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Detection of *Paragonimus* Metacercariae in the Japanese Freshwater Crab, *Geothelphusa dehaani*, Bought at Retail Fish Markets in Japan

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Metacercariae, the encysted larval stage of flukes capable of infecting the final and/or paratenic hosts, of *Paragonimus miyazakii* and of both diploid and triploid forms of *P. westermani* are found in the Japanese freshwater crab, *Geothelphusa dehaani*, which acts as the second intermediate host in Japan. This crab is known as Sawagani in Japanese and is widely distributed in Japan, from Hokkaido to Kyushu islands, including Yakushima Island. Both *Paragonimus* spp. are known to be medically important causes of human infection, although the respiratory symptoms that develop in patients vary according to the form and species of the causative lung fluke. Chronic cough with rusty-colored sputum is the most common symptom of patients infected with the triploid form of *P. westermani*, while infection with *P. miyazakii* and the diploid form of *P. westermani* usually causes pleural effusion without remarkable lesions in the lung parenchyma (1).

In Japan, the incidence of *Paragonimus* infection has increased among long-term foreign residents (2,3). It is postulated that long-term residents from Asian countries such as China, Korea and Thailand maintain their dietary habits in Japan and, thus, ingest uncooked Sawagani in their ethnic dishes. Infection of people outside of these groups who eat these dishes has also been reported. There is a need for caution regarding paragonimiasis associated with these eating habits. In some cases, the causative foodstuff included in these dishes was identified as Sawagani sold at local retail fish markets.

In the present study, we purchased Sawagani originating from three prefectures (Shizuoka Prefecture in the Tokai district, and Miyazaki and Nagasaki prefectures in the Kyushu district) at retail fish markets in the Tokyo metropolitan area between April 2004 and February 2008 and examined these crabs for the prevalence of *Paragonimus* metacercariae (Table 1). Lung fluke metacercariae were detected in 44 (17%) of 266 examined crabs. The positive crabs harbored a total of 169 metacercariae, with the average numbers of metacercariae being 3.8 and 0.64 per positive crab and per crab of the total number of crabs examined, respectively. The maximum number of metacercariae in a single crab was 23 in a crab originating in Miyazaki Prefecture that was purchased in February 2008.

Individual metacercariae isolated from the crabs were identified to the species (*P. westermani* or *P. miyazakii*) and, further, to the form (diploid or triploid) for *P. westermani*. The metacercariae of *P. miyazakii* could be morphologically discriminated from those of *P. westermani* by the presence of a membranous substance, as well as by the absence of a stylet (1). Of a total of 169 isolated metacercariae, both of these characteristics were confirmed in only 20 metacercariae, which were identified as *P. miyazakii*. The remaining metacercariae were subjected to molecular identification by PCR-restriction fragment length polymorphism (RFLP) analysis and sequencing. First, the total genomic DNA was prepared from individual metacercariae following our previously described method (4). The ITS2 region of the nuclear ribosomal DNA (rDNA) and a portion of the 16S mitochondrial rDNA were amplified by PCR using primer pairs 3S (forward: 5′-GGTA CCGGTGGATCACTCGGCTCGTG-3′) with A28 (reverse: 5′-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3′) (5) and T7-1 (forward: 5′-ATTTACATCAGTGGGCCGTC-3′) with SP6-1 (reverse: 5′-GATCCAAAAGCATGTGAAAC-3′) (6), respectively. The amplified products were treated with restriction enzymes and separated by electrophoresis on agarose gel (RFLP analysis). For the RFLP analyses, we selected restriction enzymes *Sna*BI and *Bss*SI to digest the ITS2 PCR products from *P. westermani* and *P. miyazakii* (4). We selected enzymes *Sna*BI and *Bsr*DI based on the theoretical restriction maps generated from the 16S mitochondrial rDNA sequences of diploid and triploid forms of *P. westermani* (6,7). Undigested amplicons were sequenced using the corresponding primers to verify the identification made by RFLP analy-

Table 1. Prevalence, number and species of *Paragonimus* metacercariae in Japanese freshwater crabs, *Geothelphusa dehaani*, sold at retail fish markets in the Tokyo metropolitan area, Japan

Month of purchase	Origin (Prefecture)	No. of crabs		No. of Mc[1)] detected	Species[2)] of Mc
		examined	infected		
Apr. 2004	Shizuoka	48	0	0	
Apr. 2007	Miyazaki	46	0	0	
Apr. 2007	Miyazaki	16	7	29	Pm
Apr. 2007	Nagasaki	21	5	9	Pm
June 2007	Shizuoka	35	0	0	
June 2007	Miyazaki	44	5	9	Pw (3n)
Jan. 2008	Miyazaki	30	4	6	Pm, Pw (2n)
Feb. 2008	Miyazaki	26	23	116	Pm
Total		266	44	169	

1): Metacercariae.
2): Pm, *P. miyazakii*; Pw (2n), the diploid form of *P. westermani*; Pw (3n), the triploid form of *P. westermani*.

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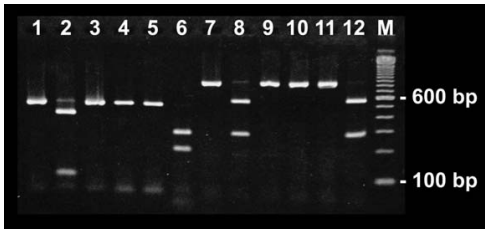

Fig. 1. RFLP patterns of PCR products amplified from the DNA of *P. westermani* metacercariae (lanes 1-3 for both the diploid and triploid forms; lanes 7-9 for the diploid form; lanes 10-12 for the triploid form) or *P. miyazakii* metacercariae (lanes 4-6). The ITS2 PCR products were untreated (lanes 1 and 4) or treated with endonucleases *Sna*BI (lanes 2 and 5) or *Bss*SI (lanes 3 and 6). The 16S rDNA PCR products were also untreated (lanes 7 and 10) or treated with endonucleases *Sna*BI (lanes 8 and 11) or *Bsr*DI (lanes 9 and 12). A 100-bp DNA ladder marker was used to estimate the size of the fragments.

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PCR amplification with the primer pair 3S and A28 generated single 520-bp products from the metacercarial DNA samples. Electrophoresis of the restriction enzyme-digested products resulted in two species-specific RFLP patterns, as previously described (4). Species identification of the metacercariae was made based on the digestion patterns of amplification products. Products that were digested with *Sna*BI to produce 2 fragments (about 420 bp and 100 bp) but remained undigested with *Bss*SI were identified as those of *P. westermani*. Products that were undigested with *Sna*BI but were digested with *Bss*SI to produce 2 fragments (about 300 bp and 220 bp; Fig. 1) were identified as those of *P. miyazakii*.

DNA samples prepared from *P. westermani* metacercariae were further analyzed to determine the form, i.e., diploid or triploid. PCR amplification of mitochondrial DNA with the primer pair SP6-1 and T7-1 produced a single 840-bp product. Restriction digestion of PCR products was used to identify the diploid and triploid forms. Products that were digested with *Sna*BI to produce 2 fragments (about 550 bp and 290 bp) but remained undigested with *Bsr*DI were identified as those of the diploid form. Products that remained undigested with *Sna*BI but were digested with *Bsr*DI to produce 2 fragments (about 560 bp and 280 bp; Fig. 1) were identified as those of the triploid form. The species and forms identified by the RFLP analyses were verified by sequencing of the respective PCR products.

Consequently, as shown in Table 1, most of the metacercariae were identified as *P. miyazakii* (157 metacercariae from 36 positive crabs), while the others were *P. westermani* (3 metacercariae from 3 positive crabs and 9 metacercariae from 5 positive crabs were of the diploid and triploid forms, respectively). However, there were no mixed infections either with *P. miyazakii* and *P. westermani* (diploid and/or triploid forms) or with both forms of *P. westermani* in any crab examined in the present study.

Sawagani from Miyazaki Prefecture were also purchased at a retail fish market in Fukuoka City in April 2008 and were examined for *Paragonimus* metacercariae. *P. miyazakii* metacercariae (35 in total) were detected in 15 of 30 examined crabs. This finding implies that Sawagani with *Paragonimus* metacercariae that are responsible for human infections are likely also sold in retail fish markets in areas other than Tokyo.

The heat resistance of *P. westermani* metacercariae within the crab hosts was investigated almost a century ago (8). The Japanese mitten crab, *Eriocheir japonicus*, which played a major role as the second intermediate host in spreading the human infection of *P. westermani* at that time in Japan was investigated (*P. miyazakii* metacercariae have never been isolated from this crab species). It was shown that boiling infected crabs at 55°C for 5 min killed all the metacercariae (8). However, to the best of our knowledge, the conditions required to kill metacercariae of *P. westermani* and *P. miyazakii* in Sawagani have not yet been well examined, although we are currently investigating these conditions. Therefore, the implementation of a health education campaign is recommended throughout Japan to emphasize that Sawagani, even those sold at retail fish markets, are potential sources of lung fluke infection in humans. Special attention should be paid to ethnic dishes that are prepared with uncooked Sawagani.

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