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*Phyllodistomum kanae* sp. nov. (Trematoda: Gorgoderidae), a bladder fluke from the Ezo salamander *Hynobius retardatus*

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Revised manuscript

***Phyllodistomum kanae* sp. nov. (Trematoda: Gorgoderidae), a bladder fluke from the Ezo salamander *Hynobius retardatus***

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(Corrected parts of the third revised manuscript are shown in red)

## ABSTRACT

The Ezo salamander, *Hynobius retardatus*, is endemic only to Hokkaido, the northernmost island of Japan. Gravid flukes of the family Gorgoderidae were discovered from the urinary bladder of *H. retardatus*. The parasites were identified as a new species named *Phyllodistomum kanae* sp. nov. In the neighboring Honshu island another bladder fluke, *Phyllodistomum patellare*, has already been found from the Japanese newt. The new species clearly differs from *P. patellare* in having a spherical ovary and very weakly lobed testes. The discovery of species of *Phyllodistomum* from urodelan amphibians is very uncommon in Eurasia. A molecular phylogeny based on 28S ribosomal DNA suggests that sphaeriid bivalves may serve as the first intermediate host for the new species.

Keywords:

Bladder fluke

*Phyllodistomum kanae* sp. nov.

Hynobiid salamander

*Hynobius retardatus*

Hokkaido

## 1. Introduction

The Japanese archipelago possesses a rich diversity of amphibians consisting of 44 species of anurans and 32 species of urodelans, in spite of its small land area (Amphibians of the World Database, American Museum of Natural History [www.amnh.org]). The great diversity of Japanese amphibians is directly correlated to the richness of their parasites. The parasitic helminths of amphibians in Japan have been investigated extensively [1-7]. Amphibian trematodes of the family Gorgoderidae found in Japan are as follows: *Phyllodistomum patellare* (Sturges, 1987) from the Japanese newt [8] and the sword-tail newt [6], *Gorgoderina kajika* (Ozaki, 1926) from the Kajika frog [2], *Gorgoderina tanigawaensis* Uchida & Itagaki, 1974 from the Japanese toad [5], and *Gorgoderina japonica* Yamaguti, 1936 from the dark-spotted frog [4]. In this report, another gorgoderid fluke is further added. The fluke was discovered from the Ezo salamander, *Hynobius retardatus* Dunn, 1923, which is endemic only to Hokkaido, the northernmost island of Japan. This salamander is a member of the Asiatic salamanders (Caudata: Hynobiidae) whose evolutionary center is considered to be North China [9].

Morphology of the Ezo salamander-derived fluke agrees with *Phyllodistomum* Braun, 1899 rather than *Gorgoderina* Looss, 1902, particularly in its broad hindbody [10]. There have been no records of gorgoderids from the Asiatic salamanders distributed all over Asia and in European Russia. Moreover, the fluke was distinct in its morphology when compared with already known related species from urodelan amphibians. In this report, a new species of *Phyllodistomum* is thus proposed for bladder flukes collected from the Ezo salamander.

## 2. Materials and methods

### 2.1. Specimen collection and morphological observation

In 4 May 2014, two *H. retardatus* adult males returning to the water to breed were captured in a small pool beside a mountain stream in Pippu town,

Hokkaido (43°56'51.9"N, 142°29'52.6"E). They were subjected to necropsy to collect endoparasites after killing with ether anesthesia. Six gravid flukes were obtained from the urinary bladder of one salamander, but the other was uninfected. Two of the flukes were flattened between glass slides, and then fixed with Alcohol-Formalin-Acetic acid (AFA) solution. The remaining worms were kept in 70% ethanol. For morphological observation, the AFA-fixed worms were stained with acetocarmine, dehydrated in an ethanol series, cleared in xylene, and finally mounted with Canada balsam. Measurements of the stained worms were used for morphometric assessment. Eggs recovered from a broken worm were also measured. All of the measurements were done under a calibrated microscope.

## *2.2. Sequencing of nuclear and mitochondrial DNA*

Genomic DNA extracted from each of two ethanol-fixed worms was used as a template for polymerase chain reaction (PCR) amplification of target genes. Experimental procedures of DNA extraction, PCR, and direct sequencing of amplicons were the same as those reported previously [11]. The primers digl2 and 1500R [12] were used for amplification of nuclear 28S rRNA gene (rDNA) including variable domains D1-D3, and the primers JB3 and JB4.5 [13] for mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*). Each of the PCR primers was also used as a sequencing primer.

## *2.3. Phylogenetic analysis*

DNA sequences of gorgoderid species related to the Ezo salamander-derived fluke were retrieved from DDBJ/EMBL/GenBank databases. Nucleotide data sets of nuclear 28S rDNA and mitochondrial *cox1* were prepared using the multiple aligner MAFFT [14]. Sites including gaps were completely removed from the alignments. The genetic software MEGA 6 [15] was used to find nucleotide substitution models and to estimate phylogenetic trees by maximum likelihood (ML) analysis. Midpoint rooted ML trees were generated from both the data sets with 500 bootstrap repetitions under

appropriate substitution models.

### 3. Results

#### 3.1. A new species (*Trematoda: Gorgoderidae*)

##### 3.1.1. Morphological description

*Phyllodistomum kanae* sp. nov. (Fig. 1).

Measurements of gravid flukes were based on two specimens stained with acetocarmine. The specimens were observed ventrally.

Body typically pyriform, 4.0 - 4.9 mm long by 2.0 - 2.1 mm wide. Forebody slender. Hindbody foliate, without crenulate margin. Neck region (posterior to oral sucker), 0.49 - 0.54 mm wide. Mouth ventral. Oral sucker sub-terminal, 0.48 - 0.49 mm width by 0.51 - 0.55 mm length. Ventral sucker pre-equatorial, 0.83 - 0.85 mm width by 0.60 - 0.70 mm length, obviously larger than oral sucker.

Esophagus narrow, 0.25 - 0.39 mm long. Pharynx absent. Penetration glands present along end of esophagus. Ceca bifurcating immediately anterior to genital pore. Ceca simple, straight, tubular, 2.7 - 3.3 mm long (left) and 2.6 - 3.5 mm long (right), ending near posterior end of hindbody.

Genital pore opening between oral and ventral suckers. Both cirrus-sac and morphologically striking cirrus absent. Seminal vesicle spherical, 0.23 - 0.24 mm maximal diameter; connecting almost directly to genital pore. Testes very weakly lobed, 0.52 - 0.54 mm width by 0.69 - 0.83 mm length (left) and 0.41 - 0.52 mm width by 0.88 - 0.92 mm length (right), lying asymmetrically along ceca. Ovary spherical or very weakly lobed, 0.39 - 0.40 mm maximal diameter, lying anterior to left testis. Vitelline glands ellipsoidal, 0.16 - 0.17 mm width by 0.22 - 0.29 mm length (left) and 0.14 - 0.18 mm width by 0.23 - 0.27 mm length (right), lying anterior to ovary. Seminal receptacle absent. Uterus lengthy and tortuous, filling entire area of hindbody. Uterine eggs immature and non-operculate, 34 - 37  $\mu$ m width by 49 - 54  $\mu$ m length (range of 10 individuals).

##### 3.1.2. Taxonomic summary

*Type host:* The Ezo salamander, *Hynobius retardatus* Dunn, 1923 (Caudata: Hynobiidae).

*Site of infection:* Urinary bladder.

*Type locality:* Pippu, Hokkaido island (Ezo), Japan.

*Date of collection:* 4 May 2014.

*Collector:* M. Nakao.

*Intermediate hosts:* Unknown.

*Type specimens:* One holotype and two paratypes have been deposited in Meguro Parasitological Museum, Tokyo, Japan (MPM Coll. No. 20950). One of the paratypes is an unflattened and unstained specimen stored in 70% ethanol.

*Etymology:* The new species is named after my daughter Kana.

### 3.2. Molecular comparison with other species

Two adult flukes of the new species were individually subjected to DNA sequencing for molecular phylogenetic comparison with other gorgoderid species. Both of the two worms showed exactly the same DNA sequences of nuclear 28S rDNA and mitochondrial *cox1*. The resultant sequences (1242 bases of 28S rDNA and 396 bases of *cox1*) were deposited into DDBJ/EMBL/GenBank databases under the accession numbers AB979868 (28S rDNA) and AB979869 (*cox1*). No identical sequences could be found in the DNA databases through the basic local alignment search tool (BLAST). *Phyllodistomum brevicecum* Steen, 1938, a bladder fluke of North American freshwater fish [16], showed the relatively high BLAST scores of 97% identity to 28S rDNA and 87% identity to *cox1*.

Maximum likelihood phylogenetic trees were estimated using nuclear 28S rDNA and mitochondrial *cox1* sequences. The new species was compared with related species of fish and amphibian parasites, although there are no other genetic data for *Phyllodistomum* spp. from amphibians. The data set 28S included 803 aligned nucleotide sites from 15 taxa, whereas the data set *cox1* only consisted of 307 nucleotide sites from 8 taxa. For the phylogeny reconstruction, the nucleotide substitution models GTR+G and HKY+G were applied to the data sets 28S and *cox1*, respectively. As shown in Fig. 2, the

new species occupied a distinct position in each of the nuclear and mitochondrial trees. In the 28S rDNA tree the new species was loosely clustered with *Phyllodistomum magnificum* Cribb, 1987 [17] and *Gorgoderia cygnoides* (Zeder, 1800) [18], whereas in the *cox1* tree the new species was distantly related to *Gorgoderina* sp. [19]. However, this relationship was considered unreliable because of lack of available taxa in *cox1*.

#### 4. Discussion

The discovery of species of *Phyllodistomum* from urodelan amphibians is very uncommon in Eurasia. The new species has rich morphological features, although egg-producing and excretory systems could not be observed in the present whole-mount specimens because of the overlapping uterus filled with eggs. As shown in Table 1, the new species was compared with other related species from Japanese and North American urodelans. In North America, *Phyllodistomum americanum* Osborn, 1903 [20, 21, 22], *Phyllodistomum singulare* Lynch, 1936 [23], *Phyllodistomum solidum* Rankin, 1937 [24, 25], and *Phyllodistomum coatneyi* Meserve, 1943 [26] have been recorded from lungless salamanders (Caudata: Plethodontidae). The new species clearly differs in morphology from these North American species. In the Honshu island of Japan, the congeneric species *P. patellare* has been found from the urinary bladder of the Japanese newt [8]. Another bladder fluke from the same newt was once described as *Phyllodistomum entercolpium* Holl, 1930 [27], but subsequently synonymized with *P. patellare* [23]. The new species is easily distinguishable from *P. patellare* by comparing shapes of ovary and testes (Table 1). In the other countries of the Far East, there are no records of *Phyllodistomum* spp. from urodelans. If anuran hosts are also considered, *Phyllodistomum skrjabini* Pigulewsky, 1953 and *Phyllodistomum sinense* Wu, 1937 have been found from the Asiatic toad in China [28, 29]. The new species differs from *P. skrjabini* and *P. sinense* in having unlobed vitelline glands.

The life cycles of *Phyllodistomum* spp. generally require freshwater bivalves as the first intermediate host and arthropods as the second intermediate host [30]. In the well-studied case of *P. solidum* (a bladder fluke of the North

American dusky salamander), *Pisidium abditum* (Haldeman, 1841), a freshwater bivalve of the family Sphaeriidae, harbors the parasitic asexual stages and the resultant cercaria encysts into infectious metacercaria in naiads of several species of Odonata [31]. At the present time the life cycle of *P. kanae* sp. nov. is completely unknown. However, the present phylogenetic tree of 28S rDNA may give clues as to its life cycle. The tree demonstrated the new species to be a member of a clade containing *P. magnificum*, *G. cynoides*, *P. brevicecum*, and others. This clade appears to relate to species in which cystocercous cercariae develop in bivalves of the family Sphaeriidae [17]. Sphaeriid bivalves in Hokkaido (e.g. *Pisidium japonicum* Pilsbry & Hirase, 1908 and *Sphaerium miyadai* Mori, 1933) [32, 33] could be candidates for the first intermediate host. The Ezo brown frog, *Rana pirica* Matsui, 1991 [34], is also a possible candidate for the definitive host of the new species because the frog shares a common ecological niche with the Ezo salamander. Further field studies are needed to determine the host usage of the new species.

The genus *Phyllodistomum* is a large group consisting mainly of fish parasites together with a few amphibian parasites, while hosts of the most closely related genera *Gorgoderina* and *Gorgodera* are only found in amphibians [10]. Russian taxonomists divided *Phyllodistomum* into four subgenera, based on the shapes of whole-body and vitelline gland [28]. Recent molecular phylogenetic studies on members of the Gorgoderidae showed that *Phyllodistomum* is paraphyletic [16, 17], as also confirmed in the present study. The broad and foliate hindbody of *Phyllodistomum* is a key morphological character separating this genus from *Gorgoderina* and *Gorgodera*, and furthermore *Gorgodera* has more testes than *Phyllodistomum* and *Gorgoderina* [10]. However, the above-mentioned molecular phylogenies suggest a possibility that the shape of hindbody and the number of testes are homoplasious traits. More extensive phylogenetic analyses are required for the revision of genera of the family Gorgoderidae. The species of *Phyllodistomum* from amphibians should be added to the analyses to clarify the evolutionary history of host-switching from fishes to amphibians or vice versa.

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## FIGURE LEGENDS

Fig. 1. Ventral views of the holotype specimen of *Phyllodistomum kanae* sp. nov. The left is a macrophotograph and the right is a drawing showing the shape and location of main organs. Abbreviations are as follows: c, cecum; e, esophagus; gp, genital pore; o, ovary; os, oral sucker; pg, penetration gland; sv, seminal vesicle; t, testis; u, uterus; vg, vitelline gland; vs, ventral sucker. A scale bar represents 1 mm.

Fig. 2. Midpoint rooted phylogenetic trees of *Phyllodistomum kanae* sp. nov. and related trematodes. The trees were generated by maximum likelihood analysis using nucleotide sequences of nuclear 28S rDNA (803 sites) and mitochondrial *cox1* (307 sites). Values of nodes are bootstrap percentages. Scale bars represent the estimated number of substitutions per nucleotide site. Database accession numbers of original sequences are given in parentheses. (A) The tree of 28S rDNA including 15 taxa. (B) The tree of *cox1* including 6 taxa. Most of the taxa used are fish parasites, except the following amphibian parasites: *Gorgoderia cygnoides*, *Gorgoderina* sp., and the new species.

Fig. 1

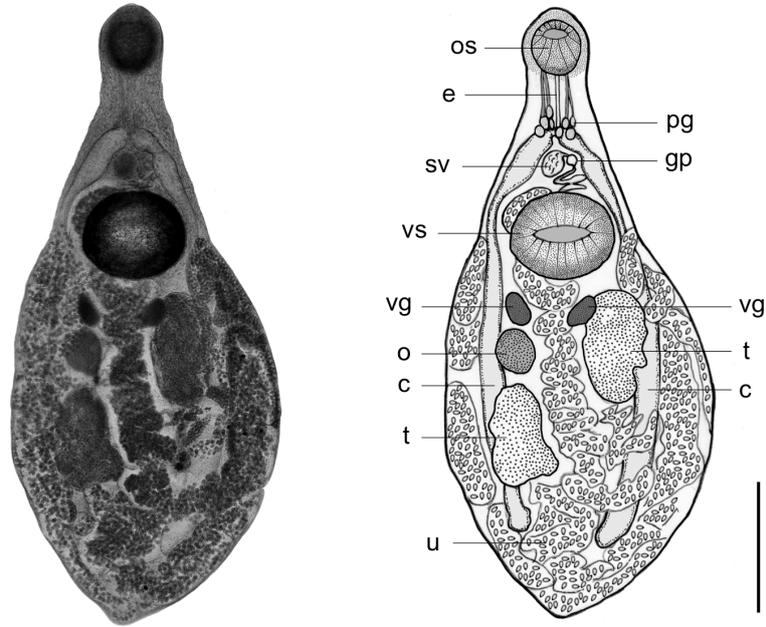


Fig. 2

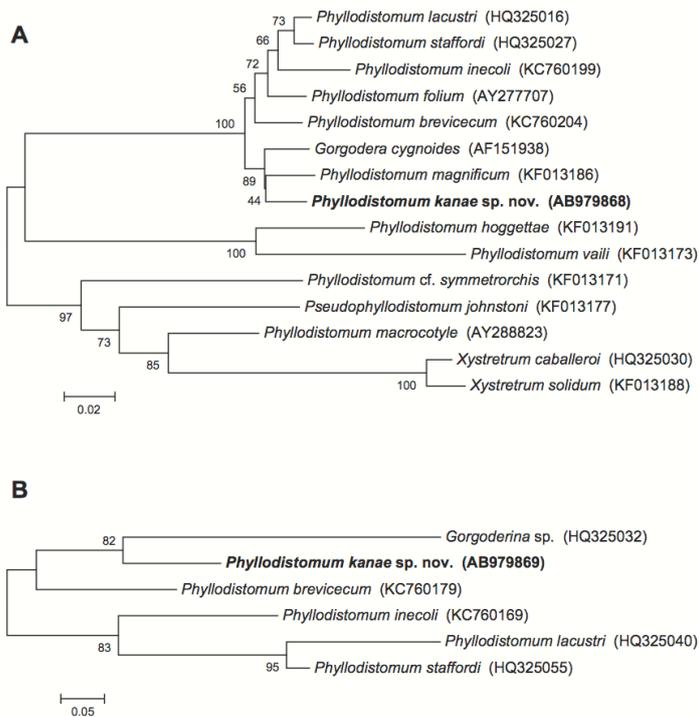


Table 1

A comparison of *Phyllodistomum* spp. from Japanese and North American urodelans, based on their original descriptions.

	<i>Phyllodistomum kanae</i> sp. nov.	<i>Phyllodistomum patellare</i> (Sturges, 1897)	<i>Phyllodistomum americanum</i> Osborn, 1903	<i>Phyllodistomum singulare</i> Lynch, 1936	<i>Phyllodistomum solidum</i> Rankin, 1937	<i>Phyllodistomum coatneyi</i> Meserve, 1943
Type locality	Hokkaido, Japan	Honshu, Japan	Minnesota	Oregon	North Carolina	Wisconsin
Type host	<i>Hynobius retardatus</i>	<i>Cynops pyrrhogaster</i> <sup>a</sup>	<i>Ambystoma maculatum</i> <sup>b</sup>	<i>Dicamptodon ensatus</i>	<i>Desmognathus fuscus</i> <sup>c</sup>	<i>Ambystoma maculatum</i>
Body length	4.0 - 4.9 mm	4.5 mm	3.5 mm	3.27 - 3.95 mm	1.82 - 2.67 mm	3.3 - 7.0 mm
Hindbody wide	2.0 - 2.1 mm	3 mm	1.4 mm	2.3 - 3.4 mm	0.76 - 1.27 mm	0.7 - 1.7 mm
Hindbody margin	Smooth	Weakly crenulate	Smooth	Crenulate	Smooth	Smooth
Ratio of sucker width <sup>d</sup>	1:1.7	1:1.2 <sup>e</sup>	1:1.5 <sup>e</sup>	1:1.2	1:1.2	1:1.4
Ovary	Spherical	Lobed	Lobed	Lobed	Smooth	Lobed
Testes	Weakly lobed	Deeply lobed	Deeply lobed	Lobed	Smooth	Deeply lobed
Vitelline glands	Ellipsoidal	Oval	Lobed	Lobed	Smooth	Deeply lobed
Uterus	Extending to hindbody margin	Extending to hindbody margin	Concentrating between ceca	Extending to hindbody margin	Extending to hindbody margin	Extending to hindbody margin

<sup>a</sup> *Cynops ensicauda* is another definitive host [6].

<sup>b</sup> *Ambystoma tigrinum* is another definitive host [21], and *Amblystoma punctatum* used in the original description is a synonym for *Ambystoma maculatum*.

<sup>c</sup> *Desmognathus monticola*, *Desmognathus ochrophaeus*, and *Desmognathus quadramaculatus* are other definitive hosts [25]

<sup>d</sup> The ratio of oral sucker to ventral sucker was calculated. Medians or means of the widths were used for the calculation.

<sup>e</sup> The values of *P. patellare* and *P. americanum* were cited from other sources [6, 22, 27].