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Dermocystid infection in Japanese fire-bellied newt, *Cynops pyrrhogaster*

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ABSTRACT. Here, we report details of a new infectious disease in wild-caught Japanese fire-bellied newts (*Cynops pyrrhogaster*), a Near Threatened species. Skin lesions consisting of numerous masses were found in the animals near Lake Biwa, Shiga Prefecture, Japan. The gross appearance of the skin lesions showed blister-, cyst-, and/or tumor-like morphology. Various sizes of skin lesions were observed on their entire body surface. Histologically, spherical basophilic cysts, including numerous spores, were observed in the dermis layer. Ultrastructural analysis indicated the presence of main bodies of flagellated zoospores within the spores. While 18s rRNA gene sequencing indicated that the skin lesions were due to dermocystid infection. To our knowledge, this is the first report of dermocystid infection in this amphibian in Japan. Further studies are needed to prevent epidemics and to establish diagnostic and treatment methods.

KEYWORDS: amphibian decline, biodiversity, *Cynops*, *Dermocystidium*, urodele

The Japanese fire-bellied newt (*Cynops pyrrhogaster*), a poisonous urodele amphibian, is endemic to Japan [26]. The adult newts live a water-dependent life and are recognized as prey for birds, mammals, and snakes that hunt in paddy fields [10, 14, 15], suggesting that they play important roles in the ecosystem. However, populations have been reported to be in decline in recent years, and the species was designated as Near Threatened in an assessment by the Ministry of the Environment of Japan 2020 (http://www.env.go.jp/en/index.html). The Japanese fire-bellied newt has also been employed as an experimental animal for embryological, regenerative medicine, and sex pheromone studies [7, 8, 16]. Despite this, many aspects of the species' biology are unresolved, such as its life history, regional differences in behavior and morphology [9, 20, 21], and susceptibility to infectious diseases.

Epidemics of infectious diseases present a survival threat to species; therefore, research into infectious diseases is important for maintaining biodiversity. Amphibian chytrid fungus disease, which currently adversely affects amphibians, has spread around the world through global transmission mechanisms and human movement [24]. Thus, amphibians are constantly at risk of this epidemic threat.

We recently found several individuals of wild-caught Japanese fire-bellied newts with skin lesions consisting of numerous masses that were suspected to be due to an infectious disease. In a previous study, Asashima *et al*. [1] reported finding a benign epithelioma on skin lesions in this species that were caused by a papillomavirus. However, the individuals we found showed swollen skin lesions that had spread over the whole body, rather than papillary lesions. Therefore, we report the morphology and genetics of the newly discovered cases and discuss the evidence that they were infected by a new disease.

MATERIALS AND METHODS

Study area and capture of C. pyrrhogaster

Individuals with skin lesions were captured in two independent surveys at a check dam in Taga Town, Shiga Prefecture, Japan (Fig. 1). The first survey was conducted on March 19, 2021, and the second was conducted on January 10, 2022. Individuals were captured using a scoop net. Experimental procedures and the care of animals were performed in accordance with the requirements of the Institutional Animal Care Committee at Nagahama Institute of Bio-Science and Technology, in compliance with National Institutes of Health guidelines.

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Histopathological examination

The lesions were fixed in Bouin's fluid or 10% neutral buffered formalin and embedded in paraffin. Four-micrometer sections were affixed to slides and stained with hematoxylin and eosin (HE). For transmission electron microscopy (TEM), the skin samples were pre-fixed in a slightly modified Karnovsky solution (2% glutaraldehyde, 2% paraformaldehyde in 0.03 M HEPES buffer, pH 7.4) at 4°C for 17 hr to preserve cell membrane structures. Following fixation, the samples were post-fixed with 1% osmium tetroxide/1.5% potassium ferrocyanide at 21–24°C for 1.5 hr [25]. Tissue samples were dehydrated and embedded in epoxy resin. Embedded specimens were sectioned at a thickness of 70 nm using Ultracut N (Reichert-Nissei, Wein, Austria), stained with 1% uranyl acetate for 20 min followed by 2% lead citrate for 2 min, and examined using a Hitachi H-7650 transmission electron microscope (Hitachi Ltd., Tokyo, Japan).

Genetic analysis

Genomic DNA was extracted from ethanol-fixed skin lesions from three individuals (two from the first survey and one from the second) using phenol/chloroform/isoamyl alcohol. Then, the18s rRNA gene was amplified by PCR using combinations of forward (CCTATCAACTTTCGATGGTAAGGTATTGGC) and reverse (CCGAAGACCTCACTAAACCATTCAATCG) primers designed to amplify *Dermocystidium* sp. 18s rRNA gene sequences [13]. The amplified conditions were 40 cycles of 98°C (10 sec), 65°C (15 sec), and 72°C (1 min). After 1.5% TAE agarose gel electrophoresis, PCR products were purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). The purified PCR products were directly sequenced using the forward and reverse primers, and the full length of 1,341 bp (excluding primers) (1,399 bp including primers) was sequenced.

Analysis by phylogenetic tree

A phylogenetic tree was constructed using the maximum likelihood method (T92+G82 model, lowest BIC and lower AICc scores among the compared models) using MEGA7 software (https://www.megasoftware.net/), as described in a previous paper [23]. Alignment was used MUSCLE program in MEGA7 software (default value: Gap open −400, Gap extend 0). Additionally, using a bootstrap method, 1,000 replicates were performed to assess the stability of the branches. *Sphaerothecum destruens* (AY267344-6, FN996945) and *S. caipira* (KU982985) were used as outgroups.

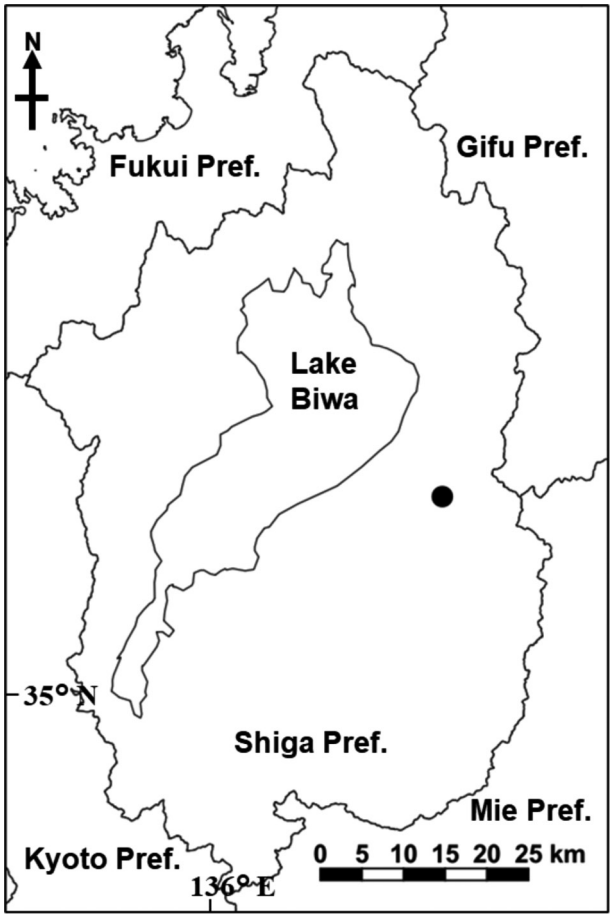

Fig. 1. Sampling site. Newts were captured at a check dam in Taga, Shiga Prefecture, Japan. The location is indicated by a filled circle.

RESULTS

Skin lesions observed in C. pyrrhogaster

In the first survey, 21 male and 16 female *C. pyrrhogaster* were captured, and skin lesions were observed in five males. In the second survey, 15 males and 10 females were captured, and skin lesions were observed in four males and one female.

The gross appearance of the skin lesions showed a blister-, cyst-, and/or tumor-like morphology (Fig. 2A–C, Supplementary Fig. 1). Various sizes of skin lesions were observed on the entire body surface (Fig. 2A–C, Supplementary Fig. 1). Histologically, many spherical basophilic cysts were observed in the dermis layer (Fig. 2D).

The cysts were composed of numerous mononuclear or multinuclear spores, and reddish-purple spores with large oval nuclei with black spots were observed close to the epidermis (Fig. 3A). TEM analysis showed the presence of the main protozoan body with characteristic paired flagella within the spores. The flagella were identified by the characteristic "9+2 structure" (nine microtubules in the periphery and two microtubules in the center) (Fig. 3B).

In an individual obtained in the second survey, a lesion was observed on the liver surface (Fig. 4A) but not on any other organs. Similar cysts were also observed in the liver lesions (Fig. 4B).

Sequence determination to Dermocystida sp.

We successfully sequenced the 1,341-bp PCR product of the 18s rRNA gene. A BLAST search of the read sequence showed homology with many species of Dermocystidia. The same sequence was detected from three individual specimens. The sequence was classified into the same clade as *Dermocyctida* sp. (Fig. 5).

DISCUSSION

The decline and extinction of amphibian populations are occurring globally, and one of the causes is the spread of infectious diseases. The present study found evidence for the presence of a dermocystid infection in the Japanese fire-bellied newt. The morphological features of the skin lesions observed in this study were similar to symptoms reported in previous papers [3–6]. The numerous spore-filled cysts in the skin, which were detected by light microscopy, were characteristic of protozoa described in the published papers. While the flagellated zoospores in the spores resembled those described in fishes [17, 18]. Gene sequence analysis indicated that the lesions on the newt skin were caused by dermocystid infection [6].

We recorded the belly/ventral surface color patterns of fire-bellied newts in a population survey carried out at the same sampling site on January 4, 2021, before sampling for the current study began. Although we did not detect prominent lesions on the ventral skin at that time, a reinvestigation of the images revealed early-stage lesions on one individual (Supplementary Fig. 1A). The size of

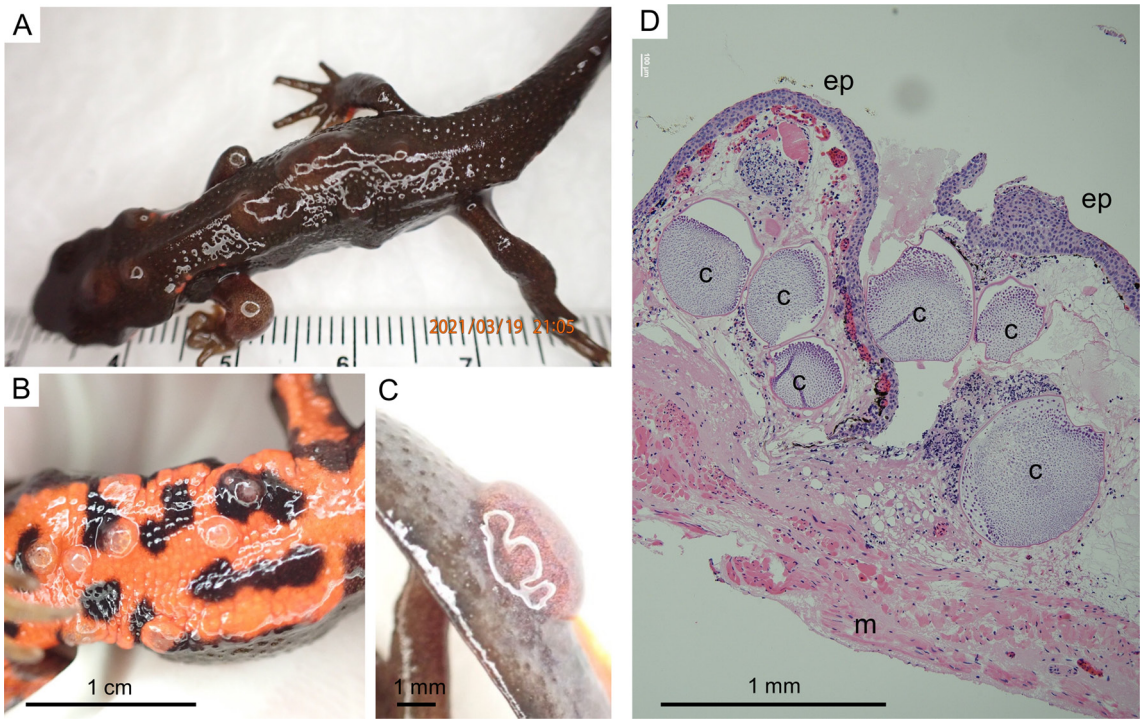

Fig. 2. Morphology of skin surface lesions. Gross appearance of the dorsolateral body (**A**), belly (**B**), tail (**C**). Skin lesions were distributed over the entire body. Scale bar, 1 cm. Micrograph of spherical basophilic cysts in skin lesions stained with HE (**D**). Specific features are indicated with lowercase letters: cyst (c), epidermis (ep), muscle (m). Scale bar, 1 mm.

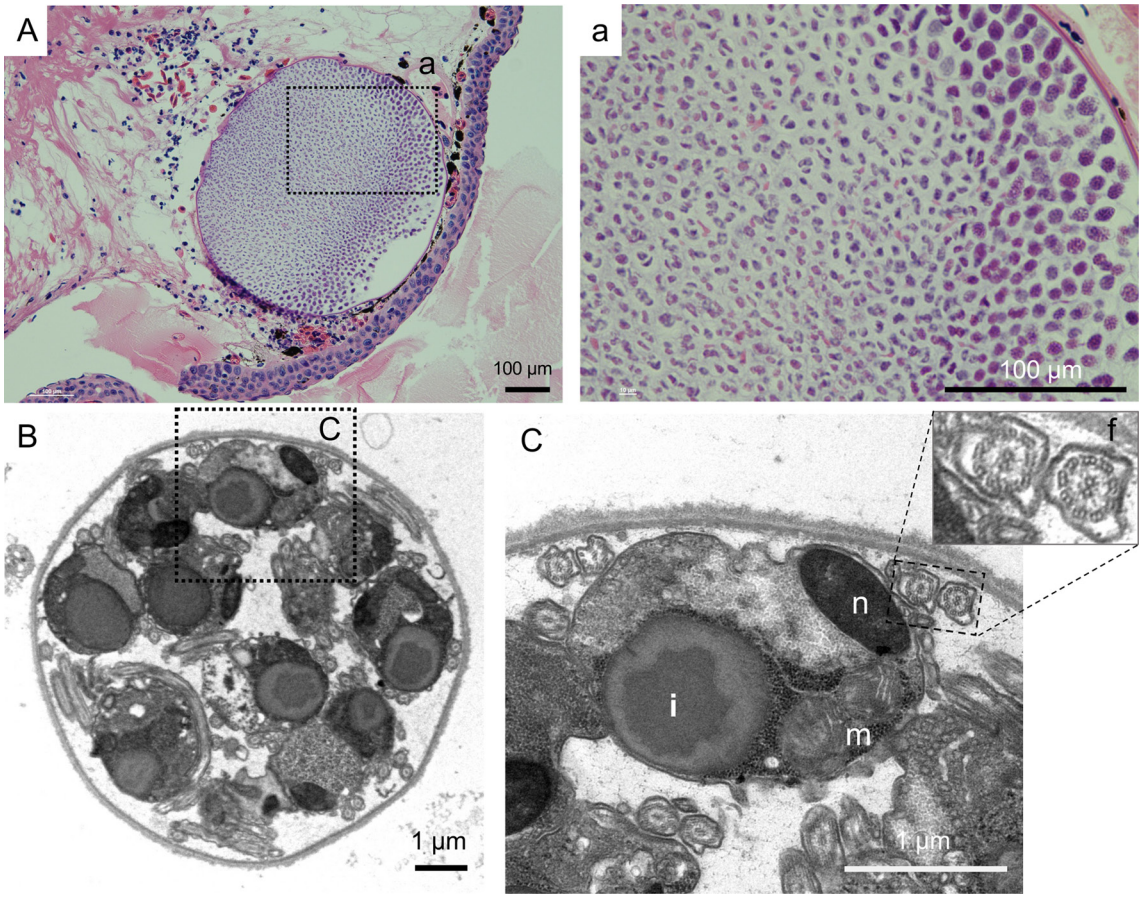

Fig. 3. Structure of spores in the spherical basophilic cysts. Cysts were composed of reddish-purple mononucleate spores with large oval nuclei and black spots close to the epidermis (**A**) and multinucleate spores far from the epidermis (**a**). Scale bar, 100 µm. Ultrastructure of mononuclear spores near the epidermis of cysts (**B**). Flagellated zoospores were observed within the spores (**C**). f, flagellum; i, inclusion body. m, mitochondria; n, nucleus. Scale bar, 1 µm. Flagella showed a characteristic 9 + 2 structure (**D**).

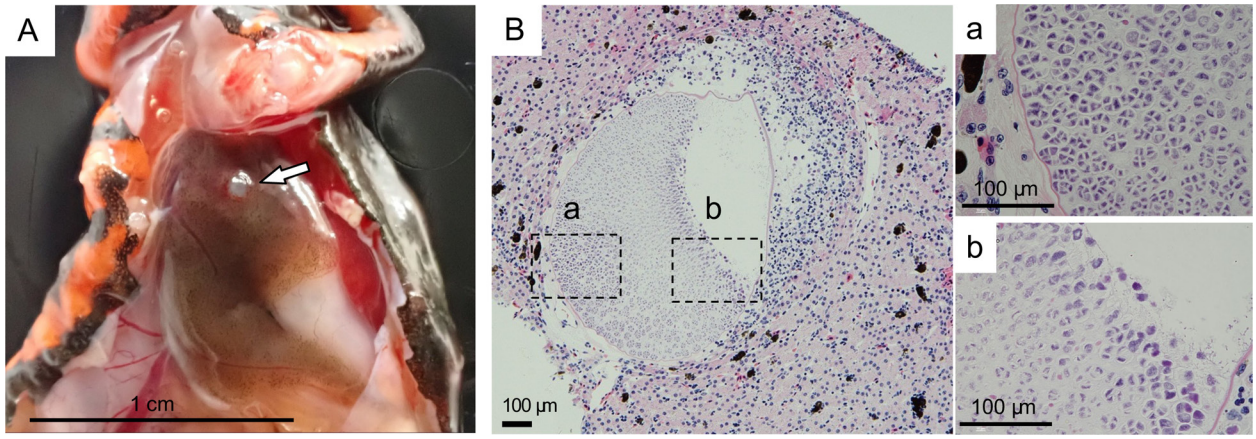

Fig. 4. Liver surface lesion. Lesion on liver surface was observed (white arrow) in a male and female newt. Micrograph of spherical basophilic cysts in liver lesions stained with HE. Cysts were composed of mononuclear (**a**) and multinuclear (**b**) spores. Scale bar, 100 µm.

the lesions had obviously progressed by March 19, 2021 (Supplementary Fig. 1B). This suggests that an outbreak of the infection had occurred before January 5, 2021, and the transmission route and source of the infection will be investigated in the future.

Dermocystid infections in newts have been mostly reported in Europe; however, in recent years, cases have also been observed in newts in North America [19], suggesting that this infection in newts is expanding in range. In addition, suspected cases of dermocystid

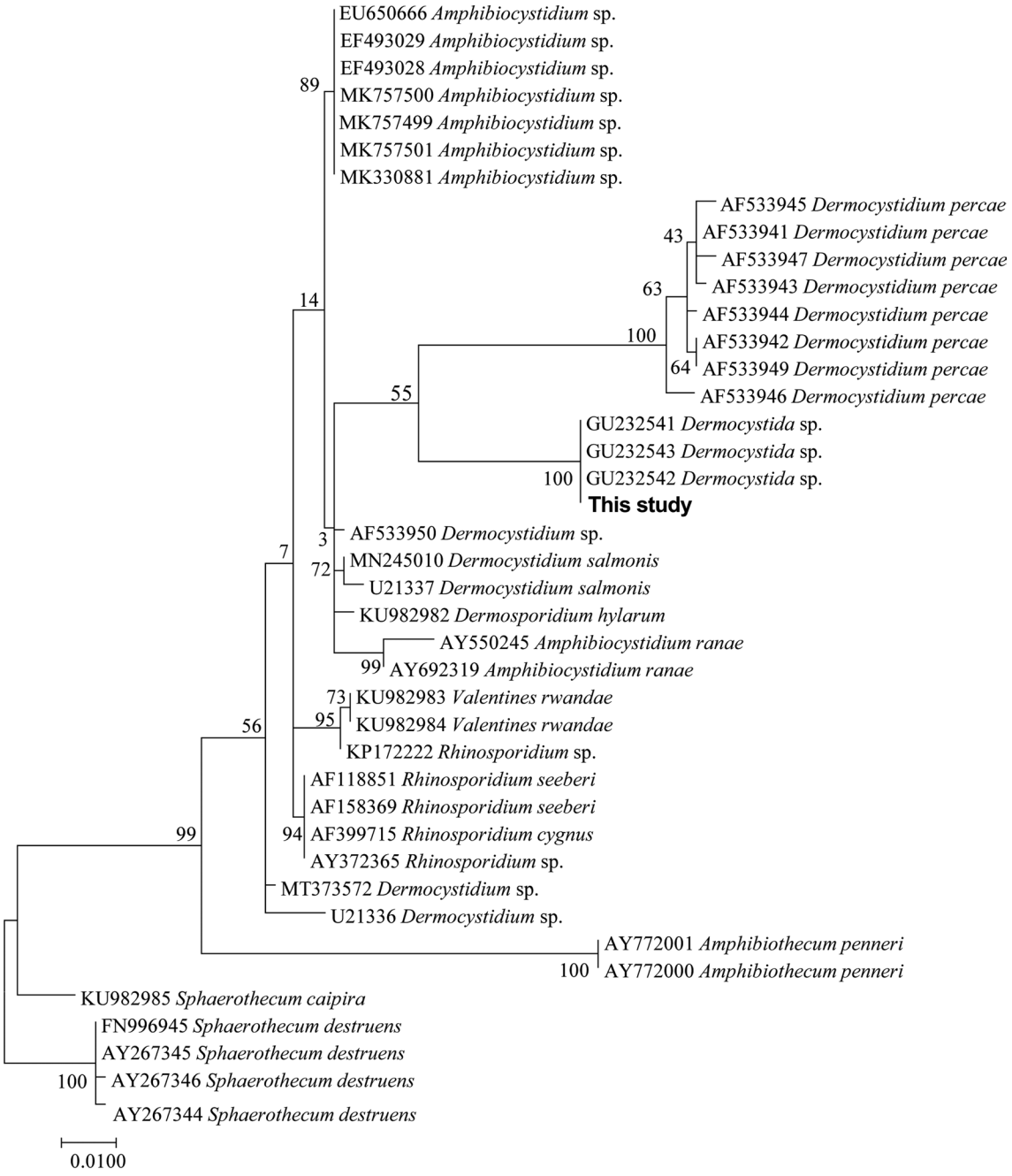

Fig. 5. Phylogenic tree of partial 18s rRNA gene sequence of dermocystid parasite detected in skin lesions (LC708043) compared with reference isolates (with accession numbers) obtained from GenBank. Phylogenetic tree was derived by maximum-likelihood analysis (MEGA 7) of a 1,400-bp region. Scale bar indicates the number of nucleotide substitutions per site. Using a bootstrap method, 1,000 replicates were performed to assess the stability of the branches. The numbers on branches represent bootstrap values for maximum likelihood.

infections in frogs have been reported in Europe [4] and South America [2]. Therefore, there is a possibility that other amphibians, as well as newts, could be infected in Japan. Because the Japanese fire-bellied newt reportedly has the potential to be terrestrial [11], newts with an infectious disease could spread the pathogen far from their aquatic habitat. We have conducted amphibian surveys in various locations and have yet to find individuals with skin lesions, such as those seen in this study, at other sites. So far, there are no other reports of dermocystid infections in Japanese fire-bellied newts. The effects of the infection on the newt's survival are also unclear.

Infection rates in male and female newts were 25% and 3.8%, respectively, suggesting that sex differences may exist. It is unclear whether this difference is due to the nature of the protozoan infection or the nature of the Japanese fire-bellied newt. Further investigation is needed to understand the disease.

Although chytrid fungus disease is ravaging amphibians worldwide [22, 24], it is symbiotic in Japan, and no mass mortality from the disease has been reported [12, 27]. Recently, however, mass mortalities of bullfrogs (*Lithobates catesbeianus*) due to ranavirus

have been observed in Japan [28], and other amphibians in Japan may be affected by this virus. Together with ranavirus, dermocystid infections may represent a new threat to Japanese amphibians.

As the area where we captured the newts is also inhabited by an arboreal frog (*Zhangixalus arboreus*) and a rare salamander (*Hynobius vandenburghi*), we must take the possibility of dermocystid infections in these species into account. To date, the route of infection and its impacts are unknown, so it is crucial that we are main our vigilance of future trends.

To our knowledge, this is the first report of dermocystid infections in the amphibian *C. pyrrhogaster*. Given the fact that amphibians help to conserve the biological diversity of their environment, the use of our research to establish epidemic prevention and infection diagnosis and treatment is of vital importance.

CONFLICT OF INTEREST. The authors declare no competing interests.

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