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Short communication

Morphological and genetic characterization of green-banded broodsacs of *Leucochloridium* (Leucochloridiidae: Trematoda) sporocysts detected in *Succinea lauta* in Hokkaido, Japan

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ABSTRACT

Green-banded broodsacs of *Leucochloridium* sporocysts were obtained from land snails, *Succinea lauta*, collected in Esashi, Esashi District, Hokkaido, Japan. The broodsacs were similar to those of *L. paradoxum*, which have never been found on the Japanese archipelago. Here, we compare morphological and molecular genetic characteristics of the green-banded broodsacs to those of *L. paradoxum*. The broodsacs described in this study and those of *L. paradoxum* showed three equal parts; however, band characteristics of the second parts differed. Moreover, the sequences of nuclear 18S and 28S rRNA genes of the *Leucochloridium* flukes were not identical with those of *L. paradoxum*, and the sequences of the mitochondrial cytochrome *c* oxidase subunit 1 gene were paraphyletic to the species. These findings suggest that the broodsacs described in this study were distinct from those of *L. paradoxum*. In the future, genetic analyses on adult flukes from avian hosts should be performed, upon precise identification according to morphology, in order to clarify relationships between adult flukes and sporocysts of *Leucochloridium* species occurring in Japan.

The genus *Leucochloridium* Carus, 1835 (Leucochloridiidae, Brachylaimoidea, Trematoda) parasitizes in the cloaca and the Bursa Fabricii of their avian final hosts and is distributed in the Holarctic region. This genus utilizes Succineidae (Pulmonata: Stylommatophora) land snails as intermediate hosts and produces vividly colored sporocyst broodsacs that occur in the ocular tentacles. Although four species, *L. japonicum* Ishii, 1932 [1], *L. sime* Yamaguti, 1935 [2], *L. cardis* Yamaguti, 1939 [3] and L. *turdi* Yamaguti, 1939 [3] have been reported in Japan, the morphology of their broodsacs remains unclear. In contrast, green- and brown-banded broodsacs have been detected in *Succinea lauta* in Japan [4] and they seem morphologically similar to those of *L. paradoxum* and *L. perturbatum* that have not been reported in Japan. Although it is known that the two species differ genetically, sequencing data of *Leucochloridium* broodsacs in Japan are so far not well resolved. In this study, green-banded broodsacs isolated from *S. lauta* in Japan were analyzed for morphological and genetic characteristics for comparison with known *L. paradoxum* broodsacs.

Two land snails infected with green-banded *Leucochloridium* broodsacs were collected in Esashi, Esashi District, Hokkaido, Japan (44°41′20.6″N 142°49′39.6″E) in 9th August 2015, and identified as *S. lauta* by their shell morphology. The sporocysts were removed from the snails and their broodsacs were morphologically observed. The metacercariae were individually isolated from two broodsacs of each snail, 10 metacercariae each (40 in total) were used for morphological observation and morphometry. The metacercariae were fixed in 70% ethanol under slight pressure using a cover glass, then stained with hematoxylin-carmine, and subsequently mounted in Canada balsam. Body length and width, oral sucker, ventral sucker and pharynx of the metacercariae were measured using an optical microscope attached to a digital camera DP26 (OLYMPUS, Tokyo, Japan) and imaging software cellSen (OLYMPAS). An image processing software, CombineZP (http://combinezp.software.informer.com/), was used for combining photographs of different focal planes. The measurements were statistically compared by *t*-test using statistical software R [5]. Moreover, respective total DNA was extracted from 10–20 pooled metacercariae using High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). The nuclear 18S and 28S rRNA genes and mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) were amplified by PCR in a final reaction volume of 50 μL, containing 1 μL of template DNA, 0.2 pM of each primer, 1.25 U of Tks Gflex DNA Polymerase (TaKaRa, BIO INC.,

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Kusatsu, Japan), and 25 µL of the reaction buffer as supplied manufacturer. The following primer sets were used: WormA and 1270R, and 930F and WormB for the 18S region [6], 28digl2 and 1500R for the 28S [7], and JB3 and CO1-R/trema for the *cox1* [8]. Thermal cycling was performed by an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, 55 °C for 15 s, and 68 °C for 30 s. The amplicons were sequenced directly in both directions with the respective PCR primers, using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3500 Genetic Analyzer (Applied Biosystems). The obtained sequences were initially assembled using the ATGC ver. 6.0.3 software (Genetyx Co., Tokyo, Japan). Phylogenetic trees were constructed employing a Maximum Likelihood method, using MEGA ver. 6 [9]. The tree included the sequences of *L. paradoxum* (GenBank accession no. KP938187 for 18S and 28S, and KP903650 and KP903661 for *cox1*), *L. perturbatum* (KP938186 for 18S, and KP903644, KP903645 and KP903664 for *cox1*), and *L. vogtianum* (KP903653—KP903655 for *cox1*). As outgroups, we used the sequences of *Urogonimus macrostomus* (JQ774503 and AY222086 for 18S, AB494468 and AY222168 for 28S, and KP903681 for *cox1*), *Brachylaima* spp. (AY222084 and AY222085 for 18S, AF184262 and LC198310 for 28S, and KP903684, KT074958, and KT074963 for *cox1*) and *Zeylanurotrema spearei* (AY222088 for 18S and AY222170 for 28S). Phylogenetic trees were constructed based on the Kimura 2-parameters with gamma distribution for 18S and 28S, and for *cox1* based on the Hasegawa-Kishino-Yano model with gamma distribution and invariant sites, which were selected with the maximum likelihood test based on Akaike's information criterion. The trees were evaluated using bootstrap tests with 1000 replications. Calculations of *p*-distances among sequences were executed using MEGA ver. 6.

The green-banded broodsacs of sporocyst were moving rhythmically in the ocular tentacles of *S. lauta* (Fig. 1 A). Eight and three broodsacs were obtained from the two snails, respectively. The broodsacs were of white or greenish ground color, of cylindrical shape, and segmented into three parts (Fig. 1 B). The first part (Fig. 1 B-1) was orange in the anterior top and had dark brown protrusions from the top to the middle area. The posterior half had one continuous, broad green band, and a dark green-dotted line was observed on the border between the first and second part. The anterior half of the second part (Fig. 1 B-2) had light green transverse striations on ground color, and the posterior half contained two intermittent broad bands which were green in the anterior half and varied from green to dark brown in the posterior half. These bands were separated from each other by a horizontal ground color line and divided into several block-like fragments by vertical ground color lines. The third part (Fig. 1 B-3) was completely of ground color with several slightly dark green striations. A tube arose from the posterior extremity of the broodsacs and reached the central branch of the sporocysts (Fig. 1 B-4). The metacercariae were almost oval and surrounded by a thin cyst wall. Well-developed oral sucker, ventral sucker, pharynx, and bilateral intestines, as well as undeveloped genital glands were observed (Fig. 1 C). The oral sucker, pharynx and ventral sucker were close to each other and overlapping one another in some specimens. The intestine bifurcated at the bottom of pharynx, and terminated at the posterior part of body. Undeveloped gonad organs were behind the ventral sucker and between two intestinal caeca; however the testes and ovary could not be precisely confirmed. On the internal edge of the oral sucker, a sawtooth-shaped structure was found (Fig. 1 D). The measurements of metacercariae are shown in Table 1. All the measurements were statistically different between metacercariae obtained from two snails (snail 1 and snail 2) ($p < 0.05$). The sequences of 18S (1638 bp; LC384421 and LC384423), 28S (1203 bp; LC384425), and *cox1* (888 bp; LC384429 and LC384430) were generated from total DNA and were identical within one host snail. However, three loci with single nucleotide polymorphism were found in the 18S sequences (0.18%) that diverged between the two snail isolates and *L. paradoxum*. The 28S sequences were also identical between the snails, but varied in eleven sites (0.87%) from those of L. *paradoxum*. The *cox1* sequences diverged in one site, with genetic distances of 2.35–3.53% to L. *paradoxum*, 15.88–17.06% to *L. perturbatum*, and 13.53–14.71% to *L. vogtianum*. In the phylogenetic trees of 18S (1630 bp) and 28S (1185 bp), the present sequences clustered with those of *L. paradoxum*. Moreover, in the *cox1* tree (170 bp), the present sequence was paraphyletic to *L. paradoxum* and constituted a sister clade with *L. vogtianum* (Fig. 2).

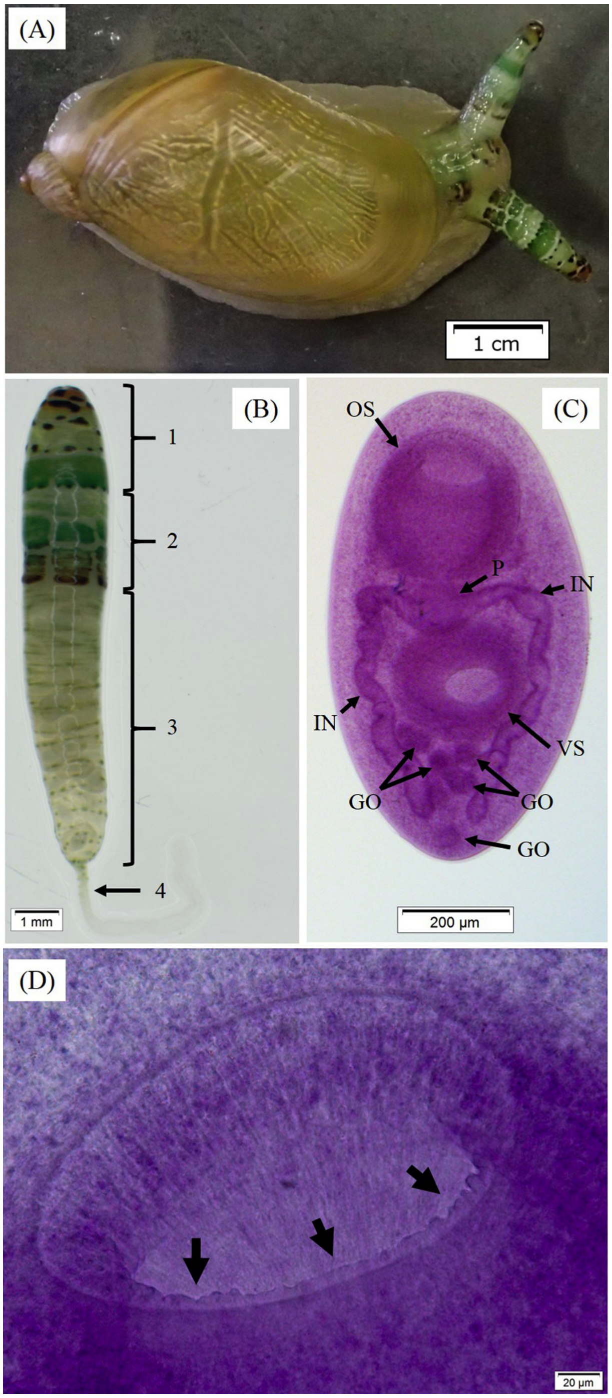

Fig. 1. Broodsacs and metacercariae of *Leucochloridium* isolated from *Succinea lauta*. (A) Green-banded broodsacs in the ocular tentacles. (B) Broodsac divided into three parts (1–3) with a tube (4) in the posterior extremity. (C) A stained metacercaria after removal of a thin cyst wall. OS: oral sucker, P: pharynx, IN: intestine, VS: ventral sucker, GO: gonad organs. (D) Sawtooth-shaped structure (arrows) on the internal edge of the oral sucker of the metacercaria. The picture was obtained using CombineZP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Measurements of *Luecochloridium* metacercaria in this study and comparison with those of a previous study.

Snail code or previous study	Body		Oral sucker		Ventral sucker		Pharynx	
	Length	Width	Length	Width	Length	Width	Length	Width
Snail 1	736.6–897.8 (819.7 ± 25.1)	348.4–524.3 (451.0 ± 21.8)	224.3–284.4 (257.4 ± 8.7)	230.9–296.0 (260.9 ± 7.6)	194.9–235.4 (210.1 ± 5.5)	200.4–262.1 (232.6 ± 7.9)	72.6–100.6 (91.0 ± 3.1)	75.2–116.7 (103.8 ± 4.5)
Snail 2	569.7–721.6 (670.6 ± 21.0)	358.6–434.7 (401.1 ± 10.6)	193.5–252.8 (227.8 ± 7.8)	198.0–251.6 (230.9 ± 5.5)	172.2–196.4 (183.8 ± 2.8)	191.3–224.8 (209.3 ± 3.6)	67.9–98.8 (79.4 ± 4.1)	80.6–108.7 (92.5 ± 3.0)
L. paradoxum [10]	0.58–0.72** (0.63 ± 0.04)	0.26–0.36** (0.29 ± 0.02)	193–150 (174 ± 9.1)	150–193 (172 ± 11.5)	136–179 (156.8 ± 9.9)	136–164 (151 ± 6.1)	57–71 (66 ± 3.3)	64–86 (73 ± 5.9)

The range values show the minimum and maximum with mean ± 95% confidence interval in parentheses. Measurements are in μm expect (*) in mm. All the measurements were statistically different between the metacercariae obtained from snail 1 and snail 2. ($p < 0.05$)

The green-banded broodsacs of *L. paradoxum* are divided into three parts and contain distinct continuous, broad green bands in the first and second parts [10]. The green-banded broodsacs in this study were also divided into three parts, however, the broad bands of the second part were different from those observed in *L. paradoxum* [10] (Fig. 1 B-2). Moreover, all the measurements of metacercariae from two snails were larger than those of *L. paradoxum* (Table 1) [10], although those measurements were statistically different between metacercariae from two snails. However, we could not clarify whether this difference is caused by *Leucochloridium* species or fixation methods [10]. Moreover, the sawtooth-shaped structure is not observed in *L. paradoxum* metacerucariae [10]. These morphological findings suggest that the present broodsacs would not be those of *L. paradoxum*. The 18S and 28S sequences of *Leucochloridium* species in this study differed from those of *L. paradoxum* and *L. pertrubatum*, although the sequences of these two species are identical, whereas their broodsacs morphology differs remarkably [11]. Furthermore, the *cox1* sequence of this study was paraphyletic to that of *L. paradoxum*, although the sequences used for the analysis were short (170 bp). These molecular results also suggested that the present broodsacs may be distinct species from *L. paradoxum*. The green-banded broodsacs have been identified and genetically characterised as *L. paradoxum* in previous studies [11–13]. However, this study showed that the green-banded broodsacs analyzed would not be *L. paradoxum* based on both the morphological and molecular characteristics. Therefore, the comprehensive analysis using morphology of sporocyst, metacercariae and adult flukes, and genetic characteristics will be necessary for species identification of *Leucochloridium*. Unfortunately, these analyses of *Leucochloridium* species occurring in Japan remain unclear. Further studies on these characteristics will clarify this *Leucochloridium* species with green-banded broodsacs.

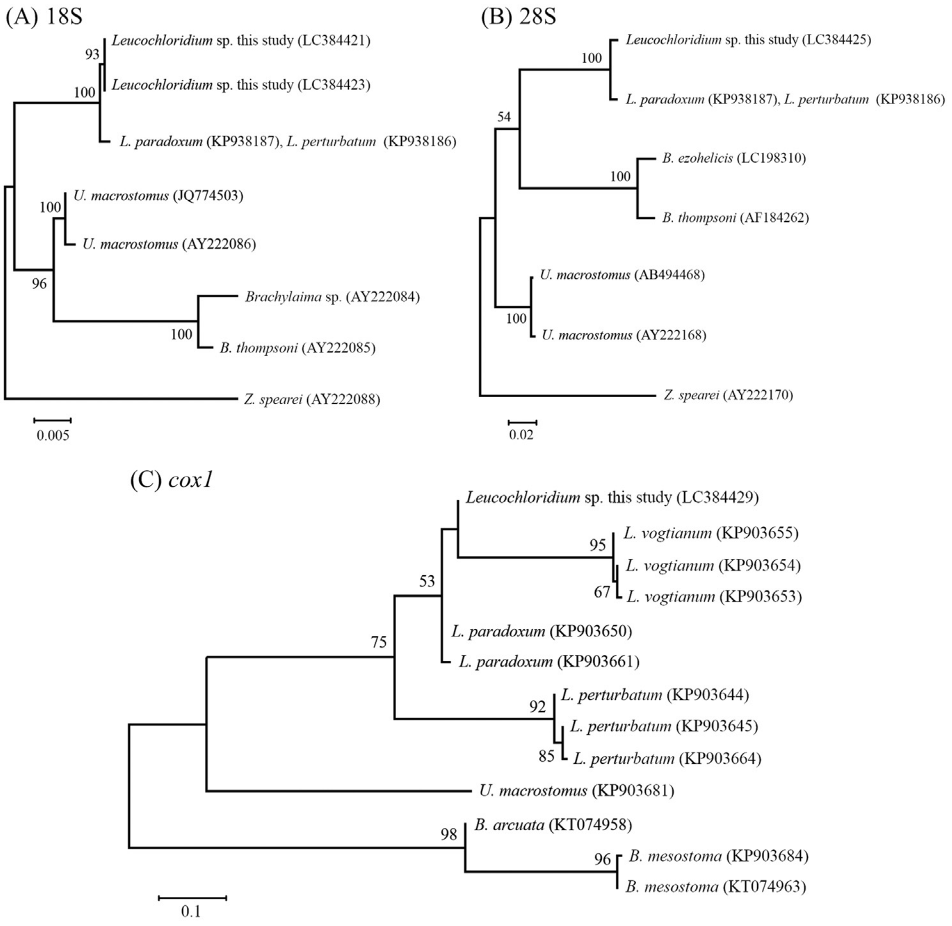

Fig. 2. Maximum likelihood trees based on the sequences of (A) a nuclear 18S rRNA gene (1630 bp), (B) a nuclear 28S rRNA gene (1185 bp), and (C) a mitochondrial cytochrome *c* oxidase subunit 1 gene marker (170 bp). Numbers near the nodes show bootstrap values (values < 50% not shown) by 1000 replications. Scale bars indicate substitutions per nucleotide.

Declarations of interest

None.

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