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Molecular Evidence of *Spirometra erinaceieuropaei* in Asian Wild Frogs (*Rana rugulosa*) from Banyuwangi City, Indonesia

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ABSTRACT

The tapeworm *Spirometra erinaceieuropaei* is the most frequently species which found in wild frog and causing a serious parasitic zoonosis known as sparganosis. This study aimed to provide molecular evidences of spargana collected from wild frogs which used as food and contribute to provide important implication for prevention and control of sparganosis. A total of 185 Asian wild frog (*Rana rugulosa*) samples were selected from food markets in Banyuwangi City, Indonesia. Molecular identification based on spargana that were collected and coding gene of mitochondrial cytochrome c oxidase 1 (*cox1*) using Polymerase Chain Reaction (PCR) method. Spargana were found in 9.1% (17/185) of the frogs and PCR analysis results identified all specimens belonging to the species *S. erinaceieuropaei*, therefore indicated that *S. erinaceieuropaei* is the major causative agent of sparganosis from frogs which sold as food in markets. These findings can be useful to the molecular diagnosis and control of *Spirometra* infections in humans and animals.

Keywords: Asian wild frog, *Rana rugulosa*, Sparganosis, *Spirometra erinaceieuropaei*.

INTRODUCTION

The species of the genus *Spirometra*, including *S. erinaceieuropaei* (*S. erinacei*), *S. decipiens*, *S. mansoni*, *S. ranarum*, and *S. mansonoides* are all intestinal parasites of canine and feline hosts (Kavana et al., 2015). The main characters in differentiation of species are the spirally coiled uterus of *S. erinaceieuropaei* and *S. decipiens* (Jeon et al., 2015). The life cycle of these parasites require 2 different intermediate hosts; the fresh water cyclops as the first intermediate host, and vertebrates such as amphibians and reptiles as the second intermediate hosts. The procercoids develop in cyclops, and the plerocercoids (spargana) develop in frogs or snakes and cause sparganosis in humans (Wiwanitkit, 2005). The tapeworm *Spirometra erinaceieuropae* is the most important species of the genus *Spirometra* tapeworms (Nakao et al., 2000). Its plerocercoid larvae (spargana) can lodge in the subcutaneous tissues and sometimes invade the abdominal cavity, eye, and central nervous system of humans causing a serious parasitic zoonosis known as sparganosis (Nithiuthai et al., 2004; Cui et al., 2011).

Sparganosis is a parasitic zoonosis endemic in Asia, Europe, and North America (Kondzior et al., 2018; Sahoo et al., 2018; Scholz et al., 2019). Humans can be infected through the consumption of contaminated water or meat from intermediate hosts or through topical application of raw, contaminated poultices to eyes and open wounds (Jeon et al., 2015; Hong et al., 2016). After entry into humans, the plerocercoid larvae (spargana) migrate to different anatomic locations, where they cause space-occupying lesions as they develop into adults. The sites spargana migrate to include skin and soft tissues, muscles, visceral organs, and the central nervous system. Clinical symptoms range from asymptomatic/mild (e.g., subcutaneous swelling) to severe (e.g., seizure and hemiparesis) depending on the site and size of lesions (Liu et al., 2015).

Sparganosis is an emerging zoonotic disease and public health challenge in Asia, potentially because of the practice of consuming wild frog meat, which is a delicacy in Indonesia (Prasetyo and Safitri, 2019). Although human sparganosis is sporadically distributed around the world, it is most frequently reported in East and Southeast Asia (Qiu and Qiu, 2009). According to a 2009 survey, more than 25% of the local wild frogs in China were infected with spargana (Li et al., 2009). Another recent report showed 104 cases of human sparganosis in China from 2000 to 2006, and more than half, or 53.9%, of cases were caused by eating snakes or frogs (Wu et al., 2007). In Thailand, over 60 sparganosis cases

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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).

In Indonesia, few cases of *Spirometra* infection have been reported from snakes in Sidoarjo and Mojokerto with total prevalence 68% and 50.85% respectively (Pranashinta et al., 2017; Yudhana et al., 2019). There have been several reports of molecular evidences for the detection of spargana infection in frogs (Jongthawin et al., 2014; Zhang et al., 2015). However, there is no molecular evidence of the parasite species in frogs in Indonesia. The aim of the present study is to provide molecular evidences of spargana collected from wild animals which used as food and consumption in Banyuwangi, East Java Province, Indonesia. These results may contribute to identify the sources of infection, which provide important implication for prevention and control of sparganosis in these areas. Moreover, we designed our analysis based on coding gene of mitochondrial cytochrome c oxidase 1 gene (*cox1*) of spagarna isolates from Asian wild frogs, *Rana rugulosa* from Banyuwangi, East Java Province, Indonesia.

MATERIALS AND METHODS

Ethical approval

This study was conducted with permission from the local agriculture department in East Java Province, Indonesia. This study was reviewed and approved by the Animal Care and Use Committee of Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia No.1.KE.190.11.2019.

Parasite samples

A total of 185 living frog specimens (*Rana rugulosa*), commonly known as Chinese edible frog or East Asian wild frog were obtained from five food markets located around the Banyuwangi City (Central, West, East, North, and South parts) East Java Province, Indonesia (114.369227 Longitude and -8.219233 Latitude).

Sparganum collection

The presence of spargana in frogs was examined according to the methods of Ooi et al. (2000). The frogs were euthanized using ethyl-ether anesthesia, weighed, and skinned. The muscles and subcutaneous tissues were carefully observed for the presence of spargana by eyes. Then, the spargana were removed from the muscles or subcutaneous tissues and put in a Petri dish containing physiological saline to observe their movement. The number of spargana collected from each infected frog were counted to estimate the intensity of sparganum infection.

DNA extraction and amplification

Total genomic DNA was extracted from individual plerocercoid sample using the extraction kit (NucleoSpin® Tissue, Macherey-Nagel, Germany) following the manufacturer's protocol. 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). Thermocybcling conditions (conducted in GeneAmp PCR System 9700, Applied Biosystems, Singapore) were as follows: 94ºC for 5 minutes; 35 cycles of 95ºC for 30 seconds, 59ºC for 30 seconds, and 72ºC for 45 seconds; with a final step at 72ºC for 10 minutes. For each PCR experiment, a negative (no DNA) and an amplicons were separated by 1% agarose gel-electrophoresis.

RESULTS

Spirometra spargana were identified in 9.1% (17/185) of the frogs (Table 1). These spargana were 1–20 cm in long and 1–1.5 mm in wide. The prevalence of sparganum infection ranged from 3.0% to 13.6%. Most host samples were infected by several spargana, and 1-3 sparganum was selected from each host sample (Table 1). Moreover, a total of 12 parasite samples were used for molecular identification. Partial sequences of the *cox1* gene were successfully amplified for each sample which shows positive bands at 467 bp (Figure 1).

PCR analysis results identified all 12 individual spargana specimens belonging to the species *S. erinaceieuropaei*. This study therefore indicated that *S. erinaceieuropaei* is the major causative agent of sparganosis from frogs which sold as street food, particularly in Banyuwangi City, Indonesia.

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Table 1. S*pirometra* and host samples from food markets in Banyuwangi City, Indonesia in 2019.

Host species	Source of samples	Number of samples	Infected host samples	Prevalence (%)
Rana rugulosa	Central Banyuwangi	35	3	8.5
Rana rugulosa	East Banyuwangi	44	6	13.6
Rana rugulosa	West Banyuwangi	39	5	5.1
Rana rugulosa	North Banyuwangi	34	2	5.8
Rana rugulosa	South Banyuwangi	33	1	3.0
Total		185	17	7.2

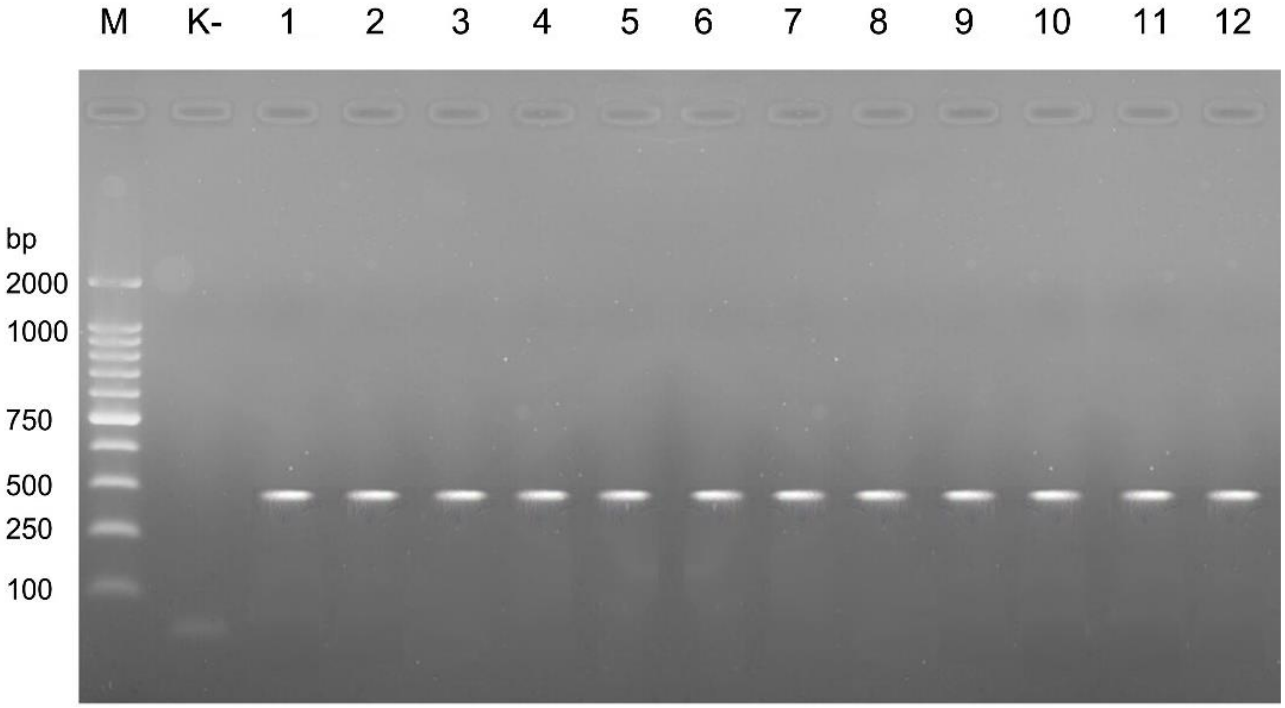

Figure 1. DNA visualization of *cox1* gene of *Spirometra erinaceieuropaei* in polymerase chain reaction products. Lane M=1:100 bp molecular weight standard, K-=Control negative.

DISCUSSION

Sparganosis is a food-borne infection caused by the migrating plerocercoid larvae of the tapeworm *Spirometra* spp. Frogs and snakes are the natural intermediate hosts for these parasites (Cui et al., 2011; Wang et al., 2011). In Chinese cuisine, which also sold in Indonesia, the bushmeat (meat of wild animals used as food) of frog and snake have an important place. The general public like this delicious bushmeat, but it poses as the potential risk of acquiring sparganosis. 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. gracilenta,* and *L. peronii* (Berger et al., 2009). There are several species in the genus *Spirometra* that can cause the disease, and the spargana of these species are often found in frogs, also there have been more than 1,700 global reports of sparganosis published (Liu et al., 2015).

Previous studies in East Java Province, Indonesia the prevalence of spargana was surveyed, but accurate species identification work was not done (Pranashinta et al., 2017; Yudhana et al., 2019).

It seems to the present study is the first report of *S. erinaceieuropaei* infection in frogs (*R. rugulosa*) from Banyuwangi City, Indonesia. Genetic diversity among *S. erinaceieuropaei* specimens from neighboring countries was discovered, and such studies should be extended in order to obtain a more complete understanding of the molecular identification of this parasite in the Asian regions. Molecular taxonomy methods based on suitable markers are well documented for identification of a group of morphologically similar parasites. These markers, such as *cox1* have been used previously 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 Diphyllobothriidae family, based on partial *cox1* sequences clearly distinguished the genus *Spirometra* from *Diphyllobothrium* because of identified the spargana samples as *S. erinaceieuropaei*. Additionally, molecular identification studies from Japan (Okamoto et al., 2007) and

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India (Sahoo et al., 2018) of *S. erinaceieuropaei* obtained in dogs found similar variations in *cox1* sequences. Future study with a larger number of samples from various localities is needed in order to clarify the genetic diversity of *S. erinaceieuropaei* in Indonesia.

CONCLUSION

In present study indicated all spargana species of frogs (*Rana rugulosa*) which sold in food markets around Banyuwangi City, East Java Province, Indonesia were confirmed as *S. erinaceieuropaei* with 9.1% (17/185) prevalence rate. The results of this study can help to control of *Spirometra* infections in humans and animals. Additionally, we also propose the control measures for Sparganosis such as periodic inspection of *S. erinaceieuropaei* infection in frogs in markets and farms are necessary and scientific propaganda should be carried out by the local governments to inform the food restriction of wild caught frogs generally.

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DECLARATIONS

Authors' contributions

AY is a supervised and project leader. RNP carried out the collection of frog samples, and MNY carried out dissection of frog samples. DKW is a data analysis and help with collected samples. All authors contributed to the drafting and revision of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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