Black-spotted pond frog *Pelophylax nigromaculatus* as a new host for the renal coccidian genus *Hyaloklossia* (Alveolata: Apicomplexa)

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**ABSTRACT**

*Hyaloklossia* Labbé, 1896 (Apicomplexa: Sarcocystidae) is a renal coccidium that infects anuran species. The genus consists of two species: *H. lieberkuehni*, recorded from *Pelophylax kl. esculentus*, *Pelophylax ribibundus*, and *Rana temporaria* in Europe; and *H. kasumiensis*, recorded from *Pelophylax porosus* in Japan. However, there have been no reports of *Hyaloklossia* in the other anurans in Japan. On June 2021, we examined a total of 58 adult frogs comprising 2 *P. p. porosus*, 23 *Pelophylax nigromaculatus*, 8 *Rana japonica*, 3 *Glandirana rugosa* (Ranidae), 13 *Fejervarya kawamurai* (Dicroglossidae), and 9 *Buergiera buergeri* (Racophorididae) for infection by *Hyaloklossia*. Microscopic examination of kidney tissues revealed a high infection incidence of 47.8% (11/23) in *P. nigromaculatus*, but the other frog species were negative for *Hyaloklossia*. Morphological and molecular analyses using nuclear ribosomal and mitochondrial genes confirmed the infective species as *H. kasumiensis.* This is a new host record for *H. kasumiensis.*

1. Introduction

The coccidian *Hyaloklossia* Labbé, 1896 comprises species that parasitize anurans. For over 120 years, the genus was considered monotypic: containing only a single species, *H. lieberkuehni* (Labbé, 1894), reported from *Pelophylax* spp. (Anura: Ranidae) in Europe (Modrý et al., 2001; Duszynski et al., 2007; Duszynski, 2021). Recently, a second species, *Hyaloklossia kasumiensis* Tokiwa, Chou, Tochigi, Katayama et Duszynski, 2021, was described from Japan in East Asia (Tokiwa et al., 2021). Genetically, *Hyaloklossia* belongs to the family Sarcocystidae and forms a sister group to other coccidia of medical and veterinary importance: *Toxoplasma* and *Neospora* in subfamily Toxoplasmatinae, *Cystoisospora* in subfamily Cystoisosporinae (Modrý et al., 2001; Oborník et al., 2002; Slapeta et al., 2003; Chou et al., 2020, 2021; Tokiwa et al., 2021). The morphological features of disporocystic and tetrasporozoic oocysts without Stieda bodies of *Hyaloklossia* are similar to those of other related species of the family Sarcocystidae with multi-host (i.e., indirect, heteroxenous, or facultatively heteroxenous) life cycle, with carnivorous animals as the definitive hosts. However, it is believed that all *Hyaloklossia* species use only a single-host (homoxenous) life cycle, with anuran species as the definitive hosts (Laveran and Mesnil, 1902; Duszynski et al., 2007). *Hyaloklossia* is thus a unique taxon with interesting aspects for studying the phylogenetics of Sarcocystidae; however, they are largely documented to be present in *Pelophylax* species in Europe, and have not been studied in endemic anurans other than *P. p. porosus* in Japan (Tokawa et al., 2021). In this study, coccidian parasites in the kidney tissues of anuran species in Japan were investigated to obtain basic knowledge about *Hyaloklossia* infection. Namely, this study aimed to investigate infection status and infection rate in six anuran species in Japan and examine *Hyaloklossia* lineages using molecular analyses.

2. Materials and methods

2.1. Sample collection and analyses

A total of 58 frogs were used in this study. They consisted of 23 black-spotted pond frogs, *Pelophylax nigromaculatus* (Hallowell, 1861); two Tokyo Daruma pond frogs, *P. porosus* (Cope, 1868); three wrinkled frogs, *Glandirana rugosa* (Temminck et Schlegel, 1838); and eight Japanese brown frogs, *Rana japonica* Boulenger, 1879 of the family Ranidae; 13 Japanese rice frogs, *Fejervarya kawamurai* (Djoung et al., 2011) of the family Dicroglossidae; and nine Kajika frogs, *Buergiera buergeri* (Temminck et Schlegel, 1838) of the family Racophorididae. All
frogs were collected on June 2021. The details of the frogs used in this study are listed in Table 1. Captured frogs were placed in laundry net bags and transported to Nippon Veterinary and Life Science University under refrigeration. All frogs were anesthetized by direct immersion in 0.3% isoflurane solution and then euthanized by cervical disruption. After measuring the length and weight of the frogs, their kidney tissue was collected and examined. All procedures were approved by the Ethical Committee for the Care and Use of Laboratory Animals at Nippon Veterinary and Life Science University (No. 2021S-45).

### 2.2. Morphological examination

Approximately half of each kidney tissue sample was homogenized using a BioMasher (Nippon, Japan), and the other half was dissected under an SZX61 stereomicroscope (Olympus, Japan). The right kidney was preserved in 10% neutral buffered formalin or 2% potassium dichromate solution. These samples were examined for the presence of sporocysts and oocysts by a BX53 optical microscope (Olympus). Photomicrographs were captured using a DP27 photomicroscope (Olympus). For measurements, ImageJ2 software (Rueden et al., 2017) was used to analyze prerecorded images captured at 1000 × magnification. The size values are reported in micrometers and are given as a range followed by the mean and standard deviation in parentheses.

Abbreviations used throughout this article are standardized (Wilber et al., 1998; Tokiwa et al., 2021); Oocyst characteristics: length (L), width (W), their ratio (L/W), microple (M), microple cap (MC), and oocyst residuum (OR); sporocyst characteristics: length (L), width (W), their ratio (L/W), Stieda body (SB), substieda body (SSB), sporocyst residuum (SR), and sporozoites (SZ).

### 2.3. DNA extraction, polymerase chain reaction (PCR), and sequencing

Genomic DNA was extracted from all sporocyst-positive samples (n = 11). A sporocyst mass collected in the kidney tissue was collected using a glass Pasteur pipette under an SZX16 stereomicroscope (Olympus) and used for DNA extraction. Genomic DNA was sequenced using the Qiagen PowerSoil DNA Isolation Kit (Qiagen, Germany) according to the manufacturer’s protocol with a 400 bp insert size. The PCR products were separated using 1.5% agarose gel electrophoresis and visualized under an LE transilluminator after staining with 0.5μg/mL of GelGreen (Bio Craft, Japan). The size of the PCR products were estimated by comparison with a 100 bp DNA plus DNA ladder (Maestrogen, Taiwan). The PCR products were sequenced by Macrogen Corp., Japan using ABI 3730xl DNA Analyzer (Thermo Fisher Scientific, USA) and the same PCR primers.

### 2.4. Genetic analysis

The obtained 18S and cox1 sequences were separately aligned using MAFFT with Q-INS-I (Katoh and Standley, 2013). Sequence similarity was analyzed using the BLASTn program available on the website of the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic trees were constructed using MEGA 11 software (Tamura et al., 2021) with cox1 sequence data of sarcocystids, obtained from the International Nucleotide Sequence Databases (INSDB; GenBank/DDBJ/EMBL). Sarcocystis rileyi (accession no. KT184389) was used as an outgroup for rooting the resulting trees. Phylogenetic trees were reconstructed using the neighbor-joining (NJ) and maximum likelihood (ML) methods. The best-fit DNA evolution model was estimated for each dataset individually using the Akaike information criterion and determined to be the Tamura-Nei model with gamma distribution. The bootstrap values for NJ and ML were obtained from 1000 to 1000 replicates, respectively.

### 3. Results and discussions

During our survey of renal coccidia in frogs, Hyaloklossia associated with two different stages of development were found in 11 of 14

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**Table 1**

| Hosts | Localities | City, Prefecture | Habitat | Latitude and longitude | No. of examined | Body size (Length (cm) and Weight (g)) |
|-------|------------|-----------------|---------|------------------------|----------------|--------------------------------------|
| Ranidae | Pelophylax nigromaculatus | Takahama, Shiga | Paddy field | 35°22′ N, 135°53′ E | 14 | 3.7–5.8 (4.7 ± 0.5) | 5.0–20.0 (10.7 ± 4.1) |
| | | | | | | 4.2–7.0 (5.7 ± 0.8) | 9.3–34.4 (17.2 ± 8.0) |
| | Pelophylax porosus porosus | Tadami, Fukushima | Paddy field | 37°21′ N, 139°18′ E | 9 | 5.0–5.9 (5.5) | 10.9–13.8 (12.4) |
| | Glandirana reconvexa | Takahama, Shiga | Paddy field | 35°22′ N, 135°53′ E | 3 | 4.5–5.8 (5.2 ± 0.5) | 14.0–20.0 (17.3 ± 2.5) |
| | Rana japonica | Mobara, Chiba | Paddy field | 35°23′ N, 139°18′ E | 8 | 2.5–3.4 (3.0 ± 0.4) | 3.0–5.0 (4.1 ± 0.9) |
| Dicroglossidae | Fejervarya kawamurai | Morigaya, Shiga | Paddy field | 35°6′ N, 135°58′ E | 7 | 3.5–4.1 (3.8 ± 0.2) | 3.6–10.0 (5.9 ± 2.3) |
| | Rhacophoridae | Takahama, Shiga | River | 35°23′ N, 135°51′ E | 9 | 3.8–4.5 (4.1 ± 0.2) | 4.0–5.0 (4.6 ± 0.5) |

**Table 2**

| Primers | Gene | Direction | Sequence (5′–3′) | References |
|---------|------|-----------|-----------------|------------|
| EF      | 18S  | Forward   | GAAGCTGGATGCTGCTATT | Kivicova et al. (2008) |
| FR      | Reverse | CTTGGCCTACTAGGCATTC | Kivicova et al. (2008) |
| Sdae_COX1_260F | cox1 | Forward | GATCTTTATGGTATATGACCC | Ogedengbe et al. (2016) |
| IRS     | Reverse | TAGTGTATCATGTAAGCGATATCCG | Gerbe (2013) |
*P. nigromaculatus* collected in a paddy in Shiga Prefecture, Japan (Table 3). Nine *P. nigromaculatus* from Fukushima and the other frog species were negative for *Hyaloklossia* infection.

The morphological characteristics of *Hyaloklossia* found in *P. nigromaculatus* were as follows. Immature oocysts (Fig. 1A–C) were bean-shaped or elongated-ovoidal, and measured L × W (n = 20): 32.3–41.5 (39.3 ± 2.9) × 18.6–23.4 (21.3 ± 1.6); L/W ratio: 1.6–2.0 (1.9 ± 0.1). Their walls were very thin and transparent. The sporont (Fig. 1A) was spheroidal with a granular cytoplasm and measured 15.9–27.8 (24.8 ± 3.3) × 15.6–19.8 (17.9 ± 1.7). Sporoblasts (Fig. 1B and C) were elongated and showed primordia of SR and SZ, but walls were very thin. The mature oocysts (Fig. 1D) were elongate and dedicate, susceptible to deformation, and measured L × W (n = 11): 35.9–42.3 (37.9 ± 1.7) × 18.6–22.5 (21.9 ± 1.3). They contained two sporocysts each. Sporocysts (Fig. 1C and D) were broadly spindle-shaped with a smooth, single layered wall and measured L × W (n = 30): 24.2–28.0 (26.2 ± 1.5) × 13.2–16.1 (14.5 ± 1.0); L/W ratio: 1.6–2.0 (1.8 ± 0.1). SB, SSB, and PSB were all absent. Sporocysts had four SZ each and an SR with a round, granular body and measured, L × W (n = 10): 6.8–10.2 (8.4 ± 0.9) × 6.5–8.5 (7.6 ± 0.7). Based on the size of its sporocyst and SR the present species was identified as *H. kasumiensis*. The original description of *H. kasumiensis* oocysts was based on a small number obtained from tissue sections of the *P. p. porosus* kidney (Tokiwa et al., 2021), and thus, the measurements were not accurate. This is the first detailed description of both the mature and immature oocysts of *H. kasumiensis*. However, the oocysts were highly polymorphic, and it was difficult to differentiate them from those of *H. lieberkuehni* reported from *P. kl. esculentus* (Laveran and Mesnil, 1902; Kazubski and Grabda-Kazubska, 1973). Furthermore, fresh kidney samples should be used for morphological observation of sporocysts; preservation in 10% formalin fixed or potassium dichromate is not recommended, as it might cause sample denaturation.

The species-level identification of *Hyaloklossia* was strongly supported by genetic analysis of its 18S sequence. Partial fragments of 18S sequences (1472 bp) of eight of the 11 samples were successfully amplified and sequenced, with 100% identity to each other. A representative sequence was deposited in the DNA Data Bank of Japan (DDBJ) under the accession no. LC669718. When compared with available sequences in INSD, the 18S sequence of *Hyaloklossia* from

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### Table 3

Summary of the 11 *Hyaloklossia*-positive adult *Pelophylax nigromaculatus* from Shiga, Japan.

| Host ID | Sex | Length (cm) | Weight (g) | Hyaloklossia Stage |
|---------|-----|-------------|------------|-------------------|
| PEL24   | Male | 4.9         | 11         | Sporocyst         |
| PEL26   | Male | 4.5         | 12         | Sporocyst         |
| PEL27   | Male | 4.9         | 10         | Sporocyst         |
| PEL28   | Male | 4.6         | 15         | Sporocyst, oocyst |
| PEL29   | Male | 4.3         | 8          | Sporocyst, oocyst |
| PEL30   | Female | 4.1    | 9          | Sporocyst         |
| PEL31   | Female | 4.3     | 6          | Sporocyst         |
| PEL32   | Female | 3.7     | 6          | Sporocyst         |
| PEL33   | Female | 5.0     | 5          | Sporocyst         |
| PEL34   | Male  | 4.9         | 10         | Sporocyst         |
| PEL36   | Male  | 4.3         | 8          | Sporocyst         |

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Fig. 1. Light microscopy of *Hyaloklossia* oocysts in the kidney of *Pelophylax nigromaculatus*. A. Immature oocyst showing the sporont with granular cytoplasm that does not fill the space inside the oocyst completely. B. An immature oocyst showing the sporoblast with very thin wall. C. An immature oocyst (left) and mature sporocyst (right). D. A mature oocyst with two sporocysts. Arrowhead and arrows indicate oocyst wall and sporocyst residuum, respectively. Scale bar = 5 μm.
Table 4
Nucleotide differences of cox1 sequences (872-bp) of Hyaloklossia kasumiensis from and Pelophylax nigromaculatus and Pelophylax porosus porosus.

| Haplotypes     | Host (sample ID) | Accession no. | Sequence positions |
|----------------|------------------|---------------|--------------------|
| Hk1            | Pp (P01)         | LC602189      | 232 233 375 548    |
| Hk2            | Pr (PEL24, PEL26, PEL30, PEL52) | LC669719 | T T A A     |
| Hk3            | Pr (PEL28, PEL29, PEL34) | LC669720 | G A C C     |
| Mixed          | Pr (PEL27)       | –             | G A A/ A/ C C     |

Pp: P. p. porosus, Pr: P. nigromaculatus.

P. nigromaculatus in this study matched with 100% identity (1449/1449 bp) with H. kasumiensis (accession no. LC602188) reported from P. p. porosus in Ibaraki, Japan. Furthermore, the sequence identity of H. lieberkuehni (accession no. AF298623) from P. kl. esculentus from Czech Republic was 99.73% (1468/1472) when compared to the sequence data obtain in the current study. The homology compared to coccidia of the other members of Sarcocystidae was less than 97.56%.

The cox1 (872 bp) sequences were obtained from the same eight samples. One sample (PEL27) was suspected to be simultaneously infected by more than one lineage of Hyaloklossia based on superimposed double nucleotide peaks on the sequence electrophograms (Supplemental Fig. 1). Multiple alignment of the other seven sequences of Hyaloklossia from P. nigromaculatus and the reference sequence of H. kasumiensis (accession no. LC602189) from P. p. porosus revealed that the GC content ranged from 37.4% to 37.7%, and 868 bp (99.5%) of these sequences were conserved, and the four sites (0.08%) were polymorphic, generating three different haplotypes. These haplotypes were designated as Hk1, Hk2, and Hk3, respectively (Table 4). Hk1 was designed for H. kasumiensis from and

dornia

H. kasumiensis

Fig. 2. -

H. kasumiensis

(Hk3). Phylogenetic relationships of the three P. nigromaculatus, PEL32) and three (PEL28, PEL29, and PEL34) samples of Japan. Hk2 and Hk3 were identified in four (PEL24, PEL26, PEL30, and

donor as Hk1, Hk2, and Hk3, respectively (Table 4). Hk1 was

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H. kasumiensis

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H. kasumiensis is highly adapted to *Pelophylax*. *Hyaloklossia*-like organisms have been reported in the kidneys of the Northern leopard frog *Lithobates pipiens* (Ranidae) in the USA and yellow-bellied toad *Bombina variegata* (Bombinatoridae) in Bulgaria (*Golemsky and Miceva*, 1975; *Levine and Nye*, 1977). This implies the existence of undescribed *Hyaloklossia* species in other frog taxa, and it is necessary to continuously survey anurans in the field and clarify host specificity through infection experiments.

Two *Pelophylax* species are found in Japan: *P. nigromaculatus* and *P. porosus*. The former is widespread in East Asia, including most of Japan, and the latter is endemic to Japan and comprises two subspecies: *P. p. porosus* in Ibaraki and HK2 and HK3 from *P. nigromaculatus* in Shiga. To clarify whether this diversity is due to host species or geographical factors, continuous investigation of the infection status and molecular characteristics of *H. kasumiensis* in *P. nigromaculatus* in East Asia and of *P. p. porosus* in western Japan is needed.

In conclusion, we reported a second definitive host for *H. kasumiensis*: the black-spotted pond frog *P. nigromaculatus*. Understanding the host-parasite relationships of *Hyaloklossia* species may be key to unraveling the phylogeny of Sarcocystidae.

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This work was supported by Japan Society for the Promotion of Science KAKENHI grant number 21K06323 (TT), the internal research grant of the National Museum of Nature and Science, Tokyo (NY), and the latter is endemic to Japan and comprises two subspecies: *P. nigromaculatus* in Ibaraki and HK2 and HK3 from *P. nigromaculatus* in Shiga. To clarify whether this diversity is due to host species or geographical factors, continuous investigation of the infection status and molecular characteristics of *H. kasumiensis* in *P. nigromaculatus* in East Asia and of *P. p. porosus* in western Japan is needed.

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**Declaration of competing interest**

No conflict of interest declared by any author.

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