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Parasitic helminths from feral raccoons (*Procyon lotor*) in Japan

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Summary

An epidemiological survey of 1688 free-ranging raccoons (*Procyon lotor*) captured on the Japanese main islands of Hokkaido, Honshu and Kyushu was undertaken to determine whether *Baylisascaris procyonis,* which provokes fatal neurological larva migrans was present; however, the worm was not detected in any of these individuals. A helminthological investigation was carried out on 229 of the captured raccoons and the following worms obtained: *Toxocara tanuki*, *Porrocaecum* sp., *Molineus legerae*, *Ancylostoma kushimaense*, *Aonchotheca putorii*, *Centrorhynchus* sp., *Centrorhynchus bazaleticus*, *C. elongatum*, Plagiorhynchidae gen sp., *Hemiechinosoma* sp., *Metagonimus takahashii*, *M. miyatai*, *Euparyphium* sp., *Plagiorchis muris*, *Brachylaima* sp., and *Taenia hydatigena*. These were the first records of *Porrocaecum* sp., *M. miyatai*, *Brachylaima* sp. and *T. hydatigena* obtained from Japanese feral raccoons. Scanning electron microscopic and/or molecular analyses were performed for both *T. tanuki* and *T. hydatigena* as these helminths both have a zoonotic counterpart amongst their families.

Key words: parasitic helminths; *Procyon lotor*; Japan

Introduction

Feral raccoons (*Procyon lotor*) are an invasive alien species in Japan and present a risk for parasitic helminth disease outbreaks. One of the most severe pathogenic agents provoked by the raccoons is the fatal neurological larva migrans caused by *Baylisascaris procyonis* (Nematoda: Ascarididae) (Kazacos, 2001). As raccoons have spread throughout Japan, a large scaled epidemiological investigation of natural helminth infection is extremely important. Initially, a screening survey that involved a naked-eye examination to determine the presence/or absence of this worm was undertaken in 1688 captured individuals. Second, 229 of these raccoons underwent a detailed helminth examination to detect minute zoonotic helminths such as genera *Strongyloides* (Nematoda: Strongyloidae) and *Echinococcus* (Cestoda: Taeniidae).

Materials and Methods

Naked-eye examination for detection of Baylisascaris procyonis

The Hokkaido Government authorized the capture of 1688 raccoons for research purposes in the Ishikari, Sorachi and Hidaka Districts (from 43°20' N to 42°30' N and from 141°15' E to 142°10' E) between April 1999 and September 2005 (Fig.1). The raccoons were transported to the Wild Animal Medical Center (WAMC) at Rakuno Gakuen University (RGU) where they were euthanized. Their bodies were measured and maturity determined by the morphological characteristics of their skulls and canines (Grau *et al*., 1970). Both speciemens from the raccoons collected in Hokkaido were registered and preserved as voucher specimens in the WAMC (RGU), Japan (Matoba *et al.*, 2006).

Entire intestinal tracts were removed from all 1688 raccoons and either stored at -20°C or fixed in 70 % ethanol prior to helminthological examination. Among the tracts, small intestines were examined by naked-eye for the presence/or absence of *B. procyonis*.

Precise helminth examination of gastro-intestines

The intestinal contents of 171 raccoons captured in Nopporo Forest Park (NFP), Ishikari District, and immediately adjacent areas of the park (43°03' N, 141°21' E) , Hokkaido Island, 27 captured in Karuizawa (36°25' N, 138°38' E), Nagano Pref., Honshu Island, 13 captured in Kobe (34°35' N, 135°13' E), Hyogo Pref., Honshu Island, and 18 captured in Sasebo (33°01' N, 129°44' E), Nagasaki Pref., Kyushu Island (Fig. 1) were examined with a dissecting microscope to detect minute helminths such as genera *Strongy-*

loides and *Echinococcus* that are less than ca. 3 mm in size. The raccoons collected from NFP were chosen as representing those from the north of Japan for this analysis as the park is located in Sapporo -the population of Sapporo is about 1,850,000 and is the capital city of Hokkaido- and is an area of natural woods that is a favored ecotourism area on Hokkaido.

Collection of intestinal samples was performed at the WAMC. Once collected, the helminths were placed in lacto-phenol solution and examined morphologically. A camera lucida (OLYMPUS Model BH2-DA) was used to measure the helminths and then voucher specimens were stored at the WAMC (RGU), Japan (Matoba *et al.,* 2006).

To achieve positive identification of male ascarid specimens, especially *Toxocara* or *Baylisascaris,* the key morphological characteristics of the cloacal region were observed. A scanning electron microscope (SEM) was also used for positive identification of this genera. Some ascarids were prepared using standard procedures (see Wiger *et al.,* 1978) and observations were made using a JEOL JSM-Sl electron microscope. Important morphological characteristics of the perianal region of these ascarids were documented photographically.

Molecular examination

Positively identified toxocarids and taeniids were used for molecular biological examination. Total DNA was isolated using an Easy-DNATM isolation kit (Invitrogen) with some modification of the manufacturer's instructions. The partial mitochondorial 12S rRNA gene was amplified by PCR from the genomic DNA using the oligonucleotide primers P60F: 5'-TTAAGATATATGTGGTACAGGATT AGATACCC-3' and P375R: 5'-AACCGAGGGTGACGG GCGGTGTGTACC-3' as reported by Dinkel *et al.* (1998). This primer set was designed for the detection of DNA of *Echinococcus multilocularis* and related tapeworms.

For the detection of DNA from genera *Toxocara*, *Toxoscaris* and *Baylisascaris*, the primers LC1F:5'-CGAGTAT CGATGAAGAACGCAGC-3' and HC2R: 5'-ATATGC TTAAGTTCAGCGGG-3' were used (Yagi *et al.,* 1999). The PCR reaction (50 µl) was performed for 45 cycles, each cycle consisted of denaturation for 1 min at 92°C, annealing for 1 min at 52°C and elongation for 1 min at 72°C. 1.25 units of AmpliTaq GoldTM (Perkin Elmer Co) were used for Taq Polymerase in each reaction. The PCR products were electrophoresed in 1.5 % agarose gel. The DNA fragment was then extracted from the agarose gel, purified using glass beads and sequenced for both DNA strands using a dye-termination kit and model 377, DNA sequencer (Perkin Elmer Co). The nucleotide sequence obtained was aligned with the sequences of the five or six known species of ascaroid nematode reported by Jacobs *et al.* (1997), Zhu *et al.* (1998) and Yagi *et al.* (1999).

Results and Discussion

1. Naked-eye detection of Baylisascaris procyonis

The raccoon roundworm (*B. procyonis*) was not isolated from the gastrointestinal contents of the 1688 individuals collected in the present survey. This worm was also not found in studies done by Miyashita (1992) and Sato and Suzuki (2006) who investigated 25 individuals captured in Osaka Pref. and 531 free-ranging raccoons captured in Wakayama Pref., Honshu Island, respectively. Thus, no raccoon roundworms have been found in the 2244 free-ranging raccoons examined to date. However, the prevalence of roundworms in captive raccoons in Japanese zoos is very high, and outbreak of the fatal neurological larva migrans caused by roundworms in captive mammals has occurred in zoos (Miyashita, 1993; Furuoka *et al*., 2003; Sato *et al*., 2002, 2003, 2005b). It is not uncommon for taxa parasites to occur in free ranging raccoons in Japan: *T. tanuki* and *Porrocaecum* sp. were obtained in the present survey and *Contracaecum rudolphii* (syn. *C. spiculigerum*) was obtained from 5 individuals in a study performed by Sato and Suzuki (2006).

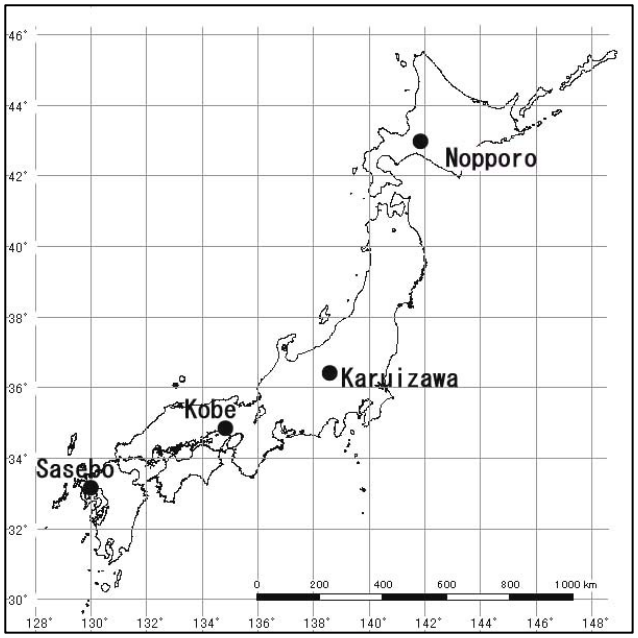

Fig.1. Geographic distribution of feral raccoons examined in the present study

2. Positive identification of toxocarid and taeniid specimens

Although *B. procyonis* was not found in the present survey, two ascarid nematodes and an immature taenid were detected in the small intestine of free-ranging raccoons captured in NFP. As the Ascariidae and Taeniidae families are included in the classification of severe zoonotic helminths, their presence was considered as a positive identification.

Ascarid specimens

One of the ascarids obtained was a mature male: body 33.7 mm in length, 0.8 mm in width; anterior extremity of the body lacked interlabia; oesophagus 3.2 mm in length with

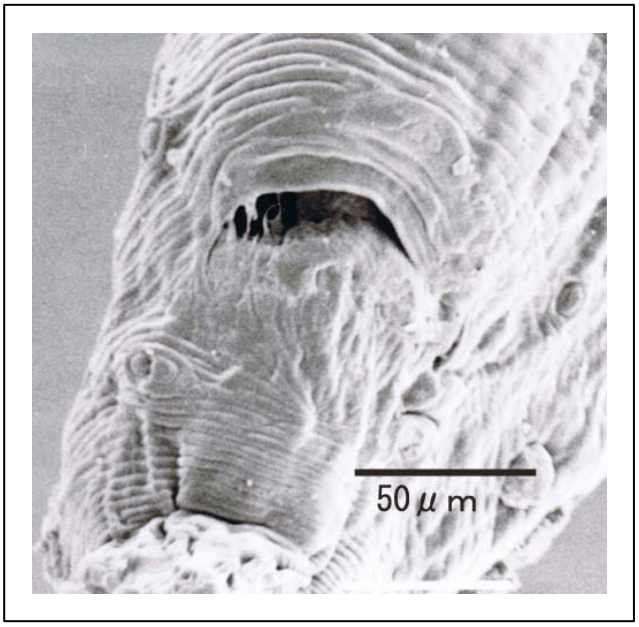

Fig.2. SEM micrograph of *Toxocara tanuki* from a feral raccoon collected in Hokkaido, Japan

cimen with five or six species of ascarid (Figs 3 – 4), this ascarid was completely identical in sequence to *T. tanuki* obtained from a raccoon dog (*Nyctereutes procyonoides*) captured in the Miyazaki Pref. and also a raccoon captured in Sapporo (Yagi *et al*., 1999).

The other ascarid specimens were in larval form, the presence of intestinal cecum meant that they were identical to the genus *Porrocaecum*. However, DNA extraction from these larvae was unsuccessful.

Ascarid nematodes from free-ranging raccoons in Japan have been reported; for example, Sato and Suzuki (2006) reported *Contracaecum rudolphi* (syn. *C. spiculigerum*) from 5 individuals. However, a method for the positive identification of ascarids should be established to support future epidemiological surveys.

Taeniid specimen

This specimen consisted of a scolex and several immature segments but was without genital organs: strobila approximately 4.2 mm in length and 1.2 mm in width; scolex with four suckers, 0.26 mm in diameter and rostellum 0.038 mm in diameter (Fig. 5), two rows of large and small hooks on the rostellum (Fig. 5), the large hooks 15 in number, 0.2 – 0.22 mm in length, and the small ones 15 in number, 0.14

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ascaroid nematode from raccoon GCAGACACAT TGAGCACTAA AATTTCGAAC GCACATTGCG CCATCGGGTT 50
Toxocara canis                 ********** ********** ********** ********** ******C*** 50
T. cati                        ********** ********** C********* ********** ******C*** 50
Toxascaris leonine             ********** ********** ********** ********** ******C*** 50
Baylisasceris procyonis        ********** ********** **A******* ********** ******C*** 50
B. transfuga                   ********** ********** **A******* ********** T********* 50
Toxocara tanuki                ********** ********** ********** ********** ********** 50

ascaroid nematode from raccoon CATTCCCGTT GGCACGTCTG GCTGAGGGTC AGA                    83
Toxocara canis                 ********** ********** ********** **T                    83
T. cati                        ********** ********** ********** ***                    83
Toxascaris leonine             ********** ********** *********T GA*                    83
Baylisasceris procyonis        ********** ********** *********T GA*                    83
B. transfuga                   ********** ********** *********T GA*                    83
Toxocara tanuki                ********** ********** ********** ***                    83
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Fig. 3. Alignment of the partial 5.8 rDNA sequences of an ascaroid nematode collected from a raccoon and 6 known species of ascaroid nematodes

The nucleotide sequences of *Toxocara canis*, *T. cati*, *Toxascaris lenonina* and *Baylisascaris procyonis* are quoted from Zhu *et al.* (1998) and *B. transfuga* and *Toxocara tanuki* are quoted from Yagi *et al.* (1999). The ascaroid nematodes from the raccoon, *Toxocara tanuki* are determined in this study. Asterisks (*) indicate sequence similarity with the raccoon ascaroid nematode. The nucleotide sequence of the ascaroid nematode has been deposited in the DDBJ/EMBL/Genbank nucleotide sequence databases with the accession number AB245964.

ventriculus, but lacked appendices or intestinal caecum in its base; spicules over 2.3 mm in length and lacked tips; cloacal region without a rough area (Fig. 2). Although this species appeared to be identical to *Toxocara tanuki*, we performed molecular analysis on this specimen. According to an alignment of the nucleotide sequences of the partial 5.8SrDNA gene and ITS-2 rDNA from the present specimen with five or six species of ascarid (Figs 3 – 4), this ascarid was completely identical in sequence to *T. tanuki* obtained from a raccoon dog (*Nyctereutes procyonoides*) captured in the Miyazaki Pref. and also a raccoon captured in Sapporo (Yagi *et al*., 1999).

The other ascarid specimens were in larval form, the presence of intestinal cecum meant that they were identical to the genus *Porrocaecum*. However, DNA extraction from these larvae was unsuccessful.

Ascarid nematodes from free-ranging raccoons in Japan have been reported; for example, Sato and Suzuki (2006) reported *Contracaecum rudolphi* (syn. *C. spiculigerum*) from 5 individuals. However, a method for the positive identification of ascarids should be established to support future epidemiological surveys.

Taeniid specimen

This specimen consisted of a scolex and several immature segments but was without genital organs: strobila approximately 4.2 mm in length and 1.2 mm in width; scolex with four suckers, 0.26 mm in diameter and rostellum 0.038 mm in diameter (Fig. 5), two rows of large and small hooks on the rostellum (Fig. 5), the large hooks 15 in number, 0.2 – 0.22 mm in length, and the small ones 15 in number, 0.14 – 0.16 mm in length, respectively. Measurements and morphological characteristics of the scorex of the present specimen were almost the same as those recorded for *Taenia hydatigena* (Abuladze, 1964). Although DNA extraction from this sample was unsuccessful and therefore, this sample could not be molecular biologically identified, the present species was identified as *T. hydatigena*.

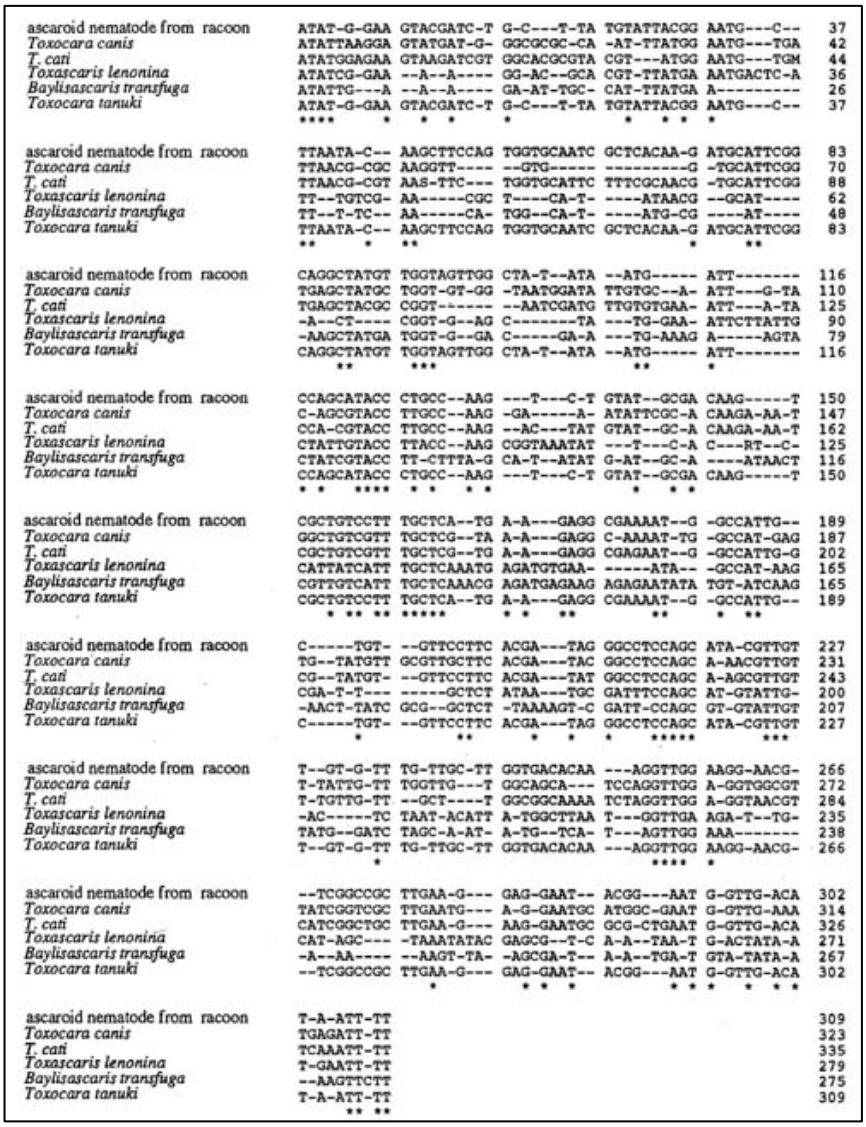

Fig. 4. Alignment of the ITS-2 rDNA sequences of an ascaroid nematode collected from a raccoon and 5 known species of ascaroid nematodes The nucleotide sequences of *Toxocara canis*, *T. cati* and *Toxascaris lenonina* are quoted from Jacobs *et al.* (1997) and *Baylisascaris transfuga* and *Toxocara tanuki* are quoted from Yagi *et al.* (1999). The raccoon ascaroid nematodes, *Toxocara tanuki* are determined in this study. Asterisks (*) indicate alignment of bases that are common to the raccoon ascaroid nematode and the 5 other ascaroid nematode species. The nucleotide sequence of the ascaroid nematode collected from a raccoon has been deposited in the DDBJ/EMBL/Genbank nucleotide sequence databases with the accession number AB245965

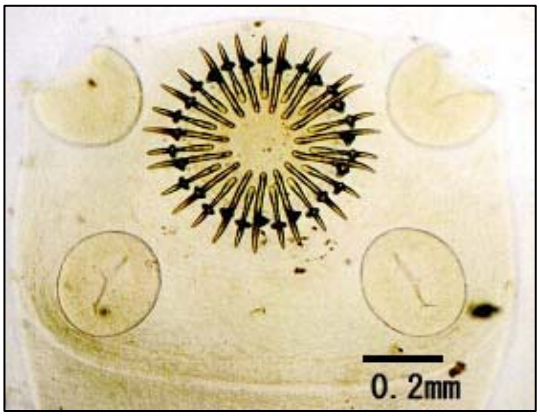

Fig. 5. Scolex of *Taenia hydatigena* obtained from a raccoon in Hokkaido

This is first report of this taeniid species collected from a raccoon in the world; however, Matoba *et al.* (2003) reported *Taenia taeniaeformis* from a free-ranging raccoon in NFP in 2001. The Taeniidae family includes the zoonotic pathogens *Echinococcus* spp. and *Taenia* spp. (Abuladze, 1964); hence, making this report important.

3. Other helminths obtained from free-ranging raccoons

Another 9 helminth species were collected from Hokkaido include: namely, 3 nematode species, *Molineus legerae* (Molineidae), *Ancylostoma kushimaense* (Ancylostomidae) and *Aonchotheca putorii* (Capilariidae); 1 acanthocephalan species, *Centrorhynchus* sp. (Centrorhynchiidae); 5 trematode species, *Metagonimus takahashii* (Metagonimidae), *M. miyatai*, *Euparyphium* sp. (Echinostomidae), *Plagiorchis muris* (Plagiorchiidae) and *Brachylaima* sp. (Brachy-

Tab.1. Gastro-intestinal helminths of free ranging raccoons in Nopporo, Hokkaido (maturity and sex of host was distinguished)

	Juvenile						Adult					
	Female(N=23)			Male(N=22)			Female(N=80)			Male(N=46)		
	Infection rate (%)	Average of parasites	Infection intensity	Infection rate (%)	Average of parasites	Infection intensity	Infection rate (%)	Average of parasites	Infection intensity	Infection rate (%)	Average of parasites	Infection intensity
NEMATODA												
Toxocara sp.	0	0	0	0	0	0	0	0	0	0	0	0
Nematoda larva (*Porrocaecum* sp.)	4.3	1	1	5	1	1	6.25	6.4	1 – 27	2.2	1	1
Molineus legerae	0	0	0	0	0	0	12.5	1.6	1 – 3	19.6	3.3	1 – 9
Ancylostoma kusimaense	0	0	0	5	1	1	12.5	2	2	0	0	0
Aonchotheca putorii	0	0	0	5	1	1	10	1.5	1 – 3	10.9	1.8	1 – 6
ACANTHOCEPHALA												
Centrorhynchus sp.	0	0	0	0	0	0	1.25	1	1	0	0	0
Plagiorhynchus ogatai	0	0	0	0	0	0	0	0	0	0	0	0
Porrorchis oti	0	0	0	0	0	0	0	0	0	0	0	0
Hemiechinosoma sp.	0	0	0	0	0	0	0	0	0	0	0	0
TREMATODA												
Metagonimus sp.	0	0	0	15	27.3	2 – 66	21.3	62.4	1 – 457	15.2	10.5	1 – 29
Euparyphium sp.	17.4	2.5	1 – 4	10	63	6 – 120	36.3	107.3	1 – 1500	41.3	23.4	1 – 183
Plagiorchis muris	0	0	0	0	0	0	2.5	1	1	0	0	0
Brachylaima sp.	3	9	1 – 25	5	3	3	0	0	0	6.5	39	1 – 73
CESTODA												
Taenia taeniaeformis	0	0	0	5	1	1	0	0	0	0	0	0
Taenia hydatigena	0	0	0	0	0	0	1.25	1	1	0	0	0

Tab.2. Gastro-intestinal helminths in free- ranging raccoons in Nopporo, Karuizawa, Kobe, and Sasebo, Japan

	Nopporo (N=171)			Karuizawa(N=27)			Kobe(N=13)			Sasebo(N=18)		
	Infection rate (%)	Average of parasites	Infection intensity	Infection rate (%)	Average of parasites	Infection intensity	Infection rate (%)	Average of parasites	Infection intensity	Infection Rate (%)	Average of parasites	Infection intensity
NEMATODA												
Toxocara sp.	0.6	0.5	1	0.0	0	0	0	0	0	0	0	0
Nematoda larva (*Porrocaecum* sp.)	4.1	4.8	1 – 27	0.0	0	0	0	0	0	0	0	0
Molineus legerae	11.2	1.1	1 – 9	3.7	2	2	15.4	1	1	6.25	1	1
Ancylostoma kusimaense	1.2	1.5	1 – 2	0.0	0	0	0	0	0	6.25	1	1
Aonchotheca putorii	8.2	1.6	1 – 6	0.0	0	0	0	0	0	0	0	0
ACANTHOCEPHALA												
Centrorhynchus sp.	0.6	1	1	3.7	1	1	7.7	1	1	25	4.75	1 – 13
Plagiorhynchus ogatai	0.0	0	0	0.0	0	0	0	0	0	12.5	1	1
Porrorchis oti	0.0	0	0	0.0	0	0	0	0	0	6.25	1	1
Hemiechinosoma sp.	0,0	0	0	0.0	0	0	0	0	0	6.25	1	1
TREMATODA												
Metagonimus sp.	16.5	43.5	1 – 457	0.0	0	0	0	0	0	6.25	32	32
Euparyphium sp.	31.8	68.4	1 – 1520	11.1	4.7	2 – 9	0	0	0	31.3	31.8	1 – 107
Plagiorchis muris	1.2	1	1	0.0	0	0	0	0	0	0	0	0
Brachylaima sp.	4.1	21	1 – 73	0.0	0	0	0	0	0	0	0	0
CESTODA												
Taenia taeniaeformis	0.6	1	1	0.0	0	0	0	0	0	0	0	0
T. hydatigena	0.6	1	1	0.0	0	0	0	0	0	0	0	0

laimiidae) (Tab. 1). *T. tanuki*, *Euparyphium* sp. and *M. takahashii* have previously been reported by Asakawa *et al.* (2000), however, our study is the first report of the other helminth species in Hokkaido, and the first report of *Porocaecum* sp., *M. miyatai*, *Brachylaima* sp. and *T. hydatigena* in Japanese feral raccoons.

The following helminths were collected from raccoons from Honshu and Kyushu: *Molineus* sp., *Metagonimus takahashii*, Echinostomidae gen. sp., *Centrorhynchus bazaleticus*, *C. elongatum*, Plagiorhynchidae gen sp, and *Hemiechinosoma* sp. (Tab. 2). Although Sato *et al.* (2005a) reported 6 acanthocephalan species that principally infest avian species in Wakayama Pref., Honshu Island, they did not find *Hemiechinosoma* sp.. The genus *Hemiechinosoma* that was collected from the present study is also a typical avian acanthocephalan species (Ryzhikov *et al.*, 1985). Previous reports of parasitic helminths collected from feral raccoons captured in Wakayama Pref. have shown that *Strongyloides procyonis* and *Physaloptera* sp. are prominent (Sato & Suzuki, 2006). As *S. procyonis* is a zoonotic nematode (Little, 1965), it was important to examine our specimens for the presence of this nematode; however, our findings were negative.

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