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Cryptosporidium proventriculi sp. n. (Apicomplexa: Cryptosporidiidae) in Psittaciformes birds

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Abstract

Cryptosporidiosis is a common parasitic infection in birds that is caused by more than 25 *Cryptosporidium* species and genotypes. Many of the genotypes that cause avian cryptosporidiosis are poorly characterized. The genetic and biological characteristics of avian genotype III are described here and these data support the establishment of a new species, *Cryptosporidium proventriculi*. Faecal samples from the orders Passeriformes and Psittaciformes were screened for the presence of *Cryptosporidium* by microscopy and sequencing, and infections were detected in 10 of 98 Passeriformes and in 27 of 402 Psittaciformes. *Cryptosporidium baileyi* was detected in both orders. *Cryptosporidium galli* and avian genotype I were found in Passeriformes, and *C. avium* and *C. proventriculi* were found in Psittaciformes. *Cryptosporidium proventriculi* was infectious for cockatiels under experimental conditions, with a prepatent period of six days post-infection (DPI), but not for budgerigars, chickens or SCID mice. Experimentally infected cockatiels shed oocysts more than 30 DPI, with an infection intensity ranging from 4,000 to 60,000 oocysts per gram (OPG). Naturally infected cockatiels shed oocysts with an infection intensity ranging from 2,000 to 30,000 OPG. *Cryptosporidium proventriculi* infects the proventriculus and ventriculus, and oocysts measure 7.4 × 5.8 μm. None of the birds infected *C. proventriculi* developed clinical signs.

Keywords: *Cryptosporidium* avian genotype III; Experimental infections; Oocyst size; PCR; Prevalence; Taxonomy

Introduction

Cryptosporidium parasites belong to the phylum Apicomplexa and infect the gastrointestinal tract of a broad range of vertebrate species (Smith et al. 2007; Sreter and Varga 2000). Currently, 41 species of *Cryptosporidium* are recognized in

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fish, amphibians, reptiles, birds, and mammals (Kváč et al. 2014; Liu et al. 2013; Qi et al. 2011). In contrast, more than 70 genotypes have been described that await the morphological and biological characterization necessary to describe them as separate species (Chalmers et al. 2018; Ryan et al. 2014; Xiao et al. 1999; Xiao and Ryan 2004).

To date, four avian *Cryptosporidium* spp. have been described in birds: *Cryptosporidium meleagridis* (Slavin 1955), *Cryptosporidium baileyi* (Current et al. 1986), *Cryptosporidium galli* (Ryan et al. 2003b) and *Cryptosporidium avium* (Holubová et al. 2016). In addition, 21 *Cryptosporidium* genotypes have been identified in more than 30 avian species worldwide (Ryan 2010), and these appear to be avian specific. They include avian genotypes I–IV and VI–IX (Helmy et al. 2017), goose genotypes I–IV (Zhou et al. 2004) and Id (Cano et al. 2016), duck genotypes I and II (Jellison et al. 2004; Morgan et al. 2001; Zhou et al. 2004) and duck genotype b (Cano et al. 2016), Eurasian Woodcock genotype (Ryan et al. 2003a), Finch genotypes I–III (Morgan et al. 2000) and YS-2017 genotype from owls (Makino et al. 2018). Mammal-specific *Cryptosporidium* spp., including *C. andersoni*, *C. hominis*, *C. muris*, *C. parvum*, *C. canis*, have been reported rarely in birds (Ferrari et al. 2018; Helmy et al. 2017; Nakamura et al. 2009; Ng et al. 2006; Oliveira et al. 2017; Qi et al. 2014; Santín et al. 2004).

Cryptosporidium meleagridis, *C. baileyi* and *C. galli* infect the intestine, lungs, and proventriculus, respectively, and have been found in several avian orders, suggesting low host specificity in avian hosts (Baroudi et al. 2013; Jellison et al. 2004; Li et al. 2015; Ng et al. 2006; Wang et al. 2014). In contrast, *C. avium*, which is closely related to *C. baileyi* and infects the same site, appears to be restricted to hosts in the order Psittaciformes (Holubová et al. 2016; Slavin 1955). Most avian *Cryptosporidium* genotypes have been detected in a small number of avian hosts, but the data are insufficient to support the conclusion that they have a narrow host range, similar to *C. avium* (Nakamura and Meireles 2015). The present study aimed to address this deficiency for *Cryptosporidium* avian genotype III, which infects the proventriculus and ventriculus of birds belonging to five orders of class Aves (Table 1). We use experimental infections to confirm that avian genotype III has a different host range than *C. galli*, and we also show that it differs from *C. galli* in other biological characteristics, oocyst morphology, and nucleotide sequence at multiple loci. Based on these and other reported data, we propose that *Cryptosporidium* avian III be recognized as a new *Cryptosporidium* species, *Cryptosporidium proventriculi* sp. n.

Material and Methods

Specimens studied

Faecal samples from pet birds owned by private breeders in the Czech Republic (n = 10), Slovakia (n = 2) and Poland (n = 3) were sampled. Each sample was collected from the floor of the bird cage immediately after defecation and placed into a separate plastic tube without fixative. The faecal consistency (loose if it took the form of the container and solid if it maintained its original shape) was noted at the time of sampling. Each animal was sampled only once. All animals were screened without previous knowledge of parasitological status. A total of 402 and 98 samples were obtained from birds in the order Psittaciformes and Passeriformes, respectively (Table 2). All samples were stored at 4 °C until used for further analysis.

Origin of specimens for transmission studies

Isolates of *Cryptosporidium proventriculi* sp. n. (previously known as *Cryptosporidium* avian genotype III) were obtained from four naturally infected adult cockatiels (*Nymphicus hollandicus*) (Czech Republic). *Cryptosporidium proventriculi* sp. n. oocysts from positive birds were pooled and used to infect an adult cockatiel, which was negative for *Cryptosporidium* DNA by PCR and was sourced from a breeder that had no *Cryptosporidium*-positive birds. Oocysts from the infected cockatiel were purified using caesium chloride gradient centrifugation (Arrowood and Donaldson 1996) and used for oocyst morphometry and infectivity studies (see Transmission studies).

Parasitological examination and oocyst preparation

All animal faeces were screened for *Cryptosporidium* oocysts using faecal smears stained with aniline-carbol-methyl violet (ACMV) (Miláček and Vítovec 1985). Faecal specimens were collected daily and stored in a 2.5% potassium dichromate solution at 4–8 °C.

Cryptosporidium oocysts from cockatiels were purified using caesium chloride gradient centrifugation prior to morphometric analyses and transmission studies (Kilani and Sekla 1987). The viability of oocysts was examined using propidium iodide (PI) staining by a modified assay of Sauch et al. (1991). Briefly, examined oocysts were washed in distilled water (DW; 10,000 oocysts in 10 μl) and mixed with 0.1 μl of PI (1% solution, SIGMA). After 30 min of incubation at room temperature in the dark, the oocysts were washed twice with DW. Oocyst viability was examined using fluorescence microscopy (filter 420 nm, Olympus IX70). Oocysts with red fluorescence were considered to be dead, and those without fluorescence were considered viable.

Oocyst morphometry

The morphology and morphometry of *Cryptosporidium proventriculi* sp. n. oocysts were examined using differential interference contrast (DIC) microscopy, brightfield microscopy following ACMV and modified Ziehl-Neelsen (ZN; Henriksen and Pohlenz 1981) staining and fluorescence microscopy following labelling with genus-specific FITC-

Table 1. Occurrence of *Cryptosporidium proventriculi* sp. n. (formerly known as *Cryptosporidium* avian genotype III) in birds from orders Psittaciformes[1], Passeriformes[2], Piciformes[3], Charadriiformes[4] and Anseriformes[5] demonstrated on the basis of molecular tools amplifying partial sequences of *Cryptosporidium* small-subunit rRNA (SSU), actin and *Cryptosporidium* oocyst wall protein (COWP) genes.

Host (scientific name)	Country	Loci for genotyping [GenBank Acc. No.]	No. of screened/ positive	References
Blue-fronted parrot[1] (*Amazona aestiva*)	Brazil	SSU[b]	NS/1	Nakamura et al. (2014)
Barred parakeet[1] (*Bolborhynchus lineola*)	Brazil	SSU [MF462155]	34/1	Ferrari et al. (2018)
Cockatiel[1] (*Nymphicus hollandicus*)	Australia	SSU [DQ650343]	NS/3	Ng et al. (2006)
	Brazil	SSU [identical with GQ227480]	8/1	Nakamura et al. (2014)
		SSU [GQ227481]	64/1	Nakamura et al. (2009)
		SSU [GU074385-87]	NS/3	Gomes et al. (2012)
		SSU [identical with GQ227480]	70/9	Ferrari et al. (2018)
	China	SSU [HM116385]	39/2	Qi et al. (2011)
	India	SSU [KX668210]	NA	unpublished
	Japan	SSU [identical with AB694729]	10/1	Iijima et al. (2018)
		SSU [AB471645] COWP [AB471653] Actin [AB471659]	4/1	Abe and Makino (2010)
Forpus sp.[1]	Brazil	SSU [MF462156]	78/12	Ferrari et al. (2018)
Galah[1] (*Eolophus roseicapilla*)	Australia	Actin [DQ650349]	NS/1	Ng et al. (2006)
		SSU[b]	13/2	Nakamura et al. (2014)
Lovebird[1] (*Agapornis* sp.)	Brazil	SSU [identical with GQ227480]	14/3	Ferrari et al. (2018)
	USA	SSU [KJ661334]	18/2	Ravich et al. (2014)
Lilian's lovebird[1] (*Agaponis lilianae*)	Japan	SSU [identical with AB694729]	5/1	Iijima et al. (2018)
Neophema sp. [1]	Brazil	SSU[b]	91/1	Ferrari et al. (2018)
Pacific parrotlet[1] (*Forpus coelestis*)	Japan	SSU [identical with AB694729]	3/1	Iijima et al. (2018)
Peach-faced lovebird[1] (*Agapornis roseicollis*)	Brazil	SSU [GQ227480]	14/1	Nakamura et al. (2009)
	Japan	SSU [AB471641] Actin [AB471655]	37/13	Makino et al. (2010)
		SSU [identical with AB694729]	29/5	Iijima et al. (2018)
Red-rumped parrot[1] (*Psephotus haematonotus*)	Brazil	SSU*	21/1	Ferrari et al. (2018)
Sun parakket[1] (*Aratinga solstitialis*)	Australia	SSU [DQ650342]	NS/1	Ng et al. (2006)
Double-collared seedeater[2] (*Sporophila caerulescens*)	Brazil	SSU[b]	10/1	Nakamura et al. (2014)
Green winged saltator[2] (*Saltator similis*)	Brazil	SSU*	152/1	Nakamura et al. (2014)
Island canary[2] (*Serinus canaria*)	Brazil	SSU[b]	498/12	Camargo et al. (2018)
Java sparrow[2] (*Padda oryzivora*)	Brazil	SSU [GU074384]	NS/1	Gomes et al. (2012)
Red-billed blue magpie[2] (*Urocissa erythrorhyncha*)	China	SSU [HM116386]	1/1	Qi et al. (2011)
Rufous-collared sparrow[2] (*Zonotrichia capensis*)	Brazil	SSU*	NS/1	Nakamura et al. (2014)
Sporophila sp.[2]	Brazil	SSU*	NS/1	Nakamura et al. (2014)

Table 1 (*Continued*)

Host (scientific name)	Country	Loci for genotyping [GenBank Acc. No.]	No. of screened/ positive	References
Saffron toucanet[3] (*Pteroglossus bailloni*)	Brazil	SSU [KU885389]	2/1	Novaes et al. (2018)
Red-billed toucan[3] (*Ramphastos tucanus*)	Brazil	SSU [KU885388]	4/2	Novaes et al. (2018)
Toco toucan[3] (*Ramphastos toco*)	Brazil	SSU [KU885387]	28/5	Novaes et al. (2018)
Seagul[4] (*Chroicocephalus brunnicephalus* and *ridibundus*)	Thailand	SSU [identical with AB694729]	70/2	Koompapong et al. (2014)
Waterbird[5,a]	Spain	SSU [KT880495-97]	265/4	Cano et al. (2016)

NS not specified.
[a] bird species was not determined.
[b] based on the duplex real-time PCR targeting the SSU gene in *Cryptosporidium* avian genotype III.

conjugated antibodies (IFA; *Cryptosporidium* IF Test, Crypto cel, Cellabs Pty Ltd., Brookvale, Australia).

Morphology and morphometry were determined using digital analysis of images (Olympus cellSens Entry 2.1, Olympus Corporation, Shinjuku, Tokyo, Japan) collected using an Olympus Digital Colour camera DP73 microscope. Length and width of oocysts (n = 100) from experimentally infected cocktails were measured under DIC at 1000 × magnification and these measurements were used to calculate the length-to-width ratio. As a control, the morphometry of *C. baileyi* (n = 100) and *C. avium* (n = 50) from experimentally infected chickens (*Gallus gallus* f. *domestica*) and budgerigars (*Melopsittacus undulatus*), respectively, were measured by the same person using the same microscope. Photomicrographs of *C. proventriculi* sp. n. oocysts observed by DIC, ACMV, ZN and IFA were deposited as a photo type at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

Molecular characterization

DNA was extracted from 200 mg of faeces, 10,000 purified oocysts, or 200 mg of tissue by bead disruption for 60 s at 5.5 m/s using 0.5 mm glass beads in a FastPrep® 24 Instrument (MP Biomedicals, CA, USA) followed by isolation/purification using a commercially available kit in accordance with the manufacturer's instructions (Exgene™ Stool DNA mini, GeneAll Biotechnology Co. Ltd, Seoul, Korea). Purified DNA was stored at −20 °C prior to being used for PCR. A nested PCR approach was used to amplify a partial region of the small ribosomal subunit rRNA (~830 bp; SSU; Xiao et al. 1999), actin (~1950 bp; Sulaiman et al. 2000), and 70 kilodalton heat shock protein genes (~515 bp; HSP70; Chelladurai et al. 2016). For the SSU fragment, primary amplification was employed with the primers 5′TTC TAG AGC TAA TAC ATG CG3′ and 5′CCC ATT TCC TTC GAA ACA GGA3′ followed by secondary amplification with the primers 5′GGA AGG GTT GTA TTT ATT AGA TAA AG3′ and 5′AAG GAG TAA GGA ACA ACC TCC A3′. For the actin fragment, primary amplification was employed with the primers 5′ATC RGW GAA GAA GWA RYW CAA GC3′ and 5′AGA ARC AYT TTC TGT GKA CAA T3′ followed by secondary amplification with the primers 5′CAA GCW TTR GTT GTT GAY AA3′ and 5′TTT CTG TGK ACA ATW SWT GG3′. For the HSP70 fragment, primary amplification was employed the primers 5′GCT CGT GGT CCT AAA GAT AA3′) and 5′ACG GGT TGA ACC ACC TAC TAA T3′ followed by secondary amplification with the primers 5′ACA GTT CCT GCC TAT TTC3′) and 5′GCT AAT GTA CCA CGG AAA TAA TC3′.

The primary PCR mixtures contained 2 μl of template DNA, 2.5 U of Taq DNA Polymerase (Dream Taq Green DNA Polymerase, Thermofisher Scientific, Waltham, MA, USA), 0.5 × PCR buffer (SSU) or 1 × PCR buffer (actin and HSP70; Thermofisher Scientific), 6 mM MgCl2 (SSU) or 3 mM MgCl2 (actin and HSP70), 200 μM each deoxynucleoside triphosphate, 100 mM each primer and 2 μL non-acetylated bovine serum albumin (BSA; 10 mg ml−1; New England Biolabs, Beverly, MA, USA) in 50 μl reaction volume. The secondary PCR mixtures were similar to those described above for the primary PCR, with the exception that 2 μl of the primary PCR product was used as the template, the MgCl2 concentration was 3 mM and no BSA was used. DNA of *C. parvum* and molecular grade water were used as positive and negative controls, respectively.

Secondary PCR products were detected by agarose gel electrophoresis, visualized by ethidium bromide staining and extracted using Gen Elute Gel Extraction Kit (Sigma, St. Louis, MO, USA). Purified secondary products were sequenced in both directions with an ABI 3130 genetic analyser (Applied Biosystems, Foster City, CA) using the secondary PCR primers and the BigDye1 Terminator V3.1

Table 2. *Cryptosporidium* species and genotypes from this study, detected by amplification of small subunit ribosomal rRNA (SSU), actin and 70 kDa Heat Shock Protein (HSP70) gene fragments in birds from the orders Psittaciformes and Passeriformes from the Czech Republic (CZK), Poland (POL) and Slovakia (SVK). Infection intensity of *Cryptosporidium* spp. is expressed as the number of oocysts per gram of faeces (OPG).

Order	Host (scientific name)	Number of screened/ positive	ID of positive animal	Country/ No. breed	Microscopical positivity (OPG)	Genotyping at the gene loci	
						SSU, ACTIN	HSP70
Psittaciformes	**African grey parrot** (*Psittacus erithacus*)	1/0	NA	CZE/1	NA	NA	NA
		2/0	NA	CZE/2	NA	NA	NA
		2/0	NA	CZE/6	NA	NA	NA
		3/0	NA	CZE/7	NA	NA	NA
		4/0	NA	CZE/8	NA	NA	NA
		4/0	NA	CZE/9	NA	NA	NA
		4/0	NA	SVK/1	NA	NA	NA
		4/0	NA	SVK/2	NA	NA	NA
	Blue-and-yellow macaw (*Ara ararauna*)	2/0	NA	CZE/2	NA	NA	NA
		4/0	NA	CZE/8	NA	NA	NA
	Blue-fronted parrot (*Amazona aestiva*)	2/0	NA	CZE/1	NA	NA	NA
		4/0	NA	CZE/2	NA	NA	NA
		4/0	NA	CZE/3	NA	NA	NA
		1/0	NA	CZE/6	NA	NA	NA
		4/0	NA	CZE/7	NA	NA	NA
		5/0	NA	CZE/8	NA	NA	NA
		2/0	NA	CZE/9	NA	NA	NA
		3/0	NA	SVK/1	NA	NA	NA
		2/0	NA	SVK/2	NA	NA	NA
	Budgerigar (*Melopsittacus undulatus*)	8/1	28471	CZE/3	No	*C. avium*	*C. avium*
		9/1	26150	CZE/1	No	*C. baileyi*	*C. baileyi*
		7/1	35382	CZE/5	No	*C. baileyi*	*C. baileyi*
		7/0	NA	CZE/2	NA	NA	NA
		5/0	NA	CZE/4	NA	NA	NA
		6/0	NA	CZE/6	NA	NA	NA
		4/0	NA	CZE/7	NA	NA	NA
		8/0	NA	CZE/8	NA	NA	NA
		7/0	NA	CZE/9	NA	NA	NA
		10/0	NA	SVK/1	NA	NA	NA
		7/0	NA	SVK/2	NA	NA	NA
	Cockatiels (*Nymphicus hollandicus*)	18/3	34758	CZE/1	Yes (2,000)	*C. proventriculi*	*C. proventriculi*
			23772		No	*C. proventriculi*	*C. proventriculi*
			25137		Yes (2,000)	*C. baileyi*	*C. baileyi*
		6/1	35508	CZE/2	No	*C. proventriculi*	*C. proventriculi*

Table 2 (*Continued*)

Order	Host (scientific name)	Number of screened/ positive	ID of positive animal	Country/ No. breed	Microscopical positivity (OPG)	Genotyping at the gene loci	
						SSU, ACTIN	HSP70
		10/2	34306	CZE/5	No	*C. proventriculi*	*C. proventriculi*
			34320		Yes (30,000)	*C. proventriculi*	*C. proventriculi*
		6/2	34759	CZE/6	Yes (2,000)	*C. proventriculi*	*C. proventriculi*
			35506		Yes (16,000)	*C. proventriculi*	*C. proventriculi*
		14/3	14384	CZE/7	Yes (2,000)	*C. proventriculi*	*C. proventriculi*
			14385		Yes (6,000)	*C. proventriculi*	*C. proventriculi*
			17618		No	*C. baileyi*	*C. baileyi*
		25/4	34751	CZE/8	Yes (8,000)	*C. proventriculi*	*C. proventriculi*
			34331	CZE/8	Yes (6,000)	*C. proventriculi*	*C. proventriculi*
			34749	CZE/8	No	*C. proventriculi*	*C. proventriculi*
			34750	CZE/8	Yes (4,000)	*C. proventriculi*	*C. proventriculi*
		20/2	34305	SK/2	Yes (24,000)	*C. proventriculi*	*C. proventriculi*
			36598	SK/2	Yes (8,000)	*C. proventriculi*	*C. proventriculi*
		8/0	NA	CZE/3	NA	NA	NA
		5/0	NA	CZE/4	NA	NA	NA
		7/0	NA	CZE/9	NA	NA	NA
		5/0	NA	SK//1	NA	NA	NA
	Red-crowned parakeet (*Cyanoramphus novaezealandiae*)	4/1	19287	CZE/3	No	*C. avium*	*C. avium*
		2/0	NA	CZE/8	NA	NA	NA
		2/0	NA	CZE/9	NA	NA	NA
	Red-fronted parrot (*Poicephalus gulielmi*)	27/3	22127	CZE/8	No	*C. proventriculi*	*C. proventriculi*
			33626	CZE/8	No	*C. proventriculi*	*C. proventriculi*
			25108	CZE/8	No	*C. proventriculi*	*C. proventriculi*
	Red-rumped parrots (*Psephotus haematonotus*)	5/0	NA	CZE/3	NA	NA	NA
		6/0	NA	CZE/9	NA	NA	NA
	Rose-breasted cockatoo (*Eolophus roseicapilla*)	5/0	NA	CZE/8	NA	NA	NA
		2/0	NA	SK/2	NA	NA	NA
	Rose-ringed parakeet (*Psittacula krameri*)	4/0	NA	CZE/8	NA	NA	NA
		2/0	NA	CZE/9	NA	NA	NA

Table 2 (*Continued*)

Order	Host (scientific name)	Number of screened/ positive	ID of positive animal	Country/ No. breed	Microscopical positivity (OPG)	Genotyping at the gene loci	
						SSU, ACTIN	HSP70
	Rosy-faced lovebird (*Agapornis roseicollis*)	6/1	26156	CZE/1	No	*C. proventriculi*	*C. proventriculi*
		4/1	37339	CZE/7	Yes (4,000)	*C. proventriculi*	*C. proventriculi*
		6/1	37244	SK/2	No	*C. proventriculi*	*C. proventriculi*
		3/0	NA	CZE/3	NA	NA	NA
		1/0	NA	CZE/4	NA	NA	NA
		2/0	NA	CZE/6	NA	NA	NA
		3/0	NA	CZE/8	NA	NA	NA
		6/0	NA	SK/1	NA	NA	NA
	Senegal parrots (*Poicephalus senegalus*)	4/0	NA	CZE/1	NA	NA	NA
		3/0	NA	CZE/2	NA	NA	NA
		2/0	NA	CZE4	NA	NA	NA
		2/0	NA	CZE/6	NA	NA	NA
		2/0	NA	CZE/7	NA	NA	NA
		5/0	NA	CZE/8	NA	NA	NA
		4/0	NA	CZE/9	NA	NA	NA
		5/0	NA	SK1	NA	NA	NA
		3/0	NA	SK/2	NA	NA	NA
	White cockatoos (*Cacatua alba*)	5/0	NA	CZE/4	NA	NA	NA
		4/0	NA	CZE/8	NA	NA	NA
		3/0	NA	CZE/9	NA	NA	NA
		3/0	NA	SK/2	NA	NA	NA
	Yellow-collared lovebird (*Agapornis personatus*)	3/0	NA	CZE/7	NA	NA	NA
		5/0	NA	CZE/8	NA	NA	NA
Passeriformes	**Gouldian finch** (*Erythura gouldiae*)	10/1	22153	POL/1	No	*C. galli*	NA
		8/1	37242	POL/2	No	*C. baileyi*	*C. baileyi*
		5/0	NA	CZE/1	NA	NA	NA
		6/0	NA	CZE/5	NA	NA	NA
		7/0	NA	CZE/10	NA	NA	NA
	Island canary (*Serinus canaria*)	25/3	30887	CZE/10	No	avian genotype I	avian genotype I
			31040		No	avian genotype I	avian genotype I
			31056		No	avian genotype I	avian genotype I

Table 2 (*Continued*)

Order	Host (scientific name)	Number of screened/ positive	ID of positive animal	Country/ No. breed	Microscopical positivity (OPG)	Genotyping at the gene loci	
						SSU, ACTIN	HSP70
	Lesser goldfinch (*Carduelis psaltria*)	10/1	19900	POL/1	No	*C. galli*	NA
		5/1	24068	CZE/10	Yes (6,000)	*C. baileyi*	*C. baileyi*
		3/1	NA	CZE/1	NA	NA	NA
		5/0	NA	CZE8	NA	NA	NA
		3/0	NA	CZE/9	NA	NA	NA
	Zebra finch (*Taeniopygia guttata*)	3/1	19418	CZE/10	Yes (10,000)	*C. baileyi*	*C. baileyi*
		4/2	24067	POL/3	No	*C. galli*	NA
		4/0	37242	POL/3	No	*C. galli*	NA

cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in 10 μl reactions.

Phylogenetic analyses

The nucleotide sequences of each gene obtained in this study were edited using the ChromasPro 2.4.1software (Technelysium, Pty, Ltd., South Brisbane, Australia) and aligned with each other and with reference sequences from GenBank using MAFFT version 7 online server with automatic selection of alignment mode (http://mafft.cbrc.jp/alignment/software/). Phylogenetic analyses were performed and best DNA/Protein phylogeny models were selected using the MEGA7 software (Guindon and Gascuel 2003; Tamura et al. 2011). Phylogenetic trees were inferred by the maximum likelihood (ML) method, with the substitution model that best fit the alignment selected using the Bayesian information criterion. The Tamura 3-parameter model (Tamura 1992) was selected for SSU and HSP70 alignments, and the general time reversible model (Tavaré 1986) was selected for actin alignment. Bootstrap support for branching was based on 1000 replications. Phylograms were drawn using the MEGA7 and were manually adjusted using CorelDrawX7. Sequences of SSU, actin and HSP70 derived in this study have been deposited in GenBank under accession numbers MK311133–MK311180.

Transmission studies

Animals

Groups of adult cockatiels, adult budgerigars (*Melopsittacus undulates*), one-days-old and 21-day-old chickens (*Gallus gallus* f. *domestica*), and eight-week-old SCID mice (*Mus mucus*; strain C.B-17), each consisting of five animals, were used for experimental infection studies. In addition, three animals from each host species were used as negative control. Three weeks prior to experimental infections, animals were screened every other day for the presence of specific DNA and oocysts of *Cryptosporidium* spp., except chickens, which were hatched under monitored conditions in the laboratory. Cockatiels and budgerigars originated from breeders with *Cryptosporidium*-free birds (Czech Republic) and SCID mice from Charles River (Germany).

Animal care

To prevent environmental contamination with oocysts, laboratory rodents were housed in plastic cages (TOP-VELAZ, Prague, Czech Republic). Chickens were housed in the plastic boxes that were appropriately sized for their age. An external source of heat was used in the first five days. Budgerigars and cockatiels were kept in bird cages, appropriate to animal species. All animals were supplied with a sterilized diet and sterilized water *ad libitum*.

Animal caretakers wore new disposable coveralls, shoe covers, and gloves every time they entered the experimental room. All wood-chip bedding, faeces, and disposable protective clothing were sealed in plastic bags, removed from the experimental room and incinerated. All housing, feeding and experimental procedures were conducted under protocols

approved by the Institute of Parasitology, Biology Centre and Central Commission for Animal Welfare, Czech Republic (Protocols No. 115/2013 and 35/2018).

Experimental design

Each animal was inoculated orally by stomach tube with 10,000 purified viable oocysts suspended in 200 μl of distilled water. Animals serving as negative controls were inoculated orally by stomach tube with 200 μl of distilled water. Faecal samples from all animals were screened daily

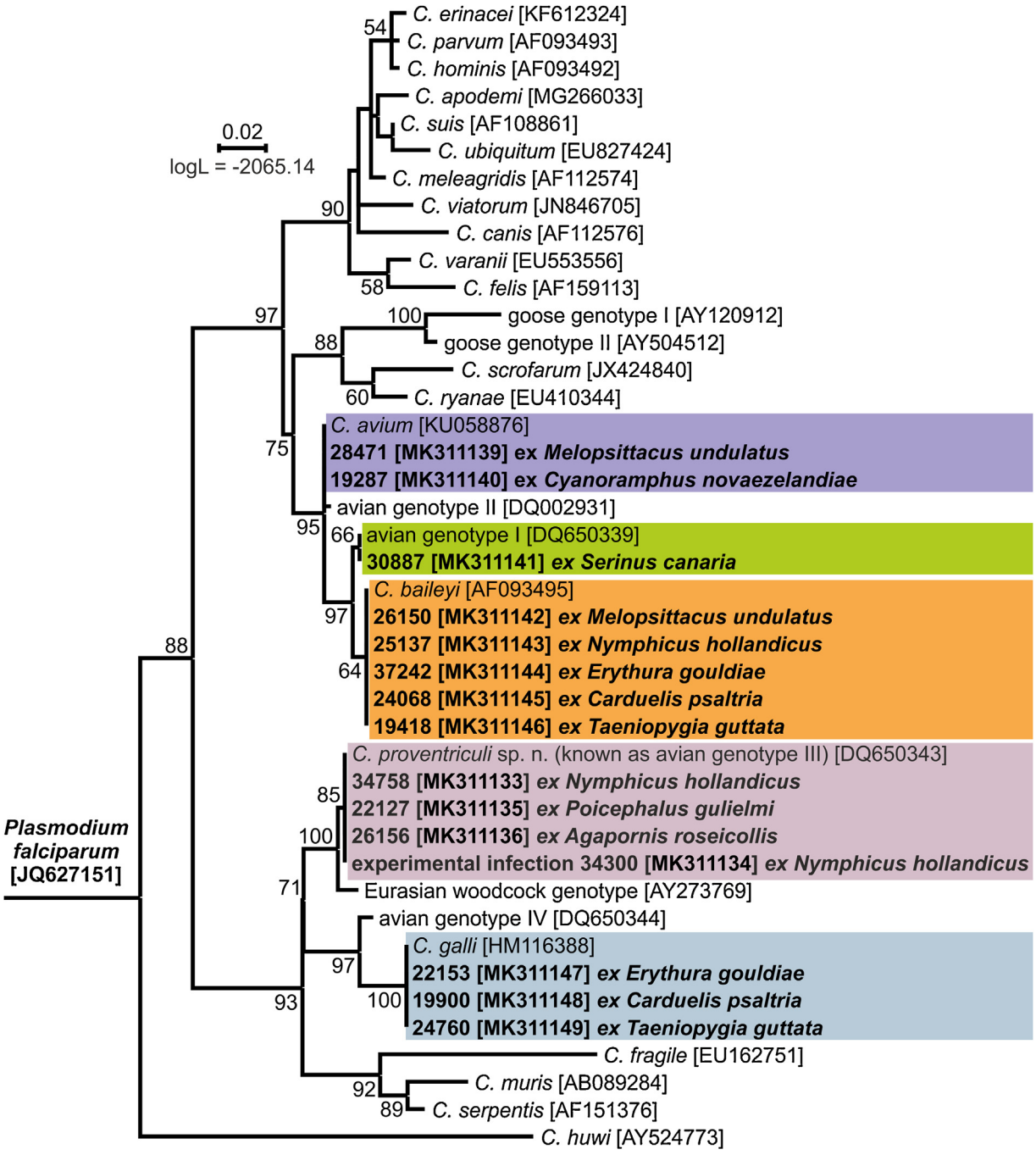

Fig. 1. Maximum likelihood tree based on partial small subunit ribosomal RNA gene sequences of *Cryptosporidium*, including sequences obtained in this study (bolded). The alignment contained 458 base positions in the final dataset. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates. The branch length scale bar, indicating the number of substitutions per site, is given in the tree. Sequences from this study are identified by isolate number (e.g. 22127).

for the presence of *Cryptosporidium* oocyst using ACMV staining and the presence of *Cryptosporidium* specific DNA was confirmed using nested PCR targeting the SSU gene. All experiments were terminated 30 days post infection (DPI). Infection intensity was reported as the number of oocysts per gram (OPG) of faeces, as previously described by Kváč et al. (2007). In addition, faecal consistency and colour and general health status were examined daily.

Histopathological and scanning electron microscopy examinations

An animal from each host group that was positive for *Cryptosporidium proventriculi* sp. n. was euthanized at 20 DPI (this time was selected based on preliminary results; data not shown). The oesophagus, ventriculus, proventriculus and entire small and large intestine was divided into 1 cm sections

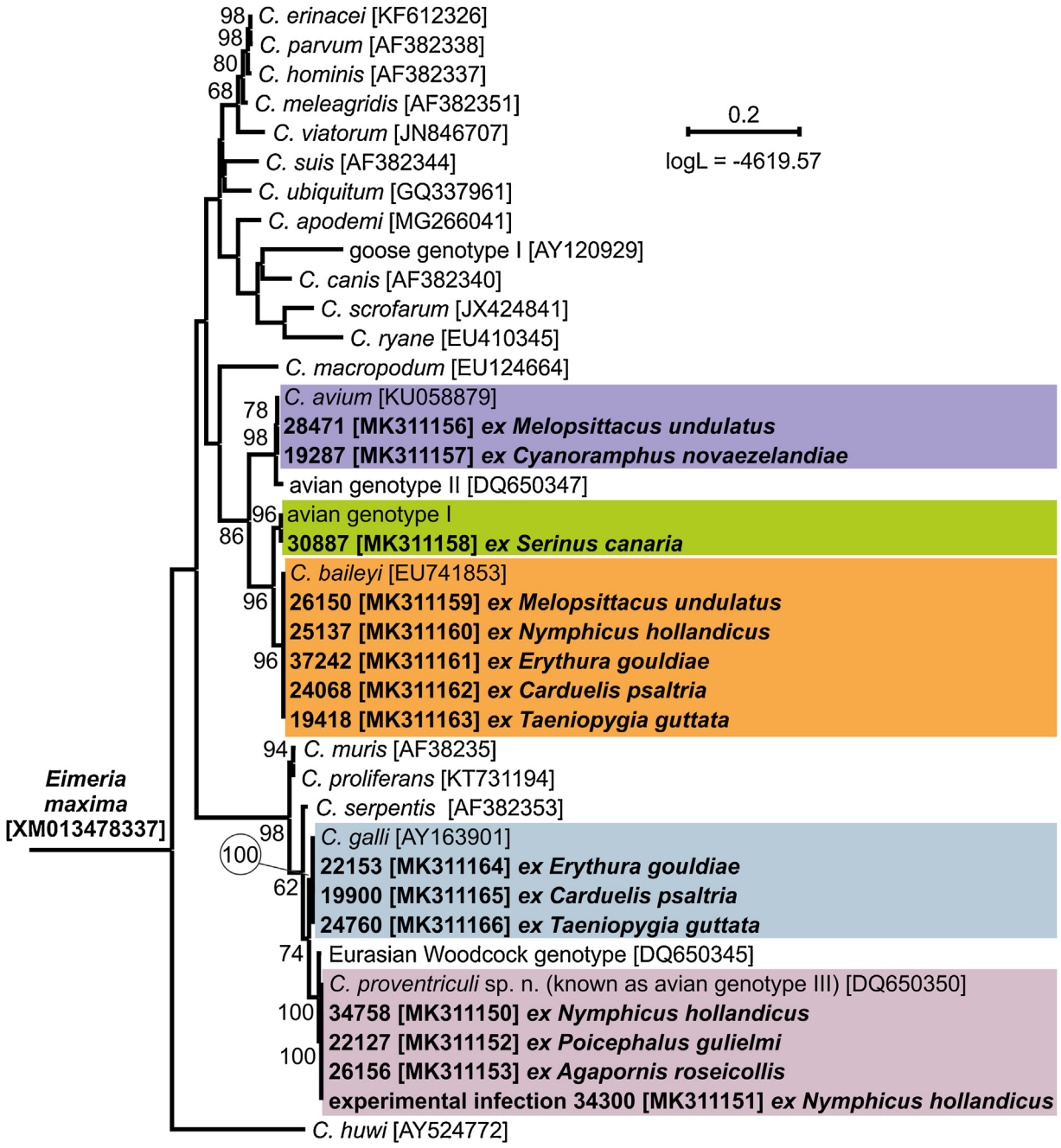

Fig. 2. Maximum likelihood tree based on partial sequences of gene coding actin of *Cryptosporidium*, including sequences obtained in this study (highlighted and bolded). The alignment contained 969 base positions in the final dataset. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates. The branch length scale bar, indicating the number of substitutions per site, is given in the tree. Sequences from this study are identified by isolate number (e.g. 22127).

and samples were processed for histology, scanning electron microscopy (SEM) and DNA sequencing. Specimens for histology were fixed in 4% buffered formalin and processed by the standard paraffin method. Sections (5 μm) for histology were stained with haematoxylin and eosin, Wolbach's modified Giemsa stain and periodic acid–Schiff stains. Specimens for SEM were fixed overnight at 4 °C in 2.5% glutaraldehyde in 0.1 M phosphate buffer, washed three times for 15 min in the same buffer, post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer for 2 h at room temperature and finally washed three times for 15 min in the same buffer. After dehydration in a graded acetone series, specimens were dried using the critical point technique, coated with gold and examined using a JEOL JSM-7401F-FE SEM.

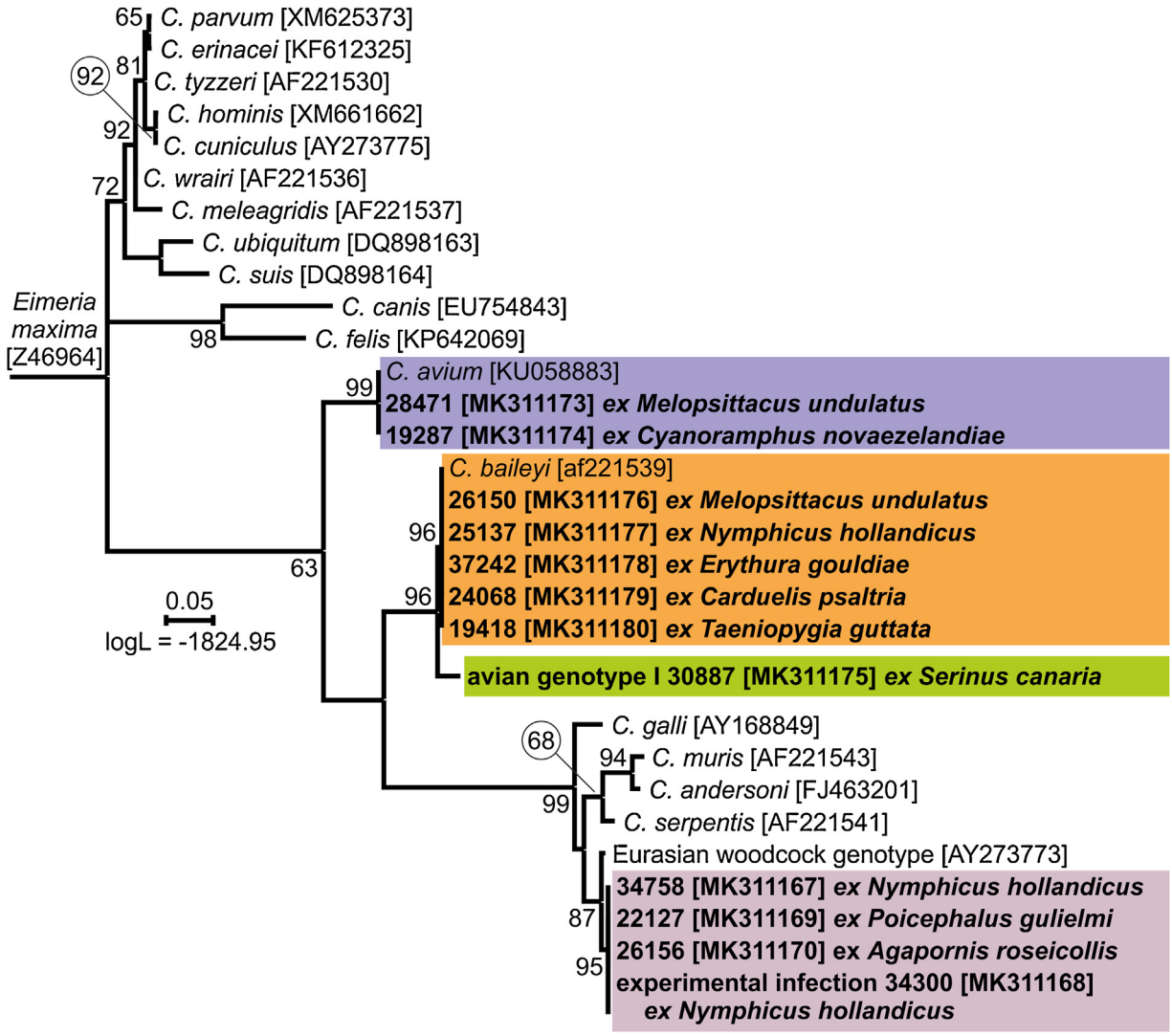

Fig. 3. Maximum likelihood tree based on partial sequences of gene coding 70 kDa Heat Shock Protein of *Cryptosporidium*, including sequences obtained in this study (highlighted and bolded). The alignment contained 488 base positions in the final dataset. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates. The branch length scale bar, indicating the number of substitutions per site, is given in the tree. Sequences from this study are identified by isolate number (e.g. 22127).

Statistical analysis

The hypothesis tested in the analysis of oocyst morphometry was that two-dimensional mean vectors of measurement are the same in the two populations being compared. Hotelling's T2 test was used to test the null hypothesis. Analyses were performed using the program R 3.5.0. (https://www.r-project.org/).

Results

Out of 500 faecal samples from Psittaciformes and Passeriformes birds, 37 (7.4%) were positive for the presence of specific DNA of *Cryptosporidium* spp. and 15 (3.0%) were microscopically positive for the presence of oocysts of *Cryptosporidium* sp. (Table 2). All microscopically positive samples were also positive using PCR.

Out of 37 birds positive for *Cryptosporidium*, 37, 37 and 33 were genotyped by sequence analysis of SSU, actin and HSP70 genes, respectively (Table 2). The remaining four positive samples failed to amplify at the HSP70 locus. Sequence analysis revealed the presence of five different *Cryptosporidium* spp., clustering with *C. avium*, *C. baileyi*, *C. galli*, *Cryptosporidium* avian genotype I and *Cryptosporidium* avian genotype III in ML trees inferred from sequences of SSU, actin and HSP70 (Figs. 1–3). *Cryptosporidium* avian genotype III is described here as a new species, *Cryptosporidium proventriculi* sp. n., and this name will be used in the following text.

Cryptosporidium avian genotype I (n = 3) and *C. galli* (n = 4) were found only in Passeriformes birds, and infected birds did not shed microscopically detectable oocysts

(Table 2). *Cryptosporidium baileyi* (n = 7) was detected in species from the orders Psittaciformes and Passeriformes, with infection intensities less than 10,000 OPG. *Cryptosporidium avium* was detected in two species from the order Psittaciformes. *Cryptosporidium proventriculi* sp. n., which was also exclusive to Psittaciformes, was detected in 21 birds from three species (Table 2), and was the most abundant species detected in this study. Out of 21 birds positive for *C. proventriculi* sp. n., 12 (57%) shed microscopically detectable oocysts, with an infection intensity ranging from 2,000 to 30,000 OPG. Naturally infected cockatiels (ID Nos. 34305, 34320, 34751 and 35506), which were the source of oocysts used in experimental infections, shed oocysts for more than five months (data not shown). None of the monitored birds had diarrhoea at the time of the screening.

Cryptosporidium proventriculi sp. n.

Fifteen cockatiels (12.1%), three red-fronted parrots (11.0%) and three rosy-faced lovebirds (9.7%) were positive for the presence of *C. proventriculi* sp. n. DNA, of which 12 cocktails and one rosy-faced lovebird shed oocysts detectable by microscopy, with infection intensity ranging from 2,000 to 30,000 OPG (Table 2). Oocyst used for experimental infections had >90% viability, determined by PI staining. SCID mice, budgerigars and chickens were not susceptible to infection with *C. proventriculi* sp. n. oocysts, as determined by PCR and microscopic examination of faecal samples and histological and molecular examination of gastrointestinal tract tissue.

Oocysts of *C. proventriculi* sp. n. were infectious for all cockatiels, with oocysts and specific DNA first detected at six DPI (Fig. 4). Following first detection, specific DNA of *C. proventriculi* sp. n. was detected in the faeces of all animals for the duration of the experiment. In contrast, oocysts were detected by microscopy almost every day (Fig. 4). The infection intensity of *C. proventriculi* sp. n. in cockatiels ranged from 4,000 to 60,000 OPG. Infected birds showed no symptoms of disease and a cockatiel necropsied at 20 DPI had no macroscopic signs of cryptosporidiosis. Developmental stages of *C. proventriculi* sp. n. were not detected by histology, but scanning electron microscopy showed the presence of developmental stages attached to the microvilli in the proventriculus and ventriculus (Fig. 5). Pathological changes were not observed.

At the SSU locus, all isolates of *C. proventriculi* sp. n., from naturally and experimentally infected birds, shared 100% identity with each other and with *Cryptosporidium* avian genotype III from different Psittaciformes hosts worldwide (e.g. HM116385, KX668210 or HM116386; see Table 1 for more details). At the actin locus, all *C. proventriculi* sp. n. isolates shared 100% identity with each other and with GenBank sequences of *Cryptosporidium* avian genotype III obtained from peach-faced lovebirds and cockatiels (e.g. AB471655–AB471658, AB471659 or DQ650350). Partial sequences of the HSP70 locus shared 100% identity with each other and shared 98.7% identity with *Cryptosporidium* Eurasian woodcock genotype (AY273773), which was the most similar sequence reported in GenBank.

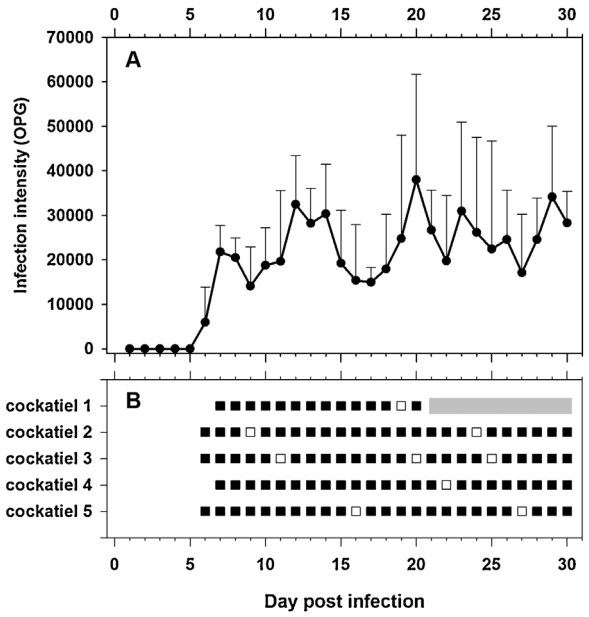

Fig. 4. Course of infection of *Cryptosporidium proventriculi* sp. n. in experimentally infected cockatiels (*Nymphicus hollandicus*). **A)** Infection intensity as number of oocysts per gram of the faeces (OPG) and **B)** daily shedding of *C. proventriculi* based on coprological and molecular examination of faces; any square indicates detection of specific DNA, black square indicates microscopic detection of oocysts and grey rectangle indicates sacrifice and dissection.

Taxonomic summary

Description: Oocysts are shed fully sporulated with four sporozoites and an oocyst residuum.

Oocysts of *C. proventriculi* n. sp. originated from naturally infected cockatiels measured 6.70–8.40 μm (mean ± S.D. = 7.35 ± 0.41 μm) × 5.10–6.3 μm (mean ± S.D. = 5.70 ± 0.32 μm) with a length/width ratio of 1.08–1.41 (mean ± S.D. = 1.23 ± 0.11) and were morphometrically identical to those recovered from experimentally infected cockatiels measured 6.60–8.40 μm (mean ± S.D. = 7.37 ± 0.44 μm) × 5.00–6.40 μm (mean ± S.D. = 5.80 ± 0.35 μm) with a length/width ratio of 1.06–1.43 (mean ± S.D. = 1.25 ± 0.10) (Fig. 6). Morphology and morphometry of other developmental stages are unknown.

Type host: cockatiel (*Nymphicus hollandicus*)

Other natural hosts: barred parakeet (*Bolborhynchus lineola*), blue-fronted parrot (*Amazona aestiva*), *Forpus* sp., galah (*Eolophus roseicapilla*), green-winged saltator (*Serinus canaria*), lovebird (*Agapornis* sp.), java sparrow (*Padda oryzivora*), peach-faced lovebird (*Agapornis roseicollis*), red-billed blue magpie (*Urocissa erythrorhyncha*), red-billed toucan (*Ramphastos tucanus*), red-fronted par-

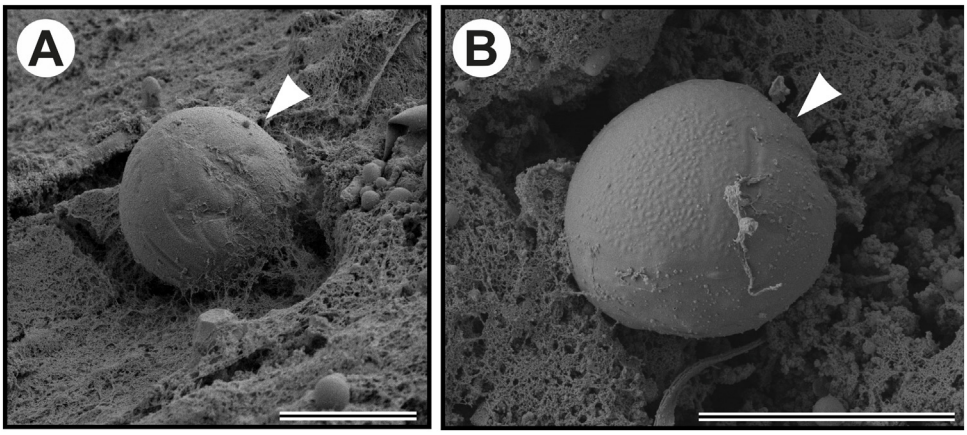

Fig. 5. Scanning electron photomicrograph of the **A)** proventriculus and **B)** ventriculus of a cockatiel (*Nymphicus hollandicus*). Attached developmental stage of *Cryptosporidium proventriculi* sp. n. (arrowhead). Bar = 10 μm.

rot (*Poicephalus gulielmi*), rosy-faced lovebird (*Agapornis roseicollis*), rufous-collared sparrow (*Zonotrichia capensis*), saffron toucanet (*Pteroglossus bailloni*), seagul (*Chroicocephalus brunnicephalus* and *ridibundus*), *Sporophila* sp., sun parakeet (*Aratinga solstitialis*) and toco toucan (*Ramphastos toco*) (see Tables 1 and 2).

Type locality: České Budějovice (Czech Republic)

Other localities: Czech Republic (České Budějovice 48°58′29″ N, 14°28′29″ E; Lanžhot 48°43′28.03″ N, 16°58′0.97″ E; Osek u Plzně 49°26′36.33″ N, 14°18′0.43″ E; Strakonice 49°15′41.39″ N, 13°54′8.63″ E and Tvrdonice 48°45′37.81″ N, 16°59′40.03″ E), Slovakia (Košice 48°42′0″ N, 21°15′0″ E and Žilina 49°13′22″ N, 18°44′24″ E), Poland (Wroclaw 51°7′0″ N, 17°2′0″ E).

Site of infection: proventriculus and ventriculus (Fig. 5)

Distribution: Australia, Brazil, China, India, Japan, Poland, Slovakia, Spain, Thailand and USA

Prepatent period: 6 DPI (cockatiel)

Patent period: at least 30 DPI in experimentally infected cockatiels and more than 5 months in naturally infected cockatiels.

Type material/hapanotype: Tissue samples in 10% formaldehyde and histological sections of infected ventriculus (nos. 176/2017 and 177/2017) and provetriculus (nos. 199/2017); genomic DNA isolated from faecal samples of naturally (isolation no. 34320) and experimentally (isolation no. 34300) infected cockatiel; genomic DNA isolated from proventriculus and ventricular and of experimentally infected cockatiel (isolation nos. 34300); digital photomicrographs nos. DIC 1–13/34300, MV 1–11/34300, IF 1–9/34300, HI 176–177, 199/2017 and SEM 199/2017) and faecal smear slides with oocysts stained by ACMV staining from experimentally infected cockatiel (nos. 10/34300, 11/34300 and 12/34300). Specimens deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

Reference sequences: Partial sequences of SSU, actin and HSP70 genes were deposited at GenBank under Acc. Nos. MK311133, MK311150 and MK311167, respectively.

Etymology: The species name is derived from the Latin noun ‘proventriculus’, because the described *Cryptosporidium* species predominantly inhabits this part of digestive tract of its hosts.

Differential diagnosis: Oocysts in faecal smears showed typical *Cryptosporidium* ACMV (Fig. 6) and Ziehl-Neelsen (Fig. 6) staining characteristics. Fixed *C. proventriculi* sp. n. oocysts are detectable with a FITC conjugated anti-*Cryptosporidium* oocyst wall antibody developed primarily for *C. parvum* (Fig. 6). Oocysts of *C. proventriculi* are larger than those of *C. avium* and *C. baileyi* ($P = 0.001$) and marginally smaller than *C. galli*. *Cryptosporidium proventriculi* sp. n. can be differentiated genetically from other *Cryptosporidium* spp. based on sequences of SSU, actin and HSP70 genes.

Discussion

Birds are naturally infected with several *Cryptosporidium* spp. While *C. meleagridis*, *C. galli* and *C. baileyi* infect a large number of birds across several avian orders, *C. avium* and most of the avian genotypes exhibit a narrow host specificity (Holubová et al. 2016). Although *C. proventriculi* sp. n. has been reported from birds in the orders Psittaciformes, Passeriformes, Piciformes and Anseriformes, it has been reported predominantly in Psittaciformes birds (Cano et al. 2016; Ferrari et al. 2018; Koompapong et al. 2014; Li et al. 2015; Ravich et al. 2014). Consistent with those reports, we found *C. proventriculi* sp. n. exclusively in Psittaciformes birds, and we identified the red-fronted parrot and rosy-faced lovebird as novel hosts in that order.

Cryptosporidiosis in birds manifests in various clinical forms depending on the species of *Cryptosporidium* and the site of infection (Nakamura and Meireles 2015). Despite the relatively large number of studies and descriptions of sev-

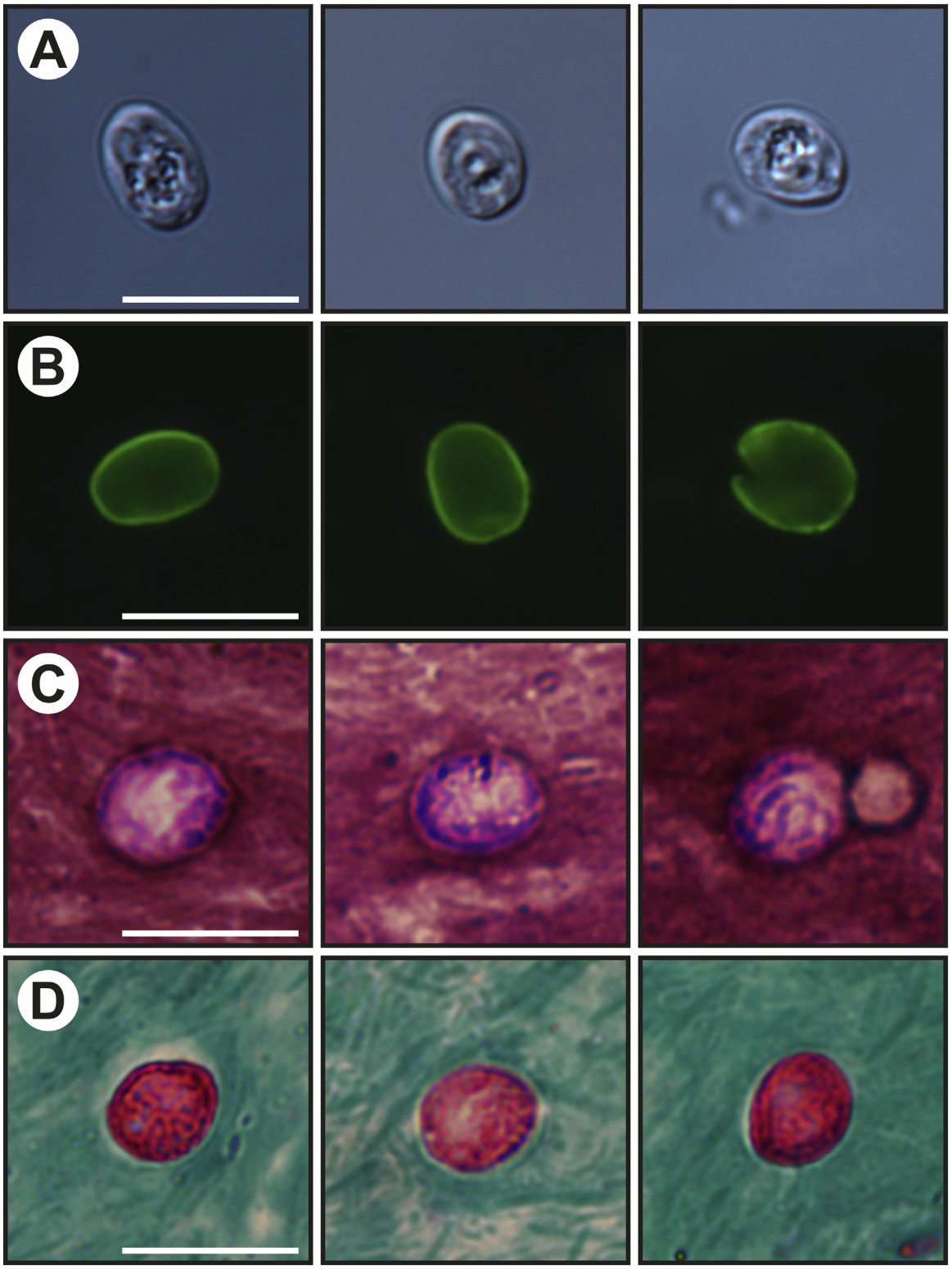

Fig. 6. *Cryptosporidium proventriculi* sp. n. oocysts visualized in various preparations: **A)** differential interference contrast microscopy, **B)** labelling with anti-*Cryptosporidium* FITC-conjugated antibody, **C)** aniline-carbol-methyl violet staining and **D)** Ziehl-Neelsen staining. Bar = 10 μm.

eral *Cryptosporidium* genotypes, knowledge of the course of infection and disease presentation of *Cryptosporidium* in birds remains quite poor. *Cryptosporidium galli* is the only well-studied gastric *Cryptosporidium* species in birds. Unlike gastric *Cryptosporidium* species infecting mammals, which generally do not cause morbidity and mortality, *C. galli* is associated with clinical disease and high mortality in birds (Blagburn et al. 1987; Blagburn et al. 1990; Morgan et al. 2001; Pavlásek 1999, 2001). However, similar to Ng et al. (2006), we found that *C. proventriculi* does not cause clinical disease or mortality in naturally or experimentally infected birds. Ravich et al. (2014) reported proventricular and ventricular cryptosporidiosis in 31 birds at necropsy and found evidence that *Cryptosporidium* was the likely cause of death in 10 of those birds. The authors identified *C. proventriculi* from samples of two birds, which were the only samples subjected to genotyping, but it is not clear whether those birds were among the 10 that likely died from cryptosporidiosis. While *C. proventriculi* could be the cause of death in those birds, infection by other *Cryptosporidium* species cannot be ruled out. Makino et al. (2010) described clinical gastrointestinal signs such as chronic vomiting, melena and weight loss in 20 out of 37 peach-faced lovebirds naturally infected with *C. proventriculi*. Radiographic examination showed enlargement of the isthmi and thickened proventricular walls in 16 of the 20 symptomatic birds. Necropsy and histopathologic examination of three dead birds showed enlargement of the proventriculus and isthmi with mucosal hypertrophy observed on the sagittal plane. The progressive weight loss with severely atrophied thoracic muscles was characteristic for all three affected birds. Histopathologically, extensive hyperplasia of the ductal epithelium of the proventricular glands was observed. Similarly, Blagburn et al. (1990) and Morgan et al. (2001) reported histopathological changes similar to those caused by *C. galli* infection in the Australian diamond firetail finch and other finches infected with *Cryptosporidium* sp. and *C. proventriculi*, respectively. In contrast, we found no histopathological changes in birds infected with *C. proventriculi*. These differences can be explained by the length of the ongoing infection. While our experiments lasted only 30 days, naturally infected birds could be infected for many months. Similarly, histopathological changes and clinical signs were observed in mammals chronically infected with the gastric species *C. proliferans*, *C. andresoni* or *C. muris* (Anderson 1987; Esteban and Anderson 1995; Kváč et al. 2016; Ozkul and Aydin 1994; Pospischil et al. 1987).

Course of infection, including prepatent and patent period, of avian-derived *Cryptosporidium* is currently known only in the four valid avian species (Current et al. 1986; Holubová et al. 2016; Ryan et al. 2003b; Slavin 1955). The prepatent period of *C. proventriculi* sp. n. (6 DPI) was similar to *C. meleagridis* and *C. baileyi*, which infect the intestine (4–8 days; Hornok et al. 1999; Lindsay et al. 1988; Rhee et al. 1991; Tůmová et al. 2002), and much shorter than the 25 days reported for *C. galli* infection in the proventriculus of chickens (Pavlásek 2001).

Oocysts of *C. proventriculi* sp. n. are morphometrically identical to those reported as *Cryptosporidium* avian genotype III (Ng et al. 2006). Although they are smaller than those of *C. galli* (Ryan et al. 2003b) and Eurasian woodcock genotype (Ryan et al. 2003a), the difference is marginal and not practically useful for differentiation (Horčičková et al. 2018).

Cryptosporidium proventriculi sp. n. is genetically distinct from other known species of *Cryptosporidium*, sharing 98.4 and 94.5% sequence identity, respectively, with *Cryptosporidium* Eurasian woodcock genotype and *C. galli* at the SSU locus and sharing 98.9 and 96.9% identity, respectively, at the actin locus. In comparison, *C. muris* and *C. andersoni* share 96–99% identity and *C. hominis* and *C. parvum* share 98–99% identity at these loci.

In conclusion, morphological, genetic, and biological data support the establishment of *Cryptosporidium* avian genotype III as a new species. According to ICZN and criteria for naming species, we propose the name *Cryptosporidium proventriculi*.

Author contributions

N.H., M.K. and B.S. conceptualised the project; Z.K., D.R., N.H. and V.Z. collected samples; N.H., M.K., V.Z. and Z.L. carried out the research; M.K., N.H. and J.M. performed phylogenetic analysis; B.S. and R.K. performed histology and electron microscopy analysis; L.H. took care of experimental animals and M.K., N.H., J.M. wrote the manuscript. All authors read and approved the final manuscript.

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