[RESEARCH NOTE]

Morphological and molecular characterization of sylvatic isolates of *Trichinella* T9 obtained from feral raccoons (*Procyon lotor*)

Tomoko Kobayashi[1,2], Yuta Kanai[3,4], Yuzaburo Oku[3,5], Yohei Matoba[1], Ken Katakura[3] and Mitsuhiko Asakawa[1*]

The genus *Trichinella* (Trichinellidae: Trichinellidea: Enoplida) has a worldwide distribution in domestic and/or sylvatic animals, and it was once believed to be a monospecific group, but this genus now comprises eight species (*Trichinella spiralis, T. nativa, T. britovi, T. murrelli, T. nelsoni, T. pseudospiralis, T. papuae, T. zimbabwensis*) and three additional genotypic variants (T6, T8, T9) that have yet to be taxonomically defined (Pozio and Zarlenga, 2005). Among them, there are two taxa of *Trichinella* in Japan, namely *Trichinella* T9 from raccoon dogs (*Nyctereutes procyonoides viverrinus* and *N. p. albus*), black bears (*Ursus thibetanus*), brown bears (*U. arctos yesoensis*) and foxes (*Vulpes vulpes schrencki*), and *T. nativa* from a red fox (*V. vulpes*) (Nagano *et al*., 1999; Kanai *et al*., 2006). Previous work has shown that raccoons (*Procyon lotor*), which have been introduced from North America since the 1970s, are involved in the sylvatic cycle of *Trichinella* in Japan (Kobayashi *et al*., 2007). We present here additional morphological and molecular analyses of these sylvatic isolates of *Trichinella* from feral raccoons in Hokkaido, Japan.

In the present study, five *Trichinella* isolates that originated from 648 feral raccoons captured in 2004 and 2005 in Hokkaido, Japan were analyzed. *Trichinella* larvae were examined by an artificial digestion method using tongue muscle (Henriksen, 1978). Briefly, individual tongue samples were digested in 1% pepsin-HCl solution with constant gentle stirring for at least 4 hr at 37ºC. After the muscle tissues had been digested, the sediment was allowed to settle and was washed several times. The sediment from the last washing was examined for larvae under a dissection microscope. The larvae were preserved in ethanol for morphological examination and as voucher specimens (Reg. Nos. AS 4324, 5342, 5417, 5498, and 5601) in the Wild Animal Medical Center, Rakuno Gakuen University, Japan. Measurements of cyst size were made from the muscular tissue placed on a glass slide under a dissection microscope. Some of the larvae collected were preserved in TE buffer at –30ºC until use, and DNA was extracted from five single larvae according to a previously described method (Bandi *et al*., 1995; Kanai *et al*., 2006). These larvae were analyzed separately by a multiplex polymerase chain reaction (PCR) following the method of Zarlenga *et al.* (1999). The five sets of primers listed in Table 1 were used for the multiplex PCR with 10 pmol/μl of each primer. Amplification was carried out using *Taq* polymerase (QIAGEN) with Minicycler™ (MJ Research) under the following conditions: preheating at 94ºC for 30 sec, annealing at 58ºC for 30 sec, and elongation at 72ºC for 1 min for 35 cycles. The PCR amplicon was separated by 2.5% agarose gel electrophoresis and stained with ethidium bromide. For further characterization, the nucleotide sequence of a partial mitochondrial cytochrome oxidase subunit I (COI) was determined. Primers (5'-CAC CCA GAA GTA TAC ATC C-3' and 5'-GTA ATA ATA GGT CTA GGG AGG-3') designed based on sequences of *T. nativa* (accession no. DQ007891) and *Trichinella* T9 (DQ 007898) were used for amplification and nucleotide sequencing. PCR was performed under the following conditions: preheating at 94ºC for 5 min, denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min, and elongation at 72ºC for 1 min for 40 cycles. PCR products were purified and directly sequenced using DTCS Quick Start Master Mix (BECKMAN COULTER™) with an automatic sequencer (CEQ™ 8000, BECKMAN COULTER™) according to the manufacturers' instructions. All sequences were aligned using GENETYX-Mac ver. 10.1.4 software.

Table 1. Primer pairs used for multiplex PCR (Zarlenga *et al*., 1999).

Primer pairs	Sequences
I	5'-GTTCCATGTGAACAGCAGT-3
	5'-CGAAAACATACGACAACTGC-3
II	5'-GCTACATCCTTTTGATCTGTT-3
	5'-AGACACAATATCAACCACAGTACA-3
III	5'-GCGGAAGGATCATTATCGTGTA-3
	5'-TGGATTACAAAGAAAACCATCACT-3
IV	5'-GTGAGCGTAATAAAGGTGCAG-3
	5'-TTCATCACACATCTTCCACTA-3
V	5'-CAATTGAAAACCGCTTAGCGTGTTT-3
	5'-TGATCTGAGGTCGACATTTCC-3

Larval cysts which formed in tongue muscles were spindle-shaped and each cyst included a single coiled larva.

[1] Department of Pathobiology, School of Veterinary Medicine, Rakuno Gakuen University, Hokkaido 060-8501, Japan.
[2] Present address: Research Team for Zoonotic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization (NARO), Kannondai, Tsukuba, Ibaraki 305-0856, Japan.
[3] Graduate School of Veterinary Medicine Laboratory of Parasitology Hokkaido University, Hokkaido 060-0818, Japan.
[4] Present address: Section of Viral Infections, Thailand-Japan Research Collaboration Center on Emerging and Re-emerging Infections (RCCERI), Nonthaburi, Thailand.
[5] Present address: Faculty of Agriculture Tottori University, Tottori 680-8553, Japan.
[*] Corresponding author, e-mail: askam@rakuno.ac.jp

Table 2. The measurements of *Trichinella* larvae obtained from the masseter of the raccoon AS4324 (in mm, n = 10).

	Max	Min	Mean	SD
Cyst length	0.47	0.3	0.336	0.046
Cyst width	0.26	0.2	0.233	0.018
Body length	1.25	0.65	1.056	0.185
Body width	0.04	0.03	0.036	0.003
Esophagus	1.12	0.46	0.774	0.036
Stichosome	0.9	0.36	0.61	0.01
Rectum	0.06	0.03	0.041	0.156

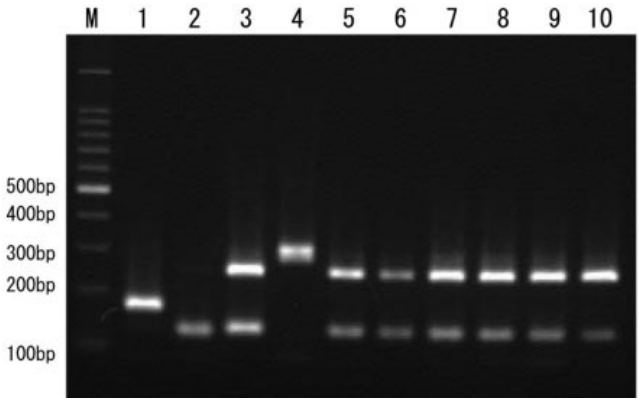

Fig. 1. Electrophoretic pattern after multiplex-PCR amplification of *Trichinella* larvae from feral raccoons and wildlife of Hokkaido Prefecture.
Lane M: 100 bp DNA ladder, lane 1: *T. spiralis* reference larva (code ISS 413), lane 2: *T. nativa* (control, Otofuke fox: Kanai *et al.*, unpublished), lane 3: T9 (control, Sapporo fox: Kanai *et al.* 2007), lane 4: *T. pseudospiralis* (code ISS 13), lane 5: AS5342, lane 6: AS5417, lane 7: AS5498, lane 8: AS4324, lane 9: AS5601, lane 10: T9 (control, Atsuma raccoon dog: Kobayashi *et al.*, unpublished)

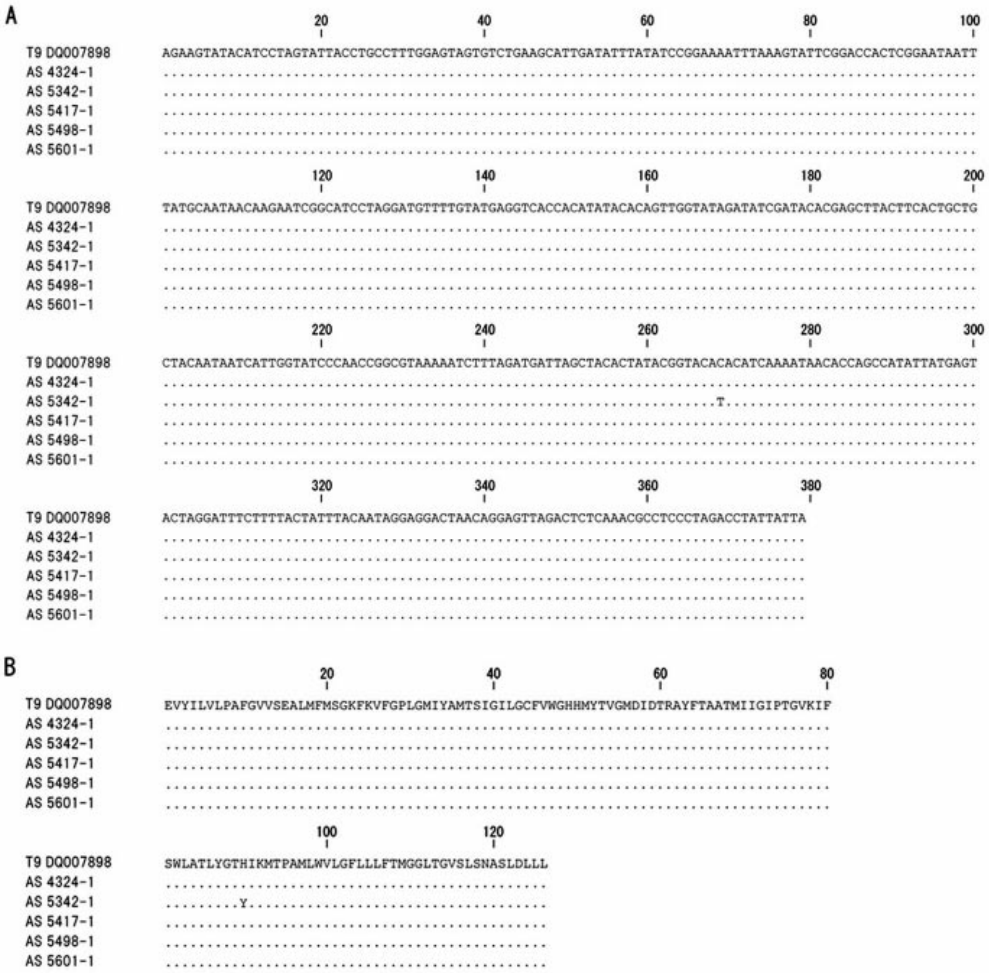

Fig. 1. Alignment of a partial COI sequence of the *Trichinella* larvae.
Nucleotide (A) and amino acid (B) alignments of a partial COI sequence of the *Trichinella* larvae of feral raccoons from Hokkaido with *Trichinella* T9 (accession number DQ007898). Bases that are identical to those of T9 are indicated by dots.

The measurements of the larvae and the cysts are shown in Table 2. Muscle larvae from infected raccoons showed two bands of 127 bp and 253 bp; a pattern specific to *T. britovi* complex (*T. britovi*, T8, T9) (Fig. 1). The nucleotide sequences of a part of the COI gene (379 bp) of larvae from the five raccoons showed highest identities (99.7-100%) to *Trichinella* T9; indeed, the COI sequences of *Trichinella* T9 from five raccoons showed little divergence (Fig. 2). Of the five samples, four (WAMC-AS nos. 4324-1, 5417-1, 5498-1, and 5601-1) had the same sequence (accession number AB 267878), which was completely identical to the previously reported sequences of *Trichinella* T9 from animals of mainland Japan (Nagano *et al*., 1999). A DNA sequence of *Trichinella* T9 from the remaining sample (WAMC-AS no. 5342-1) showed a single nucleotide polymorphism, which resulted in a single amino acid polymorphism (accession number AB 267879). DNA sequencing of the three other larvae from the same raccoons were analyzed by the same method, and showed the same pattern. *Trichinella* larvae from feral raccoons of Japan were identified by multiplex PCR and COI sequence as *Trichinella* T9.

Raccoons are widely distributed throughout North America, and have also been introduced to Russia and Western Europe. Previously, *T. murrelli* (Pozio and La Rosa, 2000) and *T. psudospiralis* (Garkavi and Gineev, 1976) have been reported in feral raccoons of North America and Russia, respectively. *Trichinella murrelli* is the etiological agent of infection in sylvatic carnivores living in temperate areas of the Nearctic region and *T. psudospiralis* is a cosmopolitan non-encapsulated species infecting both mammals and birds. However, neither *T. murrelli* nor *T. psudospiralis* but T9 has been determined in feral raccoons in Japan. Since *Trichinella* T9 has been reported only in wildlife indigenous to Japan (*i.e*., a raccoon dog from Yamagata, a black bear from Aomori, and raccoon dogs and foxes from Hokkaido) (Nagano *et al*., 1999; Kanai *et al*., 2006), the present results suggested that the raccoons tested here acquired the larvae in the natural ecosystem of Japan.

ACKNOWLEGDEMENTS

The present survey was supported in part by Grant-in-Aid (nos. 14560271, 18510205, S0891002) and by the High Technological Research Center (Rakuno Gakuen Univ.) of The Ministry of the Education, Science and Culture of Japan. We are grateful for the raccoon samples provided to us by the local government offices, EnVision, Hokkaido Forest Management Corporation, Nopporo Natural Forest Park Office, and Raccoon Research Group. We are also grateful to Y. Asakawa, K. Suzuki, G. Abe, and M. Sashika for sampling.

LITERATURE CITED

Bandi, C., La Rosa, G., Bardin, M.G., Damiani, G., Cominicini, S., Tasciotti, L. and Pozio, E. (1995) Random amplified polymorphic DNA fingerprints of the eight taxa of *Trichinella* and their comparison with allozyme analysis. Parasitology 110, 401-407.

Garkavi, A. M. and Gineev, B. L. (1976) *Trichinella* in wild animals in northern Caucasus. Voprosy Prirodnoi Ochagovosti Boleznei 8, 136-139.

Henriksen, S. A. (1978) Recovery of *Trichinella spiralis* larvae from frozen muscle samples. Acta Veterinaria Scandinavica 19, 466-468.

Kanai, Y., Nonaka, N., Katakura, K. and Oku, Y. (2006) *Trichinella nativa* and *Trichinella* T9 in the Hokkaido island, Japan. Parasitology International 55, 313-315.

Kobayashi, T., Kanai, Y., Ono, Y., Matoba, Y., Suzuki, K., Okamoto, M., Taniyama, H., Yagi, K., Oku, Y., Katakura, K. and Asakawa, M. (2007) Epidemiology, histopathology, and muscle distribution of *Trichinella* T9 in feral raccoons (*Procyon lotor*) and wildlife of Japan. Parasitology Research 100, 1287-1291.

Nagano, I., Wu, Z., Matsuo, A., Pozio, E. and Takahashi, Y. (1999) Identification of *Trichinella* isolates by polymerase chain reaction--restriction fragment length polymorphism of the mitochondrial cytochrome c-oxidase subunit I gene. International Journal for Parasitology 29, 1113-1120.

Pozio, E. and Zarlenga, D. S. (2005) Recent advances on the taxonomy, systematics and epidemiology of *Trichinella*. International Journal for Parasitology 35, 1191-1204.

Pozio, E. and La Rosa, G. (2000) *Trichinella murrelli* n. sp: etiological agent of sylvatic trichinellosis in temperate areas of North America. The Journal of Parasitology 86, 134-139.

Zarlenga, D. S., Chute, M. B., Martin, A. and Kapel, C. M. O. (1999) A multiplex PCR for unequivocal differentiation of all encapsulated and non-encapsulated genotypes of *Trichinella*. International Journal for Parasitology 29, 1859-1867.

Received January 15, 2011