

POPULATION STRUCTURE OF *ANGIOSTRONGYLUS CANTONENSIS* (NEMATODA: METASTRONGYLIDAE) IN THAILAND BASED ON PCR-RAPD MARKERS

Urusa Thaenkham, Wallop Pakdee, Supaporn Nuamtanong, Wanna Maipanich, Somchit Pubampen, Surapol Sa-nguankiat, and Chalit Komalamisra

Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Abstract. *Angiostrongylus cantonensis* is the causative agent of angiostrongyliasis, which is widely distributed throughout the world. It can specifically infect many species of intermediate and definitive hosts. This study examined the genetic differentiation and population structure using the RAPD-PCR method of parasites obtained from 8 different geographical areas of Thailand. Based on 8 primers, high levels of genetic diversity and low levels of gene flow among populations were found. Using genetic distance and neighbor-joining dendrogram methods, *A. cantonensis* in Thailand could be divided into two groups with statistically significant genetic differentiation of the two populations. However, genotypic variations and haplotype relationships need to be further elucidated using other markers.

Keywords: *Angiostrongylus cantonensis*, population structure, PCR-RAPD, Thailand

INTRODUCTION

Angiostrongylus cantonensis (rat lung-worm) is a nematode of rodents, and adult worms live inside the pulmonary arteries (Alto, 2001). Humans occasionally acquire infection by ingesting raw or undercooked snail, slug, prawn, tadpole, or contaminated vegetables, containing infective *A. cantonensis* larvae (Bronstein *et al*, 1978; Oku *et al*, 1980; Wang *et al*, 2008). The parasitic worm is neurotropic in humans and can cause eosinophilic meningitis (Alto, 2001). *A. cantonensis* is distributed

widely in 30 countries, and outbreaks are frequently reported in many countries in Asia and in the Pacific and Caribbean Islands (Wang *et al*, 2008).

Many kinds of freshwater snails (*Pila* spp, *Pomacea canaliculata*, *Biomphalaria alexandrina*, *Lymnaea natalensis*, and *Melanoides tuberculata*) and land snails (*Achatina fulica* and *Hemiplecta* spp) are reportedly paratenic hosts (Tasana *et al*, 2009). In China, *P. canaliculata* and *Ac. fulica* are believed to be closely associated with angiostrongyliasis. In Thailand, the high rate of angiostrongyliasis is linked to the custom of eating raw *Pila* snails (Wang *et al*, 2008). However, the highest infection rate (44.3%) of *A. cantonensis* is from *Ac. fulica*, while the rate among *Pila* spp is around 0.9% (Pipitgool *et al*, 1997). *Ac. fulica* is also a major source of

Correspondence: Chalit Komalamisra, Department of Helminthology, Faculty of Tropical Medicine, 420/6 Ratchawithi Road, Bangkok 10400, Thailand.

Tel/Fax: +66 (0) 2643 5600

E-mail: tmckm@mahidol.ac.th

infection worldwide (Kliks and Palumbo, 1992). *Rattus rattus* and *R. norvegicus* are considered the most common definitive hosts of this parasite, while other rodents can also serve as parasitic hosts (Cross and Chen, 2007).

The broad spectrum of specific intermediate and definitive hosts is related to the population genetic structure and diversity of disease-causing organisms. These are used to draw inferences about their evolutionary history and potential to generate trait variations that determine interactions with their hosts (Barrett *et al*, 2008). Accurate measurements of genetic variations and their distribution within host-parasite systems are an important parameters needed to assess the re-emergence of disease and the effects of infection on host mortality and parasite reproduction (Nadler, 1995).

Recently, several molecular markers have been developed to study the population genetics and diversity of various parasites such as a randomly amplified polymorphic DNA (RAPD) markers based on PCR technique (Nadler *et al*, 1995; Chrisanfova *et al*, 2000; Guizani *et al*, 2002). RAPD method is useful for studying intraspecific species where no study has previously been undertaken, with low cost and can handle extensive numbers of samples (Li *et al*, 2006). Therefore, it is possible to conduct a preliminary study of a large sample size from each population.

In this study, RAPD technique was used to examine the genetic diversity of *A. cantonensis* in various regions of Thailand that have not yet been reported. This is the first PCR-RAPD investigation of the genetic variation and intraspecific variants among 8 populations of *A. cantonensis* collected from *Ac. fulica* in Thailand.

MATERIALS AND METHODS

Sample collection

More than 200 *Ac. fulica* were collected from each of 8 different geographical areas of Thailand (Fig 1). The snails were killed and their shells were cracked. The soft tissues of individual snails were homogenized and digested with 0.25% pepsin A (BDH, Leicestershire, England) for one hour at 37°C. The digested fluid containing nematode larvae from each snail was collected using Baermann's apparatus, and L3 *A. cantonensis* larvae were identified under a dissecting microscope. The L3 larvae from each positive snail were pooled and infected into Wistar rats (*Rattus norvegicus*). After 4 months, the infected rats were sacrificed and the adult *A. cantonensis* worms were isolated from the rat pulmonary arteries. All parasite infection procedures with the rats were approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (no. FTM-ACUC001/2008). Twenty-five adult *A. cantonensis* worms from each locality were preserved in 70% EtOH at -20°C for further use.

RAPD-PCR

DNA was extracted from individual adult worms using a Genomic DNA Mini kit (Tissue) (Geneaid, Taiwan), according to the manufacturer's instructions. DNA of each worm was amplified using preselected oligonucleotide from the Operon A series (Operon Technologies, Alameda, CA). PCR was performed in 20 μ l reaction volume containing 10 μ l of TopTaq™ Master Mix Kit, 3 μ l M arbitrary primer, and 20 ng of genomic DNA. Into the reaction, MgCl₂ was adjusted to 2.5 mM for greater specificity. RAPD-PCR was conducted by heating at 95°C for 3 minutes, followed by 39 cycles of 94°C for 1 minute, 34°C for 1 minute, and 72°C for 1 minute, with

a final step at 72°C for 10 minutes. PCR amplifications were conducted in duplicate for each sample at different times in order to decrease the risk of obtaining non-reproducible RAPD bands. The PCR amplicons were separated by 2.0% agarose gel-electrophoresis, stained with ethidium bromide, and recorded by Gel Documentation instrument [G-Box (HR); Syngene, Cambridge, UK]. DNA bands were named based on the corresponding primer and estimated in molecular size in relation to 100-bp DNA marker (Biolabs, Herts, England).

Data analysis

RAPD polymorphism was scored based on the presence (1) or absence (0) of PCR amplicons, and entered into a binary data matrix. A locus was considered polymorphic only if the most common allele occurred at a frequency of ≤ 0.95 in the population (a 95% criterion). Genetic diversity was measured by the percentage of polymorphic loci (PPB), the observed number of alleles (n_a), the expected number of alleles (n_e), Nei's genetic diversity (H) (Nei, 1978) and Shannon diversity index (I) (Krebs, 1989), and gene flow (Nm) using POPGEN32 software (Yeh *et al*, 1999). A population dendrogram was constructed, based on genetic distance of Nei's, 1978 using PHYLIP version 3.5 (Felsenstein, 1989). Non-parametric analysis of molecular variance (AMOVA) and F -statistics were conducted using Arlequin population-genetics software (Excoffier *et al*, 2005) in order to determine the genetic differences and to describe the genetic structures among populations.

RESULTS

Genetic diversity

Of 30 primers, 8 (10-mers) were se-

Table 1
Sequences of 8 RAPD primers used in this study.

Primer	Sequence 5' → 3'
OPA-01	CAG-GCC-CTT-C
OPA-06	GGT-CCC-TGA-C
OPA-10	GTG-ATC-GCA-G
OPA-16	AGC-CAG-CGA-A
OPA-19	CAA-ACG-TCG-G
OPB-08	GTC-CAC-ACG-G
OPB-18	CCA-CAG-CAG-T
OPB-20	GGA-CCC-TTA-C

lected (Operon Technologies) after screening for intense and reproducible bands for further PCR amplifications. The 8 selected primers (Table 1) were used for RAPD-PCR of 112 *A. cantonensis* samples from 8 populations and 135 loci (between 165 and 1,015 bp) were obtained overall, and 116 (86%) of these loci were polymorphic. The RAPD band patterns from 8 populations are shown in Fig 2. Within a given population, however, most *A. cantonensis* had monomorphic bands (completely identical) except the population from PK (69%). There were high levels of genetic diversity among these populations [H (Nei's gene diversity)] = 0.3176 ± 0.1554 ; I (Shannon's information index) = 0.1157 ± 0.218). Gene flow among the subdivided populations was calculated according to Slatkin and Barton's formula (Slatkin and Barton, 1989), revealing a very low gene flow ($Nm = 0.059$).

Genetic distance, identity and neighbor-joining dendrogram

From the 8 *A. cantonensis* populations, Nei's genetic distance ranged between 0.069 and 1.409 (Table 2) indicated that the *A. cantonensis* populations studied could be divided into 2 major groups: group 1

Table 2

Nei's identity and genetic distance for the eight *A. cantonensis* populations in Thailand. Genetic identity values are above diagonal and genetic distances are below.

Population	KB	NK	NR	CM	LB	PC	KK	CP
KB	-	0.274	0.296	0.244	0.778	0.659	0.259	0.274
NK	1.294	-	0.918	0.896	0.452	0.735	0.881	0.896
NR	1.216	0.085	-	0.904	0.459	0.746	0.859	0.933
CM	1.409	0.109	0.101	-	0.422	0.745	0.852	0.882
LB	0.251	0.794	0.778	0.862	-	0.682	0.422	0.422
PC	0.417	0.308	0.294	0.294	0.383	-	0.755	0.768
KK	1.349	0.126	0.152	0.160	0.862	0.281	-	0.911
CP	1.294	0.109	0.069	0.126	0.862	0.264	0.093	

Abbreviations are listed in legend of Fig 1.

Table 3
AMOVA.

Source of variation	<i>d.f</i>	Sum of squares	Variance component	Percentage variation
Among groups	1	1,525.845	34.323	79.77
Among populations within a group	6	504.905	5.803	13.49
Within a population	104	301.741	2.901	6.74
Total	113	2,332.64	43.03	

Statistical analysis ($p = 0.05$)

composing of KB and LB populations and of NK, NR, CM, KK, and CP populations for group 2. However, the PK population seemed closely related to both groups. The genetic relationships are illustrated by a dendrogram, constructed by neighbor-joining method (Fig 3). The PK population was grouped with group 1. The cluster diagram clearly showed the separation of the 8 populations into 2 groups.

Genetic differentiation

The two divided groups were tested for genetic differentiation (Table 3) and as revealed by the variances among the populations relative to total variance

($F_{st} = 0.936$; $p = 0.00$), variance among populations within groups ($F_{sc} = 0.667$; $p = 0.00$), and variance among groups relative to total variance ($F_{ct} = 0.798$; $p = 0.035$), all F -statistics show significant genetic differentiation ($p < 0.05$) among the *A. cantonensis* studied for all variables. Percent variation was high between the 2 groups (80%) with low values within parasite groups (7%).

DISCUSSION

In Thailand, *A. cantonensis* is distributed in many different geographical

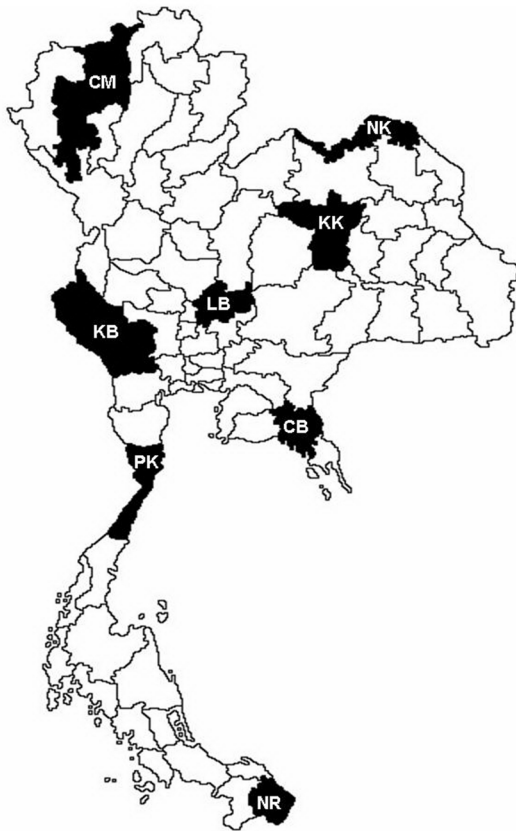


Fig 1—Eight different geographical localities in Thailand where *Ac. fulica* samples were collected. CM, Chiang Mai; NK, Nong Khai; KK, Khon Kaen; CB, Chanthaburi; KB, Kanchanaburi; LB, Lop Buri; PK, Prachuap Khiri Khan; and NR, Narathiwat.

localities (Komalamisra *et al*, unpublished), and infection has been reported in a broad spectrum of intermediate and definitive hosts (Tesana *et al*, 2009). In this study, RAPD-PCR method used to reveal the first picture of *A. cantonensis* genetic structure among 8 populations in Thailand. *A. cantonensis* can be divided into 2 major groups, based on genetic distance. Group I comprising parasite popula-

tions from Chiang Mai (CM), Nong Khai (NK), Khon Kaen (KK), Chanthaburi (CB), and Narathiwat (NR) and group II from Kanchanaburi (KB) and Lop Buri (LB) Provinces. Group I contains the majority of genotypes distributed throughout most of the country, while group II is restricted to parasite populations around central and western Thailand. Based on variance among groups relative to total variance, the two *A. cantonensis* population groups in Thailand are significantly genetically different. Moreover, genetic differentiations among parasite populations within the same group are also significantly different with high levels of genetic differentiation among the 8 populations. Genetic differentiation among *A. cantonensis*, either between or within groups, or among populations, suggested that the population structure of *A. cantonensis* in Thailand has been affected by population subdivision. The very low gene flow among the populations, high genetic diversity among populations, and a lack of genetic difference within populations, suggested that the *A. cantonensis* population may have created distinct gene frequencies and local fixation of genetic variations within their own populations (inbreeding).

The population structures of parasites are fundamentally related to their hosts. The *A. cantonensis* population in this study appeared to be increasing in genetic structure (pattern of individual genetic makeup within a population) because of local fixation of genetic variations. The sedentary nature of the definitive host, or the high morbidity of all infected hosts (both intermediate and definitive), and the large numbers of specific obligate hosts (broad spectrum of specific hosts) are factors enhancing genetic structure (Nadler, 1995). Rats and snails, which are the definitive and intermediate hosts of

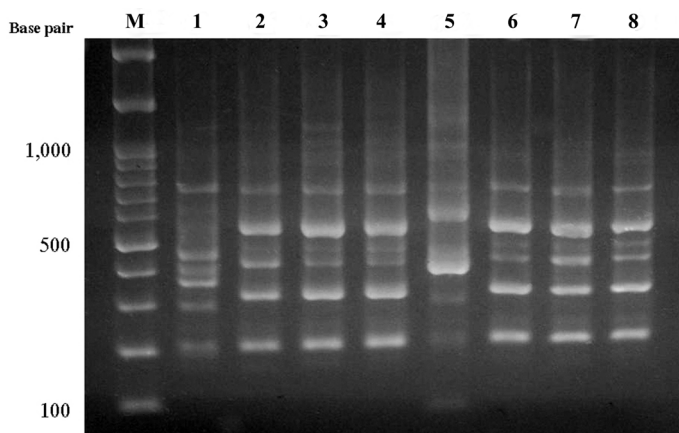


Fig 2—RAPD band patterns of 8 population of *A. cantonensis* collected from KB (lane 1), NK (lane 2), NR (lane 3), CM (lane 4), LB (lane 5), PK (lane 6), KK (lane 7), and CB (lane 8) generated by OPB-20. Primer M = 100 bp DNA ladder. Abbreviations are listed in legend of Fig 1.

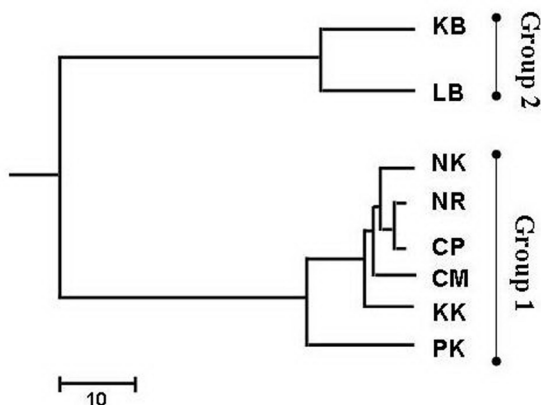


Fig 3—Genetic relationship among 8 *A. cantonensis* populations in Thailand. The cluster diagram was constructed by neighbor-joining method. The scale bar indicates genetic distance, based on RAPD polymorphism.

A. cantonensis, occupy colonies as sedentary hosts rather than disperse as vagile ones (Dowding and Murphy, 1994; Negovetic and Jokela, 2001). While *A. cantonensis*

might frequently comprise populations nearing extinction and reestablishment, each population seems genetically separate from the other. Of two major parasite genotype groups suggest group I genotypes may be older than group II, since they are the major genotypes in Thailand. The group II genotypes seem to be established in the western and central provinces of Thailand. It is possible that they are distributing into neighboring areas (from KB to PK).

A phylogeography of *A. cantonensis* and its hosts should be developed in further studies in order to permit

a clearer understanding of parasite-host relationships and genetics related to their localities. Appropriate genetic markers should be used in further studies in order to confirm the genetic differentiation among *A. cantonensis* genotypes in Thailand, which may affect levels of host mortality and parasite reproduction in the host.

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