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Faculty of Science

Isosporan oocysts in the faeces of bank voles (*Myodes glareolus*; Arvicolinae, Rodentia): real parasites, or pseudoparasites?

RNDr. Thesis

Bc. Aneta Trefancová

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Annotation:

This study concerns clarification of the origin of infections of arvicoline rodents with *Isospora* spp. based on three different approaches: phylogenetic analyses of three genes (18S rRNA, COI and COIII), morphological and morphometrical analyses, and experimental infection. Field collections, parasitological examinations of samples, microscopy, DNA extraction, PCR, and computational analyses were employed during the course of this study.

Declaration [in Czech]:

Prohlašuji, že svoji rigorózní práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury. Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své rigorózní práce, a to v nezkrácené podobě elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

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Aneta Trefancová

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Authors' contributions:

AT: ~65%

AM: ~5%

JK: ~30%

JK and AT designed the study. AT, AM and JK participated in the field studies. AT carried out coprological examination of faeces of voles, morphological and morphometrical analyses of coccidia, DNA extraction, PCR amplification, processing of obtained sequences and phylogenetic analyses, and participated in graphical editing and manuscript preparation. AT and JK designed and performed experimental infection of voles with coccidian isolates. AM provided 2 sequences of the mitochondrial COI gene, and 1 sequence of the nuclear 18S rRNA gene from the snow bunting (*Plectrophenax nivalis*). JK participated in graphical editing and manuscript preparation.

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ORIGINAL PAPER

Isosporan Oocysts in the Faeces of Bank Voles (*Myodes glareolus*; Arvicolinae, Rodentia): Real Parasites, or Pseudoparasites?

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Coccidia of the genus *Isospora*, their origin, taxonomy, and host specificity have been discussed for many years. The crucial point in question being the division of the genus, based on distinct evolutionary history and the presence/absence of the Stieda body, into the genera *Isospora* (Eimeriidae) parasitizing mainly birds and reptiles, and *Cystoisospora* (Sarcocystidae) parasitizing mammals. The description of the majority of *Isospora* species from rodents is based solely on the oocysts found in their faeces. Some of them have been described with the presence of the Stieda body, some without it, and, simultaneously, for all the described species the molecular data are entirely lacking. This study reveals the origin of isosporan oocysts found in faeces of bank voles based on morphological analyses, phylogenetic analyses, and experimental infections. Morphological analyses showed the presence of the Stieda body complex on sporocysts. Phylogenetic analyses demonstrated close phylogenetic relationships between *Isospora* from bank voles and avian isosporans. Experimental inoculations of bank voles with sporulated oocysts of *Isospora* did not result in the production of unsporulated oocysts. Hence, these organisms should be considered pseudoparasites of the bank voles/rodents (probably originating from avian *Isospora* species).

Key words: *Isospora*; *Cystoisospora*; coccidian; rodent; vole; pseudoparasite.

Introduction

The genus *Isospora* (Apicomplexa: Coccidia: Eimeriorina) was discovered and described by Schneider in 1881, being then most often detected in the faeces of birds, dogs, cats, and also rodents. However, the first described species, *Isospora rara* (Schneider 1881), was found in an invertebrate host, the gastropod *Limax* sp. (Duszynski and Upton 2001; Ghimire 2010; Levine 1982; Pellérdy 1974). From the 1970s through to the 1990s, there was a significant boom in describing new species of coccidia (particularly eimerians), the majority of descriptions being based solely on the oocysts found in the faeces of a presumed host. Moreover, in some cases, identical species were described under different names (assuming their high host

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specificity). However, the majority of these early described species have not been further confirmed by neither experimental nor molecular methods. Today, it is supposed that in some cases they may represent pseudoparasites (Ghimire 2010), i.e. parasites transferred via the paratenic/transport hosts, or accidentally passing through the gastrointestinal tract of nonspecific hosts. Pseudoparasitism may concern even the type species *I. rara*, because Schneider (1881) did not clearly prove the origin of infection in the gastropod (Ghimire 2010; Pellérdy 1974). Moreover, since then, no other *Isospora* spp. have been detected/described from the invertebrates (Ghimire 2010; Levine 1988).

Oocysts of *Isospora*-type (i.e. with two sporocysts, each containing four sporozoites), routinely found in the faeces of mammals, have for decades presented questions and conundrums (e.g. Barnard et al. 1974; Barta et al. 2005; Carreno and Barta 1999; Ernst et al. 1969; Ghimire 2010; Levine and Ivens 1990; Levine and Mohan 1960; Morrison et al. 2004; Prasad 1961; Smith 1981; Streitel and Dubey 1976). Since the discovery that isosporans infecting mammals are phylogenetically related to Sarcocystidae and that their sporocysts always lack Stieda bodies (SB), whereas isosporans infecting birds are related to Eimeriidae and possess SB (Barta et al. 2005; Carreno et al. 1998; Franzen et al. 2000; Samarasinghe et al. 2008), the situation has become even more complicated. Thus, the SB, not only regarding its presence or absence, but also its shape and size, has come to provide the key morphological trait. At present, all known *Isospora* species from mammals that lack the SB have been reassigned to the genus *Cystoisospora*, as initially proposed by Frenkel (1977). Moreover, members of the *Cystoisospora* (i.e. without SB) often have a heteroxenous life cycle with secondary intermediate/paratenic hosts (e.g. rodents) that harbour asexual, monozoic tissue cyst stages typically found in mesenteric lymph nodes, some of them also possessing extraintestinal stages occurring in various organs and tissues (Dubey 1975, 1978a, 1978b; Dubey and Frenkel 1972; Smith 1981). The sporulated oocysts of *Cystoisospora* spp. can infect both intermediate as well as definitive host.

For the majority of *Isospora* species (i.e. with SB) described from mammals, there is no knowledge on life cycles, no cross-transmission studies, nor any detailed studies on their endogenous developmental stages. The only exception is *Isospora masoni* described from the hispid cotton rat (*Sigmodon hispidus*; Rodentia: Cricetidae) by Upton et al. (1985) who carried out an experimental transmission study as well as a detailed study on its endogenous development. This species was described possessing the SB complex. Nonetheless, there are currently no molecular data verifying its classification into the genus *Isospora*. Thus, oocysts of *Isospora* found in the faeces of mammals are considered to be spurious findings from prey items – usually birds – merely passing through the mammalś gut (Ghimire 2010; Prasad 1961; Streitel and Dubey 1976). However, there are no complex studies verifying this hypothesis. A similar phenomenon was observed for example in adeleid coccidia (Adeleorina) parasitizing invertebrates, and their ability to pass through the gastrointestinal tract of vertebrates (Berto et al. 2008, 2010; Teixeira et al. 2003). This study aims to reveal the origin of *Isospora*-type oocysts found in the faeces of bank voles (*Myodes glareolus*; Rodentia: Arvicolinae) based on three different approaches – morphological analyses, experimental infections, and molecular methods.

Table 1. List of sampled localities and numbers of trapped bank voles (*M. glareolus*) in the course of 2015–2017.

Locality	2015 (no. of *M. glareolus*)	2016 (no. of *M. glareolus*)	2017 (no. of *M. glareolus*)	Total number of trapped animals
České Budějovice (South Bohemian Region)	1	10	8	19
Klášterec nad Ohří (Ústí nad Labem Region)	–	–	6	6
Litvínov (Ústí nad Labem Region)	–	8	202	210
Lužnice (South Bohemian Region)	30	20	13	63
Stružná (Karlovy Vary Region)	–	8	–	8
Třísov (South Bohemian Region)	9	–	–	9
Tymákov (Pilsen Region)	1	2	20	23
Total	41	48	249	**338**

Results

Sampling and Coprological Examination

Faeces of 338 voles from snap traps were collected from 2015 to 2017 in the Czech Republic (Table 1). Only 17/338 (5%) vole faecal samples were *Isospora*-positive, while 93/338 (27.5%) were *Eimeria*-positive. Most of the *Isospora*-positive samples were obtained in the surroundings of Tymákov (Pilsen Region), where the prevalence in 2017 surprisingly reached 34.8% (Table 2).

Table 2. List of *Isospora*-positive *M. glareolus* trapped using the classic wooden snap traps, their origin, period of collection, and intensity of infection.

Sample code	Sex	Locality	Period of collection	Intensity of infection
50_MG_PLE	♀	Plešnice (Pilsen Region)	September 2015	I++
49_MG_TYM	♂	Tymákov (Pilsen Region)	March 2016	I+++
57_MG_TYM	♂	Tymákov (Pilsen Region)	March 2016	I+++
60_MG_TYM	♀	Tymákov (Pilsen Region)	March 2016	I++
89_MG_TYM	♂	Tymákov (Pilsen Region)	May 2016	I+++
6_MG_LUZ	♀	Lužnice (South Bohemian Region)	June 2016	I+
7_MG_LUZ	♀	Lužnice (South Bohemian Region)	June 2016	I+++
P14_MG_LIT	♀	Litvínov (Ústí nad Labem Region)	October 2016	I+
9_MG_LUZ	♀	Lužnice (South Bohemian Region)	June 2017	I+++
2_MG_TYM	♀	Tymákov (Pilsen Region)	August 2017	I+++
7_MG_TYM	♂	Tymákov (Pilsen Region)	September 2017	I++
13_MG_TYM	♀	Tymákov (Pilsen Region)	September 2017	I+
18_MG_TYM	♀	Tymákov (Pilsen Region)	September 2017	I+

I+ slight infection; I++ moderate infection; I+++ heavy infection.

Table 3. List of the live-trapped *Isospora*-negative *M. glareolus* used for the experimental infections, their origin, and period of collection.

Sample code	Sex	Locality	Period of collection
11_MG_LUZ	♂	Lužnice (South Bohemian Region)	June 2017
1_MG_TYM	♀	Tymákov (Pilsen Region)	August 2017
12_MG_TYM	♂	Tymákov (Pilsen Region)	September 2017
15_MG_TYM	♀	Tymákov (Pilsen Region)	September 2017

Table 4. Coprological examination of bank voles used for the experimental infections, and schedule of the experimental infections.

	11MG_LUZ_17	1MG_TYM_17	12MG_TYM_17	15MG_TYM_17
Microscopy before the treatment with Baycox®	Negative	Coccidia-negative, eggs of *Capillaria* ++	Coccidia-negative, eggs of *Trichuris*+	Coccidia-negative, eggs of *Trichuris* +++
Baycox® 1st application	19/09/2017	19/09/2017	19/09/2017	19/09/2017
Baycox® 2nd application	22/09/2017	22/09/2017	22/09/2017	22/09/2017
Microscopy after the treatment with Baycox®	Negative	Coccidia-negative, *Capillaria* ++	Coccidia-negative, *Trichuris* +	Coccidia-negative, *Trichuris* +++
Experimental infection	10/10/2017 isolate 9MGLUZ/17	10/10/2017 isolate 2MGTYM/17	10/10/2017 isolate 9MGLUZ/17	10/10/2017 isolate 2MGTYM/17

Table 5. Isosporan species described from rodent hosts. M, micropyle; OR, oocyst residuum; OW, oocyst wall; SB, Stieda body; highlighted are those species that were described from rodents of the subfamily Arvicolinae.

Species	Type host	Type locality	Shape and size of oocysts	Shape and size of sporocysts	Presence of SB	Other
Isospora anatolicum Sayin, Dincer & Meric, 1977	*Spalax leucodon* (Spalacidae)	Asia	Spherical; 9–11 × 8–9	Ovoid; 6–9 × 4–6	No	No OR or M; smooth pale greenish yellow OW (0.8)
Isospora arvalis Mikeladze, 1973	*Microtus arvalis* (Muridae)	Russia	Subspherical; 10–12 × 8–12	Ovoid; 6–8 × 4–6	No	No OR or M; smooth colorless OW (2)
Isospora assensis Svanbaev, 1979	*Spermophilus fulvus* (Sciuridae)	Russia	Ovoid – spherical; 18–19 × 18–24	Ellipsoidal – spherical; 8–13 × 7–11	No	OR present; no M; smooth, double-contoured OW (1.5–2)
Isospora aurangabadensis Kshirsagar, 1980	*Rattus rattus* (Muridae)	India	Spherical or subspherical; 32–44 × 32–40	Ovoid, globose or elongate	Yes	No OR or M; yellowish brown OW (1.5–2)
Isospora batabatica Musaev & Veisov, 1960	*Arvicola amphibius* (Muridae)	Russia	Almost spherical or ovoid; 20–24 × 19–21	Ovoid; 9–14 × 6–9	Yes	No OR or M; smooth colorless double-contoured OW (1)
Isospora californica Davis, 1967	*Peromyscus californicus* (Muridae)	North America	Spherical to ellipsoidal or ovoid; 18–32 × 18–27	Ovoid to lemon-shaped; 13–20 × 8–13	Yes	No OR or M; smooth grey-green to light brown OW (1)
Isospora calomyscus Musaev & Veisov, 1965	*Calomyscus bailwardi* (Muridae)	Russia	Ellipsoidal; 20–23 × 16–20	Ovoid; 14–17 × 10–15	Yes	No OR or M; smooth, yellow-brown OW (1.5)
Isospora citelli Levine, Ivens & Kruidenier, 1957	*Spermophilus variegatus* (Sciuridae)	North America, Russia	Subspherical; 22–23 × 21–22	Broadly lemon-shaped; about 15 × 10	Yes	No OR or M; smooth brownish OW (1)
Isospora clethrionomydis Golemanski & Yankova, 1973	*Myodes glareolus* (Muridae)	Europe (Bulgaria)	Spherical; 23–27 × 23–27	Ovoid; 21–23 × 11–12	Yes	No OR or M; thin colorless or light yellowish OW
Isospora clethrionomysis Arnastauskiene & Maldzhiunaite, 1981	*M. glareolus* (Muridae)	Eastern Europe (Lithuania)	9–11 × 7–10	Ovoid to spindle-shaped	No record	No record

Table 5 (*Continued*)

Species	Type host	Type locality	Shape and size of oocysts	Shape and size of sporocysts	Presence of SB	Other
Isospora dawadimiensis Kasim & Al Shawa, 1985	*Jaculus jaculus* (Dipodidae)	Saudi Arabia	Ovoid or almost spherical; 22–26.5 × 20.5–22	Ellipsoidal; 12–16.5 × 9–10.5	No	No OR or M; smooth, light brown or pale green OW about (1)
Isospora dryomidis Glebezdin, 1974	*Dryomys nitedula* (Myoxidae)	Russia	Ellipsoidal; 23–26 × 20–23	Ellipsoidal; 12–15 × 9–12	No	No OR or M; smooth, colorless OW
Isospora egypti Prasad, 1960	*Meriones shawi* (Muridae)	Africa	Subspherical; 20–22 × 16–20	Ovoid; 10–12 × 6–8	Yes	No OR or M; smooth light brown OW
Isospora erythrourica Veisov, 1964	*Meriones libycus* (Muridae)	Russia	Spherical or subspherical; about 24–30 × 24-30	Ellipsoidal or spherical; 12–18 × 10–16	Probably not	No OR or M; smooth colorless – yellow–brown OW about (1.5–2.5)
Isospora freundi Yakimoff & Gousseff, 1935	*Cricetus cricetus* (Muridae)	Russia	Spherical or subspherical; 13–27 × 17–24	14 × 8–9	No	No OR or M; smooth double-contoured OW
Isospora golemanskii Levine, 1982	*Apodemus flavicollis* (Muridae)	Europe	Spherical or subspherical; 23–28 × 20-28	Ovoid; 12–16 × 8-11	No record	No OR or M
Isospora hammondi Barnard, Ernst & Stevens, 1971	*Oryzomys palustris* (Muridae)	North America	Ovoid; 24–30 × 16–21	Ellipsoidal; 13–18 × 11–15	Yes	No OR or M; smooth colorless to pinkish OW about (1)
Isospora hastingsi Davis, 1967	*Peromyscus truei* (Muridae)	North America	Ovoid; 29–33 × 22–24	Lemon-shaped	Yes	No OR or M; smooth tan or yellow-tan OW about (1.2)
Isospora krishnamurthyi Kshirsagar, 1980	*R. rattus* (Muridae)	India	Spherical or slightly ovoid; 36–48 × 35–40	Ovoid; 21–26 × 17–22	Yes	No OR or M; pale yellowish OW (1.8)
Isospora laguri Iwanoff-Gobzem, 1934	*Lagurus lagurus* (Muridae)	Russia	Ovoid; 24–32 × 16–22	16–21 × 8–13	No record	OR present; no M; thick OW
*Isospora * masoni* Upton, Lindsay, Current & Ernst, 1985	*Sigmodon hispidus* (Muridae)	North America	Ellipsoidal	Ovoid; 7–9 × 5–6	Yes	No OR or M; smooth, thin membrane-like OW

Table 5 (*Continued*)

Species	Type host	Type locality	Shape and size of oocysts	Shape and size of sporocysts	Presence of SB	Other
Isospora mcdowelli Saxe, Levine & Ivens, 1960	*Microtus pennsylvanicus* (Muridae)	North America	Spherical to subspherical; 9–11 × 8–10	Ellipsoidal; 6–8 × 4–5	No record	No OR or M; thin OW
Isospora meriones Musaev & Veisov, 1965	*Meriones vinogradovi* (Muridae)	Russia	Ellipsoidal; 18–28 × 14–24	Piriform; 10–16 × 6–10	Yes	No OR or M; smooth colorless – yellow–brown OW about (1.5-2)
Isospora mexicanasubsimi Vance & Duszynski, 1985	*Microtus mexicanus subsimus* (Muridae)	Mexico	Spherical; 21–26 × 21–26	Ovoid; 12–16 × 10–12	Yes	No OR or M; smooth OW (1.5)
Isospora ordubadica Musaev & Veisov, 1960	*Meriones persicus* (Muridae)	Russia	Ovoid or subspherical; 18–20 × 14–18	Ovoid to ellipsoidal; 10–12 × 8–10	No	No OR or M; smooth colorless OW (1)
*Isospora * peromysci* Davis, 1967	*Peromyscus maniculatus* (Muridae)	North America	Elongate or ellipsoidal; 25–43 × 14–28	Broadly ovoid; 13–21 × 11–15	No	No OR or M; smooth, pale green, tan, or light brown OW (0.8)
Isospora ratti Levine & Ivens, 1965	*Rattus norvegicus* (Muridae)	North America	Subspherical; 22–24 × 20–21	Symmetrical, broadly ovoid; about 16 × 11	Yes	No OR or M; smooth, pale tan to tan OW (1)
Isospora samsensis Svanbaev, 1979	*Spermophilus fulvus* (Sciuridae)	Russia	Ovoid; 25–32 × 22–26	Ellipsoidal; 11–13 × 8–9	No	No OR or M; smooth greenish OW (1.2–1.5)
Isospora sp.* Barnard, Ernst & Dixon, 1974	*S. hispidus* (Muridae)	North America (Alabama)	Subspherical to ovoid; 21–28 × 20–25	Ovoid; 14–18 × 9–12	Yes	No OR or M; smooth, reddish to light brown OW (1.3)
Isospora sp. Nukerbaeva & Svanbaev, 1973	*Myocastor coypus* (Myocastoridae)	Russia	Short-oval to spherical; 25–28 × 25–28	About 14 × 11	No record	OR present; no M; OW (1.5)
Isospora sp. Stout & Duszynski, 1983	*Dipodomys agilis* (Heteromyidae)	North America	Spherical or subspherical; 21–28 × 20–28	Broadly ovoid; 12–19 × 9–13	Yes	No OR or M; smooth pale yellow OW (1.6)

Table 5 (*Continued*)

Species	Type host	Type locality	Shape and size of oocysts	Shape and size of sporocysts	Presence of SB	Other
Isospora sp. * Svanbaev, 1963	*Marmota menzbieri* (Sciuridae)	Russia	Short-ovoid to spherical; 20–22 × 19–20	Ovoid; 14–15 × 7–8	No record	No OR or M; smooth, greenish; double-contoured OW (1)
Isospora spermophili Levine, 1984	*Spermophilus maximus* (Sciuridae)	Russia	Ellipsoidal, subspherical or spherical; 18–29 × 18–24	Ellipsoidal or spherical; 8–13 × 7–11	No record	OR present; no M; smooth yellow–green or brown OW (1.5–2)
Isospora tamariscini Levine, 1985	*Meriones tamariscinus* (Muridae)	Russia	Ellipsoidal or subspherical; 21–30 × 20–26	Ellipsoidal or ovoid; 12–14 × 7–10	No record	OR present; no M; smooth yellow-green OW (1.5–1.9)
Isospora teres Iwanoff-Gobzem, 1934	*L. lagurus* (Muridae)	Russia	Spherical; 24–36 × 24–36	16–21 × 8–13	No record	No OR or M
Isospora uralicae Svanbaev, 1956	*Apodemus sylvaticus* (Muridae)	Russia	Ovoid; about 26 × 22.5	Ovoid; about 14 × 9	No	No OR or M; smooth greenish double-contoured OW (1.6)
Isospora vanadica Musaev & Veisov, 1965	*Meriones persicus* (Muridae)	Russia	Ellipsoidal or ovoid; 20–28 × 14–24	Ovoid; 10–16 × 6-12	No	OR present; no M; yellowish to dark brown OW (1.5–2.5)
Isospora vinogradovi Musaev & Veisov, 1965	*M. vinogradovi* (Muridae)	Russia	Ellipsoidal, rarely ovoid; 22–28 × 18–24	Piriform; 12–16 × 8–10	Yes	OR present; no M; smooth colorless OW (2–2.5)

Using the Sherman live traps, 11 bank voles were trapped in the South Bohemian Region (Lužnice) and Pilsen Region (Tymákov). Four of them were used for the experimental infections (Table 3). All live, repeatedly examined individuals predetermined for the experimental infections, were coccidia-negative. Three of them were repeatedly positive for eggs of intestinal nematodes (*Capillaria* sp. and *Trichuris* sp.) (Table 4).

Morphology and Morphometry

Morphological analyses showed that all sporocysts in *Isospora*-type oocysts found in the faeces of bank voles possessed SB and substieda bodies (sSB). This feature reinforces their presumed phylogenetic affinity to bird isosporans (family Eimeriidae). The samples used for the morphological and morphometrical analyses are described below (MG, *Myodes glareolus*; TYM, locality Tymákov; LUZ, locality Lužnice). Out of them, 2_MG_TYM and 9_MG_LUZ were used for the experimental infections. All given measurements (including those in Table 5) are in micrometers (μm), with the means given in parentheses following the ranges.

2_MG_TYM (Fig. 1)

Oocysts were ovoidal to ellipsoidal, 24.0–29.0 × 19.0–24.0 (25.8 × 21.5) with a smooth, bi-layered oocyst wall (OW) approximately 1.2–1.4 thick. The colour of the wall was pale yellow–green. Neither micropyle (M) nor oocyst residuum (OR) were present. The oocysts possessed at least 3 distinct polar granules (PGs) of irregular shape. Sporocysts were ovoidal to ellipsoidal, 14.0–17.2 × 9.0–11.0 (15.0 × 10.1) with a thin, colorless wall. The sporocysts possessed a conspicuous nipple-like SB (1.2 × 1.0), and wide-rounded sSB. The parastieda body (pSB) was absent. The sporocyst residuum (SR) was present as a relatively compact structure composed of

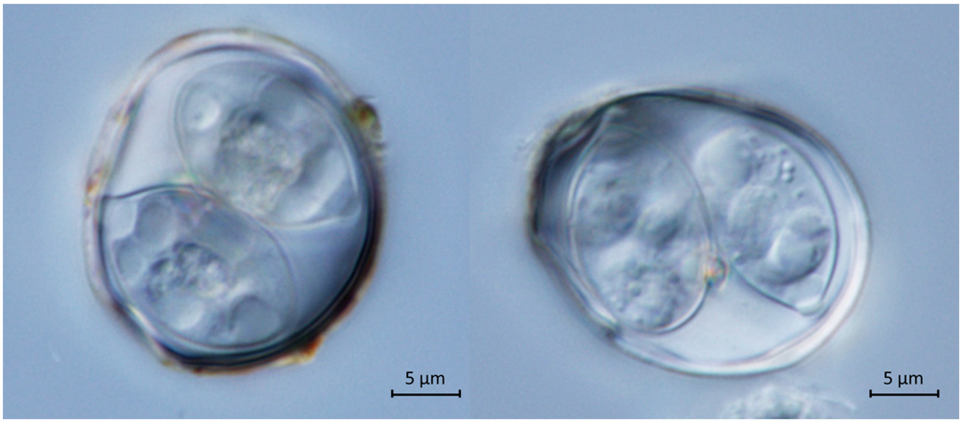

Figure 1. Sporulated oocysts obtained from *M. glareolus* isolate 2_MG_TYM (Tymákov, Pilsen Region, CZ).

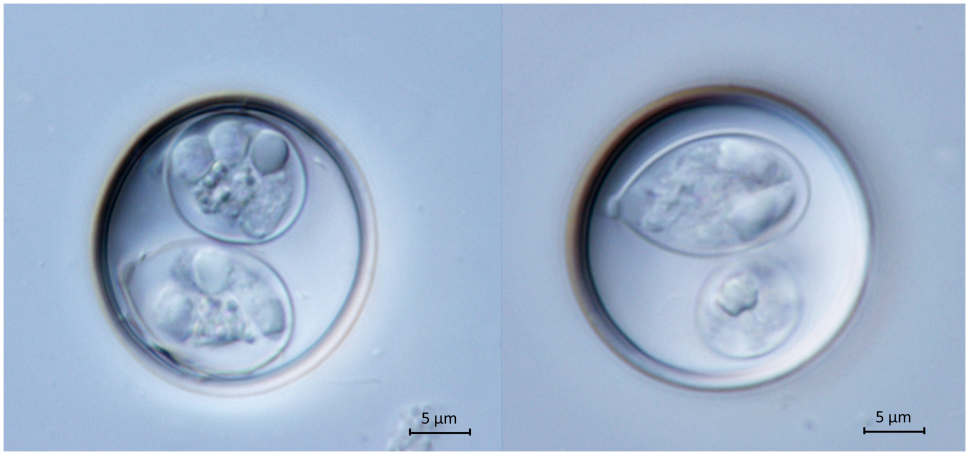

Figure 2. Sporulated oocysts obtained from *M. glareolus* isolate 49_MG_TYM (Tymákov, Pilsen Region, CZ).

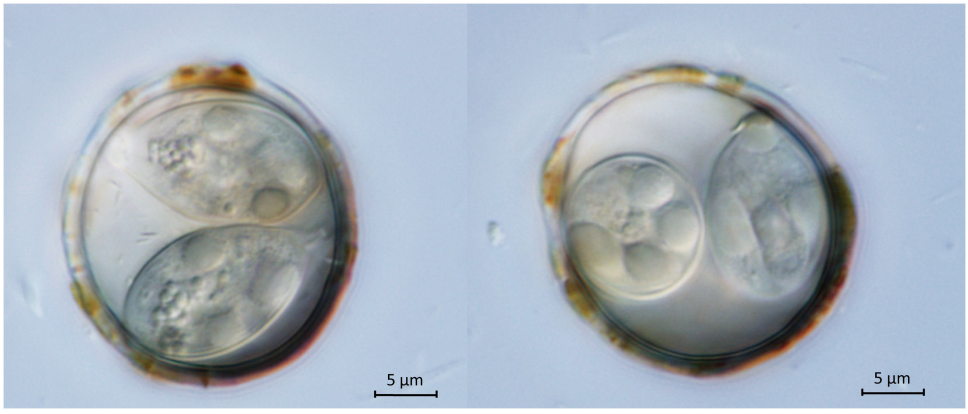

Figure 3. Sporulated oocysts obtained from *M. glareolus* isolate 57_MG_TYM (Tymákov, Pilsen Region, CZ).

large globules. Sporozoites were elongate, with globular to oval posterior and anterior refractile bodies, and the nucleus located between them (in the middle).

49_MG_TYM (Fig. 2)

Oocysts were spherical to subspherical, 23.5–28.3 × 22.6–27.8 (25.8 × 24.1) with a smooth, bi-layered and relatively thick OW (approximately 1.6). The colour of the wall was pale brown. Oocysts were without M, with a small poorly apparent compact OR. The oocysts possessed 2–4 splinter-like PGs. Sporocysts were ellipsoidal to bottle-shaped, 14.3–19.0 × 9.8–11.4 (15.9 × 10.7) with a thin, colorless wall. The sporocysts possessed nipple-like SB 1.1 × 0.7, and indistinct sSB. The pSB was absent. The SR was present as globules of various sizes scattered among SPs. SPs were elongate, with posterior and anterior globular refractile bodies, and the nucleus located between them (in the middle).

57_MG_TYM (Fig. 3)

Oocysts were spherical to subspherical, 25.8–29.3 × 24.1–27.0 (27.7 × 25.7) with a smooth, bi-layered OW approximately 1.8 thick. The colour of the wall was yellowish to brownish. Neither M nor OR were present. The oocysts possessed 2–3 PGs of irregular shape and various size. Sporocysts were ellipsoidal to bottle-shaped, 13.7–18.7 × 9.4–12.1 (16.4 × 10.7) with a thin, colorless wall. The sporocysts possessed knob-like SB 1.3 × 1.0, with rounded to conical sSB. The pSB body was absent. The SR comprised of large globules that were dispersed among SPs. SPs were elongate, with large posterior and small anterior refractile bodies of globular shape, and the nucleus located between them (in the middle).

7_MG_LUZ (Fig. 4)

Oocysts were subspherical, 25.0–30.0 × 23.0–24.0 (28.3 × 23.7) with a smooth, bi-layered and relatively thick OW (approximately 1.8). The colour of the wall was pale yellow–brown. The oocysts lacked M, and possessed a poorly apparent compact OR. A single globular PG was present. Sporocysts were ellipsoidal, 15.7–18.9 × 10.5–12.0 (17.3 × 11.3) with a thin, colorless wall. The sporocysts possessed a knob-like SB 1.2 × 1.0, and wide-rounded sSB. The pSB was absent. The SR was present as a compact structure composed of small globules. SPs were elongate, with globular to bean-shaped

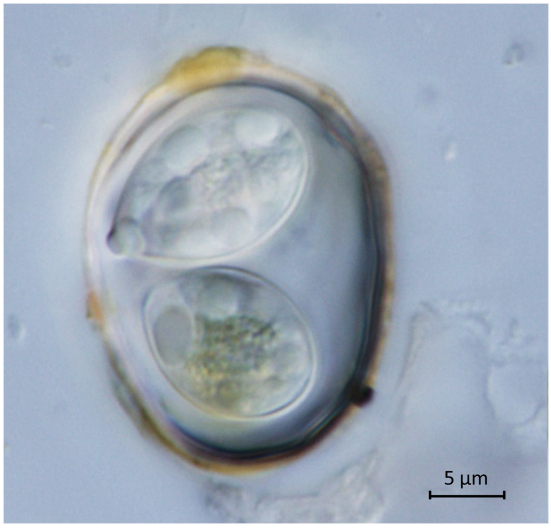

Figure 4. Sporulated oocysts obtained from *M. glareolus* isolate 7_MG_LUZ (Lužnice, South Bohemian Region, CZ).

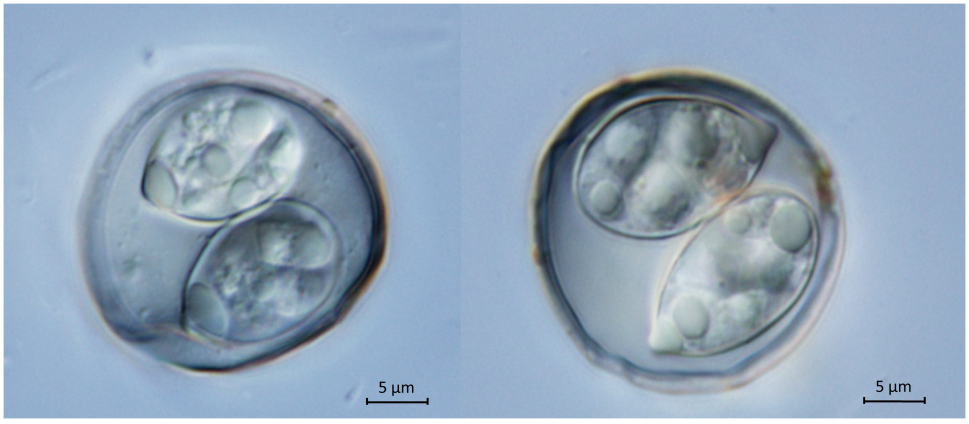

Figure 5. Sporulated oocysts obtained from *M. glareolus* isolate 9_MG_LUZ (Lužnice, South Bohemian Region, CZ).

posterior and anterior refractile bodies, and the nucleus located between them (in the middle).

9_MG_LUZ (Fig. 5)

Oocysts were subspherical to ellipsoidal, 24.0–28.4 × 19.0–25.0 (26.1 × 22.4) with a smooth, bi-layered OW approximately 1.4 thick. The colour of the wall was pale yellow-green. Neither M nor OR were present. The oocysts possessed at least 1 distinct PG of globular shape. Sporocysts were ellipsoidal, 14.0–18.6 × 9.2–12.0 (16.0 × 10.3) with a thin, colorless wall. The sporocysts possessed a conspicuous SB of triangular shape 1.2 × 1.1, and sSB of rounded shape. The pSB was absent. The SR was present in a form of compact small globules apparently surrounded by a thin membrane. SPs were elongate, with posterior and anterior refractile bodies of globular shape, and the nucleus located between them (in the middle).

Phylogenetic Analyses

Phylogenetic analyses of all 3 genes showed that *Isospora* spp. obtained from arvicoline rodents (mainly bank voles) are closely related to the isosporans of birds (family Eimeriidae).

The sequences of *Eimeria* spp., obtained mostly from bank voles trapped in the Czech Republic, were also included in the phylogenetic analyses. All phylograms showed that these eimerians formed phylogenetically distinct lineages separated from isosporans obtained from arvicoline rodents/voles/bank voles.

Furthermore, this study shows that the genus *Isospora* (Eimeriidae) is not a monophyletic, as proposed by Barta et al. (2005), but a paraphyletic taxon. These findings are best seen in the phylogenetic tree of 18S rDNA (Fig. 6), where the isosporans of reptiles (mostly from lizards) cluster almost on the basal position of the eimeriid coccidia, while isosporans of birds cluster within the family Eimeriidae, surprisingly close to the eimerians of rabbits. The paraphyly of the genus *Isospora* has also been recently proposed; based on these findings, establishing a number of new genera of eimeriid coccidia based on molecular phylogeny (monophyletic clades) has been discussed (Ogedengbe et al. 2018).

Experimental Infections

The experimental infections also supported our hypothesis of pseudoparasitism. Infections did not develop in any of the bank voles administered with the fully sporulated *Isospora*-like oocysts. It was, however, observed that for the first 4 days after the inoculation the bank voles shed more or less deformed/damaged oocysts passing through their gastrointestinal tract (Fig. 9). Based on these findings we can assume that it is not a real infection, but an accidental passage through the gastrointestinal tract of these rodents.

Discussion

The main problem of *Isospora* classification, species description, and host specificity determination is currently posed by the samples found in the faeces of mammals. Based on the relatively recent phylogenetic studies, a reclassification of the genus *Isospora* was carried out. The studies showed that isosporans infecting mammals are phylogenetically related to Sarcocystidae, while isosporans infecting

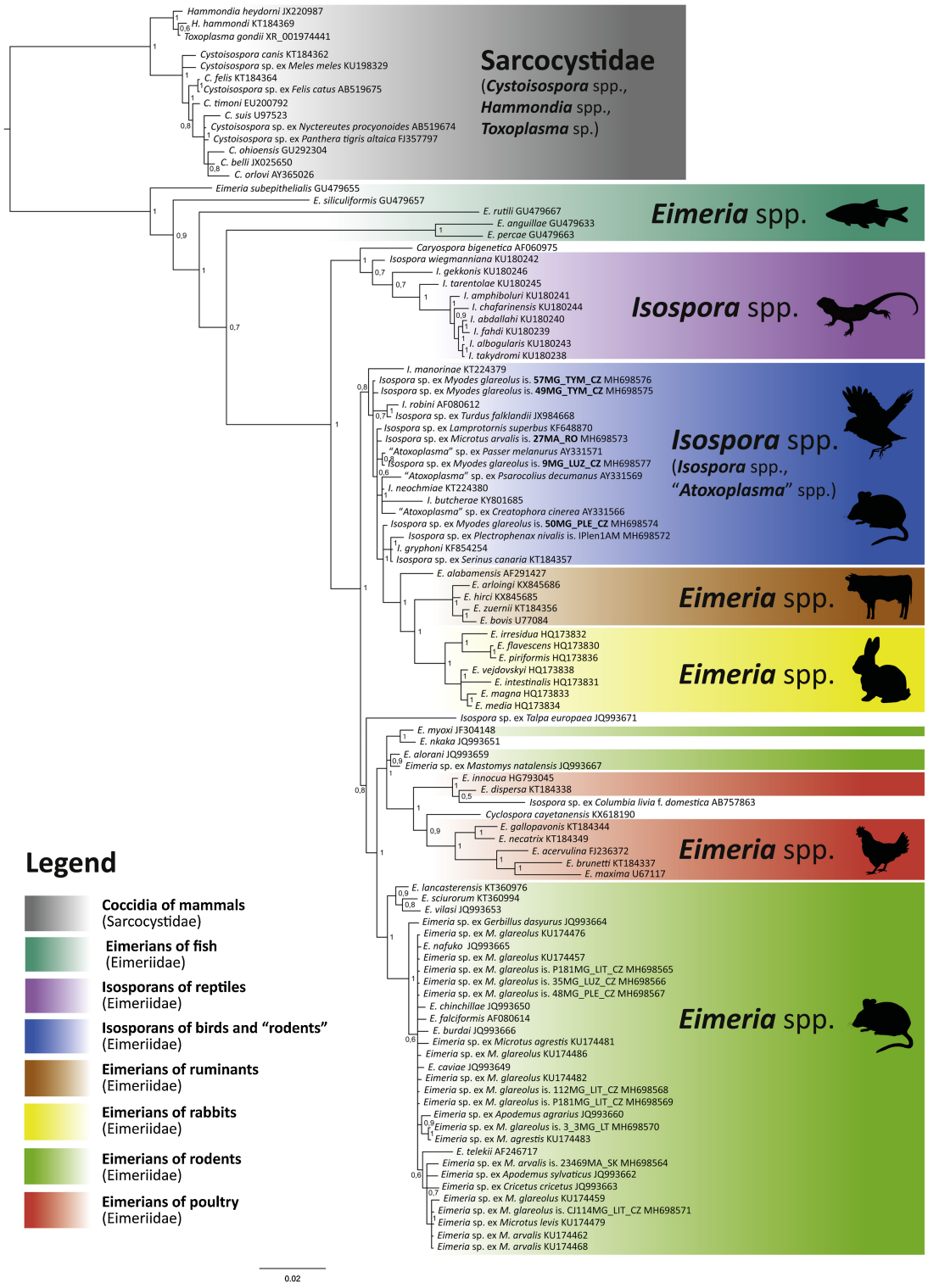

Figure 6. Phylogenetic relationships inferred by the BI analysis of the 18S rRNA sequences. Numbers at the nodes show posterior probabilities (PP); major branches are well-supported by high values of PP, and (simultaneously) all the values are higher than 0.5. The family Sarcocystidae is used as an outgroup.

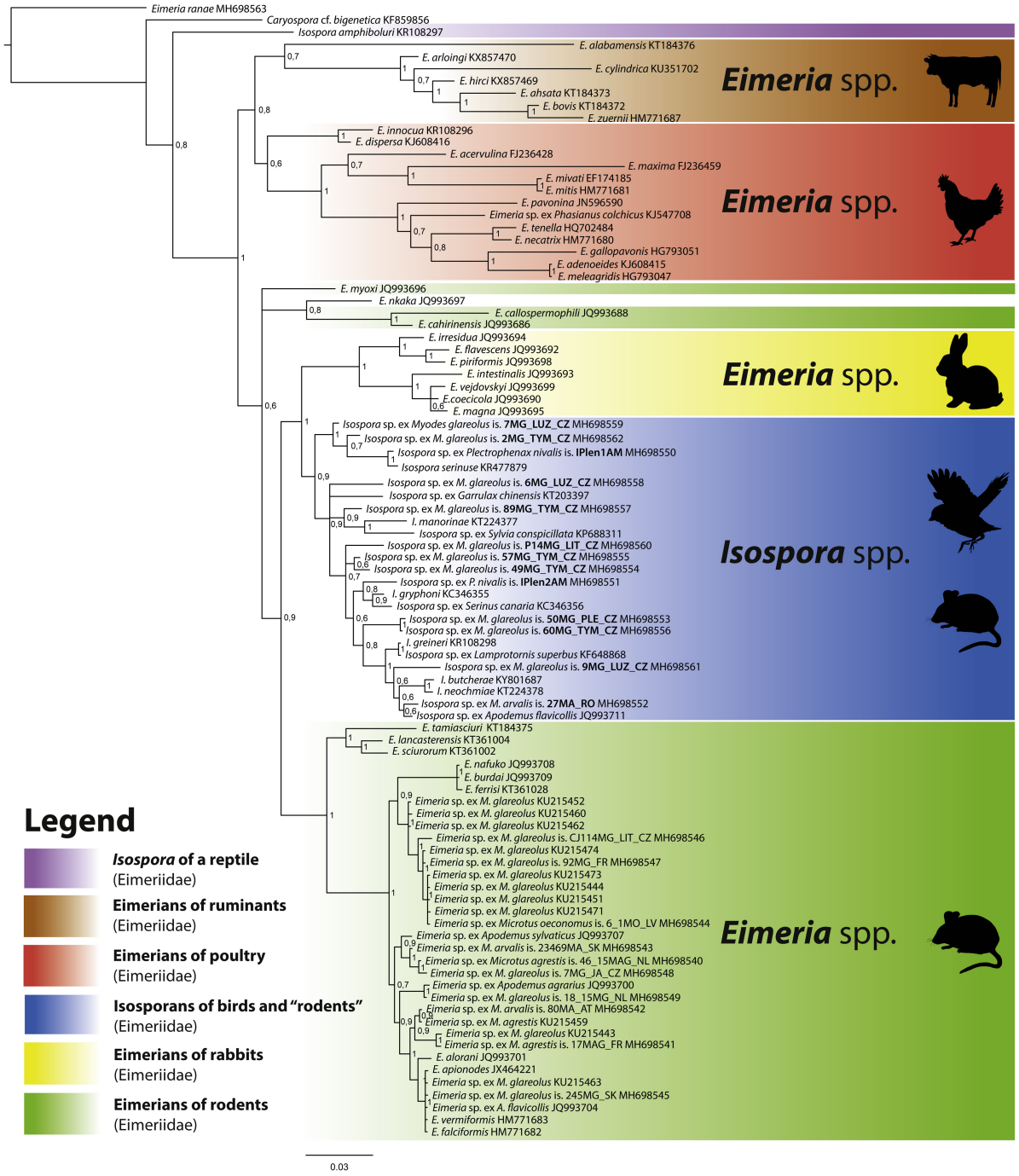

Figure 7. Phylogenetic relationships inferred by the BI analysis of the COI sequences. Numbers at the nodes show posterior probabilities (PP); major branches are well-supported by high values of PP, and (simultaneously) all the values are higher than 0.5. *Eimeria ranae* is used as an outgroup.

mainly birds are related to Eimeriidae, and the presence/absence of the SB complex was established as the key morphological trait (Barta et al. 2005; Carreno et al. 1998; Franzen et al. 2000; Jirků et al. 2002).

To date, the descriptions of coccidian species were mainly based on the morphology of oocysts occurring in the faeces of various hosts. In total, 38 *Isospora* spp. have been described from rodents till now. Out of these, 6 *Isospora* species have

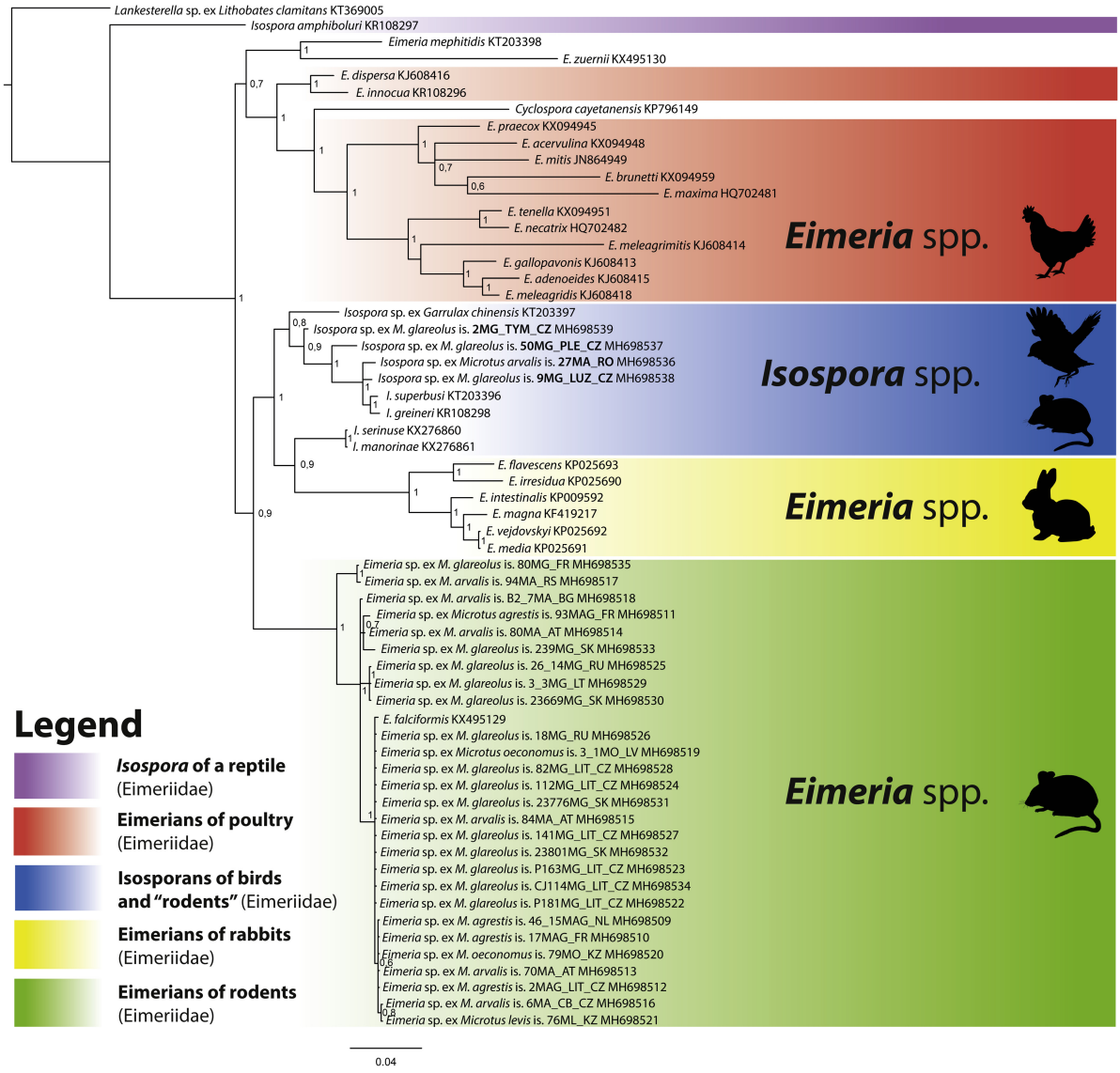

Figure 8. Phylogenetic relationships inferred by the BI analysis of the COIII sequences. Numbers at the nodes show posterior probabilities (PP); major branches are well-supported by high values of PP, and (simultaneously) all the values are higher than 0.5. *Lankesterella* sp. is used as an outgroup.

been described from voles, specifically 2 of them from bank voles. Regarding their morphology, 17 *Isospora* spp. possessing the SB complex, 12 *Isospora* spp. lacking the SB, and 9 *Isospora* spp., for which the presence of the SB complex was not recorded, have been described (Levine and Ivens 1990; Table 5). At present, all those without the SB complex have been assigned to the genus *Cystoisospora* (Carreno and Barta 1999; Carreno et al. 1998).

However, species of *Isospora* possessing the SB complex and conspicuously resembling avian representatives of the genus have been described. For instance, Levine and Mohan (1960) described an *Isospora* from cattle the oocysts of which were almost identical with the oocysts of *Isospora lacazei* originally described from an English sparrow (*Passer domesticus*; Aves: Passeriformes). Ernst et al. (1969) then described *Isospora* sp. from an opossum (*Didelphis marsupialis*; Mammalia: Didelphimorphia: Didelphidae) the oocysts of which also substantially resembled those of *I. lacazei*. The oocysts of *Isospora* sp. found in the hispid cotton rat (*S. hispidus*) by Barnard et al. (1974) also resembled isosporan oocysts from birds, furthermore, in that case, experimental infections were repeat-

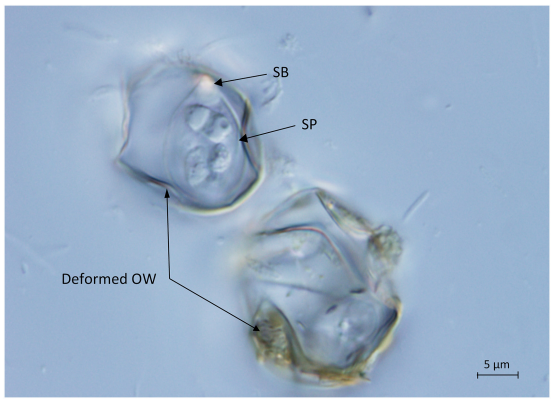

Figure 9. Deformed oocysts that passed through the gastrointestinal tract of bank voles after the administration of isolate of sporulated *Isospora*-type oocysts. OW, oocyst wall; SB, Stieda body; SP, sporocyst.

edly carried out, but with unsuccessful results. The authors assumed that the above mentioned isosporans were pseudoparasites originated from birds.

In discrepancy with the study of Carreno and Barta (1999) is the study of Upton et al. (1985), which describes the morphology and endogenous development of *Isospora* possessing the SB in detail; *I. masoni* Upton, Lindsay, Current and Ernst, 1985 was described from the hispid cotton rat based on morphology and experimental transmission. Oocysts had a thin wall, and each sporocyst possessed the SB and sSB complex. For this species, the endogenous developmental stages were identified and localized mostly in enterocytes of ileum. Extraintestinal stages were not found, hence this species was considered to be a coccidium with a monoxenic direct life cycle. The prepatent period was 4–7 days and the patent period lasted for more than 40 days. Sporulation was endogenous, the sporocysts were shed directly in the faeces. Subsequently, successful experimental transmission to the hispid cotton rat via both sporocysts and sporulated oocysts was carried out. Its endogenous sporulation, thin OW, and relatively small sporocyst size (7–9 × 5–6; Table 5) led Upton et al. (1985) to emphasize the uncertainty as to where this organism indeed belongs. Nonetheless, they placed this species in the genus *Isospora* based on the presence of SB and sSB, and also based on the apparent monoxenic life cycle. According to morphological and biological features, this coccidium cannot be unequivocally classified as coccidia either of the family Sarcocystidae or Eimeriidae. Unfortunately, there are no molecular data available for this species. Similarly, no molecular data exist on any *Isospora* spp. described from rodents. Considering all the species given in Table 5, it has not been so far determined which is a *Cystoisospora* related to coccidia of the family Sarcocystidae, and which a real *Isospora* belonging to Eimeriidae, thus, demonstrating that a resolution of the origin of *Isospora* spp. is much more complicated.

It was previously reported that unsporulated and sporulated oocysts can pass unchanged and unharmed through the intestinal tract of the mammal host (Prasad 1961; Streitel and Dubey 1976). However, the oocysts can become deformed by multiple passage. Thus, the isosporan oocysts (i.e with SB) were probably transmitted to bank voles (and also probably to other rodents) via the consumption of food contaminated by avian droppings. There remains the question of whether the oocysts of *Isospora* spp. discharged in this way (i.e passaged through the gastrointestinal tract of rodents) are subsequently able to infect the original avian host. Hypothetically, if sporocysts are intact the sporozoites inside should remain infective. The problem lies in the fact that we do not known from which bird species these isosporans originate.

Conclusion

To conclude, the hypothesis of pseudoinfection has been demonstrated based on morphological analyses, phylogenetic analyses, and experimental infection. Morphological analyses showed the presence of the SB complex on sporocysts. Phylogenetic analyses showed a very close phylogenetic relationship between *Isospora* spp. obtained from bank voles and avian isosporans. Experimental inoculations of the bank voles with sporulated oocysts of *Isospora* sp. did not result in the production of unsporulated oocysts. Hence, these organisms should be considered pseudoparasites of the bank vole/rodents. Furthermore, sporulated oocysts of some species of *Isospora* were found in the faeces of 2 live-trapped bank voles. Some of them were already partially deformed. During the further coprological examination, these rodents were coccidia-negative. The bank voles apparently ate contaminated food, and the oocysts merely passed through their gastrointestinal tracts.

Methods

Origin of the hosts and parasites: Bank voles (*M. glareolus*) were trapped in the course of 2015–2017 using classic wooden snap traps and Sherman live traps. The rodents were trapped across the Czech Republic in the South Bohemian Region

(České Budějovice, Lužnice, Třísov), Ústí nad Labem Region (Klášterec nad Ohří, Litvínov), Karlovy Vary Region (Stružná), and in the Pilsen Region (Tymákov) (Table 1).

The faeces of each individual snap-trapped vole were collected and preserved in 4% (w/v) aqueous potassium dichromate ($K_2Cr_2O_7$) solution. Live-trapped animals were placed in plastic boxes (Velaz type T II, Velaz, Prague, Czech Republic). The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of South Bohemia, and also by the Ministry of the Environment of the Czech Republic (Permit Numbers 27873/ENV/11 and 22395/2014-MZE-17214).

Coprology and morphological studies: Faecal samples collected in the field were examined for the presence of coccidian oocysts using density-gradient flotation with Sheather's sucrose solution (specific gravity 1.30) (Duszynski and Wilber 1997; Sheather 1923; Zajac and Conboy 2006). Coccidia-positive samples were allowed to sporulate on air at room temperature. The live-trapped voles were examined at least five times to ensure that they were really coccidia/*Isospora*-negative. For the determination of isosporan oocysts, an Olympus BX53 light microscope equipped with a digital camera and Olympus cellSens Standard 1.13 imaging software was used. The determination was based on the morphological and morphometrical analyses of sporulated oocysts (Berto et al. 2014; Duszynski and Wilber 1997).

DNA isolation, PCR amplification, and sequencing: Genomic DNA was extracted from the *Isospora*-positive faecal samples using the FastDNA® SPIN for Soil Kit (MP Biomedicals, LLC, Santa Ana, California, USA) following the manufacturer's protocol. PCR amplification was performed with coccidia-specific primers amplifying the gene encoding the small subunit of 18S rRNA, and mitochondrial genes for cytochrome c oxidase subunit I (COI) and III (COIII). Primers for 18S rDNA and COI were adopted from Schwarz et al. (2009) and Kvičerová et al. (2008), respectively. Sequences of primers amplifying the COIII region were provided by John R. Barta (University of Guelph, Ontario, Canada). HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) was used for all PCR reactions. PCR products were purified with alkaline phosphatase and exonuclease I enzymes (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and sequenced via the Sanger sequencing method in SEQme, s.r.o. (Dobříš, Czech Republic).

Sequence processing and phylogenetic analyses: The obtained sequences of *Isospora* spp. were verified by the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were further processed using the Sequence Scanner v2.0 (Applied Biosystems), EditSeq 5.05, and SeqMan 5.05 (DNASTAR, Inc., Madison, Wisconsin, USA) programs. Coccidian sequences of 18S rRNA, COI, and COIII genes obtained from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) together with the newly obtained sequences of our samples were used in phylogenetic analyses. The accession numbers of all sequences used in the analyses (including the newly obtained sequences) are indicated on resultant phylogenetic trees (Figs 6–8). Alignments were created in Geneious v9.1.3 (http://www.geneious.com, Kearse et al. 2012) using the MAFFT v1.3.6 algorithm with default parameters (Katoh and Standley 2013), and manually adjusted. 18S rDNA sequences were aligned in the nucleotide mode; COI and COIII sequences were aligned in the amino acid mode, then switched to nucleotide mode, and used for the analyses. Phylogenetic relationships were reconstructed using the Bayesian inference (BI) in the program MrBayes v3.2.6 (Huelsenbeck and Ronquist 2001). The best fitting evolutionary models were selected by the SMS: Smart Model Selection software (http://www.atgc-montpellier.fr/sms/, Lefort et al. 2017). BI analysis was performed using the GTR + Γ + I evolutionary model for 10 million generations for all analyses, and the trees were summarized after removing 25% burn-in. Phylogenetic trees were visualized and exported by FigTree v1.4.2 (http://tree.bio.ed.ac.uk/).

Experimental infections: For the experimental inoculations, isolates of fully sporulated oocysts of *Isospora* spp., originating from the faeces of voles trapped into the snap traps in the field samplings, were purified on the sucrose gradient.

Coccidia-negative live-trapped voles were kept individually in the plastic boxes (separated from each other), and their faeces were collected and examined daily for the presence of coccidia for 14 days; all animals were coproscopically negative by light microscopy, and were thus considered to be negative prior to the experimental infection. Moreover, before the experimental inoculation, the rodents were twice preventively treated with Baycox® (2.5% toltrazuril; Bayer Animal Health GmbH, Leverkusen, Germany) in the form of peroral suspension (46 ml/100 kg). More specifically, the first dose was administered 3 weeks before the experimental inoculation, and the second 3 days after the first dose.

Before the experimental inoculation, each animal was slightly anesthetized with diethylether (Penta s.r.o., Prague, Czech Republic). Sporulated oocysts of *Isospora* spp. were inoculated via syringe with an olive-tipped needle into the oesophagus of the anesthetized animal. After inoculation, the faeces of each animal were daily collected and examined by flotation technique with Sheather's sucrose solution until 23 days postinoculation (DPI). To obtain the faeces, the animals were individually placed into clean disinfected empty Velaz boxes (separated from each other) and left there for at least 4 h.

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