



NOTE

Parasitology

Raccoon dogs (*Nyctereutes procyonoides*) are the new natural definitive hosts of *Metagonimus hakubaensis*

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ABSTRACT. The definitive hosts of *Metagonimus hakubaensis* are reported to be hamsters, rats, mice, dogs, cats, chickens, and quails in experimental infection and Japanese water shrews in natural infection. Here we report that raccoon dogs are new natural definitive hosts of *M. hakubaensis*, based on morphological and molecular analyses of *Metagonimus* flukes collected from the host species from Aomori Prefecture, Japan. Moreover, *M. hakubaensis* recovered from raccoon dogs showed higher fecundity than those recovered from Japanese water shrews. Therefore, raccoon dogs were considered as a more suitable natural definitive host of *M. hakubaensis* than Japanese water shrews.

KEY WORDS: Japanese water shrew, *Metagonimus hakubaensis*, natural definitive host, raccoon dog, suitable definitive host

Metagonimus hakubaensis was initially described in laboratory rats orally inoculated with metacercariae from sand lampreys (*Lethenteron reissneri*) that were collected in Nagano Prefecture, Japan [10]. Previously, only Japanese water shrews (*Chimarrogale platycephala*) have been reported as the natural definitive hosts of *M. hakubaensis* [4]. In contrast, hamsters, rats, mice, dogs, cats, chickens, and quails have been reported as the experimental definitive hosts of *M. hakubaensis* [3].

Raccoon dogs (*Nyctereutes procyonoides*) belong to the family Canidae and have been reported to be natural definitive hosts of *Metagonimus yokogawai*, *Metagonimus takahashii*, and *Metagonimus miyatai*, which are closely related to *M. hakubaensis* [8, 9, 14]. This study was aimed at clarifying whether raccoon dogs are natural definitive hosts of *M. hakubaensis* based on morphological and molecular identification of *Metagonimus* flukes collected from the host animals. The host suitability of *M. hakubaensis* has been evaluated based on the number of intrauterine eggs in the flukes. The flukes from dogs and hamsters had more intrauterine eggs compared with those from other animal species; hence, dogs and hamsters were considered more suitable definitive hosts of *M. hakubaensis* [3]. This study compared the number of intrauterine eggs of *M. hakubaensis* in raccoon dogs and Japanese water shrews, which are the only natural definitive hosts of *M. hakubaensis*, and evaluated the suitability of these hosts for the flukes.

Eight raccoon dogs, designated N1–N8 and seven Japanese water shrews, C1–C7, were collected in Aomori Prefecture, Japan, during 1989–2000, and 1994–1996, respectively. In total, 2,648 *Metagonimus* flukes were recovered from the intestines of 8 raccoon dogs, ranging from 1 to 1,686 per animal (Table 1). In contrast, 5,416 *Metagonimus* flukes were recovered from the intestines of 7 Japanese water shrews (Table 1).

Of the *Metagonimus* flukes, 50 from raccoon dogs and 271 from Japanese water shrews were pressed between a glass slide and a coverslip, fixed in 70% ethanol, stained with alum carmine, and observed for morphological characteristics, such as the oral and ventral sucker size ratio, position of the testes and the uterus containing the eggs, and distribution of the vitelline follicles, which are the key morphological characters distinguishing *Metagonimus* spp. in Japan [2, 6, 7, 10–12]. The remaining flukes were preserved in 70% alcohol without being pressed and stained.

Of the 50 stained flukes from raccoon dogs, 16 had oral suckers slightly smaller than ventral suckers, and their uteri, containing the eggs, were not extended to the posterior extremity between both testes; therefore, they were identified as *M. hakubaensis* (Fig. 1 and Table 1). The remaining 34 flukes had ventral suckers significantly larger than oral suckers, and had uteri extended to the posterior extremity between both testes; therefore, they were identified as *M. miyatai* (Table 1). In contrast, of the 271 stained flukes from

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Table 1. The number of *Metagonimus* flukes recovered from raccoon dogs and Japanese water shrews

Host	Host animal code	Number of stained specimens			Number of unstained specimens	
		<i>M. hakubaensis</i>	<i>M. miyatai</i>	<i>Metagonimus</i> sp.	<i>M. hakubaensis</i>	<i>Metagonimus</i> sp.
Raccoon dog (<i>Nyctereutes procyonoides</i>)	N1	3	0	0	1,683	0
	N2	11	7	0	595	35
	N3	1	13	0	0	45
	N4	1	4	0	0	0
	N5	0	9	0	0	0
	N6	0	0	0	0	178
	N7	0	1	0	0	0
	N8	0	0	0	62	0
	Total	16	34	0	Total 2,340	258
Japanese water shrew (<i>Chimarrogale platycephala</i>)	C1	48	1	12	127	590
	C2	6	0	14	228	658
	C3	0	0	0	443	134
	C4	0	0	0	307	0
	C5	35	0	19	171	422
	C6	49	0	5	244	1,634
	C7	81	0	1	98	89
	Total	219	1	51	Total 1,618	3,527

Japanese water shrews, 219 were identified as *M. hakubaensis*, 1 as *M. miyatai*, and 51 as *Metagonimus* sp. (Table 1).

Of the unstained specimens, 2,340 flukes from raccoon dogs and 1,618 from Japanese water shrews were identified morphologically as *M. hakubaensis*, because only *M. hakubaensis* has a uterus that is not extended to the posterior extremity between both testes among *Metagonimus* spp. [10].

The mitochondrial cytochrome *c* oxidase subunit 1 (mtCOX1) DNA sequence of *M. hakubaensis* was analyzed using 17 and 12 unstained flukes from raccoon dogs and Japanese water shrews, respectively. Genomic DNA from each fluke was extracted using Quick-gDNA™ Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. A 399-bp fragment of the mtCOX1 gene was amplified by polymerase chain reaction (PCR) using primers JB3 and JB4.5 [1]. The PCR amplification consisted of one cycle of denaturation at 98°C for 3 min, followed by 40 cycles of denaturation at 98°C for 30 sec, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Finally, both strands of the PCR products were sequenced directly by Fasmac sequencing service (FASMAC, Atsugi, Japan) using either JB3 or JB4.5. Phylogenetic trees were constructed from mtCOX1 sequences using maximum likelihood as implemented in MEGA X (<https://www.megasoftware.net>). The Tamura 3-parameter model was selected for the best-fit model of maximum likelihood [13]. A bootstrap method, performed in 1,000 replicates, was used to assess the stability of the phylogenetic trees.

Six and four mtCOX1 sequence variants were detected in *M. hakubaensis* flukes from raccoon dogs and Japanese water shrews, respectively; these were registered in GenBank as accession numbers LC576452–LC576461. These variants were classified into the same clade as the reference sequences of *M. hakubaensis* (KM061415, KM061416, and KM061417) in the phylogenetic tree [5] (Fig. 2). These molecular findings strongly supported that the flukes from raccoon dogs and Japanese water shrews were *M. hakubaensis*.

The fecundity of *M. hakubaensis* recovered from raccoon dogs and Japanese water shrews were compared to determine which host species was more suitable as the natural definitive host based on the number of intrauterine eggs. The number of intrauterine eggs in a single *M. hakubaensis* specimen was counted and categorized according to Kudo *et al.* [3]. The number of intrauterine eggs was counted for 16 and 219 stained *M. hakubaensis* flukes from raccoon dogs and Japanese water shrews, respectively (Table 2). *M. hakubaensis* specimens from raccoon dogs had more intrauterine eggs compared with those from Japanese water shrews. Kudo *et al.* [3] reported that *M. hakubaensis* adults possess many eggs in highly suitable host animals. Accordingly, raccoon dogs would be more suitable definitive hosts of *M. hakubaensis* than Japanese water shrews.



Fig. 1. *Metagonimus hakubaensis* specimen recovered from a raccoon dog. Bar=100 µm.

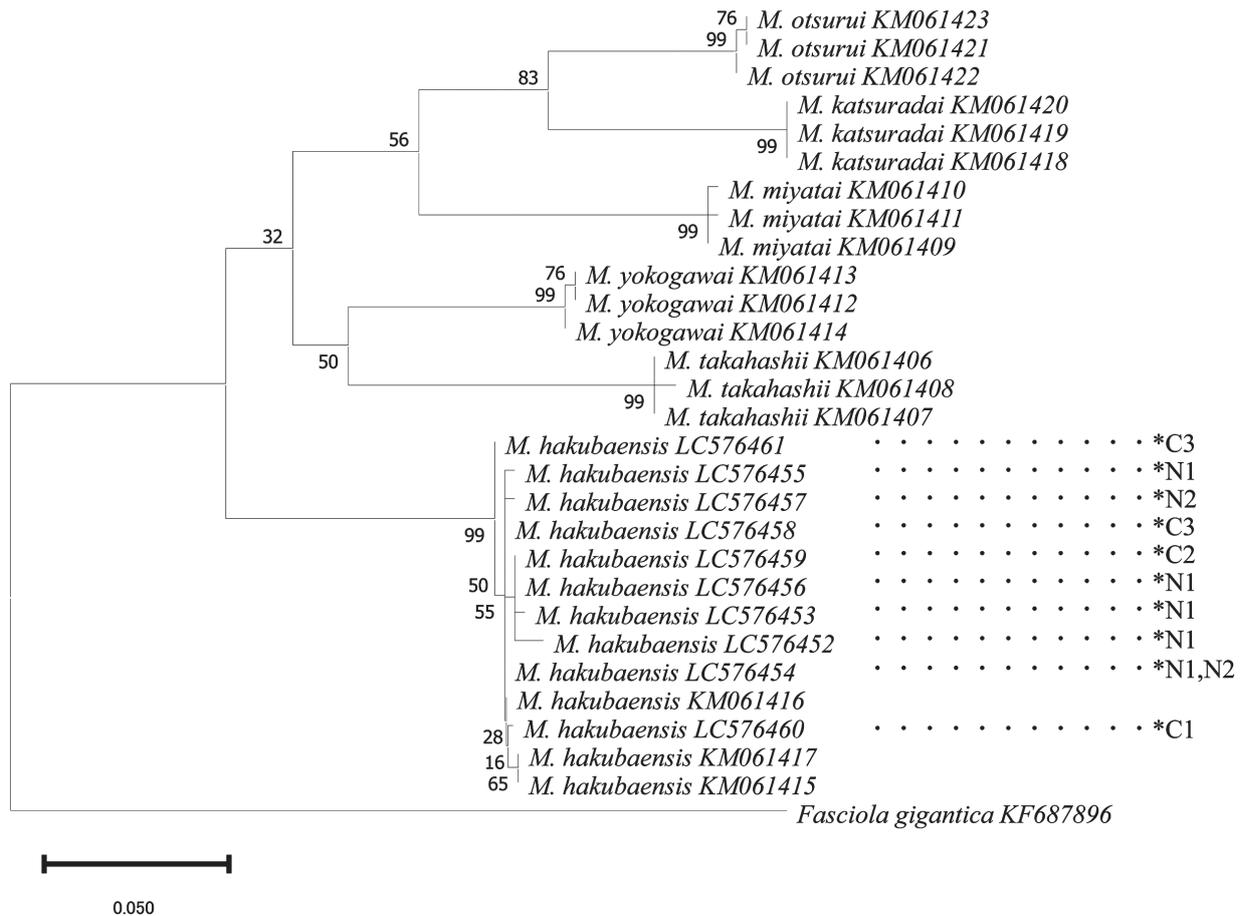


Fig. 2. The phylogenetic tree of *Metagonimus* spp. based on the dataset of mtCOX1 gene sequences. The published sequences of mitochondrial cytochrome c oxidase subunit 1 (mtCOX1) in *M. hakubaensis* (GenBank accession numbers KM061415, KM061416, and KM061417), *M. yokogawai* (KM061412, KM061413, and KM061414), *M. takahashii* (KM061406, KM061407, and KM061408), *M. miyatai* (KM061409, KM061410, and KM061411), *M. otсурui* (KM061421, KM061422, and KM061423), and *M. katsuradai* (KM061418, KM061419, and KM061420) were used [5]. A partial sequence of *Fasciola gigantica* mtCOX1 gene (KF687896) was used as a potential outgroup [15]. Branch lengths are drawn to scale, with the scale bar indicating the number of nucleotide substitutions. **M. hakubaensis* (LC576452–LC576461) recovered from raccoon dogs (N1 and N2) and Japanese water shrews (C1–C3).

Table 2. The different intrauterine egg count in *Metagonimus hakubaensis* recovered from raccoon dogs (*Nyctereutes procyonoides*) and Japanese water shrews (*Chimarrogale platycephala*)

Host	Host animal code	The number of flukes with		
		1–100 eggs/fluke	101–200 eggs/fluke	201–300 eggs/flukes
Raccoon dog (<i>N. procyonoides</i>)	N1	0	2	0
	N2	0	3	8
	N3	0	2	0
	N4	0	0	1
	Total	0	7	9
Japanese water shrew (<i>C. platycephala</i>)	C1	46	2	0
	C2	6	0	0
	C5	32	3	0
	C6	43	6	0
	C7	69	12	0
	Total	196	23	0

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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