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Molecular phylogenetic confirmation of *Gnathostoma spinigerum* Owen, 1836 (Nematoda: Gnathostomatidae) in Laos and Thailand

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Abstract: We report the molecular-phylogenetic identification of larvae of the nematode genus *Gnathostoma* Owen, 1836 collected from a snake, *Ptyas koros* Schlegel, in Laos and adult worms from the stomach of a dog in Thailand. DNA was extracted and amplified targeting the partial *cox1* gene and the ITS-2 region of ribosomal DNA. Phylogenetic analyses indicated that all five advanced third-stage larvae and seven adult worms were *Gnathostoma spinigerum* Owen, 1836. This is also the first molecular evidence of infection with *G. spinigerum* in a snake from Laos.

Keywords: ITS-2 rDNA, genotyping, parasitic nematode, fish-borne helminthoses, molecular taxonomy, South-East Asia

Gnathostomiasis is a zoonotic disease caused by nematode parasites of the genus *Gnathostoma* Owen, 1836. Dogs and cats are the main natural definitive hosts and humans are accidentally infected by acquiring larvae via ingestion of insufficiently cooked meat of the second intermediate hosts or paratenic hosts such as fish, frogs and snakes, and also by skin penetration of the larvae from such meat (Waikagul and Diaz-Camacho 2007). Human cases have been reported predominantly in Japan and Southeast Asian countries including Thailand. *Gnathostoma spinigerum* Owen, 1836 has been identified as causative agent of these infections (Waikagul and Diaz-Camacho 2007).

Gnathostoma nipponicum Yamaguti, 1941, *Gnathostoma doloresi* Tubangui, 1925 and *Gnathostoma hispidum* Fedchenko, 1972 have also been reported as human parasite in Japan (Waikagul and Diaz-Camacho 2007). In Mexico, *Gnathostoma binucleatum* Almeyda-Artigas, 1991 represents the main causative agent of gnathostomosis (Almeyda-Artigas et al. 2000). In non-endemic countries, health issues may arise from imported cases, i.e. an infection contracted while visiting an endemic country (Herman and Chiodini 2009).

Identification of worms from humans and natural hosts is traditionally done using morphological criteria (Akahane et al. 1986) but molecular approaches are useful method for identification of species of *Gnathostoma* (see Almeyda-Artigas et al. 2000, Ando et al. 2006, Jongthawin et al. 2015). However, molecular evidence of the occurrence of species of *Gnathostoma* in wildlife in Asian countries including Laos and Thailand is still lacking.

We analysed the partial sequences of mitochondrial cytochrome c-oxidase subunit I (*cox1*) gene and full-length ITS-2 region of ribosomal DNA of a *Gnathostoma* species isolated from a snake (*Ptyas korros* Schlegel; Colubridae) collected in Laos. Nucleotide sequences of *cox1* and ITS-2 of *G. spinigerum* adults recovered from the stomach of a domestic dog (*Canis lupus familiaris* Linnaeus) in Thailand and from DNA databases were used for phylogenetic analysis to explore the relationships.

One sample of *P. korros* (commonly known as the Chinese rat snake or Indo-Chinese rat snake) was bought at a local food market in Khammouane Province, Laos. The snake harboured 90 advanced third-stage larvae ($AdvL_3$) of *Gnathostoma*, five of which were microscopically iden-

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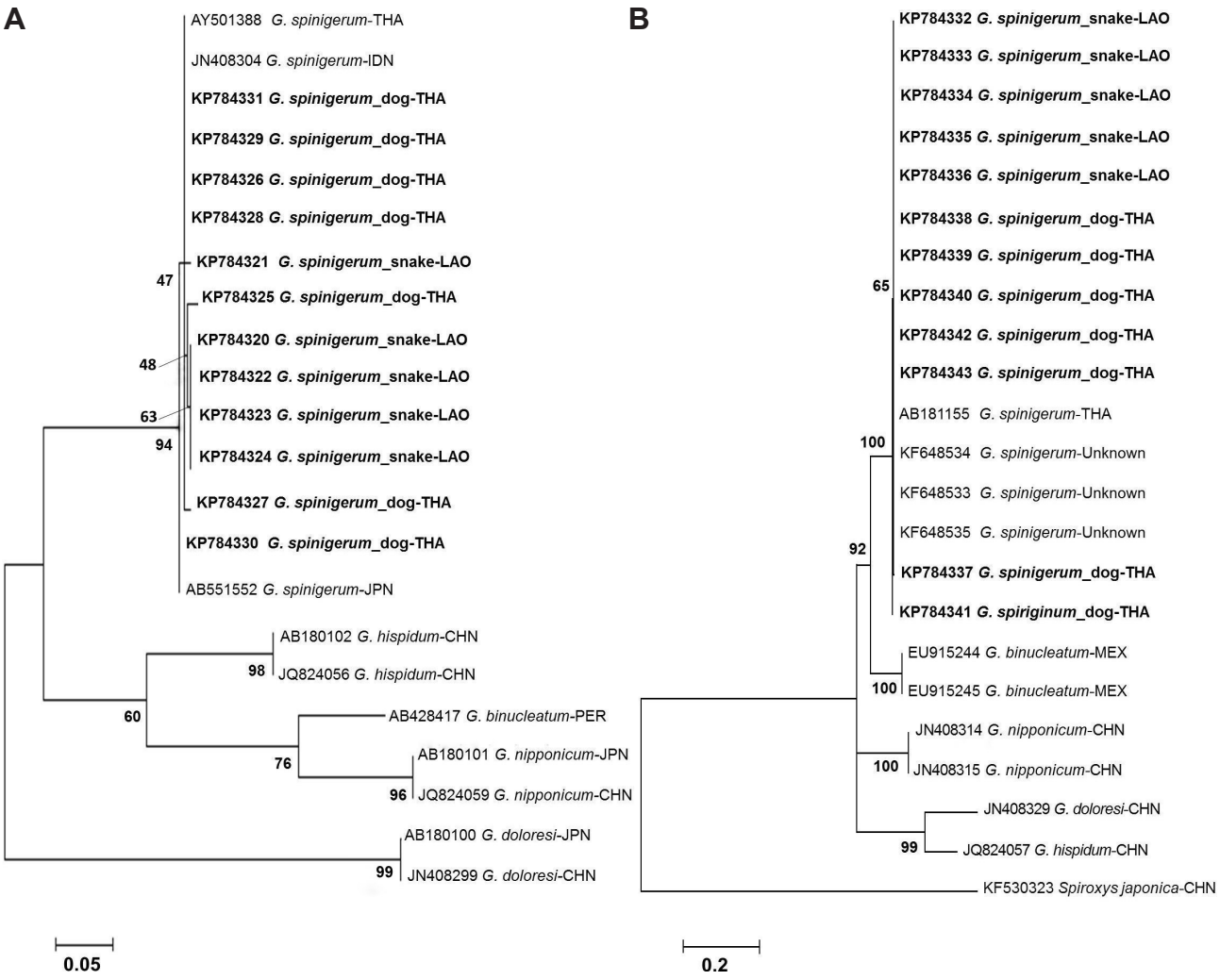

Fig. 1. Phylogenetic relationship among species of *Gnathostoma* Owen, 1836. Trees were reconstructed using the maximum likelihood method based on partial *cox1* (**A**) and ITS-2 sequences (**B**). Sequences of species of *Gnathostoma* obtained from GenBank are indicated with the accession number and the country code (ISO 3166-1 alpha-3 code). Sequences obtained in the present study are highlighted in bold. Bootstrap scores, expressed as percentages of 1 000 replications, are given at each node. Scale bars indicate substitutions per nucleotide position.

tified as *G. spinigerum* and selected for molecular identification. Seven adult worms were obtained from the stomach of a domestic dog at Animal Hospital, Faculty of Veterinary Medicine, Khon Kaen University; the parasites were microscopically identified as *G. spinigerum* based on characteristics presented by Daengsvang (1980). All parasites were preserved in 70% ethanol and kept in the freezer -80 °C before DNA extraction.

Genomic DNA was extracted from individual $AdvL_3$ and adult worms using a DNA extraction kit (NucleoSpin® Tissue, Macherey-Nagel, Germany). A partial *cox1* gene and partial 5.8S, entire ITS2 and partial 28S regions were amplified using the primers Gn_COI (forward: 5'-GCCTGCTTTTGGAATTGTTAG-3', reverse: 5'-ACGAAAACCATACAAAGTAGCCAA-3') and GS_ITS2 (forward: 5'-TGTGTCGATGAAGAACGCAG-3', reverse: 5'-TTCTATGCTTAAATTCAGGGG-3'), respectively, which were genus-specific for *Gnathostoma*.

Each polymerase chain reaction (PCR) was performed according to the previous method (Jongthawin et al. 2015). Amplicons were subjected to agarose gel electrophoresis (1.5% gel); 250 bp of *cox1* and 650 bp of partial 5.8S, entire ITS2 and partial 28S regions fragments were cut and purified. Samples for sequencing were prepared using BigDye® Terminator v3.1 cycle sequencing kit (Foster City, CA, USA) and sequenced using a 3730xI DNA Analyzer (ABI). All DNA fragments were sequenced in both directions, employing the same primers as used in each PCR.

The partial sequence of *cox1* gene and the complete sequence of the ITS-2 region from each *G. spinigerum* isolates were analysed using the BLAST-N search tool (National Center for Biotechnology Information, Bethesda, MD, USA). The new *G. spinigerum* sequences of samples from both countries were aligned with the sequences from the GenBank database (alignment length was 205 bp long for *cox1* and 472 bp for the full length of ITS-2) using the Bioedit sequence alignment editor (Hall 1999).

Phylogenetic relationships were inferred using the maximum likelihood method implemented in MEGA v6 (Tamura et al. 2013). The best substitution model for *cox1* was

Table 1. Tamura-Nei model (Tamura and Nei 1993) genetic distance matrix values based on partial *cox1* gene sequences among 12 isolates of *Gnathostoma spinigerum* Owen, 1836 and related sequences of *G. spinigerum* from the Genbank database.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 KP784320_LAO															
2 KP784321_LAO	0.010														
3 KP784322_LAO	0.000	0.010													
4 KP784323_LAO	0.000	0.010	0.000												
5 KP784324_LAO	0.000	0.010	0.000	0.000											
6 KP784325_THA	0.010	0.015	0.010	0.010	0.010										
7 KP784326_THA	0.005	0.005	0.005	0.005	0.005	0.010									
8 KP784327_THA	0.010	0.010	0.010	0.010	0.010	0.015	0.005								
9 KP784328_THA	0.005	0.005	0.005	0.005	0.005	0.010	0.000	0.005							
10 KP784329_THA	0.005	0.005	0.005	0.005	0.005	0.010	0.000	0.005	0.000						
11 KP784330_THA	0.010	0.010	0.010	0.010	0.010	0.015	0.005	0.010	0.005	0.005					
12 KP784331_THA	0.005	0.005	0.005	0.005	0.005	0.010	0.000	0.005	0.000	0.000	0.005				
13 AB551552 *G. spinigerum*_JPN	0.010	0.010	0.010	0.010	0.010	0.015	0.005	0.010	0.005	0.005	0.000	0.005			
14 AY501388 *G. spinigerum*_THA	0.005	0.005	0.005	0.005	0.005	0.010	0.000	0.005	0.000	0.000	0.005	0.000	0.005		
15 JN408304 *G. spinigerum*_IDN	0.005	0.005	0.005	0.005	0.005	0.010	0.000	0.005	0.000	0.000	0.005	0.000	0.005	0.000	

Table 2. Tamura-Nei model (Tamura and Nei 1993) genetic distance matrix values based on ITS-2 sequences among 12 isolates of *Gnathostoma spinigerum* Owen, 1836 and related sequences of *G. spinigerum* from the Genbank database.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 KP784332_LAO														
2 KP784333_LAO	0.000													
3 KP784334_LAO	0.000	0.000												
4 KP784335_LAO	0.000	0.000	0.000											
5 KP784336_LAO	0.000	0.000	0.000	0.000										
6 KP784337_THA	0.002	0.002	0.002	0.002	0.002									
7 KP784338_THA	0.000	0.000	0.000	0.000	0.000	0.002								
8 KP784339_THA	0.000	0.000	0.000	0.000	0.000	0.002	0.000							
9 KP784340_THA	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000						
10 KP784341_THA	0.002	0.002	0.002	0.002	0.002	0.003	0.002	0.002	0.002					
11 KP784342_THA	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.002				
12 KP784343_THA	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.002	0.000			
13 AB181155 *G. spinigerum*_THA	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.002	0.000	0.000		
14 KF648533 *G. spinigerum*_Unknown	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.002	0.000	0.000	0.000	

Hasegawa-Kishino-Yano (HKY) + G + I, and for the ITS2 region was Kimura-2-parameter (K2P) + I. The selected dataset and support for clusters in each tree was calculated using 1 000 bootstrap replications. Sequences of other species of *Gnathostoma* from the database were used for the alignment. Genetic distance values among isolates from Thailand and Laos were calculated using Tamura-Nei model (Tamura and Nei 1993) (Tables 1, 2, Fig. 1).

For *cox1* gene sequences, a 250 bp PCR product was amplified from all specimens. After trimming the primer sequences, five (2.4%) variable sites in the 205 bp alignment were analysed. Transitions (T↔C, n = 1; A↔G, n = 4) were more frequently observed than transversions (T↔G, n = 1; T↔A, n = 1) (the nucleotide position 94 in the analysed sequences has both transition and transversion). In the phylogenetic tree inferred from partial *cox1* sequences (Fig. 1A), all isolates from Thailand and Laos formed the same clade with known sequences of isolates of *G. spinigerum* (from Japan, Thailand and Indonesia) in the GenBank database.

For a partial 5.8S, entire ITS-2 and partial 28S regions, a 650 bp PCR product was amplified from all worms. The full-length ITS-2 region (472 bp) of each sample was aligned and two (0.4%) variable sites were detected (transitions: T↔C, n = 1; A↔G, n = 1). In the phylogenetic tree inferred from full-length ITS-2 region (Fig. 1B), all isolates from Thailand and Laos formed the same clade with known sequences of *G. spinigerum* (from undetermined Asian countries in the GenBank database and Thailand). The relationships among 12 isolates of *G. spinigerum* and related sequences were revealed by genetic distance values; 0.005–0.015 for partial *cox1* (Table 1) and 0.002–0.003 for ITS-2 (Table 2). All partial *cox1* and partial 5.8S, entire ITS-2 and partial 28S regions of *G. spinigerum* have been deposited in the GenBank database under accession No. KP784320–KP784331 and KP784332–KP784343, respectively (Fig. 1).

Some groups of people living in Thailand and Laos maintain their traditional life-style of eating local dishes prepared from raw or semi-cooked fish contaminated with infective $AdvL_3$ of species of *Gnathostoma* (see Nawa and Nakamura-Uchiyama 2004). In Laos, $AdvL_3$ of *Gnathostoma* spp. have been found in four species of edible fish and one species of frog (Vonghachack et al. 2010). We here found new molecular evidence of $AdvL_3$ of *G. spinigerum* in a snake, *P. korros*, in Khammouane province, Laos, and confirmed using molecular markers the occurrence of adults of *G. spinigerum* in Thailand.

In Thailand, the country with high prevalence of gnathostomiasis, fish, frogs, reptiles, birds and mammals have been reported to harbour $AdvL_3$ of *G. spinigerum*, wherease dogs were found to be the definitive host of this nematode (Maleewong et al. 1992). A new type of *Gnathostoma* larvae reported by Akahane et al. (1995) and Setasuban et al. (1991) may belong to either *Gnathostoma vietnamicum* Le-Van-Hoa, 1965, *Gnathostoma malaysiae* Miyazaki and Dunn, 1965 or may constitute a new species of the genus *Gnathostoma*. Using partial *cox1* gene and ITS-2 sequences as molecular markers, all samples were identified as *G. spinigerum*. Some intraspecific variation was observed in *cox1* gene sequences from two geographic regions (genetic distance; 0.005–0.015), little or no intraspecific variation was detected in the ITS-2 region (genetic distance; 0.002–0.003).

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