Parasitology International (2017.1) 66(3):240-248.

Brachylaima ezohelicis sp. nov. (Trematoda: Brachylaimidae) found from the land snail Ezohelix gainesi, with a note of an unidentified Brachylaima species in Hokkaido, Japan.

Minoru Nakao, Tsukasa Waki, Mizuki Sasaki, Jason L. Anders, Daisuke Koga, Mitsuhiko Asakawa

Brachylaima ezohelicis sp. nov. (Trematoda: Brachylaimidae) found from the land snail *Ezohelix gainesi*, with a note of an unidentified *Brachylaima* species in Hokkaido, Japan

Minoru Nakao [a,*], Tsukasa Waki [b], Mizuki Sasaki [a], Jason L. Anders [c], Daisuke Koga [d], Mitsuhiko Asakawa [e]

[a] Department of Parasitology, Asahikawa Medical University, Asahikawa, Hokkaido 078-8510, Japan
[b] Meguro Parasitological Museum, Shimomeguro, Meguro-ku, Tokyo 153-0064, Japan
[c] Graduate School of Environmental Earth Science, Hokkaido University, Sapporo, Hokkaido 060-0808, Japan
[d] Department of Microscopic Anatomy and Cell Biology, Asahikawa Medical University, Asahikawa, Hokkaido 078-8510, Japan
[e] School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan

* Corresponding author.
E-mail address: nakao@asahikawa-med.ac.jp

ABSTRACT

In the Japanese Archipelago, *Ezohelix gainesi*, a member of bradybaenid land snails, is endemic mainly to the island of Hokkaido. During July to August of 2016, a survey to detect trematode infections from *E. gainesi* was carried out at a forest city park in Asahikawa, Hokkaido. Systemic infections of the snails with sporocysts containing short-tailed cercariae were found in 5.3% of 94 individuals examined. Furthermore, most of them (90.4%) harbored non-encysted metacercariae within their kidneys. A DNA sequence identification revealed that both of the sporocyst and the metacercaria belong to an unknown species of the family Brachylaimidae. The metacercariae showed a genetic diversity with 6 haplotypes of mitochondrial DNA (mtDNA) even in the limited sampling area. A definitive host of the unknown species could not be determined, although 34 field mice (*Apodemus speciosus*) and 21 voles (*Myodes rufocanus*) from the city park were examined for intestinal parasites. To examine the adult stage, the metacercariae were perorally administrated to mice, together with anti-inflammatory treatment with methylprednisolone. Fully matured adult worms were recovered from the intestinal ileum 8 and 14 days postinfection. The gravid adults showed typical features of the genus *Brachylaima*. A morphological and biogeographical evaluation prompted us to propose *Brachylaima ezohelicis* sp. nov. for the parasite from *E. gainesi*. The autochthony of the first intermediate host and the spatial heterogeneity of mtDNA suggest that the new species found in the city park is not a recently expanded population of immigrant origin.

Keywords:
Brachylaima ezohelicis sp. nov.
Ezohelix gainesi
Hokkaido

1. Introduction

Land snails are terrestrial gastropod mollusks with or without shells, and the majority are hermaphroditic pulmonates. They have been highly diversified in the Japanese Archipelago, and the resulting fauna now includes approximately 800 species in spite of its small land area [Biodiversity Center of Japan (biodic.go.jp)]. Many of them are endemic species to Japan. Such a unique situation provides advantages to study evolutionary ecology in a geographic context. However, there have been few studies done concerning host-parasite ecological relationships between Japanese land snails and their internal organisms.

As concerns trematode parasites, members of the families Brachylaimidae [1] and Dicrocoeliidae [2] exclusively use land snails as the first intermediate host for asexual proliferation of cercarial larvae, which occurs within sporocysts grown in the hepatopancreas [3]. Land snails are also required as intermediate hosts in maintaining the life cycle of the Leucochloridiidae [4]. Especially in brachylaimid species, another land snail individual is further needed as the second intermediate host. That is, cercariae released from an infected snail invade another snail where they metamorphose into metacercariae in the kidney or the pericardial cavity [3]. Birds and mammals become definitive hosts by feeding on the second intermediate hosts. Exceptionally, amphibians and reptiles can serve as definitive hosts for a few brachylaimid species belonging to the subfamily Zeylanurotrematinae [5]. Ingested metacercariae develop into adult worms in the alimentary canal of birds and mammals, and resulting fecal eggs are fed on by snails [3]. Brachylaimids are thus strictly linked to land snails under ecological and evolutionary forces.

The family Brachylaimidae is divided into 3 or 4 subfamilies [1, 5, 6]. The subfamily Brachylaminae is the largest group, including the genera *Brachylaima*, *Glaphyrostomum*, *Postharmostomum*, *Ectosiphonus*, *Parabrachylaima*, and *Renylaima* [1, 6]. Several species of this subfamily have been recorded from birds and mammals in Japan. They are *Brachylaima syrmatici* from the copper pheasant [7], *Brachylaima eophonae* from the Japanese grosbeak [8], *Brachylaima ishigakiense* from the black rat [9], *Glaphyrostomum soricis* from

the long-clawed shrew [10], *Postharmostomum gallinum* from the domestic fowl [11], and *Brachylaima tokudai* and *Ectosiphonus orientalis* from the Japanese shrew mole [12, 13]. Records of undetermined brachylaimids are as follows: *Brachylaima* spp. from the large Japanese field mouse [14], the brown rat [15], the domestic dog [16], and the raccoon (an alien introduced into Japan) [17] and *Ectosiphonus* sp. from the long-clawed shrew [10]. Moreover, *Panopistus japonicus* of another subfamily Panopistinae has been found again from the Japanese shrew mole [13]. Nevertheless, there are only a few early reports on detection of larval brachylaimids from land snails in Japan [18, 19].

In this study, a small number of immature brachylaimids were found from intestines of Japanese toads (*Bufo japonicus formosus*) captured in a forest city park in Asahikawa, Hokkaido, the northernmost island of Japan. The infection seems to be accidental because the gravid adult worms were never seen in the other toad samples. The toad is an alien species from the Kanto region of Honshu island where there are no records of brachylaimid infections in amphibians [20]. A subsequent snail survey in the city park showed that land snails of *Ezohelix gainesi* (Bradybaenidae) are heavily infected with both sporocysts and metacercariae. A DNA sequence identification revealed that all the immature parasites detected from the toads and the snails belong to the same species. Fully matured adults were obtained through an experimental infection of immunosuppressed mice with metacercariae from *E. gainesi*. Based on morphological features of the adult specimens, we propose a new species of the genus *Brachylaima*. In this study, an additional description of *Brachylaima* sp., which has already been reported from the large Japanese field mouse *Apodemus specious* in Hokkaido [14], was also made for comparison with the new species.

2. Materials and methods

2.1. Field surveys

Field surveys were mostly conducted at Kaguraoka Park (43°44'52"N, 142°22'03"E), a forest city park located in the uptown of Asahikawa, Hokkaido. The park consists of a riverside (artificial zone) and a hillside (natural forest

zone), surrounded by residential housing. Its total land area is 40.99 ha.

An amphibian survey was carried out to examine the parasitic infections of *B. japonicus formosus* in September, 2015 and May, 2016. The captured toads were subjected to necropsy to detect parasites from internal organs. A dissection of the organs was done in Dulbecco's phosphate-buffered saline (PBS) under a binocular microscope.

The discovery of immature brachylaimids from the toads prompted a subsequent snail survey to confirm larval brachylaimids from land snails. A preliminary collection of land snails showed that *Hemipoma hakodadiense* (Helicinidae), *Succinea lauta* (Succineidae), *Cochlicopa lubrica* (Cochlicopidae), *Discus pauper* (Discidae), *Zonitoides arboreus* (Gastrodontidae), and *Acusta despecta sieboldiana* (Bradybaenidae) are thinly distributed in the riverside of the city park, but a large population of *E. gainesi* inhabits the hillside section. The snail survey, therefore, focused on *E. gainesi*. The snails were collected during July to August of 2016. Shell dimensions (height and width) were measured prior to dissection. After removing shells, each snail was dissected in PBS, focusing mainly on the hepatopancreas and the kidney. When metacercariae were found, the number of individuals were counted. To compare the prevalence of brachylaimids, *E. gainesi* was further collected from the suburbs of Asahikawa, namely Asahiyama Park (43°45'56"N, 142°28'40"E) and Arashiyama Park (43°47'36"N, 142°18'09"E). Linear distances from the city park to Asahiyama and Arashiyama are 9.3 km and 6.6 km, respectively.

Definitive hosts of brachylaimids were examined through a rodent survey in August of 2016. Rodents were captured by Sherman box traps baited with plain oatmeal (Quaker Oats Company) or small pieces of fish sausage (Nissui Company). During 5 consecutive nights, a total of 424 traps were set in the underbrush. Intestines of captured rodents were opened in PBS to examine parasites.

Most of the parasites collected from the field surveys were used for morphological observations and experimental infections. The remaining parasites were kept in 70% ethanol for later DNA analyses.

2.2. Experimental infections of mice and rats

To obtain adult worms of the unknown species, 20 to 30 metacercariae were perorally administrated to each of 7 female ICR mice. Simultaneously, 0.2 ml Depo-Medrol® (Pfizer) (corresponding to 8 mg methylprednisolone acetate) was subcutaneously injected into each of 6 mice to prevent inflammatory reactions. After 8, 14, and 21 days postinfection, two mice each were killed with ethyl ether to recover adult worms from the intestine. One mouse without methylprednisolone treatment was killed 8 days postinfection. A formalin-ether sedimentation technique [21] was used to confirm parasite egg outputs into feces.

Wild rodents of the genus *Rattus* serve as definitive hosts for many species of *Brachylaima*. Laboratory rats were used for experimental infections to check for their susceptibility to the unknown species. Three young female SD rats (6-week-old) were similarly infected with 20 to 30 metacercariae. One of the rats was simultaneously treated with subcutaneous injection of 0.3 ml Depo-Medrol®. The immunosuppressed rat and the others were killed 11 and 14 days postinfection, respectively.

2.3. DNA analyses

Templates of PCR (polymerase chain reaction) were simply prepared without purification of genomic DNA. Using a PCR thermal cycler, the small piece (approximately $1mm^3$) of sporocysts or adults was lysed in 25 µl of 0.02N NaOH at 99°C for 30 minutes. The whole body was lysed in the case of metacercariae used. One µl of each of the crude lysates was used as a template for PCR.

Both nuclear 28S ribosomal DNA (rDNA) and mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) were selected as target genes. The primer sets used are as follows: digl2 and 1500R for 28S rDNA [22] and JB3 and CO1-R trema for *cox1* [23]. A Tks Gflex DNA polymerase (TaKaRa) and the manufacturer-supplied reaction buffer, which are standardized for crude templates, were used for PCR. The PCR was run in 25 µl reaction volume including 0.25 µM each primer for 35 cycles (98°C for 10 sec, 50°C for 20 sec, and 68°C for 90 sec). When the extra bands appeared, the annealing temperature was set to 55°C. The amplicons were read 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 28S rDNA and *cox1* sequences determined were 1268 and 786 bases in length, respectively. A molecular identification of parasites was done through a similarity search for the sequences by the BLAST algorithm (ddbj.nig.ac.jp/blast).

Nucleotide data sets of 28S rDNA and *cox1* were prepared using the multiple aligner MAFFT [24]. Sequences of related taxa were retrieved from DDBJ/EMBL/GenBank databases. All gaps of the 28S rDNA alignment were removed. Phylogenetic trees were made by the neighbor-joining (NJ) method using the genetic software MEGA6 [25]. Population genetics indices were calculated using Arlequin 3.5 [26]. A network figure of *cox1* haplotypes was illustrated by TCS1.21 [27].

2.4. Morphological observations

A calibrated optical microscope with a digital camera (Axio Imager, Zeiss) was used for morphological observation. Sizes of objects were measured via their digital images using the accessory software (AxioVision).

Histological sections of infected snails were prepared to observe the internal structure of affected organs. The hepatopancreas and the kidney were kept in 10% neutral-buffered formalin, and later embedded in paraffin wax. Histological sections of 5 μm thickness were stained with hematoxylin and eosin.

Sporocysts and cercariae were mounted with PBS between glass slides and coverslips for observation in a living condition. The vital dye, neutral red, was used at approximately 0.05 % in PBS to observe the morphology of cercariae.

Non-encysted metacercariae and gravid adults were similarly observed in a living condition, and then the mounting medium between glass slides and coverslips was replaced with 10% neutral-buffered formalin. A slight pressure was added on the coverslip to arrange the posture of the worms. After removing the extra formalin, the glass slides were kept in a moisture box overnight. The resultant unstained specimens were photographed to measure the size of various parts. Using AFA (alcohol–formalin–acetic acid) solution, a post-fixation was further done overnight. Fully fixed worms were stained with acetocarmine to make permanent specimens [28].

Several gravid adults (14 days postinfection) were prepared for scanning electron microscopy (SEM). The adults were fixed with 1% glutaraldehyde in phosphate buffer (0.1M PB, pH 7.4) for 24 h. For conductive staining, the fixed samples were treated with 1% tannic acid in 0.1M PB for 3 h, rinsed in the buffer for 1h, and immersed in 1% osmium tetroxide in 0.1M PB for 3 h. The samples were then dehydrated in a graded alcohol series, transferred to isoamyl acetate, and dried in a critical point dryer (HCP-2, Hitachi). Finally, the samples were mounted onto an aluminum stub with carbon tape and coated with platinum-palladium using an ion-sputter coater (E1010, Hitachi). The tegumental surface was observed with a field emission SEM (S4100, Hitachi) at an accelerating voltage of 7kV.

One adult specimen of *Brachylaima* sp. from a field mouse (*A. speciosus*) in Sapporo, Hokkaido [14] was used for a morphological description to taxonomically evaluate the brachylaimid species in Asahikawa. The gravid adult collected on 8 June 1986 was stained with acetocarmine and has been kept in the Wild Animal Medical Center in Rakuno Gakuen University, Ebetsu, Hokkaido.

3. Results

3.1. Amphibian survey

In September, 2015, ten adult toads of *B. japonicus* were collected from Kaguraoka Park. Immature brachylaimids attached on the intestinal mucosa of two toads (20% prevalence). The number of worms found from each of the two toads was 6 and 2. The development of the worms was arrested at the level of metacercaria (Fig. 1A). Gravid adults of the brachylaimid were never seen, although four toads were further examined in May, 2016. The results indicate the toad to be an unsuitable host, and the infection seemed to be accidental due to feeding on land snails.

A DNA identification based on sequences of 28S rDNA and *cox1* reconfirmed that immature worms from toads belong to the family Brachylaimidae.

3.2. Snail survey

A subsequent snail survey disclosed an extensive epidemic of brachylaimid infections among a closed population of *E. gainesi* in Kaguraoka park. As shown in Table 1, a total of 94 land snails of *E. gainesi* were examined during July and August of 2016. Out of them, 5 snails (5.3%) were infected with sporocysts. Furthermore, metacercariae were found from 85 snails (90.4%). Numbers of metacercariae per snail ranged from 1 to 126 (mean 15.4). All of the sporocyst-positive snails were co-infected with metacercariae. The shell size of infected snails was not significantly correlated to the number of metacercariae recovered (Supplementary Fig. 1). Infected snails were also found in one other suburb park, but the prevalence was not as high (Table 1).

Photographs of the infected snails are shown in Fig. 1B to 1D. Highly branched sporocysts expanded into both the outside and inside of the hepatopancreas around the digestive tube of *E. gainesi*. Their reticular appearance was like a coral forest. The sporocysts contained many short-tailed cercariae. A few sporocysts were also seen within the kidney of severely infected snails. The inside of attacked hepatopancreas was occupied by sporocysts with their surrounding hemocytes (Supplementary Fig. 2A). Non-encysted metacercariae were found within the kidney of *E. gainesi*. Urate particles were seen in the ceca of the metacercariae, suggesting that the metacercariae are eating into the kidney tissue. In the histological sections of kidney, metacercariae were observed in the lumen encircled with hemocytes and eosinophilic substances (Supplementary Fig. 2B). No metacercariae were found from the pericardial cavity adjacent to the kidney.

Larvae (sporocyst and metacercaria) from land snails were shown to be the same species as immature brachylaimids from toads by DNA identification. A geographic variation of mitochondrial DNA (mtDNA) was not observed between the larvae from the city park and the suburb park.

3.3. Rodent survey

To determine definitive hosts of the brachylaimid species, 34 field mice (*Apodemus specious*) and 21 voles (*Myodes rufocanus*) were collected from Kaguraoka park in August, 2016. Commensal rodents (*Rattus norvegicus*,

Rattus rattus, and *Mus musculus*) could not be captured in the park during this survey period. No brachylaimid adults were found from intestines of all the captured rodents, indicating that these rodents are unsuitable as a definitive host for the target species.

3.4. DNA phylogeny and diversity

Phylogenetic relationships of brachylaimids were inferred by sequences of 28S rDNA and *cox1,* using an unknown species found in this study and related taxa published in DNA databases [29, 30, 31, 32]. The NJ phylogenetic trees of the two genes strongly suggested that the unknown species belongs to the genus *Brachylaima*, although the number of taxa available for the tree construction was insufficient (Supplementary Fig. 3).

An analysis of mtDNA mutations in *cox1* sequences showed a genetic diversity of the unknown species even in the limited area of the forest city park. Randomly selected 30 metacercariae from 15 snails were subjected to the DNA sequencing of *cox1*. The resultant population genetics indices denoted that mtDNA has kept a sequence variability without bottleneck effects (Table 2). A mtDNA haplotype network also did not result in a star-like topology due to population bottleneck (Fig. 2). The indices and the network suggest a possibility that brachylaimids distributed in the city park are not a recently expanded population of immigrant origin. It is most likely that part of an indigenous parasite population has been enclosed in the city park under a rapid urbanization.

3.5. Adult worms from experimental infections

The unknown species was able to develop into the adult stage in immunosuppressed mice after oral administration of metacercariae. The mice were examined at days 8,14, and 21 postinfection. Gravid adults were recovered from the intestinal ileum at days 8 and 14, but no parasites were found at day 21. Some of the adults contained blood in their ceca, demonstrating the parasite to be hematophagous. The number of adults from each mouse ranged from 3 to 21. In the case of the mouse without methylprednisolone treatment, only 4 immature adults were recovered at day 8. After dissection, a stool examination was carried out using feces from the large intestine. The parasite egg output into feces was

confirmed only at day 14.

Also in the experimental infections of rats, 9 gravid adults and 3 immature adults were recovered from an immunosuppressed individual at day 11 postinfection. However, no parasites were found from the other untreated group (two rats) at day 14. Rats appear to be unsuitable as a definitive host for the unknown species.

Gravid adult worms obtained through the experimental infections showed a common feature of the genus *Brachylaima*, but the detailed morphological characteristics were distinctive when compared with already known species. A new species is, therefore, proposed for an unknown brachylaimid from *E. gainesi* in Hokkaido. The parasite usage of the indigenous snail as the first intermediate host reinforces the proposal.

3.6. A new species (Trematoda: Brachylaimidae)

3.6.1. Morphological description

Brachylaima ezohelicis sp. nov. Nakao, Waki et Sasaki (Figs. 3 to 5).

Japanese name: Maimai-sango-mushi (a coral-like parasite of a land snail).

The new species was described using formalin-fixed unstained specimens of gravid adults and metacercariae. The former was from experimentally infected mice (14 days postinfection), and the latter was from naturally infected *E. gainesi*. Acetocarmine-stained adults and metacercariae were secondarily used. Eggs were obtained through a fecal examination of the mice. Cercariae from naturally infected *E. gainesi* were observed in a living condition. Number of specimens observed is as follows: adults (n=20), eggs (n=10), cercariae (n=11), and metacercariae (n=12). All specimens were observed ventrally except eggs. All measurements, unless indicated otherwise, are in μm as the mean, with range in parentheses. The detailed description of ovary complex in the adult was omitted because of the difficulty of tracing its duct system.

Adult (Figs. 3 and 4). Body cylindrical, 6.2 mm (6.9-5.5 mm) long by 1.0 mm (1.2-0.9 mm) in maximum width, covered with minute tegumental spines only on the ventral surface of the upper half. Oral and ventral suckers approximately equal in size to each other. Oral sucker subspherical, 390 (429-353) long by 361 (406-313) wide, located anterior end. Ventral sucker spherical, 360 (420-316)

long by 369 (406-334) wide, positioned on anterior quarter of body. Prepharynx short. Pharynx muscular, rounded in globular form, 209 (253-182) long by 245 (272-214) wide. Cecum undulating tubular, bifurcated immediately at postpharyngeal position, then running along bilateral borders to posterior extremity. Gonads and male genitalia occupied posterior part corresponding to about 40% of the body. Testes larger than ovary. Testes weakly lobed in irregular patterns, 703 (834-551) long by 654 (803-507) wide (anterior one), 740 (890-645) long by 585 (714-475) wide (posterior one), located between ceca. Cirrus pouch elongate-oval, containing coiled ejaculatory duct and cirrus, 308 (373-117) long by 112 (135-87) wide, vertically located in front of anterior testis. Small bursa connected cirrus pouch. Pars prostatica located at the end of seminal vesicle, not included in cirrus pouch. Seminal vesicle well-developed, winding around cirrus pouch, then descending to left side of anterior testis. Ovary subspherical or irregular in shape with smooth margin, 290 (371-230) long by 431 (562-299) wide, located between testes. Vitelline reservoir triangular, laying on ovary. Seminal receptacle spherical, 109 (168-75) long by 98 (163-57) wide, located left side of ovary. Uterus long and tortuous, occupied in intercecal field. The egg-filled tube starting from right side of anterior testis, ascending dorsally to cecum bifurcating position, then descending ventrally to genital pore in front of anterior testis. Metraterm running along the right side of cirrus pouch. Female and male genital ducts open to a common genital pore, not forming a hermaphroditic duct. Vitellarium follicular, extending bilaterally from level of posterior margin of ventral sucker to level of frontal margin of anterior testis. Excretory vesicle tubular, located between posterior extremities of ceca. Excretory pore terminal.

Egg (Fig. 5A). Shell light brown. Shape asymmetric, 32 (34-31) long by 19 (21-18) wide. Small operculum present at narrow end. Opposite end smooth or having a tiny knob. Miracidium with long cilia enveloped in thin membrane.

Cercaria (Fig. 5B). Body slender in posterior portion, 507 (586-428) long by 160 (180-134) in maximum width. Oral and ventral suckers approximately equal in size to each other. Oral sucker spherical, lacking stylet, 83 (90-73) long by 86 (92-78) wide, located on anterior end. Ventral sucker spherical, 83 (92-73) long by 85 (94-77) wide, positioned on posterior half of body. Prepharynx short.

Pharynx globular, 34 (40-30) long by 42 (45-34) wide. Cecum reverse-Y-shaped. Ending of ceca not exceeded to level of center of ventral sucker. Genital primordium located in front of tail. Tail rudimentary, 45 (56-35) long by 49 (54-44) wide.

Metacercaria (Fig. 5C). Body non-encysted, 2.9 mm (3.2-2.5 mm) long by 0.7 mm (0.7-0.6 mm) in maximum width. Oral sucker slightly larger than ventral sucker. Oral sucker spherical, 284 (307-270) long by 277 (319-248) wide, located on anterior end. Ventral sucker spherical, 259 (288-225) long by 254 (295-229) wide, positioned in anterior half of the body. Prepharynx short. Pharynx globular, 135 (150-116) long by 179 (200-140) wide. Cecum well-developed into undulating tube, bifurcated immediately at postpharyngeal position, then running along bilateral borders to posterior extremity. Immature gonads located between posterior ceca. Genital pore unobservable. Immature testes irregular in shape, 191 (273-116) long by 202 (264-121) wide (anterior one), 229 (305-148) long by 163 (217-123) wide (posterior one). Immature ovary irregular in shape, 151 (191-117) long by 159 (237-114) wide, located between immature testes. Immature uterus visible in midline of body. The thin uterus running along right side of anterior testis, overpassing ventral sucker, then returning to early genitalia in front of anterior testis. Immature vitelline follicles present in bilateral margins of mid-body.

3.6.2 Taxonomic summary

Type host: *Ezohelix gainesi* (Pilsbry, 1900).

Sites of infection: Hepatopancreas (sporocyst) and kidney (metacercariae).

Type locality: Asahikawa, Hokkaido, Japan.

Collector: M. Nakao.

Date of collection: 13 July 2016.

Definitive hosts: Unknown. The laboratory mouse (*Mus musculus*) or rat (*Rattus norvegicus*) can be used as alternative hosts under immunosuppressive condition.

Type specimens: One holotype and 10 paratypes have been deposited in Meguro Parasitological Museum, Tokyo, Japan under MPM collection numbers 21301 (1 holotype adult), 21302 (7 paratype adults), and 21303 (3 paratype

metacercariae).

Differential molecular markers: The parasite DNA sequences of nuclear 28S rDNA and mitochondrial *cox1* have been deposited into DDBJ/EMBL/GenBank databases under accession numbers LC198310 (28S rDNA) and LC198311 to LC198316 (*cox1*).

Etymology: The new species is named after the generic name of the first intermediate host.

3.7. Description of a related species from a rodent in Hokkaido

Another *Brachylaima* sp. found from *A. specious* in Sapporo, Hokkaido [14] was compared with the new species in Asahikawa. Morphological features of the rodent-derived adult worm were distinct from those of the new species as shown in the following description. The specimen was observed ventrally. All measurements, unless indicated otherwise, are in μm. The specimen has been deposited as a voucher in Meguro Parasitological Museum, Tokyo, Japan (MPM collection number 21304).

Adult (Fig. 6). Body allantoid, 4.4 mm long by 0.9 mm in maximum width. Anterior body surface spinose. Oral and ventral suckers approximately equal in size to each other. Oral sucker subspherical, 358 long by 330 wide, located on anterior end. Ventral sucker spherical, 318 long by 319 wide, located approximately 30% of body length from anterior end. Prepharynx absent. Pharynx globular, 154 long by 220 wide. Cecum tubular, bifurcated immediately at postpharyngeal position, then running straight along bilateral borders to posterior extremity. Gonads and male genitalia positioned in posterior quarter of body. Testes and ovary approximately equal in size to one another. Testes spherical with smooth margin, 330 long by 336 wide (anterior one), 330 long by 294 wide (posterior one), located between posterior ceca. Cirrus pouch small, 143 long, lying in front of anterior testis. Ovary spherical, 298 long by 316 wide, located between testes. Uterus long and tortuous, occupied in intercecal field. The egg-filled tube starting from right side of anterior testis, ascending dorsally to cecum bifurcating position, then descending ventrally to genital pore in front of anterior testis. Vitellarium follicular, extending bilaterally from level of posterior margin of ventral sucker till level of anterior margin of ovary.

The rodent-derived adult specimen clearly differs from the new species, particularly in having small spherical testes and ovary, straight tubular ceca, and widely distributed vitelline follicles. The apparent difference indicates that at least two species of *Brachylaima* are distributed in Hokkaido, depending on their different life cycles.

4. Discussion

The distribution of parasites is controlled in various environments by host-parasite ecological relationships, depending on the degrees of the parasite's infectivity to hosts and the host's susceptibility to parasites. Each trematode species individually uses a particular snail as the obligate first intermediate host. This interaction is highly specific and results in a strong natural selection on the parasites [33]. The distribution of trematodes is, therefore, restricted to limited areas in which susceptible snails are distributed. In the Japanese Archipelago, the land snail *E. gainesi* is endemic to Hokkaido and small limited areas of the Tohoku region in northern Honshu island [34], as well as the southern part of the Kurile Islands [35]. The nonexistence of *E. gainesi* in neighboring continental areas leads us to speculate that allopatric speciation occurred in this snail together with its commensal parasites. Indeed, we discovered the novel trematode *B. ezohelicis* sp. nov. from *E. gainesi* in Hokkaido. The new species was at first thought to be an immigrant species due to the recent anthropogenic introduction of host animals, because the parasite showed morphological similarities to already known species from rodents of the genus *Rattus* [9, 36, 37, 38, 39]. However, the population genetics analysis of the parasite mtDNA suggests that the new species is originally indigenous to Hokkaido. Several reports [37, 40, 41] demonstrated experimentally that *Brachylaima* spp. show a strict host specificity for their first intermediate hosts. Namely, each species is highly specific to a single snail species in producing cercariae, but the range of the second intermediate host expands to include several snail species. An exclusive relationship between brachylaimids and land snails seems to have established in every locality during a long evolutionary process. The present data clearly showed that *E. gainesi*, indigenous to

Hokkaido, serves as the first intermediate host for *B. ezohelicis* sp. nov. This evidence also prompted us to reject a hypothesis that the new species is of recent immigrant origin.

The morphological identification of *Brachylaima* is very difficult, because members of the genus are quite similar to each other even in their adult stage. The upper intercecal field of *B. ezohelicis* sp. nov. is fully occupied with coiled uterus up to the postpharyngeal position. This feature could be used to separate the new species from others whose anterior uterine loop turns back at the posterior margin of the ventral sucker (e.g., *B. tokudai* [12], *Brachylaima chiapensis* [42], *Brachylaima thompsoni* [42], and *Brachylaima microti* [43]). However, the uterine loops of many species extend beyond ventral sucker, as similar to the new species. In Japan, *Brachylaima* spp. have hitherto been described only in very small numbers. These are *B. syrmatici* [7] and *B. eophonae* [8] from birds and *B. tokudai* [12] and *B. ishigakiense* [9] from mammals. Among them, *B. eophonae* and *B. ishigakiense* are morphologically very similar to *B. ezohelicis* sp. nov. However, the new species clearly differs from *B. eophonae* in having larger testes and shorter fields of bilateral vitellarium. The differentiation of *B. ishigakiense* from the new specie was the most difficult, because the description of *B. ishigakiense* was made using contracted adult worms recovered from formalin-fixed rats [9]. A morphometrical assessment, therefore, had to be avoided in *B. ishigakiense*. In this species, the presence of receptaculum seminis uterinum (i.e., the lack of a seminal receptacle) [9] could be a unique differential point due to the new species having a well-developed seminal receptacle. Comparative checkpoints between *B. ezohelicis* sp. nov. and other closely related species in different countries are summarized in Table 3. Rodents of the genera *Rattus* and *Mus* serve as definitive hosts for all of the related species. As compared with them, morphometric measurements of the new species are mostly larger, particularly in its testes. Among species listed in Table 3, *B. cribbi* [39] and *B. ratti* [36] are very similar to the new species. However, the new species keeps distinctive characters such as lobed testes and limited distribution of tegumental spines (Fig. 4). The short ceca of larval cercaria can also be used as a distinctive character from *B. cribbi* [39]. These comparative checkpoints indicate that large lobed testes are the most specific to

B. ezohelicis sp. nov., but its shapes are too variable to use as a key character for species identification (Supplementary Fig. 4).

The definitive hosts of *B. ezohelicis* sp. nov. could not be determined through a rodent survey of Kaguraoka park. The survey showed that the field mouse *A. speciosus* and the vole *M. rufocanus* are negative for brachylaimid infections. Experimental infections of mice and rats also suggest that rodents are unsuitable hosts for the new species. In the city park, the other common mammals are limited mainly to the long-clawed shrew *Sorex unguiculatus*, the Ezo red squirrel *Sciurus vulgaris orientis*, and the red fox *Vulpes vulpes*. Shrews serve as a definitive host for *Brachylaima* spp. in North America [44] and Europe [45]. However, a sequential parasite survey of shrews in Hokkaido [46] did not lead to the discovery of *Brachylaima*. Infections of *Brachylaima* spp. also have been found from squirrels [47] and foxes [48] in Europe, although their prevalences were at low levels. Concerning birds, many species of the orders Passeriformes, Piciformes, Accipitriformes, and Strigiformes are observed in the city park as migrants or residents. There are unfortunately no referential studies on ecological relationships between wild birds and *Brachylaima* in Asia. If *B. ezohelicis* sp. nov. is susceptible to both birds and mammals, the recognition of the definitive host will cause confusion. Indeed, some species of *Brachylaima* have a broad host range from birds to mammals [49]. An ecological survey to specify the main predators of *E. gainesi* would give clues into understanding the missing link between *B. ezohelicis* sp. nov. and its definitive hosts.

The present study indicates that another species of *Brachylaima* is distributed in Hokkaido, using the field mouse *A. speciosus* as a definitive host. A simple sketch of *Brachylaima* sp. from the brown rat *R. norvegicus* in Hokkaido (Y. Tada, unpublished data from a master thesis of Graduate School of Veterinary Medicine, Hokkaido University, 1974) depicted its straight tubular ceca, bilateral vitellarium extending to the level of posterior margin of anterior testis, and small spherical testes and ovary. These characteristics are completely identical to those of *Brachylaima* sp. presented in this study (Fig. 6). This coincidence suggests that *Brachylaima* sp. can utilize a wide range of rodents as definitive hosts. In the Russian Far East, *Brachylaima apodemi* has been found from the striped field mouse *Apodemus agrarius* [50]. *Brachylaima*

sp. in Hokkaido clearly differs from *B. apodemi*, because the vitellarium field of *B. apodemi* starts from the level of pharynx. The land snail fauna of Hokkaido is relatively simple with a small number of species [Biodiversity Center of Japan (biodic.go.jp)]. The major candidates of the first intermediate host for *Brachylaima* sp. in Hokkaido are included in the families Clausiliidae (*Vitriphaedusa micropeas hokkaidoensis* and *Euphaedusa rowlandi*), Discidae (*D. pauper*), and Bradybaenidae (*A. despecta sieboldiana*, *E. gainesi*, *Ainohelix editha*, *Karafthelix blakeana,* and *Euhadra brandtii sapporo*). After confirming the first and second intermediate hosts, *Brachylaima* sp. in Hokkaido will be redescribed as a new species in combination with its larval morphology and DNA differential markers.

The prevalence of larval brachylaimid infections in *E. gainesi* snails was much higher in Kaguraoka park than in other suburb parks. The high prevalence observed in the city park was likely associated with its geographic location. The surrounding residential area possibly confines *E. gainesi* and definitive hosts within the city park, resulting in the increased opportunity of snail infection with parasite eggs. In addition, the high population density of *E. gainesi* in the city park is responsible for an efficient propagation of the infection from one individual (the first intermediate host) to others (the second intermediate host). The infected snail releasing cercariae acts as a hyper spreader to many other snails. One of the major costs for *Brachylaima* cercariae released from the first intermediate host is that of searching for the second intermediate host. In this study, we showed that *B. ezohelicis* sp. nov. utilizes *E. gainesi* as both the first and the second intermediate hosts. The benefit of this life cycle-strategy of the new species is likely that the released cercariae can reach the second intermediate host by short distance migration, because land snails of the same species generally form colonies as a result of species-specific microhabitat preferences [51, 52]. Moreover, an exogenous self-infection of the first intermediate host by self-released cercariae cannot be disregarded, given that metacercariae do not develop inside the sporocysts, opposite to what happens in ancient brachylaimids [32].

A morphological similarity among closely related taxa is responsible for misidentification of species and its consequent taxonomic confusion. Many old

species of *Brachylaima* were erected by only observations of morphologically similar adult worms, without connection with larval stages in land snails. The incomplete observation might have caused the overestimation of identical populations into different species or the underestimation of different populations into synonyms. Because of strict specificity of the first intermediate host, the group of *Brachylaima* seems to contain many morphologically indistinguishable cryptic species. To correctly identify species, a cercarial chaetotaxy has been introduced into the taxonomy of *Brachylaima* [37], and guarantees beneficial effects on describing new species [39, 40, 41]. The cercarial chaetotaxy of *B. ezohelicis* sp. nov. is expected to be described in the near future.

The use of DNA sequencing is desirable for species identification to obtain a reliable result from specimens of any developmental stages. In this study, we showed a very simple method for preparing PCR templates. By using this method, we could determine parasite DNA sequences even in a single metacercaria or a small portion of sporocysts. This method is beneficial for both taxonomical and ecological studies of trematodes. Based on the DNA sequencing and conventional morphological observation, we are now categorizing trematodes from Japanese land snails. The results will be an important foundation in clarifying the parasite fauna of the families Brachylaimidae, Dicrocoeliidae and Leucochloridiidae in Japan. The integration of DNA barcoding and morphological approaches [53] is essential for identifying cryptic species of the trematodes and reorganizing their systematics.

Acknowledgements

MN and JLA thank Itsuro Koizumi (Graduate School of Environmental Earth Science, Hokkaido University) and his laboratory members for collecting rodents in Asahikawa. MN greatly acknowledges Hirotaka Katahira (Faculty of Bioindustry, Tokyo University of Agriculture) for providing unpublished data on rodent infections of *Brachylaima* sp. in Hokkaido. Thanks are also due to anonymous reviewers for their valuable suggestions in preparing this article. The collections of toads, snails, and rodents in Kaguraoka Park were permitted by the municipal office of Asahikawa (permission nos. 505 and 134). The rodent

collection was further accepted by the Hokkaido government. This study was supported in part by a research grant from the Akiyama Life Science Foundation in 2015.

References

[1] T. Pojmańska, Family Brachylaimidae Joyeux & Foley, 1930, in: D.I. Gibson, A. Jones, R.A. Bray (Eds.), Keys to the Trematoda, Vol. 1, CAB International and The Natural History Museum, London 2002, pp. 37-43.

[2] T. Pojmańska, Family Dicrocoeliidae Looss, 1899, in: D.I. Gibson, A. Jones, R.A. Bray (Eds.), Keys to the Trematoda, Vol. 3, CAB International and The Natural History Museum, London 2008, pp. 233-260.

[3] O.W. Olsen, Animal Parasites, Their Life Cycles and Ecology (3rd Edit.), University Park Press, Baltimore, 1974 (562 pp.).

[4] T. Pojmańska, Family Leucochloridiidae Poche, 1907, in: D.I. Gibson, A. Jones, R.A. Bray (Eds.), Keys to the Trematoda, Vol. 1, CAB International and The Natural History Museum, London 2002, pp. 47-51.

[5] T.H. Cribb, P.D. Barton, *Zeylanurotrerna spearei* sp. n. (Digenea: Brachylaimidae) from the cane toad, *Bufo marinus*, in Australia, Zool. Scripta 20 (1991) 207-213.

[6] W.F. Sirgel, S. Mas-Coma, *Renylaima capensis* n. gen., n. sp. (Trematoda: Brachylaimidae) from the urinary system of the shrew *Myosorex varius* Smuts, 1832 (Insectivora: Soricidae), Parasitol Res. 106 (2010) 1443-1453.

[7] S. Yamaguti, Studies on the helminth fauna of Japan. Part 5. Trematodes of birds, III, Jpn. J. Zool. 6 (1935) 159-182.

[8] S. Yamaguti, Studies on the helminth fauna of Japan. Part 32. Trematodes of birds, V, Jpn. J. Zool. 9 (1941) 321-341.

[9] H. Kamiya, M. Machida, *Brachylaima ishigakiense* n. sp. (Trematoda, Brachylaimidae) from roof rat, *Rattus rattus* Linnaeus, Bull. Natn. Sci. Mus. Tokyo Ser. A (Zool.) 3 (1977) 125-129.

[10] M. Asakawa, H. Kamiya, M. Ohbayashi, Studies on the parasite fauna of Insectivora. V. Three trematodes from the Japanese *Sorex* spp., J. Rakuno

Gakuen Univ. 13 (1988) 21-27.

[11] S. Yamaguti, *Postharmostomum* Witenberg, 1923, in: S. Yamaguti, Synopsis of Digenetic Trematodes of Vertebrates Vol. I, Keigaku Publishing Co., Tokyo, 1971, pp. 517-518.

[12] S. Yamaguti, Helminth fauna of Mt. Ontake. Part 2. Trematoda and Cestoda, Acta Medica Okayama 8 (1952) 1-16.

[13] T. Kifune, M. Harada, I. Sawada, Tremadode parasites of the Japanese shrew moles (*Urotrichus* and *Dymecodon*) with descriptions of two new species, Med. Bull. Fukuoka Univ. 19 (1992) 101-108.

[14] M. Asakawa, M. Ohbayashi, Genus *Heligmosomoides* Hall, 1916 (Heligmosomidae: Nematoda) from the Japanese wood mice *Apodemus* spp. I. A taxonomical study on four taxa of the genus *Heligmosomoides* from three species of the Japanese *Apodemus* spp., J. Coll. Dairying 11 (1986) 317-331.

[15] Y. Tada, Parasitic helminthes of house rats in Hokkaido, especially on the *Angiostrongylus cantonensis* Chen, 1935 (in Japanese), J. Hokkaido Vet. Med. Assoc. 19 (1975) 95-98.

[16] R. Asato, H. Hasegawa, S. Kuniyoshi, T. Higa, Prevalence of helminthic infections in dogs in Okinawa Prefecture, Japan, Jpn. J. Parasitol. 34 (1985) 501-506.

[17] Y. Matoba, D. Yamada, M. Asano, Y. Oku, K. Kitaura, K. Yagi, F. Tenora, M. Asakawa, Parasitic helminths from feral raccoons (*Procyon lotor*) in Japan, Helminthologia 43 (2006) 139-146.

[18] J. Asada, I. Miyata, Studies on the cercariae in land snails, *Bradybaena similaris* and *Acusta sieboldiana* (in Japanese), Tokyo Iji Shinshi 3121 (1939) 492-497.

[19] Y. Kurisu, Studies on the trematodes parasitic in land snails in Japan, especially on the cercariae (in Japanese), Kumamoto Igakkai Zasshi 6 (1930) 1318-1331.

[20] K. Saito, H. Takeichi, N. Minami, A new record of *Bufo japonicus formosus* (Bufonidae, Amphibia) in Hokkaido, Japan (in Japanese), Bull. Asahikawa Mus. 2 (1996) 21-23.

[21] S. Uga, K. Tanaka, N. Iwamoto, Evaluation and modification of the

formalin-ether sedimentation technique. Trop. Biomed. 27 (2010) 177-184.

[22] S.S. Curran, V.V. Tkach, R.M. Overstreet, Phylogenetic affinities of *Auriculostoma* (Digenea: Allocreadiidae), with descriptions of two new species from Peru, J. Parasitol. 97 (2011) 661-670.

[23] O. Miura, A.M. Kuris, M.E. Torchin, R.F. Hechinger, E.J. Dunham, S. Chiba, Molecular-genetic analyses reveal cryptic species of trematodes in the intertidal gastropod, *Batillaria cumingi* (Crosse), Int. J. Parasitol. 35 (2005) 793-801.

[24] K. Katoh, D.M. Standley, MAFFT multiple sequence alignment software version 7: improvements in performance and usability, Mol. Biol. Evol. 30 (2013) 772–780.

[25] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725–2729.

[26] L. Excoffier, H.E.L. Lischer, Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows, Mol. Ecol. Res. 10 (2010) 564-567.

[27] M. Clement, D. Posada, K. A. Crandall, TCS: a computer program to estimate gene genealogies, Mol. Ecol. 9 (2000) 1657-1660.

[28] M. Nakao, *Phyllodistomum kanae* sp. nov. (Trematoda: Gorgoderidae), a bladder fluke from the Ezo salamander *Hynobius retardatus*. Parasitol. Int. 64 (2015) 314-318.

[29] V.V. Tkach, J. Pawlowski, J. Mariaux, Z. Swiderski, Molecular phylogeny of the suborder Plagiorchiata and its position in the system of Digenea, in: D.T.J. Littlewood, R.A. Bray (Eds.), Interrelationships of the Platyhelminthes, Taylor & Francis, London 2001, pp. 186-193.

[30] P.D. Olson, T.H. Cribb, V.V. Tkach, R.A. Bray, D.T. Littlewood, Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda), Int. J. Parasitol. 33 (2003) 733-755.

[31] P. Heneberg, J. Sitko, J. Bizos, Molecular and comparative morphological analysis of central European parasitic flatworms of the superfamily Brachylaimoidea Allison, 1943 (Trematoda: Plagiorchiida), Parasitology 143 (2016) 455-474.

[32] W.F. Sirgel, P. Artigas, M.D. Bargues, S. Mas-Coma, Life cycle of *Renylaima capensis*, a brachylaimid trematode of shrews and slugs in South Africa: two-host and three-host transmission modalities suggested by epizootiology and DNA sequencing, Parasit. Vectors 5 (2012) 169.

[33] K.C. King, J. Jokela, C.M. Lively, Trematode parasites infect or die in snail hosts, Biol. Lett. 7 (2011) 265-268.

[34] Y. Mori, J. Yokoyama, M. Kawata, A. Davison, S. Chiba, Evidence of introgressive hybridization between the morphologically divergent land snails *Ainohelix* and *Ezohelix*, Biol. J. Linnean Soc. 115 (2015) 77-95.

[35] T. Pearce, L. Prozorova, Y. Kuwahara, Terrestrial Mollusca on the Kuril Islands: previous records and problems for study, Bull. Russ. Far East Malacol. Soc. 6 (2003) 89-101.

[36] S.C. Baugh, Contributions to our knowledge of digenetic trematodes, IV, Z. Parasitenkd. 21 (1962) 502-512.

[37] S. Mas-Coma, I. Montoliu, The life cycle of *Brachylaima ruminae* n. sp. (Trematoda: Brachylaimidae), a parasite of rodents, Z. Parasitenkd. 72 (1986) 739-753.

[38] T.A. Wheeler, M. Roberts, M. Beverley-Burton, D.G. Sutton, *Brachylaima apoplania* n. sp. (Digenea: Brachylaimidae) from the Polynesian rat, *Rattus exulans* (Rodentia: Muridae), in New Zealand: origins and zoogeography, J. Parasitol. 75 (1989) 680-684.

[39] A.R. Butcher, D.I. Grove, Description of the life-cycle stages of *Brachylaima cribbi* n. sp. (Digenea: Brachylaimidae) derived from eggs recovered from human faeces in Australia, Syst. Parasitol. 49 (2001) 211-221.

[40] M. Gracenea, O. González-Moreno, Life cycle of *Brachylaima mascomai* n. sp. (Trematoda: Brachylaimidae), a parasite of rats in the Llobregat delta (Spain), J. Parasitol. 88 (2002) 124-133.

[41] P. Segade, C. Crespo, N. García, J.M. García-Estévez, C. Arias, R. Iglesias, *Brachylaima aspersae* n. sp. (Digenea: Brachylaimidae) infecting farmed snails in NW Spain: morphology, life cycle, pathology, and implications for heliciculture, Vet. Parasitol. 10 (2011) 273-286.

[42] J.E. Ubelaker, M.D. Dailey, Taxonomy of the genus *Brachylaima* Dujardin

(Trematoda: Digenea) with description of *B. chiapensis* sp. n. from Peromyscus guatemalensis in Mexico, J. Parasitol. 52 (1966) 1062-1065.

[43] F.J. Kruidenier, V. Gallicchio, The orthography of the Brachylaimidae (Joyeux and Foley, 1930); *Brachylaime microti* sp. nov.; *B. rauschi* McIntosh, 1950; and an addendum to Dollfus' (1935) list of *Brachylaime* (Trematoda: Digenea), Trans. Amer. Microscop. Soc. 78 (1959) 428-441.

[44] V.H. Oswald, Helminth parasites of the short-tailed shrew in central Ohio. Ohio J. Sci. 58 (1958) 325-334.

[45] C. Feliu, S. Mas-Coma, I. Montoliu, J. Gallego, *Brachylaima simoni* sp. n. (Trematoda: Brachylaimidae), a parasite of the shrew *Crocidura russula* (Hermann, 1780) (Insectivora: Soricidae) from the Pityusic island of Ibiza (Balearics), Helminthologia 23 (1986) 65-72.

[46] K. Mitsuhashi, A. Kawahara, M. Asakawa, First record of parasitic helminths from *Sorex minutissimus* with a special reference to helminthological review of the genus *Sorex* on Hokkaido, Japan (in Japanese), J. Rakuno Gakuen Univ. 38 (2013) 57-62.

[47] A. Diakou, E. Kapantaidakis, D. Youlatos, Endoparasites of the European ground squirrel (*Spermophilus citellus*) (Rodentia: Sciuridae) in central Macedonia, Greece, J. Nat. Hist. 49 (2015) 359-370.

[48] D.T. Richards, S. Harris, J.W. Lewis, Epidemiological studies on intestinal helminth parasites of rural and urban red foxes (*Vulpes vulpes*) in the United Kingdom, Vet. Parasitol. 59 (1995) 39-51.

[49] S. Yamaguti, *Brachylaima* Dujardin, 1843, in: S. Yamaguti, Synopsis of Digenetic Trematodes of Vertebrates Vol. I, Keigaku Publishing Co., Tokyo, 1971, pp. 730-732.

[50] E.V. Nadtochii, Helminth fauna of rodents in the Far-East (in Russian), Uchenye Zapiski Dal'nevostochnyi Gosudarstvennyi Universitet (Parazitologicheskie i Zoologischeskie issledovaniya na Dal'nem Vostoke) 16 (1970) 62-80.

[51] K. Hylander, C. Nilsson, J. B. Gunnar, T. Göthner, Differences in habitat quality explain nestedness in a land snail meta‐community. Oikos 108 (2005) 351-361.

[52] P. Sólymos, R. Farkas, Z. Kemencei, B. Páll-Gergely, F. Vilisics, A. Nagy,

E. Hornung. Micro-habitat scale survey of land snails in dolines of the Alsó-hegy, Aggtelek National Park, Hungary, Mollusca 27 (2009) 169-173.

[53] S.A. Nadler, G.P. De León, Integrating molecular and morphological approaches for characterizing parasite cryptic species: implications for parasitology, Parasitology 138 (2011) 1688-1709.

Figure Legends

Fig. 1. Brachylaimids found from toads and land snails. A) Immature worms from the intestine of *Bufo japonicus formosus*. Scale bar 0.5 mm. B) Branched sporocysts grown on the hepatopancreas of *Ezohelix gainesi*. Scale bar 1 mm. C) Sporocysts containing cercariae. Scale bar 200 μm. D) Metacercatiae emerged from the cut surface of the kidney of *E. gainesi*. Scale bar 3 mm.

Fig. 2. Frequencies of mtDNA haplotypes and their statistical parsimony network in a brachylaimid population found in a forest city park of Asahikawa. The haplotypes were named in alphabetical order. The size of ovals indicates the frequency of the haplotypes. Small circles show hypothetical haplotypes. A shaded oval represents a hypothetical ancestor.

Fig. 3. *Brachylaima ezohelicis* sp. nov. from an experimentally infected mouse (14 days postinfection). The ventral view of an adult worm (holotype). Scale bar 1 mm. Abbreviations are as follows: c, cecum; cp, cirrus pouch; ed, excretory duct; ev, excretory vesicle; gp, genital pore; m, metraterm; o, ovary; os, oral sucker; p, pharynx; sv, seminal vesicle; t, testis; u, uterus; vd, vitelline duct; vr, vitelline reservoir; vs, ventral sucker.

Fig. 4. Scanning electron microscope photographs of *Brachylaima ezohelicis* sp. nov. A) The ventral view of an adult worm. Arrows indicated a central area from which minute tegumental spines disappeared. Scale bar 1mm. B) The lateral view. The spines disappeared from lateral to back fields. Scale bar 100 μm. C) The spines. Scale bar 5 μm.

Fig. 5. Larval stages of *Brachylaima ezohelicis* sp. nov. A) Egg from feces of an experimentally infected mouse. Scale bar 10 μm. B) Cercaria from a naturally infected snail. Scale bar 100 μm. C) Metacercaria from a naturally infected snail. Scale bar 500 μm.

Fig. 6. *Brachylaima* sp. from a naturally infected field mouse (*Apodemus*

speciosus) in Hokkaido. Scale bar 1 mm.

Supplementary Fig. 1. A relationship between the shell size of infected snails (*Ezohelix gainesi*) and the number of metacercariae recovered from the snails.

Supplementary Fig. 2. Histological sections of infected snails (*Ezohelix gainesi*). A) The hepatopancreas occupied with sporocysts containing cercariae. B) The kidney parasitized with metacercariae. Scale bars of both photos represent 500 μm.

Supplementary Fig. 3. Molecular phylogenetic trees of an unknown species and related taxa of the families Brachylaimidae and Leucochloridiidae. The trees were made from DNA data sets by the neighbor-joining method with settings of K2-parameter model, a gamma shape of 0.5, and 500 bootstrap replicates. Values of nodes are bootstrap percentages. Scale bars represent divergence values. Nucleotide database accession numbers are shown in brackets. A) The nuclear 28S rDNA tree including 6 taxa (1196 nucleotide sites examined). B) The mitochondrial *cox1* tree including 5 taxa (168 nucleotide sites examined).

Supplementary Fig. 4. The morphological variation of testes of *Brachylaima ezohelicis* sp. nov. The shape of the testes varied from an oval with smooth margin to an irregular form with lobes. Scale bar 1 mm.

Fig.1

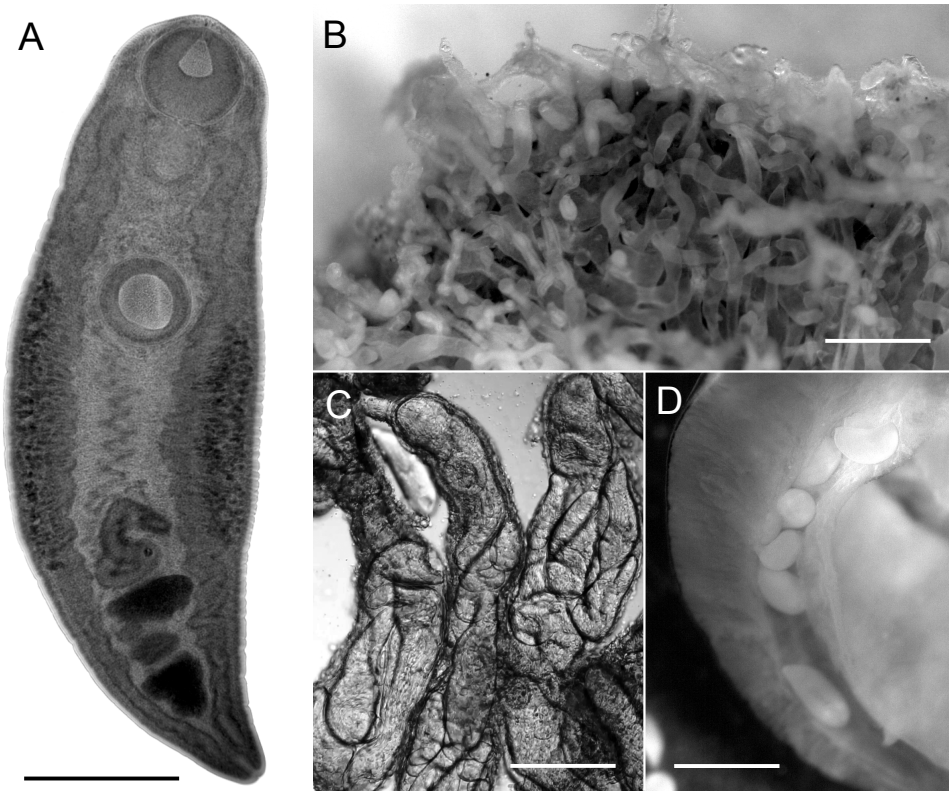


Fig. 2

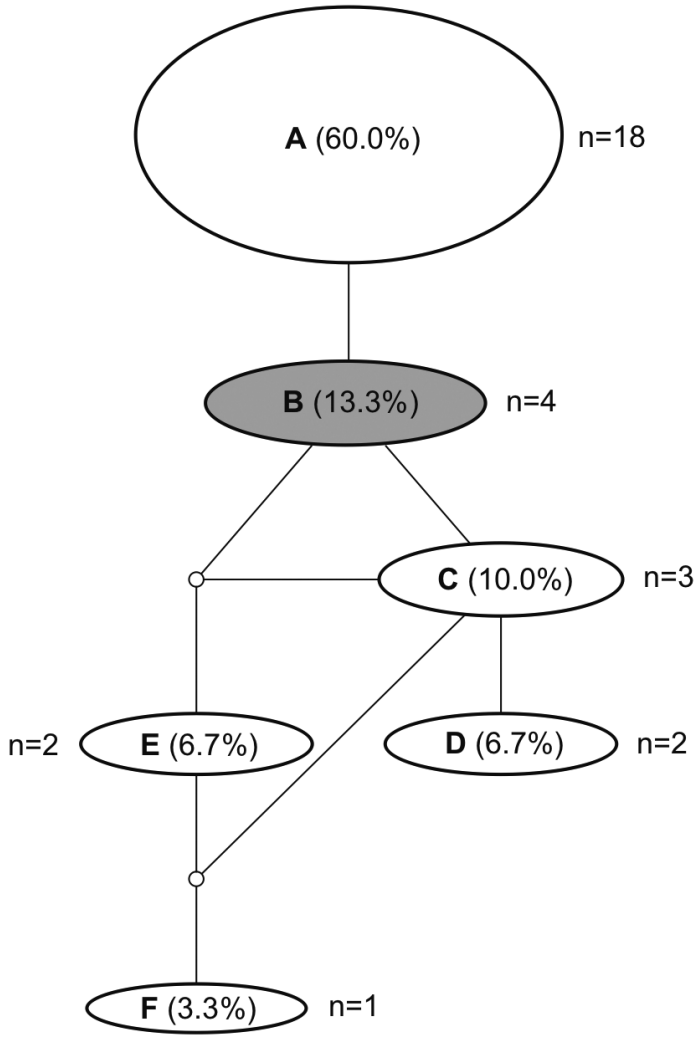


Fig. 3

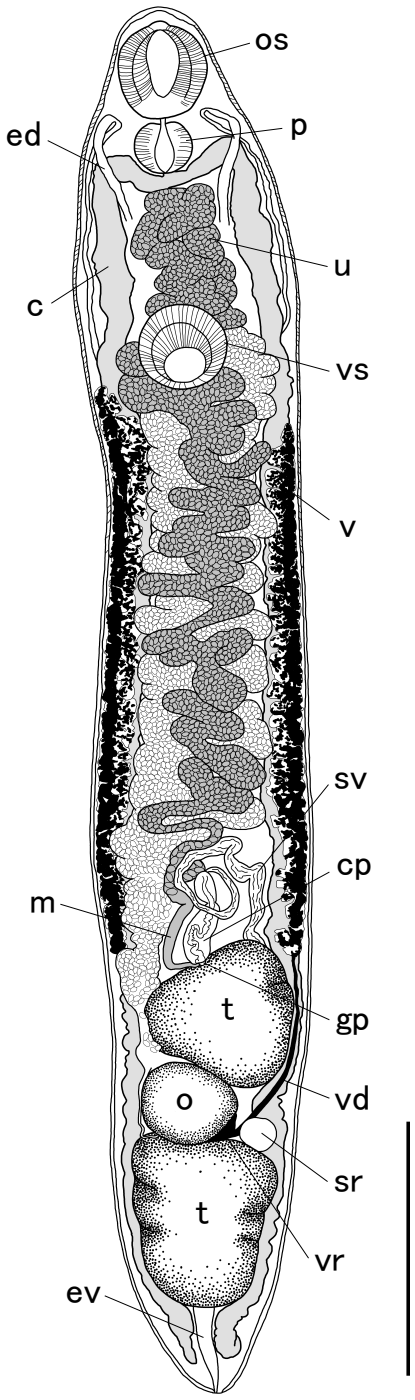


Fig. 4

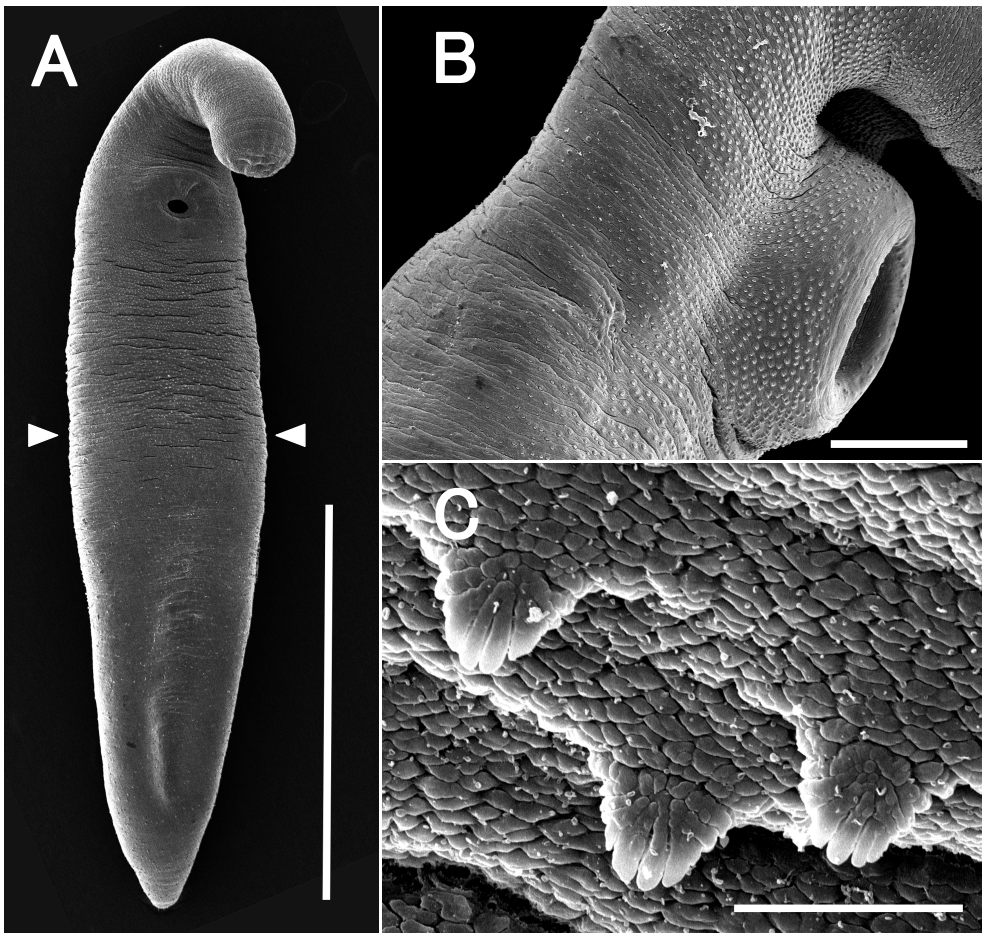


Fig. 5

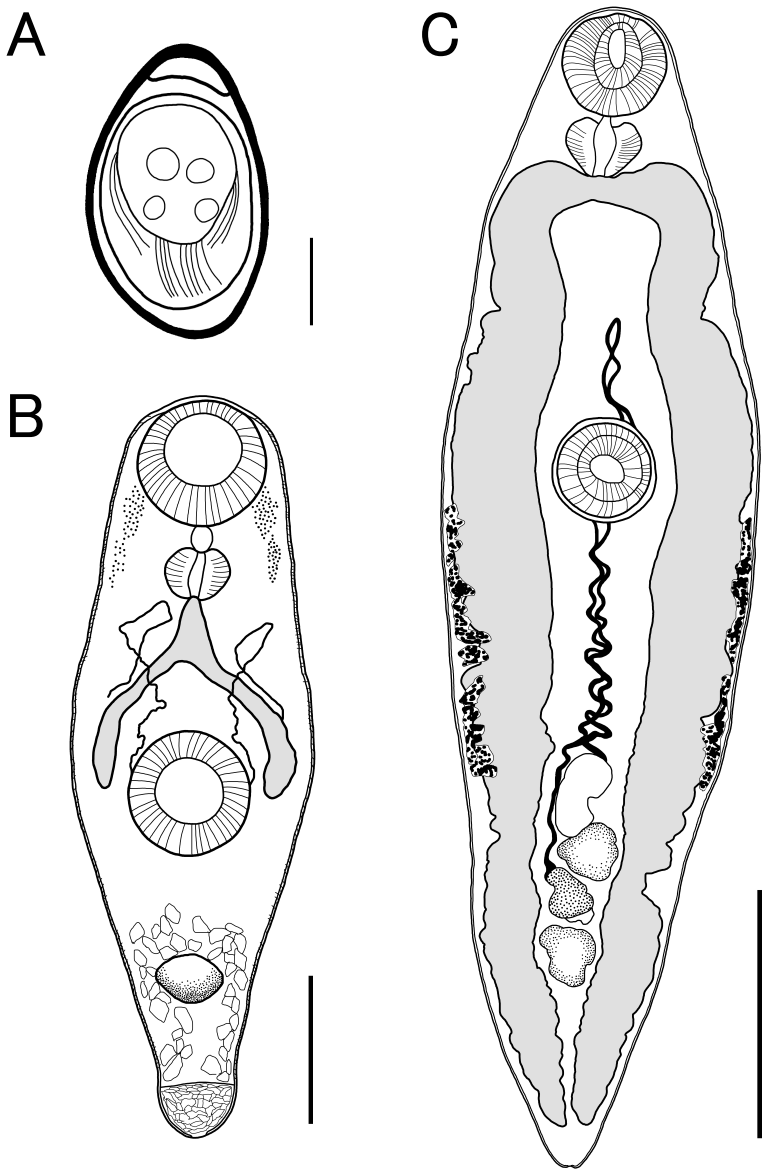
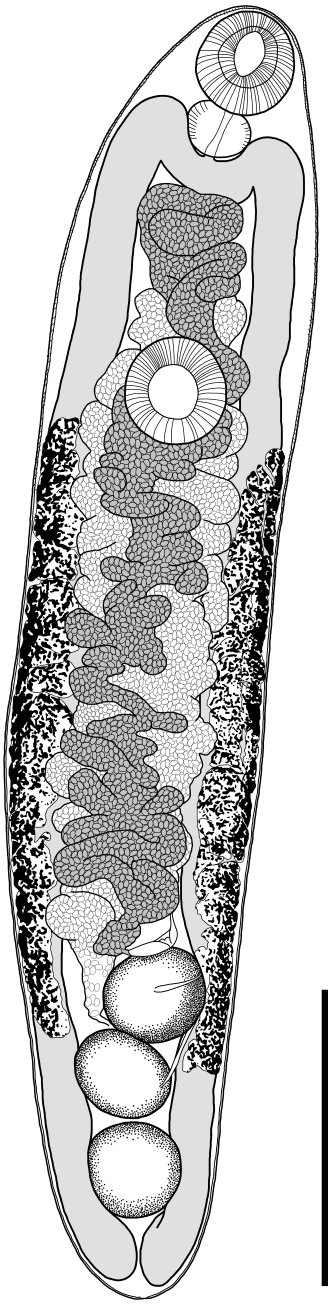
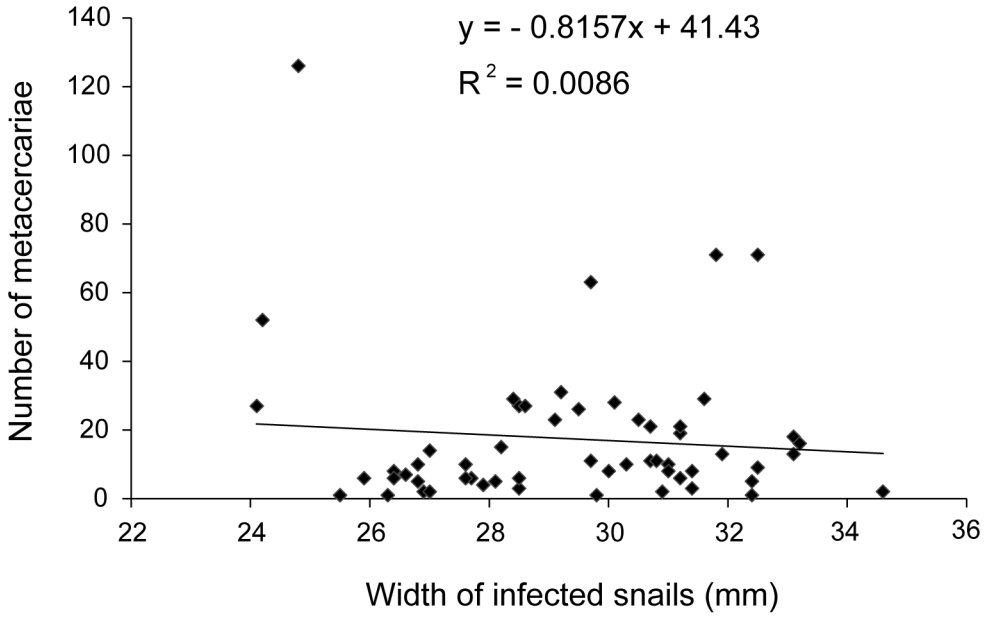


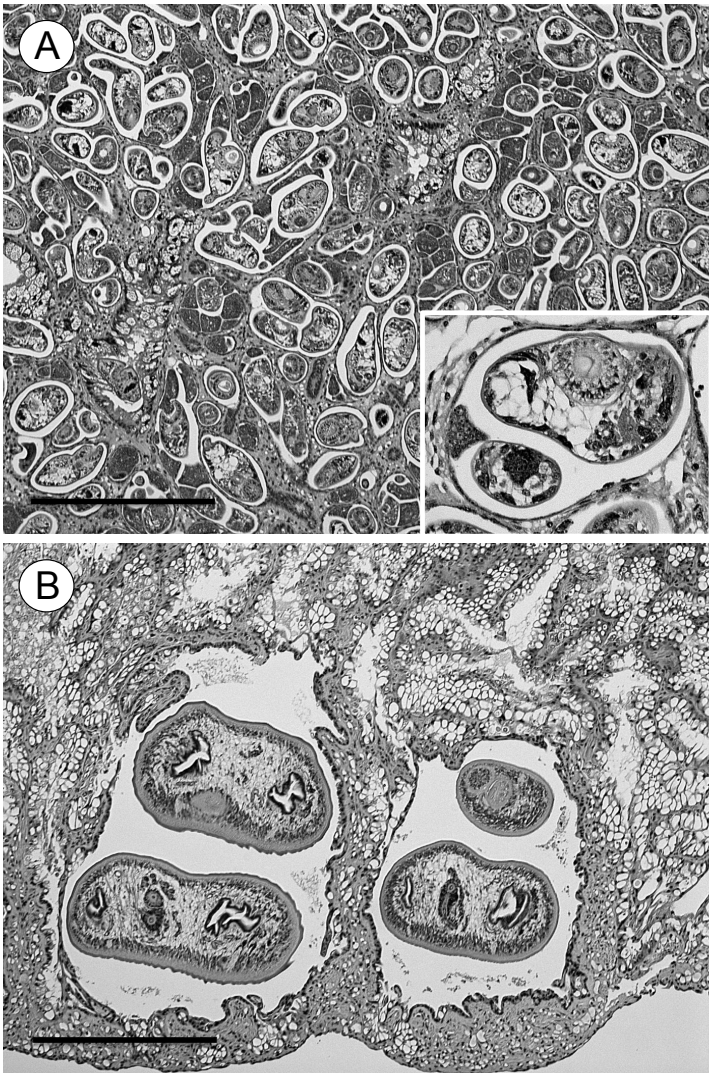
Fig. 6



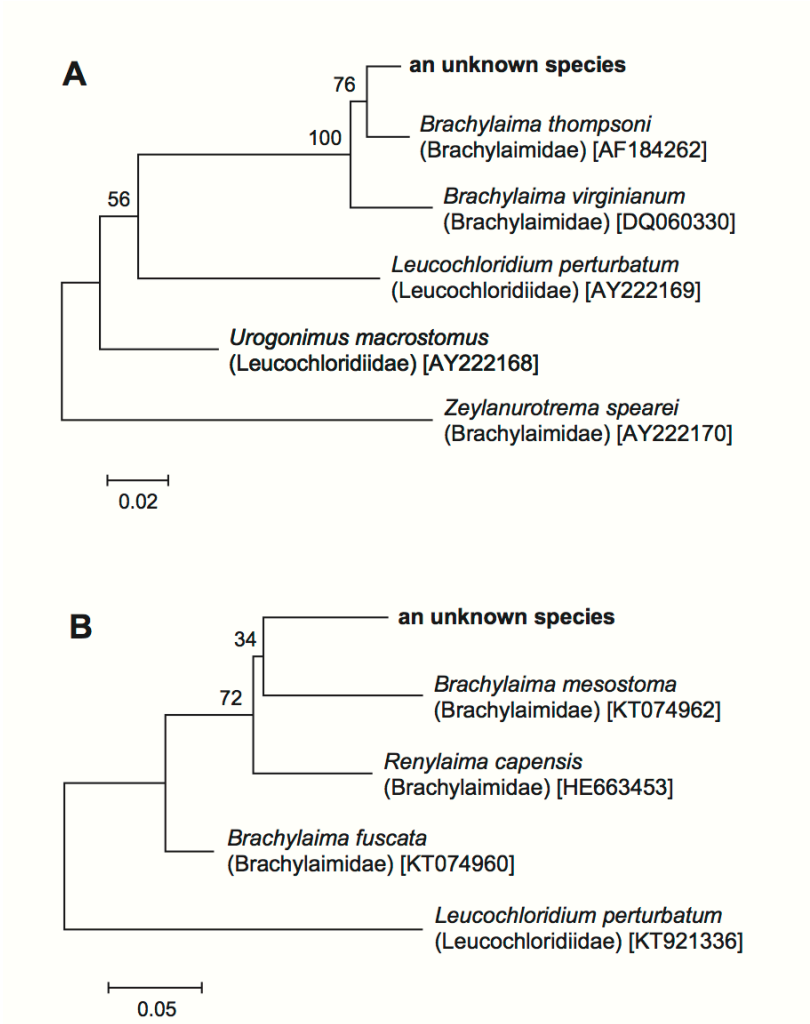
S_Fig. 1


y = - 0.8157x + 41.43
R 2 = 0.0086
Number of metacercariae
Width of infected snails (mm)
0
20
40
60
80
100
120
140
22
24
26
28
30
32
34
36

S_Fig. 2



S_Fig. 3


A
an unknown species
76
Brachylaima thompsoni
(Brachylaimidae) [AF184262]
100
Brachylaima virginianum
(Brachylaimidae) [DQ060330]
56
Leucochloridium perturbatum
(Leucochloridiidae) [AY222169]
Urogonimus macrostomus
(Leucochloridiidae) [AY222168]
Zeylanurotrema spearei
(Brachylaimidae) [AY222170]
0.02
B
an unknown species
34
Brachylaima mesostoma
(Brachylaimidae) [KT074962]
72
Renylaima capensis
(Brachylaimidae) [HE663453]
Brachylaima fuscata
(Brachylaimidae) [KT074960]
Leucochloridium perturbatum
(Leucochloridiidae) [KT921336]
0.05

S_Fig. 4

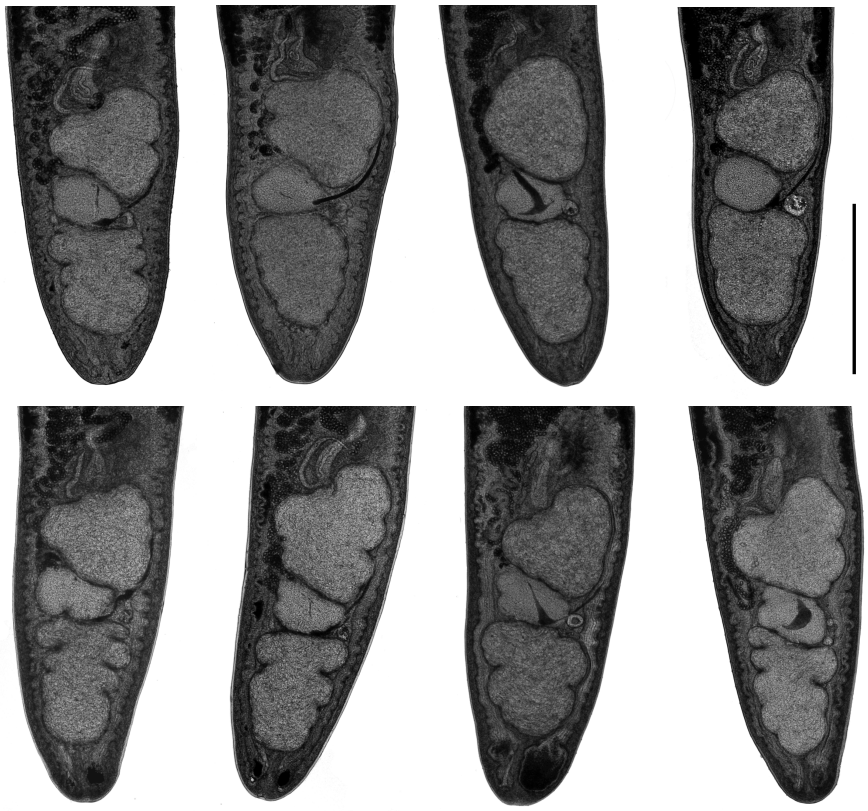


Table 1

Infections of bracylaimid larvae in land snails (*Ezohelix gainesi*) from the Kaguraoka Park, a forest city park of Asahikawa and other comparative areas (the Asahiyama and Arashiyama Parks).

Parks	No. snails examined [a]	No. infected with sporocysts (%)	No. infected with metacercariae (%)	Mean ± s.d. (range) [b]
Kaguraoka	94	5 (5.3)	85 (90.4)	15.4 ± 19.7 (126 - 1)
Asahiyama	21	0 (0)	0 (0)	
Arashiyama	7	0 (0)	2 (28.6)	12.0 ± 15.6 (23 - 1)

[a] The snails were collected in July and August of 2016.

[b] The mean number of metacercariae with standard deviation was computed in infected snails.

Table 2
Population genetics indicies of mtDNA (*cox1*) sequences from brachylaimid metacercariae in a forest city park of Asahikawa.

No. of worms examined	No. of haplotypes	Haplotype diversity	Nucleotide diversity	Tajima's D
30	6	0.623	0.002	0.104 [a]

[a] not significant.

Table 3
A morphological comparison of *Brachylaima ezohelicis* sp. nov. with closely related species (*Brachylaima cribbi*, *Brachylaima apoplania*, *Brachylaima ratti*, *Brachylaima ruminae*, and *Brachylaima mascomai*) using rodents of the genera *Rattus* and *Mus* as definitive hosts.

Checkpoints [a]	*B. ezohelicis*	*B. cribbi*	*B. apoplania*	*B. ratti*	*B. ruminae*	*B. mascomai*
1. Total body [size]	6.2 x 1.0 mm	5.0 x 0.7 mm	3.2 x 0.4 mm	5.8 x 1.2 mm	3.2 x 0.6 mm	3.5 x 0.4 mm
2. Distribution of ventral tegumental spines	From anterior end to half of body	From anterior end to posterior testis	Entire body	From anterior end to anterior testis	From anterior end to ovary	From anterior end to genital pore
3. Oral sucker [size]	390 x 361 μm	259 x 279 μm	204 x 172 μm	363 μm (diameter)	217 x 208 μm	238 x 218 μm
4. Ventral sucker [size]	360 x 369 μm	277 x 267 μm	181 x 160 μm	455 μm (diameter)	213 x 195 μm	218 x 208 μm
5. Position of ventral sucker	Anterior quarter of body	Anterior quarter of body	Anterior quarter of body	Anterior third of body	Anterior quarter of body	Anterior quarter of body
6. Pharynx [size]	209 x 245 μm	157 x 169 μm	110 x 117 μm	165 x 231 μm	97 x 115 μm	117 x 149 μm
7. Outline of ceca	Undulating	Undulating	Undulating	Tubular	Undulating	Undulating
8. Anterior testis [size]	703 x 654 μm	419 x 353 μm	215 x 199 μm	462 x 544 μm	292 x 265 μm	252 x 297 μm
9. Posterior testis [size]	740 x 585 μm	417 x 323 μm	241 x 189 μm	495 x 511 μm	295 x 268 μm	248 x 319 μm
10. Shape of testes	Weakly lobed	Oval	Oval	Oval	Oval	Oval
11. Ovary [size]	290 x 431 μm	217 x 261 μm	141 x 140 μm	313 x 495 μm	217 x 193 μm	152 x 181 μm
12. Shape of ovary	Irregular	Oval	Oval	Oval	Oval	Oval
13. Cirrus pouch [size]	308 x 112 μm	Unknown	134 μm long	207 x 124 μm	Unknown	Unknown
14. Seminal receptacle	Present	Present	Absent [b]	Absent [b]	Present	Present
15. Egg [size]	32 x 19 μm	29 x 17 μm	23 x 13 μm	30 x 17 μm	27 x 16 μm	25 x 12 μm
16. End of cercarial ceca	Not overpassing ventral sucker	Overpassing ventral sucker	Unknown	Unknown	Not overpassing ventral sucker	Overpassing ventral sucker
References cited	Present study	[39]	[38]	[36]	[37]	[40]

[a] Numerical vales of each species are shown in average, excepting those of *B. ratti* (in maximum).
[b] Seminal receptacle is undescribed in *B. apoplania* and *B. ratti*.