. cantonensis and A. costaricensis grouped with A. vasorum. Likewise the congeners Metastrongylus and Onchocerca genera could also be clearly differentiated. The present study added two new definitive hosts (Bandicota savaii and Rattus lesuea) and three new localities (Ma Hong Son in the north, Tak in the west, and Phang Nga in the south) for A. malaysiensis in Thailand, indicating its wide occurrence in the country. Three CYTB haplotypes were found in the Thailand samples of A. malaysiensis. In addition to differentiation of congeners, CYTB gene marker could be used for determining the genetic diversity of a given population/taxon.

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1. Introduction

Angiostrongylus cantonensis (Nematoda: Angiostrongylidae) is an important emerging pathogen causing human angiostrongyliasis with thousands of cases in many parts of the world (Kliks and Palumbo, 1992; Wang et al., 2012). Its typical endemic regions were Asia and the Pacific but has now spread to many other regions of the world, including Africa, Australia, Caribbean islands and the Americas (Eamsobhana, 2014).

Human infections with A. cantonensis are accidental or incidental in nature, as a result of ingestion of the third-stage larvae in intermediate hosts, paratenic hosts or contaminated raw or undercooked vegetables (Eamsobhana, 2014). The outcome may be fatal because of cerebral hemorrhage and eosinophilic meningitis for which treatment is generally symptomatic and supportive in nature (Eamsobhana, 2014). Over the years there have been extensive laboratory and clinical studies (Graeff-Teixeira et al., 2009) and development of immunodiagnosis (Eamsobhana and Yong, 2009).

Genetic aspects of A. cantonensis and related taxa have been explored vis-a-vis systematics and phylogenetics. The molecular gene markers include the nuclear 66 kDa protein gene (Caldeira et al., 2003; Eamsobhana et al., 2010a), internal transcribed spacers (Jeffries et al., 2009; Foronda et al., 2010; Liu et al., 2011; Lee et al., 2014), small subunit ribosomal RNA (Fontanilla and Wade, 2008; Eamsobhana et al., 2014), and mitochondrial cytochrome c oxidase subunit I (Jeffries et al., 2009; Eamsobhana et al., 2010b; Foronda et al., 2012; Foronda et al., 2015).

http://dx.doi.org/10.1016/j.actatropica.2015.04.020
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et al., 2010; Monte et al., 2012; Tokiwa et al., 2012; Lee et al., 2014). To-date there is only a single report on genetic variation of A. cantonensis using cytochrome b (CYTB) nucleotide sequences (Dusititipon et al., 2014).

Studies on population genetic structure of A. cantonensis and congeners are lacking. We report here CYTB haplotype diversity in A. cantonensis from different geographical locals and its genetic relationship with congeners.

2. Materials and methods

2.1. Angiostrongylus worms

Adult worms of A. cantonensis were collected from the pulmonary arteries of wild caught rodents in Thailand and China (Table 1), and from two lab strains (Hawaii and Thailand–Khon Kaen strains). All the Angiostrongylus malaysiensis adult worms were obtained from field rats in Thailand and Malaysia, including two new definitive hosts Bandicota savilei and Rattus luteus (Table 1).

The Khon Kaen (Thailand) and Hawaii isolates of A. cantonensis were maintained in the Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok (Eamsobhana et al., 2010b). A. cantonensis adult worms homogenates in FTA card from Guangxi, China were a gift of Dr. Hongman Zhang, while A. costaricensis adult specimens preserved in RNA later (RNA stabilization solution) were a gift from Dr. Elizabeth Abrahams, Department of Parasitology, University of Costa Rica.

Individual worm homogenate of A. cantonensis and A. malaysiensis from Thailand was applied and dried onto the FTA card for subsequent DNA preparations. Adult worms of A. malaysiensis from Peninsular Malaysia were preserved in absolute ethanol until DNA was extracted.

2.2. DNA extraction, PCR amplification and DNA sequencing

The FTA card method (Whatman BioScience) following the manufacturer’s instruction was employed for DNA preparation of the isolates from Thailand and the two lab strains (Eamsobhana et al., 2010b). The genomic DNAs of ethanol preserved worms were isolated using G-spin™ Total DNA Extraction Mini Kit (iNTRON Biotechnology, Inc, Korea) (Lim et al., 2012).

The DNA amplification by polymerase chain reaction was conducted using the previously described primers cyt b-F: 5’-TGATAGACGAAATTAGACG-3’ and cyt b-R: 5’-ATCAACTTACATTACAGAAC-3’ (Dusititipon et al., 2014). The thermocycler was programmed to incubate the samples for 5 min at 94 °C, followed by 35 cycles, each at 94 °C for 1 min, at 52 °C for 1 min, at 72 °C for 2 min, and a final extension at 72 °C for 5 min.

PCR products were analysed by electrophoresis on a 1.0% agarose mini gel pre-stained with SYBR SAFE DNA gel stain (Invitrogen, USA) and visualized under UV light. The target DNA fragments were isolated and purified using a LaboPass PCR purification kit (Cosmo Genetech, Seoul, Korea). Samples were sequenced by a commercial company using BigDye® Terminator v3.1 Cycle Sequencing Kit and analyzed on ABI PRISM® 377 Genetic Analyzer.

2.3. Cytochrome b nucleotide sequences from GenBank

Representative cytochrome b nucleotide sequences of A. cantonensis from Thailand and China, A. costaricensis, Angiostrongylus vasorum, Aeluromononos anubis, Metastrongylus pudendotectus, Metastrongylus salmi, Paraflagiloides normani, Protostrongylus rufescens, Brugia malayi, Onchocerca volvulus, and Setaria digitata were obtained from the GenBank (Table 2; Fig. 1).

2.4. Sequence alignment and phylogenetic analysis

Sequences from this study were edited and assembled using ChromasPro v.1.5 (Technelysium Pty Ltd., Australia) software followed by multiple sequence alignment with ClustalX (Thompson et al., 1997) program. The resulting alignment was subsequently trimmed using BioEdit v.7.0.5.3 (Hall, 1999). Kaksan 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 (Akaiek, 1973) and the Bayesian Information Criterion (Schwarz, 1978), respectively. Phylograms were constructed 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 et al., 2001). Two independent
Table 2

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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 v4.6.1.0 (http://www.fluxus-engineering.com).

3. Results

3.1. Phylogenetic relationships and genetic divergence

The aligned CYTB sequences consisted of 861 characters, of which 408 were constant, 377 were parsimony informative and 76 were parsimony uninformative. The phylogenetic trees constructed using the BI and ML methods had similar topology for the geographical isolates of A. cantonensis and A. malayensis (Fig. 1; Appendix Fig. A1). The Angiostrongylus taxa were grouped into three distict clades (Fig. 1)—1 A. cantonensis; 2 A. malayensis; and 3 A. costaricensis and A. vasorum.

The 42 sequences of A. cantonensis formed a clade with high support values. This clade consisted of three sub-clades: (a) 38 Thailand sequences; (b) 1 China-Fuzian and 2 Hawaii sequences; and (c) 2 China-Guangxi sequences. The second clade of Angiostrongylus taxa consisted of A. malayensis sequences of Thailand and Malaysia (Fig. 1).

The uncorrected ‘p’ distances for A. cantonensis and A. malayensis are summarized in Appendix Table A1. The Thailand geographical isolates of A. cantonensis had a ‘p’-distance of 0–2.93% (Table A1), while the Thailand and China isolates had a ‘p’-distance of 3.05–5.52%. The ‘p’-distance between the two closely related species A. cantonensis and A. malayensis was 8.34–9.19%, while A. malayensis isolates had a ‘p’-distance of 0–0.12%.

3.2. Haplotype diversity and nucleotide diversity

Eighteen CYTB haplotypes were revealed in the A. cantonensis sequences from Thailand, China and Hawaii (Tables 1 and 2; Fig. 2). The 38 Thailand sequences from 15 geographical localities were represented by 15 haplotypes—4 haplotypes in Northern region (AC3, AC5, AC10, AC12); 1 haplotype in Northeast region (AC11); 3 haplotypes in Western region (AC1, AC2, AC8); 2 haplotypes in Eastern region (AC13, AC15); 7 haplotypes in Central region (AC1, AC4, AC6, AC7, AC9, AC12, AC14); and 1 haplotype in Southern region.

(AC10). The three haplotypes of China (AC16, AC18) and Hawaii (AC17) sequences were not found in Thailand.

Four CYTB haplotypes were present in the A. malaysiensis sequences (Table 1, Fig. 2). Haplotype AM1 was the commonest (5 out of 8 specimens) and represented by one specimen in Tak (W. Thailand), and two specimens each in Phang Nga (S. Thailand) and Malaysia. Haplotype AM2 was found only in Mae Hong Son (N. Thailand), haplotype AM4 only in Tak, and haplotype AM3 only in Malaysia.

The haplotype/gene diversity for the 62 taxa of A. cantonensis, A. malaysiensis and the outgroups was 0.9736 ± 0.0082, and the nucleotide diversity was 0.106147 ± 0.051287. For the 50 taxa of A. cantonensis and A. malaysiensis used for generating haplotype network, the gene diversity was 0.9559 ± 0.0118 and the nucleotide diversity 0.041632 ± 0.020452.

Fig. 1. Bayesian inference (BI) phylogenetic tree of Angiostrongylus cantonensis and congeners based on mitochondrial cytochrome b nucleotide sequences.

4. Discussion

Mitochondrial DNA is considered to be effective in uncovering potential cryptic species when sequence data of small sample sizes are used (Blouin, 2002). Cytochrome b, a component of respiratory chain complex III (EC 1.10.2.2), is a commonly used mitochondrial gene for species identification and determination of phylogenetic relationships (Castresana, 2001). Despite its potential usefulness, there appears only a single study devoted to CYTB nucleotide sequences of Angiostrongylus worms (Dusitsittipon et al., 2014). Nonetheless, it has been used to analyze phylogenetic relationships of other nematodes, e.g. Baylisascaris Schroederi (Zhou et al., 2013) and Globodera spp. (Madani et al., 2010).

This study investigated the genetic relationships of geographical isolates of A. cantonensis (Thailand, China and Hawaii) and A. malaysiensis (Thailand and Malaysia) based on CYTB nucleotide sequences. Some CYTB haplotypes of A. cantonensis appeared to be confined to specific localities in Thailand, e.g. AC3 for Chiang Rai and AC5 for Chiang Mai in the north, AC8 for Kanchanaburi in the west, and AC11 for Khoon Ken as well as AC13 and AC15 for Mahasarakham in the northeast region (Fig. 2). However the sample size of each locality was too small for conclusive inference of distinct phylogeographic patterns.

The CYTB haplotypes of Thailand isolates were genetically distinct from those of China and Hawaii. This concurs with the findings based on cytochrome c oxidase subunit I (COI) nucleotide sequences (Eamsobhana et al., 2010b). However, CYTB nucleotide sequences revealed distinct genetic difference between the Fujian and Guangxi isolates of China. The close genetic affinity of Hawaii and China-Fujian isolates may reflect the movement of the parasite between these two sites.

The present phylogenetic analysis based on CYTB nucleotide sequences revealed distinct genetic differences among A. cantonensis, A. malaysiensis, A. costaricensis and A. vasonum (Fig. 1).
The clustering of *A. cantonensis* with *A. malaysiensis* concurs with the findings based on the mitochondrial COI gene (Eamsobhana et al., 2010b) and the nuclear small subunit (SSU) ribosomal DNA sequences (Eamsobhana et al., 2014). Likewise, the grouping of *A. costaricensis* with *A. vasorum* agrees with the results based on COI gene (Eamsobhana et al., 2010b) and 12 mitochondrial protein-coding genes (Jabbar et al., 2013), in contrast to *A. vasorum* grouping with *A. cantonensis* (Gasser et al., 2012).

A previous study reported 11 CYTB haplotypes in 91 *A. cantonensis* worms from eight localities in Thailand (Dusitsittipon et al., 2014). The present study added six localities from northern to southern regions of Thailand. This larger geographical coverage produced a total of 15 CYTB haplotypes for *A. cantonensis* (Tables 1 and 2; Fig. 2). Three additional CYTB haplotypes were present in non-Thai isolates—two for China and one for Hawaii. More extensive sampling is needed to determine population subdivision and aspects of phylogeography and epidemiology.

The smaller geographical coverage and small sample size of the congener *A. malaysiensis* yielded three CYTB haplotypes for three localities in Thailand, with haplotype AM2 being confined to Mae Hong Son and haplotype AM4 for Tak (Table 1; Fig. 2). Two CYTB haplotypes were present in the Malaysian sample of *A. malaysiensis*, with haplotype AM1 found in Thailand but AM3 only in Malaysia. A more intensive and extensive sampling may reveal greater haplotype diversity and the phylogeographical structure of *A. malaysiensis* as well as *A. cantonensis*.

In sum, we studied the phylogeny of *A. cantonensis*, *A. costaricensis*, *A. malaysiensis* and *A. vasorum* based on the mitochondrial CYTB nucleotide sequences. *A. cantonensis* was closer related to *A. malaysiensis* while *A. costaricensis* was closer related to *A. vasorum*. The geographical isolates of *A. cantonensis* from north, east, central and south Thailand showed considerable haplotype diversity. They were however genetically distinct from *A. cantonensis* isolates of Hawaii and China. We also report for the first time the

![Fig. 2. Haplotype networks of Angiostrongylus cantonensis and A. malaysiensis based on CYTB nucleotide sequences generated by NETWORK software. Circle represents haplotype and sizes are relative to the number of individuals sharing the specific haplotype.](https://example.com/fig2.png)
occurrence of *A. malaysiensis* in north, west and south Thailand, and *B. saveliei* and *K. losea* as new rodent definitive hosts. In addition to species identification and delimitation, the CYT B gene could be useful for studying the population genetics and epidemiology of *A. cantonensis* and other *Angiostrongylus* taxa.

**Q5 Uncited reference**

Shono (2000).

**Acknowledgements**

This study was funded in part by MoHE-HIR Grant (H-50001-00-A000025) and the University of Malaya (H-5620009). We thank the University of Malaya and Faculty of Medicine Siriraj Hospital, Mahidol University for providing various research facilities and other support. The authors wish to thank the anonymous reviewer for critical suggestions that helped to improve previous version of this article.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.actatropica.2015.04.020](http://dx.doi.org/10.1016/j.actatropica.2015.04.020)

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