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Cryptosporidium ratti n. sp. (Apicomplexa: Cryptosporidiidae) and genetic diversity of *Cryptosporidium* spp. in brown rats (*Rattus norvegicus*) in the Czech Republic

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Research Article

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Abstract

The diversity and biology of *Cryptosporidium* that is specific for rats (*Rattus* spp.) are not well studied. We examined the occurrence and genetic diversity of *Cryptosporidium* spp. in wild brown rats (*Rattus norvegicus*) by microscopy and polymerase chain reaction (PCR)/sequencing targeting the small subunit rDNA (*SSU*), actin and *HSP*70 genes. Out of 343 faecal samples tested, none were positive by microscopy and 55 were positive by PCR. Sequence analysis of *SSU* gene revealed the presence of *Cryptosporidium muris* ($n = 4$), *C. andersoni* ($n = 3$), *C. ryanae* ($n = 1$), *C. occultus* ($n = 3$), *Cryptosporidium* rat genotype I ($n = 23$), *Cryptosporidium* rat genotype IV ($n = 16$) and novel *Cryptosporidium* rat genotype V ($n = 5$). Spherical oocysts of *Cryptosporidium* rat genotype I obtained from naturally-infected rats, measuring 4.4–5.4 μm × 4.3–5.1 μm, were infectious to the laboratory rats, but not to the BALB/c mice (*Mus musculus*) nor Mongolian gerbils (*Meriones unguiculatus*). The prepatent period was 3 days post infection and the patent period was longer than 30 days. Naturally- and experimentally-infected rats showed no clinical signs of disease. Percentage of nucleotide similarities at the *SSU*, actin, *HSP*70 loci between *C. ratti* n. sp. and the rat derived *C. occultus* and *Cryptosporidium* rat genotype II, III, IV, and V ranged from 91.0 to 98.1%. These genetic variations were similar or greater than that observed between closely related species, i.e. *C. parvum* and *C. erinacei* (93.2–99.5%). Our morphological, genetic and biological data support the establishment of *Cryptosporidium* rat genotype I as a new species, *Cryptosporidium ratti* n. sp.

Introduction

The genus *Cryptosporidium* comprises obligate protozoan parasites that predominantly inhabit the gastrointestinal epithelium of humans and other vertebrate animals (Fayer, 2010). *Cryptosporidium* has been under intensive investigation for more than 40 years and the enormous diversity in the genus has been revealed by genotyping studies conducted over the past 20 years. Studies on *Cryptosporidium* in humans and livestock have predominated due to the clinical and economic importance of cryptosporidiosis in these hosts (Robertson *et al*., 2014; Kváč *et al*., 2014*b*). Research on *Cryptosporidium* spp. in wild animals has increased significantly in the last decade, expanding our knowledge of genetic diversity in the genus, but the biological properties of these parasites in wildlife remain poorly studied (Ren *et al*., 2012; Li *et al*., 2015; Kváč *et al*., 2018; Tan *et al*., 2019; Wei *et al*., 2019). Recent studies indicate that rodents, which represent about 40% of the mammalian diversity, are predominantly parasitized by host-specific *Cryptosporidium* spp. with unknown biology (Lv *et al*., 2009; Feng *et al*., 2011; Ng-Hublin *et al*., 2013; Stenger *et al*., 2017; Čondlová *et al*., 2019). Today, 45 valid *Cryptosporidium* species and a similar number of genotypes have been reported (Holubová *et al*., 2020). *Cryptosporidium muris* and *C. proliferans* have a broad host range in the order Rodentia. In contrast, a narrow host specificity has been reported for *C. alticolis* and *C. microti* in voles, *C. apodemi* and *C. ditrichi* in apodemus mice, *C. homai* and *C. wrairi* in guinea pigs, *C. tyzzeri* in house mice, *C. rubeyi* in ground squirrels and *C. occultus* in rats (Tyzzer, 1910; Vetterling *et al*., 1971; Ren *et al*., 2012; Li *et al*., 2015; Kváč *et al*., 2016, 2018; Zahedi *et al*., 2017; Čondlová *et al*., 2018; Horčičková *et al*., 2018). Additionally, a large number of *Cryptosporidium* genotypes have been reported in rodents (Kváč *et al*., 2014*b*).

Representatives of the genus *Rattus*, which are globally distributed, with the exception of the polar region (Reid, 2007; Thomson *et al*., 2018), have been reported as hosts of several

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Cryptosporidium spp. (Table 1). The recently described *C. occultus* is specific for rat hosts (Kváč *et al.*, 2018). Other species, *C. muris*, *C. parvum*, *C. tyzzeri*, *C. scrofarum*, *C. meleagridis*, *C. erinacei*, *C. ubiquitum* and *C. viatorum*, reported in rats are host-specific for other hosts. Rats probably represent minor host or the presence of these *Cryptosporidium* species is the result of the mechanical transmission of oocysts through the digestive tract (Kváč *et al.*, 2009; Lv *et al.*, 2009; Ng-Hublin *et al.*, 2013; Tan *et al.*, 2019). Rats are frequently parasitized with *Cryptosporidium* rat genotypes I-IV, which have been reported in rats in Asia, Australia and South America (Table 1). As yet, there is no comprehensive genotyping study from Europe or North America and there is no knowledge of their biological properties including oocyst size, course and location of infection, or pathogenicity, etc. In the course of the study, we obtained an isolate of *Cryptosporidium* rat genotype I and examined its biological, morphological and genetic characteristics in detail. Our data showed that *Cryptosporidium* rat genotype I is genetically and biologically distinct from valid *Cryptosporidium* species and we propose to name it as a *Cryptosporidium ratti* n. sp.

Materials and methods

Area and specimens studied

A total of 343 wild rats (*Rattus norvegicus*) were trapped using metal pedal or life traps at 16 localities in the Czech Republic over the period 2016–2019 (Fig. 1). Traps were checked every 3 hours and trapped animals were removed and transported to the Institute of Parasitology, Biology Centre CAS (PaU). Faecal samples from deceased rats were collected from the rectum during dissection. Live rats were individually housed with sterilised bedding, food and water. The feces of alive rats were collected individually for several days, each sample was individually examined for the presence of *Cryptosporidium* oocysts by the aniline–carbol–methyl violet staining (Miláček and Vítovec, 1985) followed by microscopic examination at 1000 × magnification (light microscope Olympus BX51, Tokyo, Japan), and specific DNA, by polymerase chain reaction (PCR)/sequencing targeting the small subunit ribosomal RNA gene (*SSU*) (below). If at least one sample was *Cryptosporidium* positive, the rat was considered positive. Alive rats that were negative for *Cryptosporidium* spp. were sacrificed humanly. *Cryptosporidium* positive rats were kept for several weeks and their feces were collected daily.

Molecular characterization

Total genomic DNA was extracted from 200 mg of feces or 100–200 mg of tissue specimens using a PSP spin stool DNA Kit (Invitek, Stratec, Berlin, Germany) followed by bead disruption for 60 s at 5.5 m s^{-1} using 0.5 mm glass beads in a FastPrep®-24 Instrument (MP Biomedicals, CA, USA). Purified DNA was stored at −20 °C prior to amplification by PCR. Fragments of the *SSU*, actin and the 70 kDa heat shock protein (*HSP*70) genes were amplified by nested PCR using published protocols and primers (Xiao *et al.*, 1999; Sulaiman *et al.*, 2000, 2002; Jiang *et al.*, 2005). Some PCR conditions were slightly modified from their original publications as previously described by Holubová *et al.* (2019). DNA of *C. proliferans* and molecular grade water were used as positive and negative controls, respectively. The secondary PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized following staining with ethidium bromide. Amplicons were purified using the GenElute™ Gel Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA) and sequenced in both directions using the secondary PCR primers at a commercial laboratory (SEQme, Dobříš, Czech Republic).

Phylogenetic analysis

The nucleotide sequences of each gene obtained from naturally- and experimentally-infected animals were verified by BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi), edited using Chromas Pro 2.1.4 (Technelysium, Pty, Ltd., South Brisbane, Australia) and aligned with reference sequences obtained from GenBank using BioEdit v.7.0.5 (Hall, 1999). The alignments were end-trimmed and used in the phylogenetic analyses. Phylogenetic trees were inferred using the maximum likelihood (ML) method, with the substitution model that best fits the alignment selected using the Bayesian information criterion in MEGAX software. The robustness of the phylogeny was tested with 1000 bootstraps. Phylograms were edited for style using CorelDrawX7. Sequences have been deposited in GenBank under the Accession Numbers (Acc. nos.): MT504538-MT504544 for *SSU*, MT507482-MT507485 for *HSP*70, and MT507486-MT507491 for actin.

Origin of Cryptosporidium ratti *n. sp. isolate*

An isolate of *C. ratti* n. sp. was obtained from a wild-caught rat (isolate 29 356; Rat 0) trapped at locality no. 16 (Telč). The rat was individually housed with sterile bedding and provided with sterile food and water. The bedding was changed every second day. Oocysts were purified using cesium chloride gradient centrifugation (Arrowood and Donaldson, 1996) and used for morphometry and phylogenetic analysis (*SSU*, actin and *HSP*70 genes). Oocysts obtained from Rat 0 were used to infect a single 1-week-old rat (Rat 1). Oocysts of *C. ratti* n. sp. obtained from Rat 1 were purified using cesium chloride gradient centrifugation and their viability was examined using propidium iodide (PI) staining by a modified assay of Sauch *et al.* (1991). They were used for morphometry and phylogenetic analysis and for experimental infection of other animals (see the transmission studies section). The oocysts were stored in PBS at 4–8 °C for a maximum of 3 weeks.

Transmission studies

Five 1-week-old and 8-week-old severe combined immunodeficiency (SCID) mice (strain C.B-17), BALB/c mice (*Mus musculus*), Mongolian gerbils (*Meriones unguiculatus*) and laboratory rats (*Rattus norvegicus*, strain Wistar Han) were used for transmission studies. Three animals from each host species/strain were used as negative controls. All experimental 1-week- and 8-week-old animals were inoculated by oesophageal tube with 10 000 purified oocysts of *C. ratti* n. sp. (Rat 1 origin) suspended in 50 and 200 μL of sterile PBS, respectively. Animals used as negative controls were inoculated with the same volume of sterile PBS. For a week prior to infection, faecal samples from all experimental animals were screened daily for the presence of *Cryptosporidium* oocysts and DNA using aniline–carbol–methyl violet staining and nested PCR targeting the *SSU* gene, respectively. To prevent environmental contamination with oocysts, laboratory rodents were housed in plastic cages and supplied with a sterilized diet (TOP-VELAZ, Prague, Czech Republic) and sterilized water *ad libitum*. Starting on the second-day post infection, faecal samples from each animal were screened daily for the presence of *Cryptosporidium* oocysts and DNA using aniline–carbol–methyl violet staining and nested PCR targeting the *SSU* gene, respectively. One animal from each experimental group was euthanatized at 10 and 20 days post infection (DPI). Tissue specimens from the oesophagus, stomach, small intestine and large intestine (the entire tract was divided into 1 cm-long sections), trachea, lungs, liver and kidney were sampled and

Table 1. Diversity of *Cryptosporidium* spp. in rat (*Rattus* sp.), brown rat (*Rattus norvegicus*), Asian house rat (*Rattus tanezumi*), Australian swamp rat (*Rattus lutreolus*), and Malayan black rat (*Rattus rattus diardii*) based on microscopic and molecular detection

Species/genotype of *Cryptosporidium*	Host	Detection method	Reference sequence (SSU) GenBank	Country	Reference
C. parvum	*R. norvegicus*	Microscopy	–	Japan	Iseki (1986)
	R. norvegicus	Microscopy	–	England	Webster and Macdonald (1995)
	R. norvegicus	PCR	AB271070	Japan	Kimura *et al.* (2007)
	Rattus sp.	PCR-RFLP	HQ651732	Iran	Bahrami *et al.* (2012)
	R. norvegicus	PCR	AB986579-81	Iran	Saki *et al.* (2016)
	R. tanezumi *R. norvegicus*	PCR	EU331237[a]	China	Zhao *et al.* (2015)
C. muris	*R. norvegicus*	Microscopy	–	Japan	Iseki (1986)
	R. tanezumi *R. norvegicus*	PCR	JX485397	Philippines	Ng-Hublin *et al.* (2013)
	R. rattus	PCR	JQ313975	Brazil	Silva *et al.* (2013)
	R. tanezumi *R. norvegicus*	PCR	EU245045[a]	China	Zhao *et al.* (2015)
	R. norvegicus	PCR	AB697054[a]	China	Zhao *et al.* (2019)
C. tyzzeri	*R. tanezumi* *R. norvegicus*	PCR	GQ121024	China	Lv *et al.* (2009)
C. scrofarum	*R. tanezumi* *R. norvegicus*	PCR	JX485403	Philippines	Ng-Hublin *et al.* (2013)
C. occultus	*R. tanezumi*	PCR	JX485388	Philippines	Ng-Hublin *et al.* (2013)
	R. norvegicus	PCR	MG699179	Czechia	Kváč *et al.* (2018)
	R. norvegicus	PCR	HQ822146[a]	China	Zhao *et al.* (2018)
	R. tanezumi *R. norvegicus*	PCR	MG699179[a]	China	Zhao *et al.* (2019)
C. meleagridis	*R. norvegicus*	PCR	AB271063	Japan	Kimura *et al.* (2007)
C. erinacei	*R. tanezumi*	PCR	KF612324[a]	China	Zhao *et al.* (2019)
C. ubiquitum	*R. norvegicus*	PCR	KC962124[a]	China	Zhao *et al.* (2018)
C. viatorum	*R. lutreolus*	PCR	MG021320	Australia	Koehler *et al.* (2018)
Rat genotype I	*R. norvegicus*	PCR	JX485398	Philippines	Ng-Hublin *et al.* (2013)
	R. norvegicus	PCR	FJ205699[a] JN172971[a] KP883289[a] GQ183517[a]	China	Zhao *et al.* (2018)
	R. norvegicus	PCR	AB271061 AB271062 AB271066 AB271068	Japan	Kimura *et al.* (2007)
	R. rattus	PCR	KP883292 KP883289	Iran	unpublished
Rat genotype II	*R. tanezumi*	PCR	GQ121025	China	Lv *et al.* (2009)
	R. rattus	PCR	JX294358	Australia	Paparini *et al.* (2012)
	R. tanezumi *R. norvegicus*	PCR	JX485400	Philippines	Koehler *et al.* (2018)
Rat genotype III	*R. tanezumi* *R. norvegicus*	PCR	GQ121026	China	Lv *et al.* (2009)
	R. rattus	PCR	JX294361	Australia	Paparini *et al.* (2012)
	R. tanezumi *R. norvegicus*	PCR	JX485389	Philippines	Ng-Hublin *et al.* (2013)
	R. rattus	PCR	KF176349	Brazil	Silva *et al.* (2013)
	R. tanezumi *R. norvegicus*	PCR	JX294371[a]	China	Song *et al.* (2015)
Rat genotype IV		PCR	JX485394	Philippines	Ng-Hublin *et al.* (2013)

(*Continued*)

Table 1. (*Continued.*)

Species/genotype of *Cryptosporidium*	Host	Detection method	Reference sequence (SSU) GenBank	Country	Reference
	R. tanezumi *R. norvegicus*				
	R. norvegicus	PCR	JN172970 MG917670[a] MG917671[a]	China	Zhao *et al.* (2018)
	R. tanezumi *R. norvegicus*	PCR	JN172970[a] KY483983[a] MG917670[a] AY737584[a]	China	Zhao *et al.* (2019)
	R. norvegicus	PCR	AB271067 AB271071 AB271072	Japan	Kimura *et al.* (2007)
Isolate BR8	*R. norvegicus*	PCR	AB271064	Japan	Zahedi *et al.* (2017)
Cryptosporidium sp.	*Rattus* sp.	Histology	–	Korea	Seoki *et al.* (2005)
	R. norvegicus	Microscopy	–	Iran	Gholipoury *et al.* (2016)
	R. rattus	Microscopy	–	Indonesia	Prasetyo (2016)
	R. rattus *R. norvegicus*	Microscopy	–	Japan	Yamaura *et al.* (1990)
	R. rattus *R. norvegicus*	Microscopy	–	Iran	Mirzaghavami *et al.* (2016)
	R. norvegicus *R. rattus* *diardii*	Microscopy	–	Malaysia	Tijjani *et al.* (2020)

[a]Indicates the sequence obtained in the paper has not been stored in the GenBank database and was identical to a sequence published previously.

Fig. 1. Sampling locations across the study area in the Czech Republic. Sample site numbers indicate the name of locations and coordinates are in brackets: (1) Březnice (49.556628, 13.954390), (2) Chyšná (50.545694, 13.437376), (3) Cizkrajov (49.0303555, 15.390124), (4) České Budějovice (48.974749, 14.453704), (5) Český Krumlov (48.813194, 14.321542), 6) Hodětín (49.251090, 14.547873), (7) Kardašova Řečice (49.18.2636, 14.848994), (8) Lidéřovice (49.064462, 15.373599), (9) Praha (50.074130, 14.522609), (10) Protivín (49.196654, 14.216850), (11) Přibyslav (49.580047, 15.739454), (12) Pyšely (49.875659, 14.680111), (13) Řevnov (49.475599, 14.632047), (14) Telč (49.184339, 15.472545), (15) Věžovatá Pláně (48.776780, 14.408550) and (16) Zmišovice (49.496220, 15.188810).

processed for PCR targeting the *SSU* gene, histology and scanning electron microscopy. Specimens for histology and electron microscopy were processed according to Holubová *et al.* (2019). All experiments were terminated at 30 DPI. Faecal consistency, faecal colour and animal behaviour were examined daily. Animals received standard care at the Institute of Parasitology (IP) (Holubová *et al.*, 2019). All housing, feeding and experimental procedures were conducted under protocols approved by the IP and the Central Commission for Animal Welfare, Czech Republic (protocol nos. 55/2014, 35/2018 and MZP/2019/630/1411).

Morphometric analysis

Oocyst size was determined using digital analysis of images (Olympus cellSens Entry 2.1 software, Olympus Corporation, Shinjuku, Tokyo, Japan) collected using an Olympus Digital Colour Camera DP73 (Olympus). The length and width of *C. ratti* n. sp. oocysts from naturally- (Rat 0) and experimentally-infected animals (20 oocysts from each isolate) were examined using differential interference contrast (DIC) microscopy at 1000 × magnification (Olympus IX70, Tokyo, Japan). These measurements were used to calculate the length-to-width ratio. Samples containing purified *C. parvum* oocysts (calf origin) were used as a size control. Oocyst size was measured using the same microscope and by the same person. Each slide was screened using a meandering path to prevent repeated measurement of an oocyst. Additionally, faecal smears with oocysts of *C. ratti* n. sp. and *C. parvum* (data not shown) were stained by modified Ziehl-Neelsen (ZN; Henriksen and Pohlenz, 1981) and labelled with a Cy3-labeled mouse monoclonal antibody targeting the *Cryptosporidium* oocyst outer wall antigenic sites (A400Cy2R-20X, Crypt-a-Glo, Waterborne, Inc, New Orleans, LA, USA).

Statistical analysis

Differences in *Cryptosporidium* spp. oocysts size were tested using Hotelling's multivariate version of the 2 sample *t*-test, *package ICSNP: Tools for Multivariate Nonparametrics* (Nordhausen *et al.*, 2018) in R 4.0.0. (R Core Team, 2019). The hypothesis tested was that two-dimensional mean vectors of measurement are the same in the two populations being compared.

Results

A total of 343 faecal samples were obtained from trapped brown rats at 16 localities were tested for the presence of *Cryptosporidium* spp.

Table 2. *Cryptosporidium* spp. in wild brown rats (*Rattus norvegicus*) at localities in the Czech Republic

				Genotyping at the loci		
Locality	Number examined/positive	Isolate ID	Microscopically positive (OPG)	*SSU*	Actin	*HSP*70
1	4/0	–	–	–	–	
2	39/11	15 824	No	*C. ratti* n. sp.	*C. ratti* n. sp.	*C. ratti* n. sp.
		15 825	No	Rat genotype V	NA	NA
		15 826	No	*C. ratti* n. sp.	NA	NA
		15 828	No	*C. ratti* n. sp.	*C. ratti* n. sp.	*C. ratti* n. sp.
		15 832	No	*C. ratti* n. sp.	NA	NA
		16 108	No	Rat genotype V	*C. ryanae*	NA
		16 109	No	*C. ratti* n. sp.	*C. ratti* n. sp.	*C. ratti* n. sp.
		16 115	No	*C. ratti* n. sp.	*C. ratti* n. sp.	NA
		16 116	No	*C. ratti* n. sp.	*C. ratti* n. sp.	*C. ratti* n. sp.
		16 858	No	*C. ratti* n. sp.	*C. ratti* n. sp.	NA
		16 863	No	*C. ratti* n. sp.	*C. ratti* n. sp.	*C. ratti* n. sp.
3	13/2	30 870	No	*C. occultus*	*C. occultus*	*C. occultus*
		29 340	No	*C. ryanae*	*C. ryanae*	NA
4	52/9	22 929	No	*C. occultus*	*C. occultus*	*C. occultus*
		21 353	No	Rat genotype IV	Rat genotype IV	NA
		21 364	No	*C. occultus*	*C. occultus*	*C. occultus*
		25 724	No	*C. muris*	*C. muris*	*C. muris*
		25 725	No	*C. muris*	*C. muris*	*C. muris*
		25 727	No	Rat genotype IV	Rat genotype IV	NA
		25 728	No	Rat genotype IV	Rat genotype IV	NA
		25 729	No	*C. muris*	*C. muris*	*C. muris*
		25 730	No	*C. muris*	*C. muris*	NA
5	30/0	–	–	–	–	–
6	4/0	–	–	–	–	–
7	2/0	–	–	–	–	–
8	30/13	29 300	No	Rat genotype IV	Rat genotype IV	NA
		29 301	No	Rat genotype IV	NA	NA
		29 302	No	Rat genotype IV	NA	NA
		29 303	No	*C. ratti* n. sp.	*C. ratti* n. sp.	*C. ratti* n. sp.
		29 307	No	Rat genotype IV	NA	NA
		29 309	No	*C. ratti* n. sp.	*C. ratti* n. sp.	*C. ratti* n. sp.
		29 311	No	*C. andersoni*	*C. andersoni*	*C. andersoni*
		29 312	No	Rat genotype IV	NA	NA
		29 315	No	Rat genotype IV	Rat genotype IV	NA
		29 321	No	Rat genotype IV	NA	NA
		29 330	No	*C. ratti* n. sp.	NA	NA
		30 591	No	*C. ratti* n. sp.	*C. occultus*	NA
		30 593	No	Rat genotype IV	NA	NA
9	52/1	16 360	No	*C. andersoni*	*C. andersoni*	*C. andersoni*
10	1/0	–	–	–	–	–
11	10/2	24 650	No	*C. ratti* n. sp.	NA	NA
		24 651	No	*C. ratti* n. sp.	*C. ratti* n. sp.	NA
12	16/1	16 978	No	*C. andersoni*	*C. andersoni*	*C. andersoni*

(*Continued*)

Table 2. (*Continued.*)

Locality	Number examined/positive	Isolate ID	Microscopically positive (OPG)	Genotyping at the loci		
				SSU	Actin	*HSP*70
13	4/2	23 492	No	*C. ratti* n. sp.	*C. ratti* n. sp.	*C. ratti* n. sp.
		26 823	No	Rat genotype IV	NA	NA
14	57/10	29 344	No	Rat genotype V	NA	NA
		29 353	No	*C. ratti* n. sp.	NA	NA
		29 354	No	Rat genotype IV	Rat genotype IV	NA
		29 355	No	*C. ratti* n. sp.	NA	NA
		29 356[a]	No	*C. ratti* n. sp.	*C. ratti* n. sp.	*C. ratti* n. sp.
		29 359	No	*C. ratti* n. sp.	NA	NA
		29 364	No	Rat genotype IV	NA	NA
		29 366	No	*C. ratti* n. sp.	*C. ratti* n. sp.	NA
		30 592	No	Rat genotype V	NA	NA
		30 576	No	Rat genotype V	NA	NA
15	1/0	–	–	–	–	–
16	28/4	15 461	No	*C. ratti* n. sp.	Rat genotype IV	NA
		15 571	No	*C. ratti* n. sp.	*C. ratti* n. sp.	*C. ratti* n. sp.
		21 654	No	Rat genotype IV	Rat genotype IV	NA
		21 655	No	Rat genotype IV	Rat genotype IV	NA

Oocysts were quantified by microscopy and reported per gram of feces (OPG). Fragments of the small subunit rDNA (*SSU*), actin and heat shock protein 70 (*HSP*70) genes were amplified by PCR. **NA** indicates PCR amplification failure.
[a]Isolate of *Cryptosporidium* rat genotype I used for experimental studies.

(Table 2). *Cryptosporidium*-specific DNA was detected in 55 samples by nested PCR targeting the *SSU* gene. None of the samples was positive for *Cryptosporidium* oocysts by microscopy. Out of the 55 *Cryptosporidium*-positive rats, 55, 36 and 19 were genotyped by sequence analysis of the *SSU*, actin and *HSP*70 genes, respectively. The remaining positive samples failed to amplify at the actin ($n = 19$) and *HSP*70 ($n = 36$) loci (Table 1). ML trees constructed from *SSU* sequences showed the presence of *C. muris* ($n = 4$), *C. andersoni* ($n = 3$), *C. ryanae* ($n = 1$), *C. occultus* ($n = 3$), *C. ratti* n. sp. ($n = 23$) and *Cryptosporidium* rat genotype IV ($n = 16$). Five isolates clustered in a novel group, which we have named *Cryptosporidium* rat genotype V. This group was closely related to *C. ratti* n. sp. and *Cryptosporidium* rat genotypes II and III (Fig. 2, Table 2). For the actin gene, isolates of *C. occultus*, *C. ryanae*, *C. muris*, *C. andersoni*, *C. ratti* n. sp. and *Cryptosporidium* rat genotype IV shared 100% sequence identity with sequences of *Cryptosporidium* spp. previously reported (Fig. 3). Actin sequences were not detected in any of the samples that were positive for *Cryptosporidium* rat genotype V at the *SSU* locus. A mixed infection was detected in three samples – isolate 16 108 was positive for *C. ryanae* at actin and for *Cryptosporidium* rat genotype IV at *SSU*; isolate 30 591 was positive for *C. occultus* at actin and for *C. ratti* n. sp. at *SSU*; and isolate 15 461 was positive for *Cryptosporidium* rat genotype IV at actin and for *C. ratti* n. sp. at *SSU* (Table 1, Fig. 3). None of the samples with mixed infection were successfully sequenced at the *HSP70* locus. At the *HSP*70 gene, none of the isolates positive for *Cryptosporidium* rat genotype IV or V was amplified. Likewise, 13 of the 23 positive for *C. ratti* n. sp. and one of the four positive for *C. muris* failed to be amplified at the *HSP*70 gene (Table 2, Fig. 4). The sequences of individual *Cryptosporidium* species and genotypes detected in this study were identical to each other (Figs 2–4).

Purified oocysts of *C. ratti* n. sp. from Rat 0 (isolate 29 356) trapped at locality no. 14 did not infect 8-day-old BALB/c mice ($n = 3$); whereas, an 8-day-old rat (Rat 1) was successfully infected. The oocysts purified from experimentally-infected 1-week- and 8-week-old rats (below) were morphometrically identical to oocysts recovered from RAT 0 and RAT 1. The sequences of the *SSU*, actin and *HSP*70 genes obtained from Rat 1 were identical to those of Rat 0 (isolate 29 356). Oocysts recovered from Rat 1 were used for the description of oocyst morphometry, as well as transmission and molecular studies.

Cryptosporidium ratti n. sp. oocysts (Rat 1 origin) were only infectious for 1-week- and 8-week-old rats (Fig. 5). All rats started to shed *Cryptosporidium* oocysts detectable by PCR at 4–5 DPI. Microscopically detectable infection was not observed in any rat. The presence of specific *C. ratti* n. sp. DNA in faecal specimens was more often detected in rats infected at 1-week-old (21 times during the experiment) compared to rats infected at 8-weeks-old (16–18 times, Fig. 5). All rats remained infectious until the end of the experiment (Fig. 5). Examination of the gastrointestinal tract tissue of 1-week- and 8-week-old rats at 10, 20 and 30 DPI by PCR, histology and electron microscopy revealed the presence of specific DNA and developmental stages of *C. ratti* n. sp. in the jejunum and ileum. Developmental stages were scattered on an isolated villus (Fig. 6). The lamina propria in the jejunum was sporadically slightly edematous, but these changes were probably not related to the *Cryptosporidium* infection. A slight multiplication of goblet cells on infected villi was observed in the posterior part of the ileum. One-week- and 8-week-old BALB/c and SCID mice, as well as gerbils experimentally inoculated with oocysts of *C. ratti* n. sp. (Rat 1 origin), did not develop infections detectable in feces by microscopy or PCR. These animals also had no endogenous stages detectable by histology or electron microscopy. All groups of rats, mice and gerbils used as negative controls remained uninfected.

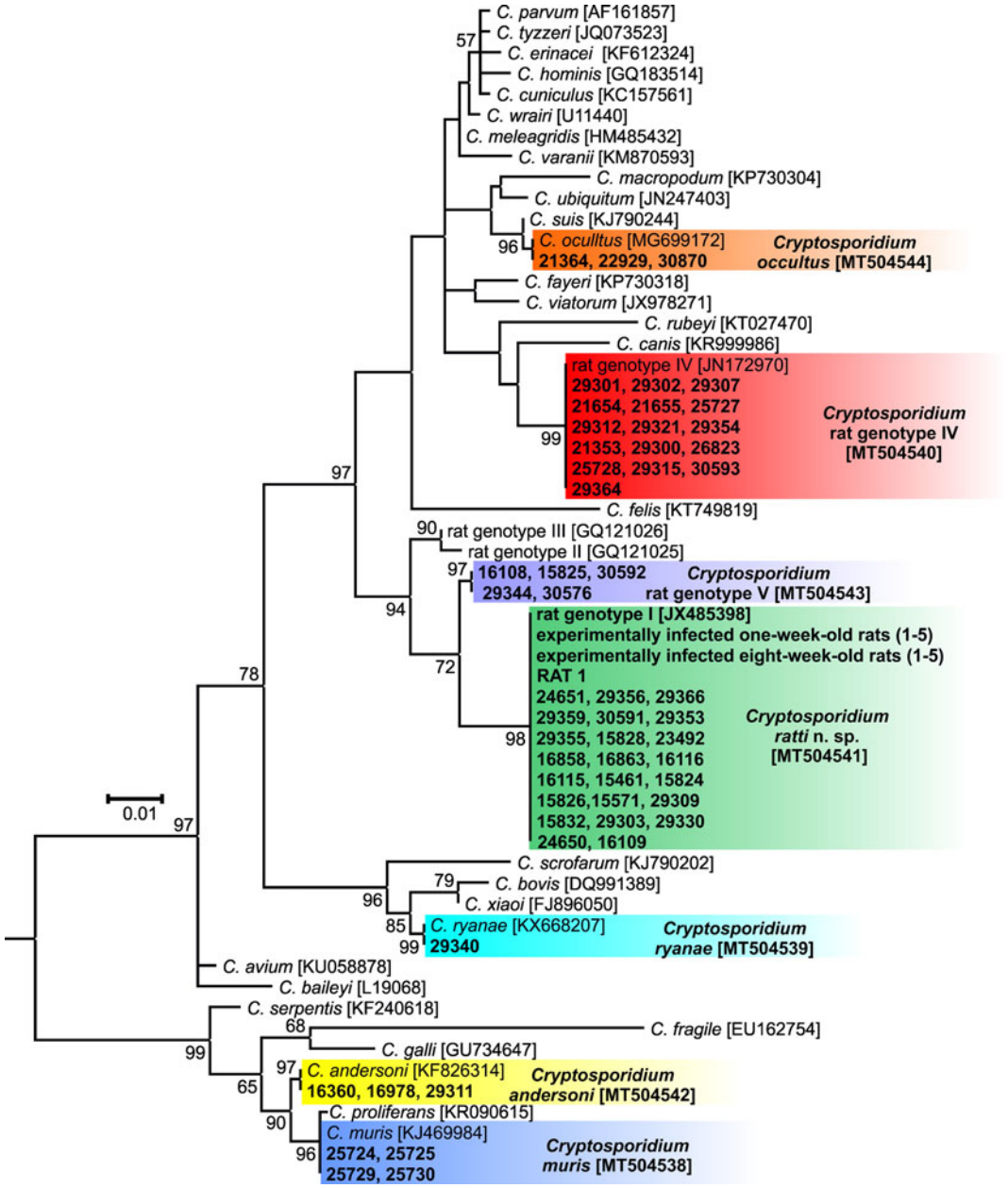

Fig. 2. Maximum likelihood tree based on partial sequences of the gene encoding the small subunit rRNA (*SSU*), including sequences obtained from naturally- and experimentally-infected hosts in this study. Tamura's 3-parameter model was applied, using a discrete Gamma distribution and invariant sites. The robustness of the phylogeny was tested with 1000 bootstraps and the numbers at the nodes represent the bootstrap *P* values with more than 50% bootstrap support. The branch length scale bar, indicating the number of substitutions per site, is included. Sequences obtained in this study are identified by isolate number (e.g. 29 356). The GenBank Accession number is in the bracket. *Cryptosporidium* species and genotypes detected in this study are colour-coded. The tree was rooted with the *SSU* sequence of *Plasmodium falciparum* (JQ627151) and the root was removed from the figure.

Taxonomic summary

Family Cryptosporidiidae Léger, 1911

Genus Cryptosporidium Tyzzer, 1907

Cryptosporidium ratti n. sp.

Syn: *Cryptosporidium* rat genotype I ex *Rattus norvegicus* of Zhao *et al.* (2018), Japan Kimura *et al.* (2007) and Philippines Ng-Hublin *et al.* (2013); *Cryptosporidium* sp. rat genotype rat193 ex *Rattus norvegicus* (Gen Bank no. JN172971, unpublished); *Cryptosporidium* environmental sequence clone ECUST628 from wastewaters of Feng *et al.* (2009); *Cryptosporidium* sp. 2162 ex *Boa constrictor* subsp. *ortoni* of Xiao *et al.* (2004); *Cryptosporidium* sp. rat genotype from raw water of Chalmers *et al.* (2010), *Cryptosporidium* sp. 18 and 23 ex *Rattus rattus* (Gen Bank no. KP883292 and KP883289, respectively, unpublished).

Type-host: *Rattus norvegicus* (Berkenhout, 1769) (Rodentia: Muridae), brown rat.

Other natural hosts: *Rattus rattus* (Linnaeus, 1758), black rat.

Type-locality: Telč (49.184339N, 15.472545E), Czech Republic.

Other localities: Chyšná (50.545694N, 13.437376E), Czech Republic; Lidéřovice (49.064462N, 15.373599E), Czech Republic; Přibyslav (49.580047N, 15.739454E), Czech Republic; Řevnov (49.475599N, 14.632047E), Czech Republic; Zmišovice (49.496220N, 15.188810E), Czech Republic.

Type-material: Histological sections of infected jejunum (nos. 181–183/2016) and ileum (nos. 184–189/2016); scanning electron microscopy specimens of infected jejunum (nos. 181–183/2016) and ileum (nos. 184–189/2016); genomic DNA isolated from faecal samples of naturally- (isolate 29 356) and experimentally- (isolate 16 848) infected rats; genomic DNA isolated from

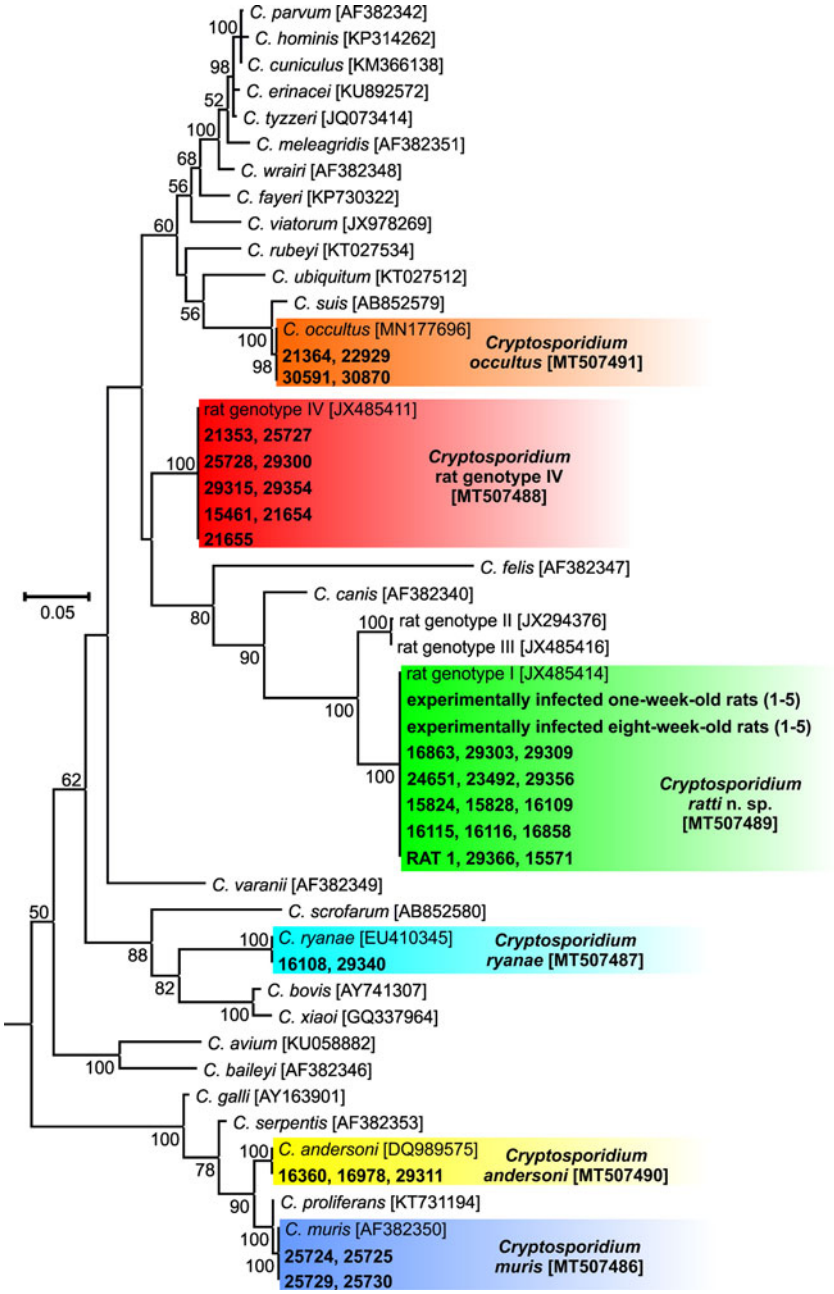

Fig. 3. Maximum likelihood tree based on partial sequences of the actin gene. The General Time Reversible model was applied, using a discrete Gamma distribution and invariant sites. The robustness of the phylogeny was tested with 1000 bootstraps and the numbers at the nodes represent the bootstrap *P* values with more than 50% bootstrap support. The branch length scale bar, indicating the number of substitutions per site, is included. Sequences obtained in this study are identified by isolate number (e.g. 29 356). The GenBank Accession number is in the bracket. *Cryptosporidium* species and genotypes detected in this study are colour-coded. The tree was rooted with the actin sequence of *Eimeria maxima* (XM013478337) and the root was removed from the figure.

jejunum and ileum of experimentally-infected rat (isolate 44 331); faecal smear slides with oocysts stained by ACMV and ZN staining (nos. 6/16848 and 15/16853). Specimens deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

Site of infection: Jejunum and ileum (present study, Fig. 6).

Distribution: As *Cryptosporidium* rat genotype I ex *Rattus norvegicus*: China (Zhao *et al.*, 2018), Japan (Kimura *et al.*, 2007) and Philippines (Ng-Hublin *et al.*, 2013); *Cryptosporidium* sp. 2162 ex *Boa constrictor* subsp. *ortoni* in USA (Xiao *et al.*, 2004); *Cryptosporidium* sp. 18 and 23 ex *Rattus rattus* in Iran; *Cryptosporidium* sp. rat genotype from raw water in the UK (Chalmers *et al.*, 2010).

Prepatent period: *Rattus norvegicus*: 4–5 DPI.

Patent period: At least 30 DPI in all experimentally infected rats (*Rattus norvegicus*)

Representative DNA sequences: Representative nucleotide sequences of *SSU* (MT504541), actin (MT507489) and *HSP*70 (MT507483) genes were saved in the GenBank database.

ZooBank registration: To comply with the regulations set out in Article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:59E724AA-5CBB-4E81-96C3-397D858E782D. The LSID for the new name *Cryptosporidium ratti* is urn:lsid:zoobank.org:act:C42A2AFD-7DB1-4B2E-AA37-3FAC0B069A26.

Etymology: The species name *ratti* is derived from the Latin noun "rattus" (meaning rat).

Description: Oocysts obtained from fresh feces specimens ex *Rattus norvegicus* (isolate 29 356) were spherical measuring 4.4–5.4 × 4.3–5.1 μm (4.9 ± 0.2 × 4.6 ± 0.2 μm) with a length to width ratio of 1.0–1.1 (1.1 ± 0.1) (Fig. 7). The oocyst wall was smooth and colourless, composed of a single layer. Micropyle and polar granule were absent, oocyst residuum was present, composed of numerous small granules and one spherical globule. Four sporozoites were present within each oocyst. Morphology and morphometry of other developmental stages are unknown.

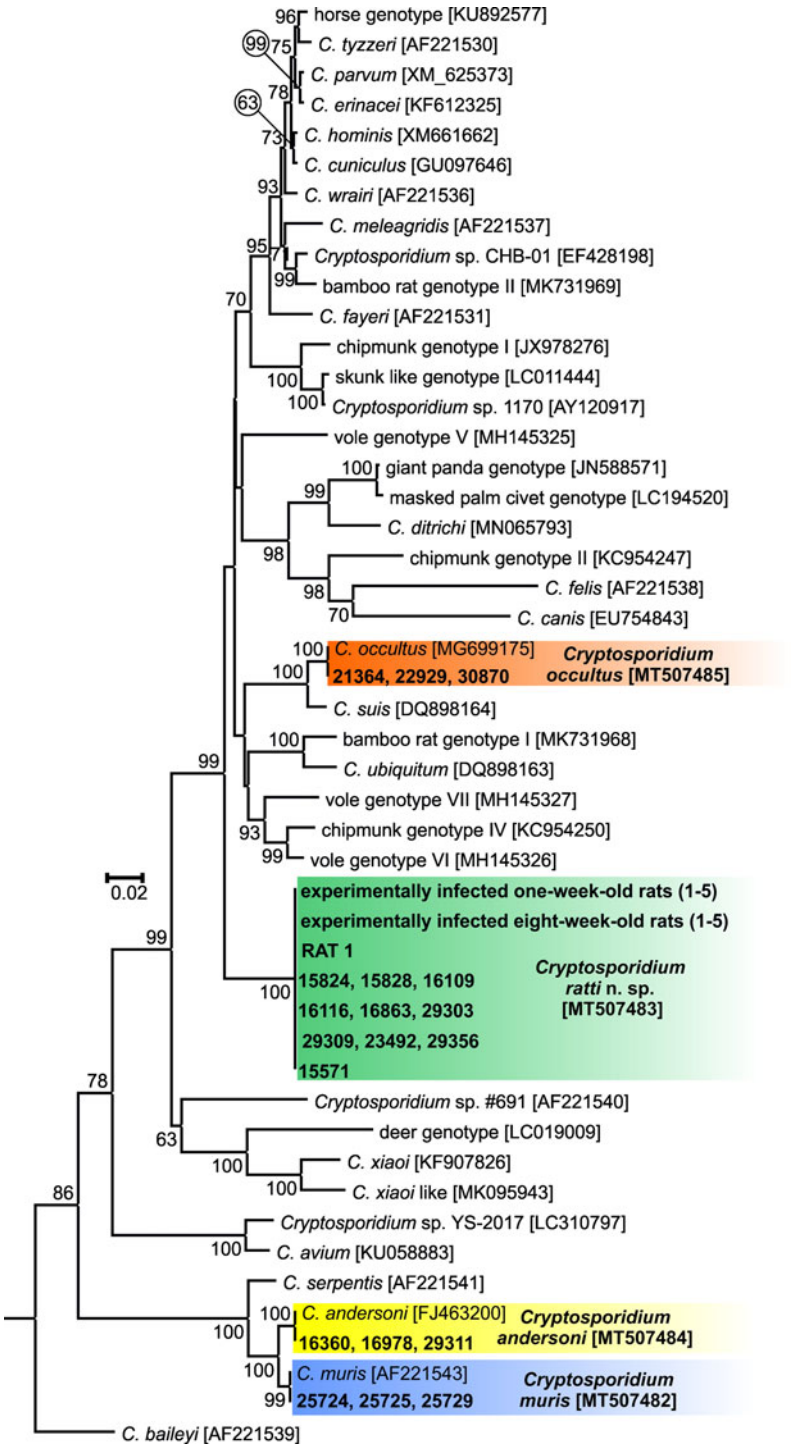

Fig. 4. Maximum likelihood tree based on partial sequences of the Heat Shock Protein 70 (*HSP70*) gene. The General Time Reversible model was applied, using a discrete Gamma distribution. The robustness of the phylogeny was tested with 1000 bootstraps and the numbers at the nodes represent the bootstrap *P* values with more than 50% bootstrap support. The branch length scale bar, indicating the number of substitutions per site, is included. Sequences obtained in this study are identified by isolate number (e.g. 29 356). The GenBank Accession number is in the bracket. *Cryptosporidium* species and genotypes detected in this study are colour-coded. The tree was rooted with the *HSP70* sequence of *Eimeria maxima* (Z46964) and the root was removed from the figure.

Remarks: Oocysts of *Cryptosporidium ratti* n. sp. showed typical *Cryptosporidium* ACMV and ZN staining characteristics and cross-react with immunofluorescence reagents developed primarily for *C. parvum*. There were no statistically significant size differences between oocysts from naturally infected rat and oocysts obtained from experimentally infected rat which measured 4.5–5.4 × 4.5–5.0 μm (4.9 ± 0.3 × 4.7 ± 0.2 μm) with a length/width ratio of 1.0–1.1 (1.1 ± 0.1) ($T^2 = 4.26$, $df_1 = 2$, $df_2 = 35.62$, $P = 0.1408$). Oocysts of *C. ratti* n. sp. are smaller than those of *C. parvum* ($T^2 = 18.88$, $df_1 = 2$, $df_2 = 27.88$, $P = 0.009$) and *C. occultus* ($T^2 = 30.38$, $df_1 = 2$, $df_2 = 28.24$, $P < 0.0001$). *Cryptosporidium ratti* n. sp. can be differentiated genetically from other *Cryptosporidium* species based on the sequences of *SSU*, actin and *HSP70* genes. Percentage of nucleotide similarities at the *SSU* locus between *C. ratti* n. sp. and the rat derived *C. occultus* and *Cryptosporidium* rat genotype

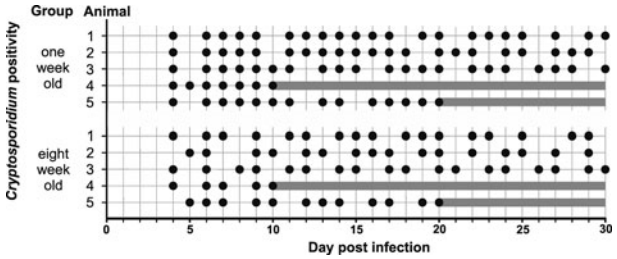

Fig. 5. Course of infection of *Cryptosporidium ratti* n. sp. in 1-week- and 8-week-old rats (*Rattus norvegicus*) based on microscopic and molecular (*SSU*) examination of feces. Grey circle indicates the detection of oocysts by microscopy, black circle indicates the detection of specific DNA by PCR. Grey line represents absence of rat due to sacrificing at 10 or 20 days post infection.

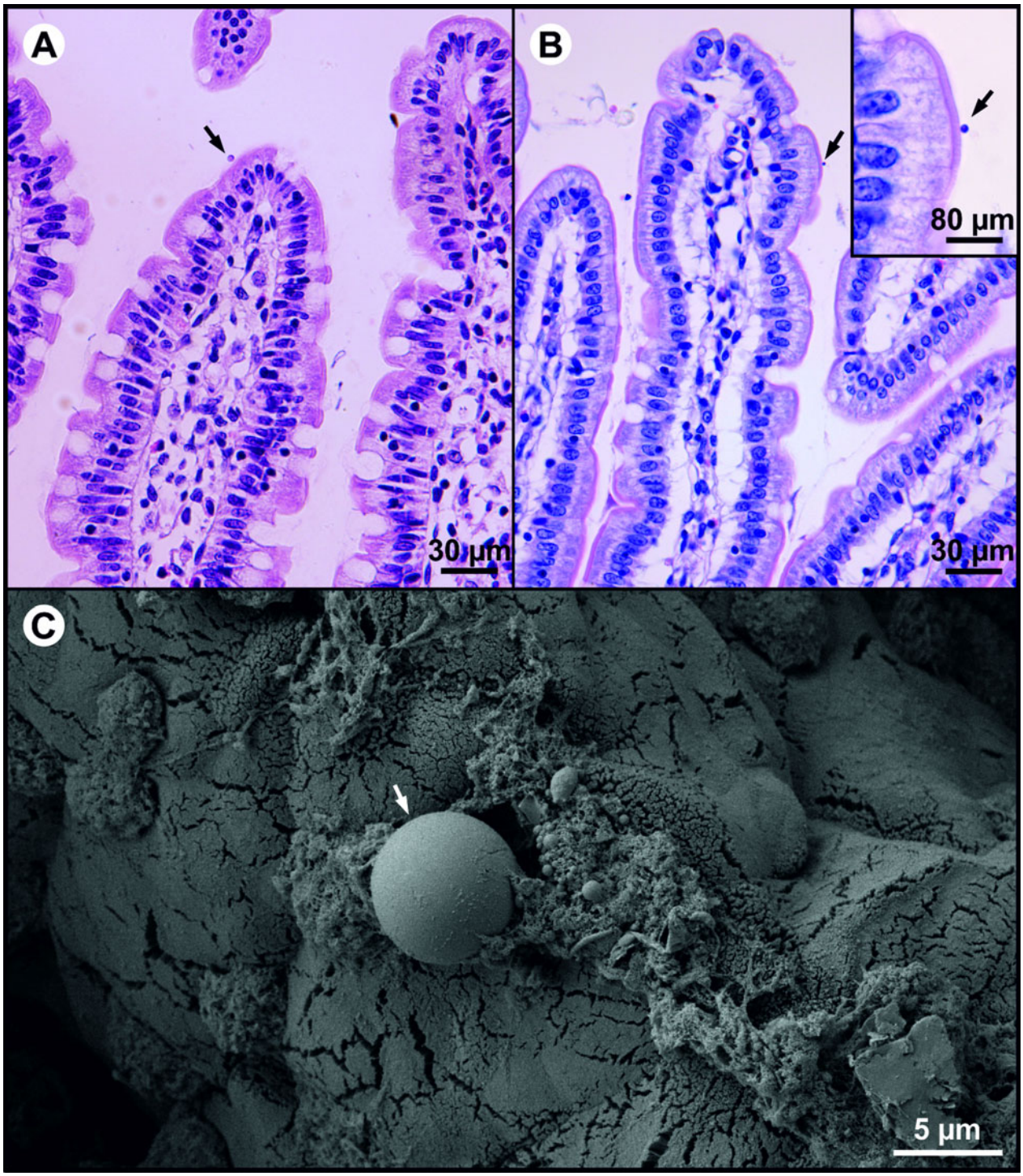

Fig. 6. Presence of developmental stages of *Cryptosporidium ratti* n. sp. (arrow) on jejunal mucosal epithelium in rat (*Rattus norvegicus*) infected at 1-week-old and sacrificed 10 days post infection. (A) and (B) histological sections stained by hematoxylin eosin, (C) scanning electron microphotograph. Scale bar is included in each figure.

II, III, IV and V was 94.4, 96.1, 96.8, 94.2 and 98.1%, respectively (Table 3). At the actin locus, *C. ratti* n. sp. shared 89.3, 94.0, 94.1 and 84.4% sequence identity, respectively, with *C. occultus* and rat derived *Cryptosporidium* genotype II, III and IV (Table 3). At the HSP70 locus, *C. ratti* n. sp. exhibited 91.0% sequence identity with *C. occultus* (Table 3).

Discussion

At least 17 *Cryptosporidium* spp. has been detected in rats worldwide (Kimura *et al.*, 2007; Lv *et al.*, 2009; Ng-Hublin *et al.*, 2013; Zhao *et al.*, 2015; Koehler *et al.*, 2018; Kváč *et al.*, 2018). The high number of detected species and genotypes in rats compared to other vertebrates may be explained by the frequent presence of non-rat-host-specific *Cryptosporidium* spp. It is possible that in cases of the presence of non-rat-host-specific *Cryptosporidium* spp., we detected only DNA from the mechanical transmission, as has been previously reported in other studies (Crawshaw and Mehren, 1987; Graczyk *et al.*, 1996; Kváč *et al.*, 2012). This presumption is supported by the fact that most of the non-rat-host-specific species come from either farm animals or from animals that are the prey of rats. In this study, we found *C. ryanae* and *C. andersoni* in rats trapped on dairy farms (data not shown). Similarly, Ng-Hublin *et al.* (2013) consider the

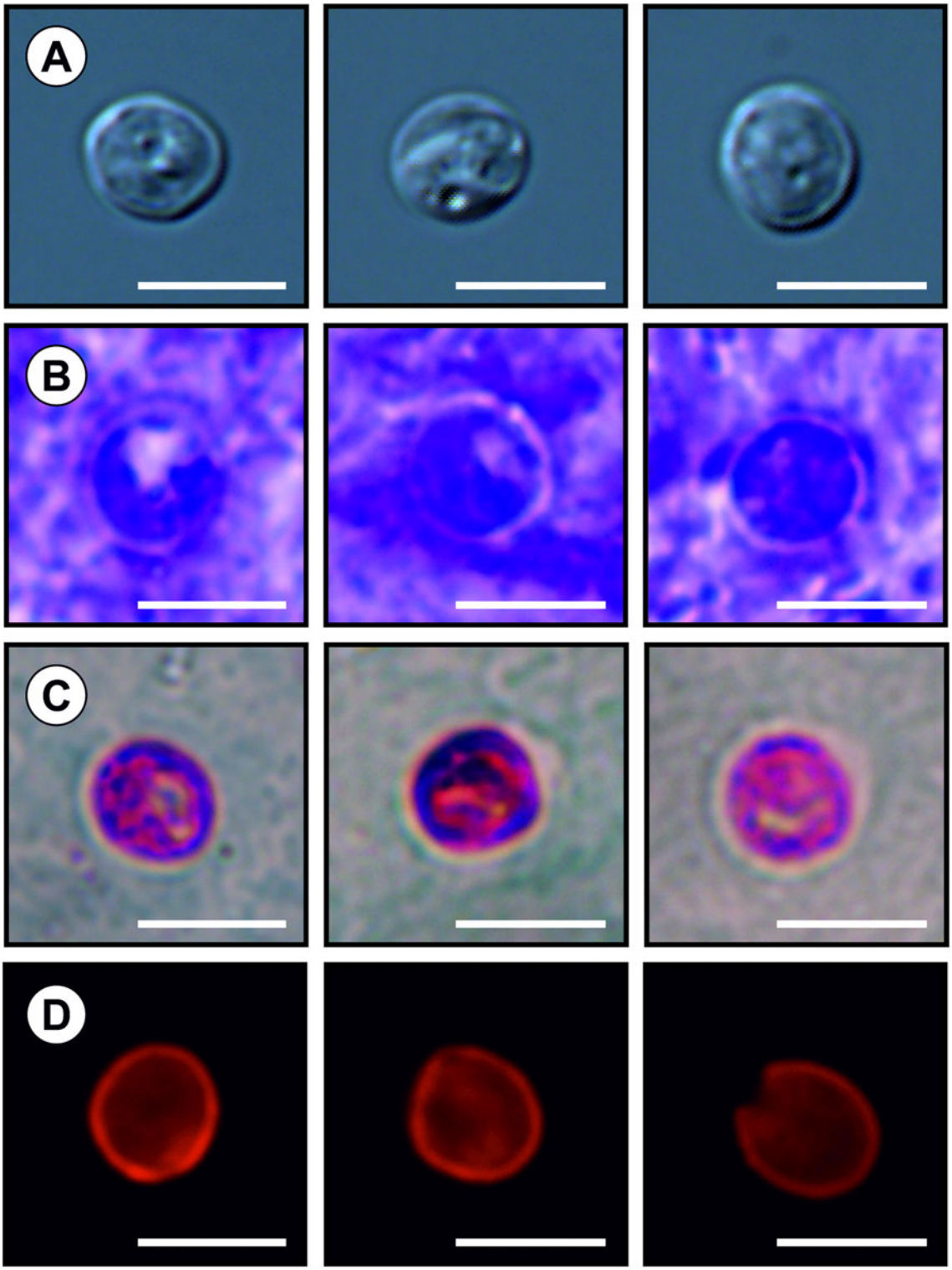

Fig. 7. Oocysts of *Cryptosporidium ratti* n. sp. (A) differential interference contrast microscopy, (B) aniline-carbol-methyl violet staining, (C) Ziehl-Nielsen staining, (D) labelling with antibody reagent consisting of a Cy3-labeled mouse monoclonal antibody made against *Cryptosporidium* oocyst outer wall antigenic sites. Bar = 5 μm.

occurrence of *C. scrofarum* in rats in the Philippines to be mechanical transmission, as pig entrails are present at the markets and pigs are raised in the villages close to the rice fields where the rats were trapped. Also, detection of *C. ratti* n. sp., rat-specific *Cryptosporidium*, in *Boa constrictor* subsp. *ortoni* by Xiao *et al.* (2004) probably represent mechanical passage after the snake caught the infected rat. It is worth noting that all non-rat-host-specific *Cryptosporidium* species – *C. meleagridis*, *C. erinacei*, *C. ubiquitum*, *C. tyzzeri* and *C. viatorum* – have only been found in some studies and rarely to a high degree within them. For the most part, one to three positive rats were detected, as in this study (Kimura *et al.*, 2007; Koehler *et al.*, 2018; Zhao *et al.*, 2018, 2019). These results show a random distribution of these *Cryptosporidium* spp. rather than adaptation to the host (Tan *et al.*, 2019). The presence of *C. parvum* in most of the studies is not surprising, as it lacks host specificity. This shows that rats are susceptible, although not the typical hosts, which are livestock (Nydam *et al.*, 2001). Comparable to previous studies, we found a low occurrence of *C. muris*, a species with broad host specificity within rodents, which suggests, as in the case of *C. parvum*,

Table 3. Percentage of nucleotide similarities between *Cryptosporidium ratti* n. sp. and selected closest and furthest *Cryptosporidium* species and *Cryptosporidium* rat genotypes II-V at small subunit ribosomal RNA (*SSU*), actin and 70 kDa heat-shock protein (*HSP70*) genes

	Gene locus		
Species/genotype	*SSU*	Actin	*HSP70*
C. andersoni	89.0	80.5	81.5
C. avium	92.4	80.3	84.3
C. baileyi	92.0	81.0	85.1
C. bovis	91.1	82.9	NC
C. canis	93.7	89.3	84.0
C. felis	92.1	83.9	82.9
C. galli	88.6	80.1	NC
C. hominis	94.3	83.1	93.2
C. muris	89.1	80.3	81.9
C. occultus	94.4	89.3	91.0
C. parvum	92.4	83.1	92.4
C. rubeyi	92.6	84.7	91.0
C. ryanae	92.4	82.7	NC
C. scrofarum	91.0	82.5	NC
C. suis	94.4	84.2	90.9
C. ubiquitum	94.2	84.7	90.3
C. xiaoi	91.4	82.7	86.5
Rat genotype II	96.1	94.0	NA
Rat genotype III	96.8	94.1	NA
Rat genotype IV	94.2	84.4	NA
Rat genotype V	98.1	NA	NA

NA, sequences are not available; NC, partial sequence does not cover sequence of *C. ratti* n. sp.

that rats are natural but not typical hosts. The frequent occurrence and high prevalence of *Cryptosporidium ratti* n. sp. (previously known as *Cryptosporidium* rat genotype I) and *Cryptosporidium* rat genotypes II-IV in previous as well as this study and the fact that these *Cryptosporidium* spp. have very rarely or never been detected in other hosts could imply that this species is host-specific for rats (Kimura *et al.*, 2007; Lv *et al.*, 2009; Paparini *et al.*, 2012; Ng-Hublin *et al.*, 2013; Silva *et al.*, 2013; Kváč *et al.*, 2018; Zhao *et al.*, 2018). Additionally, the finding that rats are susceptible to *C. ratti* n. sp. infection under experimental conditions, while mice and gerbils are not, supports the narrow host specificity of this species.

In contrast to other studies from Asia, Australia and South America, we did not detect any *Cryptosporidium* rat genotypes II and III (Lv *et al.*, 2009; Paparini *et al.*, 2012; Ng-Hublin *et al.*, 2013; Silva *et al.*, 2013; Zhao *et al.*, 2019). Given that this work is the first comprehensive study from Europe, it would not be appropriate to draw conclusions regarding the absence of these genotypes in the Czech Republic. Further studies are needed. Similarly, Čondlová *et al.* (2019) detected *Cryptosporidium* apodemus genotypes I and II across Europe, including the Czech Republic, in a 2019 study, although both genotypes were missing in their study performed in the Czech Republic in 2018 (Čondlová *et al.*, 2018). On the other hand, the absence of *Cryptosporidium* rat genotypes II and III may be suggestive of patterns of geographical distribution of these genotypes.

The novel *Cryptosporidium* rat genotype V, which we found in five animals from two locations, has never been detected in other hosts or wastewater. Repeated detection in independent samples more than t2 years apart (data not shown) may indicate that *Cryptosporidium* rat genotype V is infectious to rats. More studies are needed to confirm that this genotype is specific for rats and to explain why it was not detected in previous studies.

Although the diagnostic methods using microscopy are still frequently used for differentiation among species due to their simplicity and low cost, it is difficult to distinguish among the various *Cryptosporidium* species and genotypes because the size variability of the oocysts is small and the oocyst size of most *Cryptosporidium* genotypes is unknown. Although, oocyst size of *C. ratti* n. sp. difference from other *C. occultus* ($5.2 \times 4.9\,\mu$m), *C. parvum* ($5.3 \times 4.7\,\mu$m), it would be difficult to differentiate it microscopically from these and other *Cryptosporidium* species reported in rats. For example, *C. tyzzeri* ($4.6 \times 4.2\,\mu$m), *C. meleagridis* ($5.2 \times 4.6\,\mu$m), *C. erinacei* ($4.9 \times 4.4\,\mu$m), *C. ubiquitum* ($5.0 \times 4.7\,\mu$m) and *C. viatorum* ($5.4 \times 4.7\,\mu$m) have morphometrically similar oocysts (Lindsay *et al.*, 1989; Fayer *et al.*, 2010; Elwin *et al.*, 2012; Ren *et al.*, 2012; Kváč *et al.*, 2014*a*; Kváč *et al.*, 2018).

Cryptosporidium ratti n. sp. is genetically distinct from valid *Cryptosporidium* species at *SSU*, actin and *HSP70* and did not exhibit sequence heterogeneity. At the *SSU* locus, *C. ratti* n. sp. formed a separate cluster with *Cryptosporidium* rat genotype II, III and V and was closely related to *C. felis* with nucleotide similarities of 96.2, 96.0, 98.1, and 92.1%, respectively. These genetic variations were greater than that observed between close related species, i.e. *C. occultus* and *C. suis* (99.5%) or *C. muris* and *C. andersoni* (99.0%), and similar to that observed between distinct related species, i.e. *C. parvum* and *C. erinacei* (93.2%) or *C. alticolis* and *C. ditrichi* (96.1%). At actin locus, *C. ratti* n. sp. clustered together with *C. canis*, *C. felis*, and *Cryptosporidium* rat genotypes II and III with nucleotide similarity of 89.3, 83.9, 94.0 and 94.1%, respectively. These genetic variations are greater than those between i.e. *C. parvum* and *C. eriancei* (99.5%) and similar to those between *C. ryanae* and *C. bovis* (88.9%). There are missing nucleotide sequences of several *Cryptosporidium* species at the *HSP70* locus. Analyses of the *HSP70* locus indicate that *C. ratti* n. sp. and *C. occultus*, the rat-specific *Cryptosporidium* species, shared a nucleotide similarity of 91.0%. In comparison, i.e. *C. parvum* and *C. erinacei* share 99.2% similarity and i.e. *C. parvum* and *C. andersoni* 88.0%.

The prepatent period of *C. ratti* n. sp. was 4–5 DPI, which is consistent with *C. occultus* in rats (4–5 DPI) and other intestinal *Cryptosporidium* spp.: for example, *C. alticolis* in voles (3–4 DPI), *C. parvum* in calves (2–7 DPI), *C. tyzzeri* in mice (4–7 DPI), *C. xiaoi* in sheep (7–8 DPI), and *C. scrofarum* in pigs (4–6 DPI) (Tzipori *et al.*, 1983; Fayer and Santín, 2009; Ren *et al.*, 2012; Kváč *et al.*, 2013, 2018; Horčičková *et al.*, 2018). Unlike *C. occcultus*, which causes a massive infection of the colonic epithelium but low shedding of oocysts, *C. ratti* n. sp. causes a weak infection of the small intestine and the intensity of oocyst shedding matches the intensity of the developmental stages observed in the epithelium (Kváč *et al.*, 2018). A similar relationship between oocyst secretion and gastrointestinal involvement has been observed in other *Cryptosporidium* species infecting the small intestine (Ren *et al.*, 2012; Kváč *et al.*, 2013; Li *et al.*, 2015; Čondlová *et al.*, 2018; Holubová *et al.*, 2019).

Cryptosporidium spp. are often considered to be a cause of diarrheal diseases of humans and animals (Naciri *et al.*, 1999; Morgan-Ryan *et al.*, 2002; Rašková *et al.*, 2013; Chappell *et al.*, 2015). The faecal samples from trapped wild rats and from those experimentally infected with *C. ratti* n. sp. had solid consistency and none of the animals exhibited gastrointestinal symptoms related to *Cryptosporidium* infection. This is consistent with the results of previous studies that have found that rats and other wild animals rarely develop clinical cryptosporidiosis (Kimura

et al., 2007; Ren et al., 2012; Ng-Hublin et al., 2013; Silva et al., 2013; Li et al., 2015; Song et al., 2015; Ježková et al., 2016; Stenger et al., 2017).

Based on the results of this and previous studies, it has been shown that *Cryptosporidium* rat genotype I is biologically and molecularly different from other *Cryptosporidium* species and represents a separate species within the genus *Cryptosporidium*. Therefore, we propose the name *Cryptosporidium ratti* n. sp.

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Conflict of interest. None of the authors has any competing interests in the manuscript.

Ethical standards. Not applicable.

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