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Cryptosporidium testudinis sp. n., *Cryptosporidium ducismarci* Traversa, 2010 and *Cryptosporidium* tortoise genotype III (Apicomplexa: Cryptosporidiidae) in tortoises

RNDr. Thesis

Bc. Jana Ježková

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Annotation:

This study was focused on biology and diversity of *Cryptosporidium* infecting terrestrial tortoises from family Testudinidae. Presence of *Cryptosporidium* oocysts and specific DNA in faecal samples was established by microscopy and PCR. Analyses based on small subunit of rRNA, *Cryptosporidium* oocyst wall protein and actin genes confirmed the existence of three monophyletic lineages/species of *Cryptosporidium* infecting *Testudo* tortoises. In this study we described prepatent and patent period, intensity of infection and morphology of oocyst of *Cryptosporidium* tortoise genotype I and *C. ducismarci*.

Declaration [in Czech]

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Ježková J., 2017: *Cryptosporidium testudinis* sp. n., *Cryptosporidium ducismarci* Traversa, 2010 and *Cryptosporidium* tortoise genotype III (Apicomplexa: Cryptosporidiidae) u želv.

Abstrakt:

Porozumění rozmanitosti druhů *Cryptosporidium* Tyzzer, 1910 u želv zůstává neúplné z důvodu malého počtu studií u těchto hostitelů. Cílem této studie bylo charakterizovat genetickou diverzitu a biologii kryptosporidií čeledi Testudinidae Batsch. Vzorky trusu byly odebírány individuálně ihned po defekaci a byly vyšetřeny mikroskopicky na přítomnost kryptosporidií použitím barvení anilin-karbol-methyl violetí a pomocí PCR amplifikace a sekvenční analýzy cílené na malou ribozomální podjednotku rRNA (SSU), *Cryptosporidium* oocyst wall protein (COWP) a aktin gen. Z 387 vzorků trusu ze 16 druhů želv patřících do 11 rodů bylo 10 vzorků pozitivních mikroskopicky a 46 bylo pozitivních pomocí PCR. Všechny vzorky, které byly mikroskopicky pozitivní byly i PCR pozitivní. Sekvenční analýza amplifikovaných genů odhalila přítomnost *Cryptosporidium* tortoise genotype I (n = 22), *C. ducismarci* Traversa, 2010 (n = 23) and tortoise genotype III (n = 1). Fylogenetická analýza pro SSU, COWP a aktin gen ukázala, že *Cryptosporidium* tortoise genotype I a *C. ducismarci* jsou geneticky odlišné od dříve popsaných druhů *Cryptosporidium*. Oocysty *Cryptosporidium* tortoise genotype I měřící 5,8–6,9 × 5,3–6,5 μm, jsou morfologicky odlišné od *C. ducismarci* měřící 4,4–5,4 × 4,3–5,3 μm. Oocysty *Cryptosporidium* tortoise genotype I a *C. ducismarci* získané z přirozeně infikované želvy čtyřprsté (*Testudo horsfieldii* Gray) byly infekční pro stejný druh želvy, ale ne pro vodní želvy (*Mauremys reevesii* [Gray]), užovku proužkovanou (*Thamnophis sirtalis* [Linnaeus]), zebřičky pestré (*Taeniopygia guttata* [Vieillot]) a SCID myši (*Mus musculus* Linnaeus). Prepatentní perioda byla pro *Cryptosporidium* tortoise genotype I 11 dní po infekci (DPI) a pro *C. ducismarci* 6 DPI; patentní perioda byla delší než 200 dní pro obě kryptosporidie. Přirozeně nebo experimentálně infikované želvy nevykazovaly žádné klinické příznaky nemoci. Na základě morfologických, genetických a biologických dat byl *Cryptosporidium* tortoise genotype I ustanoven jako nový druh *Cryptosporidium testudinis* sp. n. a byla potvrzena platnost *C. ducismarci* jako samostatného druhu rodu *Cryptosporidium*.

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Cryptosporidium testudinis sp. n., *Cryptosporidium ducismarci* Traversa, 2010 and *Cryptosporidium* tortoise genotype III (Apicomplexa: Cryptosporidiidae) in tortoises

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Abstract: Understanding of the diversity of species of *Cryptosporidium* Tyzzer, 1910 in tortoises remains incomplete due to the limited number of studies on these hosts. The aim of the present study was to characterise the genetic diversity and biology of cryptosporidia in tortoises of the family Testudinidae Batsch. Faecal samples were individually collected immediately after defecation and were screened for presence of cryptosporidia by microscopy using aniline-carbol-methyl violet staining, and by PCR amplification and sequence analysis targeting the small subunit rRNA (SSU), *Cryptosporidium* oocyst wall protein (COWP) and actin genes. Out of 387 faecal samples from 16 tortoise species belonging to 11 genera, 10 and 46 were positive for cryptosporidia by microscopy and PCR, respectively. All samples positive by microscopy were also PCR positive. Sequence analysis of amplified genes revealed the presence of the *Cryptosporidium* tortoise genotype I (n = 22), *C. ducismarci* Traversa, 2010 (n = 23) and tortoise genotype III (n = 1). Phylogenetic analyses of SSU, COWP and actin gene sequences revealed that *Cryptosporidium* tortoise genotype I and *C. ducismarci* are genetically distinct from previously described species of *Cryptosporidium*. Oocysts of *Cryptosporidium* tortoise genotype I, measuring 5.8–6.9 µm × 5.3–6.5 µm, are morphologically distinguishable from *C. ducismarci*, measuring 4.4–5.4 µm × 4.3–5.3 µm. Oocysts of *Cryptosporidium* tortoise genotype I and *C. ducismarci* obtained from naturally infected Russian tortoises (*Testudo horsfieldii* Gray) were infectious for the same tortoise but not for Reeve's turtles (*Mauremys reevesii* [Gray]), common garter snake (*Thamnophis sirtalis* [Linnaeus]), zebra finches (*Taeniopygia guttata* [Vieillot]) and SCID mice (*Mus musculus* Linnaeus). The prepatent period was 11 and 6 days post infection (DPI) for *Cryptosporidium* tortoise genotype I and *C. ducismarci*, respectively; the patent period was longer than 200 days for both cryptosporidia. Naturally or experimentally infected tortoises showed no clinical signs of disease. Our morphological, genetic, and biological data support the establishment of *Cryptosporidium* tortoise genotype I as a new species, *Cryptosporidium testudinis* sp. n., and confirm the validity of *C. ducismarci* as a separate species of the genus *Cryptosporidium*.

Keywords: morphology, transmission studies, taxonomy, new species, molecular phylogeny

The genus *Cryptosporidium* Tyzzer, 1910 comprises species of protist parasites that infect epithelial cells in the microvillus border of the gastrointestinal tract of all classes of vertebrates and the bursa of Fabricius and other organs in birds (Ryan and Xiao 2014). Although species of *Cryptosporidium* have been under intensive investigation for more than 30 years, research has been heavily biased towards species infecting humans, livestock and other mammals, with comparatively little attention paid to cryptosporidia in other vertebrates (Kváč et al. 2014a, Robertson et al. 2014). Within the class Reptilia, the biology and diversity of species of *Cryptosporidium* have been described best in snakes and lizards (see Kváč et al. 2014a); in contrast, knowledge of *Cryptosporidium* in tortoises remains poor.

The first report of cryptosporidia in a tortoise described the microscopic detection of oocysts in the faeces of an Indian star tortoise, *Geochelone elegans* (Shoepff), kept in a zoo in the USA (Heuschele et al. 1986). Between 1988 and 1998, in studies using bright field or fluorescence microscopy as detection methods, oocysts of cryptosporidia

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Table 1. Occurrence of species of *Cryptosporidium* Tyzzer, 1910 in tortoise and turtles demonstrated on the basis of microscopically and molecular tools amplifying partial sequences of SSU, actin, *Cryptosporidium* oocyst wall protein and HSP70 and presence of tortoise specific *Cryptosporidium* in other reptiles and environmental samples.

Groups	Host	*Cryptosporidium* spp.	Country	Sequences (GenBank association number)	References
Tortoises	*Astrochelys radiata* (Shaw) (radiated tortoise)	*Cryptosporidium* sp.	USA	Not available	Raphael et al. (1997)
	Geochelone elegans (Schoepff) (Indian star tortoise)	*C. testudinis* sp. n.	USA Portugal	SSU (AY120914), Actin (AY120931)	Xiao et al. (2004a) Alves et al. (2005)
		Cryptosporidium sp.	USA	Not available	Heuschele et al. (1986)
		Cryptosporidium sp.	USA	Not available	Raphael et al. (1997)
		Cryptosporidium sp.	NS	Not available	Graczyk et al. (1998)
		Cryptosporidium sp.	USA	Not available	Graczyk and Cranfield (1998)
	Gopherus polyphemus (Daudin) (gopher tortoise)	*Cryptosporidium* sp.	USA	Not available	Robinson et al. (2010) McGuire et al. (2013)
	Indotestudo sp.	*Cryptosporidium* sp.	USA	Not available	Graczyk et al. (1998)
	Malacochersus tornieri (Siebenrock) (Pancake tortoise)	*C. ducismarci* Traversa, 2010	USA	SSU (GQ504270)	Griffin et al. (2010)
	Testudo hermanni Gmelin (Hermann's tortoise)	*C. testudinis* sp. n.	Spain	SSU (EU553585)	Richter et al. (2012) Pedraza-Diaz et al. (2009)
		C. ducismarci	Spain	Sequences unpublished	Alves et al. (2005)
		Cryptosporidium sp.	UK	Not available	Hedley et al. (2013)
	Testudo horsfieldii Gray (Russian tortoise)	*C. testudinis* sp. n.	USA	HSP70 (FJ429632), SSU (GQ504268)	Griffin et al. (2010)
		C. ducismarci		SSU (GQ504269)	
	Testudo kleinmanni Lortet (Egyptian tortoise)	*Cryptosporidium* sp.	USA	Not available	Graczyk et al. (1998)
	Testudo marginata Schoepff (marginated tortoise)	*C. ducismarci*	Italy	SSU (EF547155), COWP (EF519704)	Traversa et al. (2008)
		C. parvum Tyzzer, 1912		Sequences unpublished	
Turtles	*Chelonia mydas* (Linnaeus) (green turtle)	*Cryptosporidium* sp.	USA	Not available	Graczyk et al. (1997)
	Clemmys muhlenbergi (Schoepff) (bog turtle)	*Cryptosporidium* sp.	NS	Not available	Graczyk and Cranfield (1998)
Other reptiles	*Python regius* (Shaw) (ball python)	*C. testudinis* sp. n.	Spain	SSU (EU553590)	Pedraza-Diaz et al. (2009)
		C. ducismarci		SSU (EU553591)	
	Chamaeleo calyptratus Duméril et Duméril (veiled chameleon)	*C. ducismarci*	Spain	SSU (EU553587)	Pedraza-Diaz et al. (2009)
Environmental sample – water sample		*C. testudinis* sp. n.	USA	SSU (EU825744)	Yang et al. (2008)

NS – origin of the host (country) was not specified in the manuscript.

were detected in faecal samples of various tortoise species (e.g. Bourdeau 1988, Graczyk et al. 1997, Raphael et al. 1997, Graczyk and Cranfield 1998). In 2002, sequence analysis of the small subunit rRNA gene (SSU) was used to describe the *Cryptosporidium* tortoise genotype (later called *Cryptosporidium* tortoise genotype I) in captive Indian star tortoises (Xiao et al. 2002, 2004a).

Subsequent molecular studies showed rare occurrences of *C. parvum* Tyzzer, 1912 and the frequent occurrence of *Cryptosporidium* tortoise genotype I and *Cryptosporidium* tortoise genotype II (also known as *Cryptosporidium* sp. CrIT20) in Testudines, an order comprising turtle and tortoise families (Table 1). In 2010, Traversa proposed the name *Cryptosporidium ducismarci* Traversa, 2010 for *Cryptosporidium* tortoise genotype II (Traversa 2010), but data on oocyst morphology, which are required for the adequate description of a new *Cryptosporidium* species (see Xiao et al. 2004b), were not reported in that study. As a result, many authors do not consider *C. ducismarci* to be a valid species (Ryan and Xiao 2014).

In the present paper, the most comprehensive survey of *Cryptosporidium* infection in tortoises to date is provided. We undertook this study to determine the experimental transmission, oocyst morphology and molecular characteristics of *Cryptosporidium* tortoise genotype I and *C. ducismarci*. Based on the collective data from this and other studies, which show that *Cryptosporidium* tortoise genotype I is genetically distinct from known *Cryptosporidium* species, we describe this genotype as a new species. We also provide previously unreported data on *C. ducismarci*, which is recognised as a valid species.

MATERIALS AND METHODS

Specimens studied

Tortoise species owned by private breeders, pet shops and zoological gardens in the Czech Republic were sampled for the present study. Fresh faecal samples were collected from the floor (box, terrarium) immediately after defecation and each sample was placed into a separate plastic tube without fixative.

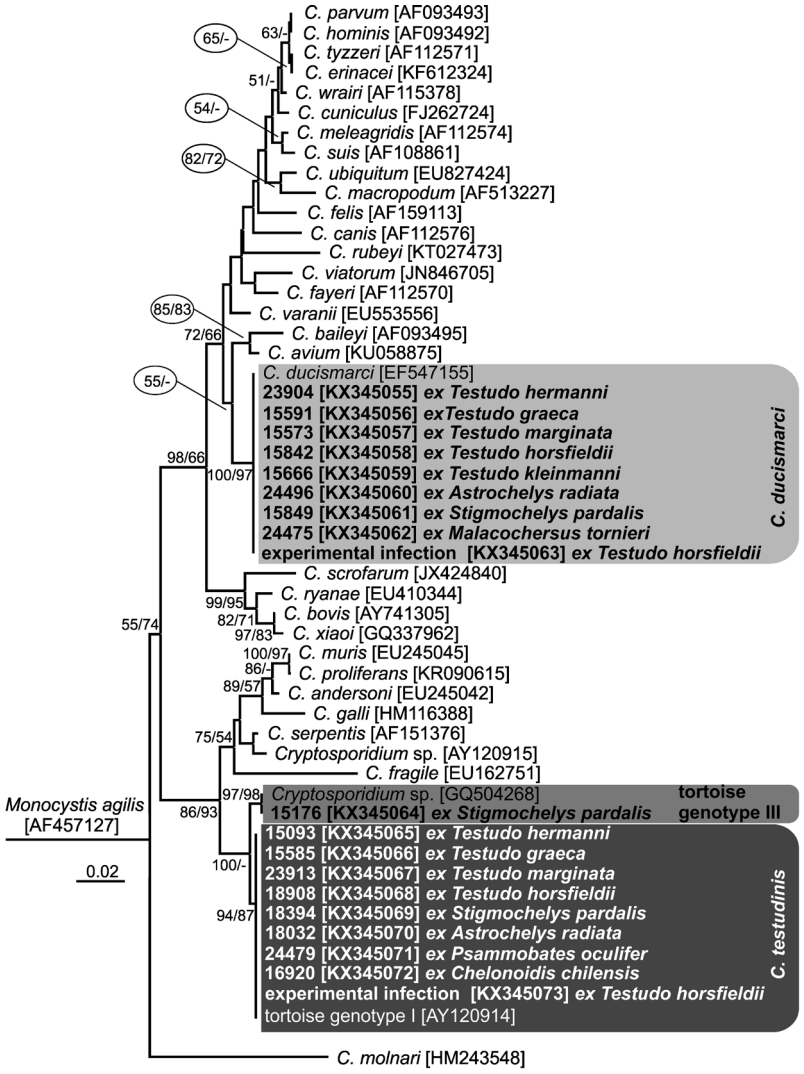

Fig. 1. Maximum likelihood tree based on partial small subunit ribosomal RNA gene sequences of species of *Cryptosporidium* Tyzzer, 1910, including *Cryptosporidium testudinis* sp. n., *Cryptosporidium ducismarci* Traversa, 2010 and *Cryptosporidium* tortoise genotype III. Sequences from this study are bolded. Numbers at the nodes represent the bootstrap values (ML/MP) gaining more than 50% support. Branch length scale bar indicate number of substitution per site.

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.

Oocysts of *Cryptosporidium* tortoise genotype I and *C. ducismarci* were originally isolated from faecal samples of naturally infected Russian tortoises (*Testudo horsfieldii* Gray). The tortoise infected with *Cryptosporidium* tortoise genotype I was kept by a private owner in Nové Hrady (Czech Republic) and the tortoise infected with *C. ducismarci* originated from a pet shop in České Budějovice (Czech Republic).

All samples were examined by microscopy for the presence of oocysts of cryptosporidia following aniline-carbol-methyl violet (ACMV) staining (Miláček and Vítovec 1985). Infection intensity was expressed as the number of oocysts per gram of faeces (OPG). Oocysts originated from pooled faecal samples of an infected Russian tortoise were purified using caesium chloride gradient centrifugation (Arrowood and Donaldson 1996) and used in morphological, experimental transmission and molecular studies. The viability of oocysts was examined using propidium iodide staining by an assay of Sauch et al. (1991). Purified oocysts were stored in phosphate-buffered saline at 4 °C.

Morphological evaluation

Oocysts of *Cryptosporidium* tortoise genotype I and *C. ducismarci* were examined using differential interference contrast (DIC) microscopy following ACMV staining and fluorescence microscopy following labelling with genus-specific FITC-conjugated antibodies (IFA; *Cryptosporidium* IF Test, Crypto cel, Cellabs Pty Ltd., Brookvale, Australia). Morphometry was measured using digital analysis of images (M.I.C. Quick Photo Pro v.3.1 software; Promicra, s.r.o., Praha, Czech Republic) collected using an Olympus Digital Colour Camera DP73. Length and width of oocysts (n = 30) were measured under DIC at 1 000× magnification and the shape index of each oocyst was calculated. As a control, the morphometry of *C. parvum* (n = 30) from a naturally infected 7-day-old Holstein calf (*Bos taurus* Linnaeus) was measured by the same person using the same microscope. Photomicrographs of *Cryptosporidium* tortoise genotype I and

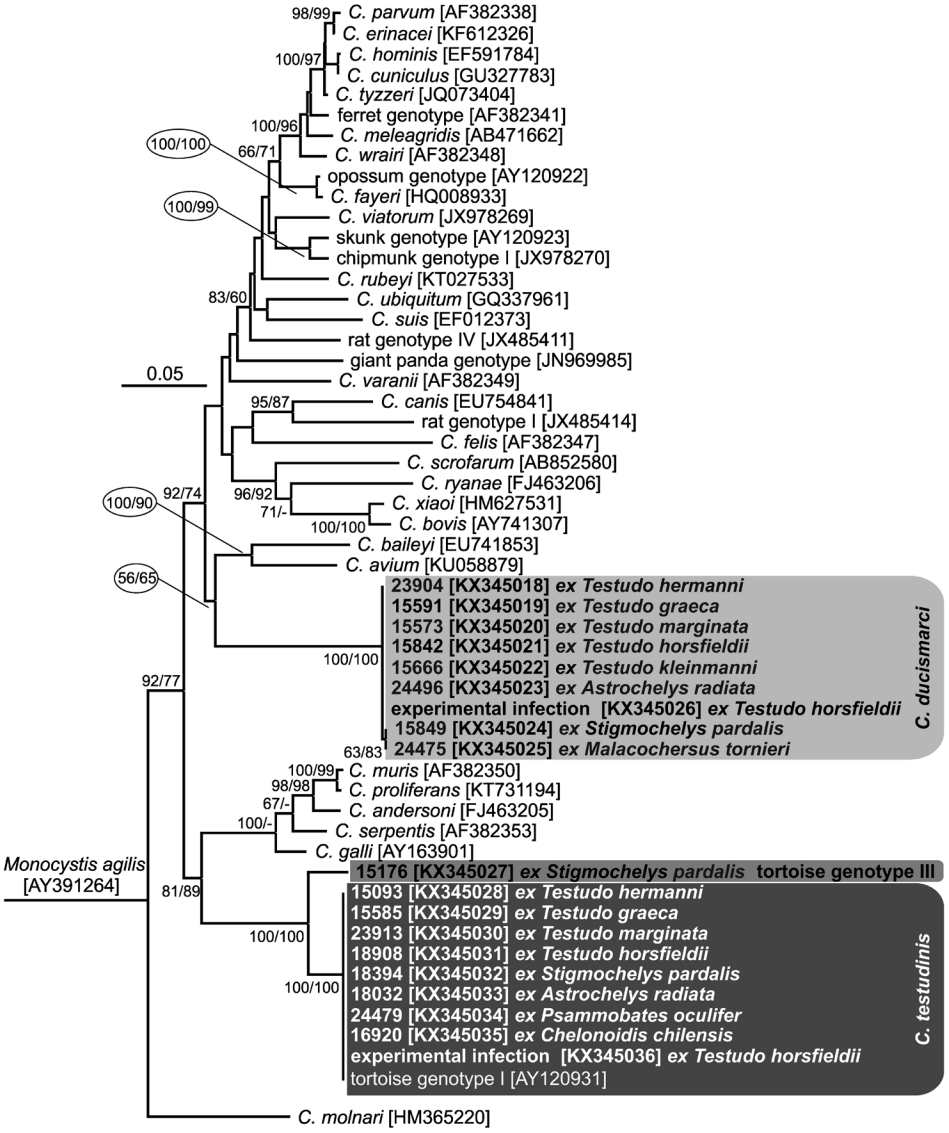

Fig. 2. Maximum likelihood tree based on partial actin gene sequences of species of *Cryptosporidium* Tyzer, 1910, including *Cryptosporidium testudinis* sp. n., *Cryptosporidium ducismarci* Traversa, 2010 and *Cryptosporidium* tortoise genotype III. Sequences from this study are bolded. Numbers at the nodes represent the bootstrap values (ML/MP) gaining more than 50% support. Branch length scale bar indicate number of substitution per site.

C. ducismarci oocysts observed by DIC, ACMV and IFA were deposited as a phototype at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic (acronym IPCAS).

Molecular study

DNA was extracted from 200 mg of faeces by bead disruption for 60 s at 5.5 m/s using 0.5 mm glass beads in a Fast Prep® 24 Instrument (MP Biomedicals, Santa Ana, CA, USA) followed by isolation/purification using a commercially available kit in accordance with the manufacturer's instructions (PSP Spin stool DNA Kit, STRATEC Molecular GmbH, Birkenfeld, Germany). Purified DNA was stored at -20 °C prior to being used for PCR. A nested PCR approach was used to amplify a region of the SSU (~ 830 bp; Xiao et al. 1999, Jiang et al. 2005), actin (~ 1 066 bp; Sulaiman et al. 2002) and *Cryptosporidium* oocyst wall protein (COWP) (~ 375 bp; Kváč et al. 2016). Both primary and secondary PCR reactions were carried out in a volume of 20 µl. The primary reaction contained 2 µl of genomic DNA (or PCR water as a negative control) and the secondary reaction contained 2 µl of the primary reaction as template. DNA of *C. parvum* was used as positive control. Secondary PCR products were detected by agarose gel (2.0%) electrophoresis, visualised by ethidium bromide staining (0.2 µg/ml) and extracted using QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany). Sequencing was carried out in both directions using an ABI 3130 sequencer analyser (Applied Biosystems, Foster City, CA). Amplification and sequencing of each locus were repeated two times.

Nucleotide sequences were edited using the programme ChromasPro 1.7.6 (Technelysium, Pty, Ltd., South Brisbane, Australia) and aligned with each other and with reference sequences (Figs. 1–3) from GenBank (www.ncbi.nlm.nih.gov/blast) using MAFFT version 7 online server with automatic selection of alignment mode (http://mafft.cbrc.jp/alignment/software/). Alignment adjustments were made manually to remove artificial gaps using BioEdit 7.0.5.3 (Hall 1999). Phylogenetic analyses were per-

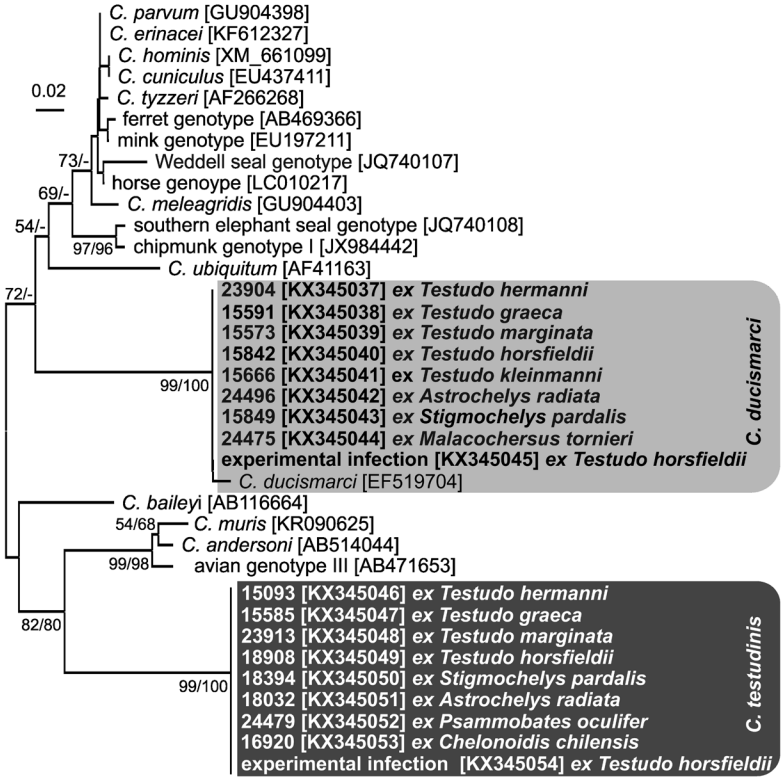

Fig. 3. Maximum likelihood tree based on partial *Cryptosporidium* oocyst wall protein gene sequences of *Cryptosporidium* spp., including *Cryptosporidium testudinis* sp. n. and *Cryptosporidium ducismarci* Traversa, 2010. Sequences from this study are bolded. Numbers at the nodes represent the bootstrap values (ML/MP) gaining more than 50% support. Branch length scale bar indicate number of substitution per site.

formed and the best DNA/Protein phylogeny models were selected using the MEGA6 software (Guindon and Gascuel 2003, Tamura et al. 2013). Phylogenetic trees were inferred by maximum likelihood (ML) and maximum parsimony (MP) methods. Bootstrap support for branching was based on 1 000 replications. Obtained phylograms were edited for style using CorelDrawX7. Sequences have been deposited in GenBank under the accession numbers KX345018–KX345073.

Experimental infections

An adult Russian tortoise, three juvenile Reeve's turtles (*Mauremys reevesii* [Gray]), three 8-week-old SCID mice (*Mus musculus* Linnaeus), an adult common garter snake (*Thamnophis sirtalis* [Linnaeus]) and three adult zebra finches (*Taeniopygia guttata* [Vieillot]) were used for experimental infection studies with *Cryptosporidium* tortoise genotype I or *C. ducismarci*. Three weeks prior to experimental infections, animals were screened daily for the presence of specific DNA and oocysts of cryptosporidia.

Each animal was inoculated orally with 10 000 purified, viable oocysts suspended in 200 µl of distilled water. All animals were sampled daily from 3 to 50 days post infection (DPI) and *Cryptosporidium*-positive animals were additionally sampled weekly from 50 to 200 DPI. Faecal samples were screened for the presence of specific DNA and oocysts of cryptosporidia using ACMV staining and nested PCR amplifying fragment of SSU gene, respectively. Consistency and colour of faeces and intensity of the infection (OPG) were determined for each sample.

RESULTS

A total of 387 faecal samples were examined from 16 terrestrial tortoise species belonging to 11 genera (Table 2). Ten samples were positive by microscopy, with an infection intensity ranging from 1 000–4 000 OPG. *Cryptosporidium*-specific DNA was detected in all microscopy-positive samples and 36 samples that were microscopy-negative. In total, cryptosporida were detected in 10 of 16 tortoise species examined (Table 2).

Phylogenetic analysis of SSU, actin, and COWP sequences using ML and MP methods revealed three distinct clusters among isolates of cryptosporidia from tortoises in the present study. Sequences within clusters shared 99.8–100% identity with each other (Figs. 1–3). One of the clusters included *Cryptosporidium* tortoise genotype I, previously isolated from an Indian tortoise in the USA, and isolates from 22 tortoises belonging to eight different species in the present study.

A second cluster included *C. ducismarci*, previously reported from a marginated tortoise (*Testudo marginata* Schoepff) in Italy, and isolates from 23 tortoises of eight different species in the present study. A third cluster included a single isolate from a Leopard tortoise (*Stigmochelys pardalis* [Bell]) in the present study and an isolate from a Russian tortoise in the USA. The isolate in this cluster was most closely related to *Cryptosporidium* tortoise genotype I, sharing 98.8% and 95.5% similarity at SSU and actin loci, respectively. We named this isolate *Cryptosporidium* tortoise genotype III (Fig. 1–3, Table 2). A COWP

Table 2. Diversity of species of *Cryptosporidium* Tyzzer, 1910 in faecal samples of various species of tortoises detected by microscopy and PCR analysis of the SSU, actin and *Cryptosporidium* oocyst wall protein genes.

Tortoise	n	*Cryptosporidium* spp.	Positive MIC/PCR	SSU	Actin	COWP	Sample ID
Astrochelys radiata (Shaw) (radiated tortoise)	22	*C. testudinis* sp. n.	1/1	$+^{1}$	$+^{1}$	$+^{1}$	18032
		C. ducismarci Traversa, 2010	1/2	$+^{2}$	$+^{2}$	$+^{1}$	24496
Centrochelys sulcata (Miller) (sulcata tortoise)	16	-	0/0	-	-	-	-
Chelonoidis carbonaria (Spix) (red-footed tortoise)	2	-	0/0	-	-	-	-
Chelonoidis chilensis (Gray) (Chaco tortoise)	1	*C. testudinis* sp. n.	0/1	$+^{1}$	$+^{1}$	$+^{1}$	16920
Chersina angulata (Schweigger) (angulate tortoise)	3	-	0/0	-	-	-	-
Geochelone elegans (Schoepff) (Indian star tortoise)	6	-	0/0	-	-	-	-
Kinixys belliana (Gray) (bell's hinge-back tortoise)	1	-	0/0	-	-	-	-
Malacochersus tornieri (Siebenrock) (pancake tortoise)	5	*C. ducismarci*	0/1	$+^{1}$	$+^{1}$	$+^{1}$	24475
Psammobates oculifer (Kuhl) (serrated tortoise)	4	*C. testudinis* sp. n.	0/2	$+^{2}$	$+^{2}$	$+^{1}$	24479
Stigmochelys pardalis (Bell) (leopard tortoise)	30	*C. testudinis* sp. n.	0/1	$+^{1}$	$+^{1}$	$+^{1}$	18394
		C. ducismarci	0/1	$+^{1}$	$+^{1}$	$+^{1}$	15849
		tortoise genotype III	1/1	$+^{1}$	$+^{1}$	-	15176
Testudo graeca Linnaeus (Greek tortoise)	57	*C. testudinis* sp. n.	1/2	$+^{2}$	$+^{1}$	$+^{1}$	15585
		C. ducismarci	0/1	$+^{1}$	$+^{1}$	$+^{1}$	15591
Testudo hermanni Gmelin (Hermann's tortoise)	122	*C. testudinis* sp. n.	0/11	$+^{11}$	$+^{8}$	$+^{5}$	15093
		C. ducismarci	1/7	$+^{7}$	$+^{5}$	$+^{4}$	23904
Testudo horsfieldii Gray (Russian tortoise)	28	*C. testudinis* sp. n.	1/1	$+^{1}$	$+^{1}$	$+^{1}$	18908
		C. ducismarci	1/4	$+^{4}$	$+^{3}$	$+^{1}$	15842
Testudo kleinmanni Lortet (Egyptian tortoise)	23	*C. ducismarci*	2/3	$+^{3}$	$+^{3}$	$+^{2}$	15666
Testudo marginata Schoepff (marginated tortoise)	64	*C. testudinis* sp. n.	0/3	$+^{3}$	$+^{2}$	$+^{1}$	23913
		C. ducismarci	1/4	$+^{4}$	$+^{1}$	$+^{1}$	15573
Terrapene carolina (Linnaeus) (common box turtle)	3	-	0/0	-	-	-	-
Total	387	*C. testudinis* sp. n.	3/22	22	17	12	-
		C. ducismarci	6/23	23	17	12	-
		tortoise genotype III	1/1	1	1	0	-

MIC – light microscopy; PCR – polymerase chain reaction; + positive results by PCR; - negative result by PCR; upper indices indicate number of successfully sequenced amplicons from positive animals.

sequence was not obtained from *Cryptosporidium* tortoise genotype III.

Two morphotypes of oocysts were detected in screened faecal samples. On the basis of morphometrics, oocysts of *Cryptosporidium* tortoise genotype I were revealed to be larger than oocysts of *C. ducismarci* (see below, Fig. 4). Based on the presented data, we propose *Cryptosporidium* tortoise genotype I as a new species, whose description is presented below. We also provide previously unreported data on *C. ducismarci* to confirm its validity.

Cryptosporidium testudinis sp. n. Figs. 4, 5

ZooBank number for species:
urn:lsid:zoobank.org:act:9161FADD-7008-48FF-9ADB-59DB60524BB9

Description. Oocysts are shed fully sporulated with 4 sporozoites and oocyst residuum inside. Sporulated oocysts (n = 30) measure 5.8–6.9 µm (mean = 6.4 µm) × 5.3–6.5 µm (mean = 5.9 µm) with length/width ratio of 1.1 ± 0.05 (Fig. 4). Morphology and morphometry of other developmental stages unknown.

Type host: Russian tortoise (*Testudo horsfieldii* Gray).

Type locality: Nové Hrady, Czech Republic (private breeder).

Site of infection: Location in the host unknown.

Other hosts: chaco tortoise (*Chelonoidis chilensis* [Gray]), Greek tortoise (*Testudo graeca* Linnaeus), Hermann's tortoise (*Testudo hermanni* Gmelin), Indian star tortoise (*Geochelone elegans*), leopard tortoise (*Stigmochelys pardalis*), marginated tortoise (*Testudo marginata*), radiated tortoise (*Astrochelys radiata* [Shaw]) and serrated tortoise (*Psammobates oculifer* [Kuhl]).

Distribution: USA, Austria (predicted based on authors' affiliations), Portugal and Spain.

Material deposited: Slides with oocysts and DNA are deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic. Partial sequences of SSU, actin and COWP genes were deposited at GenBank (Acc. Nos. KX345028–KX345036, KX345046–KX345054 and KX345065–KX345073, respectively).

Etymology: The species name *testudinis* is derived from the Latin noun 'testudo' (meaning a tortoise).

Differential diagnosis. Oocysts are larger than those of *C. ducismarci*, have similar ACMV staining to other cryptosporidia and cross react with immunofluorescence reagents developed primarily for *C. parvum*. It can be differentiated genetically from other cryptosporidia based on sequences of SSU, actin or COWP genes.

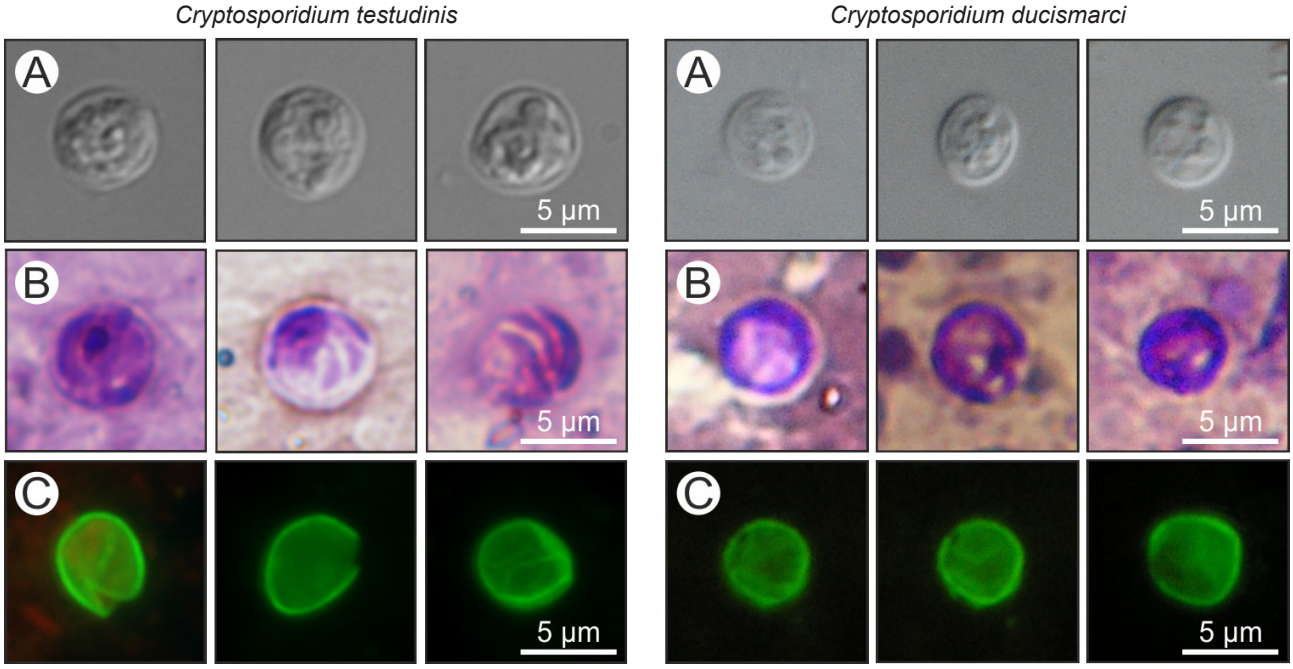

Fig. 4. Ooocysts of *Cryptosporidium testudinis* sp. n. and *Cryptosporidium ducismarci* Traversa, 2010 originating from Russian tortoises (*Testudo horsfieldii* Gray). Oocysts visualised in various preparations. **A** – differential interference contrast microscopy; **B** – stained by aniline-carbol-methyl violet; **C** – stained by anti-*Cryptosporidium* FITC-conjugated antibody.

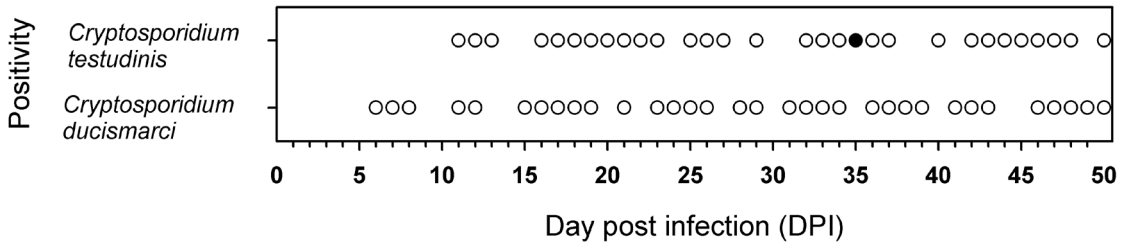

Fig. 5. Course of infection of *Cryptosporidium testudinis* sp. n. and *Cryptosporidium ducismarci* Traversa, 2010 in Russian tortoise (*Testudo horsfieldii* Gray) based on coprological and molecular examination of faeces. Circles indicate detection of specific DNA, black circle indicates microscopic detection of oocysts.

Remarks. Experimental infection was established in a Russian tortoise (*Testudo horsfieldii*), but not Reeve's turtles (*Mauremys reevesii*), a common garter snake (*Thamnophis sirtalis*), zebra finches (*Taeniopygia guttata*) or SCID mice (*Mus musculus*). Specific DNA of *C. testudinis* was first detected in faeces 11 DPI. Intermittent shedding was detected in daily samples up to 50 DPI (Fig. 5) and in weekly samples up to 200 DPI, at which point screening was terminated (data not shown). Oocysts of *C. testudinis* were not detected by microscopy during the experimental infectivity studies, with the exception of a sample obtained at 35 DPI, which had an infection intensity of 1 000 OPG. All naturally and experimentally infected tortoises from the present study exhibited growth that was typical of their size and weight. No lethargy or inappetence was reported. None of the faecal samples was diarrhoeal.

Cryptosporidium ducismarci Traversa, 2010 Figs. 4, 5

Redescription. Oocysts are shed fully sporulated (four sporozoites and oocyst residuum inside) and measure 4.4–5.4 µm (mean = 5.0 µm) × 4.3–5.3 µm (mean = 4.8 µm) with length/width ratio of 1.1 ± 0.03 (n = 30).

Differential diagnosis. Oocysts of *C. ducismarci* are smaller than those of *C. testudinis* and indistinguishable from those of *C. parvum*, have similar ACMV staining to other species of *Cryptosporidium* and cross react with immunofluorescence reagents developed primarily for *C. parvum*.

Material deposited: Slides with oocysts and DNA are deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic. Partial sequences of SSU, actin and COWP genes were deposited at GenBank (Acc. Nos. KX345018–KX345026, KX345037–KX345045 and KX345055–KX345063.)

Remarks. In 2008, a novel *Cryptosporidium* genotype named *Cryptosporidium* tortoise genotype II was genetically characterised in different species of tortoises (Traversa et al. 2008, Griffin et al. 2010). Based on the finding that *Cryptosporidium* tortoise genotype II had different SSU and COWP gene sequences than other cryp-

tosporidia, Traversa (2010) proposed the name *Cryptosporidium ducismarci*. However, the original description lacked description of oocyst morphology. Therefore, it was not be considered as a valid species by some authors. This article redescribes *C. ducismarci* by providing additional morphological, biological and molecular data to support its validity as a separate species. Experimental infection was established in a Russian tortoise (*Testudo horsfieldii*), but not Reeve's turtles (*Mauremys reevesii*), a common garter snake (*Thamnophis sirtalis*), zebra finches (*Taeniopygia guttata*) and SCID mice (*Mus musculus*).

Specific DNA of *C. ducismarci* was first detected in faeces at 6 DPI. Intermittent shedding was detected in daily samples up to 50 DPI (Fig. 5) and in weekly samples up to 200 DPI, at which point screening was terminated (data not shown).

DISCUSSION

Cryptosporidium testudinis, *C. ducismarci* and *Cryptosporidium* tortoise genotype III were detected in 5%, 5% and 0.3% of tortoises in the present study, respectively, which is comparable to data provided by Traversa et al. (2008) and Richter et al. (2012) for *C. testudinis*, i.e. 5% and 8% and *C. ducismarci* reported by Richter et al. (2012), i.e. 15%. It should be noted that all these studies were performed on captive tortoises and the occurrence in wild animals is not known.

The morphology of oocysts of *C. testudinis* and *C. ducismarci* is typical of those of species of *Cryptosporidium*. Although their size ranges and shape index mostly overlap (Fayer et al. 2010), oocysts of *C. testudinis* are significantly larger than those of *C. ducismarci*, which makes it possible to distinguish these species microscopically. In contrast to the oocysts of other gastric species of *Cryptosporidium*, including *C. serpentis*, which are oval (Cranfield and Graczyck 1994), oocysts of *C. testudinis* are spherical. Other characteristics of oocysts of *C. testudinis* and *C. ducismarci*, including thickness of the wall, its inner structure and ability to be detected using *Cryptosporidium*-specific FITC-conjugated antibodies, did not distinguish *C. testudinis* and *C. ducismarci* from other species of *Cryptosporidium* (see Kváč et al. 2014b, 2016, Robinson et al. 2010).

Despite reports of *C. testudinis* (reported as *Cryptosporidium* tortoise genotype I) in a ball python, *Python regius* (Shaw), and a veiled chameleon, *Chamaeleo calyptratus* Duméril et Duméril (Pedraza-Diaz et al. 2009), our and other studies confirm that the species of *Cryptosporidium*, that is described herein as *C. testudinis* and *C. ducismarci*, are specific to tortoises (Xiao et al. 2004a, Alves et al. 2005, Traversa et al. 2008, Griffin et al. 2010, Richter et al. 2012, present study). Graczyk and Cranfield (1998) demonstrated that an uncharacterised *Cryptosporidium* inoculum, prepared from the combined faeces of a naturally infected Indian start tortoise and bog turtle, *Glyptemys muhlenbergii* (Schoepff), was infectious for black rat snakes, *Pantherophis obsoletus* (Say in James).

We found that infections by *C. testudinis* and *C. ducismarci* produced no clinical signs in tortoises, which contrasts with previous reports of symptoms such as weight loss, weakness, lethargy, pneumonia, apathy, depression, innapetence, dehydration, diarrhoea and edema of the head and neck in tortoises infected with *C. testudinis* (referred as *Cryptosporidium* tortoise genotype I) or *C. ducismarci* (referred as *Cryptosporidium* tortoise genotype II) (Heuschele et al. 1986, Graczyk et al. 1998, Alves et al. 2005, Griffin et al. 2010). Most published studies were carried out on sick or otherwise weakened tortoises; therefore, the clinical signs could have been due to the presence of other pathogens or immunodeficiency.

The study by Traversa et al. (2008) supports the absence of clinical signs during infection by *C. testudinis*. In their study, only tortoises infected with *C. parvum* (referred as *C. pestis*) had diarrhoea and dysorexia. Likewise only two of eight *Cryptosporidium*-positive Hermann's tortoises suffered diarrhoea (Richter et al. 2012). These tortoises were also positive for *Escherichia coli*, *Proteus* sp. (both sensitive to doxycycline only), *Hexamita* spp. and oxyurids (Pharyngodonidae), supporting the co-infection hypothesis. This is further supported by the finding that a Russian tortoise and pancake tortoise infected with *C. ducismarci* and other pathogens such *Helicobacter* spp. showed moderate changes of the small intestine characterised by diffuse hyperplasia of the mucosa and low infiltration of lymphocytes in *lamina propria* (Griffin et al. 2010).

Until now, the course of *Cryptosporidium* infection in tortoises has not been described. We first detected the presence of specific DNA of *C. testudinis* and *C. ducismarci* in the faeces of Russian tortoises at 11 and 6 DPI, respectively. However, using microscopy, oocysts of *C. testudinis* were detected in the faeces only after 35 days, and *C. ducismarci* was never detected in the faeces by this approach. This is probably due to low number of oocysts being shed and the low sensitivity of microscopy relative to PCR. The difference in sensitivity of PCR and microscopy should be considered when comparing prepatent periods from different studies. For example, using microscopy to detect oocyst shedding, Cranfield and Graczyk (1994) reported a prepatent period of 12 weeks for *C. serpentis* Levine, 1980 in snakes. It is likely that the prepatent period would have been considerably shorter if they had used PCR.

Similar to other host-adapted *Cryptosporidium* spp., such as *C. scrofarum* Kváč, Kestřánová, Pinková, Květoňová, Kalinová, Wagnerová, Kotková, Vítovec, Ditrich, McEvoy, Stenger et Sak, 2013 in pigs, *C. tyzzeri* Ren, Zhao, Zhang, Ning, Jian, Wang, Lv, Wang, Arrowood et Xiao, 2012 in mice, *C. erinacei* Kváč, Hofmannová, Hlásková, Květoňová, Vítovec, McEvoy et Sak, 2014 in hedgehogs, and *C. bovis* Barker et Carbonell, 1974 and *C. ryanae* Fayer, Santín et Trout, 2008 in cattle, infections caused by *C. testudinis* and *C. ducismarci* are characterised by low oocyst shedding for a prolonged period without clinical disease (Fayer et al. 2005, 2008, Ren et al. 2012, Kváč et al. 2013, 2014b). In contrast, infections by *C. varanii* Pavlásek, Lávičková, Horák, Král et Král, 1995 and *C. serpentis*, reptile-adapted species specific for members of the order Squamata, result in high oocyst shedding and mostly cause severe and even fatal diseases (Brown-

stein et al. 1977, Cranfield and Graczyk 1994, Kimbell et al. 1999, Terrell et al. 2003, Pasmans et al. 2008, Paiva et al. 2013).

Previous studies and our phylogenetic analyses based on SSU, actin and COWP gene sequences showed that *C. testudinis* and *C. ducismarci* are genetically distinct from known species. At the SSU locus, *C. testudinis* and *C. ducismarci* exhibit 6.8% and 2.3% genetic distance from *C. fragile* Jirků, Valigurová, Koudela, Křížek, Modrý et Šlapeta, 2008 and *C. varanii*, respectively. At the actin locus, *C. testudinis* and *C. ducismarci* exhibit 15.3% and 17.0% distance from *C. serpentis* and *C. varanii*, respectively. At the COWP locus, *C. testudinis* and *C. ducismarci* exhibit 20.6 and 22.5% genetic distance from *C. muris* and *C. meleagridis* Slavin, 1955, respectively. These differences are much greater than those between closely related *Cryptosporidium* species. For example, distances between *C. parvum* and *C. tyzzeri* are 0.6%, 1.3% and 0.6%, respectively, and distances between *C. muris* Tyzzer, 1907 and *C. andersoni* Lindsay, Upton, Owens, Morgan, Mead et Blagburn, 2000 are 0.70%, 3.4% and 2.5% at the SSU, actin and COWP loci, respectively.

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