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Phylogenetic relationships of rat lungworm, *Angiostrongylus cantonensis*, isolated from different geographical regions revealed widespread multiple lineages

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ABSTRACT

We conducted a pilot survey of genetic variation of *A. cantonensis* using small subunit (SSU) ribosomal (r) RNA and mitochondrial cytochrome *c* oxidase subunit I (*coxI*) gene sequences. Two distinct SSU genotypes (G1 and G2) were identified among 17 individual *A. cantonensis* worms from 17 different geographical localities in Japan, Mainland China, Taiwan, and Thailand. The partial *coxI* sequences were determined for 83 worms from 18 different geographical localities from Japan, Mainland China, Taiwan, and Thailand. Phylogenetic analysis showed eight distinct *coxI* haplotypes (ac1 to ac8). In 16 out of 18 localities, only a single *coxI* haplotype was found. However, in two localities, two *coxI* haplotypes coexisted. The common haplotypes found were: haplotype ac1 (Tokyo, Chiba, Kanagawa, Amamioshima Island, and Taichung), haplotype ac2 (Ishikawa, Shenzhen, and Lianjiang), haplotype ac5 (the Okinawa and the Ogasawara Islands), and haplotype ac7 (Miyagi, Aichi, and Kanagawa). Each of these regions is separated from the others by high mountain ranges or oceans. In addition, the lower genetic variation and particular geographical distribution of *A. cantonensis* in each location could indicate a founder effect, which may have resulted from multiple independent origins, and suggests that haplotypes migrated from endemic areas via human-related transportation.

1. Introduction

Angiostrongylus (*Parastrongylus*) *cantonensis* (Chen, 1935) is a nematode parasite normally found in rats and mollusks, which serve as final and intermediate hosts, respectively [1]. Various organisms such as frogs, toads, shrimps, land crabs, land planarians, fishes and monitor lizards serve as paratenic hosts [2–5].

Angiostrongyliais is an emerging infectious disease among humans, domestic, and wildlife animals [6]. *A. cantonensis* is endemic in Asia, the Pacific Islands, the Caribbean Islands, USA, and Africa. However, endemic regions are now thought to be more widely distributed [6,7]. The spreading of the worm is considered to be linked to the dispersal of invasive organisms. The spatial distribution of *A. cantonensis* was closely associated with the spread of the intermediate hosts, *Achatina* (*Lissacathina*) *fulica* in the Pacific Islands [8,9], Mainland China [10] and Brazil [11,12]. Another possibility could be due to introduction of rats infected with *A. cantonensis* [13,14]. In Japan, *A. cantonensis* was first found in the southern-most part of the Ryukyu Islands [15], and subsequent investigations have revealed the widespread occurrence of *A. cantonensis* in Japan [1,16]. Despite growing evidence that the occurrence of *A. cantonensis* is increasing, there is little information on how this occurs.

The phylogeny inferred from cytochrome *c* oxidase subunit I (*coxI*) revealed three distinct lineages among *A. cantonensis* collected from Thailand, Hawaii, and Mainland China [17]. Similar result was observed among *A. cantonensis* collected from Brazil and Mainland China [18]. These results showed promise for distinguishing between different geographical isolates. However, it has been difficult to infer the phylogeographical patterns due to the paucity of information. In addition, there is no information about the phylogenic diversity of *A. cantonensis* in Japan.

In the present study, we collected *A. cantonensis* worms from Japan, Mainland China, Taiwan, and Thailand. We analyzed the

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small subunit (SSU) ribosomal (r) RNA gene to identify nematode species, and also determined a partial sequence of the *coxI* gene to assess the variability of different geographical isolates.

2. Materials and methods

2.1. Sample collection

The rat lungworms, morphologically identified as belonging to the *Angiostrongylus* group [19,20] were collected between July 2008 and January 2011 from definitive hosts, *R. norvegicus* and *R. rattus*, captured using live-catch rat-traps in Japan and Taiwan. After euthanasia, the wild rats were necropsied to collect adult worms from their pulmonary arteries or first stage (L1) larvae from their lung tissue. Giant African land snails, *Achatina fulica* (Ferussac, 1821), were collected from the Ogasawara and the Okinawa Islands, Japan, and fixed in 70% ethanol in the field and preserved at −30 °C until DNA extraction. To recover third stage larvae (L3) of *A. cantonensis* from each snail, each lung was examined under microscopy [21]. The tree slug, *Limax marginatus* Muller 1774, was also captured from Kanagawa, Japan. L3 larvae were isolated from the tissues using Baermann's apparatus. *A. cantonensis* collected from three sites in Mainland China and one site in Thailand were kindly provided by RL. Zhang (Shenzhen Center for Disease Control and Prevention, China) and C. Komalamisra (Mahidol Univ., Thailand), respectively. These worm specimens were preserved in 70% ethanol until DNA extraction.

All animal experiments in this study were performed in accordance with the Guidelines for Animal Use and Experimentation of Tokyo Medical and Dental University (100170), and the Japanese Law of Animal Welfare and Care.

2.2. DNA extraction, PCR amplification and sequencing

Genomic DNAs were extracted using the NaOH direct lysis protocol [22]. The nuclear SSU rRNA and mitochondrial *coxI* genes were selected as target for the identification of nematode species [23–26] and the investigation of intraspecific variations [17,18,27,28], respectively. Partial SSU rRNA gene was amplified by PCR using universal primer sets (5′-AAAGTTAAGCCATGCATG-3′/5′-CATTCTTGGCAAATGCTTTCG-3′) [29]. Specific primers for the partial *coxI* (5′-TTTGTTTTGATTTTTTGGTC-3′/5′-AGGATAAATCTAAATACTTACGAGGA-3′) were designated to amplify the *coxI* sequence of *A. cantonensis* (GenBank GQ398121). The amplification conditions for SSU rRNA gene consisted of an initial denaturation at 94 °C for 2 min, followed by 25 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min. The *coxI* amplification involved initial denaturation at 94 °C for 5 min, and 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, followed by a final extension step at 72 °C for 2 min. Each 20 μl reaction mixture consisted of 14.8 μl of water, 2 μl of 10× reaction Buffer (Bioneer Co., Korea), 1.6 μl of dNTPs mix (10 mM, each dNTPs 2.5 mM), 0.2 μl of each primer (1.6 μM), 0.1 μl of *Top* DNA polymerase (0.5 U/μM) (Bioneer Co., Korea), and 1 μl of sample DNA. PCR reactions were analyzed by electrophoresis on a 1.5% agarose gel and visualized by UV illumination with ethidium bromide staining.

The PCR products were purified by Exosap-IT (GE Healthcare, England). Sequencing was performed using the BigDye Terminator ver 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instruction, and the sequence was read using an ABI3100 automatic sequencer (Applied Biosystems, USA). The sequences were visualized by electropherograms and manually corrected using Finch TV software (Geospiza, USA).

2.3. Data analysis and phylogenetic tree construction

The SSU and *coxI* gene sequences were aligned and edited using the ClustalW in MEGA ver 5.0.5 [30] at the default settings. The resultant alignment was used to calculate the nucleotide compositions and *p*-distance using MEGA ver 5.0.5 software. The best-fit models of DNA evolution were estimated for each data set separately (SSU and *coxI*), using Akaike Information Criterion (AIC) implemented in MODELTEST 3.7 [31]. The best-fit models for SSU and *coxI* were GTR + G and GTR + G + I, respectively. The Maximum likelihood (ML) analysis was performed by using PAUP 4.0b10 software [32]. ML tree was evaluated using the nonparametric bootstrap methodology based on 2000 for ML (MLB). The Bayesian interference was performed using MrBayers 3.1.2 [33]. The Markov chain Monte Carlo analysis was run for two million to estimate the posterior probabilities (BPP). This number of generations led to convergence in all analyses as estimated by split frequencies under 0.01. Trees were sampled every 100th generation, discarding the first 5000 of sampled trees as burn-in. The potential scale reduction factor for all parameters approached 1.0. To further explore relationships among the *A. cantonensis* haplotypes, minimum spanning network for the partial *coxI* sequences were constructed using the statistical parsimony [34] in TCS 1.2 [35]. The network estimation was run at 90% connection limit. Haplotype frequencies in the network were not used for this study.

A BLAST search (National Center of Biotechnology Information public databases; http://blast.ncbi.nlm.nih.gov/Blast.cgi) was carried out to elucidate any similarities with obtained sequences and previously published sequences. For the SSU rRNA gene, *A. cantonensis*

Table 1
Angiostrongylus cantonensis samples (n = 83) used in the present study.

Locality	No. of individuals	Worm stage	Origins	Date	Genotype	Haplotype (n)
Chiba, **Chiba**, Japan	15	Adult	10 *Rattus norvegicus*	2009	G1	ac1 (15)
Kawasaki, **Kanagawa**, Japan	3	L3	1 *Limax marginatus*	2008	G1	ac1 (3)
Yokohama, **Kanagawa**, Japan	4	Adult	1 *Rattus norvegicus*	2010	G1	ac1 (2), ac7 (2)
Minato-ku ,**Tokyo**, Japan	2	Adult	1 *R. norvegicus*	2009	G1	ac1 (2)
Hahajima Island, the OgasawaraIsland, Japan	8	L3	8 *Achatina fulica*	2010	G1	ac5 (8)
Chichijima Island, the Ogasawara Island, Japan	2	Adult, L1	2 *R. rattus*	2009	G1	ac5 (2)
Chichijima Island, the Ogasawara Island, Japan	6	Adult, L1, L3	Laboratory maintained	-	-	ac5 (6)
Sendai, **Miyagi**, Japan	4	Adult	2 *R. norvegicus*	2009	G2	ac7 (4)
Kanazawa, **Ishikawa**, Japan	4	Adult	1 *R. rattus*	2011	G1	ac2 (3), ac3(1)
Nagoya, **Aichi**, Japan	6	Adult	2 *R. norvegicus*	2004, 2009	G2	ac7 (6)
Amamioshima Island, the Nansei Islands, Japan	4	L1, Adult	2 *R. rattus*	2009	G1	ac1 (4)
Naha, **Okinawa Island**, the Nansei Islands, Japan	10	Adult	6 *R. norvegicus*	2010	G1	ac5 (10)
Nanjo, **Okinawa Island**, the Nansei Islands, Japan	1	L3	1 *Achatina fulica*	2008	G1	ac5 (1)
Taichung, Taiwan	4	Adult	3 *R. norvegicus*	2010	G1	ac1 (4)
Shenzhen, Guangdong, China	3	Adult	3 *R. norvegicus*	2010	G1	ac2 (3)
Wenzhou, Zhejiang, China	2	Adult	Laboratory maintained	-	G2	ac6 (2)
Lianjiang, Fujian, China	3	Adult	Laboratory maintained	-	G1	ac2 (3)
Bangkok, Thailand	2	L1	Laboratory maintained	-	G1	ac4 (2)
	83					