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Analysis of sequence variation in *Gnathostoma spinigerum* mitochondrial DNA by single-strand conformation polymorphism analysis and DNA sequence☆

Charinthon Ngarmamonpirat[a], Jitra Waikagul[a,*], Songsak Petmitr[b], Paron Dekumyoy[a], Wichit Rojekittikhun[a], Malinee T. Anantapruti[a]

[a]*Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand*
[b]*Department of Tropical Nutrition and Food Science, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand*

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Abstract

Morphological variations were observed in the advance third stage larvae of *Gnathostoma spinigerum* collected from swamp eel (*Fluta alba*), the second intermediate host. Larvae with typical and three atypical types were chosen for partial cytochrome *c* oxidase subunit I (COI) gene sequence analysis. A 450 bp polymerase chain reaction product of the COI gene was amplified from mitochondrial DNA. The variations were analyzed by single-strand conformation polymorphism and DNA sequencing. The nucleotide variations of the COI gene in the four types of larvae indicated the presence of an intra-specific variation of mitochondrial DNA in the *G. spinigerum* population.

Keywords: *Gnathostoma spinigerum*; Mitochondrial DNA; Cytochrome *c* oxidase subunit I; Polymerase chain reaction–single-strand conformation polymorphism

The genus *Gnathostoma* comprises 23 species widely distributed in Asia, and Central and South America. Morphology differentiation among species is based mainly on the characteristics of the body spines, in particular the cephalic hooklets. The morphology, number of hooklets and the number of rows of hooklets on the head bulb are distinct between species [1]. Advance third stage larva (AL3) of *Gnathostoma spinigerum* has four circular rows of hooklets which has a single pointed spine on an oblong base. Among AL3 collected from the swamp eels in 1998, the hooklets variation features were found [2]. The morphological identification is difficult and a DNA sequencing method should be used to confirm species.

Recently, sequence variations within a region of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene was reported in many species of helminths such as *Capillaria*, *Echinococcus*, *Oesophagostomum*, *Schistosoma* and *Spirometra.* The partial COI region was amplified and revealed sequence variations by polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) and DNA sequencing [3–7]. The PCR–SSCP approach was applied to detect this genetic variation efficiently [8].

In this study, 565 (493 typical and 72 atypical) AL3 of *G. spinigerum* were collected from livers of swamp eels. They were divided into four groups: typical hooklets, lobed or branched hooklets, typical hooklets with additional spines and a spiral row of hooklets (Fig. 1). Each AL3 was identified and kept at −20 °C. The partial COI genes of these larvae were studied by PCR–SSCP and the sequence variations were analyzed.

Genomic DNA from 2 to 10 individual larvae of each group were prepared: typical hooklets (T1–10), atypical

☆ The COI sequence of *Gnathostoma spinigerum* reported in this paper has been submitted to the GenBank with accession number AY501388.

* Corresponding author. Tel.: +662 3549100; fax: +662 6435600.
E-mail address: tmjwk@mahidol.ac.th (J. Waikagul).

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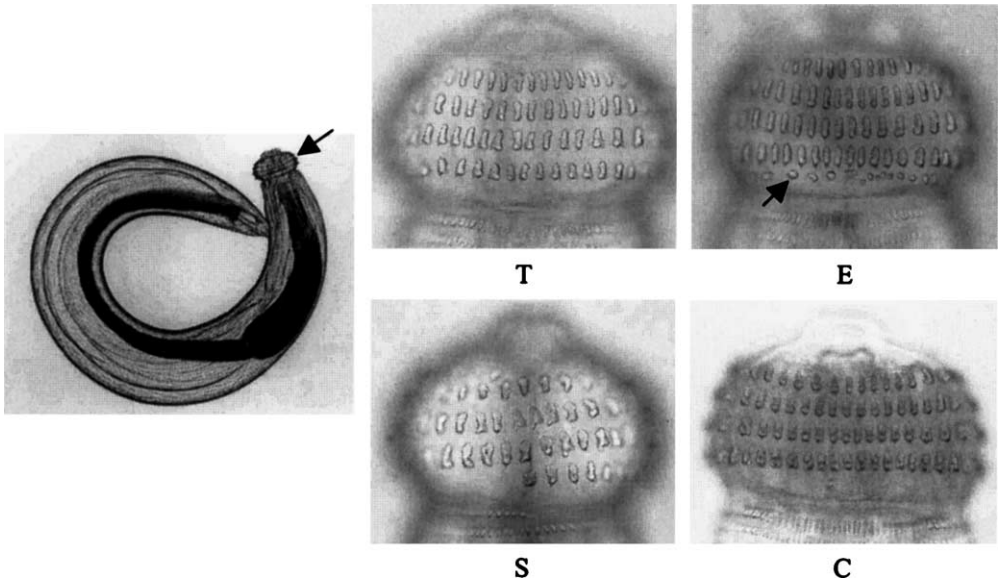

Fig. 1. Advanced third stage larva of *G. spinigerum*, cephalic bulb (big arrow) in higher magnification showing variation of hooklet features. T=typical hooklets, E=with additional spines (small arrow), C=lobed hooklets, S=spiral row of hooklets.

lobed hooklets (C1–10), additional rudimental spines (E1–10) and a spiral row of hooklets (S1–2) by using QIAamp® DNA mini kit (QIAGEN, Germany). PCR amplification was performed in 50 μl of mixture containing 10× PCR buffer, 250 μM dNTPs, 4 mM $MgCl_2$, and 1 U of Taq polymerase (DyNAzyme™ II; Finnzyme, Finland). This reaction used 100 pmol primer JB3 (5′ TTT TTT GGG CAT CCT GAG GTT TAT 3′) and JB 4.5 (5′ TAA AGA AAG AAC ATA ATG AAA ATG 3′). The PCR reaction was amplified in a Mastercycler® personal PCR machine (Eppendorf, Germany) with initial denaturation at 94 °C for 5 min, then 40 cycles of denaturation at 94 °C for 30 s, primer annealing at 58 °C for 1.30 min, extension at 72 °C for 30 s. The PCR reaction was followed by final extension at 72 °C for 5 min.

PCR products were electrophoresed in 2% agarose gel. The amplicon size of the COI fragment is 450 bp. Nucleotide variations in the COI gene were screened by SSCP [3], and then visualized by silver stain [9]. SSCP analysis revealed two bands in all individuals. However, distinct profiles were detected in some samples, particularly T7 and E1. No variation in SSCP profile was demonstrated within the group of larvae having lobed or spiral row hooklets (Fig. 2). There was no relationship between SSCP profiles and the morphology of *G. spinigerum* AL3, while genetic variability in COI and NDI genes within and among populations of *Echinococcus granulosus* from China and Argentina was reported by SSCP [4]. Moreover, different SSCP patterns of nematode from different hosts, *Capillaria* from marsupials and rodents [5], and *Oesophagostomum bifurcum* from humans and Mona monkey [6], were demonstrated. The SSCP analysis in this study revealed distinct profiles of the amplified products with 450 bp among some individuals. However, this method was not capable of fully demonstrating nucleotide variation in mtDNA among *G. spinigerum* AL3. Thus, DNA sequencing should be performed to reveal a nucleotide variation.

The amplicon fragment was purified using NucleoSpin® Extract (Macherey-Nagel, Germany) and sequenced (MWG-Biotech, Germany). Among 32 larvae, sequence analysis showed 9 different types of COI sequence. A common sequence was shared by 23 larvae. The sequence of the COI partial gene T5 was submitted to GenBank (accession number AY501388). The other eight types were T1, T6, T7, E1, E2, E6, C4 and S2. Three of ten (30%) larvae, each with typical features and with additional spines, had different types of COI sequence. 10% of larvae with lobed hooklets showed different types of COI sequence. One of two larvae with a spiral row of hooklets had different types of COI sequence. The common sequence nucleotide (nt) variations in the COI fragments for four different feature types of AL3 are summarized in Table 1.

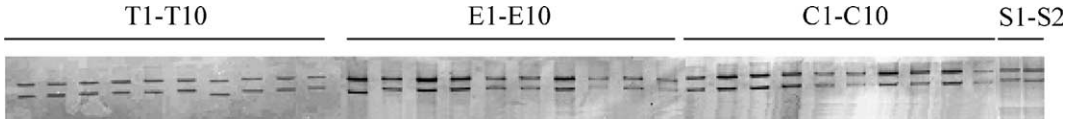

Fig. 2. Single-strand conformation polymorphism profiles of individual advanced third stage larvae of *G. spinigerum* with four different hooklet features. T=typical hooklets, E=with additional spines, C=lobed hooklets, S=spiral row of hooklets.