NEW FORM OF *PARAGONIMUS WESTERMANI* DISCOVERED IN THAILAND: MORPHOLOGICAL CHARACTERISTICS AND HOST SUSCEPTIBILITY

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Abstract. During an intensive field survey for *P. westermani* in southern Thailand, a new form of *Paragonimus* metacercariae was isolated. In this study, we referred to this new form as *P. westermani*-like, as it was almost identical to *P. westermani* in shape. To investigate the susceptibility of feline host to *P. westermani*-like, as well as its morphology at the adult stage, we inoculated the peritoneal cavity of a cat with 60 *P. westermani*-like metacercariae. Morphological examination revealed that the adult *P. westermani*-like recovered from the lungs had a six-lobed ovary, a spermatozoa-filled seminal receptacle, and singly spaced cuticular spines. These findings indicated that the morphological features of *P. westermani*-like were fundamentally identical to those of *P. westermani* (diploid type) at the adult stage. The susceptibility of feline hosts to *P. westermani*-like was different from that of *P. westermani*. To determine the proper taxonomic status of *P. westermani*-like, we have been investigating the phylogenetic relationships between *P. westermani*-like and *P. westermani* in southern Thailand.

INTRODUCTION

Paragonimus westermani is widely distributed in Asia (Miyazaki, 1991). Individuals from different geographical regions (or countries) show variations in animal and/or human susceptibility, although they share almost identical morphological features at both the adult and metacercarial stages (Blair *et al*, 1998). This implies that they form a complex of cryptic species (Blair *et al*, 1997).

During an intensive field survey for *Paragonimus* in southern Thailand (Rangsiruji *et al*, 2005), we collected another form of *Paragonimus westermani* metacercariae from freshwater crabs, *Phricotelphusa aedes*. These crabs simultaneously acted as the second intermediate host of *P. westermani*. Metacercariae of newly isolated *Paragonimus* were almost identical to those of *P. westermani* in shape, but were much smaller. For descriptive purposes, we refer to this new form as *P. westermani*-like.

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In this study, we inoculated a cat with *P. westermani*-like metacercariae isolated from freshwater crabs, *Phricotelphusa aedes*, in order to identify the susceptibility of feline hosts. The morphological characteristics of *P. westermani*-like at the adult stage were also compared with those of *P. westermani*.

MATERIALS AND METHODS

Identification of freshwater crabs

The captured freshwater crabs, belonging to the family Potamidae, were identified as *Phricotelphusa aedes* according to the method of Naiyanetr (1988).

Isolation of *Paragonimus* metacercariae

Between January and May 2003, we collected 922 freshwater crabs, *Phricotelphusa aedes*, from mountain streams in the Phanom District of Surat Thani Province, Thailand. We examined the crabs for metacercariae, as described previously (Rangsiruji *et al*, 2005). Isolated metacercariae were placed on glass slides and gently pressed under a coverglass for morphological observation and measurement.

Worm recovery from test animal

We inoculated the peritoneal cavity of a cat

with 60 *P. westermani*-like metacercariae. The cat was then treated with prednisolone (20 mg/kg) at 7-day intervals and was necropsied 148 days after inoculation. We examined the whole body of the cat for worms, as described previously (Sugiyama *et al*, 1984). Recovered worms were pressed between two glass slides, fixed in 70% ethanol, stained with borax carmine, and mounted with Canada balsam for morphological observation and measurement.

DNA amplification and sequencing of ITS2 region

We prepared DNA samples from individual *P. westermani* and *P. westermani*-like metacercariae (five metacercariae each). The ITS2 region of the nuclear ribosomal DNA was amplified by PCR and sequenced, as described previously (Sugiyama *et al*, 2002). The primers used were 3S: 5'-GGTACCGGTGGATCACTCGGCTCGTG-3' (forward: Bowels *et al*, 1995) and A28: 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3' (reverse: Blair *et al*, 1997). We aligned and compared sequences using GENETYX-WIN software (ver 7.0, Software Development, Tokyo, Japan).

RESULTS

New crab intermediate host of *Paragonimus* in southern Thailand

We captured 922 freshwater crabs (Fig 1) from mountain streams in the Phanom District of Surat Thani Province. The crabs were positive for *P. westermani* metacercariae; this is the first report of this crab species serving as a second intermediate host of *P. westermani*. *P. westermani*-like metacercariae were also isolated from the same crab species captured at the same sites.

Morphology of *P. westermani*-like metacercariae from crabs

We isolated 89 *P. westermani*-like metacercariae from the crabs. All were spherical in shape and had thin walls (Fig 2). The thickness of the cyst wall in 30 specimens ranged from 4-14 μm, with an average of 8.7 μm. The longitudinal and transverse diameters of the cyst ranged from

Fig 1- Freshwater crabs, *Phricotelphusa aedes*, which serve as the second intermediate host of both *P. westermani* and *P. westermani*-like in southern Thailand.

Fig 2- Photomicrograph of fresh *P. westermani* metacercaria. Bar is 100 μm.

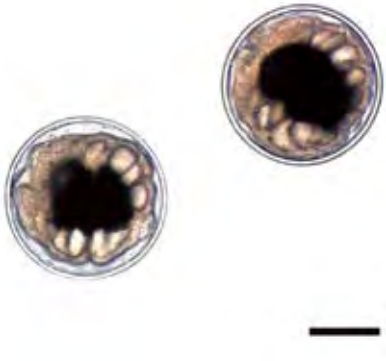

Fig 3- Photomicrograph of fresh *P. westermani*-like metacercariae. Bar is 100 μm.

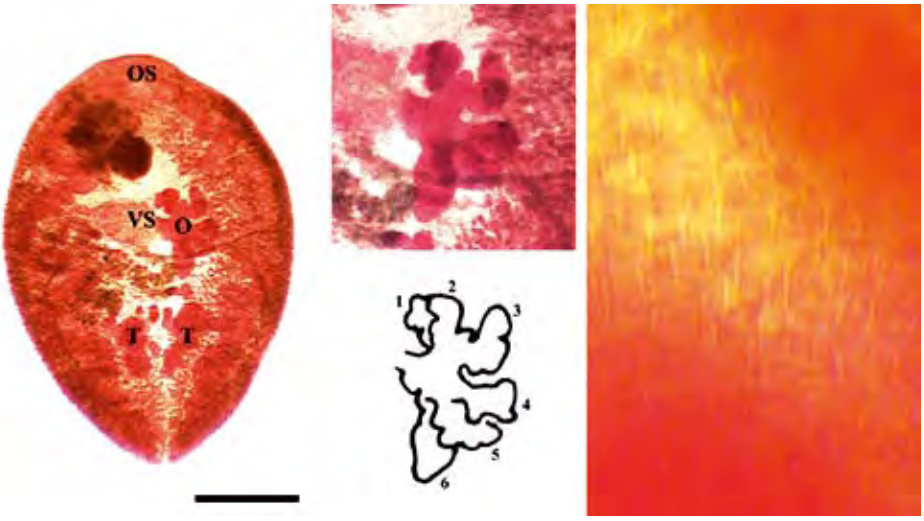

Fig 4- Adult worm of *P. westermani*-like from a cat inoculated with metacercariae. The worm had six-lobed ovary and singly spaced cuticular spines.

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Pw1 ------------------------------------------------------------ 060
Pw2 TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAACA 060
PwL ............................................................ 060

Pw1 ----------------ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG 120
Pw2 TCGACATCTTGAACGC.............................................. 120
PwL ............................................................ 120

Pw1 TCGGCTTATAAACCATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT 180
Pw2 ............................................................ 180
PwL .........G...T.............................................. 180

Pw1 GATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTATGGCGTTTCCCTAACAT 240
Pw2 ............................................................ 240
PwL ..........................................G......C.......... 240

Pw1 ACTCGCGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTG 300
Pw2 ............................................................ 300
PwL .....G...................................................... 300

Pw1 GCTCAGTAAATGATTTATGTGCGCGTTTCGCTGTCCTGTCTTCATCTGTGGTTCATGTTG 360
Pw2 ............................................................ 360
PwL .......G.T..G..............C.............................T...... 360

Pw1 CGCGTGGTCTGCGTTCGATGCTGACCTACGTATGTGCCATGTGGTCCATTCTTCTGACCT 420
Pw2 ............................................................ 420
PwL ..............................................T.......C....... 420

Pw1 CGGATTAGACGTGAGTACC---------------------- 463
Pw2 ...................CGCTGAACTTAAGCATATCACTAA 463
PwL .....C..................................... 463
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Fig 5- Aligned sequences of the ITS2 region from *P. westermani* strain Thailand (AF159604, Pw1), *P. westermani* (Pw2) and *P. westermani*-like (PwL). Identical bases are represented by dots. Hyphen indicates missing data. Numbers refer to nucleotide sequence length.

212-252 μm and from 204-240 μm, respectively, with an average of 227 x 221 μm.

The metacercariae of *P. westermani*, also isolated from the same crab hosts, were spherical in shape and had thick walls (Fig 3). The thickness of the cyst wall in five specimens ranged from 19-37 μm, with an average of 28.2 μm. The longitudinal and transverse diameters of the cysts ranged from 458-510 μm and from 438-501 μm, respectively, with an average of 492 x 480 μm.

Morphology of an adult worm

On postmortem examination of the test cat, 148 days after inoculation, 13 worms were recovered; 2 from the lungs (being paired in the worm cyst), 2 from the pleural cavity, and 9 from the liver. The worms from the lungs and pleural cavity were identified as either adults (one each, with eggs in the uterus) or pre-adults (without eggs), while the worms from the liver remained in the juvenile stage.

The size of the adult worm from the lung was 3.95 mm in length and 2.83 mm in width. The transverse diameters of the oral and ventral suckers measured 504 μm and 500 μm, respectively. The adult worm had a six-lobed ovary and singly spaced cuticular spines (Fig 4). The seminal receptacle was filled with spermatozoa.

ITS2 sequence analysis

The ITS2 region was amplified from DNA samples of individual *P. westermani* and *P. westermani*-like metacercariae using the consensus primers 3S and A28. Sequence analysis of the PCR products revealed that the aligned ITS2 region was 463 bp in length for both *P. westermani* and *P. westermani*-like samples. Pairwise comparison of the sequences showed 13 (2.8%) nucleotide differences (Fig 5). Similarity searches of the nucleotide databases GenBank/EMBL/DDBJ revealed that the ITS2 sequences of *P. westermani* were identical to those found in the databases under the accession number AF159604 for the *P. westermani* strain Thailand. However, the sequences of *P. westermani*-like did not exhibit a striking similarity to any of those found in the databases.

DISCUSSION

In this study, we observed adult *P. westermani*-like samples obtained from a cat that was inoculated with the metacercariae. The adult had an ovary that was simply divided into six lobes, a seminal receptacle filled with spermatozoa, and cuticular spines arranged singly. These morphological features at the adult stage are in good agreement with the description of *P. westermani* (Thai strain) (Sugiyama *et al*, 2001; Binchai *et al*, 2005). With regard to the morphology of metacercariae, other than the size, the features of *P. westermani*-like were almost identical to those of P. *westermani*. Therefore, it can be concluded that *P. westermani*-like should be classified as *P. westermani*, or as one of the members (a cryptic species) of the *P. westermani* complex (Blair *et al*, 1997), based on the anatomical similarities.

We investigated the susceptibility of feline hosts to *P. westermani*-like by experimental infection, and compared the results with those of *P. westermani*. From the cat experimentally infected with *P. westermani*, worms were detected only in the lungs or pleural cavity. The worms recovered were identified as adults or at least pre-adults (Binchai *et al*, 2005). In contrast, as shown in this study, juvenile *P. westermani*-like lodged predominantly in the liver, while some matured into adults in the pleural cavity or lungs. These findings suggested that the susceptibility in cats differed between *P. westermani* and *P. westermani*-like. The susceptibility of feline hosts to *P. westermani* was also examined using worms from Malaysia (Habe *et al*, 1996). About half of the worms recovered were identified as juvenile worms, but the principal domicile of the juveniles was not the liver but the skeletal muscles.

Molecular comparison based on ITS2 sequences revealed that there were a few nucleotide differences (2.8%) between *P. westermani* (*P. westermani* strain Thailand) and *P. westermani*-like. Therefore, in order to determine the proper taxonomic status of *P. westermani*-like, we need to investigate the detailed phylogenetic relationships between *P. westermani*-like and *P. westermani*. In terms of the susceptibility of *P.*

westermani-like, information regarding host-parasite relationships, particularly relating to the first intermediate hosts, is required. Studies into these issues are currently underway (Binchai *et al*, 2007).

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