Brachylaima asakawai sp. nov. (Trematoda: Brachylaimidae), a rodent intestinal fluke in Hokkaido, Japan, with a finding of the first and second intermediate hosts

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**A B S T R A C T**

In the 1970's and 1980's, an unknown species of the genus Brachylaima (Trematoda: Brachylaimidae) had been recorded from the intestines of Rattus norvegicus and Apodemus speciosus in Hokkaido, Japan. The rodent fluke was characterized in extending a bilateral vitellarian till the level of posterior margin of anterior testis and in keeping almost the same-sized spherical ovary and testes. In this study, the rodent fluke was rediscovered from A. speciosus, Apodemus argenteus, and Myodes rufocanus in Hokkaido. The resultant parasite collection enabled us to make a mitochondrial DNA (mtDNA) barcode for tracking its intermediate hosts. The metacercaria of the rodent fluke was detected frequently from the kidney of three species of land snails (Discus pauper, Succinea lauta, and Ainohelix editha). However, its sporocyst with cercariae was found only from the hepatopancreas of D. pauper, a fairly small snail. The wide-spectrum of the second intermediate host seems to increase the chance of transmitting the parasite to various mammals and birds. The use of indigenous land snails as the first and second intermediate hosts, the distinctiveness of the mtDNA sequence, and the characteristic morphology of all the developmental stages prompted us to propose Brachylaima asakawai sp. nov. for the rodent intestinal fluke in Hokkaido. The present field survey suggests that the life cycle of the new species is primarily dependent on a predator-prey relationship between rodents and D. pauper.

1. Introduction

Members of the family Brachylaimidae (Trematoda) have a unique life cycle [1,2]. They exclusively use land snails as the first intermediate host for asexual proliferation of cercarial larvae. After the parasite eggs are ingested by the first intermediate host, reticular sporocysts grow in the hepatopancreas and produce a huge number of cercariae. The short-tailed cercaria released into the environment invades the kidney or pericardial cavity of another land snail (the second intermediate host) in order to metamorphose into non-encysted metacercariae. The intermediate host specificity of brachylaimids has been well studied in the genus Brachylaima. Several species of Brachylaima are the strictest when selecting the first intermediate host, namely each of Brachylaima spp. uses only a particular species of snails to produce cercariae [3–5]. In contrast, a number of species of snails can serve as the second intermediate host. The species richness of land snails, their low mobility, and the strict compatibility between host and parasite seem to be influential in causing the regional species diversity of Brachylaima.

In Japan, only 4 species of Brachylaima have been described many years ago, although approximately 800 species of land snails occur in the archipelago [6]. The recognized species are Brachylaima syrmati (Yamaguti) [7] and Brachylaima eophonae Yamaguti [8] from birds and Brachylaima tokudai Yamaguti [9] and Brachylaima ishigakiense Kamiya et Machida [10] from mammals. The former three species were found from Honshu (the largest main island) and the latter one from Ishigaki island (the southern part of Ryukyu islands). Several studies also recorded unidentified species of Brachylaima in Japan [11–15]. We recently described a new species, Brachylaima esohelidis Nakao, Waki et Sasaki, from Eohelix gainesi (Pilsbry), a common land snail in Hokkaido (the northernmost island) [16]. Our report further characterized an...
unidentified species of *Brachylaima* from rodents in Hokkaido, because older studies [11,13] included no morphological information about the species. A retrospective search on the original data in the literatures suggested that an intestinal brachylaimid from the brown rat, *Rattus norvegicus* (Berkenhout) [11] was identical to that from the large Japanese field mouse, *Apodemus speciosus* (Temminck) [13]. The rodent fluke was characteristic in extending bilateral vitellaria to the level of the posterior margin of anterior testis and in keeping almost the same-sized spherical ovary and testes. Since there are no morphologically related species in neighboring countries, the rodent fluke seems to be an undescribed species [16].

In this study, the rodent fluke was rediscovered in Hokkaido from *A. speciosus* and from novel definitive hosts of the small Japanese field mouse, *Apodemus argenteus* (Temminck), and the grey red-backed vole, *Myodes rufocanus* (Sundevall). The newly found specimens allowed us to determine mitochondrial DNA (mtDNA) sequences for DNA barcoding. A field survey using the DNA barcoding revealed which land snails participate in keeping the life cycle of the rodent fluke. The aims of this study are (i) to clarify the first and second intermediate hosts, (ii) to elucidate the genetic diversity of the parasite, and (iii) to describe a new species using adult worms originated from experimental infections of mice. During the field survey, we noticed that two additional unknown species of *Brachylaima* further prevail in land snails in Hokkaido. The DNA information of these unknown species is also reported.

### 2. Materials and methods

#### 2.1. Field surveys and laboratory investigations

During June to August 2016, rodents were captured using Sherman traps in forest lands around Tokachigawa-Onsen, Otofuke, Hokkaido (Fig. 1). The sampling sites consisted of 4 mountainsides and 1 riverside. All the trapped rodents were sacrificed by cervical dislocation, and their intestines were opened in Dulbecco’s phosphate-buffered saline (PBS) to find parasitic helminths. After counting the number of the parasites, they were kept in 70% ethanol for molecular analyses. During fixation, some of the trematodes were flattened by pressure between a microscope slide and a coverslip, allowing for later morphological identification.

During June to September 2017, land snails were collected by hand picking from leaves of such plants as the Kuril bamboo, *Sasa kurilensis* (Rupr.), the Asian skunk-cabbage, *Lysichiton camtschatcensis* (L.), and the giant butterbur, *Petasites japonicus* (Siebold et Zucc.), in forest lands of Asahikawa, Biei, Furano, Otofuke, Ozora, and Sapporo (Fig. 1). The snail collection was also done from pieces of decayed wood in leaf litter layer. There were a total of three sampling sites in Asahikawa and two in Sapporo, but only one site was set for each of the other localities. The shell size of the collected snails was measured prior to dissection. Each snail was crushed between thick grass plates and then dissected in PBS under a stereomicroscope. The kidney and hepatopancreas were broken by fine-tipped forces for detecting metacercaria and sporocyst, respectively. The number of metacercariae per each infected snail was counted to assess the intensity of infection (worm burden). After microscopic observation on living organisms of sporocysts, cercariae, and metacercariae, these larvae were preserved in 70% ethanol or 10% neutral-buffered formalin for later examination. Part of the metacerciae were used for experimental infections in mice.

#### 2.2. DNA barcoding and phylogenetic analyses

Alkaline lysates of ethanol-fixed samples were prepared using a previously reported method [16]. In brief, the whole body of a metacercaria or an approximately 1 mm³ piece of sporocyst and adult was lysed in 25 μl of 0.02 N NaOH at 99 °C for 30 min. One μl of the lysate was used as a template for polymerase chain reaction (PCR). The TkS Gillex™ DNA polymerase (Takara) was employed for PCR, together with the manufacturer-supplied reaction buffer. For genus-level identification, nuclear 28S ribosomal DNA (rDNA) was targeted using the primer set dig1 and 1500R [17]. For species-level identification (DNA barcoding), mitochondrial cytochrome c oxidase subunit 1 (cox1) was amplified using the primer set JB3 and CO1-R trema [18]. The PCR was run for 40 cycles (98 °C for 10s, 50 °C for 20s, and 68 °C for 90s) in a total volume of 25 μl including 0.25 μM of each primer. The PCR amplification was sequenced using BigDye terminator cycle sequencing kit and ABI genetic analyzer 3500 (Applied Biosystems). Each of the PCR primers was used as a sequencing primer. The lengths of 28S rDNA and cox1 fragments determined were 1268 and 786 base pairs (bp), respectively.

The multiple aligner MAFFT [19] was employed to prepare the nucleotide datasets of 28S rDNA and cox1. Comparative sequences of related taxa were retrieved from DDBJ/ENA/GenBank databases. All sites of the alignments including gaps were used for analyses. The genetic software MEGA7 [20] chose substitutional models for both the datasets, and computed the values of pairwise divergence under K2-parameter model with a gamma setting of 0.5. Phylogenetic trees were made by maximum likelihood (ML) method of MEGA7. The robustness of the trees was tested by bootstrapping with 500 replicates. A network figure of cox1 haplotypes was illustrated by TCS1.21 [21], and population genetics indices were calculated by DnaSP 6 [22].

#### 2.3. Experimental infections

To obtain adult worms, 30 to 60 metacercariae were administered perorally to each of 9 female ICR mice. Simultaneously, 8 mice were subcutaneously injected with 0.2 ml Depo-Medrol® (Pfizer) to prevent inflammatory reactions, and one mouse remained uninjected as a control. The injected drug was equivalent to 8 mg methylprednisolone acetate. All the mice were sacrificed 14 days post infection to recover adult worms. As reported previously [16], the resultant adults were fixed with 10% neutral-buffered formalin or 1% glutaraldehyde in phosphate buffer (0.1 M PB, pH 7.4) for later morphological