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Hyaloklossia Labbé, 1896 (Alveolata: Apicomplexa) in frogs: Description of a new species and proposing a new subfamily to accommodate these enigmatic parasites

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ABSTRACT

Hyaloklossia Labbé ,1896 (Alveolata: Apicomplexa) is a monotypic genus of renal coccidia found in anurans, particularly in the edible frog *Pelophylax* kl. *esculentus* (Amphibia: Anura: Ranidae), distributed in different parts of Europe. Here we propose a new *Hyaloklossia* species from the Tokyo daruma pond frog, *Pelophylax porosus porosus*. The coccidium detected in the renal tissue of *P. p. porosus* shared some morphological characteristics with the type species, *Hyaloklossia lieberkuehni* (Labbé, 1894), reported from *P.* kl. *esculentus*. However, in addition to size differences in several oocyst and sporocyst features between these parasites, phylogenetic analysis of gene fragments from two nuclear ribosomal loci and the mitochondrial cytochrome *c* oxidase subunit 1, exposed distinct genetic differences between *H. lieberkuehni* and our new species. Although our analysis validated the monophyly of *Hyaloklossia* with some members of the Toxoplasmatinae Biocca, 1957, Cystoisosporinae Frenkel et al., 1987, and Eumonosporinae Chou et al., 2021 (Sarcocystidae Poche, 1913), comparison of genetic differences between *Hyaloklossia* species from *P. p. porosus* and *H. lieberkuehni* revealed the presence of a greater number of polymorphisms than that observed when comparing inter-species (*Heydornia* spp., *Besnoisita* spp.) or inter-genus (*Toxoplasma* vs. *Neospora, Neospora* vs. *Hammondia,* and *Neospora* vs. *Heydornia*) variabilities among members of the Sarcocystidae. This indicates that *Hyaloklossia*, as re-erected and defined by Modrý et al. (2001, Int. J. Syst. Evol. Microbiol. 51, 767–772), with its homoxenous life cycle, requires placement in its own subfamily. Thus, we propose a new subfamily, Hyaloklossiinae n. subfam., to accommodate two species, *H. lieberkuehni* from Europe and *Hyaloklossia kasumienesis* n. sp. which we describe here from *P. p. porosus* in Japan.

1. Introduction

In true numbers of species, the Apicomplexa may be the largest taxonomic group of extant organisms on Earth except, perhaps, for the Fungi (Adl et al., 2007). Many apicomplexans are pathogens to humans and their companion, domestic, wild, and other food animals and all animal species are thought to host at least one apicomplexan species and most likely have several species either unique to them or that they share with congeners and/or with sympatric species, or both. We might, therefore, expect that we know a great deal about this group vis-à-vis the species in this group, their biology and, perhaps, even have a clear understanding of their basic phylogenetic relationships (Morrison, 2009). Unfortunately, in terms of biodiversity, the Apicomplexa is probably the least studied and most poorly known group of all parasite lineages, with 6000 to 7000 named species, which is perhaps only 0.1% of the estimated total number of species (Morrison, 2009). They are all unicellular, mostly intracellular, endoparasites that are hard to find and, when found and studied, they have a limited suite of morphological, ultrastructural, cyst-forming, and life history patterns, a combination that makes them among the most difficult and undesirable organisms to work with (Morrison, 2009).

As a case in point, there are ~8120 extant species of amphibians on

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Earth (Frost, 2020), but only 45 amphibian species (0.5%) have been examined for apicomplexans and from them, 52 apicomplexan species and 38 other forms (*species incertae sedis*, *species inquirendae*, etc.) have been documented in the literature (Duszynski et al., 2007; Duszynski, 2021). Unfortunately, this demonstrates that our knowledge of apicomplexan species richness is based on very limited, non-exhaustive sampling, which does not allow estimates for species richness at any scale. Similarly, taxa used to construct phylogenetic trees need to represent adequate sampling to provide a conclusive case for determining and understanding phylogenetic relationships, but such taxon sampling for the Apicomplexa is distressing because molecular sequences and molecular data are available for only a trivial subset of known species.

Within the coccidia (Apicomplexa: Alveolata), Sarcocystidae Poche, 1913 is one of the better studied families. Currently, it includes four subfamilies: Sarcocystinae Poche, 1913 (*Sarcocystis, Flenkelia*), Toxoplasmatinae Biocca, 1957 (*Toxoplasma, Hammondia, Heydornia, Neospora*, *Besnoitia*), Cystoisosporinae Frenkel et al., 1987 (*Cystoisospora*), and Eumonosporinae Chou et al., 2021 (*Eumonospora*), along with the genera *Hyaloklossia* and *Nephroisospora* (Frenkel et al., 1979; Smith, 1981; Sercundes et al., 2016; Duszynski et al., 2018; Chou et al., 2020; Chou et al., 2021). *Hyaloklossia* is a monotypic genus that is clearly distinguishable from other genera of Sarcocystidae based on its homoxenous (direct) life cycle, gametogony and sporogony in frog renal tissues, and unusual oocyst morphology (Modrý et al., 2001; Duszynski et al., 2007; Duszynski, 2021). Based on these observations, *Hyaloklossia* is an independent lineage from other members of the Sarcocystidae (Modrý et al., 2001; Šlapeta et al., 2003).

The edible frog, *Pelophylax* kl. *esculentus* (L., 1758) (Amphibia: Anura: Ranidae), which is widespread in Europe, is the most common definitive host for *Hyaloklossia* (Laveran and Mesnil, 1902; Nöller, 1923; Kazubski and Grabda-Kazubska, 1973; Vojtková, 1976). The northern leopard frog, *Lithobates pipiens* (Schreber, 1782) (Anura: Ranidae), found in the USA, the yellow-bellied toad, *Bombina variegata* (L., 1758) (Anura: Bombinatoridae), found in Bulgaria, the European common frog, *Rana temporaria* L., 1758 (Anura: Ranidae), and the marsh frog, *Pelophylax ridibundus* (Pallas, 1771) (Anura: Ranidae), found in Europe, also have been identified as definitive hosts of *H. lieberkuehni* (Henry and Leblois, 1911; Walton, 1949; Golemansky and Miceva, 1975; Levine and Nye, 1977; Duszynski et al., 2007). Here, we describe a case of *Hyaloklossia* infection in the kidney of a Tokyo daruma pond frog, *Pelophylax porosus porosus* (Cope, 1868) (Anura: Ranidae), which has not been reported as a host in previous studies. Morphological and phylogenetic analyses were performed to compare the form we studied to *H. lieberkuehni* and these comparisons allow us to propose a new *Hyaloklossia* species. Furthermore, the taxonomic position of *Hyaloklossia* within the Sarcocystidae is reconsidered, and a new subfamily is proposed to accommodate it.

2. Materials and methods

2.1. Sample collection and examination

Nine Tokyo daruma pond frogs captured in July 2020 in Lake Kasumigaura, Ibaraki, Japan (35°58′ 43.3″ N, 140°34′49.0″ E) were examined. These frogs were sacrificed by overdosing with pentobarbital sodium and provided to us after being used in practical training for veterinary students All experiments were performed in accordance with the approved protocols (Ethical Committee for the Care and Use of Laboratory Animals at Nippon Veterinary and Life Science university: 2019-j8). All frogs examined appeared to be 3-year-old adults (6 males and 3 females), with body length 5.8–7.2 cm and weight 19–31 g.

2.2. Morphological examination

After harvesting the left kidney from each frog, half of the tissue was homogenized using BioMasher (Nippi, Japan) and the other half was dissected under observation with an Olympus SZX16 stereomicroscope (Olympus, Japan). The intestinal contents were smeared directly on to glass slides. Tissue samples from the liver, lung, spleen, right kidney, and digestive tracts were fixed in 10% neutral buffered formalin and processed into blocks by paraffinization for routine histopathological examination. Serial sections (5 μm) were stained using hematoxylin and eosin (H&E). Blood specimens collected from the heart were smeared and stained using Diff-Quick stain (Sysmex, Japan). These specimens were examined for apicomplexan parasites with an Olympus BX53 optical microscope (Olympus, Japan) using differential interference contrast. Photomicrographs were captured using a DP27 photomicroscope (Olympus) or NanoZoomer-SQ Digital slide scanner (Hamamatsu Photonics, Japan). For measurements, the ImageJ2 software (Rueden et al., 2017) was used to analyze pre-recorded images captured at 1000×. All values are reported in micrometers and given as a range followed by the mean and standard deviation in parentheses. Abbreviations used throughout are standardized (Wilber et al., 1998); oocyst characters: length (L), width (W), their ratio (L/W), micropyle (M), micropyle cap (MC), oocyst residuum (OR); sporocyst characters: length (L), width (W), their ratio (L/W), Stieda body (SB), substieda body (SSB), sporocyst residuum (SR), sporozoites (SZ), and nucleus (N) in SZ.

2.3. DNA extraction, PCR amplification, and sequencing

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

Three genetic loci, nuclear small subunit ribosomal RNA (18S), nuclear large subunit ribosomal RNA (28S), and mitochondrial cytochrome *c* oxidase subunit I (*cox1*), were amplified using specific primers and amplification conditions (Table 1). 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 30–40 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 28S and *cox1*), followed by a hold step at 4 °C.

The PCR products were separated by electrophoresis in 1.5% agarose gels and visualized under an LED transilluminator after staining with GRGreen (Bio Craft, Japan). The size of the PCR products was estimated by comparison with a 100-bp DNA plus DNA ladder (Maestrogen, Taiwan). The PCR products were purified using ExoSAP-IT (Applied Biosystems, USA) and dispatched to a sequencing service provider (Macrogen Corp., Japan) and analyzed using an ABI 3730xl DNA Analyzer (Thermo Fisher Scientific, USA) with the same PCR primers.

2.4. Genetic analysis

Sequence similarity was studied separately using the BLASTN program available in the website of the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/Blast.cg). To determine whether the genetic variation detected between *Hyaloklossia* species from *P. p. porosus* and *H. lieberkuehni* corresponded to intraspecific and/or interspecific variation, the 18S, 28S, and *cox1* sequences of closely related or sister taxa were obtained from the International Nucleotide Sequence Databases (INSD) (Table 2), and divergence was analyzed using the MEGA X software (Kumar et al., 2018). The sequences were aligned by using MAFFT with Q–INS–I (Katoh and

Table 1
Primers and PCR conditions used in this study.

Primer	Gene	Direction	Sequence (5′ to 3′)	Cycles	Annealing	References
EIF1	18S	Forward	GCTTGTCTCAAAGATTAAGCC	30	60 °C	Power et al. (2009)
EIR3		Reverse	ATGCATACTCAAAGATTACC			Power et al. (2009)
KL1	28S	Forward	TACCCGCTGAACTTAAGC	35	45 °C	Schrenzel et al. (2005)
KL3		Reverse	CMACCAAGATCTGCACTAG			Schrenzel et al. (2005)
Sdae_COX1_260F	*cox1*	Forward	GATCTTTATGTTYTTRATGCC	40	50 °C	Ogedengbe et al. (2016)
Sdae_COX1_1147R		Reverse	CATTACCCATAACYACACC			Ogedengbe et al. (2016)

Table 2
Species and sequence used for comparing 18S and 28S distances.

Subfamily	Genus	Species	Accession nos.	
			18S	28S
Toxoplasmatinae	*Toxoplasma*	*T. gondii*	L24381	L25635
	Hammondia	*H. hammondi*	AH008381	AF101077
	Heydornia	*H. heydorni*	KT184370	AF159240
		H. triffitae	GQ984223	KJ396594
	Neospora	*N. caninum*	AJ271354	AF001946
	Besnoitia	*B. besnoiti*	AF109678	AF076866
		B. bennetti	AY665399	AY778965
		B. tarandi	MH217579	AY616164
		B. darlingi	GU479631	–
		B. jellisoni	AF291426	AF076868
Cystoisosporinae	*Cystoisospora*	*C. canis*	KT184368	–
		C. belli	DQ060683	–
		C. ohioensis	GU292305	–
		C. felis	KT184364	U85705
		C. suis	KF854252	–
Eumonosporinae	*Eumonospora*	*E. henryae*	LC595644	LC595645
		E. neofalconis	KT037081	–
		E. danceloe	KJ634019	–
Incertae subfamiliae	*Hyaloklossia*	*H. lieberkuehni*	AF298623	AF513499

Standley, 2013), with application of the same algorithm in all cases. The gaps and missing data were eliminated. The percentage of sequence difference was computed using uncorrected pairwise genetic distance (*p*-distance) as the proportion of nucleotide sites at which the two sequences differed. The average distance was also calculated to compare genetic distance at subfamily-level.

Phylogenetic trees were constructed by the neighbor joining (NJ) and maximum likelihood (ML) methods using MEGA X with 18S, 28S, and *cox1* sequence data of sarcocystids. The best-fit DNA evolution models were 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 (NJB) and ML (MLB) were obtained from 1500 (for NJ) and 1000 (for ML) replicates, respectively.

3. Results

3.1. Description of *Hyaloklossia kasumiensis n. sp*

3.1.1. Type host
Amphibia (Anura: Ranidae), *Pelophylax porosus porosus* (Cope, 1886), Tokyo daruma pond frog.

3.1.2. Type locality
Japan: Ibaraki, Lake Kasumigaura (35°58′43.3″N, 140°34′49.0″ E).

3.1.3. Other hosts
None to date.

3.1.4. Geographic distribution
Known only from the type locality.

3.1.5. Unsporulated and sporulated oocysts
Immature oocysts (Fig. 3B–D) were elongated-ovoidal or bean-shaped; in tissue sections they measured L × W (n = 3): 25–30 × 15–25. The oocyst wall was thin, single-layered; M, MC, OR: all absent. Prior to sporulation, the sporont was spheroidal with granular cytoplasm and an elongate N that was usually at the margin (Fig. 3B & C) and one of them showed cytoplasmic cleavage (Fig. 3C). Two sporoblasts formed within the oocyst and were spheroidal with granular cytoplasm and an elongate nucleus that was usually at its margin (Fig. 3D).

3.1.6. Sporocysts and sporozoites
Sporocysts (Fig. 4A–C) were broadly spindle-sharped with a smooth, single-layered wall, and measured L × W (n = 20): 24.6–29.1 (27.1 ± 1.4) × 14.0–16.6 (15.5 ± 0.8); L/W ratio: 1.5–2.0 (1.8 ± 0.1); SB, SSB, PSB: all absent; sporocyst with four SZ that measured L × W (n = 34): 16.4–23.3 (20.7 ± 2.0) × 3.3–4.9 (4.1 ± 0.4); L/W ratio: 3.9–6.8 (5.1 ± 0.8); each SZ had a rounded end enclosing the N and a tapered end; SR: present as a round, granular body and measured, L × W (n = 38): 7–10 (8.4 ± 1.1) × 6–8 (7.3 ± 0.6).

3.1.7. Prevalence
This species was recovered from one of nine (11%) specimens of the type species; the type host was an adult female (body length 6.0 cm, weight 20.0 g).

3.1.8. Sporulation
Endogenous.

3.1.9. Prepatent and patent periods
Unknown.

3.1.10. Site of infection
Mature sporocysts (Fig. 1) were found in the kidney. Most of the sporocysts formed agglomerates in the interstitium (Fig. 1A and B, 2, 3A), and some were detected within renal endothelial cells (Figs. 1C and 2). A few immature and mature oocysts were observed in the kidney tissues (Figs. 1D, 3B-E). No coccidial stages were detected in the other tissues examined (or in specimens from other frogs).

3.1.11. Cross-transmission
None to date.

3.1.12. Material deposited
A holotype slide (kidney tissue, H&E) with oocysts and sporocysts from one *P. p. porosus* were deposited in the Meguro Parasitological Museum, Meguro, Tokyo, Japan, accession number, MPM Col. No. 21755. Representative sequences were deposited in the DNA Data Bank of Japan, accession numbers LC602188 (18S), LC602187 (28S) and LC602189 (*cox 1*).

3.1.13. Zoobank registration number
urn:lsid:zoobank.org:pub:E2BC99ED-CEA2-4EA5-914F-2E79A5C265A2.

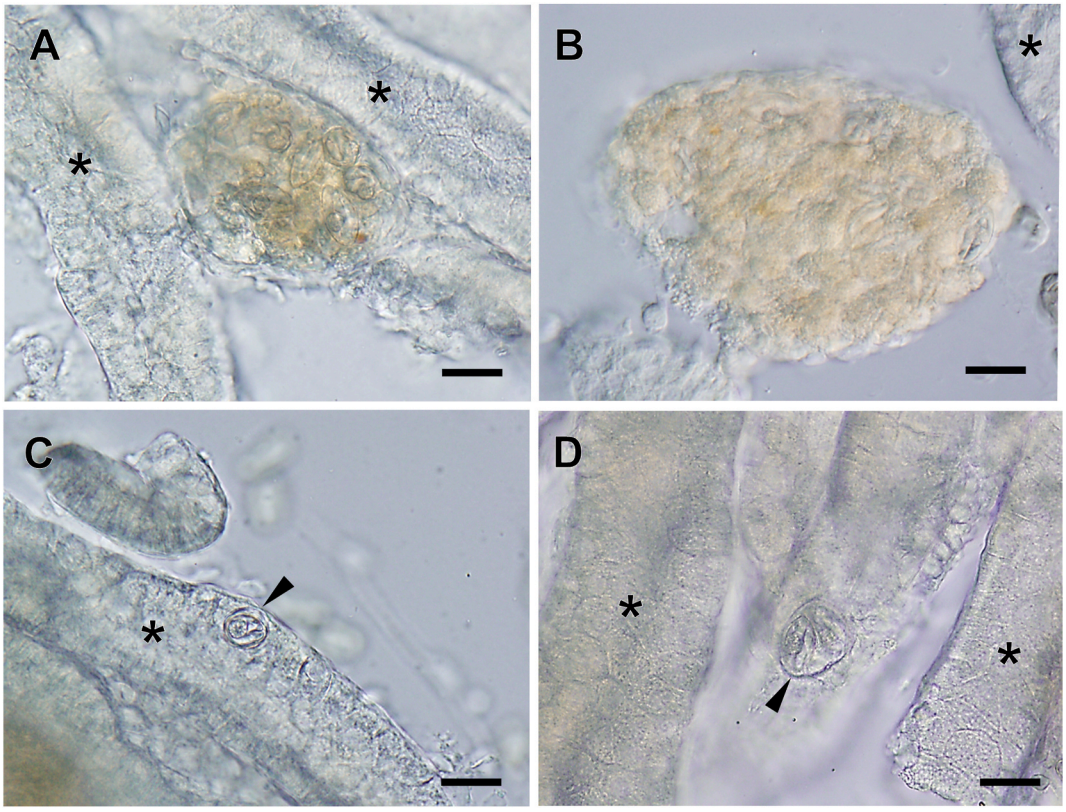

Fig. 1. Light microscopy of *Hyaloklossia* sporocysts in the kidney of *Pelophylax porosus porosus*. (A) Mature sporocysts within a cyst-like structure are visible in the renal interstitium. (B) Squash preparation of kidney showing numerous immature sporocysts. Note the granular cytoplasm of the sporoblasts (sporonts) and the barely visible membrane surrounding them. (C) A sporocyst (arrowhead) in the renal endothelial cell. (D) Mature oocyst (arrowhead) with two sporocysts in the renal endothelial cell. Asterisk indicates renal tubules. Bars = 25 μm.

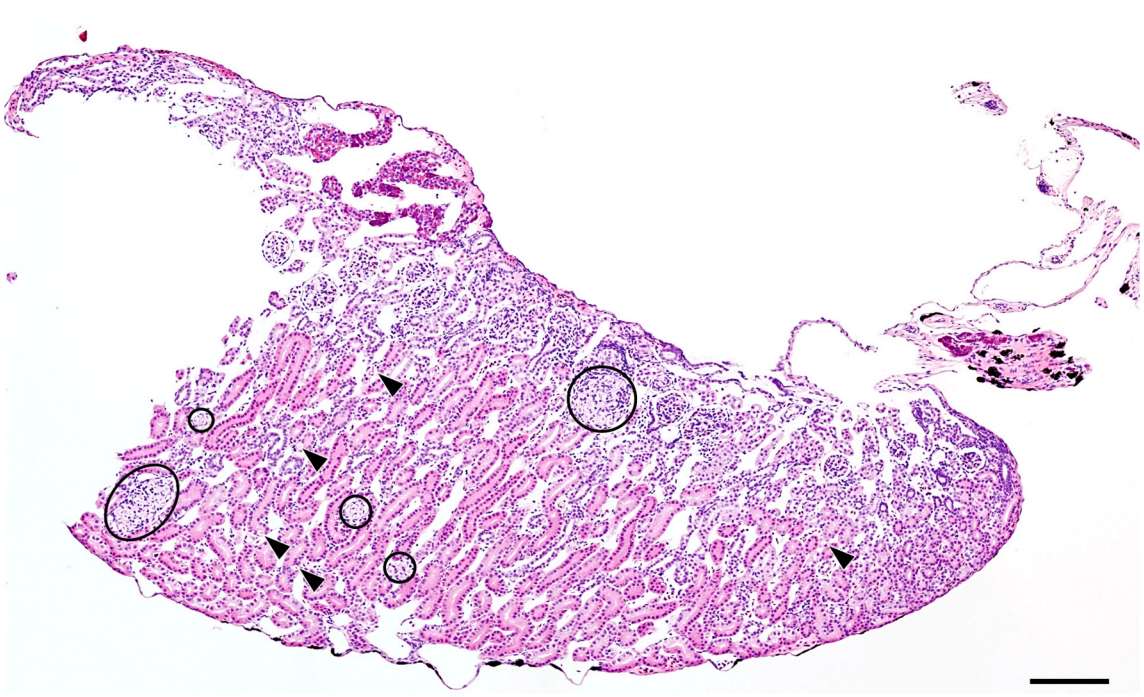

Fig. 2. Light microscopy of hematoxylin and eosin-stained sections of renal tissue of *Pelophylax porosus porosus*. *Hyaloklossia* sporocysts and/or oocysts congregated in a diffused manner in the renal interstitium (circles), and some were found solitarily in renal epithelial cells or in the lumen (arrowheads). Bar = 200 μm. A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM06312.

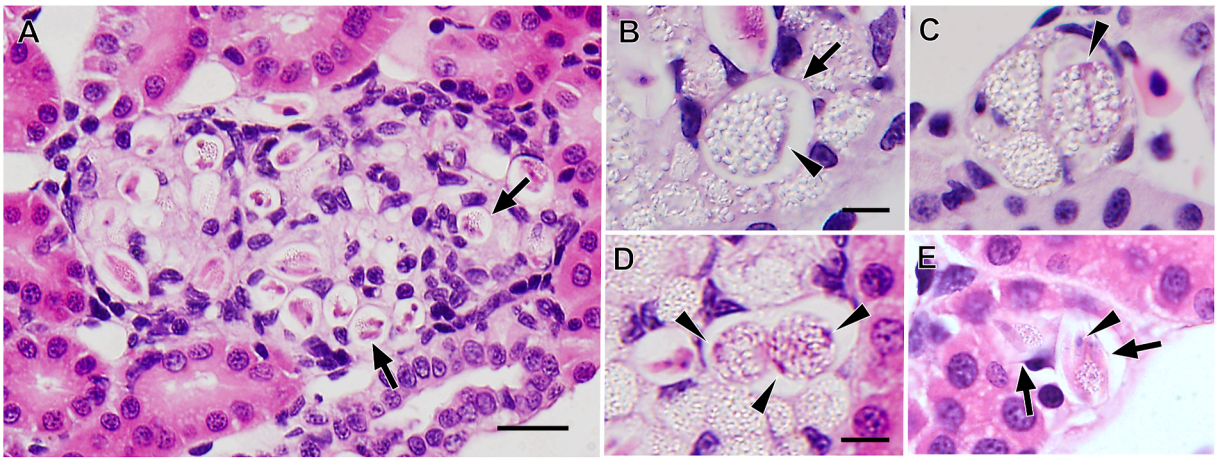

Fig. 3. Light microscopy of hematoxylin and eosin-stained sections of renal tissues of *Pelophylax porosus porosus.* (A) Mature sporocysts in the renal interstitium. Arrows indicate the transverse section of sporocysts showing four sporozoites with nuclei and a granular sporocyst residuum. (B and C) Immature oocysts. Note the very thin oocyst wall (arrows), sporonts with granular cytoplasm, and nuclei distributed at the cell margin (arrowheads). (D) Immature oocysts with two sporoblasts each and containing two polar nuclei (arrowheads). (E) Mature oocysts in renal epithelial cell showing two sporocysts with elongated sporozoites with circular nuclei (arrowhead) and a granular sporocyst residuum. Arrows indicate the sporocyst wall. Bars = 50 μm (A) and 10 μm (B–E).

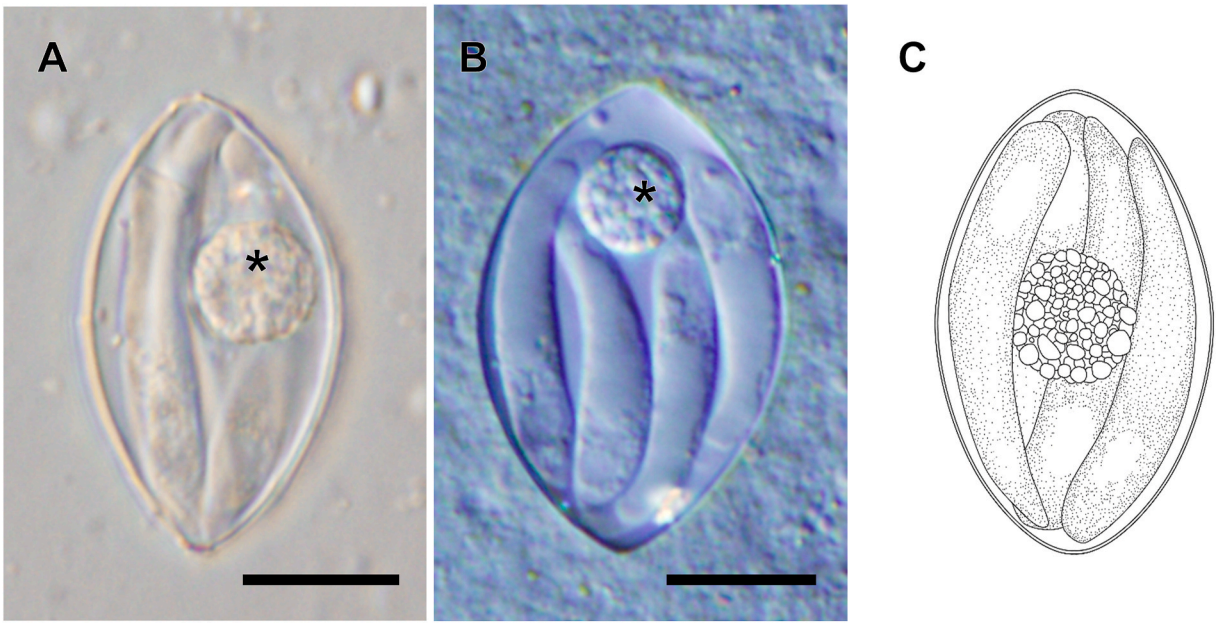

Fig. 4. Mature sporocysts of *Hyaloklossia* in the kidney of *Pelophylax porosus porosus.* (A) Light microscopy of a mature sporocyst in homogenized kidney tissue. (B) Nomarski interference contrast microscopy of a mature sporocyst in squash preparation of renal tubular tissue showing the presence of four spindle-shaped sporozoites. (C) Composite line drawing. Bar = 10 μm. Asterisk: sporocyst residuum.

3.1.14. Etymology

Derived from the ancient name, "kasumi-no-ura" of Lake Kasumigaura and *-ensis* (L., belonging to, from) to reflect where the type host was collected.

3.1.15. Remarks

The morphological features of oocysts and sporocysts are of importance as taxonomic keys that influence the description of coccidian species (Tenter et al., 2002; Berto et al., 2014). Here we compares the morphological characteristics of our species alongside the available data of its closest relative, *H. lieberkuehni*, from *P.* kl. *esculentus,* in France (Labbé, 1894, 1896; Laveran and Mesnil, 1902), Czechoslovakia (Vojtková, 1976), Poland (Kazubski and Grabda-Kazubska, 1973), the Czech Republic (Modrý et al., 2001), and from *B. variegata,* in Bulgaria (Golemansky and Miceva, 1975) (Table 3). Levine and Nye (1977) also reported *H. lieberkuehni* (which they called *Isospora lieberkuehni*) from *L. pipiens,* in the USA, but they only found "merozoites" in the cytoplasm of kidney tubule epithelial cells, so their data are not included in Table 3. When we look at all structural dimensions available (Table 3) we see that our form has slightly smaller oocysts, in length but not width, than the oocysts described from all *P.* kl. *esculentus* from Europe. We also note that the *Hyaloklossia* species detected in *B. variegata* might constitute a third *Hyaloklossia* species owing to the significant differences in the dimensions of its oocysts (20.5 × 15.0) and sporocysts (10.5 × 9.0) *vs.* those of *H. lieberkuehni* in the Czech Republic (35–45 × 20–25 and 25–30 × 14–16, respectively); confirmation of the other stages is necessary to confirm correct identification and gene sequencing would also help resolve this issue. The oocysts of *Hyaloklossia* species are exceptionally thin, irregular, and fragile, which makes them difficult to compare. In contrast, sporocysts are resistant structures. The sporocyst

Table 3
Countries of origin, hosts, and mensural summation of morphological structures known from all reports on the characteristics of *Hyaloklossia* in their reported host species in Europe *vs.* Asia: *Bombia variegata, Pelophylax* kl. *esculentus,* and *Pelophyla porosus porosus*.

Localities	Europe					Asia
	France	Poland	Czechoslovakia	Czech Republic	Bulgaria	Japan
Host species	*P.* kl. *esculentus*	*P.* kl. *esculentus*	*P.* kl. *esculentus*	*P.* kl. *esculentus*	*B. variegata*	*P. p. porosus*
Oocyst						
L	na	35–36	37	35–45	20.5	25–30
W	na	20–22	20	20–25	15.0	15–25
Sporocyst						
L	20–22, 25–30	25–30 (27.1)	21–27 (23)	25–30	10.5	24.6–29.1 (27.1)
W	na	12.5–16 (14.8)	12–15 (13)	14–16	9	14.0–16.6 (15.5)
L/W	na	na	na	na	na	1.5–2.0 (1.8)
Sporocyst residuum						
L	na	9.5–17	12	na	na	6.6–10.2 (8.4)
W	na	7–12	10	na	na	6.2–8.4 (7.3)
Sporozoite						
L	15–20, 25	17–21	15–18 (16)	17–21	na	16.4–23.3 (20.7)
W	na	3–4	2–3 (2)	3–4	na	3.3–4.9 (4.1)
L/W	na	na	na	na	na	3.9–6.8 (5.1)
References	Labbé, 1894; Labbé, 1896; Laveran and Mesnil (1902)	Kazubski and Grabda-Kazubska, 1973	Vojtková (1976)	Modrý et al. (2001)	Golemansky and Miceva (1975)	This study

na = not available.

of *H. kasumiensis* in *P. p. porosus* was slightly wider than that of *H. lieberkuehni* reported by both Kazubski and Grabda-Kazubska (1973) and Modrý et al. (2001); thus, it has a smaller L/W ratio. Finally, the sporocyst residuum in our form is smaller than that of *H. lieberkuehni* sporocysts reported by Kazubski and Grabda-Kazubska (1973) and Vojtková (1976) (see Table 3). Based on our structural and molecular results, as well as geographical isolation, we consider the coccidian described in *P. p. porosus* as new to science.

3.2. Sequence analyses of Hyaloklossia spp

3.2.1. Sequence similarity

The partial 18S gene sequence (1449 bp) of *H. kasumiensis* was 99.7% identical (1445/1449 bp) with that of *H. lieberkuehni* (accession no. AF298623) reported from the edible frog in the Czech Republic (Modrý et al., 2001) but similarity with other genera in the Sarcocystidae was less than 97.5%.

The partial 28S sequence (1422 bp) of *H. kasumiensis* revealed 98.6% identity (955/969 bp) with that of *H. lieberkuehni* (accession no. AF513499), also from the edible frog in the Czech Republic (Modrý et al., 2001). However, *H. kasumiensis* displayed similarity values < 90.8% identity with other genera in the Sarcocystidae.

A partial *cox1* sequence (910 bp) also was identified from *H. kasumiensis* and it had 87.9%–87.3% identity with the sequence from *Cystoisospora* spp. (nos. MF774038, MF774037, KT184385, LC377843, KU184384). The *cox1* sequence of *H. lieberkuehni* was not reported in the INSD, and therefore, was unavailable for comparison.

Accumulated nucleotide substitutions in the 18S and 28S genes between *H. kasumiensis* from *P. p. porosus* in Japan and *H. lieberkuehni* from the edible frog in the Czech Republic were 0.23% and 1.76%, respectively (Table 4). Some pairs of congeners among the other sarcocystids showed up to 1.20% divergence in the 18S sequence and 3.35% divergence in the 28S sequence. However, several pairs showed divergence values lower than or equal to the divergences observed between *H. kasumiensis* and *H. lieberkuehni.* In particular, the inter-specific divergence for both 18S and 28S genes between our species and *H. lieberkuehni* was higher or equivalent to that observed for *Heydornia heydorni* vs. *Heydornia triffittae* (18S: 0%, 28S: 0.35%) and *Besnoitia bennetti* vs. *Besnoitia tarandi* (18S: 0.23%, 28S: 0.35%) (Table 4). Furthermore, the inter-genus divergence between *Toxoplasma gondii* vs. *Neospora caninum* (18S: 0.23%), *N. caninum* vs. *Hammondia hammondi* (18S: 0.23%, 28S: 1.41%), and *N. caninum* vs. *H. heydorni* (18S: 0.23%) was lower than that observed between *H. kasumiensis* and *H. lieberkuehni*.

Table 4
The inter-specific genetic distance.

Species pair	18S	28S
Hyaloklossia		
H. lieberkuehni vs. *H. kasumiensis*	0.0023	0.0176
Heydornia		
H. heydorni vs. *H. triffitae*	0.0000	0.0035
Besnoitia		
B. besnoiti vs. *B. bennetti*	0.0069	0.0053
B. besnoiti vs. *B. tarandi*	0.0046	0.0018
B. besnoiti vs. *B. darlingi*	0.0115	nc
B. besnoiti vs. *B. jellisoni*	0.0092	0.0300
B. bennetti vs. *B. tarandi*	0.0023	0.0035
B. bennetti vs. *B. darlingi*	0.0092	nc
B. bennetti vs. *B. jellisoni*	0.0069	0.0335
B. tarandi vs. *B. darlingi*	0.0069	nc
B. tarandi vs. *B. jellisoni*	0.0046	0.2998
B. darlingi vs. *B. jellisoni*	0.0023	nc
Cystoisospora		
C. canis vs. *C. belli*	0.0053	nc
C. canis vs. *C. ohioensis*	0.0084	nc
C. canis vs. *C. felis*	0.0069	nc
C. canis vs. *C. suis*	0.0046	nc
C. belli vs. *C. ohioensis*	0.0061	nc
C. belli vs. *C. felis*	0.0092	nc
C. belli vs. *C. suis*	0.0023	nc
C. ohioensis vs. *C. felis*	0.0120	nc
C. ohioensis vs. *C. suis*	0.0054	nc
C. felis vs. *C. suis*	0.0069	nc
Eumonospora		
E. henryae vs. *E. neofalconis*	0.0077	nc
E. henryae vs. *E. danceloe*	0.0061	nc
E. neofalconis vs. *E. danceloe*	0.0077	nc

nc = not calculated.

3.2.2. Phylogenetic analysis

In our phylogenetic trees, members of the Toxoplasmatinae formed a monophyletic clade (clade I) that divided into two clades (Fig. 5A–C). Clade Ia included *Toxoplasma, Hammondia, Heydornia,* and *Neospora*

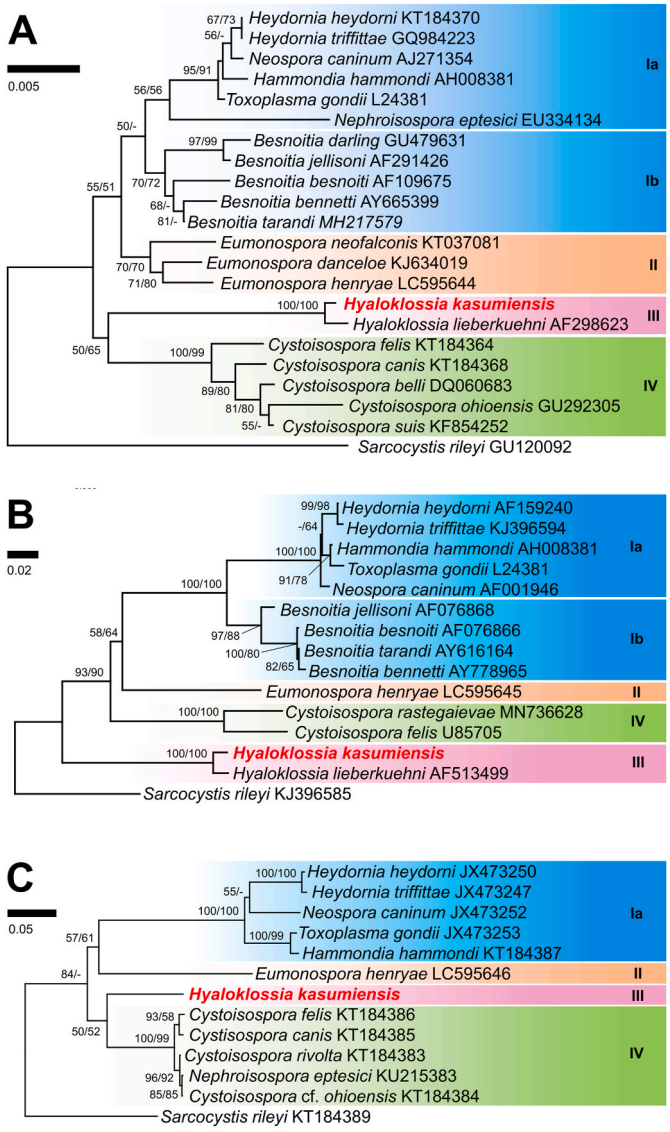

Fig. 5. Phylogenetic trees of coccidian parasites belonging to the subfamilies Toxoplasmatinae, Eumonosporinae, and Cystoisosporinae and related taxa using 18S (A), 28S (B), and *cox1* (C) sequence data. *Sarcocystis rileyi* (Sarcocystidae: Sarcocytinae) was used as an outgroup. The nodes are labeled using support from the bootstrap values obtained for neighbor joining (left) and maximum likelihood (right) methods. The unlabeled nodes and hyphens indicate support <50. Scale bars represent the substitutions per site.

with high bootstrap values [18S: 95 (NJB)/91 (MLB); 28S: 100 (NJB)/100 (MLB); *cox1*: 100 (NJB)/100 (MLB)], whereas clade Ib composed of *Besnoitia* spp. had moderate to high bootstrap values [18S: 70 (NJB)/72 (MLB); 28S: 97 (NJB)/88 (MLB)]. The Eumonosporinae clade (clade II) was monophyletic and a sister group of the Toxoplasmatinae (clade Ia + Ib) with low to moderate bootstrap values [18S: 55 (NJB)/51 (MLB); 28S: 58 (NJB)/64 (MLB); *cox1*: 57 (NJB)/61 (MLB)]. *Hyaloklossia* spp. (clade III) and Cystoisosporinae (clade IV) were monophyletic groups, respectively. With respect to the 18S and *cox1* sequences, these groups collectively formed a monophyletic clade (III + IV) with moderate bootstrap values (above 50%), whereas with respect to the 28S sequence, clade III showed the formation of an early branch at the monophyletic clade comprising Ia + Ib + II + IV. The phylogenetic positions of *Nephroisospora* were unstable, and it was placed in clade I (based on 18S sequence) or clade IV (based on *cox1* sequence).

3.3. Description of a new subfamily

Phylum Apicomplexa Levine, 1970
Class Conoidasida Levine, 1988.
Order Eucoccidiorida Legar and Duboscq, 1910.
Suborder Eimeriorina Léger, 1911
Family Sarcocystinae Poche, 1913
Subfamily Haloklossiinae n. subfam. Tokiwa, Chou, Tochigi, Katayama, et Duszynski (2021).

Diagnosis: Members exhibit a homoxenous life cycle and undergo endogenous sporulation in the kidneys of anurans. Oocysts with two sporocysts each with four sporozoites. Stieda and substieda bodies absent. Sporocyst residuum present.

Type genus: *Hyaloklossia* Labbé, 1896

4. Discussion

Hyaloklossia was first discovered in the edible frog by Lieberkühn (1854) and later was named *Klossia lieberkühni* by Labbé (1894). Two years later, Labbé (1896) established the monotypic genus *Hyaloklossia* for this species. However, the genus then was treated as a junior synonym of *Diplospora* Labbé, 1893) by Laveran and Mesnil (1902) and Minchin (1903) and, later, as *Isospora* Schneider, 1881 (Nöller, 1923; Siekmüller, 1924; Walton, 1949; Kazubski and Grabda-Kazubska, 1973; Pellérdy, 1974; Golemansky and Miceva, 1975). Eventually, *Hyaloklossia* was re-established and its defining characters emended based on its morphological and phylogenetic characteristics (Modrý et al., 2001). Notably, phylogenetic analysis using sequences of nuclear ribosomal DNA (Modrý et al., 2001; Šlapeta et al., 2003; Dubey et al., 2004; Garner et al., 2006; Jirků et al., 2009; Morrison, 2009; Gubbels et al., 2020) and apicoplast DNA (Oborník et al., 2002; Janouškovec et al., 2019) suggested that *Hyaloklossia* is more closely related to heteroxenous cyst-forming genera in the Sarcocystidae Poche, 1913, than it is to any genus in the Eimeriidae Minchin, 1903.

Laveran and Mesnil (1902) experimentally infected frogs with *H. lieberkuehni* and demonstrated its homoxenous life cycle. The sporozoites excysted from their sporocysts in the intestinal tract and migrated through the bloodstream. Vascular endothelial cells were the primary targets, and merogony was detected in organs receiving high blood flow, such as the lungs, kidney, liver, and spleen. Merozoites then localized in the kidneys to undergo gametogony, followed by sporogony. These stages then accumulated in glomerular capillaries, and often appeared in the interstitium by disrupting the Bowman's capsule. Oocysts are fragile, and resistant sporocysts are shed in the urine and eventually transmitted to other animals, particularly omnivorous tadpoles present in the aquatic environment. In our study, a small number of mature sporocysts were detected in the renal epithelium. Since sporocysts in the interstitium are unlikely to be excreted in urine, we presume that this intra-epithelial stage, had moved from the capillary to the epithelium, for urine excretion.

Ribosomal 18S and 28S gene sequences have been used to distinguish among various coccidian species and clarify the phylogenetic relationship among and between species (Power et al., 2009; Schrenzel et al., 2005; Chou et al., 2020). However, only minor interspecies differences have been observed among various members of Toxoplasmatinae (*Toxoplasma, Neospora, Hammondia, Heydornia, Besnoitia*), Cystoisosporinae (*Cystoisosporinae*), and Eumonosporinae (*Eumonospora*). In fact, the phylogenetic distances observed between *H. kasumiensis* and *H. lieberkuehni* were greater than those observed among several species or genera of the Toxoplasmatinae. Based on these results, we concluded that the genetic variation observed between our species and *H. lieberkuehni* corresponded to differences at inter-specific levels. Analysis of the *cox1* sequence from *H. lieberkuehni* from the edible frog in Europe and cross-species infection experiments are necessary to confirm whether or not our form is a cryptic species.

The development of pathogens in renal tissue has been observed in

other taxa of Eimeriorina as well, such as in members of *Pseudoklossia* (Aggregatidae), several *Eimeria* species (e.g., *E. truncata, E. fraterculae,* and *E. gaviae*) and *Margolisiella* Desser & Bower, 1977 in the Eimeriidae, and in *Nephroisospora eptesici* from the Sarcocystidae (Montgomery et al., 1978; Leighton and Gajadhar, 1986; Friedman et al., 1995; Desser and Bower, 1997; Wünschmann et al., 2010). The kidney receives a high blood flow, and renal endothelial cells could serve as the primary target for these coccidians that invade the bloodstream. Therefore, the occurrence of coccidia in the kidney is not unique to the genus *Hyaloklossia*, but might be a case of plesiomorphy. As indicated in phylogenetic trees, *Hyaloklossia* appears abruptly among genera within the Sarcocystidae, most of which have developed a heteroxenous, predator-prey life cycle involving final (carnivore) and intermediate/paratenic (herbivore/omnivore) hosts. Meanwhile, *Hyaloklossia* species exhibit less diversity for definitive host specificity than other sarcocystids and do not require an intermediate host during their life cycle. It is reasonable to suppose that *Hyaloklossia* species have a recent common ancestor with sarcocystids that parasitizie carnivores, and their ability to form cysts in intermediate/paratenic hosts that is observed in other sister taxa has been lost secondarily during host switching.

The morphological features of disporocystic and tetrasporozoic oocysts without Stieda bodies and the phylogenetic characteristics reported here and by others, indicates that *Hyaloklossia* belongs to the Sarcocystidae; however, *Hyaloklossia* can easily be distinguished from the other four subfamilies with a heteroxenous or facultatively heteroxenous life cycles. Thus, we suggest that *Hyaloklossia* belongs to a lineage independent from other members of the Sarcocystidae and propose a new subfamily to accommodate them.

Declaration of competing interest

None.

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