The Journal of Veterinary Medical Science

NOTE

Parasitology

First report of *Paragonimus skrjabini miyazakii* metacercariae in *Geothelphusa dehaani* (Sawagani) occurring in Iwate Prefecture, Japan

Yuma OHARI[1,2], Yuma SUZUKI[1], Toshiyuki SHIBAHARA[3] and Tadashi ITAGAKI[1,2]*

[1)]Laboratory of Veterinary Parasitology, Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan
[2)]Department of Pathogenetic Veterinary Science, United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[3)]Laboratory of Parasitology, Faculty of Veterinary Medicine, Okayama University of Science, Ikoinooka 1-3, Imabari, Ehime 794-8555, Japan

J. Vet. Med. Sci.
81(8): 1109–1112, 2019
doi: 10.1292/jvms.19-0164

Received: 22 March 2019
Accepted: 2 June 2019
Advanced Epub: 11 June 2019

ABSTRACT. Paragonimiasis is an important food-borne zoonosis caused by *Paragonimus* flukes and is endemic to western Japan. However, there have been few epidemiological studies in the Tohoku district of northeastern Japan. In this study, *Paragonimus* metacercariae (mc) was detected in *Geothelphusa dehaani* (Japanese freshwater crab or Sawagani) in Iwate Prefecture. Out of the 207 Sawagani collected from 35 localities, 12 individuals from six localities were infected with *Paragonimus* mc. The mc were identified as *P. skrjabini miyazakii* based on the sequences of nuclear ribosomal internal transcribed spacer 2 and mitochondrial cytochrome *c* oxidase I. This is the first report of *P. s. miyazakii* mc infection in Sawagani in Iwate Prefecture.

KEY WORDS: *cox1*, *Geothelphusa dehaani*, nuclear ribosomal internal transcribed spacer 2, Iwate Prefecture, *Paragonimus skrjabini miyazakii*

Paragonimiasis is one of the severe food-borne parasitic zoonosis caused by *Paragonimus* flukes, parasitizing the lungs of carnivora and/or omnivorous mammals and humans [2]. In Japan, two *Paragonimus* species, *P. westermani* (including the diploid and triploid forms) and *P. miyazakii*, were recognized as infectious agents in humans [11], and the latter species has been recently referred to as *P. skrjabini miyazakii* [3, 6]. Both the species require two intermediate hosts; the first is a freshwater snail and the second is a freshwater crab [7]. Human paragonimiasis is caused by consuming the uncooked freshwater crabs infected with metacercariae (mc) [7]. Therefore, many epidemiological studies on *Paragonimus* infection in crabs [9] have been carried out from southwestern to central Japan where human paragonimiasis is endemic [8]. On the other hand, epidemiological studies in Tohoku district, northeastern Japan have been limited [9], and there has been no report on *Paragonimus* infection in freshwater crabs in Iwate Prefecture, Japan. This study is the first to report the detection of *P. s. miyazakii* metacercariae (mc) in the fresh water crabs, *Geothelphusa dehaani,* in this region.

A total of 207 Japanese freshwater crabs *G. dehaani*, also called "Sawagani" in Japanese, were collected from mountain streams from 35 localities in Iwate Prefecture from September to October 2016 and from April to September 2017 (Fig. 1, Table 1). The crabs were individually crushed and digested with stirring at 37°C for 15–30 min in 100 m*l* digestive solution (1,000 m*l* distilled water, 1.4 m*l* hydrochloric acid (Wako Pure Chemical Industries, Osaka, Japan), 1.3 g pepsin (proteolytic activity 1:10,000) (MP Biomedicals Inc., Solon, OH, U.S.A.)). The resultant solutions were filtered by a tea strainer for removing shell debris, and the sediments were repeatedly washed with 0.85% NaCl solutions. The resultant sediments were examined for mc under a stereo microscope. The detected mc were measured in diameter using an optical microscope with a digital camera DP26 and imaging software cellSens (Olympus, Tokyo, Japan). The mc were excysted with tweezers and total DNAs were extracted using High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). Polymerase chain reaction (PCR) was performed for the nuclear ribosomal internal transcribed spacer 2 (ITS2) and the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene. The primers used were 3S and A28 for ITS2 [1], and JB3 and JB4.5 for *cox1* [4]. PCR was performed in a final volume of 50 μl containing 1 μl of template DNA, 0.25 μM of each primer, 1 unit of Tks Gflex DNA polymerase (TaKaRa, Kusatsu, Japan), and 25 μl of

*Correspondence to: Itagaki, T.: itagaki@iwate-u.ac.jp

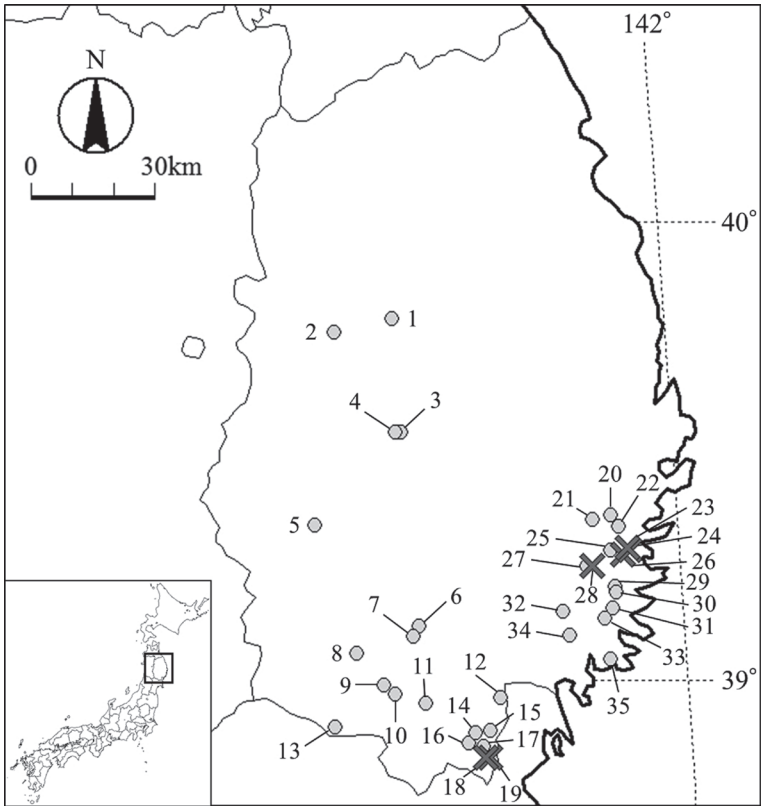

Fig. 1. Map of Iwate Prefecture showing the sampling localities. The black crosses and gray circles show localities from where *Paragonimus skrjabini miyazakii* metacercariae infected and non-infected Sawagani, respectively, were collected. The numbers indicate the locality numbers shown in Table 1.

manufacturer's supplied reaction buffer. Thermal cycling conditions were as follows: 1 min at 94°C, followed by 35 cycles at 98°C for 10 sec, 60°C (ITS2) or 55°C (*cox1*) for 15 sec, and 68°C for 30 sec. PCR amplicons were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), and then sequenced in both directions with the same single PCR primers, using a BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA, U.S.A.) in an ABI 3500 Genetic Analyzer (Applied Biosystems, Waltham, MA, U.S.A.). The resulting sequences were initially assembled using ATGC ver. 6.0.3 (Genetyx Co., Tokyo, Japan) and aligned using Clustal W [14]. The accession numbers of reference sequence used for homology calculations were as follows: *P. s. miyazakii* (ITS2: AB713405, AY618742, U96912; *cox1*: AY618807, AY618809, AY618812-AY618814, AY618816, AY618817, AY618820-AY618823, AY618830, AY618833, U97215), *P. westermani* (ITS2: AB354214, U96907, U96908, U96909, U96910; *cox1*: AB354223, AB354225, AY140671, AY140672, AY140676, AY140682, AY140686, AY140695, U97205), *P. ohirai* (ITS2: U96911; *cox1*: AF008189, U97214) and *P. s. skrjabini* (ITS2: U96913, AY618734; *cox1*: AB325522, AB703455, AY618759-AY618761, AY618783, AY618786, AY618788, AY618789, AY618793-AY618795, AY618798, AY618800, AY618801, AY618805, U97216). A phylogenetic tree based on the *cox1* sequences was constructed employing a Maximum Likelihood method [13]. The tree was constructed based on the Hasegawa-Kishino-Yano model [5] with a proportion of invariant sites, which were selected with the maximum likelihood test based on Akaike's information criteria and evaluated using bootstrap tests with 1000 replications. These analyses were performed using MEGA6 [13].

A total of 39 *Paragonimus* mc were detected in 12 Sawagani from 6 localities (Table 1). The mc were almost circular and their mean diameter (n=25) was 437.5 μm (range 377–485 μm). No stylet was found on their oral suckers. The determined ITS2 sequences (461 bp) were registered to GenBank (LC461214-LC461216) with a single mutation site at the nucleotide position 267. The mutation site was a C in LC461214 (9 mc), a T in LC461215 (3 mc), and heterozygous in LC461216 (27 mc). The homology calculated using the 361 bp ITS2 sequences and ignoring two gap sites was 99.72–100% with *P. s. miyazakii*, 98.61–100% with *P. s. skrjabini*, 92.24% with *P. ohirai*, and 90.86–92.80% with *P. westermani*. The *cox1* sequences (396 bp) were identical among all the mc and registered to GenBank (LC461217). The *cox1* sequence homology (340 bp) was 97.65–100% with *P. s. miyazakii*, 89.71–91.47% with *P. s. skrjabini*, 86.76–87.05% with *P. ohirai*, and 80.00–85.29% with *P. westermani*. In the phylogenetic tree constructed with *cox1* (340 bp), the sequence from this study clustered with those of *P. s. miyazakii* with 100% bootstrap value, and the *P. s. miyazakii* clade was paraphyletic with the *P. s. skrjabini* clade (Fig. 2).

The Sawagani has been reported as the second intermediate host of *P. s. miyazakii* and diploid *P. westermani* in Japan [9]. Although the mean diameter of mc obtained in this study was similar to that of both the species [10], the stylet generally observed in *P. westermani* mc was not observed in this study. The ITS2, which is a useful marker for species discrimination irrespective of

Table 1. Number of collected crabs and detected metacercariae in each locality

No. in Fig. 1	Locality			No. of collected crabs (No. of positives)	Code of metacercariae (Total No. of metacercariae)
	Name	Latitude (N)	Longitude (E)		
1	Takizawa	39.7888130	141.2286940	2 (0)	
2	Morioka	39.7604520	141.0618990	3 (0)	
3	Shiwa	39.5426320	141.2350930	7 (0)	
4	Shiwa	39.5430830	141.2203890	2 (0)	
5	Kitakami	39.3402410	140.9774910	4 (0)	
6	Oshu	39.1197140	141.2550460	9 (0)	
7	Oshu	39.0972990	141.2368660	13 (0)	
8	Oshu	39.0609210	141.0739780	6 (0)	
9	Hiraizumi	38.9911820	141.1459760	8 (0)	
10	Ichinoseki	38.9719680	141.1760270	13 (0)	
11	Ichinoseki	38.9526270	141.2620780	12 (0)	
12	Ichinoseki	38.9638740	141.4719730	4 (0)	
13	Ichinoseki	38.8989690	141.0029290	12 (0)	
14	Ichinoseki	38.8863490	141.3945950	4 (0)	
15	Ichinoseki	38.8913540	141.4371810	2 (0)	
16	Ichinoseki	38.8661980	141.3749440	10 (0)	
17	Ichinoseki	38.8580380	141.4173890	9 (0)	
18	Ichinoseki	38.8341680	141.4207840	8 (1)	Gd094mc02 (1)
19	Ichinoseki	38.8322430	141.4341460	6 (1)	Gd085mc01 (1)
20	Kamaishi	39.3613240	141.8132759	1 (0)	
21	Kamaishi	39.3513811	141.7600270	12 (0)	
22	Kamaishi	39.3374470	141.8330840	1 (0)	
23	Kamaishi	39.2909711	141.8544840	13 (4)	Gd179mc01, Gd180mc01, Gd182mc01, Gd186mc01 (4)
24	Kamaishi	39.2857761	141.8617921	2 (1)	Gd175mc01-Gd175mc04 (4)
25	Kamaishi	39.2837900	141.8072040	6 (0)	
26	Kamaishi	39.2755640	141.8410420	5 (4)	Gd140mc01, Gd141mc01-Gd141mc03, Gd142mc01-Gd142mc12, Gd144mc01-Gd144mn04 (20)
27	Kamaishi	39.2501310	141.7362790	2 (0)	
28	Kamaishi	39.2495400	141.7542680	1 (1)	Gd132mc01-Gd132mc09 (9)
29	Kamaishi	39.2065830	141.8118979	4 (0)	
30	Kamaishi	39.1943590	141.8151961	4 (0)	
31	Ofunato	39.1582100	141.8036351	2 (0)	
32	Ofunato	39.1516700	141.6631762	4 (0)	
33	Ofunato	39.1357050	141.7797031	2 (0)	
34	Ofunato	39.0997160	141.6773439	10 (0)	
35	Ofunato	39.0470749	141.7874241	4 (0)	
	Total			207 (12)	

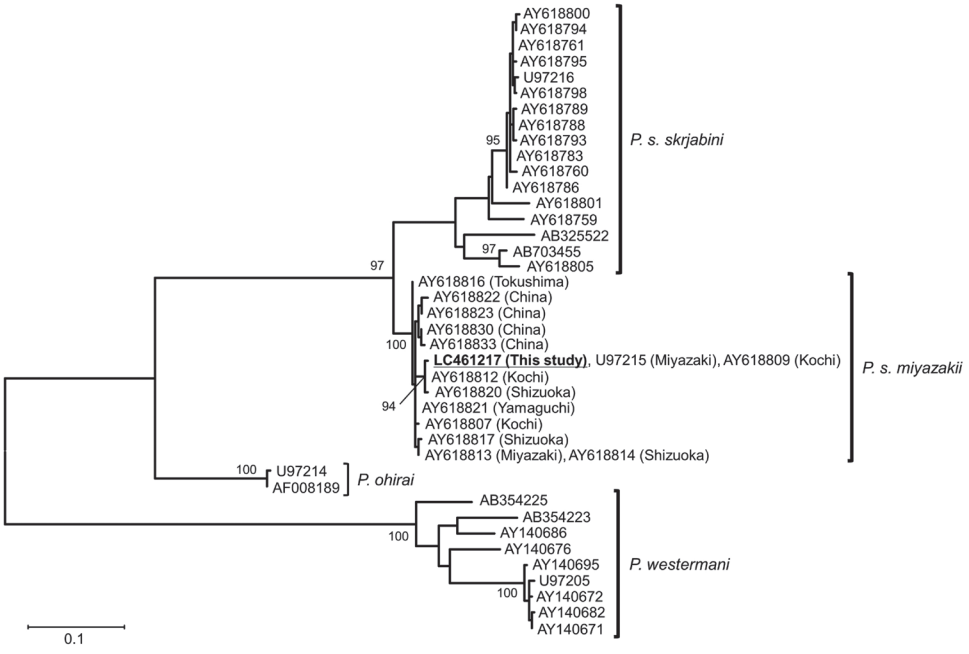

Fig. 2. A maximum likelihood tree constructed based on *cox1* seuences of *Paragonimus* species. The numbers near the nodes indicate bootstrap values (>90%). The scale bar indicates the number of substitutions per sequence position.

the life cycle stages of the lung flukes [1, 12], showed that the present mc were more closely related to *P. s. miyazakii* and *P. s. skrjabini,* members of *P. skrjabini* complex [3], than to *P. westermani* and *P. ohirai*. Moreover, the *cox1* sequence was completely consistent with those (U97215, AY618809) of *P. s. miyazakii* previously detected in Japan, belonged to the *P. s. miyazakii* clade in the phylogenetic tree, and was clearly distinct from the *P. s. skrjabini* clade with high bootstrap value (Fig. 2). The relationships between morphology and genetic characteristics including *cox1* marker among *Paragonimus* species have been confirmed in previous studies [1, 3]. From these results, the mc in this study were identified as *P. s. miyazakii*. This is the first report of *P. s. miyazakii* infection in Sawagani occurring in Iwate Prefecture, Japan and it suggests a possible risk of human paragonimiasis up on consumption of uncooked Sawagani in this region. The first intermediate and definitive hosts of *P. s. miyazakii* in this region remain unknown; therefore, further studies are needed to elucidate the life cycle of *P. s. miyazakii* in Iwate Prefecture.

ACKNOWLEDGMENTS. We are grateful to the members of the Department of Animal Risk Management, Faculty of Risk and Crisis Management, Chiba Institute of Science.

REFERENCES

1. Blair, D., Agatsuma, T., Watanobe, T., Okamoto, M. and Ito, A. 1997. Geographical genetic structure within the human lung fluke, *Paragonimus westermani*, detected from DNA sequences. *Parasitology* **115**: 411–417. [Medline] [CrossRef]
2. Blair, D., Xu, Z. B. and Agatsuma, T. 1999. Paragonimiasis and the genus Paragonimus. *Adv. Parasitol.* **42**: 113–222. [Medline] [CrossRef]
3. Blair, D., Chang, Z., Chen, M., Cui, A., Wu, B., Agatsuma, T., Iwagami, M., Corlis, D., Fu, C. and Zhan, X. 2005. *Paragonimus skrjabini* Chen, 1959 (Digenea: Paragonimidae) and related species in eastern Asia: a combined molecular and morphological approach to identification and taxonomy. *Syst. Parasitol.* **60**: 1–21. [Medline] [CrossRef]
4. Bowles, J., Blair, D. and McManus, D. P. 1995. A molecular phylogeny of the human schistosomes. *Mol. Phylogenet. Evol.* **4**: 103–109. [Medline] [CrossRef]
5. Hasegawa, M., Kishino, H. and Yano, T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* **22**: 160–174. [Medline] [CrossRef]
6. Irie, T., Yamaguchi, Y., Doanh, P. N., Guo, Z. H., Habe, S., Horii, Y. and Nonaka, N. 2017. Infection with *Paragonimus westermani* of boar-hunting dogs in Western Japan maintained via artificial feeding with wild boar meat by hunters. *J. Vet. Med. Sci.* **79**: 1419–1425. [Medline] [CrossRef]
7. Kawashima, K. 2003. Biology of lung flukes. pp. 165–182. *In*: Progress of Medical Parasitology in Japan (Otsuru, M., Kamegai, S. and Hayashi, S. eds.), Meguro Parasitological Museum, Tokyo.
8. Nagayasu, E., Yoshida, A., Hombu, A., Horii, Y. and Maruyama, H. 2015. Paragonimiasis in Japan: a twelve-year retrospective case review (2001–2012). *Intern. Med.* **54**: 179–186. [Medline] [CrossRef]
9. Nishida, H. and Shibahara, T. 2003. Epidemiology of paragonimiasis. pp. 201–217. *In*: Progress of Medical Parasitology in Japan (Otsuru, M., Kamegai, S. and Hayashi, S. eds.), Meguro Parasitological Museum, Tokyo.
10. Nishida, H., Shibahara, T., Torii, M., Okamoto, K., Sakai, M. and Gyoten, J. 1987. Studies on the lung flukes, the genus *Paragonimus* (Trematoda: Troglotrematidae), found from the freshwater crab, *Geothelphusa dehaani* in northern part of Kyoto Prefecture, Japan II. Geographical distribution of the lung flukes in the districts Yosa, Miyazu, Kasa and Maizuru. *Nihon Seibutsu Chiri Gakkai Kaiho* **42**: 15–21 (in Japanese with English abstract).
11. Otsuji, Y. 2003. Paragonimiasis. pp. 183–200. *In*: Progress of Medical Parasitology in Japan (Otsuru, M., Kamegai, S. and Hayashi, S. eds.), Meguro Parasitological Museum, Tokyo.
12. Sugiyama, H., Morishima, Y., Kameoka, Y. and Kawanaka, M. 2002. Polymerase chain reaction (PCR)-based molecular discrimination between *Paragonimus westermani* and *P. miyazakii* at the metacercarial stage. *Mol. Cell. Probes* **16**: 231–236. [Medline] [CrossRef]
13. Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30**: 2725–2729. [Medline] [CrossRef]
14. Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673–4680. [Medline] [CrossRef]