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NOTE

Parasitology

The first detection of *Dicrocoelium chinensis* sporocysts from the land snail *Aegista vulgivaga* in Gifu Prefecture, Japan

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ABSTRACT. Trematodes of the genus *Dicrocoelium* are one of the most common parasites in ruminant animals; however, their life cycles in Japan are unclear. To find the sporocysts of *D. chinensis* in the natural field, we sampled 269 land snails (14 species) at a location with high level infection of sika deer in Gifu Prefecture, Honshu Island, Japan in autumn between 2017 and 2019. During the sampling period, we found mother sporocysts in the hepatopancreas of *Aegista vulgivaga* and *Cyclophorus herklotsi*. DNA barcoding based on the sequences of cytochrome *c* oxidase subunit 1 showed that the sporocysts from *A. vulgivaga* belonged to *D. chinensis*, indicating that this snail has potential as the first intermediate host of *D. chinensis* at this location.

KEY WORDS: *Aegista vulgivaga*, *Dicrocoelium chinensis*, DNA barcode, first intermediate host, sporocyst

Trematodes belonging to the genus *Dicrocoelium* Dujardin, 1845, are common parasites in ruminant animals. The flukes cause occasional economic losses to the livestock industry due to liver disorders; unless their infection levels are high, they generally produce mild symptoms [19]. Typically, *Dicrocoelium* spp. occupy three hosts during their life cycle [3, 11, 12]. Adult flukes, which are found in mammalian definitive hosts, release eggs into the feces of their hosts. Each egg contains a miracidium, which hatches when the egg is eaten by the first intermediate host land snails, such as *Cochlicopa lubrica* (O. F. Müller, 1774). The miracidium then migrates to the snail's hepatopancreas (digestive gland) and develops into a mother sporocyst. Many daughter sporocysts develop inside the mother sporocyst, and subsequently release infective cercariae. The cercariae included in slime balls leave the host and develop into metacercariae when they are fed on by ants belonging to the genus *Formica* Linnaeus, 1758. The infective metacercariae are then encysted in the abdomen of the ants and invade the definitive mammalian hosts via oral transition when the ants are ingested during grazing.

The genus *Dicrocoelium*, including *Dicrocoelium chinensis* (Sudarikov and Ryjikov, 1951) Tang and Tang, 1978 and *Dicrocoelium dendriticum* (Rudolphi, 1819), has been detected over a wide area of Japan, except in small islands such as the Nansei Islands [7, 14, 21, 22, 30, 31]. *Dicrocoelium chinensis* has been mainly reported in sika deer, *Cervus nippon* Temminck, 1838, and occasionally in Japanese serow, *Capricornis crispus* (Temminck, 1844) [7, 21, 22, 31], over a wide area of Honshu Island. However, the intermediate hosts of the fluke, *D. chinensis,* have not been confirmed in this country. Despite no reports of *Dicrocoelium* sporocysts in any wild Japanese land snails, Japanese veterinary parasitology textbooks describe the land snail *C. lubrica* as the first intermediate host of *Dicrocoelium* spp. in Japan [8, 9]. Itagaki [9] briefly described the fact that daughter

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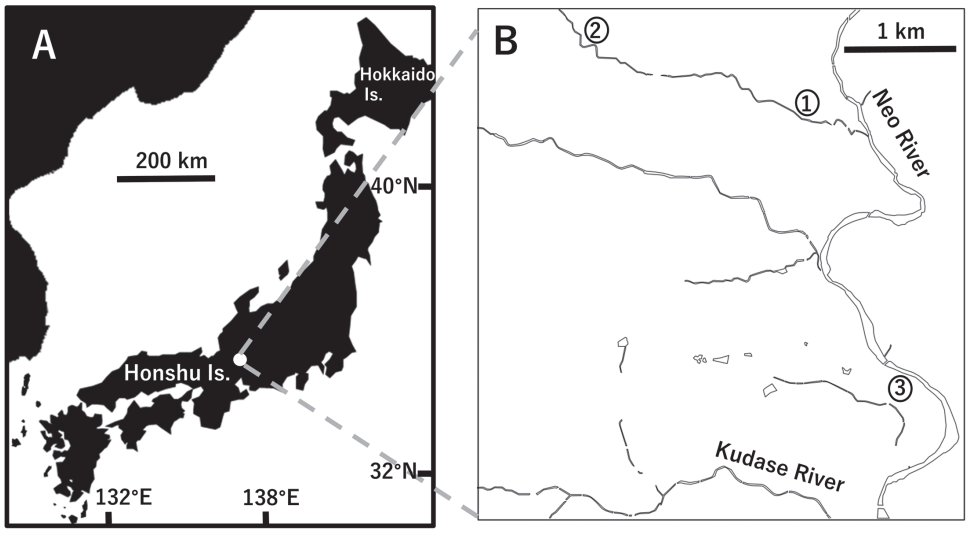

Fig. 1. Study locations. Numbers in circles represent the location numbers in the Supplementary Table 1. The latitude and longitude of each location are as follows: Location 1: 35°33'40"N 136°38'02"E. Location 2: 35°34'08"N 136°36'21"E. Location 3: 35°31'54"N 136°38'42"E.

sporocysts were developed in *C. lubrica* after a challenge experiment with the eggs of *Dicrocoelium* sp.; however, no paper was cited for the experiment, and no evidence was described for the genus identification of the sporocysts. Moreover, *C. lubrica* is distributed mainly on Hokkaido Island and is rare or absent in other areas, including Honshu Island [1, 2], even though *D. chinensis* has been found in wild ruminants on this island [7, 21, 22]. Thus, the aim of this study was to find wild land snails infected with *D. chinensis* sporocysts at a location in Gifu Prefecture, Honshu Island, Japan, where the fluke infection has been reported in sika deer [7], and *C. lubrica* presence has not been reported [2].

Fluke surveys were carried out at three locations in Tanigumitakashina and Taniguminagase in Ibigawa-cho, Ibi-gun, Gifu Prefecture, Japan, during the autumn and winter seasons of 2017 to 2019 (Fig. 1). We chose this area because a high prevalence of *D. chinensis* infection is reported in sika deer harvested by local hunters in this area (the dissection records in this area reported 22% prevalence based on 118 individuals hunted between December 1, 2017, and January 17, 2018). Locations 1 and 2 were situated in the forest, and location 3 was in a residential area 10 m from the forest edge (Fig. 1). To sample adult flukes, we obtained a sika deer that was commercially hunted using a snare trap at location 2 on December 7, 2019. The liver of the sika deer was frozen before being transported to the laboratory. The liver was then thawed at room temperature and subsequently dissected to obtain flukes. The flukes had bilateral testes and were identified as adult *D. chinensis* based on the morphological observations described by Otranto *et al*. [20] (Fig. 2A). Among adult flukes, two individuals were stained with acetocarmine after being fixed in 99% ethanol, mounted on slides, and deposited at Meguro Parasitological Museum with accession numbers MPM Coll. No. 21725a-b. A tissue sample from one unstained adult fluke was used for DNA barcoding analysis, as described later. Further, a total of 269 land snails from three locations (Supplementary Table 1) were transported to the laboratory for dissection under a stereomicroscope. Of these land snails, five individuals of *Aegista vulgivaga* Schmacker & Boettger, 1890 (Camaenidae Pilsbry, 1895) (Fig. 2B, 2C) and one of *Cyclophous herklotsi* Martens, 1861 (Cyclophoridae Gray, 1847) were found to be infected with sporocysts (Fig. 2D). These sporocysts were observed under a microscope and fixed in 99% ethanol for preservation. All the sporocysts from *A. vulgivaga* were immature (Fig. 2E), and the one from *C. herklotsi* contained mortal cercariae.

Tissue samples from fixed sporocysts and an unstained adult fluke were used for DNA analyses. Species identification using sporocyst morphology is difficult; however, DNA barcoding based on a partial sequence of cytochrome *c* oxidase subunit 1 (COI, ca. 300–800 bp) can be used to distinguish trematode species [15–17, 26–28]. For the analysis, DNA from the adult fluke and sporocysts was eluted using alkaline lysate (NaOH, 0.02 N) or the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), and the obtained samples were used as templates for a polymerase chain reaction (PCR) using the primers JB3 and CO1-R trema for COI [13] as described previously [15]. DNA sequencing was subsequently outsourced to Eurofins Genomics Inc. (Tokyo, Japan).

We amplified DNA fragments (841–898 bp) derived from the adult fluke tissue and sporocysts from three *A. vulgivaga* and one *C. herklotsi.* No amplification was obtained from the sporocysts found in two *A. vulgivaga*, possibly because of sample degradation due to poor storage conditions. Alignment datasets were prepared using the BioEdit Sequence Alignment Editor [5] together with the sequences of Japanese *Dicrocoelium* spp. retrieved from the DNA DataBank of Japan/European Nucleotide Archive/GenBank databases. These sequences (291 bp) were used for comparison and species delimitation based on divergence values computed in MEGA X [10] with the default setting of *p*-distance (Table 1). Based on a comparison between adult flukes, the divergence values of intraspecific variation in *D. chinensis* were less than 0.007 (99.3–100% similarity), and the value between two *Dicrocoelium* species was 0.110 (89.3% similarity). Sequences divergences of not more than 0.007 (not less than 99.3% similarity) were observed between the three adult flukes and the sporocysts from *A. vulgivaga*. However, the sporocysts from *C. herklotsi* differed from *Dicrocoelium* spp., with divergence values ranging from 0.210 to 0.227 (77.3–79.0% similarity). Next, we performed a phylogenetic analysis of COI using the 291-bp sequences of all samples, except for the sporocysts from *C. herklotsi*. A phylogenetic tree was constructed based on the maximum-likelihood (ML) method in MEGA X using the Hasegawa-Kishino-Yano

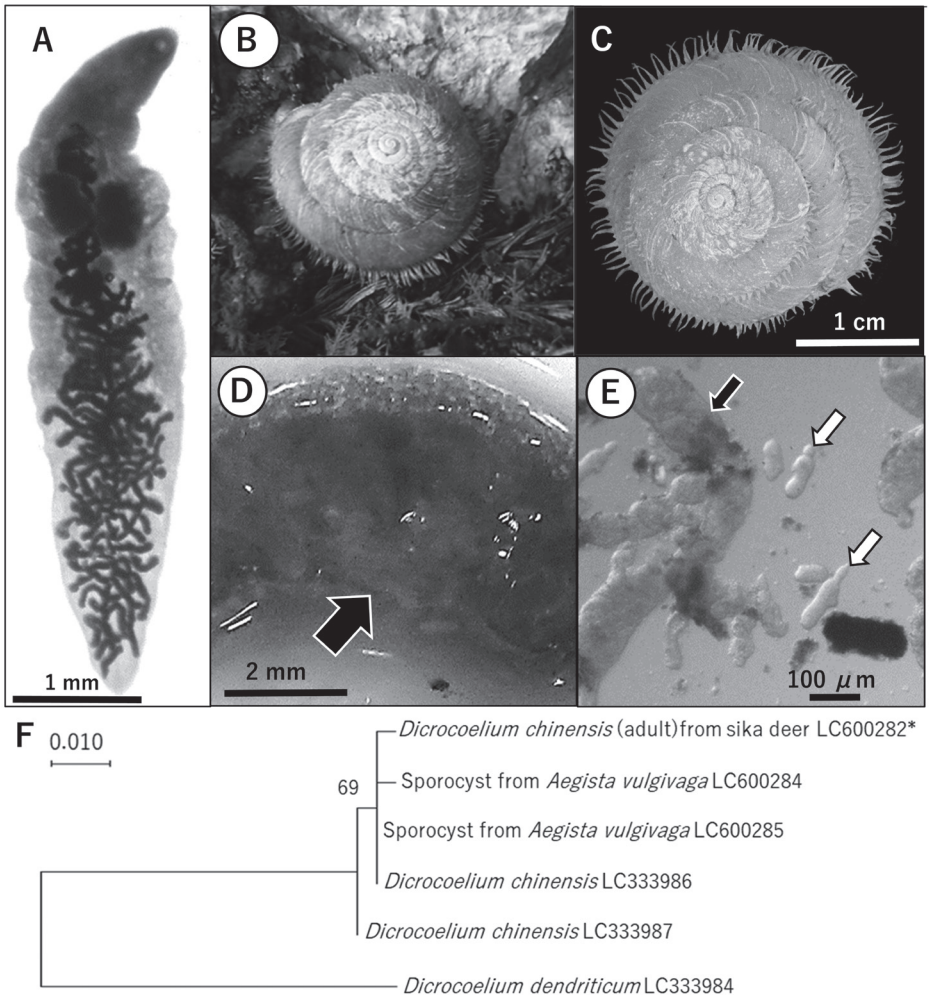

Fig. 2. *Dicrocoelium chinensis* and the host snails sampled in the study. A. Adult *D. chinensis* sampled from the liver of sika deer. B. *Aegista vulgivaga* in the field. C. Shell specimen of *A. vulgivaga*. D. Sporocysts (arrow) in the gonad of *A. vulgivaga*. E. Sporocysts from *A. vulgivaga*. Black and white arrows represent a mother sporocyst and an immature cercaria, respectively. F. A maximum-likelihood phylogenetic tree of sporocysts and *Dicrocoelium* spp. in Japan, inferred from partial nucleotide sequences of cytochrome *c* oxidase subunit 1 (COI). Bootstrap values greater than 50% are shown. Database accession numbers are shown after the sample names. The sequence of adult *D. chinensis* (LC600282) obtained in this study was identical to that of a sporocyst from *A. vulgivaga* (LC600283).

Table 1. Values of *p*-distance of mitochondrial cytochrome *c* oxidase subunit 1 among the *Dicrocoelium* isolates and sporocysts sampled in this study

Species or sporocyst number	Host	Genbank no.	*p*-distance						
			1	2	3	4	5	6	7
1 *Dicrocoelium chinensis*, adult (this study)	*Cervus nippon*	LC600282							
2 *Dicrocoelium chinensis*, adult (#1, Japan)	*Cervus nippon*	LC333986	0.003						
3 *Dicrocoelium chinensis*, adult (#2, Japan)	*Cervus nippon*	LC333987	0.007	0.003					
4 *Dicrocoelium dendriticum*, adult (#1, Japan)	*Cervus nippon*	LC333984	0.110	0.107	0.103				
5 Sporocyst #1 (this study)	*Aegista vulgivaga*	LC600283	0	0.003	0.007	0.110			
6 Sporocyst #2 (this study)	*Aegista vulgivaga*	LC600284	0.007	0.003	0.007	0.110	0.007		
7 Sporocyst #3 (this study)	*Aegista vulgivaga*	LC600285	0.003	0	0.003	0.107	0.003	0.003	
8 Sporocyst #4 (this study)	*Cyclophorus herklotsi*	LC600286	0.227	0.223	0.220	0.210	0.227	0.227	0.223

model [6] with reference sequences of related taxa retrieved from GenBank database. The reliabilities of the trees were tested with 1,000 bootstrap replicates [4]. In the tree, the two *Dicrocoelium* species were divided, and the sporocysts from *A. vulgivaga* formed a clade with the adults of *D. chinensis* (Fig. 2F). These results indicate that the sporocysts from *A. vulgivaga* were *D. chinensis,* and that this snail has the potential to be the first intermediate host of *D. chinensis* at the sampling site. The COI sequences of sporocysts from *A. vulgivaga* and the adult *D. chinensis* were deposited in GenBank with accession numbers LC600283-LC600285 and LC600282, respectively. The sporocysts from *A. vulgivaga* were mounted on slides with Hoyer's medium and deposited at Meguro Parasitological Museum with the accession number MPM Coll. No. 21724.

We could not identify the sporocyst from *C. herklotsi*, even at the genus or family level, because GenBank did not include sufficient closely related COI gene sequences (e.g., 80.8% similar to *Plagiorchis maculosus*: GenBank accession number NC042482). Further, sporocysts and cercariae have few species-specific morphological features. Therefore, to identify the sporocysts at a higher taxonomic level, we PCR-amplified 28S rDNA using the primers dig12 and 1500R [25] and performed phylogenetic analysis using sequences from related species retrieved from GenBank databases in MEGA X with the ML method and a General Time Reversible model [18], supported by 1,000 bootstrap replicates. The 28S rDNA tree showed that the sporocyst is probably a member of the suborder Xiphidiata Olson, Cribb, Tkach, Bray & Littlewood, 2003, because the sequence from the sporocyst formed a clade with other species of the suborder Xiphidiata (bp=80, Supplementary Fig. 1). The 28S rDNA and COI sequences of the sporocyst from *C. herklotsi* were deposited in GenBank as Xiphidiata, fam. gen. sp. with accession numbers LC600287 and LC600286, respectively.

In this study, we detected sporocysts of *D. chinensis* from *A. vulgivaga*. *Aegista vulgivaga* may acts as the first intermediate host of *D. chinensis* in Central Honshu Island, where the snail is distributed, because *A. vulgivaga* is a major species living in the litter layer of forests and grasslands [1] though the feeding habits of this snail species have not been examined. However, these habitats provide frequent opportunities for the ingestion of the feces of definitive hosts by *A. vulgivaga*. In China, three camaenid snails, *Bradybaena similaris* Férussac, 1822, *Bradybaena virgo* Pilsbry, 1927, and *Cathaica fasciola* Draparnaud, 1801, have been reported to act as the first intermediate hosts of the fluke [23, 24, 29]. In these studies, cercariae were identified as *D. chinensis* based on their morphology ; however, the morphological identification of cercariae is very difficult because larval trematodes have few species-specific morphological features. Moreover, the sporocysts and cercariae of unknown dicrocoeliid species have been detected in *B. similaris* [27]. Therefore, the sporocysts from these reported three hosts should be re-confirmed using the DNA barcoding method, even though *D. chinensis* may have a wide host range and can infect many snail species in the family Camaenidae.

In Iwate Prefecture, Japan, located in the northern area of *A. vulgivaga* distribution, the habitats of *D. dendriticum* and *D. chinensis* do not overlap as the former was exclusively detected in Japanese serow from the mountainous western region, and the latter was found in sika deer and occasionally in Japanese serow from the eastern coastal areas [21]. If *A. vulgivaga* can act as an intermediate host of *D. chinensis*, the isolation of the two *Dicrocoelium* species is probably a consequence of the distribution of their definitive hosts, since *A. vulgivaga* has been recorded in both the mountainous and coastal areas of this prefecture [2]. Using DNA barcoding, we detected sporocysts of *D. chinensis* from wild land snails in Japan for the first time. This information could be highly useful for parasite control and highlights the possibility of using DNA barcoding to trace trematode species and life cycle patterns. However, the first and second intermediate hosts of the two *Dicrocoelium* species in Japan remain unclear. This study is the beginning of a large survey to investigate the life cycles of *Dicrocoelium* flukes in this country.

CONFLICTS OF INTEREST. The authors declare that they have no conflicts of interest.

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REFERENCES

1. Azuma, M. 1995. Colored illustrations of the land snails of Japan, Enlarged and Revised Edition, Hoikusya, Osaka.
2. Biodiversity Center of Japan. 2010. Distribution of Wild Animals in Japan. https://www.biodic.go.jp/kiso/atlas/ [accessed on Feb. 2, 2021].
3. Dawes, B. 1968. The trematoda: with special reference to British and other European forms, CUP Archive, Cambridge.
4. Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783–791. [Medline] [CrossRef]
5. Hall, T. A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acid. S.* **41**: 95–98.
6. Hasegawa, M., Kishino, H. and Yano, T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* **22**: 160–174. [Medline] [CrossRef]
7. Hayashi, K., Tang, W., Ohari, Y., Ohtori, M., Mohanta, U. K., Matsuo, K., Sato, H. and Itagaki, T. 2017. Phylogenetic relationships between *Dicrocoelium chinensis* populations in Japan and China based on mitochondrial nad1 gene sequences. *Parasitol. Res.* **116**: 2605–2609. [Medline] [CrossRef]
8. Ishii, T. 1998. Veterinary parasitology and parasite physiology 2. Helminths and others, Kodansha scientific Tokyo.
9. Itagaki, T. 2019. Trematode. pp. 88–117. In: Doubutsu Kiseichubyogaku, 4th ed. (Itagaki, T. and Fujisaki, K. eds.), Asakura-shoten, Tokyo.
10. Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol.* **35**: 1547–1549. [Medline] [CrossRef]
11. Krull, W. H. and Mapes, C. R. 1952*a*. Studies on the biology of Dicrocoelium dendriticum (Rudolphi, 1819) Looss, 1899 (Trematoda: Dicrocoeliidae), including its relation to the intermediate host, Cionella lubrica (Müller). III. Observations on the slimeballs of Dicrocoelium dendriticum. *Cornell Vet.* **42**: 253–276. [Medline]
12. Krull, W. H. and Mapes, C. R. 1952*b*. Studies on the biology of *Dicrocoelium dendriticum* (Rudolphi, 1819) looss, 1899 (Trematoda:

Dicrocoeliidae), including its relation to the intermediate host, *Cionella lubrica* (Müller). VII. The second intermediate host of *Dicrocoelium dendriticum*. *Cornell Vet.* **42**: 603–604. [Medline]
13. Miura, O., Kuris, A. M., Torchin, M. E., Hechinger, R. F., Dunham, E. J. and Chiba, S. 2005. Molecular-genetic analyses reveal cryptic species of trematodes in the intertidal gastropod, *Batillaria cumingi* (Crosse). *Int. J. Parasitol.* **35**: 793–801. [Medline] [CrossRef]
14. Nakamura, T., Nakahari, J., Machida, N., Kiryu, K. and Machida, M. 1984. Dicroceliasis in the wild Japanese serow, Capricornis crispus. *Nippon Juigaku Zasshi* **46**: 405–408. [Medline] [CrossRef]
15. Nakao, M., Waki, T., Sasaki, M., Anders, J. L., Koga, D. and Asakawa, M. 2017. *Brachylaima ezohelicis* sp. nov. (Trematoda: Brachylaimidae) found from the land snail *Ezohelix gainesi*, with a note of an unidentified *Brachylaima* species in Hokkaido, Japan. *Parasitol. Int.* **66**: 240–249. [Medline] [CrossRef]
16. Nakao, M., Sasaki, M., Waki, T., Iwaki, T., Morii, Y., Yanagida, K., Watanabe, M., Tsuchitani, Y., Saito, T. and Asakawa, M. 2019*a*. Distribution records of three species of *Leucochloridium* (Trematoda: Leucochloridiidae) in Japan, with comments on their microtaxonomy and ecology. *Parasitol. Int.* **72**: 101936. [Medline] [CrossRef]
17. Nakao, M., Sasaki, M., Waki, T. and Asakawa, M. 2019*b*. *Pseudoleucochloridium ainohelicis* nom. nov. (Trematoda: Panopistidae), a replacement for *Glaphyrostomum soricis* found from long-clawed shrews in Hokkaido, Japan, with new data on its intermediate hosts. *Species Divers.* **24**: 159–167. [CrossRef]
18. Nei, M. and Kumar, S. 2000. Molecular Evolution and Phylogenetics, Oxford University Press, New York.
19. Otranto, D. and Traversa, D. 2002. A review of dicrocoeliosis of ruminants including recent advances in the diagnosis and treatment. *Vet. Parasitol.* **107**: 317–335. [Medline] [CrossRef]
20. Otranto, D., Rehbein, S., Weigl, S., Cantacessi, C., Parisi, A., Lia, R. P. and Olson, P. D. 2007. Morphological and molecular differentiation between *Dicrocoelium dendriticum* (Rudolphi, 1819) and *Dicrocoelium chinensis* (Sudarikov and Ryjikov, 1951) tang and tang, 1978 (Platyhelminthes: Digenea). *Acta Trop.* **104**: 91–98. [Medline] [CrossRef]
21. Ohtori, M., Aoki, M. and Itagaki, T. 2014. Distinct distribution of *Dicrocoelium dendriticum* and *D. chinensis* in Iwate Prefecture, Japan, and a new final host record for *D. chinensis*. *J. Vet. Med. Sci.* **76**: 1415–1417. [Medline] [CrossRef]
22. Taira, K., Shirasaka, S., Taira, N., Ando, Y. and Adachi, Y. 2006. Morphometry on lancet flukes found in Japanese sika deer (*Cervus nippon centralis*) captured in Iwate Prefecture, Japan. *J. Vet. Med. Sci.* **68**: 375–377. [Medline] [CrossRef]
23. Tang, C., Tang, Z., Tang, L., Cui, Q., Lu, H. and Qian, Y. 1983. Studies on the biology and epizootics of *Dicrocoelium chinensis* in the eastern inner Monggol autonomous region. *Dong Wu Xue Bao* **29**: 340–348.
24. Tang, C., Tang, L., Wang, P., Shi, H., Sai, Q., Wen, Z. and Luo, Y. 1985. Investigations on the biology of a few *Dicrocoelium* species from sheep on Qinghai Plateau. *Dong Wu Xue Bao* **31**: 254–262.
25. Tkach, V. V., Kudlai, O. and Kostadinova, A. 2016. Molecular phylogeny and systematics of the Echinostomatoidea Looss, 1899 (Platyhelminthes: Digenea). *Int. J. Parasitol.* **46**: 171–185. [Medline] [CrossRef]
26. Waki, T. 2017. Diversity of terrestrial mollusks and their helminths in artificial environments in Yoyogi Park, Tokyo, Japan. *J. Asia-Pac. Biodivers.* **10**: 254–256. [CrossRef]
27. Waki, T., Nakao, M., Hayashi, K., Ikezawa, H. and Tsutumi, N. 2018. Molecular and morphological discrimination of dicrocoeliid larvae (Trematoda: Digenea) from terrestrial mollusks in Japan. *J. Parasitol.* **104**: 660–670. [Medline] [CrossRef]
28. Waki, T., Sasaki, M., Mashino, K., Iwaki, T. and Nakao, M. 2020. *Brachylaima lignieuhadrae* n. sp. (Trematoda: Brachylaimidae) from land snails of the genus *Euhadra* in Japan. *Parasitol. Int.* **74**: 101992. [Medline][CrossRef]
29. Wu, B. H., Xida, S. and Song, C. C. 1991. Fauna of Zhejiang (Trematoda), Zhejiang Science and Technology Publishing House, Hang Zhou.
30. Yagisawa, M. 1978. Studies on zoonotic helminths from mammals in northern Honshu, Japan. *Hirosaki Igaku* **30**: 239–284.
31. Yamaguti, S. 1933. Transactions 1. Studies on the helminth fauna of Japan. Part1 Trematodes of birds, reptiles and mammals. *Jpn. J. Zool.* **5**: 1–134.