International Journal for Parasitology: Parasites and Wildlife 26 (2025) 101020

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

A new genus and species of isosporoid coccidium from captive green tree frogs, *Ranoidea caerulea* (Anura: Hylidae)

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ARTICLE INFO

Keywords:
Amphibia
Coccidia
Litoria caerulea
18S
COI

ABSTRACT

A new genus and a new species of isosporoid coccidium (Apicomplexa: Conoidasida: Eimeriorina) are described and illustrated from green tree frogs (*Ranoidea caerulea*) (Anura: Hylidae) imported from Papua New Guinea and Indonesia. The described species has disporocystic and tetrasporozoic oocysts without a Stieda body. Nine species originally belonging to the genus *Isospora* Schneider, 1881 in the family Eimeriidae Minchin, 1903 described from Anura are recognized as members of the new genus and new combinations of the species names are proposed. The phylogenetic analyses of partial gene fragments of 18S rRNA and mitochondrial cytochrome *c* oxidase subunit 1 genes from isosporoid oocysts from green tree frogs suggested that isosporoid oocysts without Stieda bodies from anurans should be placed in the Sarcocystidae and no longer belong in the Eimeriidae.

1. Introduction

Amphibia are classified into the orders Anura (frogs and toads), Urodela (salamanders and newts), and Gymnophina (caecilians). The total number of extant amphibian species is approximately 8700; of these, approximately 90% belong to the order Anura (Amphibia Web, 2024). There are now about 57 valid species of apicomplexans of the suborder Eimeriorina (Alveolata: Apicomplexa) identified in Amphibia and these are distributed in four dissimilar genera; *Eimeria* Schneider, 1875 (40); *Goussia* Labbé, 1896 (3); *Hyaloklossia* Labbé, 1896 (2), and *Isospora* Schneider, 1881 (12) (Labbé, 1894, 1896; Pellérdy, 1965, 1974; Upton et al., 1993; Duszynski et al., 2007; McAllister and Upton, 2008; Jirků et al., 2009; McAllister et al., 2014; Cao et al., 2017; Tokiwa et al., 2021). Members of *Isospora* and *Hyaloklossia*, with disporocystic and tetrasporozoic oocysts, are often lumped under a generic title referred to as isosporoid coccidia. However, we now know that isosporoid coccidia with Stieda bodies on their sporocysts should be classified as *Isospora* species in the Eimeriidae Minchin, 1903 while those isosporoid coccidia without Stieda bodies should be placed in the Sarcocystidae Poche, 1913 (Carreno and Barta, 1999; Barta et al., 2005). Currently, there are 12 "*Isospora* species" listed that infect amphibians, 10 in Anura and two in Urodela (Duszynski et al., 2007; Cao et al., 2017). Of these species, three possess a Stieda body while the remaining nine do not. This suggests that, in addition to *Isospora,* another lineage of isosporoid coccidia is involved in the infection of amphibians.

In this study, tetrasporozoic and disporocystic oocysts lacking Stieda bodies were recovered from the fecal specimens of pet green tree frogs, *Ranoidea caerulea* (White, 1970) (syn. *Litoria caerulea* White, 1790) (Anura: Hylidae) native to regions of Oceania and surrounding areas. In Japan, it was widely known among veterinarians that wild imported green tree frogs are naturally infected with isosporoid coccidia, but the details remained unclear. Phylogenetic analyses using DNA sequences revealed that the isosporoid coccidium we detected in *R. caerulea* did not belong to the family Eimeriidae but rather to the Sarcocystidae clade. Thus, we propose a new genus for these species in amphibians and describe a novel anuran isosporoid species within this genus. In addition, we propose new replacement names (*nomina nova*) for the other nine isosporoid coccidia that parasitize amphibians.

2. Materials and methods

2.1. Sample collection and examination

Oocysts were detected in the feces of an adult pet *R. caerulea* in Chiba, Japan, during a health examination in December 2023, which led to the diagnosis of a coccidia infection. The frog was purchased from a

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https://doi.org/10.1016/j.ijppaw.2024.101020
Received 30 July 2024; Received in revised form 11 November 2024; Accepted 12 November 2024
Available online 14 November 2024

pet shop in Japan in November 2023 and belonged to one of the authors' households. The frog was caught in the wild in Papua New Guinea. Fecal samples were collected weekly from November 2023 to January 2024. Additionally, fecal samples were collected from two additional wild adult *R. caerulea*, which were imported from Indonesia and kept at a pet store in Japan to detect oocysts in April 2024. To identify the species, the fecal samples were preserved in 15-ml centrifuge tubes with tap water and stored at room temperature for morphological observation or 4 °C for genetic analysis until examination.

2.2. Morphological examination

Oocysts were observed using a BX41 microscope (Olympus, Tokyo, Japan) with direct fecal smear technique, and photomicrographs were captured using a DP74 photomicroscope (Olympus). Measurements were made using CellSens software (Olympus) under an oil immersion objective. The data are presented in micrometers as the mean, followed by the range in parentheses. Descriptive methods followed Duszynski and Wilber (1997), and standardized abbreviations for structural characteristics of both oocysts and sporocysts followed Wilber et al. (1998); oocyst characters: length (L), width (W), their ratio (L/W), micropyle (M), micropyle cap (MC), oocyst residuum (OR), polar granule (PG); sporocyst characters: length (L), width (W), their ratio (L/W), Stieda body (SB), substieda body (SSB), para-Stieda body (PSB), sporocyst residuum (SR), sporozoites (SZ), and refractile body (RB) and nucleus (N) in SZ. Line drawings were made using Fire Alpaca (ver. 2.0; PGN Inc., Tokyo, Japan).

2.3. Molecular analysis

Genomic DNA was extracted from oocysts using a Power Soil DNA isolation kit (MoBio Laboratories, USA) as previously described (Tokiwa et al., 2017). The resulting DNA specimens were used as templates for PCR amplification; double-distilled water was used as the negative control.

A partial fragment of the 18S rRNA gene (*18S*) and the mitochondrial encoded cytochrome *c* oxidase subunit 1 gene (*COI*) were amplified using the primer sets EF (5′-GAACTGCGAATGGCTCATT-3′)/ER (5′-CTTGC GCCTACTAGGCATTC-3′) (Kvicerová et al., 2008) and Sdae-Cox1_260F (5′-GATCTTTATGTTYTTRATGCC-3′)/Sdae-Cox1_1147R (5′-CATTACCC ATAACYACACC-3′) (Ogedengbe et al., 2018), respectively. The PCR mixture contained 2.5 μl of 10 × Ex Taq buffer (Takara Bio Inc., Shiga, Japan), 0.2 mM dNTPs, 0.2 μM of each primer, 1 U Ex Taq polymerase (Takara Bio, Inc.), and 1 μl DNA extract in a total volume of 20 μl. PCR conditions were as follows: initial denaturation at 94 °C for 5 min; 35 cycles at 94 °C for 30 s, 54 °C (*18S*) or 50 °C (*COI*) for 1 min, and 72 °C for 1 min; and extension at 72 °C for 7 min (*18S*) or 1 min (*COI*). Amplified DNA was electrophoresed in 2% agarose gel and visualized using a light-emitting diode transilluminator. The PCR products were sent to a sequencing service (Macrogen Corp., Kyoto, Japan) and analyzed using an ABI3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) using the same primers as those used for PCR amplification. The sequences were visualized by electropherograms and manually corrected using MEGA 11 software (Tamura et al., 2021).

2.4. DNA sequence analyses

Sequence similarity was determined separately using the BLASTN program and the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/Blast.cg).

Reference sequences were obtained from the DDBJ/ENA/GenBank databases for phylogenetic analyses. MAFFT (Katoh and Toh, 2010) was used for multiple alignments of *18S* sequences, whereas ClustalOmega (Sievers and Higgins, 2018) was used for *COI* sequences. Both MAFFT and ClustalOmega analyses were performed using Geneious Prime (geneious.com). Obvious errors were detected through visual inspection of each alignment. The nucleotide sequence divergence was calculated using the dataset of closely relates species shown in Supplement 1, based on the uncorrected pairwise genetic distance (*p*-distance) with MEGA 11 software.

Phylogenetic analyses were performed via Bayesian inference (BI) using MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001) and maximum likelihood (ML) using PHYML 3.3.2 (Guindon et al., 2010). *18S* was analyzed using BI only, while *COI* was analyzed both ML and BI methods. Furthermore, jModelTest 2.1.10 (Darriba et al., 2012) was used to evaluate the best-fit model and parameters for the analyses of *18S* and *COI* alignments based on the Akaike Information Criterion. This resulted in the general time-reversible model, which accounts for the proportion of invariant sites (+*I*) with gamma-distributed rate variation among sites (+*G*) and was the best-fit model for all datasets. The BI analysis consisted of 1.1×10^6 generations of Markov Chain Monte Carlo searches containing four chains, a heated chain temperature of 0.2, and a burn-in of 100,000 generations. ML bootstrap analysis of 500 replicates was performed to estimate node support. *Goussia bayae* was used as an outgroup for both *18S* and *COI* datasets.

3. Results

3.1. Taxonomic summary and description

3.1.1. Description of the new genus

Phylum Apicomplexa Levine, 1970
Class Conoidasida Levine, 1988
Family Sarcocystidae Poche, 1913
Subfamily Hyaloklossinae Tokiwa, Chou, Tochigi, Katayama & Duszynski, 2021
Batrachospora n. gen.

Diagnosis: Coccidia that develop in Amphibia species and produce oocysts with two sporocysts, each with four SZ; SB, SSB, and PSB are all absent in sporocysts; exogenous sporulation, but in some species, oocysts may be semi-sporulated in fresh feces; to date, only parasites of Anura.

Synonyms: *Isospora* Schneider, 1881 *pro parte*, *Diplospora* Lavier, 1941 *pro parte*.

Etymology: Ancient Greek *βᾰτρᾰ́χος* (batrachus "frog,") + Greek *σπορᾰ́* (spora, "seed"). Sex: female.

Compendium of *Batrachospora* species of anurans (9):

Batrachospora brumpti (Lavier, 1941) n. comb.
Synonym: *Isospora brumpti* Lavier, 1941, *Diplospora brumpti* (Lavier, 1941) Grasse, 1953
Type host: *Bufotes viridis* (Laurenti, 1768) (Bufonidae)

Batrachospora cogginsi (Bolek, Janovy & Irizarry-Rovira, 2003) n. comb.
Synonym: *Isospora cogginsi* Bolek, Janovy & Irizarry-Rovira, 2003
Type host: *Pseudacris triseriata triseriata* (Wied-Neuwied, 1839) (Bufonidae)

Batrachospora cruzi (Pinto & Vallim, 1926) n. comb.
Synonym: *Isospora cruzi* Pinto & Vallim, 1926
Type host: *Scinax crospedospilus* (Lutz, 1925)
Other hosts: *S. fuscovarius* (Lutz, 1925), *S. nasicus* (Cope, 1862), *S. ruber* (Laurenti, 1768) (Hylidae)

Batrachospora fragosum (Upton & McAllister, 1988) n. comb.
Synonym: *Isospora fragosum* Upton & McAllister, 1988
Type host: *Gastrophryne olivacea* (Hallowell, 1856) (Microhylidae)

Batrachospora kukunoris (Cao, Shang, Yang, Zhang, Duszynski, Zhang, Zhu & Bian, 2017) n. comb.
Synonym: *Isospora kukunoris* Cao, Shang, Yang, Zhang, Duszynski, Zhang, Zhu & Bian, 2017
Type host: *Rana kukunoris* Nikolskii, 1918 (Ranidae)

Batrachospora neos (Yakimoff & Gousseff, 1936) n. comb.
Synonym: *Isospora neos* Yakimoff & Gousseff, 1936
Type host: *Rana arvalis* Nilsson, 1842 (Ranidae)
Batrachospora stomatici (Chakravarty & Kar, 1944) n. comb.
Synonym: *Isospora stomaticae* Chakravarty & Kar, 1944, *Isospora stomatici* (Chakravarty & Kar, 1944) Levine, 1985
Type host: *Duttaphrynus stomaticus* (Lütken, 1864) (Bufonidae)
Batrachospora wenyoni (Ray & Das Gupta, 1935) n. comb.
Synonym: *Isospora wenyoni* Ray & Das Gupta, 1935
Type host: *Duttaphrynus melanostictus* (Schneider, 1799) (Bufonidae)
Batrachospora wladimirovi (Yakimoff, 1930) n. comb.
Synonym: *Isospora wladimirovi* Yakimoff, 1930
Type host: *Hyla arborea* L. (Hylidae)

Remarks: The genus *Isospora* was created for *Isospora rara* with disporocystic oocysts detected in the feces of European slugs. The schematic drawing of the original description shows a tetrasporozoic sporocyst with a distinct SB (Schneider, 1881). Labbé (1893) proposed the genus *Diplospora* for isosporoid oocysts detected in the feces of passerine birds. Lieberkühn (1854) found an isosporoid coccidium in the kidney of *Pelophylax* frogs and called it "psorosperm." Labbé (1894) named this species *Klossia lieberkuhni*, and two years later, he proposed the genus *Hyaloklossia* (Labbé, 1894, 1896). Laveran and Mesnil (1902) treated *Hyaloklossia* and *Diplospora* as junior synonyms of *Isospora*. Clearly, the taxonomic placement of vertebrate coccidia that produce oocysts with two sporocysts, each with four SZ, into appropriate genera has been a controversial theme since the genus *Isospora* was first named by Schneider (1881). Structural and molecular data accumulated since that time, allows us to recognize *Isospora* as a paraphyletic group of species of at least two major lineages. One has sporocysts with a SB, and usually a SSB, and these mostly monoxenous species are placed in the Eimeriidae. The second group, with sporocysts that lack both SB and SSB, has species that are now placed in multiple genera within the Sarcocystidae. Barta et al. (2005) tried to explain some of the early historic tangles but pointed out that even the International Commission on Zoological Nomenclature (ICZN) has not been able to sort out which species are legitimate members of *Isospora*.

Fourteen valid species of coccidia with isosporoid-type oocysts have been reported in amphibians prior to this study (see Table 1, in Duszynski et al., 2007; Cao et al., 2017; Tokiwa et al., 2021). Two species are in the *Hyaloklossia*, namely, *H. lieberkuehni* (Labbé, 1894) and *H. kasumiensis* Tokiwa et al., 2021 (Tokiwa et al., 2021, 2022a, 2022b), while the remaining 12 species were described as belonging to *Isospora*. Of these species, 12/14 (85.7%) were detected from anuran hosts, while the other two were reported in urodelan hosts and 11/12 (91.7%) of the species from anuran hosts have neither SB nor SSB in their sporocysts but both of the species from uroderan hosts do have SB. *Hyaloklossia* can be identified from other isosporoid-type coccidia by its site of infection in the kidneys and the shedding of mature sporocysts.

3.1.2. Description of the new species

***Batrachospora caeruleae* n. sp. (Figs. 1 and 2)**

Type host: Green tree frog, *Ranoidea caerulea* (White, 1790); Amphibia; Anura; Hylidae.

Type locality: Unknown. The oocysts were recovered from adults of *R. caerulea* imported into and maintained in Chiba, Japan as pet animals.

Other hosts: None to date.

***Unsporulated and sporulated oocysts*:** Spheroidal sporont (Figs. 1A and 2A) with granular cytoplasm. Two subspheroidal sporoblasts (Fig. 2B) with granular cytoplasm within oocyst. Mature oocyst (Figs. 1B, C, 2C) spheroidal to subspherical, and measured L × W: 19.8 × 18.8 (18–22 × 17–21.5); L/W ratio: 1.05 (1.0–1.1). Oocyst wall single-layered, smooth, colorless; thickness, 0.6 (0.4–0.7). M, MC, OR, and PG all absent.

Sporocysts and sporozoites: Sporocyst wall single-layered, thin, smooth, and colorless. Sporocyst (Figs. 1D–F, 2D) ellipsoidal, and measured L × W: 12.1 × 9.9 (11-13 × 9–10.5); L/W ratio: 1.2 (1.1–1.4). SB, SSB, and PSB absent. SR with numerous small and dispersed granules initially occupying large portion of sporocyst, gradually coalescing, and eventually forming spheroidal to subspheroidal body composed of coarse granules with void in center. SZ short, sausage-shaped, curved, and oriented horizontally or diagonally to the long axis of sporocyst. Refractile body and N invisible under ordinary light microscopy.

Site of infection: Unknown. Oocysts were recovered from fecal samples.

Sporulation: Exogenous. In fresh feces, some oocysts contained sporonts, whereas most contained sporoblasts, both filled with numerous granules. By 48 h, immature SZ were visible within the dispersed granules of sporoblasts. A few mature oocysts with SZ and aggregated SR were observed at 72 h.

Prevalence: Found in 3/3 (100%) specimens of the type host examined for oocysts.

Cross-transmission: None to date.

Pre-patent and patent period: Unknown. We can only note that oocysts were detected during the sampling period (>2 month).

ZooBank registration: 571E690D-242A–4A17-883E-5AEF0D18913B.

Etymology: The species name is derived from the host's specific epithet.

Material deposited: Phototypes (Bandoni and Duszynski, 1988) were deposited at the Meguro Parasitological Museum, Meguro, Tokyo, Japan, under MPM Coll. Nos. 25324 (mature oocyst) and 25325 (sporocyst). Sequences representing *18S* (1347-bp) and *COI* (871-bp) were deposited to the DNA Data Bank of Japan (DDBJ) under accession nos. LC842307, LC842308, respectively.

Remarks: This is the type species for the genus *Batrachospora*. The coccidian obtained from one frog from Papua New Guinea and two frogs from Indonesia had consistent morphology of mature oocyst and were identified as the same species, *B. caeruleae*. Table 1 lists the *Isospora* species with non-SB sporocyst that we incorporated into our proposed new genus, the remaining *Isospora* species with SB on their sporocysts, their structural dimensions, the location of their endogenous development and their sporulation (if known) and their amphibian hosts and countries from which they were described.

Oocysts of *B. caeruleae* most closely resembles those of *B. cruzi* detected in snouted tree frogs (*Scinax* spp.) in South America (Pinto and Vallim, 1926; Carini, 1936) and *B. wladimirovi* detected from European tree frogs (*Hyla arborea*) in Caucasia (Yakimoff, 1930a, 1930b). Oocysts of *B. caeruleae* are slightly more spheroidal than those of *B. cruzi* and *B. wladimirovi* such that they have slightly different L/W ratios (1.1 vs 1.2 for both) and sporocysts of *B. caeruleae* are smaller and somewhat differently shaped than those of the other two species (12 × 10 vs 14 × 14 and 13 × 9, respectively). However, the published descriptions of both species are inadequate and re-descriptions of both are needed. Rzepczyk (1976) reported three oocyst morphotypes found in *R. caerulea*. The first, found in 6/12 (50%) frogs, had only unsporulated oocysts that were, 39 × 33; the second morphotype, found in 3/12 (25%) frogs, also had unsporulated oocysts that were, 28 × 25; but her third morphotype, found in 2/12 (17%) frogs, was sporulated with tetrasporozoic and disporocystic oocysts that measured, 16.8–20.7 × 14.0–16.8. The size of oocysts of the third morphotype overlaps those of *B. caeruleae,* but they are slightly smaller with a larger L/W ratio.

3.2. Phylogenetic analysis of Batrachospora caeruleae and related taxa

A 1347-bp fragment of the *18S* and a 871-bp fragment of *COI* of *B. caeruleae* were amplified and sequenced. The sequences obtained from three green tree frogs were completely identical for both the *18S* and *COI*. The partial *18S* sequence from *B. caeruleae* shared a 97.9–98.0% similarity (100% query cover) to *Hyaloklossia* spp. (accession nos. AF298623, LC669718, and LC602188) and 97.7–97.9% similarity (100% query cover) to *Eumonospora* (syn. *Caryospora*) (accession

Table 1
List of species of *Batrachospora*, *Hyaloklossia*, and *Isospora* from amphibians.

Species	Host (Order, Family)	Oocyst L × W (L/W ratio)	Sporocyst L × W (L/W ratio)	SB	Site of infection	Shedding stage	Sporulation	Locality	References
Batrachospora brumpti (Lavier, 1941)	*Bufotes viridis* (Anura, Bufonidae)	24 × 16 (1.5)	15-19 × 10-14	Absent	Intestine	Oocyst	Exogenous	Syria, Turkmenistan	Lavier, 1941
Batrachospora cogginisi (Bolek et al., 2003)	*Pseudacris triseriata triseriata* (Anura, Bufonidae)	19.3 × 15.1 (1.3)	13.3 × 9.9	Absent	Intestine	Oocyst	Exogenous	USA	Bolek et al., 2003
Batrachospora cruzi (Pinto & Vallim, 1926)	*Scinax crospedospilus*, *S. fuscovarius, S. nasicus*, *S. ruber* (Anura, Hylidae)	20.7 × 17.2 (1.2)	14 × 13.8	Absent	Intestine	Oocyst	Presumably exogenous	Brazil	Pinto and Vallim, 1926
Batrachospora fragosum (Upton & McAllister, 1988)	*Gastrophryne olivacea* (Anura, Microhylidae)	18.5	12.7 × 10.9 (1.2)	Absent	Unknown[a]	Oocyst	Exogenous or endogenous	USA	Upton and McAllister, 1988
Batrachospora kukunoris (Cao et al., 2017)	*Rana kukunoris* (Anura, Ranidae)	14.2 × 10.2 (1.4)	8.7 × 7.2 (1.2)	Absent	Unknown[a]	Oocyst	Exogenous	China	Cao et al., 2017
Batrachospora neos (Yakimoff & Gousseff, 1936)	*Rana arvalis* (Anura, Ranidae)	26 × 22.4 (1.0–1.1)	12.6–13.6 × 7.2–10.8	Absent	Intestine	Oocyst	Exogenous	Poland	Kazubski and Grabda-Kazubska, 1973
Batrachospora stomatici (Chakravarty & Kar, 1952)	*Duttaphrynus stomaticus* (Anura, Bufonidae)	25.5 × 17.5 (1.5)	15.4–17.6 × 11.0	Absent	Intestine	Oocyst	Exogenous	India	Chakravarty and Kar, 1952
Batrachospora wenyoni (Ray & Das Gupta, 1935)	*Duttaphynus melanostictus* (Anura, Bufonidae)	16-20 × 11-14	8 × 4	Absent (in line drawing)	Unknown[a]	Oocyst	Exogenous	India	Ray and Das Gupta, 1935; Duszynski et al., 2007
Batrachospora wladimirovi (Yakimoff, 1930)	*Hyla arborea* (Anura, Hylidae)	21.4 × 17.6 (1.2)	13 × 9	Absent (in line drawing)	Unknown[a]	Oocyst	Unknown	Caucasia	Yakimoff, 1930a, b
Batrachospora caeruleae Tokiwa et al., 2024	*Ranoidea caerulea* (Anura, Hylidae)	19.8 × 18.8 (1.1)	12.1 × 9.9 (1.2)	Absent	Unknown[a]	Oocyst	Exogenous	Japan	This study
Hyaloklossia lieberkuehni (Labbé, 1894)	*Pelophylax* kl. *esculentus* (Anura, Ranidae)	35-41 × 20-25	25-30 × 14-16	Absent	Kidney	Sporocyst	Endogenous	Europe	Modrý et al., 2001
Hyaloklossia kasumiensis Tokiwa et al., 2021	*Pelophylax porosus porosus*, *Pelophylax porosus brevipodus*, *Pelophylax nigromaculatus* (Anura, Ranidae)	39.3 × 21.3 (1.9)	27.1 × 15.5 (1.8)	Absent	Kidney	Sporocyst	Endogenous	Japan	Tokiwa et al., 2021, 2022a, 2022b
Isospora delicatus Upton & McAllister, 1988	*Pseudacris streckeri streckeri*, *P. illinoensis* (Anura, Bufonidae)	15.8 × 15.7 (1.0)	13.5 × 8.0 (1.7)	Present	Unknown[a]	Oocyst	Endogenous	USA	Upton and McAllister, 1988
Isospora hightoni Upton et al., 1993	*Plethodon albagula* (Urodela, Plethodontidae)	22.9 × 22.8 (1.0)	16.6 × 11.1 (1.5)	Present	Unknown[a]	Oocyst	Exogenous	USA	Upton et al., 1993
Isospora jeffersonianum Doran, 1953	*Ambystoma jeffersonianum* (Urodela, Ambystomatidae)	18.5–22.5 (1.0)	45.5–16 × 7-8	Present	Unknown[a]	Oocyst	Exogenous	USA	Doran, 1953
Isospora hylae Mesnil, 1907, *species inquirenda*	*Hyla arborea* (Anura, Hylidae)	30-35 × 20-25	23 × 17	na	Intestine	Oocyst	Endogenous	France	Mesnil, 1907
Isospora ranae (Rivolta, 1878), *nomen nudum*	*Pelophylax* kl. *esculentus* (Anura, Ranidae)	na	na	na	na	Oocyst	na	na	Rivolta, 1878; Pellérdy, 1965, 1974

na: not available.
[a] The oocysts were recovered from the feces or rectal contents.

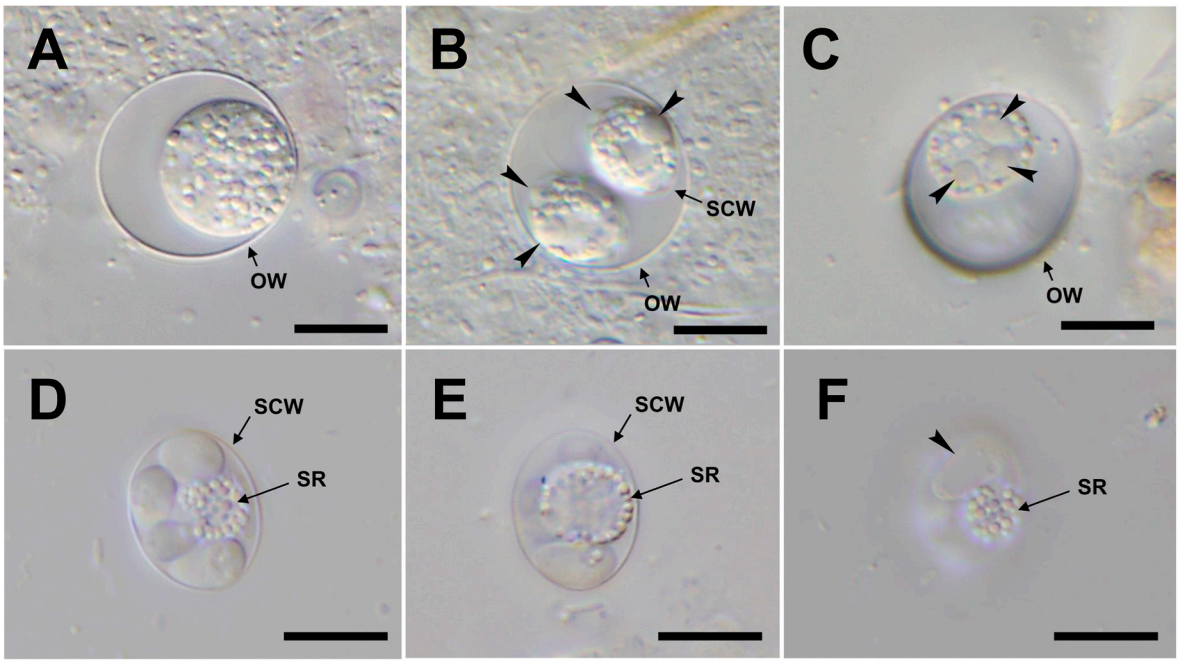

Fig. 1. Nomarski interference contrast photomicrographs of oocysts and sporocysts detected from green tree frog (*Ranoidea caerulea*). A: sporont with a granular cytoplasm. B: mature oocyst with two sporocysts with sporozoites. C: sporocyst with sporozoites (transverse plane) and scattered granules. D–E: sporocyst released from mature oocysts. Arrowhead: sporozoite; OW: oocyst wall; SCW: sporocyst wall; SR: sporocyst residuum. Scale bar = 10 μm.

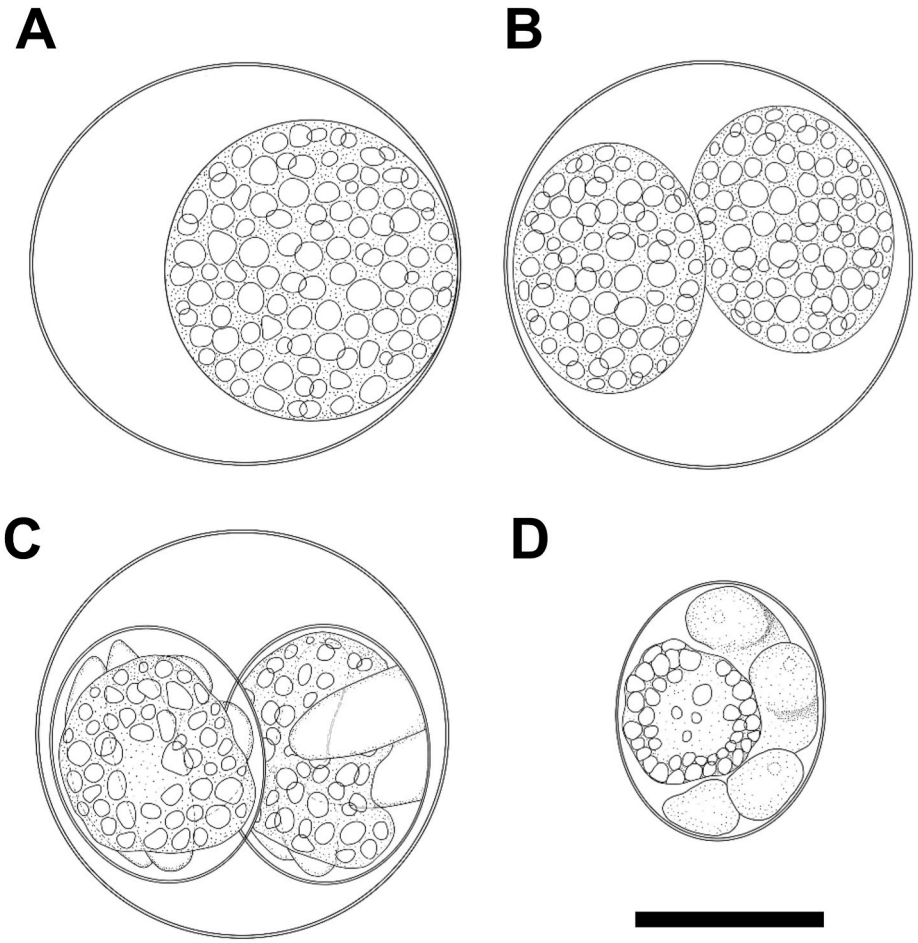

Fig. 2. Line drawings of oocysts and sporocysts detected from green tree frog (*Ranoidea caerulea*). A: sporont, B: sporoblasts, C: mature oocyst, D: sporocyst with four sporozoites. Scale bar = 10 μm.

Table 2
Summary of distances (%) based on the p-distance model. The lower left section shows inter-generic distances based on *18S*, while the upper right section shows inter-generic distances based on *COI*. Bold values indicate intra-generic distances (*18S*/*COI*).

Subfamily	Genus	Tox	Ham	Hey	Neo	Bes	Eum	Cys	Hya	Bat
Toxoplasmatinae	*Toxoplasma*	**nc/nc**	1.4	8.0	7.0	nc	23.6	21.1	22.6	20.9
	Hammondia	0.4	**nc/nc**	10.1	9.8	nc	23.5	21.7	22.5	21.4
	Heydornia	0.5	0.1	**0/0.7**	7.1	nc	25.4	22.1	22.3	21.5
	Neospora	0.6	0.1	0.1	**nc/nc**	nc	25.1	21.1	21.3	20.8
	Besnoitia	0.2	1.2	1.2	1.2	**0.8/nc**	nc	nc	nc	nc/nc
Eumonosporinae	*Eumonospora*	1.5	1.4	1.2	1.3	1.1	**nc/nc**	19.5	20.6	19.7
Cystoisosporinae	*Cystoisospora*	2.1	1.8	1.8	1.8	1.9	2.0	**0.7/1.5**	12.5	12.4
Hyaloklossinae	*Hyaloklossia*	3.0	2.7	2.7	2.7	2.6	2.1	2.7	**0.3/nc**	5.4
	Batrachospora	3.1	2.7	2.7	2.7	2.6	2.0	2.5	1.5	**nc/nc**

nc: not calculated, Tox: *Toxoplasma*, Ham: *Hammondia*, Hey: *Heydornia*, Neo: *Neospora*, Bes: *Besnoitia*, Eum: *Eumonospora*, Cys: *Cystoisospora*, Hya: *Hyaloklossia*, Bat: *Batrachospora*.

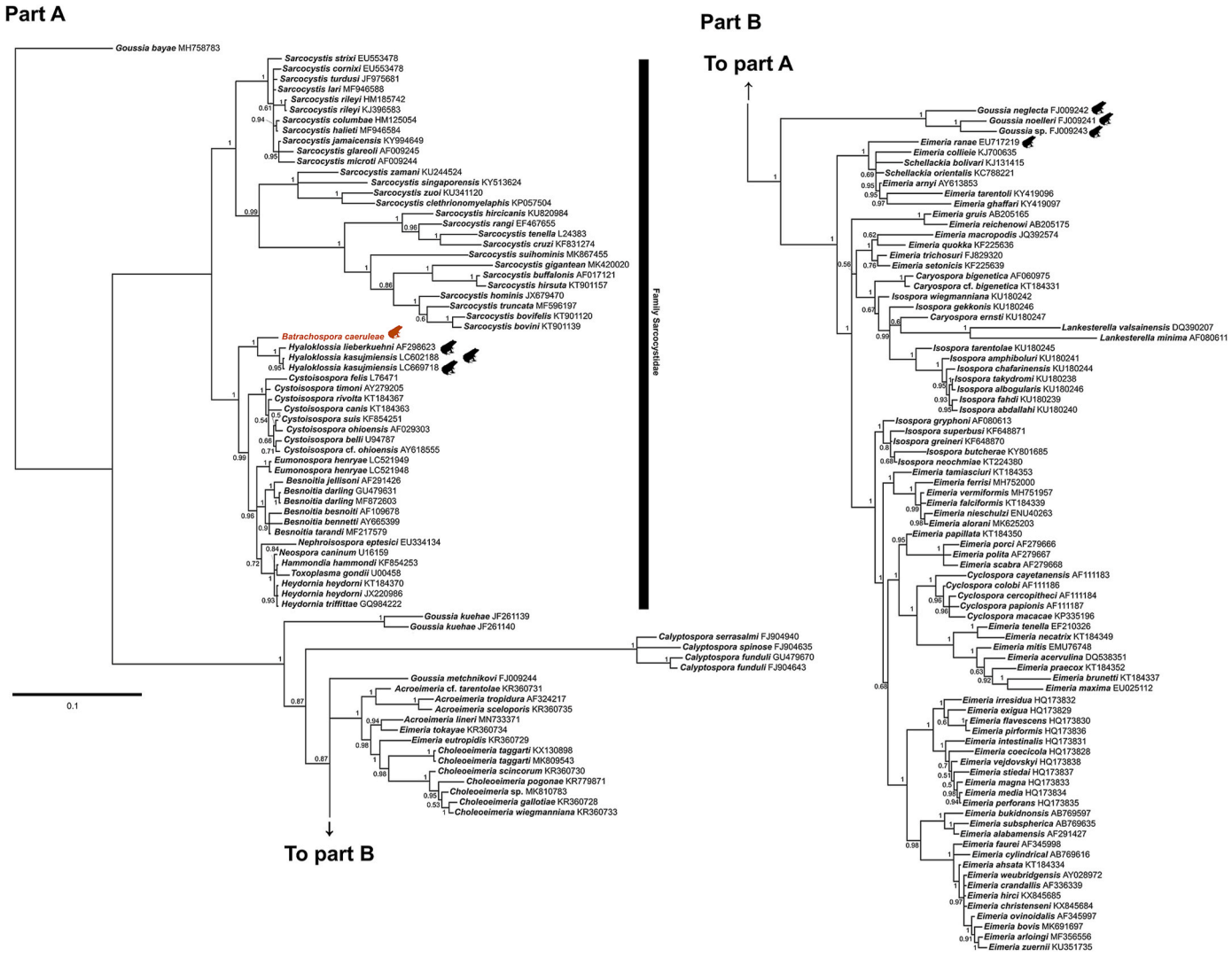

Fig. 3. Bayesian phylogenetic trees based on *18S* sequences. Nodal values indicate Bayesian posterior probabilities. Silhouettes indicates coccidia parasitizing anurans. The scale bar represents substitutions per site.

nos. MN629229, MN629230, KT037081, and KJ634019). Sequence comparison of *COI* showed highest similarity of 94.4–94.6% (100% query cover) to *H. kasumiensis* (LC602189, LC669719, and LC669720). The lowest evolutionary distance with *Batrachospora* was found with *Hyaloklossia*, with values of 1.5% for *18S* and 5.4% for *COI* (Table 2). These values are similar to the intergeneric distance in Toxoplasmatinae (≤1.2% for *18S* and 1.4–10.1% for *COI*), and were higher than the interspecific distance in Sarcocystidae (≤0.8% for *18S* and ≤1.5% for *COI*).

Phylogenetic trees based on the *18S* (Fig. 3) and *COI* sequences (Fig. 4) showed that *B. caeruleae* was clearly separated from *Isospora* spp. (Eimeriidae) which possesses SB. In both trees, members of the Sarcocystidae formed a monophyletic clade that divided into two clades: one consisting of *Sarcocystis* (Sarcocystinae) and the other consisting of

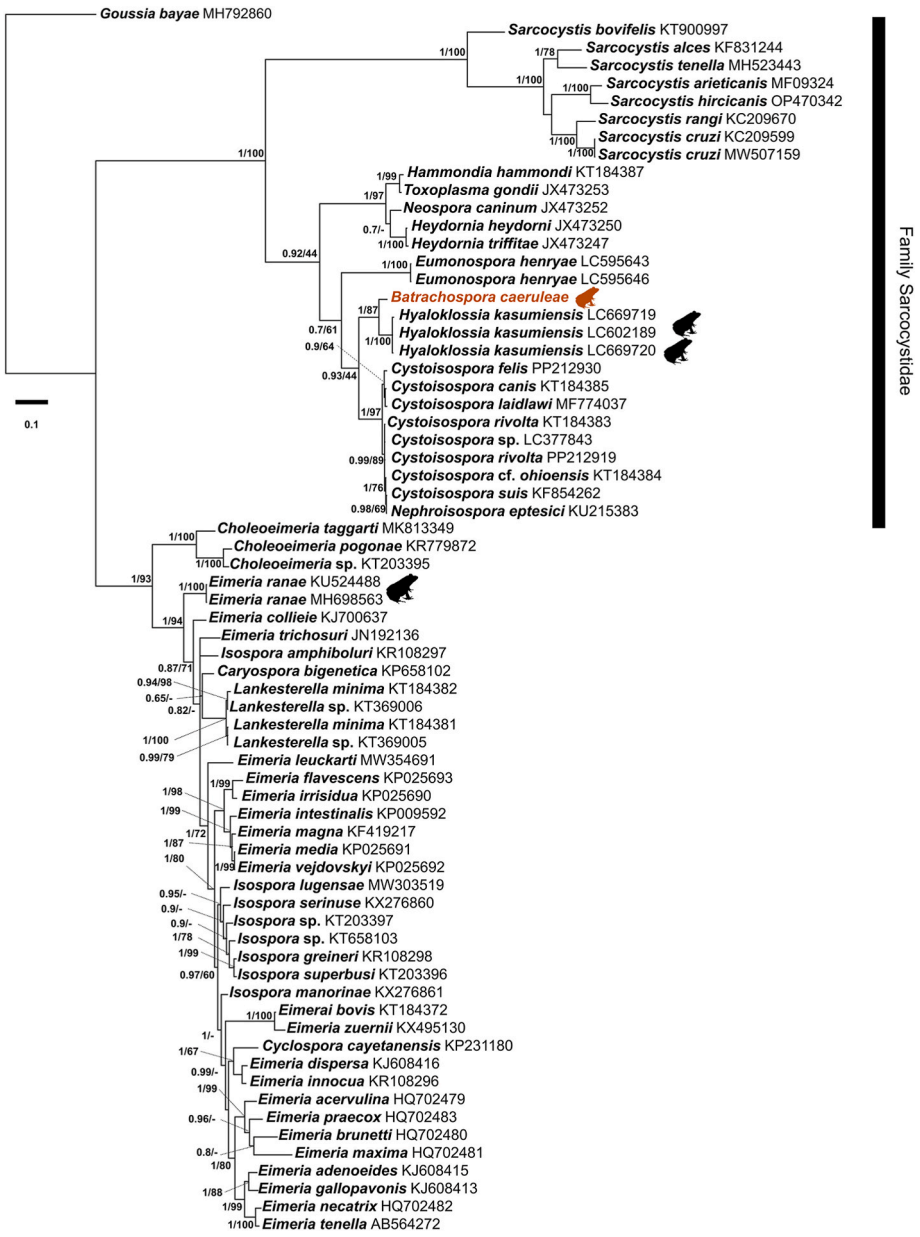

Fig. 4. Bayesian phylogenetic trees based on *COI* sequences. Nodal values indicate Bayesian posterior probabilities (≥0.65, left) and maximum likelihood bootstrap percentage (≥60, right). Silhouettes indicates coccidia parasitizing the Anura. The scale bar represents the substitutions per site.

Toxoplasma, Hammondia, Heydornia, Neospora and *Besnoitia* (Toxoplasmatinae), *Eumonospora* (Eumonosporinae), *Cystoisospora* (Cystoisosporinae), and *Hyaloklossia* (Hyaloklossinae). *Batrachospora* were sister to *Hyaloklossia* with high posterior probabilities (*18S*: 1, *COI*: 1) and bootstrap values (*COI*: 87), but the phylogenetic positions of this clade was unstable, and it diverged earlier in *18S* tree and formed a monophyletic group with *Cystoisospora* in *COI* tree.

4. Discussion

In this study, ten coccidian species with isosporoid-type oocysts and sporocysts without Stieda bodies, nine previously described and one new species, were incorporated into a new genus, *Batrachospora,* within the Sarcocystidae (Table 1). Phylogenetic analyses of partial gene sequences of fragments of *18S* and mitochondrial *COI* genes from isosporoid oocysts from three imported frogs in Japan suggested that isosporoid oocysts without Stieda bodies from anuran hosts no longer belonged in the Eimeriidae. The similarities in the number of sporocysts or sporozoites may represent convergent evolution, whereas the absence of Stieda bodies in Sarcocystidae signifies an important synapomorphy (Franzen et al., 2000; Jirků et al., 2002; Upton, 2002; Barta et al., 2005; Schrenzel et al., 2005; Berto et al., 2014; Chou et al., 2020, 2021).

The complete lifecycle of *Batrachospora* spp. is unknown although the endogenous developmental stages of a few species (*B. brumpti, B. cogginsi, B. cruzi, B. neos, B. stomatici*) has been observed in the epithelial cells of the intestines (Lavier, 1941; Carini, 1936; Chakravarty and Kar, 1952; Kazubski and Grabda-Kazubska, 1973). Although there is no detailed description of the sporulation time in *B. cruzi* and *B. fragosum,* most *Batrachospora* species extracted from feces show immature semi-sporulated oocysts. Furthermore, the oocyst of *Batrachospora* has a thin and fragile wall, suggesting that it may not survive outside the aquatic environment (Duszynski et al., 2007). As adult anurans are carnivorous, the opportunity to ingest oocysts in an aquatic environment is markedly limited. This suggests that tadpoles may be involved in the transmission of *Batrachospora* (Bolek et al., 2003), as observed in *H. lieberkuehni*, *Eimeria* spp., and *Goussia* spp. (Nöller, 1923; Paperna

et al., 1997; Jirků et al., 2009). Herbivorous tadpoles can ingest mature sporocysts while feeding on algae and detritus at the bottom of the pond. *Goussia* infects only tadpoles, and the infection is lost during metamorphosis (Paperna et al., 1997; Jirků et al., 2009). Contrastingly, *Batrachospora, Eimeria*, and *Hyaloklossia* infections have been detected in adult frogs (Bolek et al., 2003; Tokiwa et al., 2022a, 2022b). Thus, the possibility of an indirect life cycle involving intermediate or paratenic hosts cannot be ruled out. However, it seems more likely that the infection is maintained even after the tadpoles undergo metamorphosis. We hypothesize that stagnant freshwater environments such as ponds, paddy fields, ditches, and water tanks may be important foci of infection for *Batrachospora* species.

Funding

Founding for this study was provided by Japan Society for Promotion of Science KAKENHI Grant Number 21K06323, Japan (T. Tokiwa).

CRediT authorship contribution statement

Toshihiro Tokiwa: Writing – review & editing, Writing – original draft, Visualization, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Riona Morizane:** Resources, Formal analysis. **Shyun Chou:** Writing – review & editing, Formal analysis, Data curation. **Donald W. Duszynski:** Writing – review & editing, Writing – original draft, Validation, Supervision.

Conflict of interest

The authors have no conflict of interest to report.

Acknowledgments

We would like to thank Dr. Kenichi Tamukai (Den-en-chofu Animal Hospital, Tokyo, Japan) for providing the idea that inspires this research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2024.101020.

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