

# A NEW RECORD OF *PARAGONIMUS* OTHER THAN *P. WESTERMANI* IN SOUTHERN THAILAND

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**Abstract.** Field surveys of *Paragonimus* in Surat Thani Province, southern Thailand, revealed a new record of a lung fluke species other than *P. westermani*. The metacercariae were obtained from the crab, *Ranguna smalleyi*. The cysts of the metacercariae were spherical in shape and the larval body in the cysts contained pinkish granules. Fully mature adult worms were obtained from experimental infections with a rat and a ferret. The adult worms from the two host animals resembled each other, except for size, and had the anatomical characteristics of *P. bangkokensis*, i.e. the cuticular spines were arranged mainly in groups, the ovaries were highly branched, while the testes were more simply divided. Chromosomal preparations of the testes showed a haploid number of 11. As no sequence data of *P. bangkokensis* has been deposited in the GenBank/EMBL/DBJ nucleotide database, the ITS2 region was sequenced using the metacercariae as starting material. A similarity search of *P. bangkokensis* ITS2 sequence using the BLAST program revealed that there was only one base difference between this population and *P. harinasutai* occurring in central Thailand. The result may suggest a close relationship between *P. bangkokensis* and *P. harinasutai*. This is the first description of *Paragonimus* species other than *P. westermani* occurring in southern Thailand.

## INTRODUCTION

At present, there are at least 6 species of *Paragonimus* in Thailand: *Paragonimus westermani*, *P. siamensis*, *P. heterotremus*, *P. bangkokensis*, *P. macrorchis*, and *P. harinasutai* (Blair *et al.*, 1999). *Paragonimus bangkokensis*, the fourth species discovered in Thailand, was reported in the central part (Nakhon Nayok: Miyazaki and Vajrasthira, 1967, and Saraburi: Srisont *et al.*, 1997) as well as in the eastern part (Chantaburi: Srisont *et al.*, 1997). In China, He *et al.* (1982) published their first discovery of this species in Hainan Island. To date, studies concerning *P. bangkokensis* in Thailand have included morphology (Sugiyama *et al.*, 1989; Komalamisra *et al.*, 2004) and molecular genetics (Intapan *et al.*, 2004). In all studies mentioned, only specimens obtained from central Thailand were employed.

Studies of the second intermediate hosts of *P. bangkokensis* revealed 3 species of waterfall crabs: *Potamiscus smithianus*, *Larnaudia beusekomae*, and *Apotamonautes hainanensis*. There was one report of *P. bangkokensis* natural infection in Indian mongooses.

Experimental infections were carried out and adult worms were obtained from cats and a bandicoot (Miyazaki and Vajrasthira, 1967; He *et al.*, 1982).

In the present study, we focused on *P. bangkokensis* occurring in southern Thailand. We extensively surveyed the distribution of the metacercariae in waterfall crabs in Surat Thani Province. Experimental infections with metacercariae were carried out in mammalian hosts, other than cats and bandicoots. Morphological, chromosomal, and molecular data were obtained either from the metacercarial or adult stages, or both.

## MATERIALS AND METHODS

### Collection of crab intermediate hosts

Waterfall crabs were collected along mountain streams in Pa Nom district, Surat Thani Province during the period January 2003-May 2004. The crabs were identified as *Ranguna smalleyi* according to Naiyanetr (1988). These crabs were further examined for *Paragonimus* metacercariae.

### Examination of *Paragonimus* metacercariae

The crabs were ground and digested with pepsin (1%, w/v) and concentrated hydrochloric acid (0.7%, v/v). The samples were filtered through wire sieves (pore sizes 300 and 500  $\mu$ m), and 0.9% physiological saline was added. They were allowed to stand for approximately one hour and the sediment was

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examined under a stereomicroscope for *Paragonimus* metacercariae. Morphological observations and measurements of the metacercariae obtained were carried out.

#### Adult worms from test rat and ferret

The metacercariae were infected into a Wistar rat and a ferret to raise them to adult worms. The feces of these test animals were examined for *Paragonimus* eggs after approximately 60 days using the formalin-ether concentration method. The results of the experimental infections are shown in Table 1. To prepare permanent specimens for morphological observation, recovered worms were pressed between two glass slides, fixed in 70% ethanol, stained with borax carmine, and mounted with Canada balsam.

#### Chromosomal preparations from adult worms

The Giemsa air-dried preparation of chromosomes from testes followed Sugiyama *et al* (1985). Photomicrographs of a total of 30 cells with well-spread meiotic metaphase chromosomes were taken and a haploid number of chromosomes obtained.

#### DNA amplification and sequencing of the ITS2 region

DNA samples were prepared from the metacercariae, as previously described by Sugiyama *et al* (2002). DNA was amplified by PCR using primers 3S (Bowles *et al*, 1995) and A28 (Blair *et al*, 1997) for the ITS2 region of the nuclear ribosomal DNA. The conditions for the PCR reactions followed Sugiyama *et al* (2002). The resultant PCR products were sequenced using the corresponding primers and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) on an automated DNA sequencer (ABI310, Applied Biosystems). The consensus sequence obtained was compared to sequences in a database (<http://www.ncbi.nlm.nih.gov/BLAST/>).

## RESULTS

#### New crab intermediate host of *P. bangkokensis*

In January 2003 and May 2004, field surveys of *P. bangkokensis* were carried out in Surat Thani Province. A total of 1,001 freshwater crabs were examined. These crabs, belonging to the family Potamidae, called *Ranguna smalleyi* (Fig 1), are recorded as a new crustacean host of *P. bangkokensis*.

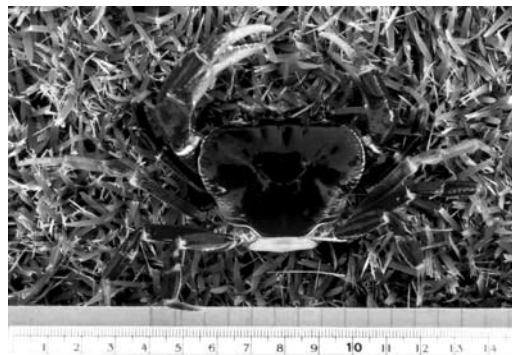


Fig 1- *Ranguna smalleyi*, a new crab intermediate host of *P. bangkokensis* collected in southern Thailand.

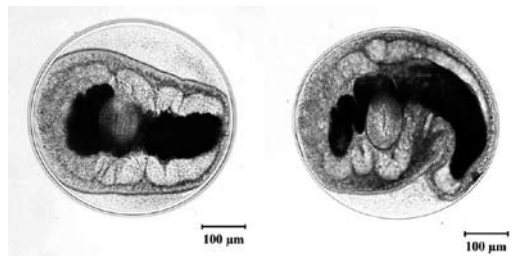


Fig 2- *Paragonimus bangkokensis* metacercariae obtained in southern Thailand. Scale bar = 100  $\mu$ m.

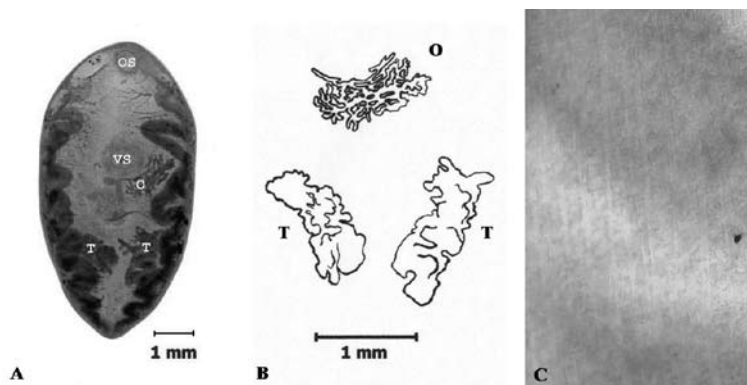


Fig 3- Adult worm obtained from a test rat. A: Photomicrograph of the adult worm (OS: oral sucker, VS: ventral sucker, O: ovary, T: testes). B: Drawing of the ovary and testes. C: Photomicrograph of cuticular spines. Scale bar = 1 mm.

Table 1  
Experimental infections of rat and ferret with metacercariae of *P. bangkokensis*.

Test animals	Dose of mc <sup>(a)</sup>	Duration of infection (days)	Rate of mc <sup>(a)</sup> recovered as adult flukes (%)	No. of adult flukes recovered		
				Lungs	Liver	Total
Rat	11	111	18	1	1	2
Ferret	7	239	43	3	-	3

<sup>(a)</sup> mc = metacercariae

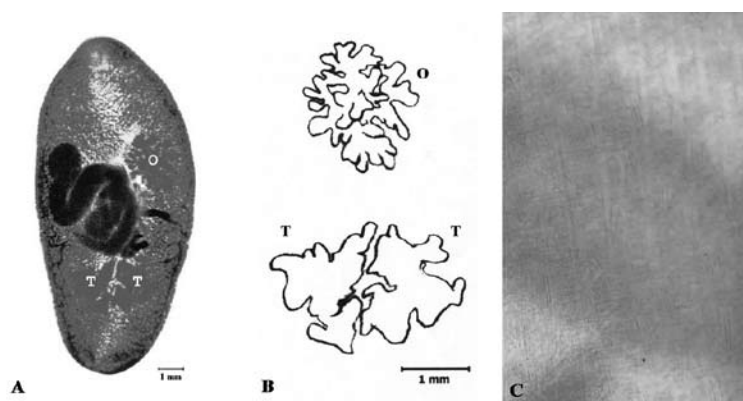


Fig 4- Adult worm obtained from a test ferret. A: Photomicrograph of the adult worm (O: ovary, T: testes). B: Drawing of the ovary and testes. C: Photomicrograph of cuticular spines. Scale bar = 1 mm.

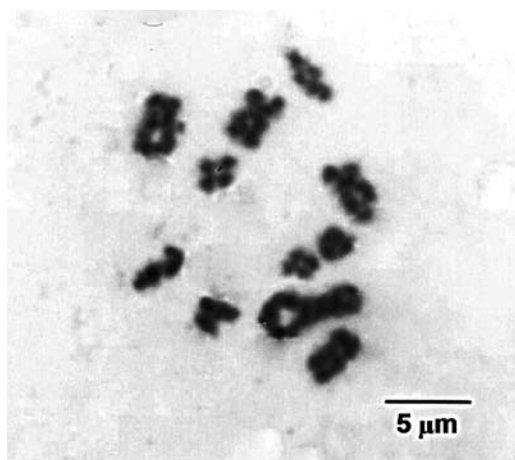


Fig 5- A photomicrograph of the meiotic metaphase chromosomes with a haploid number of 11 (n = 11). Scale bar = 5 μm.

#### Characteristics of metacercariae

A total of 55 metacercariae were recovered from all crabs examined. Fig 2 shows representative metacercariae, which were spherical in shape; the inner membrane was approximately 13 μm thick. In

25 fresh specimens, excluding the outer membrane, the longitudinal and transverse diameters of the inner cyst ranged from 521-379 x 469-365 μm (average 437 x 422 μm). The larval bodies contained pinkish granules.

#### Adult worms from test animals; morphology and number of chromosomes

After *Paragonimus* eggs were detected in feces, the rat and ferret were sacrificed. Table 1 illustrates the number of adult flukes obtained at necropsy.

Five adult worms were obtained from the rat and ferret; thus, these mammals became the new definitive hosts of *P. bangkokensis*. In 3 mounted specimens of adult worms from the rat (Fig 3A) and ferret (Fig 4A), the sizes of the worms were different. Two specimens from the rat had an average size of 7.60 x 3.75 mm, with length 7.5-7.7 mm and width 3.7-3.8 mm. The average transverse diameters of the oral and ventral suckers measured 590 μm and 597 μm, respectively. The ovary was highly branched, while the testes were much more simply divided (Fig 3A and 3B). The cuticular spines were arranged in groups (Fig 3C). One specimen from the ferret showed a larger body size, with length and width 13.40 x 6.30 mm. The transverse diameters of the oral and ventral suckers were 1.06 and

**Pb: TGTCGATGAAGAGCGCAGCCAAC TGTGTGAATTAATGTGAACTGCATACTGCTTTGAACA**  
**Ph: TGTCGATGAAGAGCGCAGCCAAC TGTGTGAATTAATGTGAACTGCATACTGCTTTGAACA**

**Pb: TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG**  
**Ph: TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG**

**Pb: TCGGCTTATAAACTATCGCGACGCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT**  
**Ph: TCGGCTTATAAACTATCGCGACGCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT**

**Pb: GATTTCCCCGATCTGACCTTGTGTGCGGTGGGGTGCCAGATCTATGGCGTTTTCCCTAACCT**  
**Ph: GATTTCCCCGGTCTGACCTTGTGTGCGGTGGGGTGCCAGATCTATGGCGTTTTCCCTAACCT**  
 \*

**Pb: GTCCGGGCGTACCCATGTTGTGGCTGAAGGCCTTGGTGGGGATGTGGCAACGGAATCGTG**  
**Ph: GTCCGGGCGTACCCATGTTGTGGCTGAAGGCCTTGGTGGGGATGTGGCAACGGAATCGTG**

**Pb: GCTCAGTGAATTATTTATGTGCGCGTTCCGTCGTCTTATCATCATCTATGGTTGATGCTG**  
**Ph: GCTCAGTGAATTATTTATGTGCGCGTTCCGTCGTCTTATCATCATCTATGGTTGATGCTG**

**Pb: CGTGTGGTGTGCGTCTGATGCTGACCTGAGTATGTGCCATGTGGCTCATTCTCCTGACCT**  
**Ph: CGTGTGGTGTGCGTCTGATGCTGACCTGAGTATGTGCCATGTGGCTCATTCTCCTGACCT**

**Pb: CGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA**  
**Ph: CGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA**

Fig 6- Similarity search result of the ITS2 sequences from *P. bangkokensis* (Pb) and *P. harinasutai* (Ph). An asterisk (\*) indicates a variable region.

1.18 mm, respectively. Other structures, including the ovary, testes (Fig 4A and 4B), and cuticular spines (Fig 4C) resembled those of the rat.

In the present study, chromosomal preparations of the testes from one adult worm showed many well-spread meiotic metaphase chromosomes. Fig 5 is a photomicrograph of the chromosomes with a haploid number of 11 ( $n = 11$ ).

#### Genetic variation of *P. bangkokensis* based on the ITS2 region

The result of the ITS2 sequence similarity search of *P. bangkokensis* showed only one base difference between this population and *P. harinasutai* occurring in central Thailand (GenBank accession number AF159609) (Fig 6).

#### DISCUSSION

In southern Thailand, *P. bangkokensis* metacercariae were discovered in the new crab intermediate host, *R. smalleyi*. Similar to the previous report by Miyazaki and Vajrasthira (1967), the metacercariae were spherical in shape and the average longitudinal and transverse diameters of the inner cyst were 437 x 422  $\mu\text{m}$ . The larval bodies contained pinkish granules. In addition, the morphological characteristics of the

adult worms obtained from the new mammalian hosts (the rat and ferret) were also identical to those described by Miyazaki and Vajrasthira (1967). These worms possessed a highly branched ovary, more simply divided testes, clustered cuticular spines, and a haploid chromosome number of 11. In terms of the genetic variation of *P. bangkokensis* based on the ITS2 region, there was only a single base difference between this population and *P. harinasutai* occurring in central Thailand. The result may suggest a close relationship between *P. bangkokensis* and *P. harinasutai*. This agreed with the morphological characteristics of both species, except for the arrangement of the cuticular spines.

#### ACKNOWLEDGEMENTS

The authors would like to thank Miss Tapawittra Pongpawe for technical assistance. This study was supported in part by grant from Nissan Science Foundation.

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