NOTE *Parasitology*

Distinct Distribution of *Dicrocoelium dendriticum* and *D. chinensis* in Iwate Prefecture, Japan, and a New Final Host Record for *D. chinensis*

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ABSTRACT. This study dealt with the morphological and molecular identification of *Dicrocoelium* flukes obtained from Japanese serow (*Capricornis crispus*) and sika deer (*Cervus nippon centralis*) in the twelve districts of Iwate Prefecture, Japan. *Dicrocoelium dendriticum* and *D. chinensis* were exclusively detected in the western, and coastal and eastern areas of Iwate Prefecture, respectively. This geographically distinct occurrence of the two *Dicrocoelium* species would be associated with the distribution of the final hosts, sika deer for *D. chinensis* and Japanese serow for *D. dendriticum*. This study also reports that *Capricornis crispus* is a new final host of *D. chinensis*.

KEY WORDS: *Capricornis crispus*, *Cervus nippon, Dicrocoelium chinensis*, *Dicrocoelium dendriticum*, new host record

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Lancet flukes of the genus *Dicrocoelium* parasitize in the bile duct and gall bladder of domestic and wild ruminants. Dicrocoeliasis caused by the lancet flukes produces generally mild symptoms and occasional severe economic losses in the domestic industry [6]. Three species, *Dicrocoelium dendriticum*, *D. hospes* and *D. chinensis* are recognized as the causative agents of the diseases. *Dicrocoelium dendriticum* has been reported in Europe, Asia, northern Africa and North America [8], whereas *D. hospes* is distributed in Africa [8] and *D. chinensis* in Eastern Asia and Europe [7, 9].

Dicrocoelium dendriticum(synonym for *D. lancelatum*) and *D. chinensis* occur in Japan, and the former species has been reported in the Japanese serow (*Capricornis crispus*) [5, 12], wild rabbits *(Lepus brachyurus*) [12], Japanese deer (*Sika nippon nippon*, syn. *Cervus nippon centralis*) [13] and sika deer (*Cervus nippon yezoensis*) [2], whereas the latter species has been detected in sika deer (*Cervus nippon centralis*) [9].

This study dealt with the morphological and molecular identification of *Dicrocoelium* flukes obtained from the Japanese serow and sika deer in Iwate Prefecture in northern district of Japan, and herein, we report that the two lancet flukes are distributed in the prefecture and that the Japanese serow is a new final host of *D. chinensis*.

We collected 42 lancet flukes from the bile ducts of 4 Japanese serows and 9 sika deer in the twelve districts (Morioka, Shizukuishi, Nishiwaga, Kawai, Tono, Sumita, Yamada, Otsuchi, Kamaishi, Ofunato, Rikuzentakata and Ichinoseki) of Iwate Prefecture, Japan, during 1993 and 2006, and the flukes were kept in 70% ethanol until analysis (Table 1). The flukes were morphologically identified based on the descriptions of Yamaguti [14], Tang and Tang [10], Otranto *et al*. [7] and Taira *et al*. [9], and discrimination between *D. dendriticum* and *D. chinensis* relied on the testes orientation (tandem in *D. dendriticum* and bilateral in *D. chinensis*).

Molecular identification of the flukes was performed based on the nucleotide sequence of the second internal transcribed spacer (ITS2) of nuclear ribosomal DNA. Total DNA was extracted from individual flukes using E.Z.N.A Mollusc DNA kits (Omega Bio-tek, Doraville, GA, U.S.A.) according to the manufacturer's instruction. DNA fragments were amplified by the polymerase chain reaction (PCR) using ITS2-F and ITS2-R primers [1]. PCR was performed in a 25-μl reaction volume containing 2 μl of DNA template, 0.2 mM of each dNTP, 0.1 μM of each primer, 1.25 U of Go-Taq DNA polymerase (Promega, Madison, WI, U.S.A.) and the manufacturer-supplied reaction buffer. Reaction cycles consisted of an initial denaturing step at 94°C for 90 sec, followed by 30 cycles at 94°C for 90 sec, 53°C for 90 sec and

Table 1. *Dicrocoelium* flukes used in this study

Flukes		Host	
Code	Species	Species	Location
Dd#1–Dd#7	*D. dendriticum*	Japanese serow	Morioka
Dd#8–Dd#13	*D. dendriticum*	Japanese serow	Shizukuishi
Dd#14–Dd#15	*D. dendriticum*	Japanese serow	Nishiwaga
Dc#1–Dc#2	*D. chinensis*	Japanese serow	Rikuzentakata
Dc#3–Dc#5	*D. chinensis*	Sika deer	Ofunato
Dc#6–Dc#8	*D. chinensis*	Sika deer	Kamaishi
Dc#9–Dc#11	*D. chinensis*	Sika deer	Ohtsuchi
Dc#12–Dc#13	*D. chinensis*	Sika deer	Tono
Dc#14–Dc#16	*D. chinensis*	Sika deer	Sumita
Dc#17–Dc#19	*D. chinensis*	Sika deer	Rikuzentakata
Dc#20–Dc#22	*D. chinensis*	Sika deer	Kawai
Dc#23–Dc#25	*D. chinensis*	Sika deer	Yamada
Dc#26–Dc#27	*D. chinensis*	Sika deer	Ichinoseki

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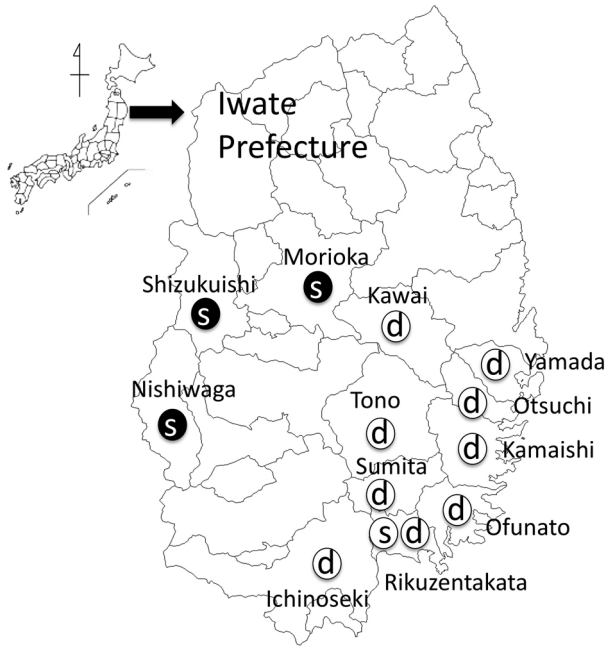

Fig. 1. Twelve districts of Iwate Prefecture in which *Dicrocoelium* flukes were obtained. Black (●) and white (○) circles show the district in which *D. dendriticum* and *D. chinensis* were detected, respectively. "s" and "d" show a Japanese serow and a sika deer, respectively.

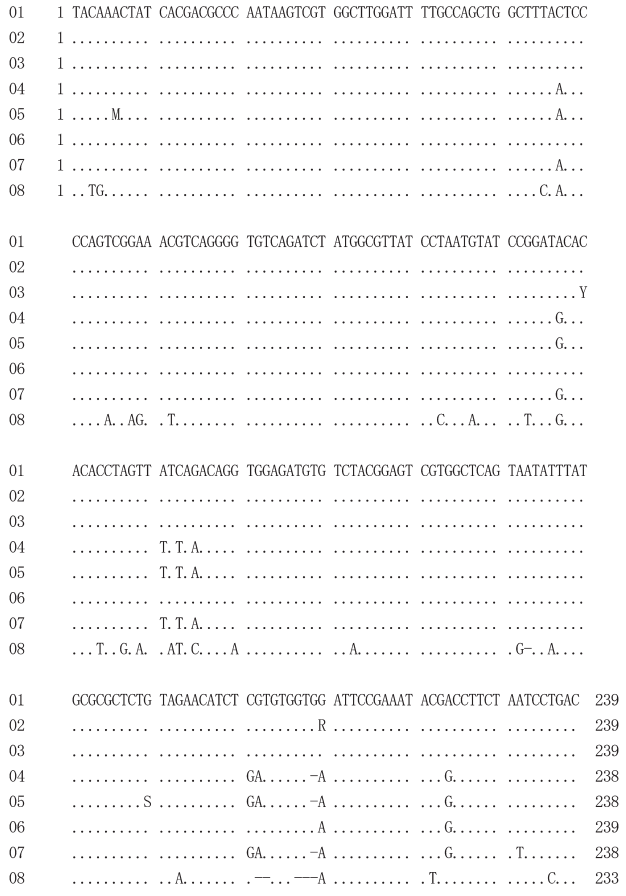

Fig. 2. Sequence alignments of the ITS2 of *D. dendriticum*, *D. chinensis* and *D. hospes*. Dots (.) show the identical nucleotide to the code 01. "-" shows deletion of nucleotide. "M", "R" and "Y" show the nucleotides, "A and C", "G and A" and "C and T", respectively. 01: Dd #1–Dd #5, Dd #7–Dd #9, Dd #11–Dd #15 (AB367789), 02: Dd #6 (AB369980), 03: Dd#10 (AB369981), 04: Dc#1–Dc#18, Dc#20–Dc#27 (AB367790), 05: Dc#19 (AB369982), 06: *D. dendriticum*(DQ379986), 07: *D. orientalis*(EF547132), 08: *D. hospes*(EF102026).

72°C for 120 sec, with a final extension at 72°C for 10 min using the GeneAmp PCR Systems 2700 (Applied Biosystems, Tokyo, Japan). PCR amplicons were precipitated with ethanol / sodium acetate and dissolved in untrapure water and directly sequenced in both directions using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.). The sequencing reactions were run on a 3500 Genetic Analyzer (Applied Biosystems). The ITS2 sequences were aligned and compared with those of *Dicrocoelium* spp. deposited in GenBank using GENETYX ver. 10.0.2 (Genetyx, Tokyo, Japan).

Of the 42 flukes, 15 (Dd#1–Dd#15) from 3 serows in the three western districts (Morioka, Shizukuishi and Nishiwaga) were morphologically identified as *D. dendriticum*, and the remaining 27 (Dc#1–Dc#27) from 9 sika deer and 1 serow in the nine eastern and coastal districts were identified as *D. chinensis*(Table 1, Fig. 1). ITS2 fragments were amplified in the total DNA of the 42 flukes. The ITS2 sequences (239bp) of *D. dendriticum* were identical exclusive of those of the 2 flukes (Dd#6 and Dd#10) from deer in the Kamaishi and Otsuchi districts, which showed heterogeneous nucleotides (Y and R) in the 120 and 210 positions, respectively, from the 5′ terminal of the ITS2 (Fig. 2). The 15 sequences of *D. dendriticum* showed high nucleotide similarities between 99.6% and 100% and similarities between 98.8% and 99.2% with the sequence (DQ379986) of *D. dendriticum* from cattle in the southern Italy. The ITS2 sequences (238bp) of *D. chinensis* were also identical exclusive of that of one fluke (Dc#19) from deer in the Rikuzentakata district, which showed heterogeneous nucleotides (M and S) in the 5 and the 190 positions from the 5′ terminal of the ITS2 (Fig. 2). The sequences of *D. chinensis* showed the similarities within the range of 98.2–99.6% with that (EF547131) of *D. chinensis* from sika deer in Austria. The nucleotide similarities between *D. dendriticum* and *D. chinensis* were within the range of 94.6–96.4%, and those among the two species and *D. hospes*(EF102026) were 86.2–87.0%. Those molecular findings strongly supported species identification based on morphological characteristics of lancet flukes in this study. The sequences of the ITS2 analyzed in this study were deposited in the DNA data bank of Japan (DDBJ) as accession numbers AB367789, AB367790 and AB369980 to AB369982.

This study reconfirmed that morphological and molecular markers (testis orientation and ITS2 sequence) clearly dif-

ferentiate *D. dendriticum* and *D. chinensis* as reported by Otranto *et al*. [7], because the results of morphological and molecular identification of the two lancet flukes were completely identical.

The present study clarified that *D. dendriticum* was detected in the western area and not in the coastal and eastern areas, while *D. chinensis* was detected in the coastal and eastern areas and not in the western area. The previous study also revealed that a single species *D. chinensis* was detected from sika deer in the coastal area of Iwate Prefecture [9]. This geographically distinct occurrence of the two *Dicrocoelium* species would be associated with the distribution of the final hosts, sika deer for *D. chinensis* and Japanese serow for *D. dendriticum*, because in Iwate Prefecture, the distribution of sika deer has been limited in the southeastern coastal area until recently, while the main occurrence of Japanese serow was previously in the Ou mountains of the western area, although the ruminant occurs throughout Iwate [3]. It is therefore, speculated that *D. chinensis* and *D. dendriticum* have spread their distribution together with sika deer and Japanese serow, respectively. Furthermore, this speculation might be also supported by the fact that *D. dendriticum* is the only species detected in Aomori prefecture in which sika deer rarely occurs [12].

Sika deer extensively occurs throughout Japan exclusive of rare occurrence in the Tohoku region and some other areas [3]; however, there have been few reports on detection of *Dicrocoelium* flukes from the ruminant in the other region. Yamaguti [13] detected *Dicrocoelium* flukes from sika deer of Kyoto Prefecture in the western region of Japan and identified them as *D. lanceatum* (syn. *D. dendriticum*). However, the author described that the right and left testes of these flukes located side by side, showing characteristic of *D. chinensis* and possibility as the species. On the other hand, Kitamura *et al*. [2] detected *D. dendriticum* from sika deer (*C. n. yezoensis*) that is a subspecies of *Cervus nippon* and definitely distributed in Hokkaido, Japan. Further, Yagi *et al*. [11] recovered *Dicrocoelium* flukes that could not be identified accurately as *D. chinensis* in morphology from wild rodents (*Clethrionomys rufocanus bedfordiae* and *C. rutilus mikado*) in Hokkaido. In this report, the authors mentioned that the testes of the flukes located obliquely or tandemly, suggesting possibility of *D. dendriticum*. These reports indicate no distribution of *D. chinensis* in Hokkaido. Interestingly, sika deer populations derived from Hokkaido and Iwate, and possibly from Kyoto, closely relate to each other in molecular phylogeny [4]. These findings indicate that *D. chinensis* has not been introduced into Hokkaido together with geographical isolation of sika deer (*C. n. yezoensis*).

The present study revealed that a Japanese serow from Rikuzentakata of the coastal area was infected with *D. chinensis*. *Dicrocoelium chinensis* has been detected in sheep in China [10], several cervid species, such as musk deer (*Moschus moschiferus*), in the Soviet Union, mouflon (*Ovis ammon musimon*) and roe deer (*Capreolus capreolus*) in Austria and Italy [7], and sika deer (*Cervus nippon centralis*) in Japan [9]. We herein report that Japanese serow (*Capricornis crispus*) is a new final host of *D. chinensis*.

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