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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

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Members of the genus *Glaphyrostomum* Braun, 1901 (Trematoda: Brachylaimidae) are parasites of birds. However, an exception occurs in *Glaphyrostomum soricis* Asakawa, Kamiya and Ohbayashi, 1988, which was described from the long-clawed shrew, *Sorex unguiculatus* Dobson, 1890, in Hokkaido, Japan. A recent DNA barcode-based trematode survey of land snails clearly showed that *Ainohelix editha* (A. Adams, 1868), a bradybaenid snail indigenous to Hokkaido, serves as the first and second intermediate hosts for a species of the genus *Pseudoleucochloridium* Pojmańska, 1959 (Panopistidae). Its adult stage was furthermore confirmed from *S. unguiculatus*. A comparison of adult morphology between *Pseudoleucochloridium* sp. and *G. soricis* revealed that both should be considered the same species. However, *Pseudoleucochloridium soricis* comb. nov. cannot be applied because *P. soricis* (Sołtys, 1952) already exists as the type species of the genus. We, therefore, propose *Pseudoleucochloridium ainohelicis* nom. nov. as a replacement name for *G. soricis*.

Key Words: Trematoda, Panopistidae, *Pseudoleucochloridium*, new replacement name, Hokkaido.

Introduction

The class Digenea (Platyhelminthes: Trematoda) is a group of obligate parasites, which have a complex life cycle involving three hosts in typical species (Cribb *et al.* 2003). The digenean trematodes asexually reproduce mainly in molluscan first intermediate hosts, and the resulting cercariae metamorphose into metacercariae in second intermediate hosts. The metacercariae mature hermaphroditic adults in vertebrate definitive hosts. The discrimination of the larvae and adults from each of the hosts is essential in conducting taxonomic and ecological studies on digenean trematodes.

Several species of trematodes belonging to the closely related families Brachylaimidae Joyeux and Foley, 1930 and Panopistidae Yamaguti, 1958 are endemic to Japan. Members of these families are unique in using land snails as the first and second intermediate hosts (Pojmańska 2002a). Each of the parasites uses a particular species or group of snails as the first intermediate host, but a wide range of snail species are involved as the second intermediate host (Mas-Coma and Montoliu 1986, 1995; Gracenea and González-Moreno 2002). Thus, the parasites play the different roles of a strict specialist and a generalist in their larval stages. The Japanese species of the two families have been infrequently recorded from shrews (Asakawa *et al.* 1988), shrew-moles (Yamaguti 1952; Kifune *et al.* 1992), rodents (Kamiya and Machida 1977), and birds (Yamaguti 1935, 1941), although their larval stages still remain undiscovered from land snails.

A recent DNA barcode-based trematode survey of land snails in Hokkaido, the northernmost island of Japan, led us to the description of two new species belonging to the genus *Brachylaima* Dujardin, 1843 (Nakao *et al.* 2017, 2018). During the continuing survey of land snails, we noticed that *Ainohelix editha* (A. Adams, 1868), an indigenous land snail in the island, serves as the first and second intermediate hosts for an unidentified species of *Pseudoleucochloridium* Pojmańska, 1959 (Panopistidae). A subsequent survey of small mammals succeeded in finding its adult stage from the long-clawed shrew, *Sorex unguiculatus* Dobson, 1890. However, this species has already been described as *Glaphyrostomum soricis* Asakawa, Kamiya, and Ohbayashi, 1988 (Brachylaimidae) from the same definitive host. A nomenclatural revision is needed to correct the taxonomic position of *G. soricis*. A new combination name, *Pseudoleucochloridium soricis* comb. nov., cannot be applied because the same name, *P. soricis* (Sołtys, 1952), already exists as the type species of the genus.

Accordingly, in this study we propose *P. ainohelicis* nom. nov. as a replacement name for *G. soricis*. The field survey

of this parasite enabled us to describe the larvae (sporocyst, cercaria, and metacercaria) from land snails and to redescribe the adult from shrews. The natural transmission of the parasite in Hokkaido was also considered based on the prevalence data of land snails, together with a discussion on the validity of both the species and the related families.

Materials and Methods

Field survey. During July and October in both 2017 and 2018, a field survey was carried out at three woody sites of Asahikawa, Hokkaido, namely Shunkodai (43.808°N, 142.355°E), Ubun (43.719°N, 142.351°E), and Tomisawa (43.746°N, 142.316°E). Land snails of *Ainohelix editha*, *Ezohelix gainesi* (Pilsbry, 1900), *Discus pauper* (Gould, 1859), and *Succinea lauta* Gould, 1859 were collected by hand picking from plant leaves and litter layers. Each snail was crushed between thick grass plates and then dissected in Dulbecco's phosphate-buffered saline (PBS) under a stereomicroscope. The heart, kidney, and hepatopancreas were broken by fine-tipped forceps to detect metacercariae and sporocysts including cercariae. The number of metacercariae per infected snail was counted to measure the intensity of infection. After microscopic observation of the living larvae, the remainder were kept in 70% ethanol or 10% neutral-buffered formalin for later analyses. In the sites of Shunkodai and Tomisawa, shrews were collected dead, through the use of pitfall and Sherman box traps. All the shrews were necropsied to detect adult trematodes from the internal organs, particularly the intestine. The adult worms recovered were also kept in ethanol or formalin.

Morphological observation. A microscope with a digital camera (Axio Imager, Zeiss) was used to observe parasite morphology. Digital photographic data were processed by the accessory software (AxioVision) to measure object sizes. Sporocysts and cercariae mounted on glass slides with PBS were observed in a living condition. The vital dye, neutral red, was used at approximately 0.05% in PBS to stain the internal structure of the cercariae. Non-encysted metacercariae and gravid adults were flattened in 10% neutral-buffered formalin between a glass slide and a coverslip. A slight pressure was added on the coverslip to arrange their posture. After removing the extra formalin, the slides were kept in a moisture box overnight. The resultant flattened worms were photographed for morphometric assessment. Line-drawing figures were made from digital photographs by using an interactive pen display (UGEE Co. Ltd.) and the software Clip Studio Paint (SELSYS, Inc.). As reported previously (Nakao *et al.* 2018), the permanent specimens of the flattened worms were made after staining with Heidenhain's hematoxylin. The type specimen of adult *G. soricis* kept in Meguro Parasitological Museum, Tokyo (no. MPM19500) was used for a comparative observation with newly obtained materials.

DNA sequencing. Polymerase chain reaction (PCR) and subsequent DNA sequencing were carried out as reported previously (Nakao *et al.* 2017). Templates of PCR were prepared from ethanol-preserved specimens without purifying DNA. A half body (metacercaria) or an approximately 1 mm^3 piece (sporocyst and adult) was lysed in 25 µl of 0.02N NaOH at 99°C for 30 min. One µl of the lysate was used as a template. The Tks Gflex™ DNA polymerase (TaKaRa) was used for PCR with the manufacturer-supplied reaction buffer. Nuclear 28S ribosomal DNA (rDNA) was amplified using the primer set digl2 and 1500R (Tkach *et al.* 2016), and mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) using the set JB3 and CO1-R trema (Miura *et al.* 2005). The latter gene is needed for DNA barcoding. The PCR was run for 40 cycles (98°C for 10 sec, 50°C for 20 sec, and 68°C for 90 sec) in a total volume of 25 µl including 0.25 μM of each primer. The PCR amplicons were 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.

Phylogenetic analyses. The nucleotide alignment datasets of 28S rDNA and *cox1* were prepared by MAFFT (Katoh and Standley 2013). The comparative sequences of related taxa were retrieved from DDBJ/ENA/GenBank databases. A phylogenetic tree of 28S rDNA was made by maximum likelihood (ML) method under the best-fit nucleotide substitution model GTR+I. The integrated software MEGA7 (Kumar *et al.* 2016) was used for the model selection and the tree construction. The robustness of the trees was tested by bootstrapping with 500 replicates. Inter- and intraspecific values of pairwise divergence between *cox1* barcode sequences were computed by MEGA7 under *p*-distance model. The *cox1* sequences of the related sympatric species, *Brachylaima ezohelicis* Nakao, Waki and Sasaki, 2017 (database accession nos. LC198311–6) and *B. asakawai* Nakao, Sasaki and Waki, 2018 (LC349002–11), were used for the comparison. A *cox1* haplotype network was illustrated by TCS (Clement *et al.* 2000), and population genetics indices were computed by DnaSP (Rozas *et al.* 2017).

Results

Sampling. During the survey period from 2017 to 2018, 657 land snails were examined for the trematode infections (Table 1). We demonstrated only one snail of *Ainohelix editha* from Shunkodai to be infected with the reticular sporocyst of *Pseudoleucochloridium ainohelicis* nom. nov. Branched tubes of the sporocyst containing cercariae occupied the hepatopancreas (Fig. 1). In contrast, the host range of the metacercaria included 4 snail species (*A. editha*, *Ezohelix gainesi*, *Discus pauper*, and *Succinea lauta*). All of the metacercariae detected were from the pericardial cavity. The metacercarial prevalence of *A. editha* was relatively high, ranging from 5.0 to 38.5%. The number of metacercariae per snail (the intensity of infection) was also high in *A. editha*, ranging from 1 to 16. Shrews were collected in October, 2018. Three individuals of *Sorex unguiculatus* (two from Shunkodai and one from Tomisawa) were available for the examination of parasites. No other species of shrews were collected. The adults of *P. ainohelicis* nom. nov. were de-

Table 1. A field survey of land snails in Asahikawa to detect larval *Pseudoleucochloridium ainohelicis* nom. nov.

Sites[a]	Periods	Land snails	No. examined	No. infected with SC (%)[b]	No. infected with MC (%)[c]	Mean no. of MC with s.d. (range)[d]
Shunkodai	Jul. 2017	*Ainohelix editha*	76	0 (0)	12 (15.8)	2.2±1.6 (1–5)
	Jul. 2018	*A. editha*	33	1 (3.0)	3 (9.1)	2.3±1.5 (1–4)
	Aug. 2018	*A. editha*	22	0 (0)	7 (31.8)	3.9±3.7 (1–12)
	Jul. 2017	*Ezohelix gainesi*	4	0 (0)	1 (25.0)	1 (1)
	Jul. 2018	*Succinea lauta*	293	0 (0)	3 (1.0)	1.7±0.6 (1–2)
Ubun	Jul. 2018	*A. editha*	13	0 (0)	5 (38.5)	6.0±6.7 (1–16)
	Sep. 2018	*Discus pauper*	38	0 (0)	1 (2.6)	1 (1)
Tomisawa	Sep. 2017	*D. pauper*	47	0 (0)	2 (4.3)	1.5 (1–2)
	Sep. 2018	*D. pauper*	82	0 (0)	4 (4.9)	2.0±1.4 (1–6)
	Oct. 2018	*D. pauper*	49	0 (0)	4 (8.2)	2.5±2.4 (1–6)
Total		*A. editha*	144	1 (0.7)	27 (18.8)	3.3±3.7 (1–16)
		D. pauper	216	0 (0)	11 (5.1)	2.0±1.6 (1–6)

[a] The coordinates of the collection sites are as follows: Sunkodai (43.808°N, 142.355°E), Ubun (43.719°N, 142.351°E), and Tomisawa (43.746°N, 142.316°E).
[b] Sporocysts (SC) containing cercariae were detected from the hepatopancreas. [c] Non-encysted metacercariae (MC) were detected from the pericardial cavity.
[d] The mean number of metacercariae with standard deviation was computed among the infected snails.

tected from all three shrews. The number of the adults per shrew was very few, ranging from 1 to 2. A total of 5 adults were obtained, but only two were gravid.

DNA barcoding. Twelve representatives of the larvae and adults from the three sites of Asahikawa (*i.e.*, 1 sporocyst from *A. editha*, 9 metacercariae from all the snail species, and 2 adults from *So. unguiculatus*) were subjected to a distance-based DNA barcoding of mitochondrial *cox1*. All of the samples were identified as *P. ainohelicis* nom. nov. The mean of the intraspecific divergence was 0.003. The related species of *Brachylaima ezohelicis* and *B. asakawai* are sympatrically distributed in the survey areas. When compared among the three species, the interspecific divergence were extremely high, ranging from 0.138 to 0.223. A parsimony network consisting of 6 *cox1* haplotypes illustrated a slightly scattered pattern, showing the absence of a dominant haplotype (Fig. 1). The population genetics indices of the 12 *cox1* sequences were as follows: haplotype diversity (0.818), nucleotide diversity (0.00362), Tajima's D (−1.41807), and Fu's FS (−0.411). Both the network and the indices suggest that no bottleneck events occurred in the recent past.

Molecular phylogeny. A ML phylogenetic tree was constructed using the 28S rDNA data set including *P. ainohelicis* nom. nov. and members of related families. The resultant tree topology was not robust, perhaps due to the lack of essential taxa (Fig. 2). However, the phylogeny suggests a possibility that the families Brachylaimidae and Leucochloridiidae are paraphyletic. The isolates of *P. ainohelicis* nom. nov. were shown to be distinct from all related species for which DNA sequences are available.

Descriptions. The specimens obtained in the field survey were used for the descriptions of larval and adult *P. ainohelicis* nom. nov. The numbers of the specimens used are 1 sporocyst (10 branches), 10 cercariae, 10 metacercariae, 2 adults, and 10 mature eggs. The eggs were obtained from the metraterm of one broken adult. All specimens were observed ventrally, excepting the sporocyst and the egg. All measurements, unless indicated otherwise, are in μm as the mean, with minimum-maximum range in parentheses. In the case of adults, only the minimum-maximum range was shown because of the low sample size.

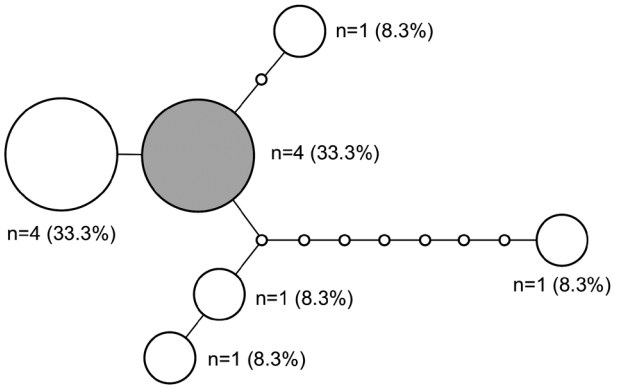

Fig. 1. Frequencies of *cox1* haplotypes and their statistical parsimony network in *Pseudoleucochloridium ainohelicis* nom. nov. All of the twelve isolates were collected in Asahikawa. The size of circles indicates the frequency of the haplotypes. Small circles show hypothetical haplotypes. The shaded circle represents the hypothetical ancestor.

Family **Panopistidae** Yamaguti, 1958
Genus ***Pseudoleucochloridium*** Pojmańska, 1959
Pseudoleucochloridium ainohelicis nom. nov.
[Japanese name: Ohguchi-mushi]
(Figs 3–6)

Glaphyrostomum soricis Asakawa, Kamiya, and Ohbayashi, 1988: 21–22, figs 1–2.

Sporocyst. Body tubular, with numerous reticular branches, grown throughout snail's hepatopancreas (Fig. 3A). Number of branches per tube variable. Tubes distinctly thick, as compared with those of *Brachylaima* species (Mas-Coma and Montoliu 1986; Gracenea and González-Moreno 2002; Nakao *et al.* 2017), 186 (171–199) in diameter, including many cercariae (Fig. 3B). Movement of tubes sluggish. Emergence point of cercariae from tubes unclear.

Cercaria. Body prolate, slender in posterior portion,

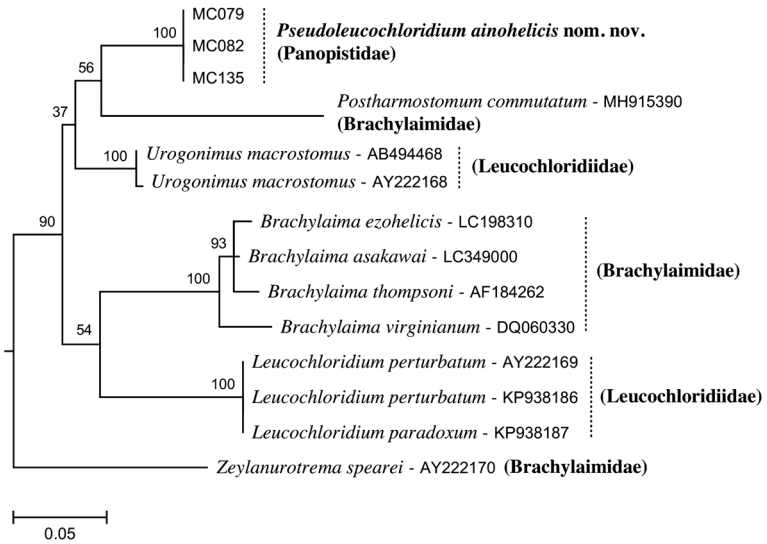

Fig. 2. A maximum-likelihood phylogenetic tree of the superfamily Brachylaimoidea inferred from 28S rDNA sequences. Three isolates of *Pseudoleucochloridium ainohelicis* nom. nov. and 11 isolates of 9 related species were included in the tree. The DNA database accession numbers are shown in the tree. *Clinostomum cutaneum* Paperna, 1964, a member of the family Clinostomidae Lühe, 1901, was used as an outgroup taxon (database accession no. GQ339114). Bootstrap percentages are shown on each node. Scale bar indicates the number of substitutions per nucleotide site.

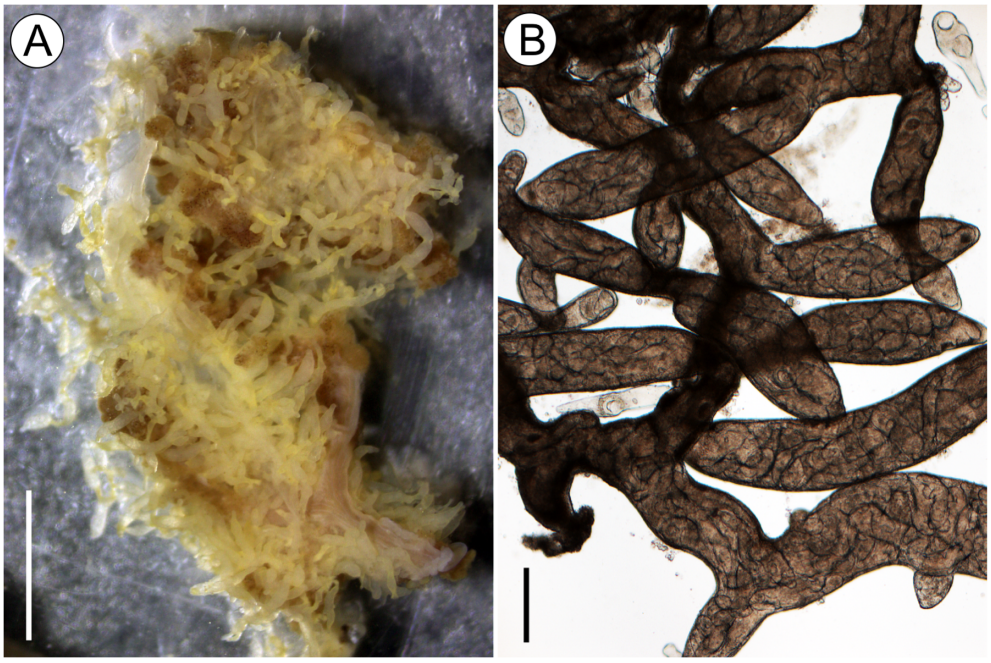

Fig. 3. The sporocyst of *Pseudoleucochloridium ainohelicis* nom. nov. from *Ainohelix editha*. A) Branched tubes in the hepatopancreas (macrophotograph). Scale bar 2 mm; B) Branched tubes containing cercariae. Scale bar 200 µm.

346 (328–376) long by 127 (112–146) in maximum width (Fig. 4A). Oral and ventral suckers equal in size. Oral sucker round, lacking stylet, 73 (68–78) long by 71 (66–80) wide, located on anterior end. Ventral sucker round, 63 (58–67) long by 66 (63–75) wide, positioned on center of body. Prepharynx very short. Pharynx globular, 25 (19–29) long by 29 (25–33) wide. Ceca thick and reverse-U-shaped, running along margin of ventral sucker. Distal end of ceca not exceeding level of center of ventral sucker. Genital primordium located in front of tail. Tail rudimentary, 29 (26–32) long by 36 (31–42) wide.

Metacercaria. Body disk-like flattened, non-encysted, 1.4 (1.3–1.5) mm long by 0.9 (0.7–1.1) mm in maximum width (Fig. 4B). Suckers especially large for body size. Oral sucker smaller than ventral sucker (a width ratio of 1 to 1.2). Oral sucker round, 328 (261–393) long by 350 (288–437) wide, located on anterior end. Ventral sucker round, 412 (333–520) long by 423 (337–543) wide, positioned on center

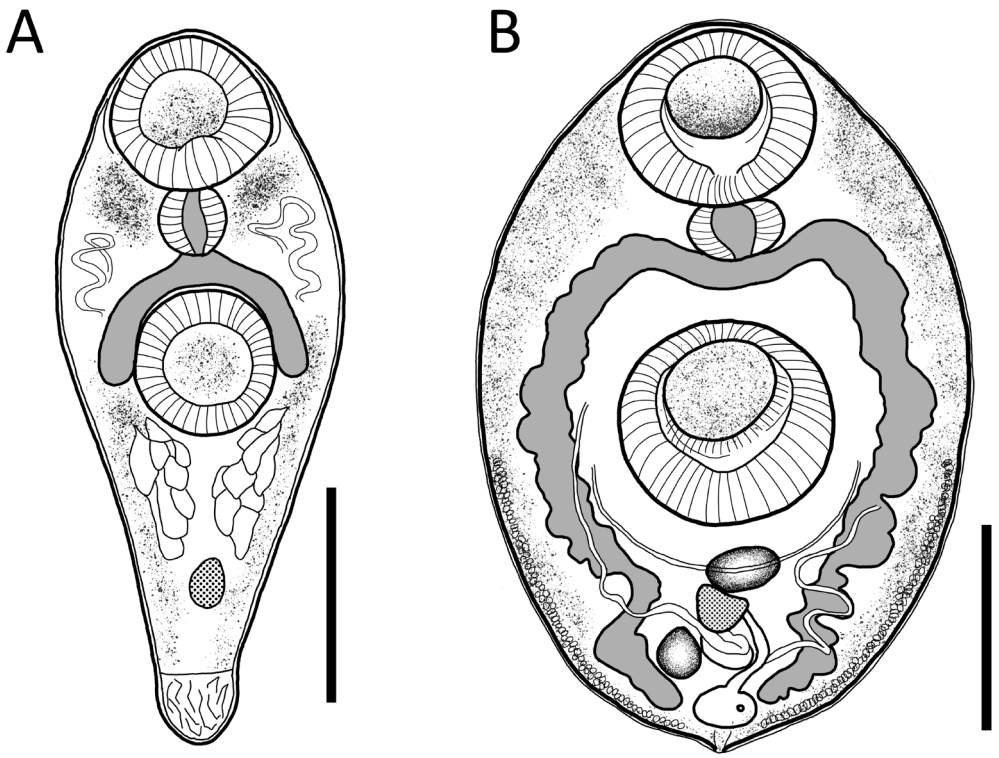

Fig. 4. The cercaria and metacercaria of *Pseudoleucochloridium ainohelicis* nom. nov. from *Ainohelix editha*. Both of the drawings are in ventral view. A) Cercaria. Scale bar 100 µm; B) Metacercaria. Scale bar 500 µm.

of body. Prepharynx very short. Pharynx oblate-shaped, 97 (77–114) long by 136 (117–165) wide. Intestine bifurcated immediately at postpharyngeal position. Each cecum thick, undulating tubular, slightly ascending and then descending bilaterally to posterior extremity. Immature gonads located between posterior ceca. Immature testes nearly spherical, 111 (91–135) long by 117 (93–167) wide (anterior one), 82 (74–96) long by 119 (102–152) wide (posterior one). Immature ovary nearly spherical, 77 (62–93) long by 118 (96–162) wide, located between immature testes. Genital pore open to ventral surface at midline, located near posterior end. Male and female genital ducts amorphous, located left-side of posterior testis. Bilateral vitelline follicles very immature, extending from middle to close to posterior extremity. Excretory pore terminal.

Adult. Body oval, 1.4–1.5 mm long by 0.7–0.8 mm in maximum width (Fig. 5). Suckers especially large for body size. Oral sucker smaller than ventral sucker (a width ratio of 1 to 1.2). Oral sucker round, 334–366 long by 359–373 wide, located on anterior end. Ventral sucker round, close to intestinal bifurcation, 420–428 long by 427–439 wide, positioned on center of body. Prepharynx very short. Pharynx oblate-shaped, 92–95 long by 134–142 wide. Intestine bifurcated immediately at postpharyngeal position. Each cecum thick, undulating tubular, slightly ascending and then descending bilaterally to posterior extremity. Mature gonads located between posterior ceca. Anterior testis immediately posterior to ventral sucker. Both testes unlobed, nearly spherical, 97–98 long by 148–161 wide (anterior one), 126–143 long by 109–151 wide (posterior one). Ovary unlobed,

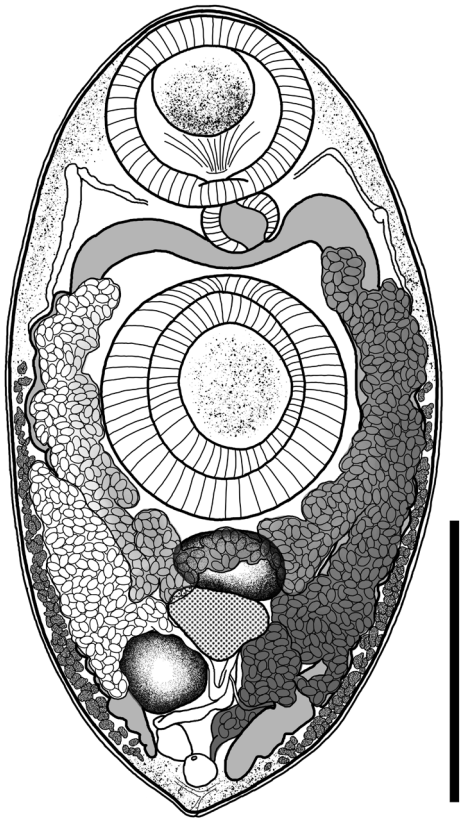

Fig. 5. The adult of *Pseudoleucochloridium ainohelicis* nom. nov. from *Sorex unguiculatus*. The drawing is in ventral view. The large suckers, M-shaped configuration of uterus, and terminally-positioned genital pore are characteristic of the genus. Scale bar 500 µm.

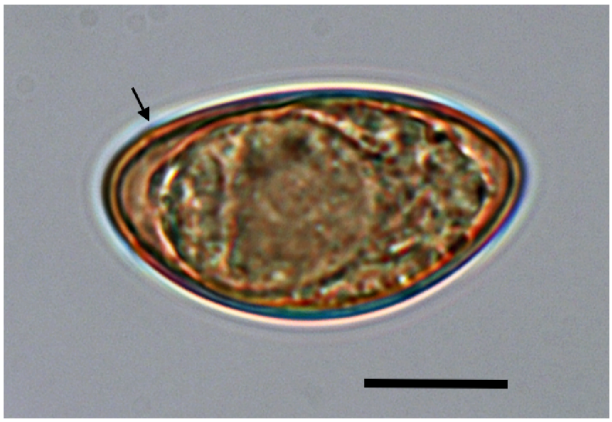

Fig. 6. The egg of *Pseudoleucochloridium ainohelicis* nom. nov. in the gravid adult. The left end is an operculum. An arrow indicates the notch of eggshell. A miracidium is visible inside. Scale bar 10 µm.

nearly spherical, 93–115 long by 160–176 wide, located between testes. Vitellarium follicular, extending bilaterally from middle to close to posterior extremity. Remnant posterior space occupied by twisting uterus, including numerous eggs. Configuration of uterus M-shaped (ascending from right side of ovary to right corner of cecum, then passing immediately beneath ventral sucker, again ascending to left corner of cecum, and finally descending to genital cavity). Genital cavity small, connecting metraterm and piriform cirrus pouch, located near posterior end. Genital pore open to ventral surface. Excretory pore terminal.

Egg. Immature eggs continuously developed in uterus, reached maturity in metraterm. Mature egg light brown, slightly asymmetric, 32 (30–34) long by 17 (16–18) wide (Fig. 6). Small operculum present. Miracidium with long cilia visible inside.

Distribution. To date, the distribution of *P. ainohelicis* nom. nov. is restricted to Hokkaido. In this study, all the developmental stages were found in Asahikawa, an inland area of Hokkaido. The parasite had been recorded as *G. soricis* from several areas of Hokkaido, namely Ebetsu, Kitami, Ozora, and Kushiro (Asakawa *et al.* 1988; Asakawa *et al.* 1992; Mitsuhashi *et al.* 2013; M. Asakawa, unpublished data).

Hosts. The land snail indigenous to Hokkaido, *A. editha*, serves as the first and second intermediate hosts for *P. ainohelicis* nom. nov.. The sporocyst and metacercaria parasitize the hepatopancreas and pericardial cavity, respectively. Other land snails, *E. gainesi*, *D. pauper*, and *Su. lauta*, are involved as the second intermediate host. All of them are endemic species mainly in northern Japan. The long-clawed shrew, *So. unguiculatus*, serves as the definitive host. The adult parasitizes the lower part of the intestine. The geographic distribution of the shrew is restricted to Hokkaido, Sakhalin, and the adjacent minor part of the Eurasian Continent (Hutterer 2005).

Etymology. The new specific name is given after the generic name of *A. editha*, an essential intermediate host in Hokkaido.

Vouchers. The specimens of *P. ainohelicis* nom. nov. used in this study have been deposited in Meguro Parasitological Museum, Tokyo under the collection numbers MPM21491 (1 adult) and MPM21492 (3 metacercariae). The holotype is also kept in the same museum (Asakawa *et al.* 1988).

Differential molecular markers. The parasite DNA sequences (28S rDNA and 6 haplotypes of *cox1*) are available for precise species identification. All of them have been deposited into DDBJ/ENA/GenBank databases under the accession numbers LC455740 (28S rDNA) and LC455741–6 (*cox1*).

Discussion

In this study, *Glaphyrostomum soricis* from shrews in Hokkaido has been renamed as *Pseudoleucochloridium ainohelicis* nom. nov. Both the genera *Glaphyrostomum* and *Pseudoleucochloridium* belong to the superfamily Brachylaimoidea, whose members use land snails as intermediate hosts (Pojmańska 2002a). Members of the genus *Glaphyrostomum* are parasites of birds (Pojmańska 2002b); *G. soricis* from shrews is the only exception to this pattern (Asakawa *et al.* 1988). The genera *Glaphyrostomum* and *Pseudoleucochloridium* share common morphological characteristics, but can be differentiated by the configuration of uterus and the position of genital pore (Pojmańska 2002b, c). In the type specimen of *G. soricis*, it was very difficult to locate the position of the genital pore, but the M-shaped configuration of the uterus was characteristic of *Pseudoleucochloridium* (Pojmańska 2002c). The present study enabled us to confirm the terminally-positioned genital pore through observation of living materials from shrews, demonstrating that *G. soricis* is a member of *Pseudoleucochloridium*. The size of suckers and the distribution of vitellarium in the type specimen of *G. soricis* are also consistent in identifying it as *P. ainohelicis* nom. nov.

Before the erection of *Pseudoleucochloridium*, the corresponding species were assigned to *Leucochloridium* Carus, 1835 because of their morphological similarities. As a result of the revision (Pojmańska 1959), the former genus is properly used for the parasites of shrews and the latter genus for the parasites of birds. As far as we know, only five species of *Pseudoleucochloridium* have been found from shrews in Eurasia. These are *P. ainohelicis* nom. nov., *P. pericardicum* Mas-Coma and Montoliu, 1995, *P. rotundus* Bychovskaya-Pavlovskaya and Kulakova, 1970, *P. skrjabini* (Shaldybin, 1953), and *P. soricis*. These species are morphologically quite similar to one another (Table 2). The sizes of oral and ventral suckers, the outline of cecum, the shape of testes and ovary, and the distribution of vitellarium are important characters to discriminate the species. The specimens of *P. ainohelicis* nom. nov. from Hokkaido are most similar to those of *P. pericardicum* from the Pyrenees, but can be differentiated by having a larger ovary, a shorter vitellarium, and strongly undulating ceca. The intraspecific genetic diversity of *P. ainohelicis* nom. nov. estimated by the sequences

Table 2. A comparison of *Pseudoleucochloridium* spp. found from shrews in Eurasia.

Checkpoints[a]	*P. ainohelicis* nom. nov.	*P. pericardicum*	*P. rotundus*	*P. skrjabini*	*P. soricis*
1. Body (size)[b]	1.5×0.8 mm	1.0×0.7 mm	1.1×1.0 mm	1.2×0.6 mm	1.7×0.9 mm
2. Oral sucker (size)[b]	0.35×0.37 mm	0.30×0.27 mm	0.34×0.41 mm	0.27 mm	0.40 mm
3. Ventral sucker (size)[b]	0.42×0.43 mm	0.36×0.31 mm	0.47×0.45 mm	0.35 mm	0.38 mm
4. Ratio of suckers[c]	1 : 1.2	1 : 1.2	1 : 1.4	1 : 1.3	1 : 0.95
5. Outline of ceca	undulating	undulating	undulating	straight	straight
6. Shape of testes	spherical	spherical	lobed	spherical	spherical
7. Shape of ovary	large, spherical	spherical	lobed	small, spherical	spherical
8. Upper limit of vitellarium	center	from center to one-third anterior	center	one-third anterior	center
9. Definitive host	*Sorex unguiculatus*	*So. araneus* *So. minutus* *N. fodiens*	*So. araneus*	*So. araneus* *Neomys fodiens*	*So. araneus*
10. 1st intermediate host	*Ainohelix editha*	*Cepaea hortensis*	unknown	unknown	unknown
11. 2nd intermediate host	*A. editha* *Ezohelix gainesi* *Discus pauper* *Su. lauta*	*C. hortensis* *Euomphalia strigella*	unknown	unknown	*Succinea putris* *Perforatella bidens*
12. Distribution	Hokkaido	Pyrenees	Ukraine	Mordovia	Poland

[a] References cited are as follows: Mas-Coma and Montoliu 1995 (*P. pericarcardicum*); Bychovskaya-Pavlovskaya *et al.* 1970 (*P. rotundus*); Shaldybin 1953 (*P. skrjabini*); Sołtys 1952 (*P. soricis*); Pojmańska 1959 (*P. soricis*). [b] Median values were computed. [c] The width or diameter ratio of oral sucker to ventral sucker was computed.

of mitochondrial *cox1* suggests that the parasite is originally indigenous to Hokkaido rather than being a recent immigrant. The endemism of the intermediate and definitive hosts in Hokkaido also supports the validity of *P. ainohelicis* nom. nov.

As shown in Table 2, land snails acting as the first intermediate host are proven only in *P. ainohelicis* nom. nov. and *P. pericardicum*. Although the sporocyst of *P. soricis* was found from Pyrenean snails (Jourdane 1976), the causative species was synonymized to *P. pericardicum* (Mas-Coma and Montoliu 1995). Snails of the second intermediate host are further confirmed in *P. ainohelicis* nom. nov., *P. pericardicum*, and *P. soricis*. It is likely that the disk-like flattened body and large suckers of the metacercaria are an adaptation to parasitize the pericardial cavity of snails. The intensity of the metacercarial infection is relatively low (*i.e.*, mostly 1 to 3 metacercariae per snail in *P. ainohelicis* nom. nov.), perhaps due to the limited space of the pericardial cavity or the high mortality of severely infected individuals.

Most of the recognized species of *Pseudoleucochloridium* are from Europe, in accordance with the distribution of the Eurasian shrew, *Sorex araneus* Linnaeus, 1758. The discontinuous finding of *P. ainohelicis* nom. nov. in the Far East suggests that more congeners may be distributed widely in Eurasia, as a result of cospeciation events among trematodes, land snails, and shrews. In particular, a remarkable speciation occurs in land snails due to their low mobility and the resulting geographical isolation (Cook 2008; Rundell and Price 2009). The strict host specificity of land snails in developing the sporocyst might exert a selective pressure on the evolution of *Pseudoleucochloridium*.

The prevalence data of *P. ainohelicis* nom. nov. in land snails has important implications for considering the transmission dynamics in natural settings. In the present survey, a total of 144 snails of *Ainohelix editha* were examined (Table 1). The sporocyst was found from only one snail (0.7%), whereas 27 snails (18.8%) harbored the metacercaria. The hepatopancreas of the sporocyst-infected snail was displaced by the proliferating reticular branches (Fig. 3A). It is, therefore, likely that the sporocyst prevalence becomes lower due to a high mortality from the dysfunction of hepatopancreas. Another possibility is that land snails rarely ingest the parasite eggs from shrews. In any case, the sporocyst-infected snail serves as a superspreader for other snails. Land snails generally aggregate with other individuals of the same species (Hylander *et al.* 2005; Sólymos *et al.* 2009). Therefore, cercariae from a sporocyst-infected snail can probably be transmitted to normal snails in a certain space where the snails aggregate. The high prevalence of metacercarial infections is maintained as a result of the snail-to-snail transmission. In this study, the metacercaria was also found from *Ezohelix gainesi*, *Discus pauper*, and *Succinea lauta*, but their prevalence was relatively lower than that of *A. editha*. The snail-to-snail transmission also occurs in individuals of other snail species sharing a common microhabitat with *A. editha*.

All of the snails involved in the transmission of *P. ainohelicis* nom. nov. mainly inhabit the litter layer of woodlands. This soil surface seems to be a major environment in which snail-to-snail transmission occurs efficiently. Shrews living in the litter layer become infected when preying on the metacercaria-infected snails. According to a helminthological review of shrews in Hokkaido (Mitsuhashi *et al.* 2013), *G. soricis* (=*P. ainohelicis* nom. nov.) was found from *So. unguiculatus*, but not from *So. caecutiens* or *So. gracillimus*. This host preference is probably due to the food habit and/or the infection susceptibility of the shrews.

The present 28S rDNA-based phylogeny of the

Brachylaimoidea is still immature because of the lack of essential taxa. However, as already reported by another study (Valadão *et al.* 2018), our result suggests that the present morphology-based classification of the corresponding families is unnatural, particularly for members of the Brachylaimidae. A large-scale taxon sampling from land snails and vertebrates is required to revise the paraphyly of the Brachylaimoidea. Based on the resultant molecular phylogeny, the erection of new families and the rearrangement of existing genera will probably be necessary.

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