

RESEARCH NOTE

MOLECULAR EVIDENCE OF *SPIROMETRA ERINACEIEUROPAEI* INFECTION IN SNAKES *PTYAS KORROS* FROM LAO PDR AND THAILAND AND FROGS *HOPLOBATRACHUS RUGULOSUS* FROM MYANMAR

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Abstract. Sparganosis is a parasitic disease in humans and animals caused by plerocercoid larvae (spargana) of the genus *Spirometra*. *Spirometra erinaceieuropaei* is the major causative agent of the disease in Asian countries. However, molecular evidence of the causative parasite species in animals remains lacking. A total of 19 spargana specimens were obtained from frogs, *Hoplobatrachus rugulosus*, collected from Myanmar and snakes, *Ptyas korros*, from Lao PDR and Thailand. A partial sequence of mitochondrial cytochrome c oxidase subunit1 gene (*cox1*) was amplified, sequenced, and the phylogenetic relationship was constructed using maximum likelihood method. Results revealed that the level of nucleotide variations in the partial *cox1* sequence (429 bp) among the spargana ranged 0-3.5%, with 15 variable sites. Phylogenetic analysis indicated that all spargana specimens were *S. erinaceieuropaei*. This is the first report of *S. erinaceieuropaei* in *P. korros* from Lao PDR and Thailand and *H. rugulosus* from Myanmar. The results emphasize the need for prevention and control of sparganosis in these regions.

Keywords: *Spirometra erinaceieuropaei*, *Hoplobatrachus rugulosus*, *Ptyas korros*, cytochrome c oxidase subunit1, spargana, sparganosis

INTRODUCTION

Sparganosis is a parasitic disease in humans and animals caused by plero-

cercoid larvae (sparganum /pl. spargana) of several diphylobothroid tapeworms belonging to the genus *Spirometra*, including *S. erinaceieuropaei*, *S. ranarum*, *S. mansonoides* and the *Sparganum proliferum* (Gray *et al*, 1999; Zhu *et al*, 2002; Lescano and Zunt, 2013). The most important causative agents are *S. mansonoides* and *S. erinaceieuropaei*, which are distributed

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in North American and Asian countries, respectively (Anantaphruti *et al*, 2011). *Spirometra* requires two intermediate hosts, the first being the copepods and the second comprising frogs, snakes or other reptiles (Lescano and Zunt, 2013). Carnivores such as dogs and cats serve as the final hosts, in which the larvae develop into adults in their small intestines (Kim *et al*, 2009; Li *et al*, 2011). Humans become infected by drinking untreated water containing infected copepods, consuming insufficiently cooked meat of the second intermediate hosts or paratenic hosts, or using such meat as poultices for medicinal or ritualistic purposes (Ooi *et al*, 2000).

Although human sparganosis is sporadically distributed around the world, it is most frequently reported in East and Southeast Asia (Cho *et al*, 1975; Qiu and Qiu, 2009; Anantaphruti *et al*, 2011). In Thailand, over 60 sparganosis cases have been reported since 1943, and the patients were almost exclusively infected with *S. erinaceieuropaei*, with only few cases of *S. proliferum* infections (Anantaphruti *et al*, 2011; Boonyasiri *et al*, 2014). There have been several reports of molecular evidences for the detection of spargana infection in Thai patients (Koonmee *et al*, 2011; Boonyasiri *et al*, 2013; 2014). However, molecular evidence of the parasite species in animals in Thailand or adjacent areas remains lacking.

The aim of the present study is to provide molecular evidences of spargana collected from animals in Thailand, Lao PDR and Myanmar, and to compare the genetic variations among the isolates obtained from geographically different areas. These results may contribute to identify the sources of infection, which provide important implication for prevention and control of sparganosis in these areas. For this purpose, we analyzed partial

sequences of mitochondrial cytochrome c oxidase 1 gene (*cox1*) of spargana isolates from frogs, *Hoplobatrachus rugulosus* and snakes, *Ptyas korros* from Lao PDR, Myanmar and Thailand. A phylogenetic tree was constructed to ascertain the species and genetic relationships among the spargana samples.

MATERIALS AND METHODS

Parasite samples

This study was conducted from November 2013 to June 2014. A total of 19 spargana specimens were obtained from snakes (*P. korros*, commonly known as Chinese rat snake or Indo-Chinese rat snake) and frogs (*H. rugulosus*; syn: *Rana rugulosa* Wiegmann or *R. tigrina* Steindachner, commonly known as Chinese edible frog, East Asian bullfrog or Taiwanese frog). Two dead snakes were obtained from two food markets in Kuchi Narai District, Kalasin Province, northeastern Thailand; three dead snakes from three food markets located in the central part of Lao PDR (Nabo and Luksong markets in Khammouane Province and a market in Vientiane capital city); and three frogs from a local market in Mong La, Shan State, western Myanmar (Fig 1A). The presence of spargana was determined according to the method of Ooi *et al* (2000). In brief, muscles of snakes and frogs were exhaustively examined for the presence of spargana by naked eye, followed by examination under a stereomicroscope (4x magnification). Then, the spargana specimens were carefully removed and placed in a Petri dish containing 0.85% saline solution to observe their viability and movement. The parasites were verified microscopically and identified as spargana of the genus *Spirometra* according to their morphological features

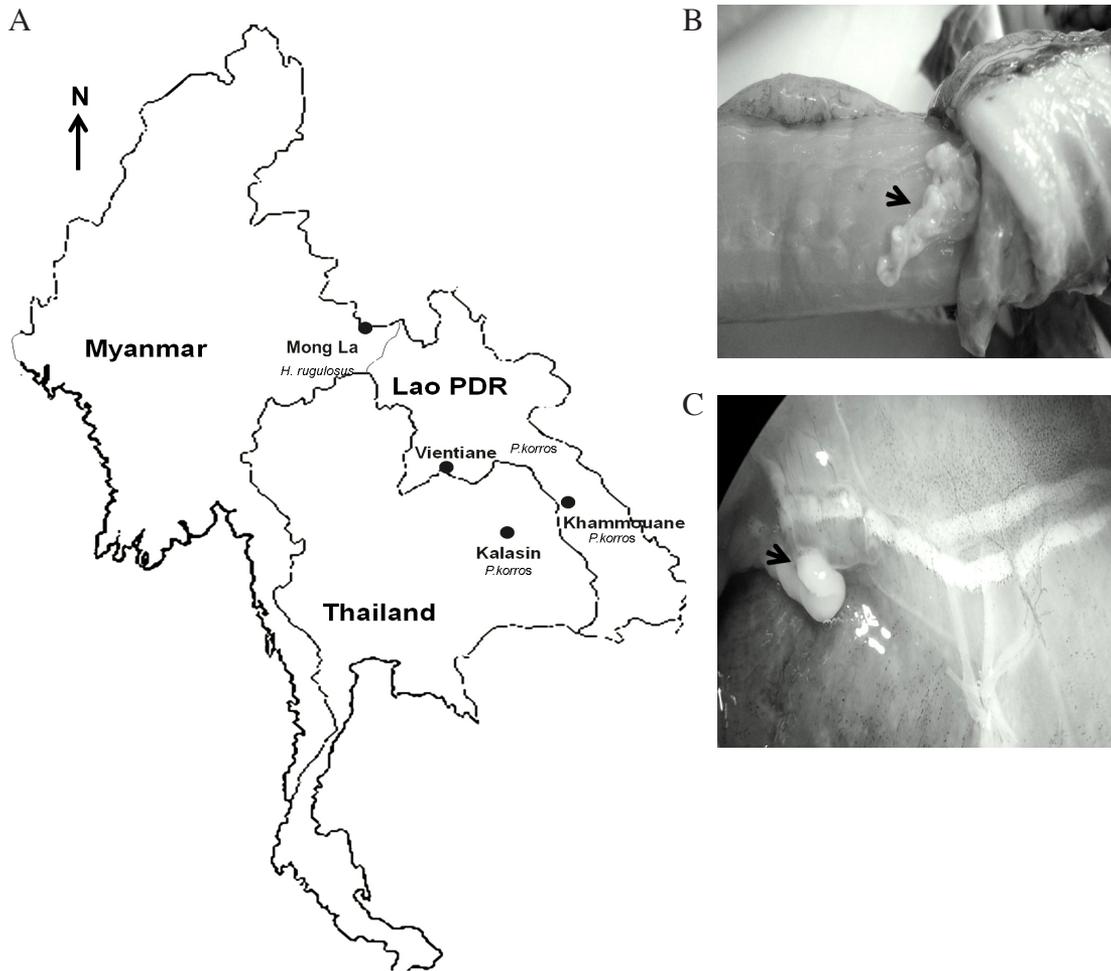


Fig 1—Geographical localities of samples collected in this study (A), and location of sparganum underneath the skin (subcutaneous connective tissue) of snake (*P. korros*) (B) and frog (*H. rugulosus*) (C). Arrow indicates sparganum.

(Mueller, 1974). They were stored in 75% ethanol and kept at -80°C until used for DNA extraction.

PCR amplification, DNA sequencing and sequence analysis

DNA was extracted directly from individual sparganum using a commercial DNA extraction kit (NucleoSpin[®] Tissue, Macherey-Nagel, Germany) according to the manufacturer's instructions. A partial sequence of *cox1* was amplified using the primers Se658-F (5'-TTT GAT CCT TTG

GGT GGT GG-3') and Se1124-R (5'-ACC ACA AAC CAC GTG TCA TG-3'), which were designed from the *cox1* gene of *S. erinaceieuropaei* (GenBank accession no. AB369250) (Boonyasiri *et al*, 2013). PCR was performed in a 25 μl of reaction volume containing 10 ng of DNA, 2.5 μl of 10X FastStart High Fidelity Reaction buffer (Roche, Mannheim, Germany), 18 mM MgCl_2 , 200 μM dNTPs, 0.2 μM each primer (Invitrogen, Carlsbad, CA), and 0.625 U FastStart High Fidelity Enzyme Blend (Roche). Thermocycling condi-

tions (conducted in GeneAmp PCR System 9700, Applied Biosystems, Singapore) were as follows: 94°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 45 seconds; with a final step at 72°C for 10 minutes. For each PCR experiment, a negative (no DNA) and a positive control (known *S. erinaceiropaei* DNA confirmed by DNA sequencing) were included. Amplicons were separated by 1% agarose gel-electrophoresis and 467 bp fragments were excised and sequenced using the Applied Biosystems 3730×I DNA Analyzer and ABI big dye Version 3.1 (Foster City, CA). DNA sequencing was conducted at First BASE Laboratories Sdn Bhd (Selangor, Malaysia).

The partial *cox1* sequences from individual *S. erinaceiropaei* specimens were analyzed using BLAST-N search (National Center for Biotechnology Information, Bethesda, MD). *S. erinaceiropaei cox1* sequences from NCBI database were aligned with our sequences (429 bp after trimming to adjust to the length of the shortest sequence) using Bioedit (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) (Hall, 1999). Phylogenetic relationship was analyzed by MEGA 6 using the ML method (Tamura *et al*, 2013). The HYK+G+I model with its parameters being selected for concatenated dataset and bootstrap support for ML tree were calculated using 1000 bootstrap replications.

RESULTS

All spargana specimens ($n = 19$) were observed underneath the skin of *P. korros* (Fig 1B) and *H. rugulosus* (Fig 1C). Two specimens were recovered from *P. korros* obtained at food markers in Kuchi Narai District, Kalasin Province, Thailand; 3 and 13 from *P. korros* at food markers in Vientiane capital city and Khummouane

Province, Lao PDR, respectively; and 1 specimen from *H. rugulosus* at a local market in Mong La, Shan State, Myanmar.

A 467 bp amplicon of *cox1* was sequenced from all 19 individual spargana. The level of nucleotide variation in *cox1* sequences among spargana isolates were 0-3.5%, with 15 variable sites in the 429 bp analyzed sequences [15/429, (3.5%) nucleotides] representing 4 groups (Fig 2). The intra-specific nucleotide variations included transitions (T↔C, $n = 11$; A↔G, $n = 2$) and transversion (T↔G, $n = 2$). To verify the spargana species, phylogenetic relationships of tapeworms in the Diphylobothriidae family were re-constructed using ML method, and the sequence of *Taenia solium* (GenBank no. AY211880) was used as an out group (Fig 3). The results revealed that all partial *cox1* sequences of spargana obtained in this study were located in *S. erinaceiropaei* clade from various geographical localities including China, Japan and Thailand, with 98%-99% similarity (BLAST-N search), confirming that the spargana specimens obtained from Lao PDR, Myanmar and Thailand were *S. erinaceiropaei*. All *S. erinaceiropaei* new partial *cox1* sequences were deposited in GenBank database (Fig 3).

DISCUSSION

Spargana of *S. erinaceiropaei* are known to infect many vertebrate species (amphibians, reptiles and mammals), but the most common intermediate/paratenic hosts are snakes and frogs. *S. erinaceiropaei* spargana are found in a variety of snake species, including *Elaphe radiata*, *E. taeniura*, *E. carinata*, *E. schrenckii*, *E. dione*, *E. rufodorsata*, *E. quadrivirgata*, *Rhabdophis tigrinus tigrinus*, *Natrix tigrina lateralis*, *Dinodon rufozonatum*, *Zamenis spinalis*, *Agkistrodon halys*, *Ptyas mucosus*, *P. korros*, *Naja*

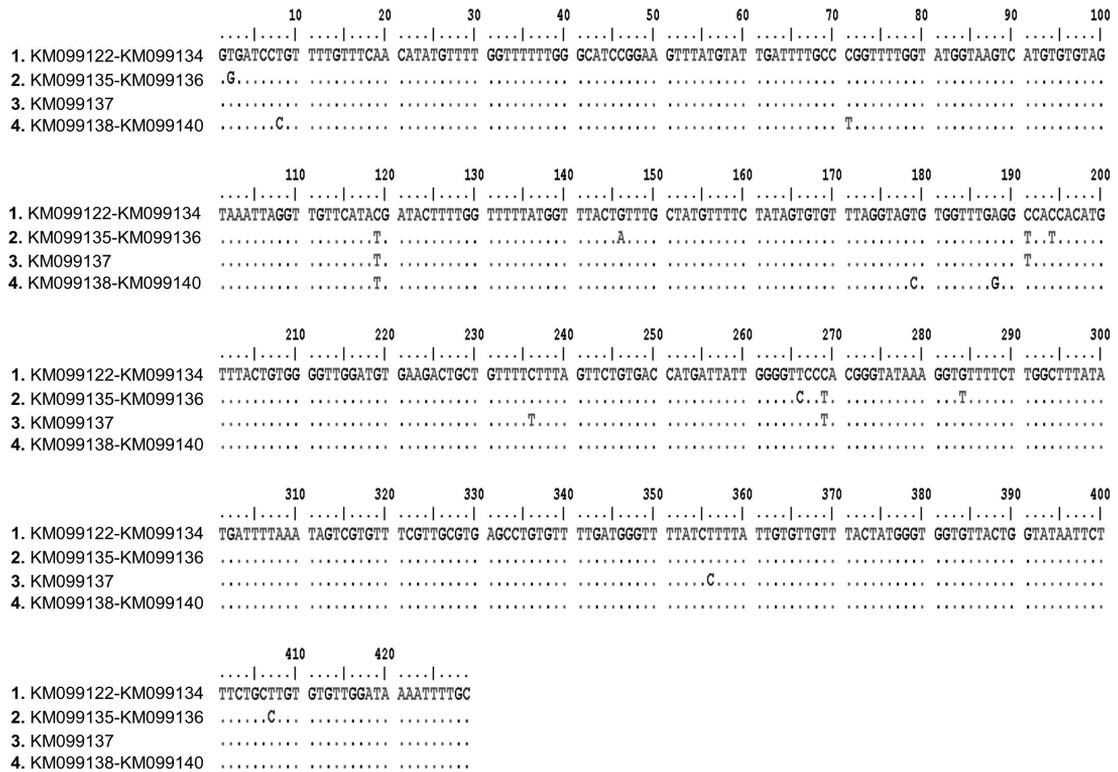


Fig 2—Four levels of nucleotide variations in the partial *cox1* sequences among spargana isolates. There are 15/429 (3.5%) nucleotides, with 11 T<-> C and 2 A<-> G transitions and 2 T<-> G transversions.

naja and *Zoacys dhumnades* (Cho *et al*, 1973; Sato *et al*, 1992; Wang *et al*, 2011). Infection rates >90% have been reported in *E. quadrivirgata* and *Rhabdophis tigrinus tigrinus* from Japan (Sato *et al*, 1992). Spargana of *S. erinaceieuropaei* have also been found in many species of wild frogs, including *Rana nigromaculata*, *R. limnocharis*, *R. temporaria* and *Bufo gargarizans* (Liu *et al*, 2010; Wei *et al*, 2014). In a survey of Australian amphibians, *S. erinaceieuropaei* also was found in a variety of tree frogs, including *Litoria caerulea*, *L. aurea*, *L. gracilentata*, and *L. peronii* (Berger *et al*, 2009).

Previous survey in several East Asian countries including Korea, China and Japan, where sparganosis is prevalent among humans, have revealed that there

still exists traditional habit of eating raw frogs or snakes meat or using such meat for medicinal reasons. In Thailand, sparganosis has been sporadically reported all around the country with the highest number of cases in the northeastern part of the country, where there is a tradition of eating food prepared from raw or semi-cooked frogs or snake meat (Anantaphruti *et al*, 2011). In addition, a number of patients have a history of applying frog flesh as a poultice for sore eyes (Anantaphruti *et al*, 2011; Boonyasiri *et al*, 2014). Although Lao PDR and Myanmar are the neighboring countries to Thailand and have similar climate and traditional lifestyle, little is known about spargana infection either in humans or animals.

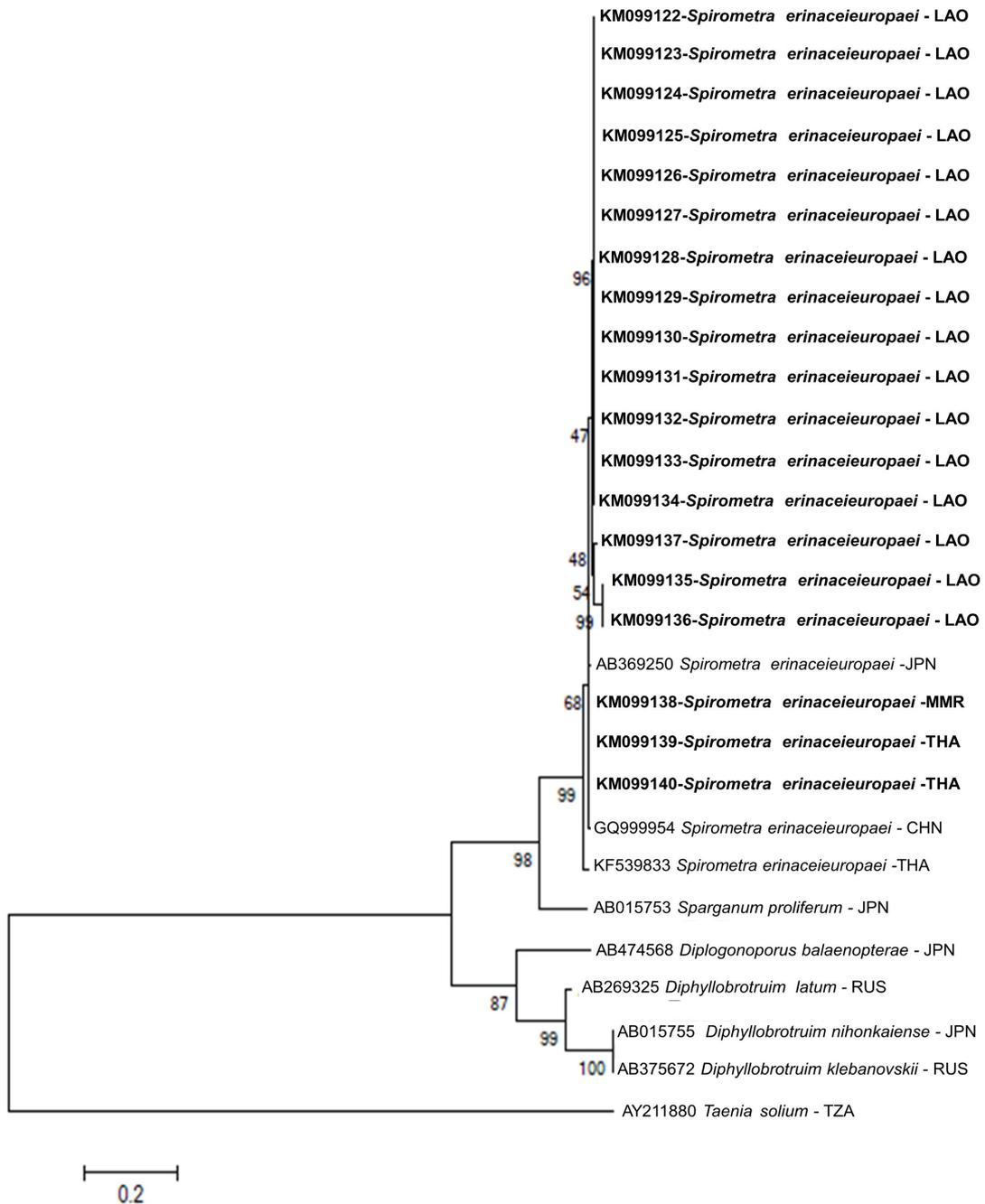


Fig 3–Phylogenetic tree of tapeworms in the Diphyllobothriidae family. The tree was re-constructed using maximum likelihood method based on partial *cox1* sequences. *Spirometra* species and other tapeworms obtained from GenBank are indicated with accession number and country code (ISO 3166-1 alpha-3 code). *Spirometra erinaceieuropaei* sequences obtained from this study are presented in bold. Bootstrap scores expressed as percentages of 1,000 replications are given at each node.

The present study is the first report of *S. erinaceieuropaei* infection in snakes (*P. korros*) from Lao PDR and Thailand, and in a frog (*H. rugulosus*) from Myanmar. Genetic diversity among *S. erinaceieuropaei* specimens from these three countries was discovered, and such studies should be extended to other neighboring countries, viz. Cambodia and Vietnam, in order to obtain a more complete understanding of the molecular phylogeny of this parasite in the Greater Mekong Subregion.

Molecular taxonomy methods based on suitable markers are well documented for identification of a group of morphologically similar parasites. These markers, such as *cox1*, *cox3* and NADH dehydrogenase subunit 3 and 4 genes (*nad3* and *nad4*), have been used for the identification of *S. erinaceieuropaei* (Zhu *et al*, 2002; Okamoto *et al*, 2007; Liu *et al*, 2010; Wang *et al*, 2011; Liu *et al*, 2012; Wei *et al*, 2014). In the present study, phylogenetic relationship of tapeworms within the Diphyllbothriidae family, based on partial *cox1* sequences clearly distinguished the genus *Spirometra* from *Diphyllbothrium* and identified the spargana samples as being *S. erinaceieuropaei*. Variations in *cox1* sequences among *S. erinaceieuropaei* isolates from different geographical localities from this study are similar to that of *cox1* of *S. erinaceieuropaei* isolated from frogs (*R. nigromaculata*) obtained from different localities of Hunan Province, China (Liu *et al*, 2010). Additionally, studies from Japan, India and Indonesia of *S. erinaceieuropaei* obtained in dogs found similar variations in *cox1* sequences (2.6%) (Okamoto *et al*, 2007). Future study with a larger number of samples from various localities is needed in order to clarify the genetic diversity of *S. erinaceieuropaei*.

In summary, all 19 spargana specimens from snake (*P. korros*) from Lao PDR

and Thailand and from frog (*H. rugulosus*) from Myanmar were confirmed as *S. erinaceieuropaei*. Campaigns warning people to avoid ingesting raw flesh of snakes and frogs or using these meats as poultices should be conducted to control and prevent sparganosis in the endemic areas of Southeast Asia.

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