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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 ridibundus,* and *Rana temporaria* in Europe; and *H. kasumiensis*, recorded from *Pelophylax porosus 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 *Buergeria buergeri* (Rhacophoridae) 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 parasitizes 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; Šlapeta 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 heteroxonous) 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 (Tokiwa 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 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* (Djong et al., 2011) of the family Dicroglossidae; and nine Kajika frogs, *Buergeria buergeri* (Temminck et Schlegel, 1838) of the family Rhacophoridae. All

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Table 1
Details of the six anuran species used in this study.

Hosts	Localities			No. of examined	Body size	
	City, Prefecture	Habitat	Latitude and longitude		Length (cm)	Weight (g)
Ranidae						
Pelophylax nigromaculatus	Takashima, 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)
	Tadami, Fukushima	Paddy field	37°21′ N, 139°18′ E	9	4.2–7.0 (5.7 ± 0.8)	9.3–34.4 (17.2 ± 8.0)
Pelophylax porosus porosus	Kamisu, Ibaraki	Paddy field	35°51′ N, 140°40′ E	2	5.0–5.9 (5.5)	10.9–13.8 (12.4)
Glandirana rugosa	Takashima, 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°28′ N, 140°18′ E	8	2.5–3.4 (3.0 ± 0.4)	3.0–5.0 (4.1 ± 0.9)
Dicroglossidae						
Fejervarya kawamurai	Moriyama, 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)
	Kamisu, Ibaraki	Paddy field	35°51′ N, 140°40′ E	6	3.5–5.0 (4.2 ± 0.6)	5.4–13.8 (8.1 ± 2.8)
Rhacophoridae						
Buergeria buergeri	Takashima, 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 used in this study.

Primers	Gene	Direction	Sequence (5′–3′)	References
EF	18S	Forward	GAAACTGCGAATGGCTCATT	Kvicerová et al. (2008)
FR		Reverse	CTTGCGCCTACTAGGCATTC	Kvicerová et al. (2008)
Sdae_COX1_260F	*cox1*	Forward	GATCTTTATGTTYTTRATGCC	Ogedengbe et al. (2016)
SR5		Reverse	TAGGTATCATGTAACGCAATATCCAT	Gjerde (2013)

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 (Nippi, 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 under 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), micropyle (M), micropyle 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 detected in the kidney tissue was collected using a glass Pasteur pipette under an SZX16 stereomicroscope (Olympus) and used for DNA extraction. Genomic DNA was extracted from the mass using Qiagen Power Soil DNA Isolation Kit (Qiagen, Germany) according to the specified procedure with a prolonged vortex time of 20 min with the TissueLyser LT (Qiagen). The sample obtained was used as a PCR template. Double-distilled water was used as negative control.

Two genetic loci, nuclear small subunit ribosomal RNA (18S) and mitochondrial cytochrome *c* oxidase subunit I (*cox1*), were amplified using specific primers (Table 2). PCR was performed using 20 μL reaction volumes, each containing 0.2 μL of TaKaRa ExTaq polymerase (TaKaRa, Japan), 2 μL of 10 × buffer, 1.6 μL of dNTPs (2.5 mM each), 0.2 μL of each primer (50 μM), 1.0 μL of the template, and 14.8 μL of double-distilled water. The thermocycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 45–60 °C for 30 s, and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 10 min (for 18S) or 5 min (for *cox1*), followed by a hold step at 4 °C.

The PCR products were separated using 1.5% agarose gel electrophoresis and visualized under an LE transilluminator after staining with GR green (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.cg).

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 (INSD; 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 1500 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

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

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

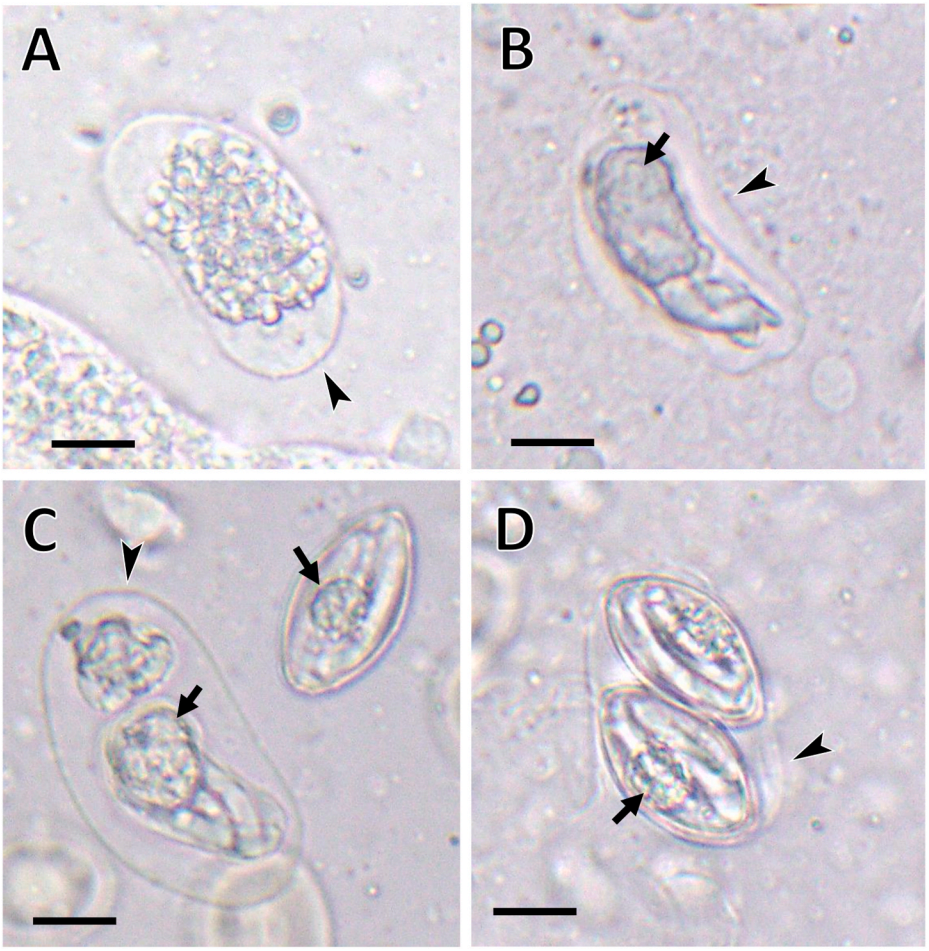

Fig. 1. Light microscopy of *Hyaloklossia* oocysts in the kidney of *Pelophylax nigromaculatus*. A. Immature oocyst showing the sporont with granular cytoplasm that does not fill the space inside the oocyst completely. B. An immature oocyst showing the sporoblast with very thin wall. C. An immature oocyst (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			
			232	233	375	548
Hk1	Ppp (P01)	LC602189	G	A	A	C
Hk2	Pn (PEL24, PEL26, PEL30, PEL32)	LC669719	T	T	A	A
Hk3	Pn (PEL28, PEL29, PEL34)	LC669720	G	A	C	C
Mixed	Pn (PEL27)	–	G	A	A/C	A/C

Ppp: *P. p. porosus,* Pn: *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 *Hyalokossia* based on superimposed double nucleotide peaks on the sequence electroprograms (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 *P. p. porosus* (type host) in Ibaraki, Japan. Hk2 and Hk3 were identified in four (PEL24, PEL26, PEL30, and PEL32) and three (PEL28, PEL29, and PEL34) samples of *P. nigromaculatus,* respectively. The *cox1* sequences were deposited in the DDBJ under the accession numbers LC669719 (Hk2) and LC669720 (Hk3). Phylogenetic relationships of the three *cox1* haplotypes of *H. kasumiensis* and related taxa are shown in Fig. 2. Topologies were consistent for both the NJ and ML trees. For brevity, we present only the NL tree and include bootstrap values for ML. Four independent clades were present in the tree, of which the *Hyaloklossia* clade became monophyletic with *Cystoisospora* + *Nephroisospora* clade; within the *Hyaloklossia* clade, Hk2 diverged first, and then Hk1 and Hk3 formed a monophyletic lineage.

Lieberkühn (1854) was first to discover coccidium in the kidney of *P.* kl. *esculentus*. Labbé (1894) later described these coccidian species reported by Lieberkühn (1854) as *Klossia lieberkühni.* However, this was soon reclassified in a new genus *Hyaloklossia* (Labbé, 1896). Several researchers have reported on the wide spread occurrence of *H. lieberkuehni* in ranids of *P.* kl. *esculentus, P. ridibundus* and *R. temporaria* in Europe (Laveran and Mesnil 1902; Nöller, 1923; Walton, 1949; Kazubski and Grabda-Kazubska 1973; Vojtková 1976; Modrý et al., 2001). Recently, *Hyaloklossia* infections were reported in *P. p. porosus*, an endemic species of Japan, and described it as *H. kasumiensis* (Tokiwa et al., 2021). *Pelophylax nigromaculatus,* which was found to be infected by *Hyaloklossia* in this study, is the second definitive host of *H. kasumiensis.* However, *P. nigromaculatus* collected in Shiga showed a high infection rate of 78.6% (11/14; 95% CI 51.7%–93.2%), whereas *Pelophylax* spp. from Fukushima ($n = 9$) and Ibaraki ($n = 2$), which are adjacent to each other and located on the east side of Japan, were negative (95% CI 0%–30.0%). The low infection rates in these areas are similar to the result of the previous study of *P. p. porosus* in Ibaraki (Tokiwa et al., 2021), the infection rate of *H. kasumiensis* was 11% (1/9; 95% CI 0%–45.7%).

The life cycle of *Hyaloklossia* is not well understood. According to Laveran and Mesnil (1902), *Hyaloklossia* sporocysts are ingested orally by omnivorous tadpoles, and sporozoites released in the intestinal tract pass through the bloodstream to invade and multiply in the lungs, kidneys, spleen, liver, and adipose tissue. Of these, merozoites that reach the kidneys undergo sporogony. However, Nöller (1913, 1923) believed that the endogenous stages reported in extraintestinal organs other than the kidneys by Laveran and Mesnil (1902) were those of *Lankesterella minima* (Apicomplexa: Eimeriorina), and those of *Hyaloklossia* are limited to the kidney. More importantly, *Hyaloklossia* has a homoxenous lifecycle and is transmitted orally to omnivorous tadpoles via their sporocysts shed in frog urine. Thus, infection is more likely to occur in water bodies such as paddy fields and water-tight ponds; so, the host habitat may have made a difference in the prevalence of *Hyaloklossia* infection.

The infectivity of *Hyaloklossia* in other frogs is not well understood. In the present study, we were unable to identify any other *Hyaloklossia*-infected individuals among *G. rugosa* (Ranidae), *F. kawamurai* (Dicroglossidae), and *B. buergeri* (Rhacophoridae). In particular, *G. rugosa* was collected from the same paddy field in Shiga where high *Hyaloklossia* infection rates were observed in *P. nigromaculatus*, indicating that

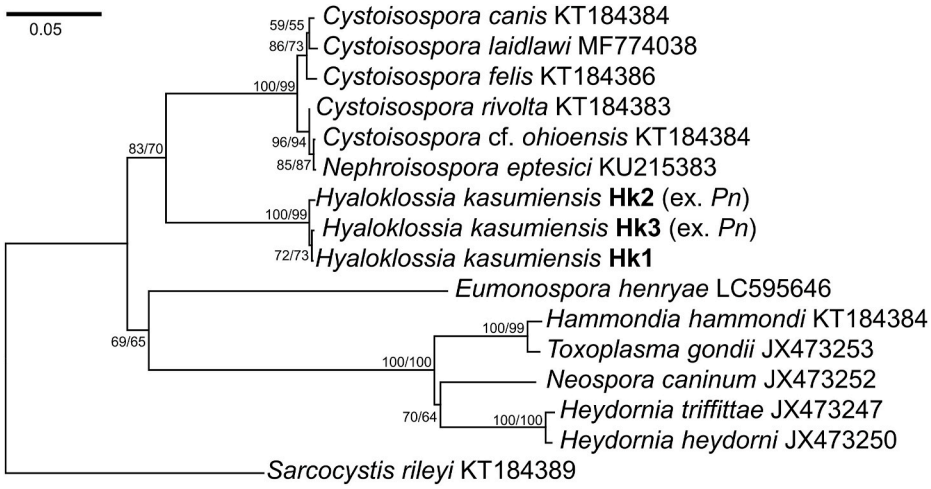

Fig. 2. Phylogenetic tree of based on *cox1* sequences of *Hyaloklossia* and related species belonging to Toxoplasmatinae (*Toxoplasma, Neospora, Hammondia, Heydornia*), Cystososporinae (*Cystoisospora*) and Eumonosporinae (*Eumonospora*) constructed using the neighbor joining method. The nodes are labeled using support from the bootstrap values obtained for the neighbor joining (left) and maximum likelihood (right) methods. *Pn*: *Pelophylax nigromaculatus.*

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 (Golemasky 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* distributed in eastern Japan and *P. p. brevipodus* distributed in western Japan (Matsui and Maeda, 2018). In the present study, in terms of intraspecific *cox1* sequence diversity in *H. kasumiensis*, haplotype Hk1 was recorded from *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. brevipodus* 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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Declaration of competing interest

No conflict of interest declared by any author.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2022.02.003.

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