

## ***Paragonimus ohirai* Metacercariae in Crabs Collected along the Arakawa River in Tokyo, Japan**

Hiromu SUGIYAMA<sup>1</sup>, Yasuyuki MORISHIMA<sup>1</sup>, Yosuke KAMEOKA<sup>2</sup>, Kyoko ARAKAWA<sup>1</sup> and Masanori KAWANAKA<sup>1</sup>

<sup>1</sup>Department of Parasitology and <sup>2</sup>Division of Genetic Resources, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku Ward, Tokyo 162-8640, Japan

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**ABSTRACT.** Brackish water crabs infected with *Paragonimus ohirai* metacercariae have been reported in various regions in Japan. However, infected crabs have not been identified in Tokyo. We therefore collected the crab, *Chiromantes dehaani*, between August 2002 and July 2003 from 12 locations along the Arakawa River that flows through Tokyo. Of the 922 captured crabs, 177 (19%) from 6 locations were infected with *Paragonimus* metacercariae. The prevalence of metacercariae at these 6 locations ranged from 5 to 89%. The number of metacercariae per infected crab ranged from 1 to 190, with an average of 13.1. The morphological features of the metacercariae and of adult worms recovered from test rats infected with metacercariae showed that the metacercariae in the infected crabs were *P. ohirai* Miyazaki, 1939. The ITS2 sequence data support this conclusion. This paper is the first description of *P. ohirai* infection of crabs in Tokyo.

**KEY WORDS:** brackish water crab, *Chiromantes dehaani*, geographical distribution, lung fluke, *Paragonimus ohirai*.

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*Paragonimus* metacercariae of an unknown species were isolated from brackish water crabs in Kumamoto Prefecture, Japan [5]. The novel species was subsequently named *P. ohirai* Miyazaki, 1939 according to the morphological features of metacercariae and adult worms from infected test mammals [6]. Since this discovery, brackish water crabs infected with *P. ohirai* metacercariae have been found in various regions of Japan [7, 8]. However, with respect to the Kanto district, *P. ohirai* metacercariae have been confirmed only in crabs found in the Boso area of Chiba Prefecture [3, 13].

Although crabs infected with *P. ohirai* have not been identified in other areas of the Kanto district, adult *P. ohirai* worms were detected in a stray dog that died in Bunkyo Ward, Tokyo, about 50 years ago [4]. The source of this canine infection remains unknown. We extensively surveyed the distribution of *P. ohirai* metacercariae in brackish water crabs at several locations along the Arakawa River that flows through metropolitan Tokyo.

### **MATERIALS AND METHODS**

**Isolation and examination of *Paragonimus* metacercariae:** Between August 2002 and July 2003, we collected 922 brackish water crabs (*Chiromantes dehaani*, the second intermediate host of *P. ohirai*; formerly referred to as *Sesarma dehaani* or *Holometopus dehaani* [9]) from 12 locations along the banks of the Arakawa River in Tokyo. To examine crabs for *Paragonimus* metacercariae, we removed the carapace and then excised and compressed the midgut gland between two 10 × 6 cm glass plates. The presence of metacercariae was examined under a stereoscopic microscope. The compressed tissues were recovered using

teasing needles or a spatula and then suspended in artificial gastric juice consisting of 1 g of pepsin (Sigma Aldrich Japan, Tokyo, Japan) in 1 l of 0.7% hydrochloric acid. The digest was filtered through 60-mesh wire sieves with a little water. The sediment in the filtrate was repeatedly washed with 0.4% NaCl and poured onto a watch glass. Metacercariae in the sediment were placed on glass slides and pressed slightly under a coverslip for morphological observations and measurements.

**Adult worms from test rats:** We infected 4 male Wistar rats with metacercariae to raise them to adult worms. The rats were necropsied 42 to 70 days after infection to recover worms from the lungs and pleural cavities. Recovered worms were compressed between two glass slides, fixed in 70% alcohol, stained with borax carmine and mounted with Canada balsam for morphological observations and measurements.

**DNA amplification and sequencing of the ITS2 region:** The ribosomal DNA ITS2 region from two metacercariae isolated from each of the 6 positive locations was amplified by PCR and sequenced as described [10]. The primers used were 3S: 5'-GGTACCGGTGGATCACTCGGCTCGTG-3' (forward) and A28: 5'-GGGATCCTGGTTAGTTTCTTTT CCTCCGC-3' (reverse). We aligned and compared sequences using the GENETYX-WIN (ver. 4.0, Software Development Co., Tokyo, Japan) program.

### **RESULTS**

**Prevalence, amount and infection intensity of *P. ohirai* metacercariae in crabs:** We identified *Paragonimus* metacercariae in 177 (19%) of the 922 crabs collected at the following locations: Senju in Adachi Ward, Yahiro in

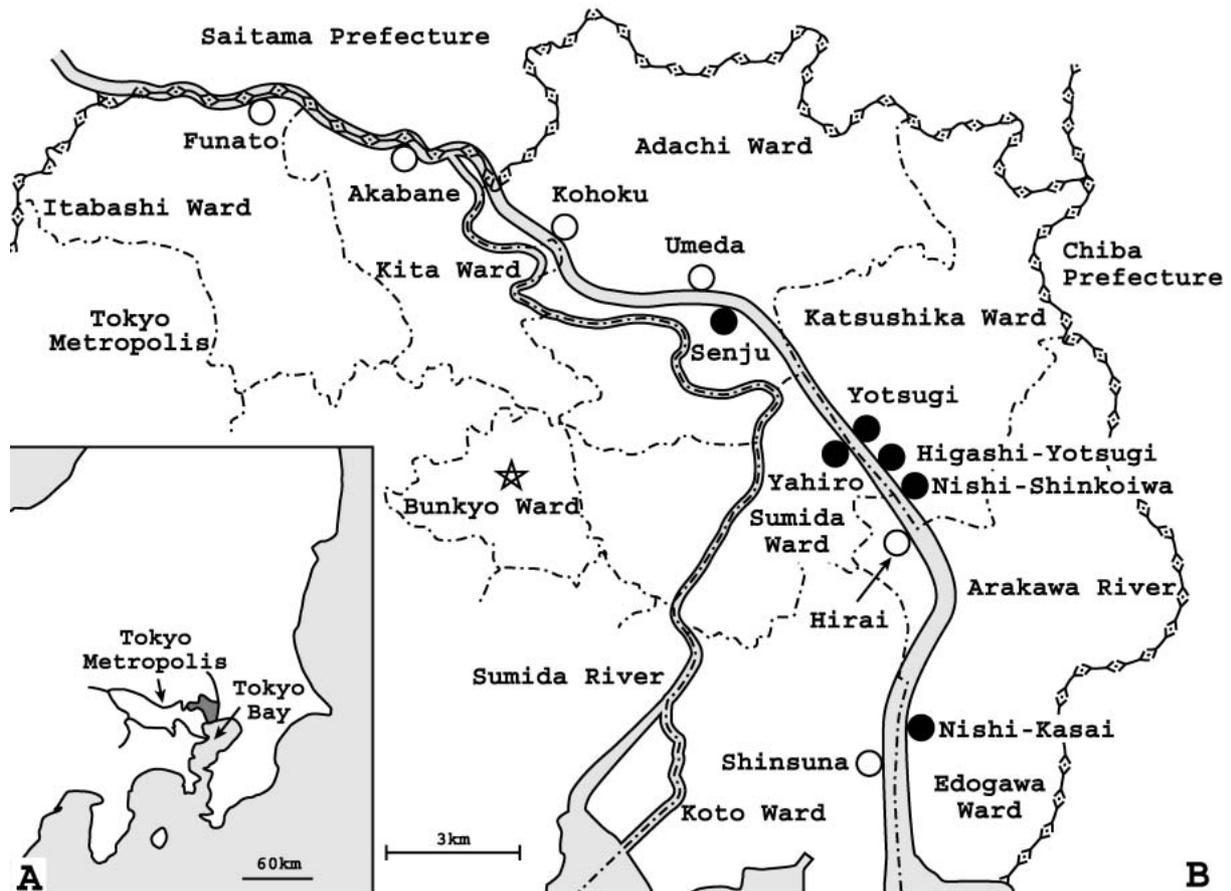


Fig. 1. Map of central Japan. (A) Area examined in this study is eastern Tokyo. (B) Details of study area showing locations where brackish water crabs, *C. dehaani*, were collected. Closed circles, locations of crabs infected with *P. ohirai*; open circles, locations of uninfected crabs; star, Hakan (formerly Koishikawa-Hara-machi) in Bunkyo Ward, where a stray dog infected with *P. ohirai* died about 50 years ago.

Table 1. Prevalence, amount and infection intensity of *P. ohirai* metacercariae in *C. dehaani* crabs from locations along the Arakawa River in Tokyo

Location		No. of crabs		% infected	Total no. of mc <sup>a)</sup> detected	No. of mc per infected crab	
		Examined	Infected			Range	Average
Itabashi Ward	Funato	139	0	—	—	—	—
Kita Ward	Akabane	27	0	—	—	—	—
Adachi Ward	Kohoku	75	0	—	—	—	—
	Umeda	93	0	—	—	—	—
Sumida Ward	Senju	36	3	8	3	1	1.0
	Yahiro	110	86	78	858	1–64	10.0
Katsushika Ward	Yotsugi	37	33	89	1,085	1–190	32.9
	Higashi-Yotsugi	50	35	70	310	1–159	8.9
	Nishi-Shinkoiwa	60	3	5	13	1–11	4.3
Edogawa Ward	Nishi-Kasai	58	17	29	42	1–10	2.5
	Hirai	76	0	—	—	—	—
Koto Ward	Shinsuna	161	0	—	—	—	—
Total		922	177	19	2,311	—	13.1

a) Metacercariae.



Fig. 2. Morphology of a metacercaria. (A) Photomicrograph of a fresh *P. ohirai* metacercaria encysted with a thin outer (O) and a thick inner (I) cyst wall, attached to the liver of the crab host. (B) Photomicrograph of the oral sucker of encysted metacercaria showing a stylet (arrow) dorsally embedded in the oral sucker.

Katsushika Ward, Yotsugi, Higashi-Yotsugi and Nishi-Shinkoiwa in Katsushika Ward, and Nishi-Kasai in Edogawa Ward (Fig. 1). The prevalence of metacercariae at these 6 locations varied from 5 to 89% (Table 1). We isolated 2,311 metacercariae from these 177 infected crabs. The number of metacercariae collected from an infected crab ranged from 1 to 190, with an average of 13.1. The average was 2.5 per crab for all crabs examined. The highest incidence (89%) of metacercarial infection and the largest number (32.9) of the mean burden per infected crab was found in Yotsugi in Katsushika Ward. We did not find *Paragonimus* metacercariae in 745 crabs collected from Funato in Itabashi Ward, Akabane in Kita Ward, Kohoku and Umeda in Adachi Ward, Hirai in Edogawa Ward and Shinsuna in Koto Ward.

We also attempted to collect crabs from the banks of the Sumida River, which diverges from the Arakawa River at Akabane in Kita Ward and flows into Tokyo Bay as one of its tributaries (Fig. 1). However, we did not capture any crabs from this river, probably because most of the banks have been sealed in concrete.

*Morphology of metacercariae from crabs:* *Paragonimus* metacercariae obtained from crabs were spherical, with thin

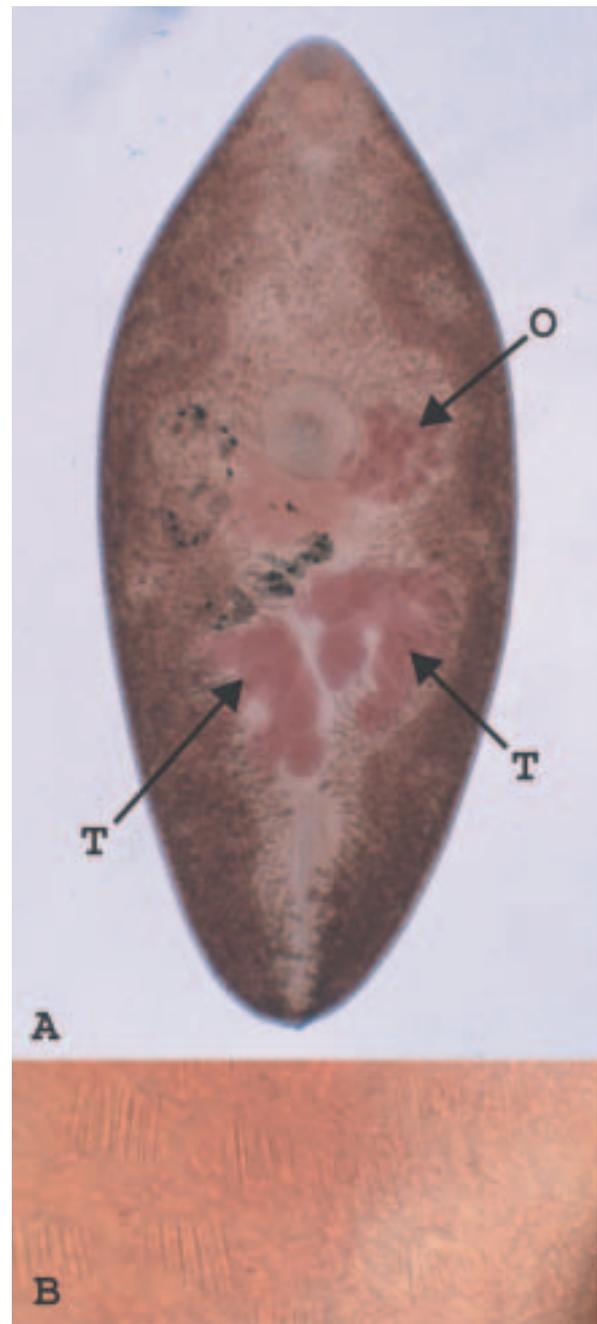


Fig. 3. Morphology of an adult worm. (A) Dorsal view of a mounted adult worm from the lung of the rat 70 days after infection, stained with borax carmine, showing the testes (T) and ovary (O). (B) Photomicrograph of cuticular spines arranged in groups.

outer and thick inner walls (Fig. 2A). The thickness of the inner cyst wall of the 50 specimens from Yotsugi ranged from 2.9 to 5.2  $\mu\text{m}$ , with an average of 4.1  $\mu\text{m}$ . The longitudinal and transverse diameter of the inner cyst freed from the outer cyst ranged from 262 to 331  $\mu\text{m}$  and from 196 to

Table 2. Experimental infection of rats with metacercariae of *P. ohirai*

Rat No.	Source of mc <sup>a,b)</sup>	Dose of mc	Duration of infection (days)	Rate of mc recovered as adult flukes (%)	No. of adult flukes recovered		
					Total	Pleural cavity	Lungs
1	Yahiro	20	42	15	3	0	3
2	Yahiro	20	70	75	15	1	14
3	Yotsugi	20	42	25	5	0	5
4	Nishi-Kasai	20	47	45	9	1	8

a) Location of Arakawa River site where metacercariae-infected crabs were isolated.

b) Metacercariae.

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001: TGTTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAGCA: TKO
001: -----: TNE

061: TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG: TKO
001: .....: TNE

121: TCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTGGCCAGCTGGCGT: TKO
045: .....: TNE

181: GATTTCCCAATCTGACCATGTGTTGGTGGGGTGCCAGATCTATGGCGTTTCCCTAACCT: TKO
105: .....: TNE

241: ATCCGGGCGTACCCATGTTGTGGCTGAAGGCCTTGGTGGGGATGTGGCAACGGAATCGTG: TKO
165: .....: TNE

301: GCTCAGTGGATTATTTATGTGCGCGTTCCGTTGTCTATCATCATCTATGGTTGATGCTG: TKO
225: .....: TNE

361: CGCATGGTGTGCGTCCGACGCCAACCTACGTATGGGCGGTGGCTCATTCTCCTGACCT: TKO
285: .....: TNE

421: CGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA: 463: TKO
345: -----: 363: TNE

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Fig. 4. Sequence of the ITS2 region. The nucleotide sequence of the ITS2 (plus flanking region) obtained from the metacercariae in this study (TKO) was aligned with the ITS2 sequence from *P. ohirai* isolated from the Tanegashima Island of Kagoshima Prefecture, Japan (TNE, accession number: U96911). Identical bases are represented by dots. A hyphen indicates no sequence data. An asterisk denotes the putative ITS2 origin and terminus, thus the ITS2 5' and 3' sequence termini are within the 5.8S and 28S rDNAs, respectively. The numbers refer to the alignment positions.

282  $\mu\text{m}$ , respectively, with an average of  $302 \times 232 \mu\text{m}$ . All larvae had a stylet in the oral sucker and red granules in the body (Fig. 2B).

**Infection of rats with metacercariae and morphology of adult worms:** We necropsied 4 rats 42 to 70 days after inoculation with *Paragonimus* metacercariae collected from 3 locations along the Arakawa River (Table 2). The lungs and pleural cavities of infected rats contained from 3 to 15 adult worms with an average of 8.0. The average size of 20 mounted adult worms from the 4 rats was  $7.4 \times 3.7 \text{ mm}$ , ranging from 6.4 to 8.8 mm in length and from 2.9 to 5.0 mm in width. The ratio of body length to width ranged from 1.5 to 2.4, with an average of 2.0. The average transverse diameters of the oral and ventral suckers were 616 and 792  $\mu\text{m}$ , respectively. The ventral sucker was always larger than the oral sucker and was situated somewhat anterior to the

middle of the worm body. The ovary was intricately ramified with a coral-like appearance (Fig. 3A). The seminal receptacle was small but filled with spermatozoa. The uterus was situated on the opposite side of the ovary. The testes, situated on both sides of the posterior part of the body, were larger than the ovary. The cuticular spines were arranged in groups (Fig. 3B).

**ITS2 sequence analysis:** We sequenced the ITS2 region of the ribosomal DNA from 12 metacercariae. Alignment of these data revealed that all of the ITS2 regions were 463 bp, with no variation in length and composition among the specimens (Fig. 4). Searches of nucleotide databases showed that the ITS2 (plus flanking region) sequence was identical to that of ITS2 from *P. ohirai*, which was isolated from the Tanegashima Island in Kagoshima Prefecture, Japan (GenBank/EMBL/DDBJ accession number: U96911) [1].

## DISCUSSION

In Japan, the lung flukes, *P. westermani*, *P. miyazakii* and *P. ohirai*, have caused symptomatic illness in humans and/or animals. These species can be differentiated by morphological differences seen in fresh metacercariae and mounted adult worm specimens [7, 8].

The *Paragonimus* metacercariae obtained in this study had thin outer and thick inner cyst walls. The stylet in the oral sucker and red granules in the body were identified in all larvae examined. The adult worms from infected rats had intricately ramified ovaries and cuticular spines arranged in groups. These morphological features are consistent only with those of the metacercarial and adult stages of *P. ohirai* [2, 11, 12]. Thus, the *Paragonimus* metacercariae found in this investigation were all identified as *P. ohirai*. The ITS2 sequence data support this conclusion.

About 50 years ago, sporadic *P. ohirai*-infection was identified in a stray dog that lived for 8 months and died in Bunkyo Ward, Tokyo [4] (Fig. 1). However, the source of this canine infection was not determined and since then, *P. ohirai* infection of its second intermediate crab host in Tokyo has not been documented. The data reported here imply that this dog might have become infected with *P. ohirai* by eating crabs from the banks of the Arakawa River.

Though the Arakawa River flows through the densely populated Tokyo metropolitan area, the natural features and abundant greenery of its lower part are protected and promoted. The riverside provides an ideal site for recreation and people often walk their dogs along its banks. In fact, while collecting crabs, we observed dogs with collars running off leash at or near the water's edge. Since we found a high prevalence of *P. ohirai* metacercariae in crabs at many locations along the Arakawa River, dogs in this area might become infected through consuming these crabs. Therefore, dog owners must be persistently educated about the benefits of obeying local regulations when using riverbanks and put dogs on a leash to prevent canine exposure to *P. ohirai*.

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