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Distribution records of three species of *Leucochloridium* (Trematoda: Leucochloridiidae) in Japan, with comments on their microtaxonomy and ecology

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ABSTRACT

Insectivorous birds serve as definitive hosts for trematodes of the genus *Leucochloridium*. The parasites exclusively use amber snails of the family Succineidae as intermediate hosts. A pulsating and colorful display of the larval broodsac in the snail's eyestalk seems to be a caterpillar mimic for attracting birds. A colored design of the broodsac is very useful for parasite identification. In Japan, characteristic broodsacs from amber snails have been recorded from 1980's, but their taxonomic discrimination from Asian, European, and North American species has not been achieved. In this study, old scientific records, sighting information on broodsacs from the general public, and direct molecular evidence by DNA barcoding clearly showed that at least three species of *Leucochloridium* are distributed in Japan. A vertical-striped broodsac found from *Succinea* sp. in Okinawa, the subtropical island of Japan, were treated as *Leucochloridium* sp., but being almost identical to that of *Leucochloridium passeri* in neighboring Taiwan. The European species of *Leucochloridium perturbatum* and *Leucochloridium paradoxum* were frequently detected from *Succinea lauta* in Hokkaido, the northernmost island of Japan. The former species was common in inland areas of Hokkaido, whereas the latter species was frequently seen in the coastal areas. A possible explanation for the parasite distribution pattern is that principal definitive hosts (migratory or resident birds) differ in each parasite. The conspecificity of *Leucochloridium variae* in North America and *L. perturbatum* in Europe and the Far East is also discussed.

1. Introduction

Members of the genus *Leucochloridium* (Trematoda: Leucochloridiidae) [1] have attracted the remarkable attention of both academic and common people, because of very strange figure and action of their larval broodsacs in land snails of the family Succineidae (known as amber snails having a thin and fragile shell). A pulsating and colorful display of the broodsac in the snail's eyestalk seems to be a caterpillar mimic for attracting insectivorous birds [2]. A host exchange occurs when the birds accidentally prey on the broodsacs containing many metacercariae. Gravid adults parasitizing in the cloaca or bursa Fabricii release eggs into the environment with the bird's excreta. After amber snails ingest the eggs, multitubular sporocysts grow from the hepatopancreas into the body cavity. Asexual multiplication of cercariae occurs in the sporocysts, and the cercariae continuously develop into encysted metacercariae. The fully developed sporocyst, namely a mature broodsac, exhibits a rhythmical activity in the eyestalk. Thus, members of *Leucochloridium* depend absolutely on birds and amber snails for keeping their life cycle [3,4]. Food and migratory habits of the birds and regional abundance of the snails directly affect the distribution of the parasites.

In several spots of the Japanese Archipelago, characteristic

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broodsacs of amber snails have been infrequently reported from 1980's mainly by malacologists. In the early phase of the discovery, the broodsacs were found from *Succinea lauta* (Succineidae) in Hokkaido, the northernmost island of Japan [5–8], *Oxyloma hirasei* (Succineidae) in Honshu, the main island of Japan [9,10], and *Succinea* sp. (an alien species from North America, formerly *Calcisuccinea* sp.) in Okinawa, the subtropical island of Japan [11]. Recently, the pulsating tentacles of snails capture the attention of internet users. The ordinary people upload the video clips and photographs on the Web as “mind-controlled zombie snails”. Both the academic and amusing information strongly suggest that two independent species, each displaying “brown-banded broodsac” or “green-banded broodsac”, are distributed in Hokkaido [12], and furthermore that “vertical-striped broodsac” in Okinawa is a quite different species. A colored design of the broodsac is very useful for parasite identification. However, the Japanese species are still unclassified, and their discrimination from Asian, European, and North American species has not been achieved. On the other hand, during 1930's to early 2010's, adults of *Leucochloridium* spp. from birds were reported in Honshu [13–15] and Hokkaido [16] independently of the larvae in amber snails. The early reports of 1930's include the species descriptions of *Leucochloridium* as follows: *L. japonicum* [13], *L. sime* [14], *L. turdi* [15], and *L. cardis* [15]. Most recently, the green-banded broodsac from Esashi, Hokkaido has been reported as *Leucochloridium* sp., based on the comparison of DNA sequences with those of European species [17]. The study is, however, insufficient with respect to morphological and genetic variations because only two infected snails were analyzed.

In this study, a large-scale snail survey was carried out to clarify the taxonomy, distribution, and species diversity of *Leucochloridium* in Japan. The main purposes of this study are 1) to definitively identify the parasites by DNA barcoding, 2) to morphologically characterize the larval stage of each species, and 3) to elucidate their geographical distribution. The infections of amber snails with immature and mature broodsacs were compared in our main fields of Asahikawa, Hokkaido, to consider the wintering of the parasites. The avifauna of the research fields was also examined to estimate possible candidates for their definitive hosts. The microtaxonomy and ecology of *Leucochloridium* spp. in Japan were discussed, based on the results of molecular phylogenetic analyses.

2. Materials and methods

2.1. Internet survey

A social networking service was used to assemble information on broodsac-pulsating snails in Japan. Using Twitter (@parasitology_as, an account of Department of Parasitology, Asahikawa Medical University), we appealed to the general public for sending the information during May to September in 2018. The sighting information including the date, locality, and color photograph was regarded as valid for registration. In 2017, a follower sent a photograph of the infected snail to us. This reliable information was exceptionally added to the result of the internet survey.

2.2. Parasite sampling and field surveys

In the period from 2016 to 2018, broodsac-pulsating snails were unsystematically collected in several localities of Hokkaido (Otaru, Sapporo, Asahikawa, Biei, Furano, and Abashiri) by ourselves or volunteers at every opportunity of seeing them. The specimens of broodsacs from Okinawa (Naha and Tomigusuku) were already deposited in Meguro Parasitological Museum, Tokyo. The infected snails were sent to the museum by volunteers in 2008, 2009, and 2015. The first year's sample from Naha has been reported in the 78th Annual Meeting of the Japanese Society of Parasitology [11]. The other samples from Naha in 2009 and Tomigusuku in 2015 were used in this study. After recording the coloration of broodsacs, all the samples (whole snails or separate broodsacs) from Hokkaido and Okinawa were kept in 70–99% ethanol.

In 2017, a preliminary snail survey was carried out in order to select suitable areas for examination of the infection prevalence. A small number of amber snails were collected in various localities of Asahikawa and Biei, Hokkaido. As reported previously [18], the snails were individually dissected in Dulbecco's phosphate-buffered saline (PBS) under a stereomicroscope. Immature broodsacs (i.e. uncolored tubular sporocysts) found from the snails were kept in 70% ethanol.

During June to August in 2018, a systematic sampling of amber snails was carried out at three sites of Asahikawa to estimate the prevalence of *Leucochloridium* infections with the mature and immature broodsacs. Amber snails were collected by hand-picking from plant leaves. Shells of the snails were measured in length by an electronic caliper, and all of them were then dissected. The mature and immature broodsacs from the snails were kept in 70% ethanol. The sampling locations were named the sites A (43.749 N,142.368E), B (43.808 N, 142.355E), and C (43.719 N, 142.351E). The sites A and B are included in the public parks, Kaguraoka and Shunkodai, respectively. The landscape of the sites A and B is a forest with marshy grounds, and that of the site C is a small hill with wood. In these sites, the ground is covered with snow from December to early April. Many amber snails appear on plant leaves from middle May to early August, and the mating of the snails is frequently seen on the leaves in June and July (M. Nakao, unpublished observations). It is likely that the gravid snails subsequently hide in the litter layer for laying eggs.

In the period from 2014 to 2018, the avifauna of Kaguraoka (the site A) and Shunkodai (the site B) Parks was assessed by members of the Asahikawa Branch of the Wild Bird Society of Japan. The authorized guide books of birds in Japan [19,20] were referred for the classification of wild birds and the evaluation of their food habits, seasonal migrations, and geographical distributions.

2.3. Parasite examination of birds

Accident carcasses of small or medium-sized insectivorous birds, which were kept in the Wild Animal Medical Center, Rakuno Gakuen University, or were directly given from bird-watchers, were examined for adult worms of *Leucochloridium*. The carcasses were preserved in a freezer or in sufficient amount of 70% ethanol. A dissection was focused mainly on the cloaca in order to find the adult worms. Intestinal tissues fixed with ethanol were broken using fine forceps. The parasites collected were subjected to morphological and molecular identification.

2.4. Morphological observation of parasites

The colored pattern of each mature broodsac was recorded as a macrophotograph. Fully-developed metacercariae from the broodsac were flattened in 10% neutral-buffered formalin between a grass slide and a coverslip for morphometric measurements. The metacercariae and adults of *Leucochloridium* spp. were stained with Heidenhain's iron hematoxylin or Schneider's aceto-carmine, dehydrated in graded ethanol series, cleared in creosote, and mounted with Canada balsam. An optical microscope with a digital camera (Axio Imager, Zeiss) was used for morphological observations. Sizes of objects were measured via their digital images using the accessory software (AxioVision). Published keys to the species of *Leucochloridium* [21,22] were used for morphological identification.

2.5. Statistical tests

The free software R (www.r-project.org) was used for statistical analyses of snails. A Fisher's exact test was employed to examine differences between the prevalence rates of snails infected with immature and mature broodsacs. A Student's *t*-test was applied to compare the

mean lengths of snails after confirming the equal variance by an F-test. The *p*-values of <0.05 were considered statistically significant.

2.6. DNA sequencing and phylogenetic analyses

The ethanol-preserved specimens of *Leucochloridium* spp. were used as sources for DNA sequencing. As reported previously [18], a whole body of the metacercaria or a piece of the adult worm was lysed in 25 µl of 0.02 N NaOH at 99 °C for 30 min. One µl of the lysate was used as a template for polymerase chain reaction (PCR). In this study, a single broodsac-derived metacercaria from each infected snail was regarded as an isolate. Mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*), nuclear 28S ribosomal DNA (rDNA), and internal transcribed spacers (ITS including both ITS1 and ITS2) in the rDNA cassette were chosen as PCR targets. The *cox1* sequences are necessary for DNA barcoding to discriminate species. The Tks Gflex DNA polymerase (TaKaRa) was employed for PCR with the manufacturer-supplied reaction buffer. Primer sets used were as follows: JB3 and CO1-R trema for *cox1* [23], and digl2 and 1500R for 28S rDNA [24]. In the case of metacercaria from Okinawa, the reverse primer CO1-R trema was replaced by JB4.5 [25]. The following original set was used for the amplification of ITS: Leuco-ITS/F (5'-ACC GAA CTT GAT CAT TTA GAG GAA GTA-3') and Leuco-ITS/R (5'-ATG GTC ACA GGC TTC GGT GCT GGG CTA-3'). The PCR was run for 40 cycles (98 °C for 10 s, 50 °C for 20 s, and 68 °C for 60–90 s) in a total volume of 25 µl including 0.25 µM of each primer. The PCR amplicons were sequenced by 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 DNA sequences determined in this study have been deposited into DDBJ/ENA/GenBank databases under the accession numbers LC466770–96 (*cox1*), LC466797–9 (28S rDNA), and LC466800–2 (ITS).

The nucleotide alignment datasets of *cox1*, 28S rDNA, and ITS were individually prepared by MAFFT [26]. The comparative sequences of related taxa were retrieved from DDBJ/ENA/GenBank databases. The data sets of *cox1*, 28S rDNA, and ITS consisted of 184, 1248 and 1008 nucleotide sites, respectively. The best-fit nucleotide substitution model of each dataset was selected by MEGA7 [27] as follows: HKY + I for *cox1*, K2 for 28S rDNA, and T92 for ITS. The midpoint-rooted phylogenetic tree of each data set was made under the substitution model by maximum likelihood (ML) method of MEGA7. The robustness of the trees was tested by bootstrapping with 500 replicates. The mean values of pairwise genetic divergence were computed by MEGA7 under *p*-distance model, using the data sets of *cox1*, 28S rDNA, and ITS.

Parsimony networks of *cox1* haplotypes were illustrated by TCS [28], and their population genetics indices were computed by DnaSP [29]. The data set of *cox1* used for these analyses was composed of 807 nucleotide sites.

3. Results

3.1. Sighting information and broodsac collection

The internet survey of pulsating broodsacs resulted in 12 sighting instances. These information were concentrated in Hokkaido. The brown-banded ones were observed in Oshamanbe, Sapporo, Kunneppu, and Rausu, and the green-banded ones in Sapporo, Asahikawa, Nemuro, Kushiro, Shiranuka, and Shizunai (Fig. 1). There was no sighting instances from the other Japanese islands, excepting a case of the vertical-striped broodsac in Tomigusuku, Okinawa. Some of the observers directly sent the infected snails to us. The gifts of the brown-banded broodsacs (3 isolates) from Oshamanbe and Rausu were available for later molecular analyses.

During three-year period from 2016 to 2018, 30 broodsac-pulsating snails were collected in several localities of Hokkaido. The infected snails were found mainly from June to July. All of the host snails were easily identified as *S. lauta*, because only this species prevails in Hokkaido [30]. Two types of the brown- and green-banded broodsacs were isolated from the snails. The brown ones (20 isolates) were obtained from Sapporo, Asahikawa, Biei, Furano, and Abashiri, and the green ones (10 isolates) were from Otaru, Sapporo, Asahikawa, and Abashiri (Fig. 1).

Museum-preserved broodsacs from 6 snails in Okinawa (Naha and

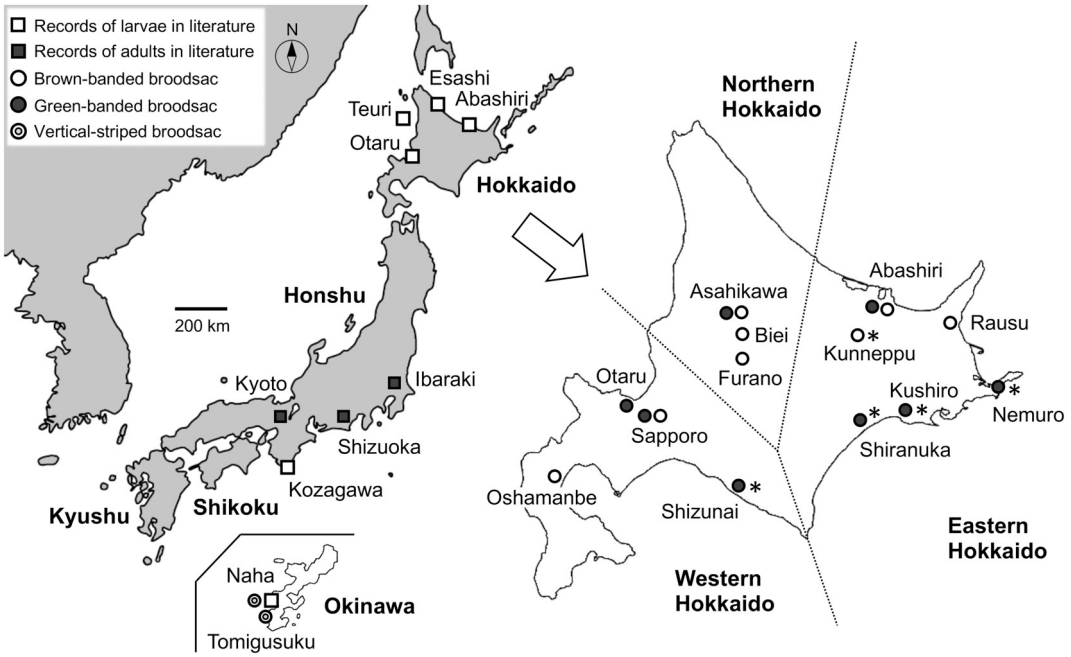

Fig. 1. A map showing the distribution of *Leucochloridium* in Japan. Bibliographic data of the parasites and the internet-based sighting information of the broodsac-pulsating snails were plotted on the map. Results of the present field survey were also shown on the map. Square points indicate the bibliographic data (open squares for larval records and closed squares for adult records). Circular points show the sighting information and the results of the field survey (open circles for brown-banded broodsac, closed circles for green-banded broodsac, and double circles for vertical-striped broodsac). Sites where the infected snails were confirmed only by photographs from volunteers are marked with asterisks. In the other circular points, the parasite samples were subjected to DNA barcode identification.

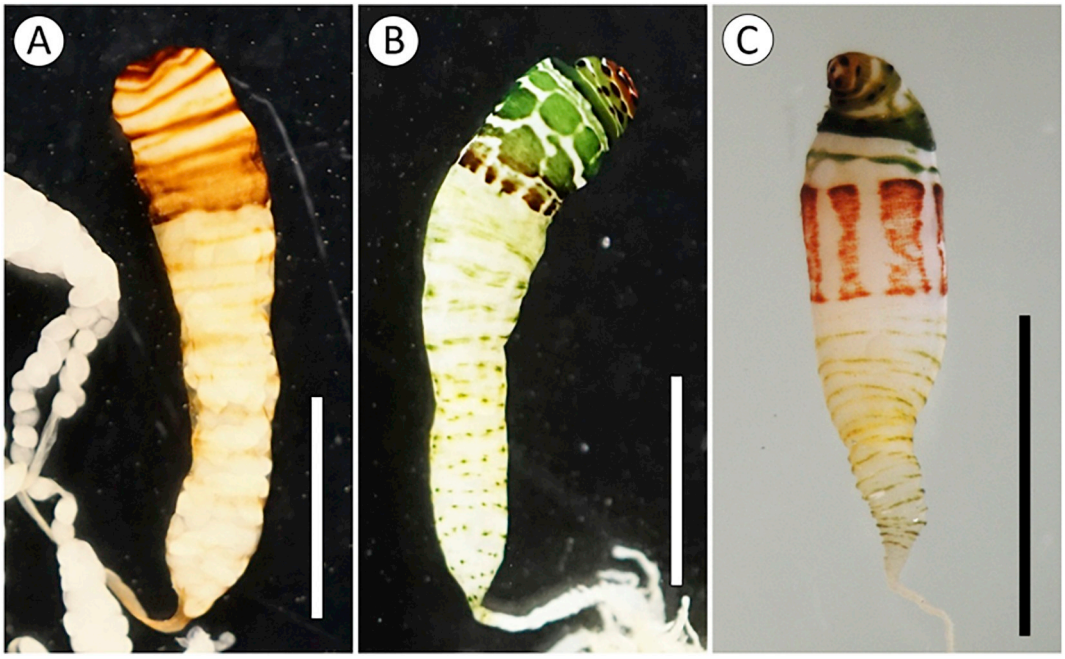

Fig. 2. Colorations of mature broodsacs removed from amber snails. Scale bars indicate 5 mm. A) Brown-banded broodsac in Hokkaido. B) Green-banded broodsac in Hokkaido. C) Vertical-striped broodsac in Okinawa. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Tomigusuku) were unique in having vertical stripes. The host snails of Okinawa were retrospectively identified as *Succinea* sp. (an alien species from North America) from the remaining shells (R. Ueshima, unpublished data). Furthermore, a preliminary snail survey of Hokkaido in 2017 resulted in obtaining 14 uncolored immature broodsacs from Asahikawa and Biei. Through these collection processes, 30 isolates of mature broodsacs from Hokkaido, 3 isolates of mature broodsacs from Okinawa, and 14 isolates of immature broodsacs from Hokkaido were available for later molecular analyses.

Overall results from the internet survey, the broodsac collection, and bibliographic records [5–8,12] clearly demonstrated that at least three species of *Leucochloridium* are distributed in Japan (Fig. 1). Furthermore, the distribution patterns of the brown- and green-banded broodsacs in Hokkaido seemed to be different from each other, because the green ones were particularly found from the coastal areas. As described in detail later, we assigned the brown-banded broodsac (Fig. 2A) to *L. perturbatum* [31], the green-banded broodsac (Fig. 2B) to *L. paradoxum* [32], and the vertical-striped broodsac (Fig. 2C) to *Leucochloridium* sp.

3.2. Prevalence of infected snails in Asahikawa

The prevalences of snails infected with immature or mature broodsacs were examined at the sites A, B, and C of Asahikawa (Table 1). From June to August in 2018, the snails were randomly collected, and their mature and immature broodsacs were individually subjected to DNA barcoding. Since the immature ones from each infected snail were colorless, all of them were molecularly identified. In the case of the mature ones, some of them were identified only by the coloration. A snail having at least one mature broodsac was judged as "mature infection", while a snail having only immature broodsacs as "immature infection". Both *L. perturbatum* and *L. paradoxum* were detected in the sites A and C. The former species was obviously dominant. The total prevalence (mature and immature infections combined) of *L. perturbatum* was 6.8% in the sites A and 6.1% in the site C, whereas that of *L. paradoxum* was relatively at low levels in both the sites. Only the immature broodsacs of *L. perturbatum* were detected at low prevalence in the site B. As shown in Table 1, the prevalence rates of the immature infection with *L. perturbatum* (5.1% in the site A and 3.5% in the site C) were higher than those of the mature infection (1.7% in the site A and 2.5% in the site C). A Fisher's exact test, however, presented that the differences were statistically insignificant ($p = 0.281$ in the site A and $p = 0.535$ in the site C).

The shell lengths of snails infected with *L. perturbatum* were compared between categories of the mature and immature infections. Combined data of the shell lengths from the sites A, B, and C were used for the comparison. The mean length with standard deviation (minimum and maximum range) of the mature group ($n = 12$) was

Table 1
Prevelence of amber snails infected with *Leucochrolidium* spp. in three collection sites of Asahikawa, Hokkaido.

Sites[a] (coodinates)	No. snails examined	No. snails infected with *L. perturbatum* (%)			No. snails infected with *L. paradoxum* (%)		
		Immature[b]	Mature[c]	Total	Immature	Mature	Total
A (43.749 N, 142.368E)	118	6 (5.1)	2 (1.7)	8 (6.8)	0 (0)	1 (0.8)	1 (0.8)
B (43.808 N, 142.355E)	615	8 (1.3)	0 (0)	8 (1.3)	0 (0)	0 (0)	0 (0)
C (43.719 N, 142.351E)	395	14 (3.5)	10 (2.5)	24 (6.1)	4 (1.0)	4 (1.0)	8 (2.0)
Total	1128	28 (2.5)	12 (1.1)	40 (3.5)	4 (0.4)	5 (0.4)	9 (0.8)

[a] Amber snails were collected during June to August in 2018.
[b] All immature broodsacs (uncolored) from each snail were identified by DNA barcoding.
[c] Some mature broodsacs (colored) from each snail were identified by the coloration, and the remainder were identified by DNA barcoding.

Table 2
Avifauna records (2014–2018) of Kaguraoka and Shunkodai Parks in Asahikawa, Hokkaido.

Orders	Families	Species[a]	Migrant[b]	Distribution[c]	Food habits
Anseriformes	Anatidae	*Aix galericulata**	Summer		Plant, insect, snail
		*Anas platyrhynchos**	Winter	Holarctic	Plant, insect, Snail
Columbiformes	Columbidae	*Streptopelia orientalis**	Summer		Omnivorous
		Treron sieboldii	Summer		Plant
Cuculiformes	Cuculidae	*Cuculus optatus**	Summer	Palearctic	Insect
		*Cuculus canorus**	Summer	Palearctic	Insect
Charadriiformes	Scolopacidae	*Actitis hypoleucos**	Summer	Palearctic	Insect
		*Scolopax rusticola**	Summer	Palearctic	Earthworm, insect
		*Gallinago hardwickii**	Summer		Earthworm, insect
Accipitriformes	Accipitridae	*Milvus migrans*	Resident	Palearctic	Carnivorous
		Accipiter nisus	Resident	Palearctic	Birds, insect
Coraciiformes	Alcedinidae	*Alcedo atthis*	Summer	Palearctic	Fish, amphibian, insect
Piciformes	Picidae	*Jynx torquilla*	Summer	Palearctic	Insect
		Dendrocopos kizuki	Resident		Insect, plant
		Dendrocopos minor	Resident	Palearctic	Insect, plant
		Dendrocopos leucotos	Resident	Palearctic	Insect, plant
		Dendrocopos major	Resident	Palearctic	Insect, plant
		Picus canus	Resident	Palearctic	Insect, plant
Falconiformes	Falconidae	*Falco subbuteo*	Summer	Palearctic	Omnivorous
		Falco peregrinus	Resident	Holarctic	Birds
Passeriformes	Laniidae	*Lanius bucephalus**	Summer		Carnivorous
	Corvidae	*Garrulus glandarius**	Resident	Palearctic	Insect, plant
		*Corvus corone**	Resident	Palearctic	Omnivorous
		*Corvus macrorhynchos**	Resident		Omnivorous
	Paridae	*Poecile palustris**	Resident	Palearctic	Insect, plant
		*Poecile varius**	Resident		Insect, plant
		*Periparus ater**	Resident	Palearctic	Insect, plant
		*Parus minor**	Resident		Insect, plant
	Alaudidae	*Alauda arvensis**	Summer	Palearctic	Plant, insect
	Pycnonotidae	*Hypsipetes amaurotis**	Resident		Plant, insect
	Cettiidae	*Cettia diphone**	Summer		Insect, plant
		*Urosphena squameiceps**	Summer		Insect
	Aegithalidae	*Aegithalos caudatus**	Resident	Palearctic	Insect, plant
	Phylloscopidae	*Phylloscopus examinandus**	Transient		Insect
		*Phylloscopus borealoides**	Summer		Insect
		*Phylloscopus coronatus**	Summer		Insect
	Zosteropidae	*Zosterops japonicus**	Summer		Insect, plant
	Bombycillidae	*Bombycilla garrulus*	Winter	Palearctic	Plant
		Bombycilla japonica	Winter		Plant
	Sittidae	*Sitta europaea**	Resident	Palearctic	Insect, plant
	Certhiidae	*Certhia familiaris**	Resident	Palearctic	Insect
	Sturnidae	*Spodiopsar cineraceus**	Summer		Insect, plant
		*Agropsar philippensis**	Summer		Insect, plant
	Turdidae	*Turdus cardis**	Summer		Insect, earthworm
		*Turdus obscurus**	Transient		Insect, plant
		*Turdus chrysolaus**	Summer		Insect, earthworm, plant
		*Turdus naumanni**	Winter		Insect, plant
	Muscicapidae	*Luscinia cyane**	Summer		Insect
		*Muscicapa dauurica**	Summer		Insect
		*Ficedula narcissina**	Summer		Insect
		*Cyanoptila cyanomelana**	Summer		Insect
	Passeridae	*Passer rutilans**	Summer		Insect, plant
		*Passer montanus**	Resident	Palearctic	Insect, plant
	Motacillidae	*Motacilla cinerea**	Summer	Palearctic	Insect
		*Motacilla alba**	Resident	Palearctic	Insect
		*Anthus hodgsoni**	Summer		Insect, plant
	Fringillidae	*Fringilla montifringilla**	Transient	Palearctic	Plant, insect
		Chloris sinica	Summer		Plant
		Carduelis spinus	Winter	Palearctic	Plant
		Carduelis flammea	Winter	Holarctic	Plant
		*Uragus sibiricus**	Summer		Plant, insect
		Loxia curvirostra	Winter	Holarctic	Plant
		*Pyrrhula pyrrhula**	Resident	Palearctic	Plant, insect
		*Coccothraustes coccothraustes**	Summer	Palearctic	Plant, insect
	Emberizidae	*Emberiza cioides**	Summer		Plant, insect
		*Emberiza spodocephala**	Summer		Plant, insect

[a] Asterisks indicate possible candidates for the definitive hosts of *Leucochloridium*.

[b] "Summer" is that birds stay in Hokkaido during spring and summer seasons for breeding. "Winter" is that birds stay in Hokkaido during autumn and winter seasons for overwintering. "Transient" is that birds temporarily stay in Hokkaido without breeding. "Resident" is that birds stay in Hokkaido without migration.

[c] Birds having a wide distribution range are marked as "Palearctic" and "Holarctic". The "Palearctic" means a continuous distribution from Europe to the Far East. The "Holarctic" means the widest distribution including Europe, Asia, the Far East, and North America.

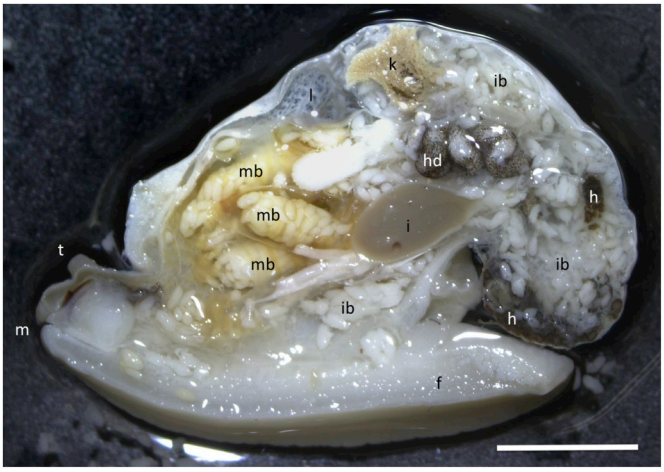

Fig. 3. A sagittal plane of *Succinea lauta* naturally infected with larval *Leucochloridium paradoxum*. Scale bar indicates 5 mm. Abbreviations are as follows: f, foot; h, hepatopancreas; hd, hermaphroditic duct; ib, immature broodsac; i, intestine; k, kidney; l, lung; mb, mature broodsac; m, mouth; t, tentacle.

18.8 ± 2.3 (14.4–22.0) mm, while that of the immature group (n = 28) was 15.3 ± 2.6 (10.4–21.1) mm. A Student's t-test showed that the mature group was significantly larger than the immature group (t = −3.9587, df = 38, p = 0.0003).

Through this prevalence survey, a total of 32 isolates of immature broodsacs and 12 isolates of mature broodsacs were available for later molecular analyses.

3.3. Avifauna of Asahikawa

Recent 5-year records of bird-watching in Kaguraoka and Shunkodai Parks of Asahikawa were compiled for estimating candidates for the definitive hosts of *L. perturbatum* and *L. paradoxum*. As shown in Table 2, 66 species of birds, encompassing 9 orders and 27 families, were recorded in the parks. Possible candidates for the definitive hosts of *Leucochloridium* were selected based on the insectivorous food habits of birds. Raptors of the Accipitriformes and Falconiformes, a kingfisher of the Coraciiformes, and woodpeckers of the Piciformes were excluded from the candidates. From members of the Anseriformes, Columbiformes, Cuculiformes, Charadriiformes, and Passeriformes, 48 species were listed as the possible candidates (Table 2). Out of them, 34 species (71%) are migratory birds and 14 species (29%) are resident birds. Nineteen species (40%) of the candidates are common in Europe, but only one candidate (the mallard, *Anas platyrhynchos*) is widely distributed in the Holarctic region.

3.4. Detection of adults from birds

A total of 30 insectivorous birds from various localities of Hokkaido were necropsied in this study. The bird samples encompassed 12 families and 18 species (Supplementary Table 1). The infection of *Leucochloridium* was confirmed in only one individual of the scaly thrush, *Zoothera dauma*, collected at Ebetsu in August 2003. Fifty-five adults were recovered from the cloaca. They reached to a fully gravid state, and one of them was identified as *L. perturbatum* by DNA barcoding.

3.5. Larval and adult morphology

The three types of mature broodsacs found in Japan were easily differentiated from one another by their coloration. The present DNA barcoding classified the brown-banded, green-banded, and vertical-striped broodsacs as *L. perturbatum*, *L. paradoxum*, and *Leucochloridium* sp., respectively. Morphological characteristics of these broodsacs are as follows:

Brown-banded broodsac (Fig. 2A). The anterior-distal end is dark brown with dots. Horizontal brown stripes (bands or rings) are prominent in the anterior part. The brown bands subsequently continue to the posterior end, but its color is too light. The size of the sac is approximately 13 mm in length.

Green-banded broodsac (Fig. 2B). The anterior-distal end is red or red-brown with patchy black spots, followed by green bands. Green square blocks and subsequent brown small blocks are prominent in the anterior part. Green dotted bands appear after the brown blocks and continue to the posterior end. The size of the sac is approximately 15 mm in length.

Vertical-striped broodsac (Fig. 2C). The anterior-distal end is brown with patchy black spots, followed by green bands. Vertical red-brown stripes are prominent in the anterior part. Their number is 10–12 per sac. Thin green bands appear after the stripe and continue to the posterior end. The size of the sac is relatively small, approximately 8 mm in length.

The brown-banded broodsac in Hokkaido showed the same design as that of *L. perturbatum* in Europe [31]. Moreover, the green-banded brood sac in Hokkaido was very similar in appearance to that of *L. paradoxum* in Europe [32]. The vertical-striped broodsac in Okinawa was almost identical to a small-sized broodsac of *L. passeri* found in

Table 3
morphological comparison of the metacercariae of *Leucochloridium perturbatum*, *Leucochloridium paradoxum*, *Leucochloridium passeri*, and *Leucochloridium* sp.

Characteristics (all measurements in μm)		*L. perturbatum* (n = 12) in Japan[a]	*L. perturbatum* (n = 47) in Europe[b]	*L. paradoxum* (n = 12) in Japan[a]	*L. paradoxum* (n = 20) in Europe	*Leucochloridium sp.* (n = 12) in Japan[a]	*L. passeri* (n = 1) in Taiwan
1. Total body	Length	1154 (1032–1227)	(480–860)	1174 (1101 – 1303)	630 (580–720)	579 (532–619)	690
	Width	577 (536–619)	(220–420)	506 (488–571)	290 (260–360)	319 (275–346)	402
2. Oral sucker	Length	347 (318–361)	(150–260)	313 (288–356)	174 (150–193)	193 (182–201)	165
	Width	349 (324–373)	(160–250)	305 (287–337)	172 (150–193)	174 (154–205)	185
3. Ventral sucker	Length	290 (270–310)	(110–290)	259 (227–286)	156 (136–179)	154 (140–171)	160
	Width	306 (287–330)	(120–290)	264 (233–307)	151 (136–164)	163 (144–190)	167
4. Ratio of suckers[c]		1.14: 1	1.01: 1	1.16: 1	1.14: 1	1.07: 1	1.11: 1
5. Pharynx	Length	110 (102–118)	(60–110)	96 (82–112)	66 (57–71)	75 (65–80)	55
	Width	128 (112–146)	(60–130)	110 (92–133)	73 (64–86)	81 (72–99)	77
6. Arrangement of gonads		Triangle	Triangle	Triangle	Triangle	Amorphous	Amorphous
7. Position of genital pore		Dorsal terminus	Dorsal terminus	Dorsal terminus	Dorsal terminus	Dorsal terminus	Undescribed
References cited		Present study	[31]	Present study	[55]	Present study	[33]

[a] Metacercariae of the three Japanese species were measured after flattening between a grass slide and a coverslip. Mean values are shown with range (in parentheses).
[b] The original description lacks mean values.
[c] Ratio of oral sucker (width) to ventral sucker (width).

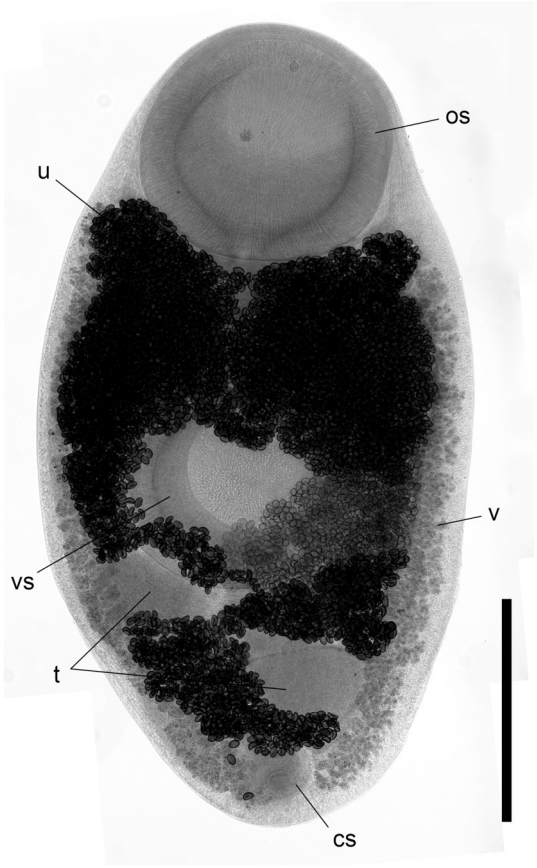

Fig. 4. An adult of *Leucochloridium* from the scaly thrush, *Zoothera dauma*. The specimen was stained with Heidenhain's ion hematoxylin. Scale bar indicates 500 μm. Abbreviations are as follows: cs, cirrus sac; os, oral sucker; t, testis; u, uterus; v, vitellarium; vs, ventral sucker. A sample from the same batch was identified as *L. perturbatum* by DNA barcoding.

neighboring Taiwan [33].

The broodsac-pulsating snails generally contained single or several colored mature broodsacs within their body. Tubular sporocysts (uncolored immature broodsacs) were coexistent with the mature broodsac (s). A whole space of the body cavity was occupied by them, and the sexual maturation of the host snail was highly suppressed (Fig. 3). Colored mature broodsacs contained approximately 100–200 metacercariae, which were enveloped with a jelly-like thick coat. Immature broodsacs contained developing cercariae, and also few or many of the encysted metacercariae. Fully developed metacercariae of the three Japanese species were compared with one another, together with those of the related foreign species (Table 3). The metacercariae of the Japanese species were drawn in Supplementary Fig. 1. The morphological discrimination of metacercariae was virtually impossible between the Japanese species of *L. perturbatum* and *L. paradoxum*. *Leucochloridium* sp. in Okinawa was similar in size to *L. passeri* in Taiwan. The development of gonad primordia were also similar in *Leucochloridium* sp. and *L. passeri*. The Japanese species of both *L. perturbatum* and *L. paradoxum* were larger in size than the corresponding European species, even after taking into consideration the artifact of flattening (Table 3). There were no differences in organ arrangements between the Japanese and European species.

Adult leucochloridiids from the scaly thrush were identified to generic level by morphological observation. The configuration of uterus, the distribution of vitellarium, and the dorsal position of genital pore proved them to be a member of *Leucochloridium* (Fig. 4). Morphometric data (means and ranges in parentheses) of the adults (n = 10) were as follows: Body ellipsoidal, 2.0 (1.8–2.3) mm in length by 1.1 (0.9–1.2) mm in maximum width. Oral sucker spherical, 0.54 (0.49–0.58) mm in length by 0.58 (0.54–0.62) mm in width. Ventral sucker spherical, 0.51 (0.44–0.56) mm in length by 0.51 (0.46–0.57) mm in width. Pharynx oval, 0.19 (0.17–0.22) mm in length by 0.24 (0.21–0.27) mm in width. The morphological identification of the adults to species was impossible, because gonads and intestinal tracts were masked by egg-filled uterus.

3.6. Molecular identification and population genetics

A total of 91 Japanese isolates of *Leucochloridium* (Supplementary Table 2) were used for molecular analyses. These are composed of 88 isolates from Hokkaido and 3 isolates from Okinawa. First of all, an unrooted ML tree was made by using DNA barcode sequences (mitochondrial *cox1*) of all the Japanese isolates. The resultant tree clearly showed that these isolates were divided into 3 clades. As shown in Fig. 5A, the topology of the clades was illustrated using the representative sequences (29 isolates), together with the published *cox1* sequences (18 isolates) of European species (*L. perturbatum*, *L. paradoxum*, *L. vogtianum*, and *L. subtilis*) [25] and a Japanese unspecified species [17]. In this DNA barcoding process, there were no discrepancies between the coloration of mature broodsacs and the assignment of species. The *cox1* sequences of European *L. perturbatum* were very similar to those of the brown-banded broodsac of Hokkaido. The sequence similarity was also observed between European *L. paradoxum* and the green-banded broodsac of Hokkaido, but these were slightly separated into two clades. The vertical-striped broodsac in Okinawa showed a sister relationship with *L. vogtianum* in Europe. The comprehensive results of the DNA barcode classification and the broodsac coloration indicated that the brown-banded, green-banded, and vertical-striped broodsacs should be assigned to *L. perturbatum*, *L. paradoxum*, and *Leucochloridium* sp., respectively. The previously reported isolate of *Leucochloridium* sp. in Hokkaido [17] was identified as *L. paradoxum*.

The pairwise genetic divergence values of *cox1*, 28S rDNA, and ITS sequences were compared among the European and Japanese species. The published sequences of 28S and ITS from the European species [34–37] were used for the comparison. The topology of each phylogenetic tree was also taken into consideration. In the sequences of *cox1*, a pairwise divergence between European and Japanese *L. paradoxum* reached to 0.024, but the value was too small to treat them as two separate species (Table 4 and Fig. 5A). The similar low value (0.016) was also observed between European and Japanese *L. perturbatum*. The other pairwise comparisons resulted in high values being at interspecific level. By contrast, the conservative sequences of 28S rDNA was unusable for the microtaxonomy of the congeneric species because of low resolution (Table 4 and Fig. 5B). In the variable sequences of ITS, the genetic diversity of the congeneric species could be depicted (Table 4 and Fig. 5C). The pairwise comparisons of ITS showed a close similarity between European and Japanese *L. paradoxum*, but there was a quite different ITS sequence of European *L. paradoxum* in DNA databases (see the clade Europe 2 in Fig. 5C). The sequences of ITS furthermore supported a sister relationship between *L. vogtianum* and *Leucochloridium* sp. in Okinawa, and an identity between European and

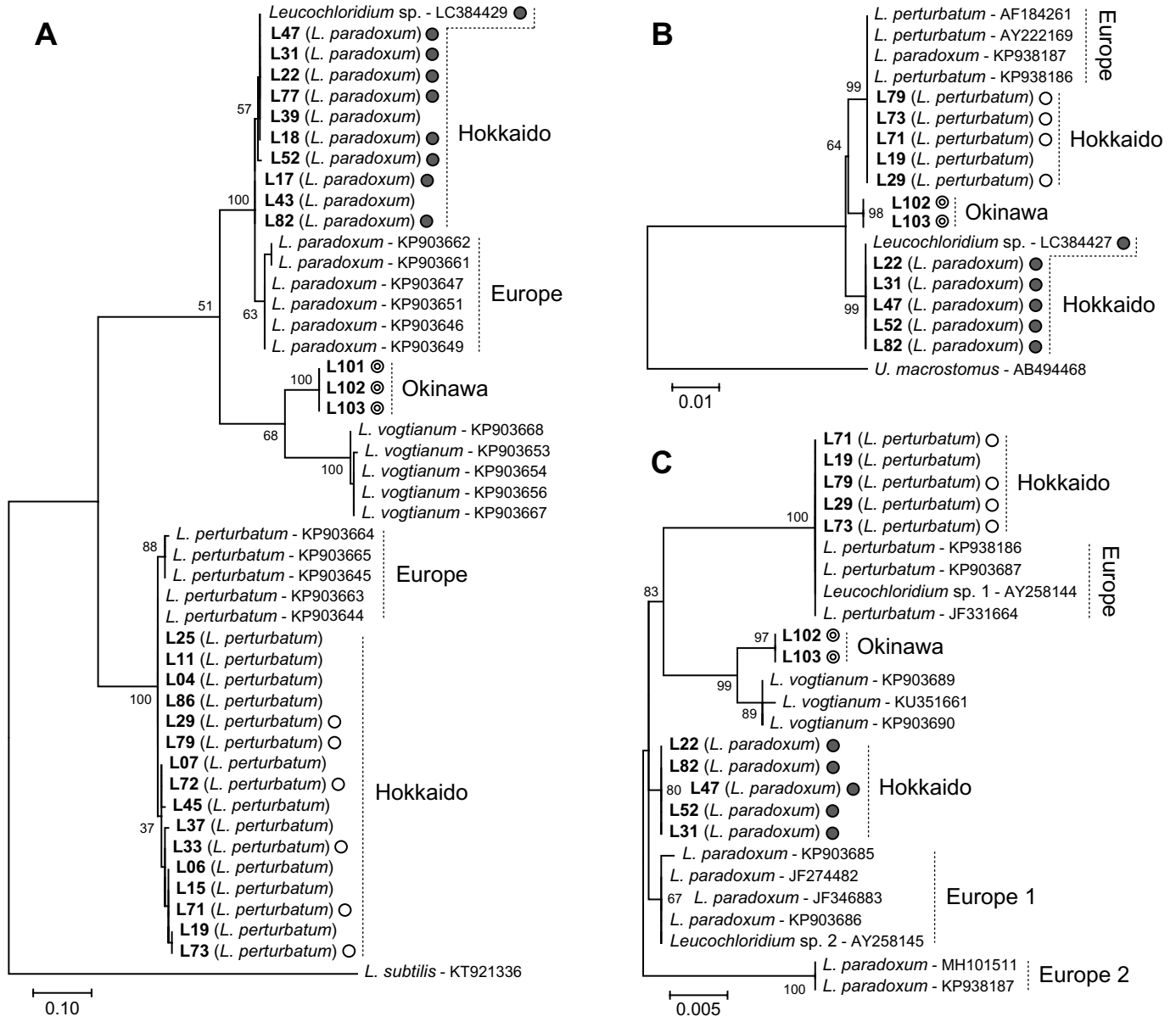

Fig. 5. Mid-point rooted maximum likelihood trees of European and Japanese species of *Leucochloridium*. Bootstrap percentages are shown on representative nodes. Scale bars indicate the number of substitutions per nucleotide site. Isolates examined in this study are shown in bold face. Open and closed circles indicate the brown- and green-banded broodsacs, respectively. Double circles denote the vertical-striped broodsac. The nucleotide accession numbers of published sequences are shown after the scientific name. A) The tree of mitochondrial *cox1*. B) The tree of 28S rDNA. C) The tree of internal transcribed spacers 1 and 2.

Japanese *L. perturbatum*.

The *cox1* haplotype networks of *Leucochloridium* in Hokkaido were figured, using all of the sequences determined in this study (70 isolates of *L. perturbatum* and 18 isolates of *L. paradoxum*). The network of *L. perturbatum* consisted of 20 haplotypes, and included two main haplotypes showing a star-like structure (Fig. 6A). Whereas, another network of *L. paradoxum* showed a relatively simple structure containing 6 haplotypes (Fig. 6B). Both of the networks included common haplotypes from far-distant localities in Hokkaido. The population genetics indices of the *cox1* sequences were presented in Table 5. The negative values of Fu's FS and Tajima's D suggested a population expansion of both *L. perturbatum* and *L. paradoxum* in Hokkaido. The haplotype and nucleotide diversities were higher in *L. perturbatum* than in *L. paradoxum*.

4. Discussion

Broodsac-pulsating snails are discovered largely by chance. The difficulty of finding infected individuals from host animals prohibits active studies on *Leucochloridium*. In this study, a large-scale survey was conducted to know a current situation about where and which species of *Leucochloridium* exist in Japan. The results clearly demonstrated that three species of *Leucochloridium* are independently distributed in northern and southern parts of Japan. The common European species of *L. paradoxum* and *L. perturbatum* were confirmed in Hokkaido, whereas another species in Okinawa.

The already known species of *Leucochloridium* in Japan must be compared with the presently confirmed species. During the 1930's, *L. japonicum* [13], *L. sime* [14], *L. turdi* [15], and *L. cardis* [15] had been

Table 4
Mean values of pairwaise genetic divergences among the European and Japanese species of *Leucochloridium*.

DNA	Alphabetical nos. of taxa		Pairwise comparisons[a]				
cox1	A. *L. paradoxum* in Europe (0.006)[b]	A.					
	B. *L. paradoxum* in Hokkaido (0.003)	**0.024**[c]	B.				
	C. *L. perturbatum* in Europe (0.009)	0.147	0.160	C.			
	D. *L. perturbatum* in Hokkaido (0.011)	0.149	0.162	**0.016**	**D.**		
	E. *L. vogtianum* in Europe (0.004)	0.156	0.148	0.161	0.165	E.	
	F. *Leucochloridium* sp. in Okinawa (0)	0.125	0.123	0.135	0.133	0.112	
28S	A. *L. paradoxum* in Europe	A.					
	B. *L. paradoxum* in Hokkaido	**0.009**	B.				
	C. *L. perturbatum* in Europe	0	0.009	C.			
	D. *L. perturbatum* in Hokkaido	0	0.009	**0**	D.		
	E. *Leucochloridium* sp. in Okinawa	0.007	0.008	0.007	0.007		
ITS	A. *L. paradoxum* in Europe 1	A.					
	B. *L. paradoxum* in Hokkaido	**0.002**	B.				
	C. *L. perturbatum* in Europe	0.016	0.015	C.			
	D. *L. perturbatum* in Hokkaido	0.016	0.015	**0**	D.		
	E. *L. vogtianum* in Europe	0.012	0.011	0.021	0.021	E.	
	F. *Leucochloridium* sp. in Okinawa	0.012	0.012	0.022	0.022	0.006	F.
	G. *L. paradoxum* in Europe 2	0.016	**0.016**	0.029	0.029	0.026	0.026

[a] The divergence values were computed under *p*-distance model.
[b] Intra-specific divergences of each taxon are shown in parentheses.
[c] Intra-specific comparisons between European and Japanese species are shown in bold face.

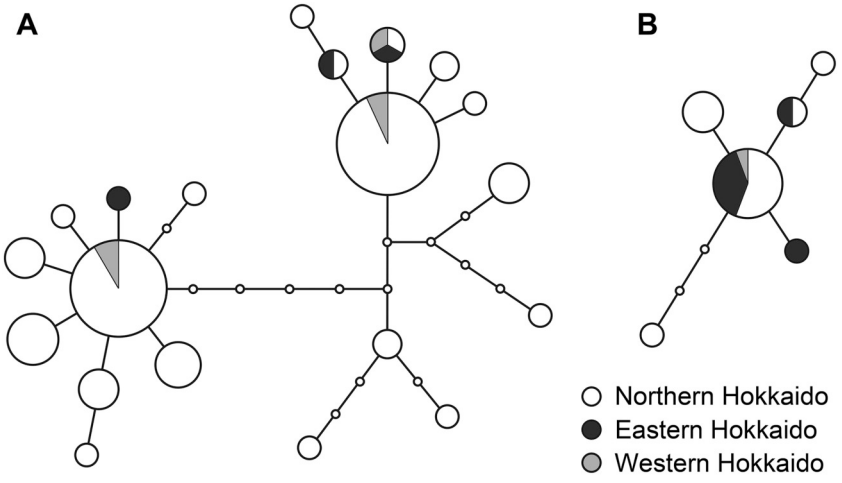

Fig. 6. Frequencies of mitochondrial *cox1* haplotypes and their statistical parsimony network in the Hokkaido populations of *Leucochloridium perturbatum* ($n = 70$) and *Leucochloridium paradoxum* ($n = 18$). The size of ovals indicates the frequency of the haplotypes. Small circles show hypothetical haplotypes. Geographic areas of the isolates examined were roughly divided into northern, eastern, and western Hokkaido (see Fig. 1). A) The network of *L. perturbatum*. B) The network of *L. paradoxum*.

described from birds in central Honshu (Fig. 1). In the trematode synopsis of Yamaguti in 1971 [38], the two genera, *Urogonimus* and *Neoleucochloridium*, were invalidated as synonyms of *Leucochloridium*. The former genus is now resurrected and the latter genus is treated as a subgenus of *Leucochloridium* [1]. As indicated by Kagan [21], the Yamaguti's species, *L. cardis* [15], is obviously a member of *Urogonimus* because of its unique configuration of uterus. In fact, a leucochloridiid found from the rustic bunting, *Emberiza rustica*, in Japan was identified as *Urogonimus macrostomus* [39]. There is no direct evidence to demonstrate relationships between the other old species in Honshu and the presently confirmed species in Hokkaido and Okinawa. Especially, the Yamaguti's species, *L. sime* [14], is undoubtedly a member of *Leucochloridium*. Yamaguti himself and also Kagan [21] stated that *L. sime* is closely related to *L. variae* [40] in North America. Pojmańska [31] further indicated the morphological similarity of adult worms among *L. sime*, *L. variae*, and *L. perturbatum*, when she described a European species of the brown-banded broodsac as *L. perturbatum*. Considering the present results in Hokkaido, *L. sime* is probably identical to *L. perturbatum*, because many birds including *Coccothraustes coccothraustes*, the type host of *L. sime*, repeatedly migrate between Hokkaido and Honshu [19,20]. The finally remaining old species, *L. japonicum* [13] and *L. turdi* [15], should be treated as *species inquirenda* because of the lack of information, although *L. turdi* was formerly treated as *Urogonimus turdi* [21].

There are still three species in tropical Asia to be examined their relatedness to *Leucochloridium* sp. in Okinawa. These are *L. muscularae* [41] and *L. passeri* [41] in Guangdong (formerly Canton), southern China, and *L. hypotaenidiarum* [42] in the Philippines. Pojmańska [22] considered *L. passeri* to be a synonym of *L. muscularae*. The vertical-striped broodsac of *Leucochloridium* sp. in Okinawa was almost identical

Table 5
Population genetics indices of mitochondrial *cox1* sequences (807 nucleotide sites) from *Leucochloridium* spp. in Hokkaido.

Species	No. isolates examined	No. haplotypes	Haplotype diversity	Nucleotide diversity	Fu's FS	Tajima's D
L. perturbatum	70	20	0.899	0.00635	−3.089	−0.72834
L. paradoxum	18	6	0.719	0.00151	−1.860	−1.36637

to a broodsac found in neighboring Taiwan [33]. The causative species of Taiwan was identified as *L. passeri* [41], based on the morphology of adults obtained from experimental infections [33]. Although the larval stage of *L. hypotaenidiarum* in the Philippines is unknown, the geographic proximity of Okinawa to Taiwan strongly supports that *Leucochloridium* sp. in Okinawa is the same as *L. passeri* in Taiwan. The closeness of Taiwan to the type locality of *L. passeri* (Guangdong) also reinforced the species identification in Taiwan. However, an unidentified status of the vertical-striped broodsac in Okinawa was kept unchanged, because the parasite has been reported from an alien snail (*Succinea* sp.) from North America [11]. Since the native amber snail, *Succinea lyrata*, is distributed in both Okinawa and Taiwan [43,44], it is highly likely that the parasite is also a native species. As far as we know, there are no records of broodsacs similar to those of Okinawa and Taiwan. Although *L. cyanocittae* in North America shows a vertical-striped broodsac [40,45,47], its coloration is quite different from that of Okinawa. The final conclusion of the species identification awaits the completion of DNA barcoding of the parasites in Taiwan and Guangdong.

The geographic distribution of *Leucochloridium* is characteristic of each continent. Species exhibiting distinctive broodsacs have been found in almost every zoogeographic region as follows: *L. perturbatum* [31], *L. paradoxum* [32], *L. subtilis* [46], and *L. vogtianum* [4] in Europe (Palearctic realm), *L. passeri* [41] in tropical Asia (Oriental realm), *L. variae* [40], *L. cyanocittae* [40,47], and *L. problematicum* [47,48] in North America (Nearctic realm), *L. flavum* [49] in Brazil (Neotropical realm), and *L. australiense* [50] in Australia (Oceanian realm). Thus, the distribution of each *Leucochloridium* species must be controlled by indigenous hosts of birds and amber snails. The avifaunas of Europe and North America are partially overlapping with one another [51]. However, many birds (e.g. members of the Passeriformes) are zoogeographically specific to each of the continents [52]. Candidate birds for the definitive hosts of *Leucochloridium* in Hokkaido are also common in Europe. In this context, the findings of *L. perturbatum* and *L. paradoxum* in Hokkaido (so far distant from Europe) are not a surprising result. Both the parasites seem to be widely and continuously distributed in Eurasia through expanding the host range. The parasite's genetic and morphological differences observed in this study should be regarded as intraspecific geographic variations. In particular, the Japanese isolates of *L. paradoxum* differ from the European ones in the appearance of broodsac and the size of metacercaria. Based on the differences, the green-banded broodsac in Hokkaido was once treated as an unspecified species of *Leucochloridium* [17].

Pojmańska [31] erected *L. perturbatum* with an emphasis on its distribution in Europe, and distinguished this species from *L. variae* in North America. Although both of them are morphologically quite similar to each other, the distinctive faunas of birds and amber snails between the Palearctic and Nearctic regions support her idea. A concept of the zoogeography-based cryptic species is accepted in this study. However, Bakke [53–55] considered that several species showing brown-banded broodsacs in both the Nearctic and Palearctic regions are conspecific. He regarded *L. fuscostriatum* [56] in North America, and *L. perturbatum*, *L. subtilis*, and *L. fuscum* [57] in Europe as the junior synonyms of *L. variae*. The DNA barcoding of brown-banded broodsacs and their related adults in North America is absolutely needed to evaluate the specific status of *L. variae*. If the *cox1* sequences of *L. perturbatum* are constantly confirmed from the North American samples, European *L. perturbatum* should be invalidated, and a resulting Holarctic distribution could be regarded as the recent geographic expansion of *L. variae* due to the long-distance invasion of birds between the continents.

The present study depicted a simple map about the distribution of *L. perturbatum, L. paradoxum,* and *Leucochloridium* sp. in Japan. At present time, there are two main focuses, Hokkaido (Palearctic realm) and Okinawa (Oriental realm), in which the parasite life cycles are maintained under quite different climates and ecosystems. However, in the other islands (Honshu, Shikoku, and Kyushu), the current parasite information is completely missing. This vacancy suggests that the maintenance of the parasite life cycles is virtually impossible in these islands probably due to the population decrease of amber snails associated with industrialization and urbanization. Indeed, it is difficult to find amber snails in these islands (T. Waki, unpublished observations). In contrast, the incredible abundance of amber snails in Hokkaido make it suitable to maintain the life cycles of *L. perturbatum* and *L. paradoxum*. The abundance of amber snails in Okinawa cannot be discussed due to the luck of field surveys. The present study strongly suggest that *L. perturbatum* and *L. paradoxum* prevail throughout Hokkaido. Moreover, we noticed that *L. perturbatum* is common in inland areas of Hokkaido, while *L. paradoxum* seems to be predominant in the coastal areas of eastern Hokkaido because of frequent sighting data of the green-banded broodsac. A possible explanation for the distribution patterns of *L. perturbatum* and *L. paradoxum* is that principal avian hosts differ in each parasite. Color preferences in selecting foods are observed in birds [58]. It can be, therefore, hypothesized that the brown or green coloration of broodsacs is selectively attractive to particular birds. In fact, the host records of *L. perturbatum* and *L. paradoxum* in Europe [25,31,32] suggest a possibility that the former is related to medium-sized insectivorous birds (e.g. members of *Turdus*) and the latter to small-sized insectivorous birds (e.g. members of *Parus* and *Cyanistes*). Behavioral ecological studies on both hosts and parasites are required to better understand true meanings of the coloration and pulsation of broodsacs.

The present study provided an important implication on the transmission dynamics of *L. perturbatum* and *L. paradoxum*. The prevalence survey of the larval infections in *S. lauta* showed that the immature broodsac-infected snails still exist even in summer when the mature broodsac-pulsating snails have emerged. The shell lengths of the immature broodsac-infected snails were smaller than those of the mature broodsac-infected snails, indicating a time-lag of the infections between them. It seems likely that the immature broodsac-infected snails overwinter in litter layers and emerge as pulsating snails in next year's seasons (spring to summer). A similar idea of wintering has already been published, based on the observation of snails (*Succinea puteris*) infected with *L. paradoxum* in Leningrad Province, Russia [59]. The following additional information are needed in order to verify the wintering hypothesis within snails: 1) the growing period of broodsac in infected snails, 2) the mortality of infected snails, 3) the prevalence of infected snails in spring (April and May), 4) the prevalence of infected birds in spring and summer, and 5) the persistence of infections in birds (i.e. the duration of the egg output). If the infection of birds is continuing for 6 to 12 months, the birds (migratory, resident, or both) can transmit the parasite to snails also in next year's seasons.

The haplotype network and population genetic analyses of mitochondrial *cox1* sequences show that *L. perturbatum* is more divergent than *L. paradoxum* in their populations of Hokkaido. The difference of the divergence suggests that *L. perturbatum* historically preceded *L. paradoxum* in the time of initial founder introduction into Hokkaido. However, a sampling bias should be considered in *L. paradoxum*, because the number of the samples examined are relatively small as compared with *L. perturbatum*. Furthermore, it is essential to consider whether the introductions of the two species into Hokkaido had been already closed or are still repeated by migratory birds. The comparative sampling of *L. perturbatum* and *L. paradoxum* from the Far East areas of the Eurasian continent is necessary to verify the present introduction of the parasites into Hokkaido. The finding of the same *cox1* haplotypes from the neighboring continent meets the minimum requirement of demonstrating the present introduction. If there are no identical haplotypes, it is highly possible that the parasites are kept only in Hokkaido without reintroduction from the continent. The wintering of the parasites within snails seems to be important in keeping the independent life cycles in the island.

The morphological identification of *Leucochloridium* is very difficult in both adult and larval stages [60], except for the coloration of

broodsacs. Also in this study, the specimens of metacercariae and adults could not be identified to species only by morphological observations. The keys to the species [21,22] were unapplicable to the fully gravid adults. Eventually, the observation of mature broodsacs and the DNA barcoding of the parasite tissues are most practical for the species identification. A systematic cataloging of broodsacs and their DNA barcodes worldwide will contribute the better understanding of the parasites, particularly in their taxonomy, evolution, and behavioral ecology.

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