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Molecular detection of filarial nematode parasites in Japanese black bears (*Ursus thibetanus japonicus*) from Iwate Prefecture, Japan

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ABSTRACT. This study aimed to detect filarial parasites in blood samples of Japanese black bears (*Ursus thibetanus japonicus*) collected from Iwate Prefecture, Japan. Positive amplicons were obtained from 26 out of 30 samples by nested PCR targeting 18S ribosomal RNA gene and first internal transcribed spacer regions. DNA sequences of *Mansonella* sp. close to *M. ozzardi* and *Dirofilaria* sp. were detected for eight and 11 positive amplicons, respectively. Co-infection was detected for the remaining seven amplicons. *Dirofilaria* sp. was identified as *D. ursi* by further genetic analysis of 5S ribosomal RNA gene sequence. The results of this study will contribute to further investigations of Japanese black bears for monitoring their risk as a reservoir of possible zoonotic filarial parasites.

KEY WORDS: filaria, Japanese black bear, PCR, phylogenetic analysis

Filarial parasites are members of the phylum Nematoda, and comprise an important species, causing a variety of disorders of medical and veterinary relevance, such as lymphatic filariasis, heartworm diseases, and onchocerciasis [11]. Some species of filarial nematodes are zoonotic parasites; for example, previous studies reported that *Dirofilaria immitis* (heartworm disease in dog and cats), *Onchocerca* sp. (subcutaneous filariasis of many animal species), and *Dirofilaria ursi* (subcutaneous filariasis of black bear) can be responsible for human pulmonary and subcutaneous infections [12–14, 16]. A human case of *D. ursi* infection was recently documented in Fukushima Prefecture, Japan [21]. Therefore, it is important to investigate the existence of filarial parasites in animals, including in wildlife.

The Japanese black bear (*Ursus thibetanus japonicus*) is one of a subspecies of the Asian black bear that inhabits the two main islands of Japan, Honshu, and Shikoku. The bears tend to inhabit broad-leaved deciduous forests, which support their diet, including grasses, berries, and nuts [5]. Due to expansion of their distribution in recent years, conflicts between bears and humans on farms and in villages have occurred. Increasing opportunities for contact with humans may cause zoonotic diseases. Previous studies reported that *Babesia* sp. and *Hepatozoon ursi* were detected from Japanese black bears [6, 8]. Moreover, in 1983, the filarial parasite species, *D. ursi*, *Tetrapetalonema* (*Tetrapetalonema*)*akitensis* sp., and *Dipetalonema* (*Chenofilaria*) *japonica* sp. were detected in blood and tissue of Japanese black bears [19]. However, genetic information regarding the filarial parasites remains limited. In this study, we collected blood samples from Japanese black bears in Iwate Prefecture, a northern region of Honshu (Tohoku area), Japan, and filarial parasites were detected using a universal nested PCR system to identify genetic

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Table 1. Profiles of Japanese black bear used in this study

Sample ID	City	Collection date	Sex	Estimated age (year)	*Mansonella* sp.	*Dirofilaria* sp.	
					18S-ITS1 genotype (accession no.)	18S-ITS1 genotype (accession no.)	5S rRNA genotype (accession no.)
289	Sumita	2011.5.9	Male	12	-	Dirofilaria 18S-ITS1-1 (LC570013)	Dirofilaria5S (LC570024)
290	Morioka	2011.6.2	Female	4	-	Dirofilaria 18S-ITS1-10 (LC570022)	Dirofilaria5S
291	Fudai	2011.7.11	Male	2	-	-	-
292	Kitakami	2011.7.14	Male	4	Mansonella 18S-ITS1-1 (LC570007)	-	-
293	Kamaishi	2011.8.10	Male	5	Mansonella 18S-ITS1-1	Dirofilaria 18S-ITS1-3 (LC570015)	ND
294	Kanegasaki	2011.8.25	Male	9	Mansonella 18S-ITS1-1	Dirofilaria 18S-ITS1-5 (LC570017)	ND
295	Morioka	2011.8.27	Female	10	Mansonella 18S-ITS1-1	-	
296	Morioka	2011.8.27	Male	15	-	Dirofilaria 18S-ITS1-6 (LC570018)	Dirofilaria5S
297	Morioka	2011.8.29	Male	10	-	Dirofilaria 18S-ITS1-9 (LC570021)	Dirofilaria5S
298	Ninohe	2011.8.28	Male	4	Mansonella 18S-ITS1-3 (LC570009)	-	-
299	Morioka	2011.9.2	Male	9	Mansonella 18S-ITS1-5 (LC570011)	-	-
300	Morioka	2011.9.4	Female	10	Mansonella 18S-ITS1-1	-	-
301	Iwate	2011.9.12	Female	4	-	-	-
302	Morioka	2011.9.12	Male	7	Mansonella 18S-ITS1-1	Dirofilaria 18S-ITS1-3	ND
305	Yamada	2012.5.1	Male	9	-	Dirofilaria 18S-ITS1-4 (LC570016)	Dirofilaria5S
306	Yamada	2012.5.3	Male	6	Mansonella 18S-ITS1-2 (LC570008)	-	-
307	Nishiwaga	2012.8.10	Male	4	-	Dirofilaria 18S-ITS1-1	Dirofilaria5S
309	Kamaishi	2012.6.1	Male	7	Mansonella 18S-ITS1-1	Dirofilaria 18S-ITS1-11 (LC570023)	Dirofilaria5S
310	Kamaishi	2012.6.1	Male	8	Mansonella 18S-ITS1-1	Dirofilaria 18S-ITS1-2 (LC570014)	ND
311	Sumita	2012.6.4	Male	4	-	Dirofilaria 18S-ITS1-1	Dirofilaria5S
312	Morioka	2012.6.14	Male	6	-	Dirofilaria 18S-ITS1-2	Dirofilaria5S
313	Morioka	2012.6.17	Female	7	Mansonella 18S-ITS1-1	Dirofilaria 18S-ITS1-2	ND
314	Kuzumaki	2012.8.10	Male	8	Mansonella 18S-ITS1-4 (LC570010)	-	-
315	Kanegasaki	2012.8.4	Female	5	-	Dirofilaria 18S-ITS1-8 (LC570020)	ND
316	Iwate	2012.8.17	Male	5	Mansonella 18S-ITS1-6 (LC570012)	-	-
318	Esashi	2012.8.7	Male	3	-	-	-
319	Yamada	2012.8.22	Male	7	Mansonella 18S-ITS1-2	Dirofilaria 18S-ITS1-7 (LC570019)	ND
321	Miyako	2012.8.27	Male	15	-	Dirofilaria 18S-ITS1-1	ND
322	Morioka	2012.8.26	Female	4	-	Dirofilaria 18S-ITS1-3	ND
327	Ninohe	2012.9.5	Male	6	-	-	-

"-" Means no fragment was amplified for the parasite. "ND" means no positive fragment could be obtained by the PCR targetting 5S rRNA.

characteristics of filarial nematodes found in the bears. A filarial parasite, *Mansonella* sp. [2], was detected from the animals for the first time. Moreover, this is the first documentation of *D. ursi* from the bears in Iwate Prefecture, Tohoku area of Japan.

Blood samples were collected from 30 Japanese black bears hunted for nuisance controls in Iwate Prefecture permitted by the prefectural government between 2011 to 2012 (Table 1). No animals were killed specifically for this study. Whole blood samples were stored at −20°C until further use. No blood smear was examined in this study because the frozen blood samples could not be used for making smears.

Total DNA was prepared from whole blood samples using Quick-gDNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) and stored at −20°C until use. A universal nested PCR was employed for partial DNA sequencing of small subunit ribosomal RNA gene (18S) and first internal transcribed spacer (ITS1) regions (18S-ITS1) [17] of filarial parasites, as described below. The first-round

PCR was performed in a 10 µl volume containing 0.5 µl of stored DNA template, 1 µl of each first primer (10 µM, FIL-1F and UNI-1R) [17], 5 µl of 2 × GoTaq G2 Hot Start Master Mix (Promega, Madison, WI, USA), and 2.5 µl of nuclease-free water. The PCR thermal cycling program consisted of an initial denaturation step for 2 min at 94°C, 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 54°C, and extension for 45 sec at 72°C, with a final extension step for 5 min at 72°C. The second-round PCR was performed using the same conditions, with 1 µl of each second primer (10 µM, FIL-2F and FIL-2R) [17] and 0.5 µl of the first PCR amplification products as template after 10-fold dilution in nuclease-free water. The second-round PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide, and then visualized under ultraviolet light. DNA fragments obtained from the second-round PCR were excised from the gel and purified using a NucleoSpin Gel and PCR Clean-up Kit (MACHEREY-NAGEL, Düren, Germany). The DNA sequences of extracted PCR fragments was determined by the Sanger dideoxy chain termination method (FASMAC, Atsugi-, Japan), using FIL-2F primer. DNA fragments from mixed parasite infections were cloned using DynaExpress TA PCR cloning kit (BioDynamics Laboratory, Tokyo, Japan), and the inserted nucleotide sequences (4 clones) were analyzed. Another PCR targeting 5S rRNA gene of filarial parasites was carried out for *Dirofilaria* sp. detected in this study. Briefly, PCR was performed in a 25 µl volume containing 2 µl of stored DNA template, 0.5 µl of Tks Gflex™ DNA Polymerase (Takara, Kusatsu, Japan), 0.75 µl of each primer (10 µM, S2 and S16) [20], 12.5 µl of 2 × Gflex Buffer (Takara), and 8.5 µl of nuclease-free water. The PCR thermal cycling program consisted of an initial denaturation step for 1 min at 94°C, 40 cycles of denaturation for 10 sec at 98°C, annealing, and extension for 30 sec at 68°C, following the manufacturer's protocol. The PCR amplicons were excised and sequenced from both directions as described above. The DNA sequences obtained by PCR in the present study were compared to reference sequences already registered in the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore/).

Positive DNA fragments of the appropriate size were produced via the universal nested PCR (26/30: 86.7%). The DNA sequences of *Mansonella* sp. close to *M. ozzardi* [18] (similarity by BLAST alignment, 88–90%) were detected in 8 of the 26 samples. The DNA sequences of *Dirofilaria* sp. homologous to *D. repens* (similarity in BLAST alignment, 89–91%) [15] were detected in 11 of the 26 samples. Mixed peaks were observed in the sequence chromatogram of the second-round PCR products from the remaining seven samples. Conventional TA cloning of the second-round PCR products revealed that the seven samples contained the DNA of both *Mansonella* sp. and *Dirofilaria* sp. The six (Mansonella 18S-ITS1-1 to 6) and 11 (Dirofilaria 18S-ITS1-1 to 11) genotypes were determined from *Mansonella* sp. and *Dirofilaria* sp., respectively, and registered with the GenBank database under accession numbers LC570007-LC570023. Genotypes detected from individual hosts are summarized in Table1. No clear association was observed between the parasite detections and the age of bears (Table 1).

A maximum likelihood (ML) tree (Fig. 1) was constructed using MEGA v.10.1.7 [9] including the resultant 18S-ITS1 genotypes of *Mansonella* sp. and *Dairofilaria* sp. with the reference sequences. The sequences of *Mansonella* sp. were included in the clade of *Mansonella* spp. (Fig. 1). *M. ozzardi* causes human mansonelliasis in central and south America, with the blackfly implicated as its vector [7]. A recent report showed that *M. ozzardi*-like nematodes can be detected in black bears (*Ursus americanus*) hunted in the USA [4]. The present study is the first report, which shows the existence of *Mansonella* sp. in Japanese black bears. Human mansonelliasis has never been reported in Japan; however, attention should be maintained to prevent possible zoonotic filariasis.

Next, we investigated the molecular species determination of *Dirofilaria* sp. detected in this study. The sequences of *Dirofilaria* sp. comprised a clade separated from the other filarial parasites (Fig. 1), and the species could not be inferred. A previous study indicated that Japanese black bears could be infected with *Tetrapetalonema akitensis*, and *Dipetalonema japonica* in addition to *D. ursi* [19]. However, no molecular information is available for the former two species. Regarding *D. ursi*, reference DNA sequences were not available for 18S-ITS1 regions. Therefore, we performed PCR targeting the 5S rRNA gene, and an identical sequence (Dirofilaria5S: accession no. LC570024) was obtained from 9 of the 18 positive samples (Table1). The ML tree for the 5S rRNA gene sequences (Fig. 2) suggested that *Dirofilaria* sp. detected in this study were closely related with those of *D. ursi* detected in black bears in the USA [10]. This result strongly suggested that *Dirofilaria* sp. detected in this study was *D. ursi*. Among the 18S-ITS1 genotypes, Dirofilaria 18S-ITS1-11 was independent from the others (Fig. 1), but no variation was observed in 5S rRNA gene (Table 1). Further research will be required to examine the nucleotide diversity of *D. ursi.*

D. ursi is a common filarial parasite of bears in the USA [3, 10], and can be found in the abdominal cavity, subcutaneous tissues, and in the submucosa of the esophagus. *D. ursi* is vectored by blackflies [1], and has the potential to subsequently infect humans. A recent study reported a human case of subcutaneous infection of *D. ursi* in Fukushima Prefecture, Japan [21]. DNA alignment analysis revealed that the sequence of *D. ursi* detected in the present study was almost identical to that obtained in the previous study [21] (Fig. 2). The previous study [21] mentioned the detection of the worm in Japanese black bears in Hyogo Prefecture, the western part of Japan. The present study, on the other hand, is the first documentation of the existence of *D. ursi* in Japanese black bears from Iwate Prefecture, Tohoku area of Japan, which includes Fukushima Prefecture.

In conclusion, we detected DNA of filarial parasites, unidentified *M. ozzardi*-like nematodes, and *D. ursi* in blood samples of Japanese black bears in Iwate Prefecture, Japan. Although the results of the present study exclusively involved molecular information regarding the parasites, we identified novel molecular information, especially for the partial gene sequence of the 18S-ITS1 region of *D. ursi*, and registered them to GenBank. However, in the future, adult worms of both species should be investigated in Japanese black bears to precisely identify the species involved by morphology, and DNA of adult worms should be analyzed to ensure the reliability of the molecular information obtained in the present study. Nevertheless, the results of the present study will contribute to further investigations to reveal filarial parasite burdens in bears for monitoring their risk as reservoirs of possible zoonotic parasites.

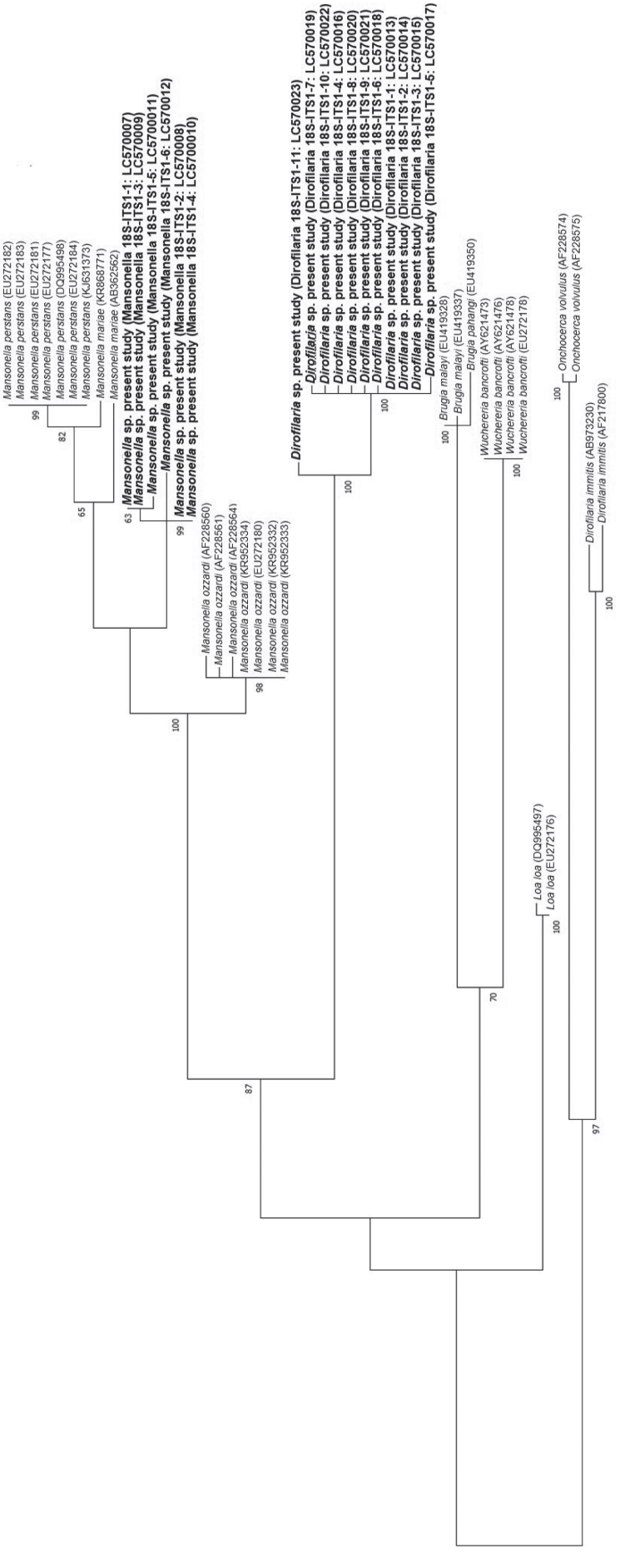

Fig. 1. Maximum likelihood tree of 18S-ITS1 region of filarial parasites detected in this study, along with reference sequences, constructed using MEGA v.10.1.7. Branch lengths correlate to the number of substitutions inferred, according to the scale bar. *Mansonella* sp. detected in this study is highlighted in bold font.

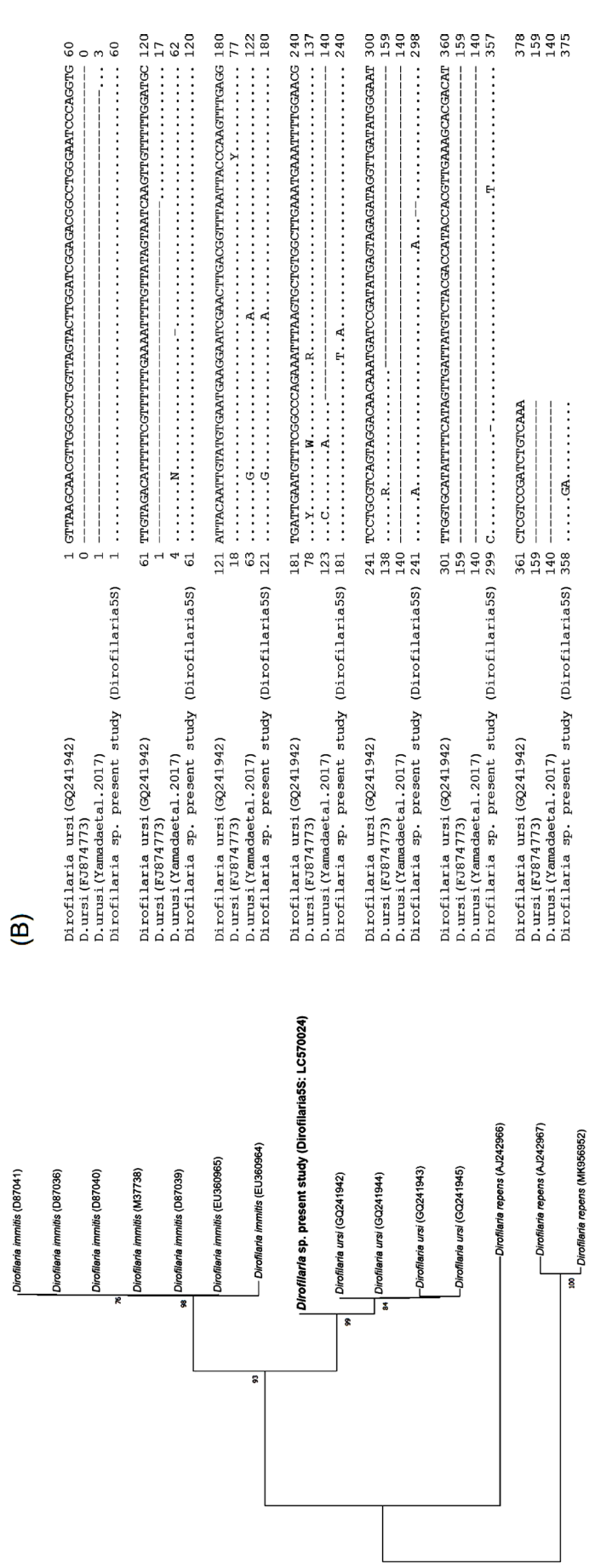

Fig. 2. (A) Maximum likelihood tree of 5S rRNA region of *Dirofilaria* sp. detected in this study, along with reference sequences, constructed using MEGA v.10.1.7. Branch lengths correlate to the number of substitutions inferred, according to the scale bar. *Dirofilaria* sp. detected in this study is highlighted in bold font. (B) Alignment of 5S rRNA region of *Dirofilaria* sp. detected in this study with those of *D. ursi* reference sequences. The sequence of *D. ursi* reported in Yamada *et al.* 2017 [21] was obtained by personal communication. A dot in the alignment indicates that the sequence is identical to that of the reference sequence on top (GQ241942). A dash indicates a deletion or no sequence (a gap) at the position.

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

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