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Molecular identification and genetic diversity of *Gnathostoma spinigerum* larvae in freshwater fishes in southern Lao PDR, Cambodia, and Myanmar

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Abstract

Gnathostomiasis, an emerging food-borne parasitic zoonosis in Asia, is mainly caused by *Gnathostoma spinigerum* (Nematoda: Gnathostomatidae). Consumption of raw meat or freshwater fishes in endemic areas is the major risk factor. Throughout Southeast Asia, including Thailand, Lao PDR, Cambodia, and Myanmar, freshwater fish are often consumed raw or undercooked. The risk of this practice for gnathostomiasis infection in Lao PDR, Cambodia, and Myanmar has never been evaluated. Here, we identified larvae of *Gnathostoma* species contaminating freshwater fishes sold at local markets in these three countries. Public health authorities should advise people living in, or travelling to, these areas to avoid eating raw or undercooked freshwater fishes. Identification of larvae was done using molecular methods: DNA was sequenced from *Gnathostoma* advanced third-stage larvae recovered from snakehead fishes (*Channa striata*) and freshwater swamp eels (*Monopterus albus*). Phylogenetic analysis of a portion of the mitochondrial cytochrome c oxidase subunit I gene showed that the *G. spinigerum* sequences recovered from southern Lao PDR, Cambodia, and Myanmar samples had high similarity to those of *G. spinigerum* from China. Sequences of the nuclear ribosomal DNA internal transcribed spacer 2 region closely resembled sequences of *G. spinigerum* from Thailand, Indonesia, the USA, and central Lao PDR. This is the first molecular evidence of *G. spinigerum* from freshwater fishes in southern Lao PDR, Cambodia, and Myanmar.

Keywords *Gnathostoma spinigerum* · Foodborne nematode · Molecular evidence · Genetic diversity · Freshwater fishes · Asian countries

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Introduction

Human gnathostomiasis is an emerging harmful food-borne parasitic zoonosis caused by *Gnathostoma* spp. Humans are accidental hosts for these nematodes (other mammals are normal definitive hosts) and acquire infection by consuming raw or undercooked meat harboring *Gnathostoma* advanced third-stage larvae (AL3). The infective larva develops incompletely in the human body but remains an AL3 or may develop to a fourth-stage larva or immature adult (Daengsvang 1981). *Gnathostoma* species need two intermediate hosts: the first is a copepod and the second can be a freshwater fishes (i.e., swamp eel, snakehead fish, loach), frog, snake, or bird (Daengsvang 1980; Nawa et al. 2015). The disease is reported around the world, for example, in Asia (China, Indonesia,

Japan, Korea, Malaysia, Myanmar, Philippines, and Thailand), Latin America (specifically Mexico, Peru, and Brazil), and the USA (Hale et al. 2003; Moore et al. 2003; Herman and Chiodini 2009; Diaz 2015; Chaves et al. 2016). *Gnathostoma spinigerum* is the main causative species in Asia, while *Gnathostoma binucleatum* is the only species proven to cause human gnathostomiasis in the USA (Nawa et al. 2015). There is traditional culture of consumption of raw or undercooked fishes in Asian countries. Among the prepared dishes are sushi and sashimi in Japan (Nawa et al. 2005); koi-pla, pla-ra, pla-som, and som-fak in Thailand (Grundy et al. 2012); koi-ga in Vietnam; and kinilaw, sabaw, and sukba in the Philippines (Waikagul and Diaz-Camacho 2007). Formerly occurring only in endemic areas where risky dietary practices persisted, gnathostomiasis (and other foodborne zoonoses) may now be found in any part of the world. This is made possible by the rapid and easy transportation of chilled fish around the world, and because cheap travel allows tourists to sample local diets far from their homelands (Eiras et al. 2018). On returning home, such tourists may present their medical practitioners with an unfamiliar set of symptoms, delaying correct diagnosis (Eiras et al. 2018).

The AL3 stages of *Gnathostoma* species can be differentiated by size, number of hooklets in each row on the head-bulb, shape and distribution of cuticular spines, the shape of, and number of nuclei in the intestinal epithelial cells (Nawa et al. 2015). However, highly experienced personnel are required to make a species determination. We therefore employed molecular tools for species identification of the *Gnathostoma* larvae in freshwater fishes in Lao PDR, Cambodia, and Myanmar. Awareness of gnathostomiasis is limited in these countries, both in the general population and among public health authorities: we wished to sound a warning on the risks of eating undercooked freshwater fishes. In humans, the larvae wander randomly in the body, causing mechanical damage to tissues and chemical damage by toxic substances released from the worms. The clinical manifestations of gnathostomiasis fall into two categories. The first is cutaneous larva migrans: the worm migrates into the subcutaneous tissues causing intermittent painful and pruritic migratory swelling. The second is visceral larva migrans: the wandering worm can be found in almost any visceral organs (Rusnak and Lucey 1993), the eyes (Funata et al. 1993; Nawa et al. 2010), the lungs (Intapan et al. 2008), and the central nervous system (CNS) (Schmutzhard et al. 1988; Katchanov et al. 2011). The presence of larvae in the CNS is particularly dangerous, sometimes resulting in death.

Previously, the identification of *G. spinigerum* AL3 was confirmed by sequence analysis of part of the mitochondrial cytochrome c oxidase subunit I (COI) gene and of the nuclear ribosomal region encompassing a portion of the 5.8S gene, ITS-2, and the 5′ end of the 28S gene. This molecular approach has been used to determine the identity of *G. spinigerum* in Thailand and Lao PDR (Ngarmamonpirat et al. 2005; Jongthawin et al. 2015, 2016). The spontaneous emergence of an adult *G. spinigerum* identified morphologically and molecular method from a Lao PDR patient was reported (Phetsouvanh et al. 2018).

Here, we obtained such molecular data from *Gnathostoma* larvae recovered from freshwater fishes bought from local food markets in southern Lao PDR, Cambodia, and Myanmar. We used COI and ITS2 sequences to identify and to evaluate the phylogenetic relationships of the recovered species. The results will provide a better understanding of the molecular epidemiology and a better means for identification of *G. spinigerum* in this region. We placed our data in a global context using sequences of those *G. spinigerum* that were publicly available in the GenBank database.

Materials and methods

Gnathostoma worms

The *Gnathostoma* AL3 samples were recovered from the liver of a swamp eel (*Monopterus albus*) bought from a local market in Champasak Province, Lao PDR; from swamp eel livers in Siem Reap Province, Cambodia; from the livers of swamp eels and snakehead fish (*Channa striata*) in Naypyidaw region, Myanmar; and from swamp eel livers in Yangon region and Bago region, Myanmar (Table 1; Fig. 1). The collection sites were located in a modified map (Central Intelligence Agency, US 2018). All fish were identified using information in Fish Base online (http://www.fishbase.org/) (Accessed Jan 23, 2018). Worms were isolated using a dissecting microscope and

Table 1 Collection sites, hosts, and molecular identification of *Gnathostoma* larvae found

Sample codes	Number of larvae found*	Host	Locations
Gs1	1	*Monopterus albus*	Champasak Province, Lao PDR
Gs2–Gs11	10	*Monopterus albus*	Siem Reap Province, Cambodia
Gs12–Gs16	5	*Monopterus albus*	Naypyidaw Region, Myanmar
Gs17	1	*Channa striata*	Naypyidaw Region, Myanmar
Gs18–Gs22	5	*Monopterus albus*	Yangon Region, Myanmar
Gs23	1	*Monopterus albus*	Bago Region, Myanmar

*All larvae were subsequently identified as *Gnathostoma spinigerum*

Fig. 1 Map showing collection sites of *Gnathostoma spinigerum* from Lao PDR, Cambodia, and Myanmar. This map was modified from a map in the World Factbook, published by the Central Intelligence Agency (Central Intelligence Agency, US 2018) (https://www.cia.gov/library/publications/the-world-factbook/geos/th.html.) Stars represent locations for sample collections (n = number of individual specimens)

Table 2 The specific primers used in this study

Primer	Sequence(5′-3′)	Size (bp)	Reference
COI gene			
GsCox1-1F	5′-ATTTGGTCTTTGGTCAGGC-3′	792	In this study
GsCox1-1R	5′-CACACAACCAATCAAACCAATC-3′		
Gn_COI-F	5′-GCCTGCTTTTGGAATTGTTAG-3′	250	Jongthawin et al. (2015)
Gn_COI-R	5′-ACGAAAACCATACAAAGTAGCCAA-3′		
GsCox1-2F	5′-GCTGCTACTATGGTGATTGCTG-3′	674	In this study
GsCox1-2R	5′-CTGCAATCAATCATACTCAACTTA-3′		
Partial 5.8S, entire ITS-2, and partial 28S regions			
GS_ITS2-F	5′-TGTGTCGATGAAGAACGCAG-3′	650	Jongthawin et al. (2015)
GS_ITS2-R	5′-TTCTATGCTTAAATTCAGGGG-3′		

stored and transported at 4 °C in normal saline (0.85% NaCl in distilled water). The larvae were carried to the Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand, where they were transferred to RNA*later* (Qiagen, Hilden, Germany) and stored at − 70 °C until used.

DNA amplification and sequencing

Genomic DNA was extracted from individual *Gnathostoma* AL3 using NucleoSpin® tissue kit (Macherey-Nagel GmbH & Co., Germany) according to the manufacturer's instructions. The DNA samples were amplified by using the SimpliAmp™ Thermal Cycler (Applied Biosystems®, Singapore). The primers are listed in Table 2. The reaction was carried out in a 25-μL volume containing 2.5 μL of 10 × high fidelity PCR buffer, 1.8 mM $MgCl_2$, 0.5 μL of dNTP mix (10 mM), 0.5 μL of each primer (10 μM), 0.125 U of Taq DNA FS high fidelity PCR system, and 5 μL of DNA template from individual worms. For COI or ITS2 sequences, reaction mixture was pre-incubated for 2 min at 94 °C, followed by 10 cycles of 94 °C for 1 min (denaturation), 40 °C for 1 min (annealing), 68 °C for 2 min (extension), then 30 cycles of 94 °C for 1 min (denaturation), 45 °C for 1 min (annealing), 68 °C for 2 min (extension), and final extension at 68 °C for 7 min. Amplicons

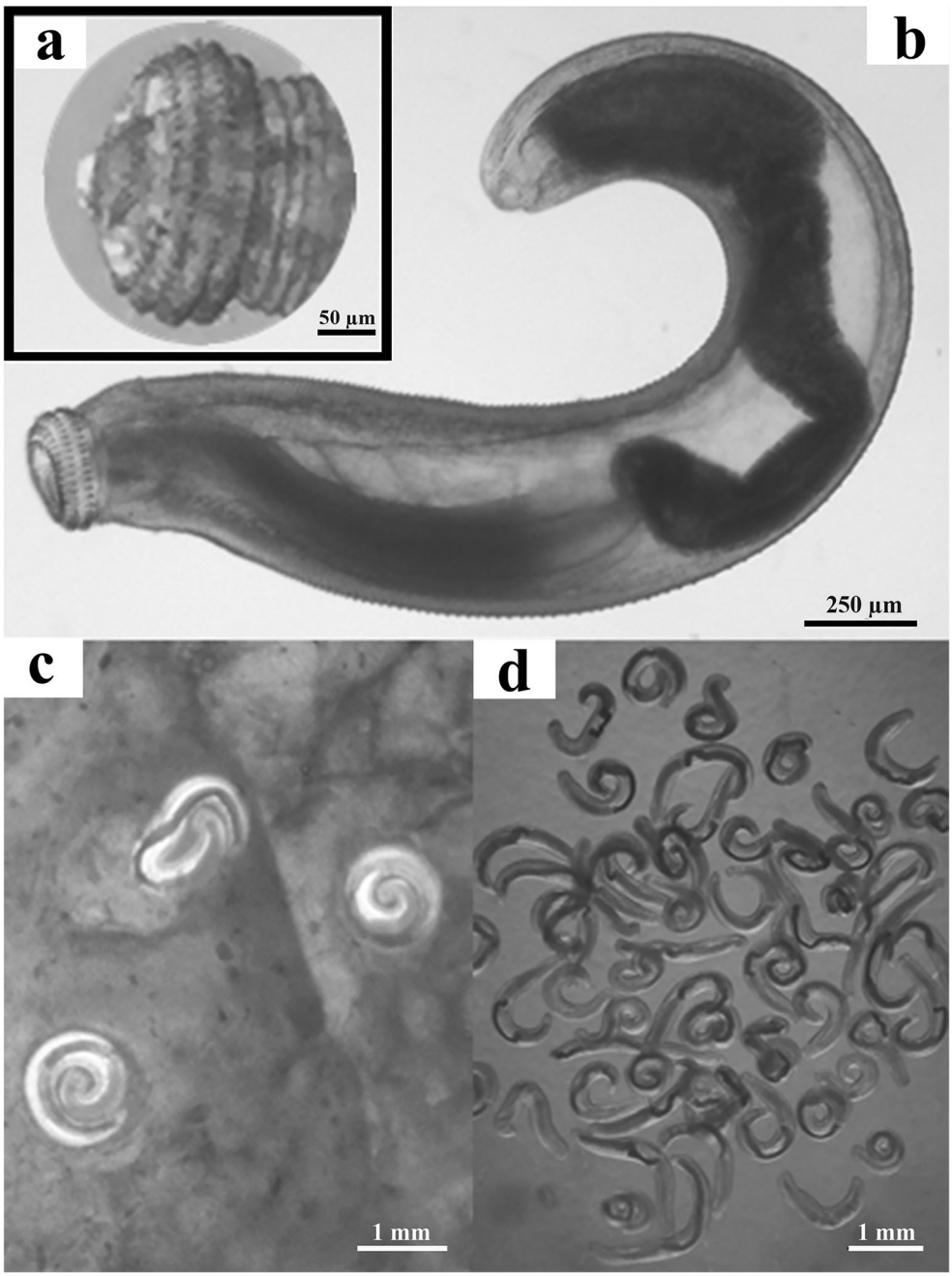

Fig. 2 Advanced third-stage larva (AL3) of *Gnathostoma spinigerum*, from the liver of a swamp eel from Champasak Province, Lao PDR (**a** (head) and **b** (whole body)). Encysted AL3 within the liver of a swamp eel purchased in Siem Reap Province, Cambodia (**c**) and AL3 recovered from swamp eel liver from Naypyidaw Region, Myanmar (**d**)

were resolved with electrophoresis in a 1% (*w*/*v*) agarose gel. DNA direct sequencing of amplified products was done using the Applied Biosystems 3730×I DNA Analyzer and ABI Big Dye Version 3.1 (Applied Biosystems, Foster City, CA) in both directions, using the PCR primers as sequencing primers.

Sequence alignment and phylogenetic analysis

The partial sequences of the COI gene (1470 bp) and the partial 5.8S, entire ITS2, and partial 28S regions (605 bp—here after abbreviated as ITS2) were aligned and compared with *Gnathostoma* sequences in the GenBank database using the multiple sequence alignment program ClustalW within BioEdit (Hall 1999). A phylogenetic tree was done using the Bayesian Information Criterion (BIC) in the program MrBayes v.3.2 (Ronquist et al. 2012). COI and ITS2 datasets were chosen using the Bayesian Information Criterion (BIC) in MEGA7 software: the lowest BIC score is considered to best describe the substitution pattern (Kumar et al. 2016). COI alignment, the Tamura-Nei model (TN93) with non-uniformity of evolutionary rates among sites using a distinct Gamma distribution (+G), and ITS2 alignment Kimura 2-

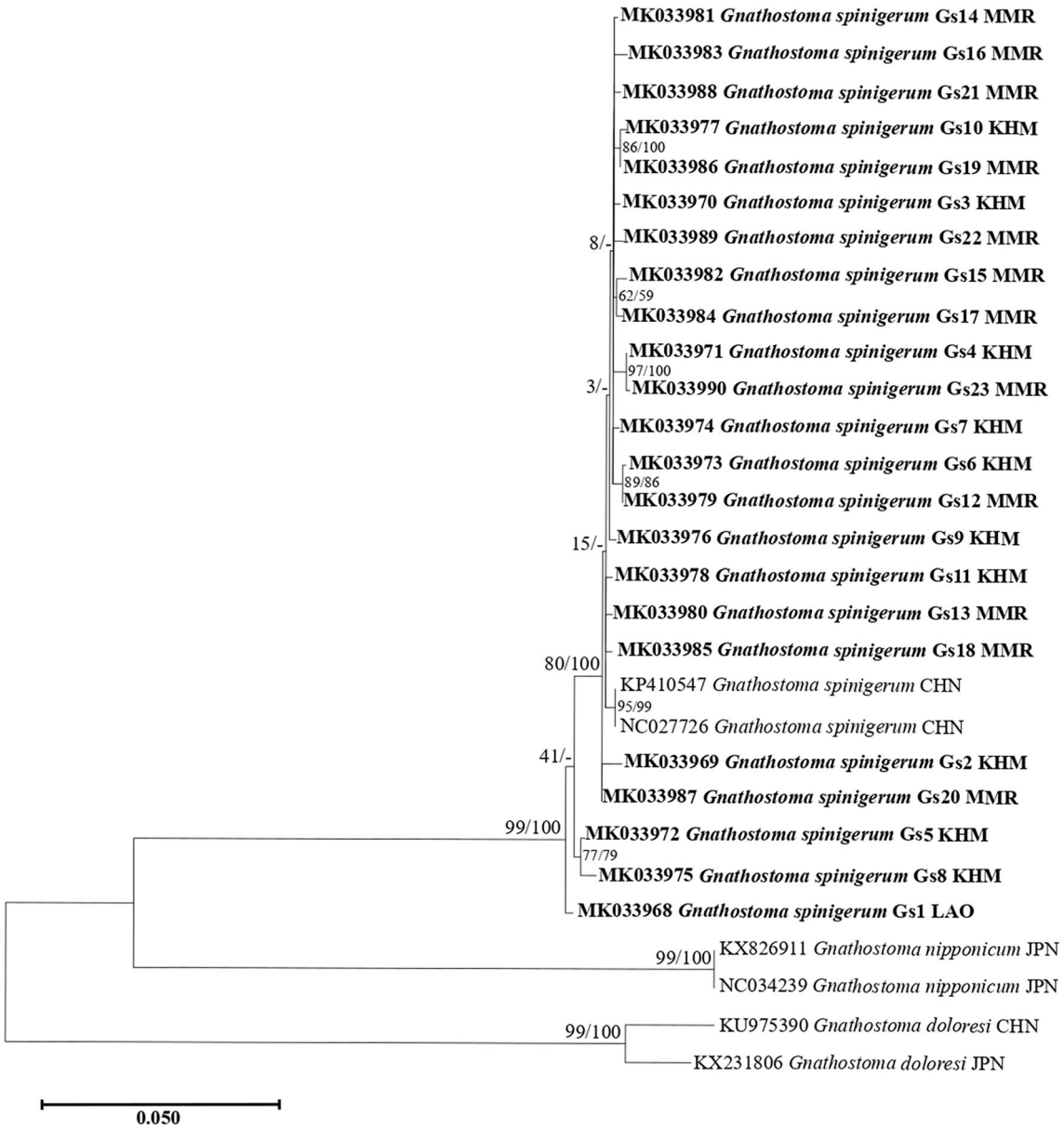

Fig. 3 Phylogenetic relationships inferred from an alignment of COI gene sequences using maximum likelihood. Sequences of *Gnathostoma* species obtained from GenBank are indicated with the accession number and the country code (ISO 3166-1 alpha-3 code). Support values (ML bootstrap (percentages of 1000 replicates)/Bayesian posterior probabilities) are shown above the branches. A dash instead of a numerical support value indicates that a particular grouping was not found by that method of analysis. Sequences obtained in the present study are highlighted in bold. Scale bars indicate substitutions per nucleotide position

parameter model (K2) with non-uniformity of evolutionary rates among sites using a distinct Gamma distribution (+G) were selected. The COI analysis was run for 210,000 generations (2 runs, each of four chains), by which time the standard deviation of split frequencies had fallen below 0.01 and sampled every 1000 generations. Examination of the output (using the "sump" command) indicated that the potential scale reduction factor was 1 for relevant parameters and that stationarity had been approached after 25% of generations. For ITS2, the best model was the K2+G model allowing for a proportion of invariable sites. Following the approach outlined above for the COI alignment, the analysis was run for 500,000 generations and the first 25% of trees discarded as burnin. Maximum likelihood (ML) method was implemented in MEGA7 (Kumar et al. 2016). Bootstrap percentage was estimated by 1000 replications.

Results

Under microscopic examination (Supplementary file 1), *Gnathostoma*-like larvae were morphologically identified as *G. spinigerum* AL3 based on criteria previously reported by Daengsvang (1980). The sizes (width × length) of AL3 were 0.35 × 2.59 mm ($n = 1$, from Lao PDR), 0.28 ± 0.03 × 2.02 ± 0.14 ($n = 10$, from Cambodia), and 0.27 ± 0.04 × 2.06 ± 0.15 ($n = 12$, from Myanmar). The head bulb of the *G. spinigerum* larva has four rows of hooklets (Fig. 2). There is a marked cephalic constriction between head bulb and body. The whole body cuticle is covered with single spines.

DNA sequences were successfully obtained from amplified products ($n = 23$; Champasak Province, Lao PDR, $n = 1$; Siem Reap Province, Cambodia, $n = 10$; Naypyidaw City, Myanmar, $n = 6$; Yangon City, Myanmar, $n = 5$; Bago City, Myanmar, $n = 1$). BLAST search results indicated that the sequences were from *G. spinigerum*. For all partial mitochondrial COI gene sequences obtained, the sequences were 99% identical (100% coverage) with *G. spinigerum* from China (GenBank accession nos. KP410547 and NC027726) (Fig. 3). For the ITS2 region, all sequences were 99–100% similar (100% coverage) with *G. spinigerum* from Thailand, Indonesia, the USA, and central Lao PDR (GenBank accession nos. AB181155, JN408321, KF648534, and KP784332) (Fig. 4).

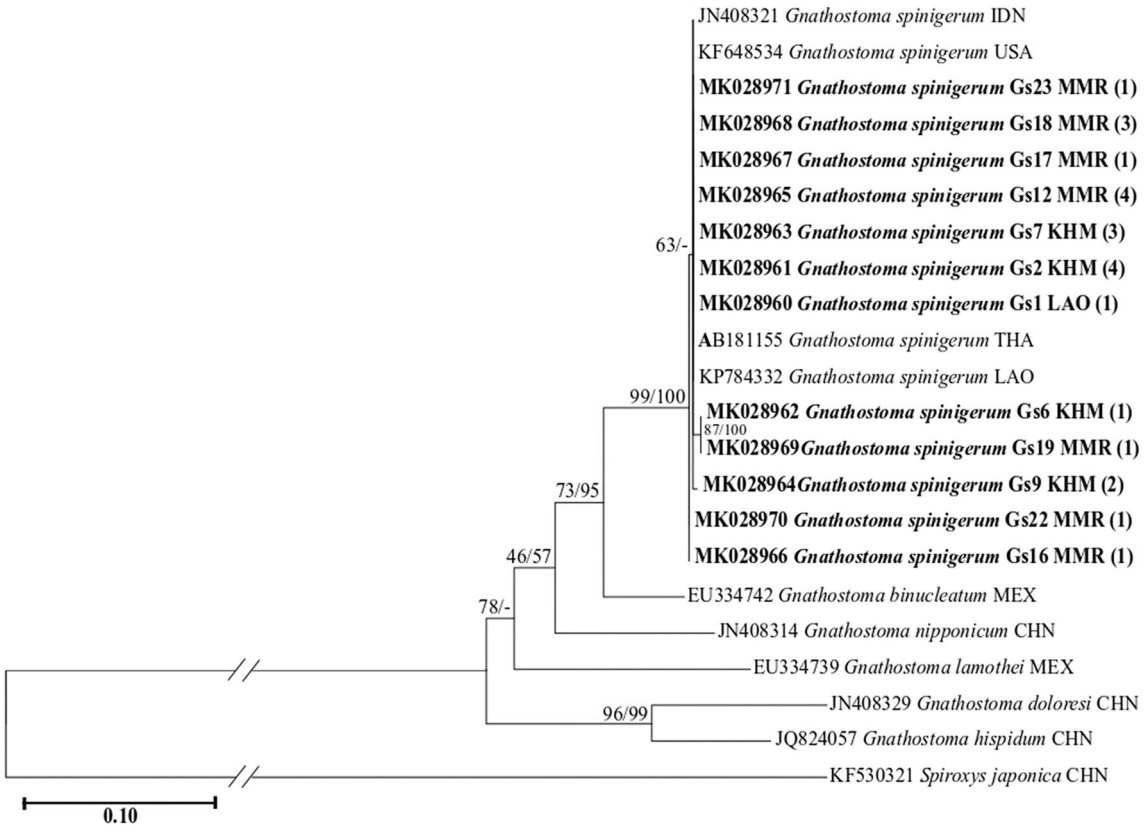

Fig. 4 Phylogenetic relationships inferred from ITS2 sequences using maximum likelihood. The sequences of *Gnathostoma* species obtained from GenBank are indicated with the accession number and the country code (ISO 3166-1 alpha-3 code). Support values (ML bootstrap (percentages of 1000 replicates)/Bayesian posterior probabilities) are shown above the branches. A dash instead of a numerical support value indicates that a particular grouping was not found by that method of analysis. Sequences obtained in the present study are highlighted in bold. Scale bars indicate substitutions per nucleotide position. Numbers in parentheses indicate larvae number revealed identical sequences

Twenty-three partial COI and ITS2 sequences were used for phylogenetic analyses (GenBank accession nos. MK033968-MK033990 for COI and MK028960-MK028971 for ITS2). The phylogenetic tree showed that *G. spinigerum* sequences from the present study have high similarity to those of *G. spinigerum* from GenBank when the COI gene (1470 bp) and ITS2 region (605 bp) were used as DNA markers. COI sequences of *G. spinigerum* clustered in a group with high bootstrap support (ML/BI 99%/100%) shown in Fig. 3. Analysis of ITS2 sequences of *G. spinigerum* similarly placed our sequences in a well-supported clade (ML/BI 99%/100%) with other sequences (Fig. 4).

Discussion

G. spinigerum is widely distributed in tropical and subtropical areas. People in rural areas in the Greater Mekong countries including Thailand, Lao PDR, Myanmar, Cambodia, Vietnam, and China (Yunnan Province and Guangxi Zhuang Autonomous Region) like to consume raw or undercooked dishes potentially harboring *Gnathostoma* AL3 (Nawa et al. 2005). The first clear report of gnathostomiasis acquired in Myanmar is that by Nomura et al. (2000). In that instance, infection was acquired by two Japanese men visiting or working in Myanmar. Later, a mass outbreak of gnathostomiasis among Korean immigrants who consumed raw freshwater fish in Myanmar was reported (Chai et al. 2003). Recently, at least ten gnathostomiasis cases from Myanmar have been reviewed without species identification (Wai et al. 2018). In Cambodia, one gnathostomiasis case with removed *G. spinigerum* worm was reported (Hem et al. 2015). In Lao PDR, 29.8% of randomly selected participant sera were sero-positive for gnathostomiasis using the immunoblot technique (Vonghachack et al. 2010). Edible fish, frogs, and snakes were reported as the second intermediate hosts for *Gnathostoma* in Lao PDR and Myanmar (Jung et al. 2008; Vonghachack et al. 2010; Chai et al. 2015; Jongthawin et al. 2016). Our molecular identification of *G. spinigerum* in snakehead fish and swamp eel should provide a warning to people living in, or travelling to, these areas about the dangers of consuming raw or undercooked freshwater fishes. We note that fish is a major protein source for the local people and that major changes in cultural practices might be required. On the basis of our literature search, this is the first molecular evidence of *G. spinigerum* in freshwater fishes in southern Lao PDR, Cambodia, and Myanmar. There is little intraspecific variation among the available COI nucleotide sequences of *G. spinigerum*, all from Thailand, China and the countries investigated here: this gene region is certainly able to discriminate *G. spinigerum* from other members of the genus. The present result supports previous findings (Jongthawin et al. 2016; Eamsobhana et al. 2017), based on COI sequences, that *G. spinigerum* recovered in Thailand and central Lao PDR is closely related. The ITS2 sequences revealed distinct differences among *G. spinigerum*, *G. binucleatum*, *Gnathostoma doloresi*, *Gnathostoma hispidum*, *Gnathostoma lamothei*, and *Gnathostoma nipponicum* as differentiated previously (Jongthawin et al. 2016), but minimal or no differences within each species. Consequently, this conserved region could be a useful genetic marker for the specific identification of *Gnathostoma* species.

Conclusions

The present results combined with previous studies indicate that *G. spinigerum* is the major species widespread in second intermediate hosts in Thailand, Lao PDR, Cambodia, and Myanmar. People in these areas should beware of consuming raw or undercooked meat potentially contaminated with the infective stage of the harmful gnathostoma worm. Prevention and control of this dangerous foodborne disease should be promoted by policy makers and public health personnel especially in travelers who visit in these areas. Local people and travelers who visit these areas should be made aware of the dangers of eating uncooked fish.

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Compliance with ethical standards

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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