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rDNA ITS sequences of *Uncinaria* spp. from Tsushima leopard cat (*Prionailurus bengalensis euptilura*)

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Summary

Uncinaria spp. parasites were collected from two Tsushima leopard cats found on Tsushima Island, Nagasaki prefecture, Japan. One *Uncinaria* sp. was observed in the large intestines, and the other was observed in the small intestines. The nematode found in the large intestines was determined to be *Uncinaria felidis* by morphological identification. The other *Uncinaria* sp. found in the small intestines was morphologically different from *U. felidis* in the short-length of prevulvar flap. After isolation of genomic DNA of these worms, a second internal transcribed spacer (ITS2) of ribosomal DNA was amplified and sequenced using PCR techniques. The ITS2 region of *U. felidis* had 222 nucleotide sequences in length. The alignments of ITS2 sequence for *Uncinaria felidis* and *Uncinaria sp.* showed one nucleotide (0.45 %) replacement. These differences may be regarded as intraspecific variation.

Keywords: Tsushima leopard; *Uncinaria*; Genomic DNA; ITS2

Introduction

Two species of *Uncinaria* were so far found in the Tsushima leopard cat (*Prionailurus bengalensis euptilura*) by morphometric investigations (Yasuda *et al.*, 1992; Yasuda *et al.*, 1993), but molecular investigations were not done yet. DNA sequencing techniques are nowadays being increasingly used to identify parasites species. The sequencing methods have often been performed on species differentiation of nematodes (Gasser *et al.*, 1996; Chilton & Gasser, 1999; Nadler *et al.*, 2000; e Silva *et al.*, 2006; Hu *et al.*, 2002; Ishibashi, 2003; Otsuka *et al.*, 2004; Mochizuki *et al.*, 2006; Palmer *et al.*, 2007). Of particular value was the development and establishment of sequencing method for ribosomal DNA (rDNA) isolated from fragments of worms and single egg of helminths (Gasser *et al.*, 1993). Internal transcribed spacers (ITS) of rDNA were proposed to be useful target to identify the lineage of species of nematodes and to provide an accurate species marker (Otsuka *et al.*, 2004, Mochizuki *et al.*, 2006). The present study on *Uncinaria* nematodes was therefore focused on screening this genetic target.

Materials and methods

Parasite isolation

The parasites used in this study were detected from two Tsushima leopard cats that were killed in road accidents. One of leopard cats was obtained in November 2006 in Sago, Tsushima city, Nagasaki prefecture (CAT1). The second cat was acquired in January 2007 in Oshika (CAT2) that is about 12 km distance from Sago (Fig. 1). Parasites were isolated from intestinal contents under a stereomicroscope and were preserved in 70 % ethanol at -20 °C. Five worms were collected in the large intestines (group herein denoted as "Worm A"), three worms were found in

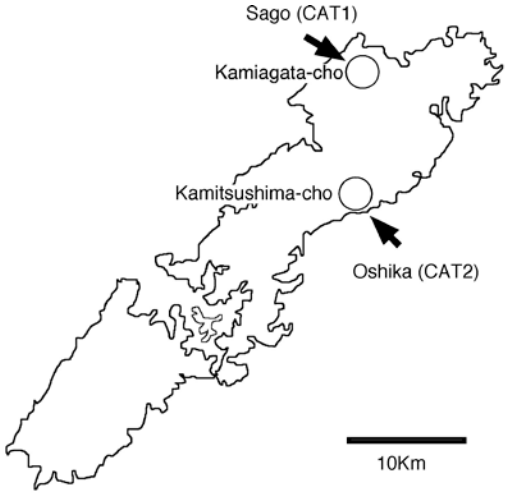

Fig. 1. Collection points of two dead Tsushima leopard cats (CAT1 and CAT2) on Tsushima Island, Japan

Table 1. Parasites used in the study

	Infected organ	Sample name	PCR product	ITS2	ID of Tsushima leopard cat
Worm A	Large intestine	3-2HoL	312bp	222bp	CAT2
		3-2hooL	312bp		CAT2
		6-HoS	312bp		CAT2
		8-HoL	312bp		CAT2
		9-UM2	312bp		CAT1
Worm B	Small intestine	3-2HoS	312bp	222bp	CAT2
		8-4UF	312bp		CAT2
		9-UM3	312bp		CAT1

the small intestines (group "Worm B") (Table 1). All worms were treated with lactophenol for morphological study under the microscope. Each worm was identified following the guidelines reported in Yasuda *et al*. (1992).

rDNA ITS sequence

Genomic DNA was isolated from a single worm by using DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) in accordance with the manufacture's protocol. The ITS2 region of the worm DNA was amplified using the following primers: NC1 (forward: 5'-ACG TCT GGT TCA GGG TTG TT-3'), and, NC2 (reverse: 5'-TTA GTT TCT TTT CCT CCG CT-3') (Gasser *et al.*, 1993). The PCR products were purified using SUPREC^{TM}-02 (Takara, Shiga, Japan). The length of the PCR product from *Uncinaria* spp. isolated from the Tsushima leopard cats was 312 bp, this region was located between partial 5.8S and 28S rRNA genes. The lengths of the ITS2 were 222 bp (Fig. 4). The nucleotide sequences were determined by an automated DNA sequencer (ABI3130xl Genetic Analyzer; Applied Biosystems) using primers NC1 or NC2 in separate reaction with BigDye Terminator v3.1 Cycle Sequencing Kit (PE Biosystems). The sequences were aligned using the GENETYX Version 8.0 (GENETYX, Tokyo).

Results

Worm A had characteristics as follows. The smallest worm was about 4 mm in length. Worms had the anterior extremity inclined dorsally. The mouth was oval, armed with a pair of ventrolateral cutting plates and a pair of dorsolateral cutting plates. A female had a large prevulvar flap and terminal spike (Fig. 2). Based on characteristics described by Hasegawa (1989), the worm A was identified as *Uncinaria felidis*.

Worm B had characteristics as follows. The smallest worm was also about 4 mm in length. The mouth was oval, with

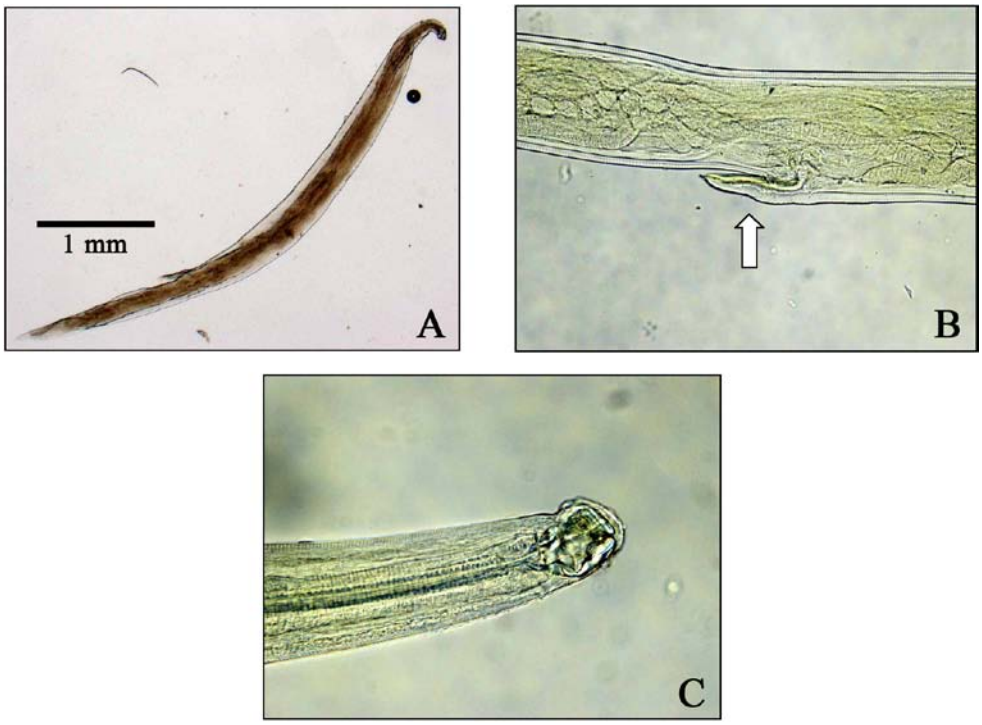

Fig. 2. Worm A photomicrographs
A - whole female worm body; B - prevulvar flap; C - anterior end

Fig. 3. Worm B photomicrographs
A - whole female worm body; B - short prevulvar flap; C - anterior end

a pair of ventrolateral cutting plates and a pair of dorsolateral cutting plates as in previous case. Female worms had a short prevulvar flap (Fig. 3). Worm B was morphologically closer to *U. maya* which was previously found in the small intestine of the Iriomote cat *Prionailurus bengalensis iriomotensis*, although *U. maya* is slightly smaller than Worm B in the body length (Hasegawa, 1989).

The 5' and 3' ends of ITS2 were determined by comparison with the ITS2 sequence of *Ancylostoma duodenale* (GenBank number: AJ001594). The obtained ITS2 sequences in examined isolates were compared with *Uncinaria stenocephala* from the arctic fox (AF194145), *Uncinaria* sp. from the California sea lion (AF217889) and *Uncinaria* sp. from the northern fur seal (AF217890), all originated from the U.S.A. The difference in the ITS2 sequence between Worm A and Worm B was associated only with one transitional nucleotide substitution G/A (i.e. in 0.45 % of bases) (Fig. 4). The differences of ITS2 sequences between Worm A and *Uncinaria stenocephala* (AF194145), *Uncinaria* sp. from California sea lions (AF217889), *Uncinaria* sp. from northern fur seals (AF217890) were 12.9 %, 15.0 %, and 14.2 %, respectively. The differences between Worm B and *Uncinaria stenocephala* (AF194145), *Uncinaria* sp. from California sea lions (AF217889), *Uncinaria* sp. from northern fur seal (AF217890) were 12.9 %, 15.5 % and 14.6 %, respectively (Table 2). Among the five worms allocated to the Worm A set and the three worms allocated to the Worm B set, each ITS2 sequence had 100 % homology.

Table 2. The ITS2 sequence differences comparing each sample with other *Uncinaria* sp. deposited in the GeneBank (%)

	***Uncinaria stenocephala* (arctic fox: AF19415)**	***Uncinaria* sp. (California sea lion: AF217889)**	***Uncinaria* sp. (northern fur seal: AF217890)**
Worm A	12.9	15	14.2
Worm B	12.9	15.5	14.6

Discussion

Given that a number of studies have demonstrated that ITS region provides accurate species markers, the intraspecific variation could be considered low in accordance with previous studies on this marker. The differences in the ITS2 sequences between *U. stenocephala* and *Uncinaria* spp. from the California sea lion and the northern fur seal were 12.4 % and 11.6 %, respectively. The distance of the ITS2 sequence between Worm A and *U. stenocephala* gave the comparable value to that measured between *U. stenocephala* from arctic foxes and *Uncinaria* spp. from California sea lions and northern fur seal. Whereas the distance between Worm A and *Uncinaria* spp. from California sea lions and northern fur seals was slightly higher than that described above for *U. stenocephala*.

In comparison with Worm A, the ITS2 sequence of Worm B had one base pair difference (Fig. 4). Worm B differed from Worm A in the intestinal location of parasitizing, and also manifested clear differences in morphological features (Figs. 2, 3). The question thus arises over whether worm A and B may be regarded as taxonomically different individuals based on these differences. It is difficult to argue that the differences associated with biotope or cat affiliation induced the single base pair difference. *U. maya* para-

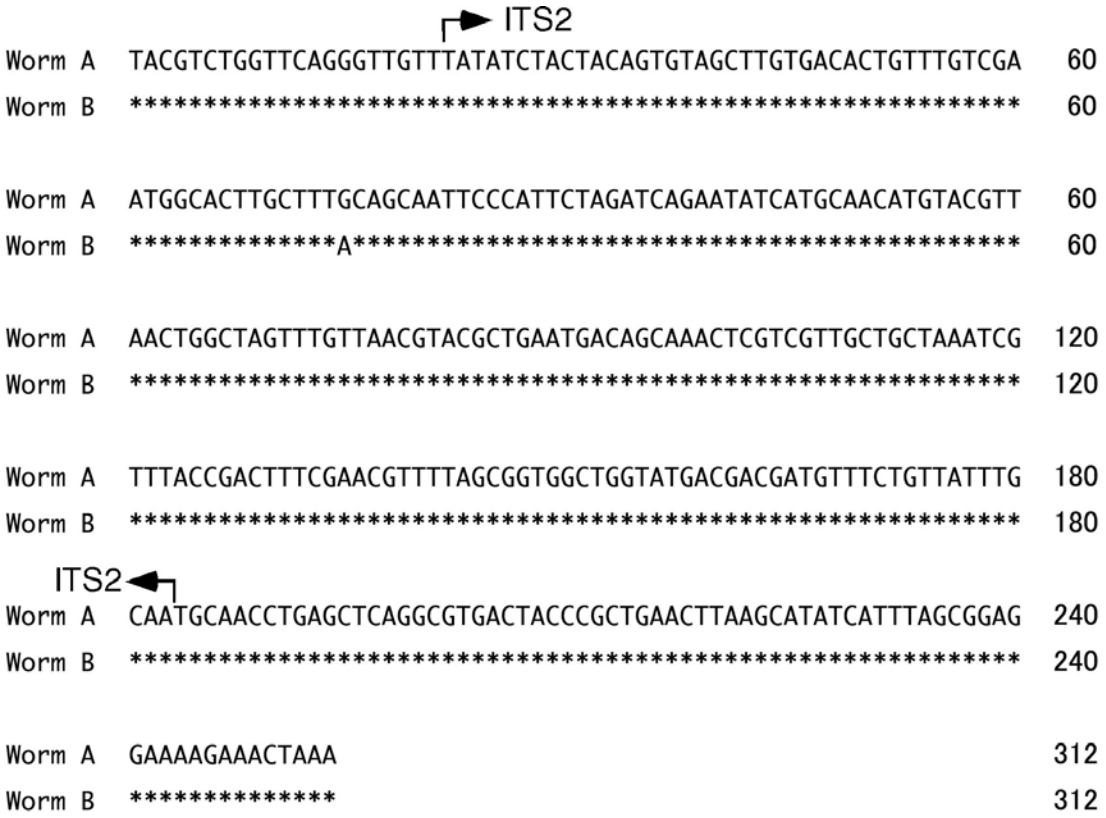

Fig. 4. The alignment of the ITS2 sequence of worm A and worm B

sitizes in the small intestine of the Iriomote cat, and it closely resembles *U. felidis* that inhabits the large intestine of the Leopard cat (Hasegawa & Asato, 1985; Hasegawa, 1989). However, *U. maya* is smaller in body length than *U. felidis* and the prevulvar flap of *U. maya* is shorter than that of *U. felidis*. Thus it has been proposed that *U. felidis* has transformed into *U. maya* with the replacement of the principal host, the Leopard cat into the Iriomote cat (Hasegawa *et al.*, 1985; Hasegawa, 1989). In our study, the 0.45 % difference in the ITS2 sequence between Worm B and Worm A (*U. felidis*) was recorded. Several published reports indicated that the differences up to 1% of ITS sequences could be regarded as attributable to intraspecific variation (Campbell *et al.*, 1995; Romstad *et al.*, 1997; Mochizuki *et al.*, 2006). Thus we presume that Worm B originally belonged to the same species as Worm A (*U. felidis*), and as in the case of *U. maya*, it mutated from Worm A (*U. felidis*) due to adaptation to Tsushima leopard cats that evolved from Leopard cats.

Nadler *et al*. (2000) described differences between *Uncinaria* sp. from California sea lions and that from northern fur seals in molecular and morphometric characters. The authors stressed that not solely morphometric approach, but also molecular techniques are needed to unequivocally classify *Uncinaria* spp. However, the rate of sequence evolution varies according to the lineages, so it is needed to set a boundary, defining the minimum amount of sequence change that merits species status in individual nematode genera (Nadler *et al.*, 2000). The data collected in the present study may contribute to the partial clarification of evolution in *Uncinaria* genus, but the more complex collection of sequence data in these organisms and from various nematode species are required to draw more precise conclusions.

In this study, the sequence data gave us suggestions about evolutionary relationships between the two morphologically different *Uncinaria* sp. from the Tsushima leopard cats. It will be of interest to further study this nematode in additional samples from other Tsushima leopard cats, and to compare the data gathered from parasites harboring by Iriomote wild cats and domestic cats.

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