

Paragonimus paishuihoensis Metacercariae in Freshwater Crabs, *Potamon lipkei*, in Vientiane Province, Lao PDR

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Abstract: Among *Paragonimus* species, *P. paishuihoensis* is one of the most mysterious and poorly understood species. Metacercariae are characterized by having a unique dendritically branched excretory bladder. However, the morphology of the adult worm remains unknown. To date, metacercariae of this species have been reported only in China and Thailand. In this study, we first found *P. paishuihoensis* metacercariae in freshwater crabs, *Potamon lipkei*, in Hinheub District, Vientiane, Lao PDR, with a prevalence of 77.7% and the average intensity of 10.3 (range 1-28) metacercariae per crab. The molecular data based on ITS2 and CO1 markers indicated that *P. paishuihoensis* from Laos and Thailand were almost completely identical and were close to members of the *Paragonimus bangkokensis*/*Paragonimus harinasutai* complex. Attempts to infect experimental animals (cats, dogs, and rats) with *P. paishuihoensis* were unsuccessful, suggesting that these animals might be unsuitable definitive hosts for the species. Further studies are necessary to elucidate the taxonomic status and life cycle of *P. paishuihoensis*.

Key words: *Paragonimus paishuihoensis*, metacercaria, freshwater crab, *Potamon lipkei*, Lao PDR

INTRODUCTION

Among about 50 nominal species of the genus *Paragonimus*, *Paragonimus paishuihoensis* Tsao and Chung 1965 is one of the most mysterious and poorly understood species because no adult has ever been obtained [1]. The metacercariae of this species, originally found in Szechuan (Sichuan) Province, China, are unique in the genus *Paragonimus* because they possess a highly branched excretory bladder [2]. After experimental infection in cats, only 1 juvenile worm was obtained [2]. Subsequently, Wang et al. [3,4] found *P. paishuihoensis* metacercariae in freshwater crabs in Yunnan Province, China. At almost the same time, Cui et al. [5] determined nuclear ribosomal second internal transcribed spacer region (ITS2) and a partial mitochondrial cytochrome c oxidase subunit 1 gene (CO1) sequenc-

es of *P. paishuihoensis* and another 4 *Paragonimus* species in China and revealed that *P. paishuihoensis* was genetically close to *Paragonimus bangkokensis*, *Paragonimus menglaensis* and *Paragonimus xiangshanensis*. In addition to those reports from China, Rangsiruji et al. [6] discovered *P. paishuihoensis* metacercariae in Chanthaburi Province, Thailand, and reported genetic similarities of this species to *P. bangkokensis* and *Paragonimus harinasutai* based on analyses of ITS2 and CO1 sequences. Here, we report morphological and molecular features of *P. paishuihoensis* metacercariae first found in freshwater crabs, *Potamon lipkei*, in Lao PDR.

MATERIALS AND METHODS

Collection of metacercariae

Paragonimus metacercariae were collected from freshwater crabs, *Potamon lipkei*, in Hinheub district, Vientiane Province, Lao PDR, by the methods described previously [7]. Nine crabs were examined individually in detail to determine the prevalence of each *Paragonimus* species and their distribution in crab tissues (Table 1). *P. paishuihoensis* metacercariae collected from

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Table 1. Prevalence and distribution of *Paragonimus* metacercariae in 9 crabs *Potamon lipkei*

<i>Paragonimus</i> species	Prevalence in crabs			Distribution of MC in tissues			
	No. infected (%)	No. of MC found (% total)	Range (mean)	Muscles	Gills	Liver	Others
<i>P. harinasutai</i>	8 (88.8)	688 (75.5)	4-225 (86)	534	85	25	44
<i>P. heterotremus</i>	5 (55.5)	140 (15.4)	4-55 (28)	129	9	-	2
<i>P. paishuihoensis</i>	7 (77.7)	72 (7.9)	1-28 (10.3)	48	6	9	9
<i>P. bangkokensis</i>	2 (22.2)	11 (1.2)	5-6 (5.5)	9	2	-	-

MC, Metacercariae

Table 2. Results of experimental infection in laboratory animals with *P. paishuihoensis* metacercariae

Host	No. of Mc given	Method of infection	Autopsy at (months post-infection)	No. of worms recovered
Cat 1 ♀	8	Peritoneal	16.5	0
Cat 2 ♀	10	Peritoneal	16.5	0
Cat 3 ♀	20	Peritoneal	11.5	0
Cat 4 ♂	6	Oral	8.5	0
Cat 5 ♂	25	Oral	7	0
Dog 1 ♀	25	Oral	5	0
Rat 1 ♀	15	Oral	2	0*
Rat 2 ♂	10	Intra-peritoneal	2	0*

*Whole body was examined. Mc, metacercariae.

additional crabs used for experimental infection were not included in Table 1. Metacercariae were identified based on morphological characteristics. Metacercariae that have a dendritic excretory bladder were regarded as *P. paishuihoensis* and were used for experimental infection and for morphological and molecular analyses.

Experimental infection to laboratory animals

Fresh metacercariae having a dendritic excretory bladder were given to 5 cats, 1 dog, and 2 rats either orally or intraperitoneally (Table 2). Fecal examination was carried out 1 month after infection and weekly thereafter. Since none of those animals became *Paragonimus* egg-positive after several months, they were autopsied at the designated time (Table 2) to examine the lungs. In case of rats, the whole body was examined using pepsin digestion.

Molecular analyses

From 3 metacercariae of *P. paishuihoensis* (codes: MC32-MC34), we amplified and sequenced the ITS2 region using the primer pair 3S and A28 [8] and a partial CO1 gene using the primer pair of CO1-1-forward and CO1-2-reverse [9]. The methods for molecular study followed those described previ-

ously [10]. The obtained sequences were deposited in the GenBank with accession no. AB679285-AB679290. In addition, we downloaded and used further sequences of *Paragonimus* spp. from GenBank, including the available sequences (ITS2: AB471886 and CO1: AB471887) of *P. paishuihoensis* from Thailand and 1 ITS2 sequence (AB370191), which was originally registered as *Paragonimus* sp. from Laos and is now re-registered as *P. paishuihoensis* by us. Two sequence data sets, ITS2 and CO1, were used to reconstruct the phylogenetic trees. Each set was aligned using Clustal-W [11] with default options. Evolutionary analyses were conducted in MEGA5 [12]. Maximum likelihood trees were reconstructed using the best models: the Kimura 2-parameter with gamma rate heterogeneity (+G) for ITS2 tree and Hasegawa-Kishino-Yano model with invariable sites (+I) for CO1 tree.

RESULTS

Morphology of *P. paishuihoensis* metacercariae

When we examined *Paragonimus* metacercariae in the freshwater crabs, *P. lipkei*, caught in Hinheub District, Vientiane, Lao PDR, we found metacercariae having an excretory bladder of dendritic appearance (Fig. 1) in addition to 3 previously reported species; *P. harinasutai*, *Paragonimus heterotremus*, and *P. bangkokensis* [13,14]. By light microscopic observations, these metacercariae were spherical in shape with 2 cyst walls (Fig. 1A,B). The outer cyst (n=8) was $702.0 \pm 46.0 \times 670.5 \pm 49.1$ μm (range 577-725 \times 631-755) in diameter, while the inner cyst (n=17) was $620.9 \pm 30.9 \times 573.9 \pm 28.8$ μm (range 518-622 \times 580-681). A distinct space was present between the 2 walls. The thickness of outer and inner cyst wall was 4.9 ± 0.3 and 5.7 ± 0.7 μm , respectively. Two metacercariae were artificially excysted (Fig. 1C) and measured. They measured $1,502 \times 737$ and $1,436 \times 533$ μm respectively; oral suckers measured 105×118 and 117×112 μm , and ventral suckers 186×211 and 158×192 μm , respectively. A branched excretory bladder runs the

length of the body. All these morphological and morphometric data of encysted and excysted metacercariae were compatible with those of *P. paishuihoensis* reported previously [2,3,6].

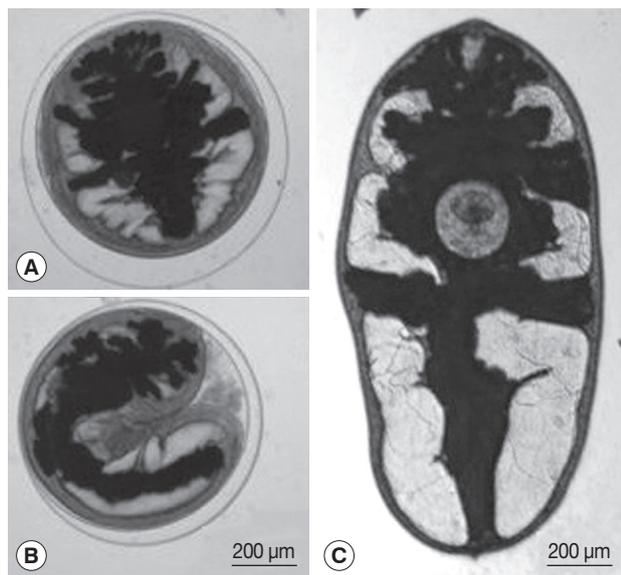


Fig. 1. Encysted form (A, B) and excysted form (C) of *P. paishuihoensis* metacercariae from crabs (*P. lipkei*) collected in Lao PDR.

Prevalence of *Paragonimus* metacercariae in crabs

In this study, 9 crabs were examined individually in detail to determine the prevalence of each *Paragonimus* species and their distributions in crab host tissues. Among 4 co-existing *Paragonimus* metacercariae in crabs, *P. harinasutai* was the most common species which was found in 8 (88.8%) crabs and accounted for 75.5% of total metacercariae found, followed by *P. heterotremus*, *P. paishuihoensis*, and *P. bangkokensis* (Table 1). Individual crab harbored metacercariae of 1-4 *Paragonimus* species. Regardless of the *Paragonimus* species, the majority of metacercariae were distributed in the muscles and hepatopancreas of the crab, and only a few were found in the gills or other tissues (Table 1).

Experimental infection

None of the 8 experimental animals (5 cats, 1 dog, and 2 rats), which were infected with *P. paishuihoensis* metacercariae, became egg-positive after infection (range of times until autopsy; 2.0-16.5 months). At autopsy, all of them were negative for *Paragonimus* worms (Table 2). No lesion suggestive of *Paragonimus* infection was found in the pleural and abdominal cavity of any experimental animal.

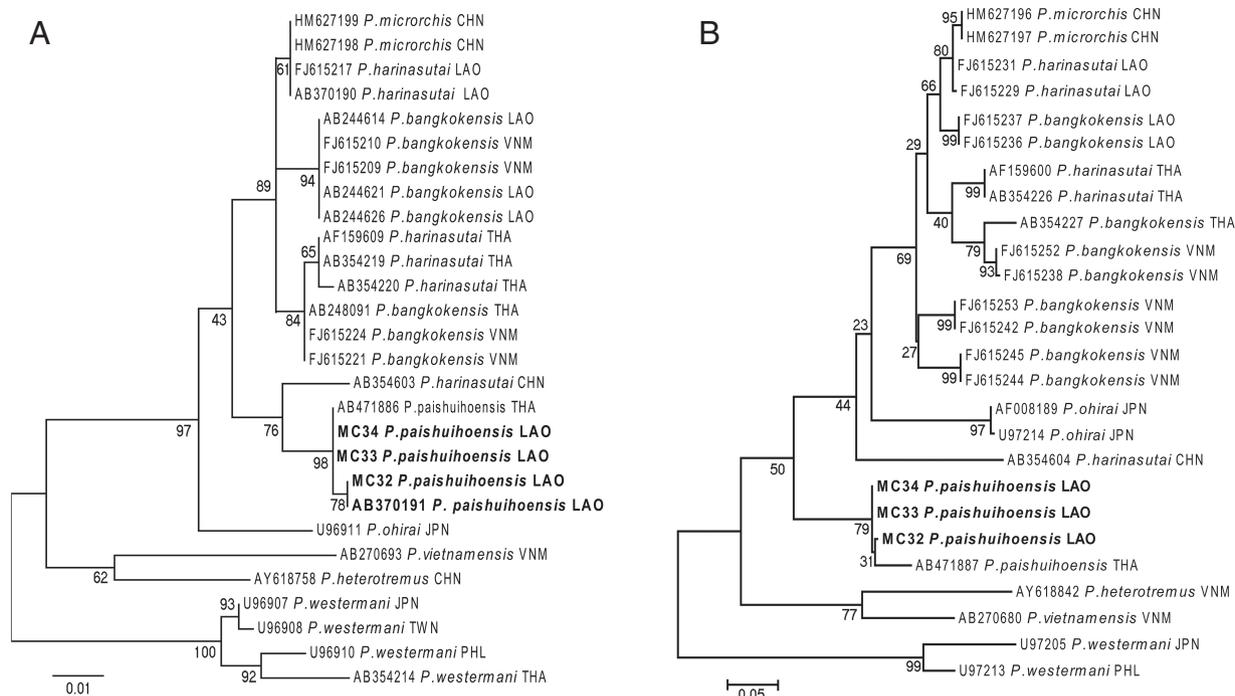


Fig. 2. The Maximum Likelihood tree constructed from ITS2 (A) and CO1 (B) sequences. Bootstrap scores (percentages of 1,000 replications) are presented for each node. The sequences obtained from the samples in this study are represented in bold, while others from DNA database are shown with the GenBank accession no., species name, and country code.

Molecular analyses

For each gene region, the sequences of *P. paishuihoensis* isolates from Lao PDR were almost completely identical with each other; only 1 and 2 nucleotide differences were observed in the ITS2 (363 bp) and CO1 (354 bp) alignments, respectively. Similarities between the Lao sequences and those from the Thai sample were 99.9% in ITS2 (in spite of an AG indel) and 96.2% in CO1 sequences. Consequently, Lao and Thai samples formed a reasonably well-supported group in trees inferred from either gene region (Fig. 2). *P. paishuihoensis* was shown to be close to the *P. bangkokensis*/*P. harinasutai* complex.

DISCUSSION

The present study clearly showed that *P. paishuihoensis* is present in Vientiane Province, Lao PDR as the third country after China [2,3,13] and Thailand [6]. *P. paishuihoensis* metacercariae were often found together with 1-3 other *Paragonimus* species: *P. harinasutai*, *P. heterotremus*, and *P. bangkokensis* in a same crab, indicating that competition among these species of metacercariae may not so strict in *P. lipkei*. Consistent with a previous report [13], *P. harinasutai* was by far the commonest species in this study area (Table 1).

Until now, fully mature adult worms of *P. paishuihoensis* have never been found in wildlife, nor in experimentally infected animals except for 1 juvenile obtained by experimental infection in a cat in China [2]. We infected cats, a dog, and rats, which are known as susceptible definitive hosts for almost all *Paragonimus* spp. [1], with *P. paishuihoensis* metacercariae, but no worms were established themselves in these hosts. This indicates that dogs, cats, and rats are not suitable definitive hosts for *P. paishuihoensis*; it might have narrow host specificity to an unknown mammalian host, or require a paratenic host. If a paratenic host is required, our results suggest that it is not the rat; no juvenile worms were found when the body of each rat was completely digested. Further studies are necessary to elucidate the life cycle of *P. paishuihoensis*.

In molecular studies, Cui et al. [5] reported that *P. paishuihoensis* obtained in Yunnan Province, China, was genetically similar to *P. xiangshanensis* and *P. bangkokensis* from China. Unfortunately, those sequence data are not registered in GenBank. To date, *P. paishuihoensis* from Thailand is represented in GenBank by only a single ITS2 and a single CO1 sequence [6]. In the present study, molecular phylogenetic analyses revealed that *P. paishuihoensis* isolates from Lao PDR and Thailand were

genetically almost completely identical with each other, thus forming a moderately to well-supported group in ITS2 and CO1 trees. Consistent with the previous reports [14,15], *P. paishuihoensis* sequences were clustered with *P. harinasutai* of China with a moderate bootstrap value of 76% to make a group separated from the rest of the *P. bangkokensis*/*P. harinasutai*. However, grouping with *P. paishuihoensis* and *P. bangkokensis*/*P. harinasutai* complex was supported by very low bootstrap values (less than 50%), especially in CO1 tree. For further investigation on taxonomic status in this group, more sampling and sequence data accumulation is necessary. Since only 1 each of ITS2 and CO1 sequence of *P. harinasutai* and none of other related species (*P. xiangshanensis* and *P. bangkokensis*) from China are available in the GenBank database, more sequence data of those species from China and also from other geographical locations would be useful for better understanding of their taxonomic status and phylogenetic relationship.

In conclusion, we demonstrated the presence of *P. paishuihoensis* metacercariae in Lao PDR, suggesting a wider distribution of this species in Indochina than previously suspected. Since the definitive hosts of *P. paishuihoensis* have not yet been identified, further studies are required to clarify the taxonomic status and life cycle of this species.

REFERENCES

1. Blair D, Xu ZB, Agatsuma T. Paragonimiasis and the genus *Paragonimus*. *Adv Parasitol* 1999; 42: 113-222.
2. Tsao WC, Chung HL. Preliminary observations on metacercariae cysts, excysted metacercariae and adolescent worm of a new species of lung fluke, *Paragonimus paishuihoensis* sp. nov. discovered in P'enghsien in Szechuan Province. *Acta Parasitol* 1965; 2: 252-256.
3. Wang WL, Lei L, Zhou BJ, Zhang X, Wang ML, Xia DG. Investigation and observation on *Paragonimus paishuihoensis* in Mengla County, Yunnan Province. *Endemic Dis Bull* 2002; 17: 62-65.
4. Wang WL, Shen YZ, Lei L, Zhou BJ, Song JL, Liu AW, Han D. Observations on the metacercariae of *Paragonimus paishuihoensis* by transmission electron microscopy. *Chinese J Zoonosis* 2005; 21: 150-152.
5. Cui AL, Chang ZS, Chen MG, Blair D, Chen SH, Zhang YN, Feng Z. Taxonomic status in DNA sequences of five species of genus *Paragonimus*. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* 2003; 21: 27-30.
6. Rangsiruji A, Binchai S, Qian BZ, Morishimia Y, Sugiyama H. A new form of *Paragonimus* metacercariae isolated from freshwater crabs: possible discovery of *P. paishuihoensis* in Thailand. *Proceedings of Joint International Tropical Medicine Meeting* 2008;

- p 137.
7. Habe S, Lai KPE, Agatsuma T, Ow Y, Kawashima K. Crab hosts for *Paragonimus westermani* (Kerbert, 1878) in Malaysia. *Jpn J Trop Med Hyg* 1993; 21: 137-142.
 8. Bowles J, Blair D, McManus DP. A molecular phylogeny of the human schistosomes. *Mol Phylogenet Evol* 1995; 4: 103-109.
 9. Thaenkham U, Waikagul J. Molecular phylogenetic relationship of *Paragonimus pseudoheterotremus*. *Southeast Asian J Trop Med Public Health* 2008; 39: 217-221.
 10. Doanh PN, Shinohara A, Horii Y, Habe S, Nawa Y, The DT, Le NT. Morphological and molecular identification of two *Paragonimus* spp., of which metacercariae concurrently found in a land crab, *Potamiscus tannanti*, collected in Yenbai Province, Vietnam. *Parasitol Res* 2007; 100: 1075-1082.
 11. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL-W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994; 22: 4673-4680.
 12. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011; 28: 2731-2739.
 13. Odermatt P, Habe S, Manichanh S, Tran DS, Duong V, Zhang W, Phommathet K, Nakamura S, Barennes H, Strobel M, Dreyfuss G. Paragonimiasis and its intermediate hosts in a transmission focus in Lao People's Democratic Republic. *Acta Trop* 2007; 103: 108-115.
 14. Doanh PN, Shinohara A, Horii Y, Yahiro S, Habe S, Vannavong N, Strobel M, Nakamura S, Nawa Y. Morphological differences and molecular similarities between *Paragonimus bangkokensis* and *P. harinasutai*. *Parasitol Res* 2009; 105: 429-439.
 15. Doanh PN, Hien HV, Nonaka N, Horii Y, Nawa Y. Co-existence of *Paragonimus harinasutai* and *Paragonimus bangkokensis* in Central Viet Nam with special emphasis on their close phylogenetic relationship. *Parasitol Int* 2012; 61: 399-404.

