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FAKULTA ZEMĚDĚLSKÁ A TECHNOLOGICKÁ

Kryptosporidie a kryptosporidióza synantropně žijících hlodavců

Disertační práce

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Prohlášení

Předkládám tímto k posouzení a obhajobě disertační práci zpracovanou na závěr doktorského studia na Fakultě zemědělské a technologické Jihočeské univerzity v Českých Budějovicích. Prohlašuji tímto, že jsem práci vypracovala samostatně, s použitím odborné literatury a dostupných zdrojů uvedených v seznamu, jenž je součástí této práce. Dále prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění, souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Fakultou zemědělskou a technologickou, elektronickou cestou ve veřejně přístupné sekci databáze STAG, provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách.

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Tato disertační práce je založena na výsledcích řady vědeckých publikací, které vznikly za účasti dalších spoluautorů. Prohlašuji, že jsem v rámci studia kryptosporidií a kryptosporidiózy synantropně žijících hlodavců provedla většinu původního výzkumu a tato práce je založena na vědeckých výsledcích, jimiž jsem hlavní autorkou nebo spoluautorkou.

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RNDr. Jana Ježková

Seznam impaktovaných publikací

Disertační práce vychází z těchto publikací:

I. Kváč M., Vlnatá G., **Ježková J.**, Horčičková M., Konečný R., Hlásková L., McEvoy J., Sak B. 2018: *Cryptosporidium occultus* sp. n. (Apicomplexa: Cryptosporidiidae) in rats. European Journal of Protistology 63: 96–104.

II. Ježková J., Prediger J., Holubová N., Sak B., Konečný R., Feng Y., Xiao L., Rost M., McEvoy J., Kváč M. 2021: *Cryptosporidium ratti* n. sp. (Apicomplexa: Cryptosporidiidae) and genetic diversity of *Cryptosporidium* spp. in brown rats (*Rattus norvegicus*) in the Czech Republic. Parasitology 148: 84–97.

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Prohlášení spoluautorů

Všichni níže uvedení spoluautoři prohlásili, že RNDr. Jana Ježková se podstatným způsobem podílela na přípravě a publikování rukopisů, ve kterých není prvním autorem, přiložených k této disertační práci. Souhlas jednotlivých spoluautorů byl elektronicky zaslán školiteli doktorandky prof. Ing. Martinu Kváčovi, Ph.D., a byl ověřen předsedou OR DSP Zoohygiena a prevence chorob hospodářských zvířat Fakulty zemědělské a technologické Jihočeské univerzity v Českých Budějovicích prof. Ing. Evou Samkovou, Ph.D.

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Předložená disertační práce zahrnuje výsledky týkající se kryptosporidií a kryptosporidiózy synantropně žijících hlodavců, které byly získány na základě multidisciplinárního přístupu zahrnujícího molekulární biologii, parazitologii, zoologii, histologii a experimentální infekce a byly publikovány v šesti vědeckých impaktovaných publikacích.

Anotace

Zástupci rodu *Cryptosporidium* (Apicomplexa) parazitují v tělech poikilotermních i homoiotermních obratlovců včetně člověka. Jedná se o jednohostitelské, jednobuněčné a epicelulární parazity. Zatímco kryptosporidiím lidí a hospodářských zvířat byla věnována celá řada studií, výzkum na dalších obratlovcích včetně synantropních hlodavců zaostává. Znalosti o biologických vlastnostech jednotlivých druhů a genotypů jsou ve většině případů nedostatečné nebo zcela chybějící. Do současné doby bylo popsáno 51 platných druhů kryptosporidií a byly detekovány nižší stovky genotypů, o kterých neexistuje dostatečné množství údajů, aby bylo možné je považovat za platné druhy. Tato disertační práce výrazně rozšiřuje znalosti o kryptosporidiích specifických pro synantropní hlodavce, zaměřuje se na jejich výskyt a diverzitu v rámci tří čeledí a 12 rodů, hostitelskou a orgánovou specifitu, morfologii vývojových stádií, patogenitu a jejich přenos. Mimo jiné, výsledky získané v této práci přispěly k popisu pěti druhů a 14 genotypů specifických pro synantropní hlodavce.

Annotation

Representatives of the genus *Cryptosporidium* (Apicomplexa) parasitize the bodies of poikilothermic and homoeothermic vertebrates, including humans. They are single-host, unicellular and epicellular parasites. While a number of studies have been devoted to cryptosporidia of humans and livestock, research on other vertebrates, including synanthropic rodents, has lagged behind. Knowledge of the biological characteristics of individual species and genotypes is in most cases inadequate or completely lacking. To date, 51 valid species of cryptosporidia have been described and lower hundreds of genotypes have been detected for which there is insufficient data to consider them as valid species. This dissertation significantly advances the knowledge of cryptosporidia specific to synanthropic rodents, focusing on their occurrence and diversity within three families and 12 genera, host and organ specificity, developmental stage morphology, pathogenicity and transmission. Among others, the results obtained in this work contributed to the description of five species and 14 genotypes specific to synanthropic rodents.

Souhrn

Předmětem předložené disertační práce je studium výskytu a diverzity kryptosporidií synantropních hlodavců. V průběhu studia bylo vyšetřeno pomocí mikroskopických a molekulárních metod 1256 vzorků trusu synantropních hlodavců ze 3 čeledí a 12 rodů z pěti zemí. Z toho 743 vzorků z České republiky, 136 ze Slovenska, 63 z Filipín, 47 z Kambodži a 267 z Thajska. Pouze 13 vzorků bylo mikroskopicky pozitivních na přítomnost oocyst kryptosporidií. Specifická DNA kryptosporidií byla detekována ve 176 případech. Fylogenetickými analýzami částečných sekvencí genů kódujících malou podjednotku rRNA (SSU), heat shock protein (HSP70), aktin, *Cryptosporidium* oocyst wall protein (COWP), thrombospondin-related adhesive protein (TRAP-C1) a 60 kDa glykoprotein (gp60) byla prokázána přítomnost 29 různých kryptosporidií. Bylo detekováno pět již dříve popsaných druhů (*C. andersoni*, *C. muris*, *C. parvum*, *C. ubiquitum* a *C. ryanae*) a 29 genotypů. Z nich 13 bylo detekováno vůbec poprvé, konkrétně 9 genotypů z hlodavců z Thajska (*Cryptosporidium* sp. THA1–9) a 4 genotypy ze syslů z České republiky (*Cryptosporidium* sp. Sc01–04). Současně byl detekován dosud nepopsaný druh specifický pro nutrie – *C. myocastoris*. Na základě biologických a molekulárních odlišností byly z dříve známých genotypů ustanoveny samostatné druhy: *C. occultus* (dříve známo jako *Cryptosporidium suis*-like, *Cryptosporidium* sp. RTA368, *Cryptosporidium* sp. W20486, *Cryptosporidium* sp. P156, *Cryptosporidium* sp. K4515, *Cryptosporidium* sp. AQ7 a *Cryptosporidium parvum* VF383), *C. ratti* (dříve známo jako *Cryptosporidium* sp. rat genotyp I), *C. sciurinum* (dříve známo jako *Cryptosporidium* sp. ferret genotyp) a *C. mortiferum*. (dříve známo jako *Cryptosporidium* sp. chipmunk genotyp I). Velikost oocyst nově popsaných druhů kryptosporidií se od sebe do jisté míry lišila. Oocysty *C. occultus* měřily 5,20 × 4,94 μm, *C. ratti* 4,90 × 4,60 μm, *C. myocastoris* 5,02 × 4,85 μm, *C. sciurinum* 5,54 × 5,22 μm a *C. mortiferum* 5,64 × 5,37 μm. Studium tkáňové specifity prokázalo vývoj *C. occultus* v tlustém střevě potkanů. Predilekčním místem *C. ratti* stejně jako *C. myocastoris* bylo jejunum a ileum potkanů, respektive nutrií. Lokalizace infekce *C. sciurinum* nebyla v naší studii prokázána a *C. mortiferum*, parazitující u veverek, bylo detekováno ve slepém a tlustém střevě. Druh *C. occultus* byl infekční pro potkany, myši a pískomily. Druhy *C. ratti* a *C. myocastoris* se zdají

být úzce hostitelsky specifické a byly infekční pouze pro potkany, respektive pro nutrie. Obdobně druh *C. sciurinum* specifický pro veverky obecné nebyl infekční pro žádného jiného modelového hostitele. U druhu *C. mortiferum* byla prokázána nejširší hostitelská specifita, byl infekční pro myši, fretky, pískomily, veverky obecné a veverky popelavé. V rámci této práce byla popsána patogenita u všech námi popsaných druhů. V případě druhů *C. occultus, C. ratti, C. myocastoris* a *C. sciurinum* nebyly pozorovány žádné klinické příznaky infekce u žádného z infikovaných hostitelů. Pouze u druhu *C. mortiferum* byla pozorována kryptosporidióza s vysokou virulencí pro veverky obecné.

Výsledky práce ukázaly, že kryptosporidie detekované u synantropních hlodavců nepředstavují významné riziko pro člověka a jím chovaná domácí a hospodářská zvířata.

Summary

The subject of the present dissertation is the study of the occurrence and diversity of cryptosporidia of synanthropic rodents. In the course of the study, 1256 faecal samples of synanthropic rodents from 3 families and 12 genera from five countries were examined using microscopic and molecular methods. Of these, 743 samples were from the Czech Republic, 136 from Slovakia, 63 from the Philippines, 47 from Cambodia and 267 from Thailand. Only 13 samples were microscopically positive for the presence of cryptosporidia oocysts. *Cryptosporidium*-specific DNA was detected in 176 cases. Phylogenetic analyses of partial sequences of the genes encoding small subunit rRNA (SSU), heat shock protein (HSP70), actin, *Cryptosporidium* oocyst wall protein (COWP), thrombospondin-related adhesive protein (TRAP-C1) and 60 kDa glycoprotein (gp60) revealed the presence of 29 different cryptosporidia. Five previously described species (*C. andersoni*, *C. muris*, *C. parvum*, *C. ubiquitum* and *C. ryanae*) and 29 genotypes were detected. Of these, 13 were detected for the first time, namely 9 genotypes from rodents from Thailand (*Cryptosporidium* sp. THA1-9) and 4 genotypes from gophers from the Czech Republic (*Cryptosporidium* sp. Sc01-04). At the same time, a previously undescribed species specific to nutria, *C. myocastoris*, was detected. On the basis of biological and molecular differences, the previously known genotypes were established as separate species: *C. occultus* (formerly known as *Cryptosporidium suis*-like, *Cryptosporidium* sp. RTA368, *Cryptosporidium* sp. W20486, *Cryptosporidium* sp. P156, *Cryptosporidium* sp. K4515, *Cryptosporidium* sp. AQ7 and *Cryptosporidium parvum* VF383), *C. ratti* (formerly known as *Cryptosporidium* sp. rat genotype I), *C. sciurinum* (formerly known as *Cryptosporidium* sp. ferret genotype) and *C. mortiferum* (formerly known as *Cryptosporidium* sp. chipmunk genotype I). The oocyst size of the newly described *Cryptosporidium* species differed to some extent from each other. Oocysts of *C. occultus* measured 5.20 × 4.94 μm, *C. ratti* 4.90 × 4.60 μm, *C. myocastoris* 5.02 × 4.85 μm, *C. sciurinum* 5.54 × 5.22 μm, and *C. mortiferum* 5.64 × 5.37 μm. Tissue specificity studies demonstrated the development of *C. occultus* in the colon of rats. The predilection sites of *C. ratti* as well as *C. myocastoris* were the jejunum and ileum of rats and nutria, respectively. The localization of *C. sciurinum* infection

was not demonstrated in our study, and *C. mortiferum*, parasitizing squirrels, was detected in the caecum and colon. The species *C. occultus* was infectious to rats, mice and gerbils. The species *C. ratti* and *C. myocastoris* appeared to be narrowly host specific and were infective only for rats and nutria, respectively. Similarly, the species *C. sciurinum* specific to red squirrels was not infectious to any other model host. *C. mortiferum* species showed the broadest host specificity and was infectious to mice, ferrets, gerbils, red squirrels and eastern gray squirrels. Pathogenicity has been described for all species described in this work. In the case of the species *C. occultus*, *C. ratti*, *C. myocastoris* and *C. sciurinum*, no clinical signs of infection were observed in any of the infected hosts. Only *C. mortiferum* was observed to have cryptosporidiosis with high virulence for red squirrels.

The results of the work showed that cryptosporidia detected in synanthropic rodents do not pose a significant risk to humans and their domestic and livestock animals.

OBSAH

1 CÍLE PRÁCE

Cílem této práce bylo studovat prevalenci, diverzitu a biologii kryptosporidií u synantropně žijících hlodavců.

Následující konkrétní dílčí cíle představují jednotlivé kroky výzkumu nezbytné pro dosažení hlavního cíle:

- Vyhodnotit výskyt a prevalenci kryptosporidiových infekcí u synantropně žijících hlodavců.

- Prokázat hostitelskou specifitu a infektivitu získaných izolátů kryptosporidií pomocí experimentálních infekcí.

- Popsat biologii získaných druhů a genotypů kryptosporidií, zejména průběh infekce, patogenitu a lokalizaci vývojového cyklu včetně popisu vývojových stádií.

- Popsat diverzitu kryptosporidií synantropně žijících hlodavců pomocí multilokusové genotypizace a fylogenetických analýz.

2 LITERÁRNÍ PŘEHLED

2.1 *Cryptosporidium* a kryptosporidióza – obecný úvod

Cryptosporidium Tyzzer, 1907 je rod mikroskopických jednobuněčných jednohostitelských epicelulárních parazitů, jejichž hostiteli mohou být zástupci všech tříd obratlovců (O'donoghue 1995, Ziegler et al. 2007). Je tomu již více než 100 let, kdy byly kryptosporidie poprvé pozorovány v žaludku a následně ve střevě myší (Tyzzer 1910, 1912). Od té doby bylo popsáno více než 50 druhů kryptosporidií a stovky genotypů detekovaných v gastrointestinálním traktu, případně i dalších orgánech svých hostitelů (Current et al. 1986, Goodstein et al. 1989, Xiao et al. 2002, Holubová et al. 2016). Kryptosporidie bývají detekovány nejen v definitivních hostitelích a jejich trusu, ale i v pitné vodě, v odpadních vodách, v půdě, na zelenině a celkově na všech předmětech a prostředí, které přišly do styku s trusem, respektive stolicí obsahující oocysty kryptosporidií (Kváč et al. 2013c, Striepen 2013, Abbas et al. 2022).

Soužití kryptosporidií a jejich hostitelů se postupem času více a více přizpůsobuje a ve většině případů se zdá, že je vztah parazita a hostitele v rovnováze. V posledních letech byla provedena řada studií, ve kterých bylo zjištěno, že většina druhů kryptosporidií nezpůsobuje žádné klinické příznaky (Turkcapar et al. 2002, Houpt et al. 2005, Vítovec et al. 2006, Kváč et al. 2014c, Ryan et Xiao 2014, Segura et al. 2015). Nicméně některé druhy mohou vyvolat onemocnění kryptosporidiózu doprovázené řadou klinických příznaků (Current et Garcia 1991, Chalmers et Davies 2010). Z pohledu člověka patří mezi nejvýznamnější druhy *C. parvum* a *C. hominis*, které vyvolávají infekci u imunodeficitních, ale i imunokompetentních jedinců (Johansen et al. 2015). U imunodeficitních jedinců mohou infekci vyvolat i další druhy kryptosporidií a mohou se rozšířit téměř do všech tkání svého hostitele (Macher 1988, Reina et al. 2016, Wesełowska et al. 2016). V zemích třetího světa je kryptosporidióza druhé nejčastější průjmové onemocnění, na které umírají zejména děti do 5 let věku, k jejichž nákaze dochází obvykle z kontaminované vody (Striepen 2013).

Kryptosporidie jsou z evolučního hlediska velmi dobře adaptovány k parazitickému způsobu života. Zejména odolnost oocyst jim umožňuje přežívat v různých podmínkách vnějšího prostředí a úspěšně odolávají i dezinfekčním

prostředkům (Uguen et al. 1997, Dumetre et al. 2012, Adeyemo et al. 2019, Chalmers et al. 2019, Zahedi et Ryan 2020).

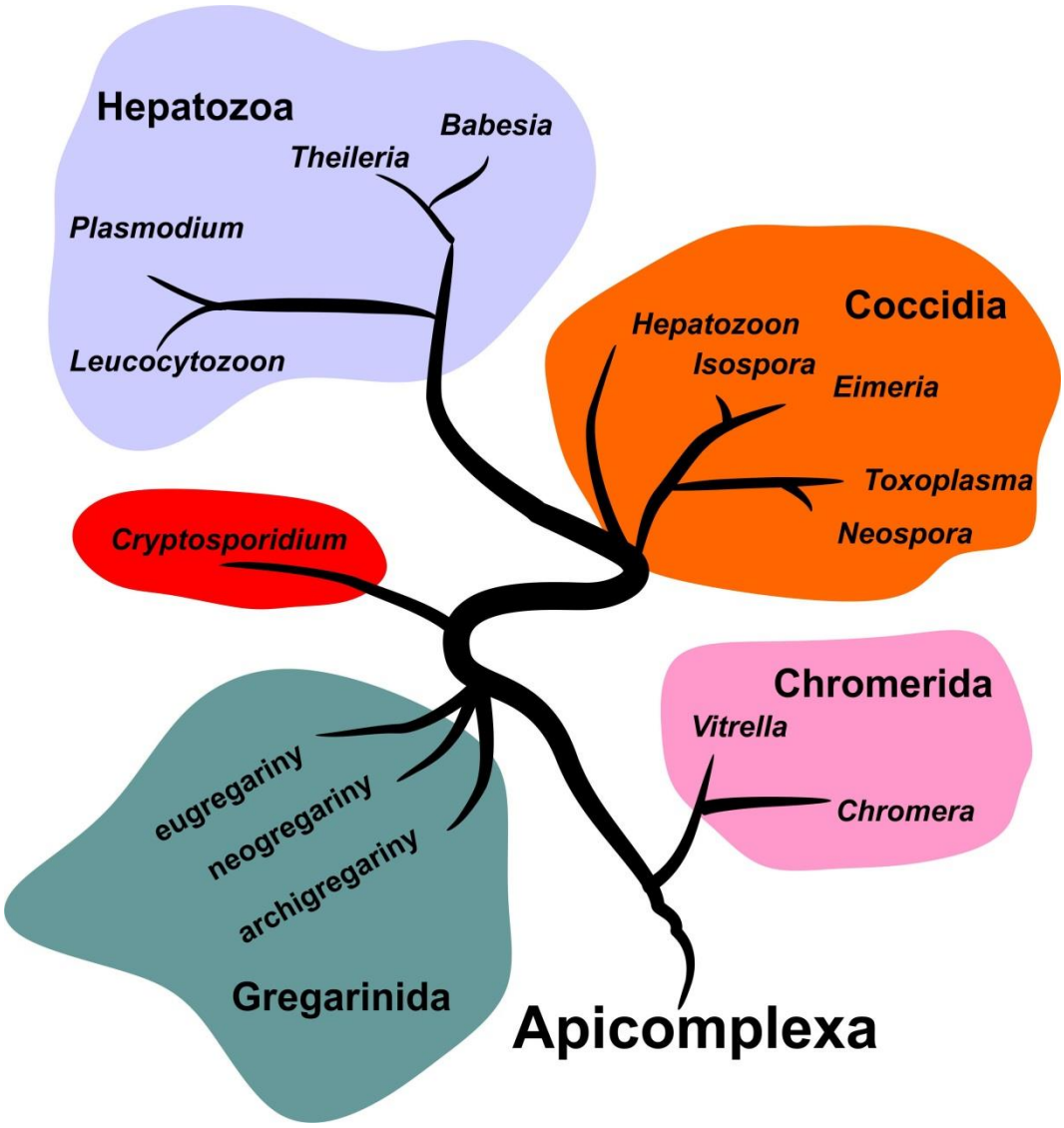
2.1.1. Klasifikace a fylogeneze kryptosporidií

Z fylogenetického hlediska jsou kryptosporidie řazeny do kmene Apicomplexa (Cavalier-Smith 2014). Původně byly kryptosporidie řazeny mezi kokcidie, nicméně již Tyzzer při popisu prvních druhů kryptosporidií upozornil na některé odlišnosti kryptosporidií od kokcidií, přesto je však ke kokcidiím přiřadil (Tyzzer 1910). Mezi kryptosporidiemi a kokcidiemi lze nalézt několik odlišností: (i) endogenní stádia kryptosporidií nejsou lokalizována v cytoplazmě, ale jsou lokalizována na povrchu buňky (epicelulární lokalizace), ii) kryptosporidie jsou na hostitelskou buňku připojeni tzv. feeder organelou, iii) u kryptosporidií nenalezneme morfologické struktury, jako jsou sporocysta, mikropyle a pólová čepička, které jsou typické pro oocysty kokcidií, iv) v rámci životního cyklu dochází u kryptosporidií ke vzniku dvou odlišných typů oocyst, v) kryptosporidie mají na rozdíl od kokcidií schopnost autoinfekce hostitele (Ryan et al. 2014). Na přelomu tisíciletí byly provedeny první fylogenetické studie, jejichž závěrem bylo, že kryptosporidie mají nejen z biologického, ale i z fylogenetického hlediska blíže ke gregarinám (Fayer et al. 1997, Carreno et al. 1999). Mezi společné biologické znaky kryptosporidií a gregarin patří morfologická stavba organel, které slouží k přichycení k hostitelské buňce a nepřítomnost plastidového genomu (Valigurová et al. 2007, Cavalier-Smith 2014).

V roce 2014 byla publikována fylogenetická studie, ve které byly kryptosporidie zařazeny do třídy Gregarinomorphea, a do skupiny Cryptogregarina (Cavalier-Smith 2014). V roce 2019 Adl et al. (2019) tuto třídu rozšířili na podtřídu Cryptogregarinorida v rámci Gregarinasina. Nicméně v posledních čtyřech letech bylo publikováno několik fylogenomických analýz zahrnující kryptosporidie a gregariny, které vyvrací validitu skupiny Cryptogregarina (Janouškovec et al. 2019, Mathur et al. 2019, Salomaki et al. 2021). Ukazuje se, že kryptosporidie pravděpodobně představují samostatnou monofyletickou skupinu v rámci kmene Apicomplexa a je zjevně nutná další taxonomická revize (Obrázek 1). Rod *Cryptosporidium* byl také navržen jako nejstarší divergující linie v rámci

Apicomplexa (Salomaki et al. 2021), zatímco jiné studie naznačují více nezávislých původů (Mathur et al. 2019).

Obrázek 1. Ilustrace postavení kryptosporidií v rámci kmene Apicomplexa.

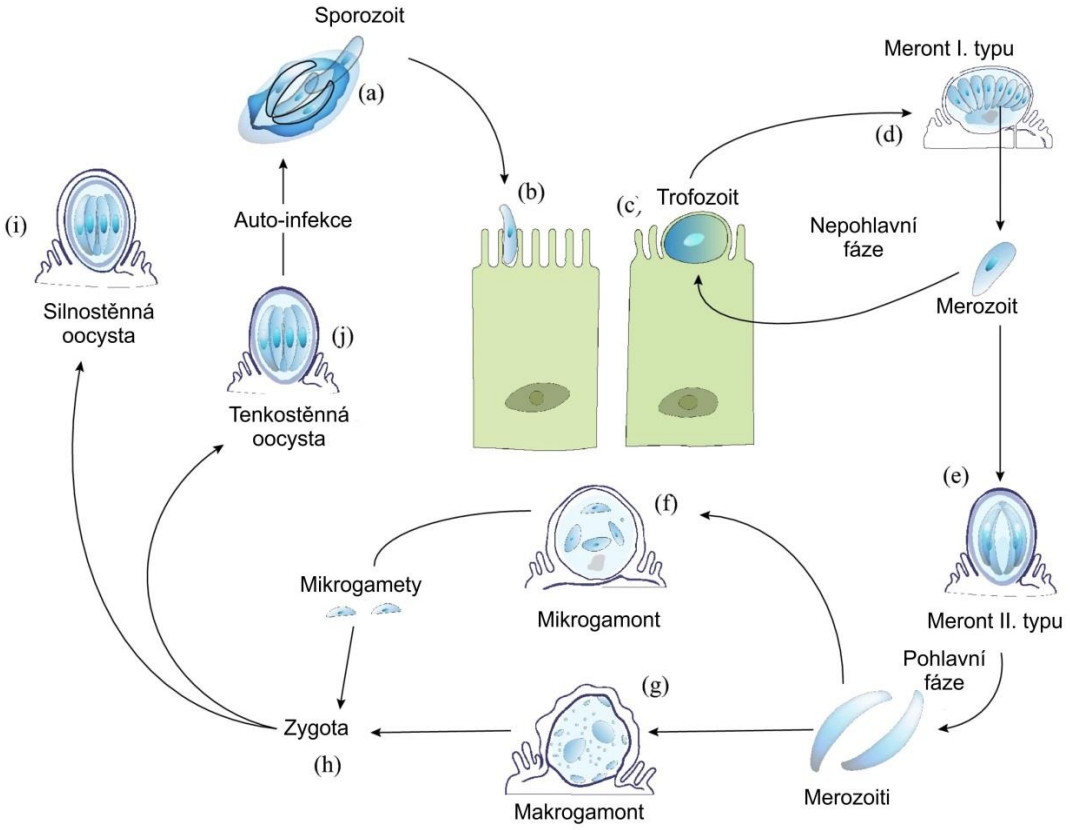


2.1.2 Vývojový cyklus a morfologie kryptosporidií

Vývojový cyklus kryptosporidií zahrnuje vždy pouze jednoho hostitele a dělí se na čtyři fáze: excystace, merogonie, gametogonie a sporogonie, v rámci nichž parazit prodělává asexuální (merogonie) a sexuální (gametogonie) rozmnožování (Obrázek 2). Aby mohl celý cyklus úspěšně proběhnout, je nezbytné pozření infekce schopných oocyst kryptosporidií vnímavým hostitelem. V případě, že oocystu pozře netypický hostitel, dojde buď k excystaci a pokusu sporozoitů o infekci hostitele,

který však končí nezdarem, nebo oocysty projdou zažívacím traktem tohoto hostitele bez ztráty životaschopnosti (Graczyk et al. 1996, 1998). Množství infekce schopných oocyst není pro infekci rozhodující (Zambriski et al. 2013, Melicharová et al. 2014). Uvádí se, že nižší desítky oocyst jsou dostatečné pro vyvolání infekce (Okhuysen et Chappell 2002).

Obrázek 2. Grafické znázornění vývojového cyklu *Cryptosporidium parvum* (Dhal et al. 2022, upraveno).



Po pozření oocyst hostitelem dojde k excystaci. To znamená, že z oocysty jsou uvolněni čtyři sporozoiti, kteří napadají buňky epitelu trávicího traktu případně jiné orgány. Každý sporozoit obsahuje haploidní jádro s osmi chromozomy (Blunt et al. 1997). Obvykle kryptosporidie infikují gastrointestinální trakt, konkrétně žaludek a střevo, nicméně byly detekovány i v jiných orgánech, a to zejména u ptáků, kde se druh *C. baileyi* může vyskytovat v plicích, ledvinách a Fabriciově burze (Current et al. 1986). Další odlišnost ve vývojovém cyklu byla popsána u druhů *C. molnari* a *C. huwi* parazitujících u kostnatých ryb. Vývojová stadia kryptosporidií těchto druhů byly detekovány uvnitř žaludeční sliznice, případně uvnitř buněk střevního epitelu. Část vývojového cyklu těchto druhů tedy probíhá uvnitř buněk a ne

epicelulárně, jak je tomu obvykle u kryptosporidií ostatních obratlovců (Sunnotel et al. 2006, Ryan et al. 2015).

Uvolnění sporozoiti z excystovaných oocyst se aktivně pohybují, vyhledávají a napadají hostitelskou buňku (Wetzel et al. 2005). Žaludeční druhy kryptosporidií jsou adaptovány na kyselé pH žaludku, zatímco sporozoiti střevních druhů nejsou schopni přežívat při takto nízkém pH (Widmer et al. 2007). Při kontaktu sporozoita s hostitelskou buňkou dochází k vychlípení plazmatické membrány hostitelské buňky a prodlužování mikrovilů, které postupně obklopují sporozoita až do samotného vzniku parazitoforního vaku. S hostitelskou buňkou je sporozoit propojen pomocí tzv. feeder organely (Goebel et Braendler 1982, Yoshikawa et Iseki 1991, Valigurová et al. 2007, Melicharová et al. 2014).

Následující fáze vývojového cyklu je nazývána merogonie, během níž se jádro trofozoita asexuálně rozmnožuje. Vzniká meront I. typu, který obsahuje 6–8 merozoitů. Tito merozoiti I. typu napadají další buňky a opět dávají vzniknout merontům I. typu, případně merontům II. typu obsahujícím již pouze 4 merozoity (Aydin 1997). Pouze z merozoitů II. typu se vyvíjí mikrogamonty a makrogamonty. Nicméně nezbytnost a přítomnost merozoitů II. typu byla zpochybněna při *in vitro* experimentech (English et al. 2022). Mikrogamonti se mnohonásobně dělí a dávají vzniknout až 16 pohyblivým mikrogametám (samčí pohlavní buňky). Makrogamonti jsou jednojaderní a vyvíjí se pouze v jednu makrogametu (samičí pohlavní buňka), která bývá aktivně vyhledávána mikrogametami. Mikrogameta penetruje do makrogamety a tím dojde k oplodnění. Takto oplodněná makrogameta se vyvíjí a dozrává v oocystu (Current et al. 1986, Fayer et Ungar 1986, Current et Reese 1987, Valigurová et al. 2008).

Všechny oocysty kryptosporidií obsahují čtyři sporozoity a jedno velké reziduální tělísko (Uni et al. 1987). U druhu *C. parvum* byl prokázán vznik dvou typů oocyst. Tenkostěnné oocysty způsobující autoinfekci a silnostěnné oocysty, které jsou vylučovány trusem do vnějšího prostředí, kde jsou schopné okamžité infekce nového hostitele (Current et Reese 1987, Blunt et al. 1997, Sunnotel et al. 2006, Widmer et al. 2007). Díky robustní stavbě buněčné stěny jsou silnostěnné oocysty velmi dobře adaptovány na přežití ve vnějším nepříznivém prostředí (Jenkins et al. 2010).

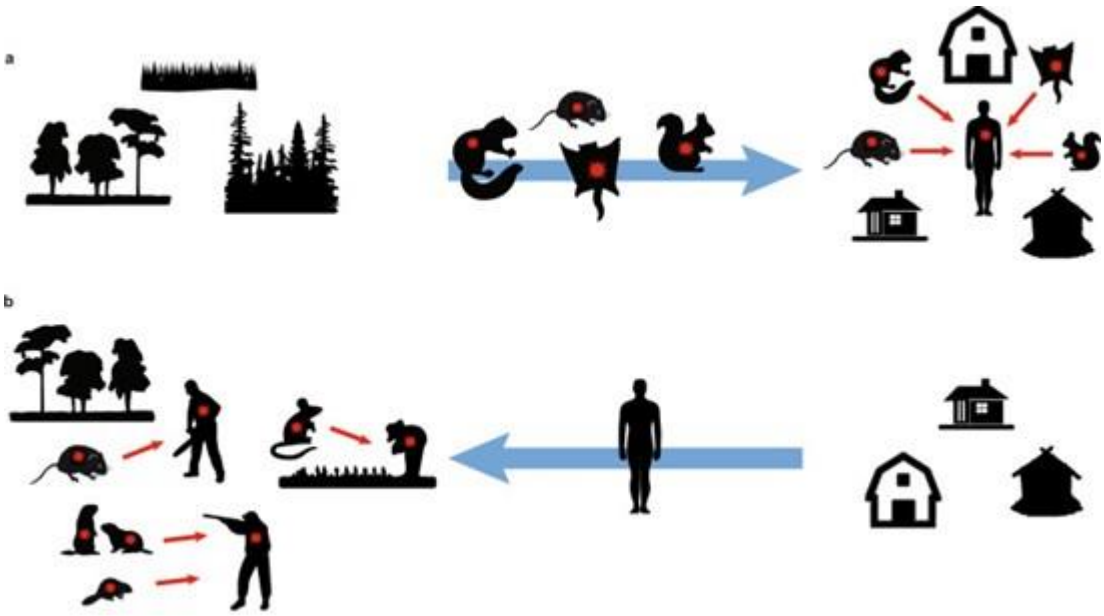
Morfologické rozdíly ve tvaru a velikosti oocyst jsou u většiny kryptosporidií natolik malé, že je nelze využít jako spolehlivý diferenciální znak pro rozlišení

jednotlivých druhů (Jex et al. 2008, Kváč et al. 2013a, Kváč et al. 2014a, Holubová et al. 2016, Holubová et al. 2019). Oocysty střevních druhů kryptosporidií jsou sférického tvaru s velikostí 4,0–6,5 µm (Tyzzer 1912, Fayer et al. 2008), oocysty žaludečních druhů jsou pak oválné o velikosti 6,5–9,0 µm (Ryan et Xiao 2014).

2.1.3 Zdroj kryptosporidií, jejich šíření a prevalence

Kryptosporidie jsou do vnějšího prostředí vylučovány trusem, respektive stolicí infikovaných hostitelů. Ve vnějším prostředí se mohou pasivně šířit a zachovávat si svoji infektivitu pro další potenciální hostitele, například pomocí hlodavců, či dalších živočichů (Obrázek 3). Zdrojem kryptosporidií je v první řadě již zmiňovaný trus, či stolice a dále všechny typy vod, do kterých se oocysty dostaly, půda a další kontaminované předměty a potraviny, jako zelenina a ovoce (Li et al. 2020c, Daraei et al. 2021, Abbas et al. 2022).

Obrázek 3. Cesty zvyšující riziko přenosu patogenů přenášených hlodavci (Ecke et al. 2022, upraveno).



Z 24 druhů a genotypů kryptosporidií infekčních pro člověka je pouze *C. hominis* hostitelsky specifický pro lidi, pět jich je specifických pro hospodářská zvířata a sedm pro volně žijící hlodavce (Ortega et Kváč 2013, Zajaczkowska et al. 2022). K

přenosu kryptosporidií mezi hlodavci a člověkem dochází nejčastěji dvěma způsoby. V prvním případě dochází k přenosu z druhů, které pronikají do lidských obydlí a jejich okolí (Obrázek 3a), nebo lidé se vydávají do přirozených stanovišť hlodavců, nebo využívají hlodavce jako přírodní zdroj (Obrázek 3b).

Tělesná hmotnost hlodavců může dichotomicky zvyšovat riziko přenosu patogenů. Zatímco hlodavci s vysokou tělesnou hmotností jsou často loveni pro kožešinu nebo maso, mnoho hlodavců s nízkou tělesnou hmotností má méně vyvinuté strategie imunitní obrany a vykazují velké populační výkyvy, což má za následek vznik nákazových ohnisek. Období vysoké populační hustoty hlodavců jsou často spojována s hojným šířením hlodavců do prostředí obývaných člověkem. Vzhledem k tomu, že patogeny jsou často spojovány se synantropními hlodavci a s hlodavci obecně, je riziko nákazy člověka kvůli blízkému vztahu s hlodavci zvýšeno (Jones et al. 2008, Shrivastava et al. 2017).

Daraei et al. (2021) provedl studii, ve které využil data všech dostupných publikací a prokázal celkovou prevalenci kryptosporidií ve vodě 36 %. Nejvyšší prevalence byla odhalena v odpadních vodách (46,9 %), dále v povrchových vodách (45,3 %), v přírodních vodách (31,6 %), v pitné vodě (25,5 %), ve vodě z nádrží (24,5 %), v podzemních vodách (18,8 %), v bazénové vodě (7,5 %) a v mořské vodě 0,2 %). Zjištěná prevalence kryptosporidií ve vodě ovšem nemusí znamenat, že se z vody může někdo nakazit, jelikož řada používaných detekčních metod není schopna odhalit, zda se ve vodě vyskytují životaschopné a tím pádem infekce schopné oocysty. Může se jednat o neinfekční oocysty, případně může být zachycena pouze specifická DNA (Efstratiou et al. 2017, Daraei et al. 2021).

2.2 Diverzita kryptosporidií

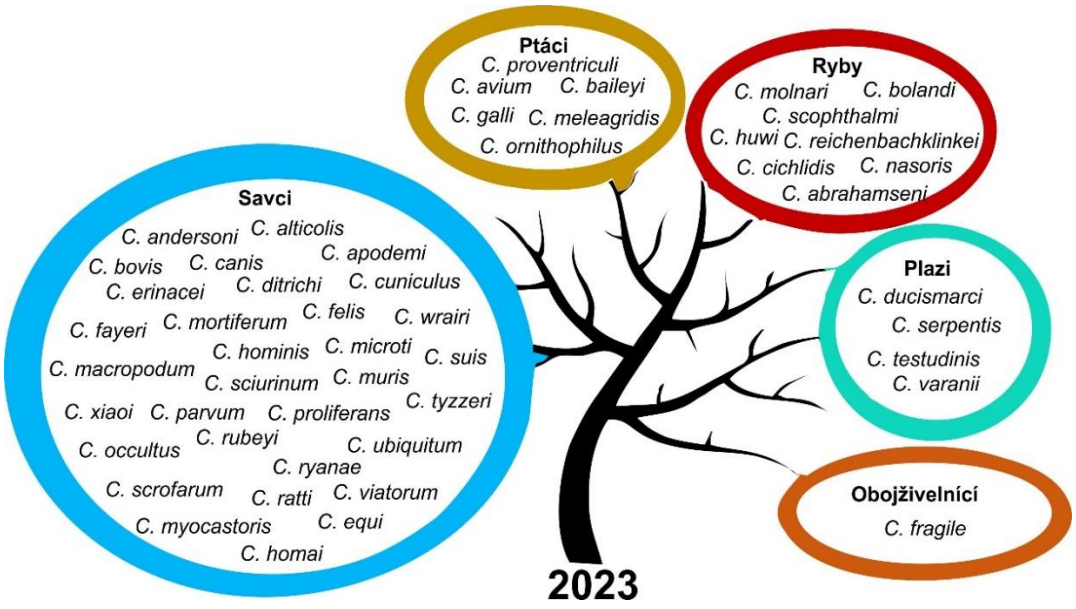
Pojem hostitelská specifita lze chápat jako rozmezí živočišných druhů, které je parazit schopen využívat jako své hostitele. Druhy kryptosporidií se od sebe liší v hostitelském spektru. Některé druhy kryptosporidií jsou úzce, jiné zase široce hostitelsky specifické. Mezi druhy s nejširší hostitelskou specifitou patří druhy *C. parvum* a *C. ubiquitum*, které jsou s největší pravděpodobností infekční pro všechny savce včetně člověka (Dupont et al. 1995, Fayer 2004, Rašková et al.

2013, Li et al. 2014a). Mezi druhy s úzkou hostitelskou specifitou patří *C. wrairi*, *C. hominis*, *C. ryanae*, *C. scrofarum*, *C. suis* a řada dalších (Morgan-Ryan et al. 2002, Kváč et al. 2013a, Ifeonu et al. 2016). I úzce hostitelsky specifické druhy mohou příležitostně parazitovat v nespecifickém hostiteli. Jako příklad lze uvést infekci druhem *C. erinacei*, kterým byl infikovaný imunokompetentní člověk. Nicméně typickým hostitelem tohoto druhu kryptosporidie je ježek (Kváč et al. 2014c). Koevoluce parazita s hostitelem a hostitelsko-geografická segregace vedly ke vzniku různých druhů a subtypů, které se vyskytují v různých částech světa (Chen et al. 2023b). Příkladem je výskyt různých subtypů druhu *C. parvum*. Některé subtypy se vyskytují celosvětově, zatímco jiné byly detekovány pouze v Severní Americe (subtypy IIaR2) nebo v Evropě (subtyp III) (Hijjawi et al. 2022, Chen et al. 2023b).

Diverzita kryptosporidií je obrovská, mnohem větší, než bylo předpokládáno a každý další nový popis posouvá naše znalosti. Zatímco na konci 20. století bylo známo jen několik desítek druhů a genotypů do této doby bylo popsáno 51 platných druhů kryptosporidií a byly detekovány stovky genotypů, které byly od platných druhů odlišeny na základě odlišností v sekvenci genu kódujícího malou podjednotku rRNA a částečně podle hostitelské specifity (Obrázek 4) (Kváč et al. 2014b).

Ještě v nedávné době se předpokládalo, že genetická diverzita a populační struktura kryptosporidií v daném hostiteli je omezena na 1–4 specifické druhy (Feng et al. 2018), nicméně nedávné studie, včetně této práce ukazují, že jeden hostitel může být parazitován i desítkami různých kryptosporidií (Čondlová et al. 2018, Holubová et al. 2020, He et al. 2022, Jia et al. 2022, Wang et al. 2022a, Huang et al. 2023). Zásadní překážku při detekci těchto multi-infekcí představuje problém odlišení kolonizujících/replikujících se parazitů od pasáže/kontaminace (Feng et al. 2018).

Obrázek 4. Druhy kryptosporidií infikující jednotlivé skupiny obratlovců k roku 2023.



2.2.1 Tkáňová specifita

Tkáňová specifita je až na výjimky specifickou vlastností daného druhu a genotypu kryptosporidie. U vnímavých imunokompetentních hostitelů je predilekční místo infekce neměnné a jednotlivé kryptosporidie se od sebe liší svou tkáňovou specifitou. Zmíněná výjimka nastává u imunodeficitních a oslabených hostitelů, u kterých může docházet k šíření infekce i do dalších tkání a orgánů (Current et al. 1986, Macher 1988, Wang et al. 2014, Wesełowska et al. 2016).

Podle lokalizace infekce v trávicím traktu lze kryptosporidie rozdělit do dvou monofyletických, morfologicky odlišných linií, a to na střevní a žaludeční kryptosporidie (Xiao et al. 2002, 2004, Kváč et al. 2013c). V rámci střevních druhů odlišujeme druhy s afinitou k tenkému, slepému a tlustému střevu. U řady druhů kryptosporidií, kde je známá lokalizace v trávicím traktu, bylo zjištěno, že se nejčastěji vyvíjí v tenkém střevě, konkrétně v duodenu a jejunu (Čondlová et al. 2018, Horčičková et al. 2019, Ježková et al. 2021b). U menší části kryptosporidií byl zjištěn vývoj ve slepém nebo tlustém střevě (Čondlová et al. 2018, Horčičková et al. 2019). U ptačích kryptosporidií byla zjištěna multiorgánová lokalizace. Přestože byla u některých druhů kryptosporidií detekována lokalizace mimo trávicí trakt, všechny druhy a genotypy kryptosporidií parazitují primárně v trávicím traktu svých hostitelů

(O'donoghue 1995, Thompson et al. 2005). Tyto druhy se mohou vyjma zažívacího traktu vyskytovat v plicích, ledvinách, močovodech a Fabriciově burze (Current et al. 1986, Curtiss et al. 2015, Holubová et al. 2016). Lokalizace v hostiteli je obvykle odvozována dle příslušnosti k dané fylogenetické linii – střevní nebo žaludeční, nicméně fylogenetická pozice v rámci linie střevních kryptosporidií nekoresponduje s tkáňovou specifitou této skupiny (Čondlová et al. 2018, Kváč et al. 2018, Horčičková et al. 2019, Ježková et al. 2021a, 2021b).

2.3 Synantropie a synantropní živočichové

Za synantropii je obecně z biologického hlediska považován stav, kdy volně žijící živočich, případně rostlina, žije v těsné blízkosti člověka, nebo v krajině, která je do jisté míry člověkem modifikována a má z toho prospěch. Takoví živočichové či rostliny žijí obvykle uprostřed měst, vesnic a dalších sídel, která jsou do jisté míry udržovaná člověkem. Lze sem zahrnout parky, zahrady, rybníky, krajnice silnic i smetiště (Petráčková et Kraus 2000, Johnson et Klemens 2005). Synantropní živočichové využívají lidských sídel zejména k ochraně proti jejich přirozeným predátorům, případně i odlovu člověkem, jelikož na nehonebních pozemcích nemohou být loveni. Příkladem takových živočichů, kteří odlovu touto cestou unikají, jsou na našem území například vrány, holubi, nutrie a zajíci (Gade 2010, Dipineto et al. 2013).

Obecně lze synantropní živočichy rozdělit na eusynantropní, kteří jsou přímo vázání na lidská obydlí a na hemisynantropní, kteří žijí ve volné přírodě, ale v případě kontaktu s člověkem se stávají potenciálně synantropními (Rosický et Weise 1951).

Jak vyplývá z výše uvedeného rozdělní, tak i hlodavce lze rozdělit do dvou skupin, a to na eusynantropní, tzv. pravé synantropní hlodavce, kam patří potkani, krysy a myši. Druhá skupina hemisynantropních hlodavců, tzv. příležitostní synantropní hlodavci jsou tvořeni hraboši, myšicemi, hryzci a norníky. Za určitý druh synantropie lze považovat i soužití dalších hlodavců, jako veverek, nutrií a syslů, kteří nejsou na člověka významně vázáni, ale mohou příležitostně z lidské přítomnosti a činnosti profitovat (Rosický et Weise 1951, Dubinský et al. 1995).

2.3.1 Synantropní hlodavci jako zdroj zoonotických patogenů

Hlodavci představují z hlediska evoluce největší a zároveň nejmladší skupinu savců zahrnující 2000–2700 druhů, jejichž předkové se zřejmě podobali křečkům. Přibližně 40–42 % všech savců je kategorizováno právě jako členové této skupiny. Čeleď Muridae zahrnuje dokonce více než 1100 druhů, což představuje asi čtvrtinu všech savců (Aplin et al. 2003, Witmer 2005, Wolff 2007). Vyskytují se zejména v Africe, Eurasii, Austrálii a některé druhy byly pomocí člověka introdukovány po celém světě (Harrison et Bates 1991, Wilson 2005, Amr et al. 2018). Navzdory morfologické a ekologické rozmanitosti, sdílejí všichni hlodavci jeden společný znak a tím je diprotodontní chrup, který je specializován k hlodání, při němž se protistojné řezáky vzájemně obrušují. Toto samoostření je jedním z faktorů ohromného evolučního úspěchu hlodavců (Wolff 2007).

Hlodavci, kteří jsou hojní, celosvětově rozšíření a žijící v blízkosti člověka mohou představovat jeden z možných rezervoárů kryptosporidií pro člověka a domácí či hospodářská zvířata. Vzhledem k úzkému vztahu, který synantropní hlodavci vykazují s lidskou populací a domácími zvířaty, jsou považováni za významné přenašeče původců bakteriálních, virových či parazitárních onemocnění. Z bakteriálních původců lze jmenovat leptospiry, salmonely, listerie, rickettsie, dále původce tularémie (*Francisella tularensis)*, tuberkulózy (*Mycobacterium tuberkulosis*) nebo moru (*Yersinia pestis*), původce virových onemocnění jako hantaviry, virus lymfatické choriomeningitidy (LCMV), a dále parazity jako například *Trichinella spiralis*, *Toxoplasma gondii* a v neposlední řadě jsou i původci kryptosporidiózy (Meerburg et al. 2009, Battersby 2015).

2.3.2 Kryptosporidie synantropních hlodavců

Kryptosporidiím hlodavců obecně byly dosud věnovány stovky studií (PubMed, www.ncbi.nlm.nih.gov). Zatímco některé rody/čeledě hlodavců jsou studovány častěji, například myši, krysy, potkani, myšice, hraboši a křečci (Lv et al. 2009, Rašková et al. 2013, Mohebali et al. 2017, Stenger et al. 2017, Čondlová et al. 2018, Zhao et al. 2018, Čondlová et al. 2019, Garcia-Livia et al. 2022, Hancke et Suarez 2022), u jiných jsou k dispozici jen ojedinělá sdělení nebo neexistují žádné dostupné informace (Pacini et al. 2023). Příkladem poslední skupiny mohou být hlodavci

obývající africký kontinent. Bohužel neexistuje dostatek informací o globálním výskytu kryptosporidií právě u synantropních hlodavců. Jaká je tedy skutečná prevalence a diverzita kryptosporidií u synantropních hlodavců z celosvětového hlediska je stále otevřenou otázkou. Výsledky metaanalytické studie Taghipour et al. (2020) ukázaly, že v Americe hlodavci hrají významnou roli rezervoárů kryptosporidií s možností infekce člověka. V současné době bylo celosvětově u hlodavců detekováno 17 druhů a 57 genotypů kryptosporidií (Tabulka 1). Z této podmnožiny zástupců rodu *Cryptosporidium* byl u osmi prokázán přenos na člověka (Tabulka 2).

Tabulka 1. Druhy a genotypy kryptosporidií detekované u hlodavců.

Druh/genotyp *Cryptosporidium*	**Typický hostitel (*latinský název*)**	**Synantropie**	**Citace**
C. myocastoris	Nutrie (*Myocastor coypus*)	Ano	Ježková et al. (2021a)
C. ratti	Potkan (*Rattus norvegicus*)	Ano	Ježková et al. (2021b)
C. microti	Hraboš (*Microtus arvalis*)	Ano	Horčičková et al. (2019)
C. alticolis	Hraboš (*Microtus arvalis*)	Ano	Horčičková et al. (2019)
C. occultus	Potkan (*Rattus norvegicus*)	Ano	Kváč et al. (2018)
C. apodemi	Myšice (*Apodemus agrarius*)	Ano	Čondlová et al. (2018)
C. ditrichi	Myšice (*Apodemus flavicollis*)	Ano	Čondlová et al. (2018)
C. homai	Morče (*Cavia porcellus*)	Ne	Zahedi et al. (2018)
C. proliferans	Hlodoun (*Tachyoryctes splendens*)	Ne	Kváč et al. (2016)
C. rubeyi	Sysel (*Spermophilus beecheyi*)	Ano	Li et al. (2015)
C. sciurinum	Veverka (*Sciurus vulgaris*)	Ano	Prediger et al. (2021)
C. viatorum	Člověk a hlodavci	ND	Elwin et al. (2012)
C. tyzzeri	Myš (*Mus musculus*)	Ano	Ren et al. (2012)
C. ubiquitum	Skot a hlodavci	Ano	Fayer et al. (2010)

Tabulka 1. Druhy a genotypy kryptosporidií detekované u hlodavců (pokračování).

Druh/genotyp *Cryptosporidium*	**Typický hostitel (*latinský název*)**	**Synantropie**	**Citace**
C. wrairi	Morče (*Cavia porcellus*)	Ne	Vetterling et al. (1971)
C. muris	Myš (*Mus musculus*)	Ano	Tyzzer (1907, 1910)
C. mortiferum	Veverka (*Sciurus vulgaris*)	Ano	Tůmová et al. (2023)
Bamboo rat genotyp I	Hlodoun (*Rhizomys sinensis*)	Ne	Wei et al. (2019)
Bamboo rat genotyp II	Hlodoun (*Rhizomys sinensis*)	Ne	Wei et al. (2019)
Bamboo rat genotyp III	Hlodoun (*Rhizomys sinensis*)	Ne	Li et al. (2020a)
Beaver genotyp I	Bobr (*Castor canadensis*)	Ne	Feng et al. (2007)
Beaver genotyp II	Bobruška (*Aplodontia rufa*)	Ne	Li et Atwill (2021)
Beaver genotyp III	Bobruška (*Aplodontia rufa*)	Ne	Li et Atwill (2021)
Chipmunk genotyp II	Čipmank (*Tamias striatus*)	Ano	Stenger et al. (2015a)
Chipmunk genotyp III	Burunduk (*Eutamias sibiricus*)	Ano	Lv et al. (2009)
Chipmunk genotyp IV	Čipmank (*Tamias striatus*)	Ano	Stenger et al. (2015a)
Chipmunk genotyp V	Křečík (*Phodopus sungorus*)	Ne	Chen et al. (2021b)
C. ubiquitum-like	Hlodoun (*Rhizomys sinensis*)	Ne	Li et al. (2020b)
Deer mouse genotyp I	Křeček (*Peromyscus* sp.)	Ne	Xiao et al. (2002)
Deer mouse genotyp II	Křeček (*Peromyscus* sp.)	Ne	Feng et al. (2007)
Deer mouse genotyp III	Křeček (*Peromyscus* sp.)	Ne	Feng et al. (2007)
Deer mouse genotyp IV	Křeček (*Peromyscus* sp.)	Ne	Feng et al. (2007)
Deer Mouse isolate 1543 Pero NA	Křeček (*Peromyscus* sp.)	Ne	Stenger et al. (2017)
Deer Mouse 1799 Pero NA	Křeček (*Peromyscus* sp.)	Ne	Stenger et al. (2017)
Hamster genotyp	Křečík (*Phodopus sungorus*)	Ne	Lv et al. (2009)
Mouse genotyp II	Myš (*Mus domesticus*)	Ano	Foo et al. (2007)
Mouse genotyp (Naruko genotyp)	Myšice (*Apodemus speciosus*)	Ano	Murakoshi et al. (2013)

Tabulka 1. Druhy a genotypy kryptosporidií detekované u hlodavců (pokračování).

Druh/genotyp *Cryptosporidium*	**Typický hostitel (*latinský název*)**	**Synantropie**	**Citace**
Mouse genotyp (KSFM)	Myšice (*Apodemus* sp.)	Ano	Song et al. (2015)
Mouse genotyp (CR 159)	Myš (*Mus musculus domesticus*)	Ano	Garcia-Livia et al. (2020)
Mouse genotyp (CR 72)	Myš (*Mus musculus domesticus*)	Ano	Garcia-Livia et al. (2020)
Muskrat genotyp I (W7)	Ondatra (*Ondatra zibethicus*)	Ne	Xiao et al. (2002)
Muskrat genotyp II	Ondatra (*Ondatra zibethicus*)	Ne	Zhou et al. (2004)
Rat genotyp II	Krysa (*Rattus tanezumi*)	Ano	Lv et al. (2009)
Rat genotyp III	Krysa (*Rattus tanezumi*)	Ano	Lv et al. (2009)
Rat genotyp IV	Krysa (*Rattus tanezumi*)	Ano	Ng-Hublin et al. (2013a), Ng-Hublin et al. (2013b)
Rat genotyp BR8	Potkan (*Rattus norvegicus*)	Ano	Kimura et al. (2007)
Rat genotyp (CR72)	Krysa (*Rattus rattus*)	Ano	Garcia-Livia et al. (2020)
Rat genotyp (CR159)	Krysa (*Rattus rattus*)	Ano	Garcia-Livia et al. (2020)
Shrew genotyp	Rejsek (*Blarina brevicauda*)	Ne	Feng et al. (2007)
Squirrel genotyp I (Sbey03a)	Sysel (*Spermophilus beecheyi*)	Ano	Atwill et al. (2004)
Squirrel genotyp II (Sbey03b)	Sysel (*Spermophilus beecheyi*)	Ano	Atwill et al. (2004)
Squirrel genotyp III (Sbld05a)	Sysel (*Spermophilus beldingi*)	Ano	Pereira et al. (2010)
Squirrel genotyp IV (Sbld05d)	Sysel (*Spermophilus beldingi*)	Ano	Pereira et al. (2010)
Squirrel genotyp V (Sbey11e)	Sysel (*Spermophilus beecheyi*)	Ano	Li et al. (2015)
Vole genotyp	Hraboš (*Microtus pennsylvanicus*)	Ano	Feng et al. (2007)
Vole genotyp 1	Hraboš (*Microtus arvalis*)	Ano	Stenger et al. (2017), Horčičková et al. (2019)
Vole genotyp II	Hraboš (*Microtus arvalis*)	Ano	Stenger et al. (2017), Horčičková et al. (2019)
Vole genotyp III	Hraboš (*Microtus arvalis*)	Ano	Stenger et al. (2017), Horčičková et al. (2019)
Vole genotyp IV	Hraboš (*Microtus arvalis*)	Ano	Horčičková et al. (2019)
Vole genotyp V	Hraboš (*Microtus arvalis*)	Ano	Stenger et al. (2017), Horčičková et al. (2019)

Tabulka 1. Druhy a genotypy kryptosporidií detekované u hlodavců (pokračování).

Druh/genotyp *Cryptosporidium*	**Typický hostitel (*latinský název*)**	**Synantropie**	**Citace**
Vole genotyp VI	Hraboš (*Microtus arvalis*)	Ano	Stenger et al. (2017), Horčičková et al. (2019)
Vole genotyp VII	Hraboš (*Microtus arvalis*)	Ano	Horčičková et al. (2019)
Vole genotyp 1947 Mipe NA	Hraboš (*Microtus pennsylvanicus*)	Ano	Stenger et al. (2017)
Vole genotyp 1962 Mipe-NA	Hraboš (*Microtus pennsylvanicus*)	Ano	Stenger et al. (2017)
Vole genotyp 1763 Mipe NA	Hraboš (*Microtus pennsylvanicus*)	Ano	Stenger et al. (2017)
Vole genotyp Mrb001	Hraboš (*Myodes rufocanus bedfordiae*)	Ano	Nepublikováno
Vole isolate 12438 Mygl EU	Norník (*Myodes glareolus*)	Ano	Stenger et al. (2017)
Vole isolate 10482 Mygl EU	Norník (*Myodes glareolus*)	Ano	Stenger et al. (2017)
Vole isolate 2035 Myga NA	Hraboš (*Myodes gapperi*)	Ano	Stenger et al. (2017)
Qinghai vole genotyp	Hraboš (*Microtus fuscus*)	Ano	Zhang et al. (2018)
Cryptosporidium sp. Sc01	Sysel (*Spermophilus citellus*)	Ano	Ježková et al. (2023)
Cryptosporidium sp. Sc02	Sysel (*Spermophilus citellus*)	Ano	Ježková et al. (2023)
Cryptosporidium sp. Sc03	Sysel (*Spermophilus citellus*)	Ano	Ježková et al. (2023)
Cryptosporidium sp. Sc04	Sysel (*Spermophilus citellus*)	Ano	Ježková et al. (2023)

Tabulka 2. Druhy a genotypy kryptosporidií hlodavců s prokázaným zoonotickým potenciálem.

Druh/genotyp *Cryptosporidium*	**Typický hostitel**	**Citace**
C. occultus	Potkan obecný (*Rattus norvegicus*)	Ong et al. (2002), Xu et al. (2020)
C. ditrichi	Myšice lesní (*Apodemus flavicolis*)	Beser et al. (2020), Lebbad et al. (2021)
C. viatorum	Člověk a hlodavci*	Elwin et al. (2012), Wu et al. (2020), Xu et al. (2020), Sardar et al. (2021)
C. tyzzeri	Myš (*Mus musculus*	Sulaiman et al. (2005), Rašková et al. (2013), Garcia et al. (2020)
C. ubiquitum	Skot (*Bos taurus*), hlodavci, primáti	Fayer et al. (2010), Kifleyohannes et al. (2022), Alderisio et al. (2023)
C. muris	Myš (*Mus musculus*) a další hlodavci	Petrincová et al. (2015), Ayinmode et al. (2018), Guy et al. (2021)
C. mortiferum	Veverka (*Sciurus vulgaris*) a další hlodavci	Feltus et al. (2006), Insulander et al. (2013), Lebbad et al. (2013), Guo et al. (2015), Xu et al. (2019), Bujila et al. (2021), Lebbad et al. (2021), Tůmová et al. (2023)
C. parvum	Skot (*Bos taurus*)	Robertson et al. (2014)
Cryptosporidium sp. mink genotyp	Norek americký (*Mustela vison*)	Ng-Hublin et al. (2013b)

* Typický hostitel není dosud znám

3 KOMENTÁŘ K VÝSLEDKŮM

Veškeré získané výsledky (obrázky, grafy a tabulky), použitý materiál a metodiky je možné nalézt v přiložených publikacích.

3.1 Prevalence kryptosporidií synantropních hlodavců

V rámci doktorského studia bylo získáno a vyšetřeno 1256 vzorků synantropních hlodavců z 5 zemí (Česká republika, Slovensko, Filipíny, Kambodža a Thajsko). Všichni vyšetření hlodavci patří do řádu Rodentia a dále jsou diverzifikováni do tří čeledí a 12 rodů (Tabulka 3). Z toho 743 vzorků pocházelo z České republiky, 136 ze Slovenska, 63 z Filipín, 47 z Kambodži a 267 z Thajska. Počet pozitivních vzorků na kryptosporidie byl u potkanů a krys 131 (18,2 %), u veverek 26 (10 %), u syslů osm (6,3 %) a u nutrií 11 (7,3 %), Tyto výsledky odpovídají průměrné promořenosti volně žijících hlodavců pro daný rod (Deng et al. 2020, Ni et al. 2021, Garcia-Livia et al. 2022).

Tabulka 3. Počet vyšetřených synantropních hlodavců a na kryptosporidie pozitivních vzorků trusu v rámci všech provedených studií, které jsou podkladem této práce.

Čeleď	Rod	Počet vyšetřených	Počet pozitivních
Muridae	*Rattus* (588), *Apomys* (10), *Bandicota* (19), *Mus* (51), *Berylmys* (5), *Niviventer* (21), *Cannomys* (2), *Maxomys* (16), *Leopoldamys* (8)	720	131
Sciuridae	*Sciurus* (258), *Spermophilus* (128)	386	34
Myocastoridae	*Myocastor* (150)	150	11
Celkem		**1256**	**176**

Procento infikovaných synantropních hlodavců se napříč různými studiemi do značné míry liší a to od nuly až po 63 % pozitivních jedinců (Kellnerová et al. 2017, Deng et al. 2020, Garcia-Livia et al. 2020, Spencer et Irwin 2020, Garcia-Livia et al. 2022, Hancke et Suarez 2022). Značné rozdíly v prevalenci, ve srovnání našich a publikovaných dat, mohou být ovlivněny řadou faktorů. Nejčastější příčinou je stanovení prevalence z různě velkých skupin vyšetřovaných jedinců. Velké rozdíly jsou způsobeny studiemi, které jsou založené na vyšetření pouze malého množství jedinců, což celkovou prevalenci do jisté míry může značně zkreslit, obzvlášť pokud se jedná skupiny zvířat žijících v zajetí nebo z omezeného množství lokalit. Naše

i předešlé studie ukázaly, že lokalita má vliv na celkové promoření populace hostitelů (Kellnerová et al. 2017, Deng et al. 2020, Taghipour et al. 2020, Chen et al. 2021a, Lv et al. 2022). Jako příklad lze uvést distribuci kryptosporidií u syslů obecných v České republice. Kryptosporidie byly detekovány pouze na pěti z 39 vyšetřovaných lokalit (**příloha VI**).

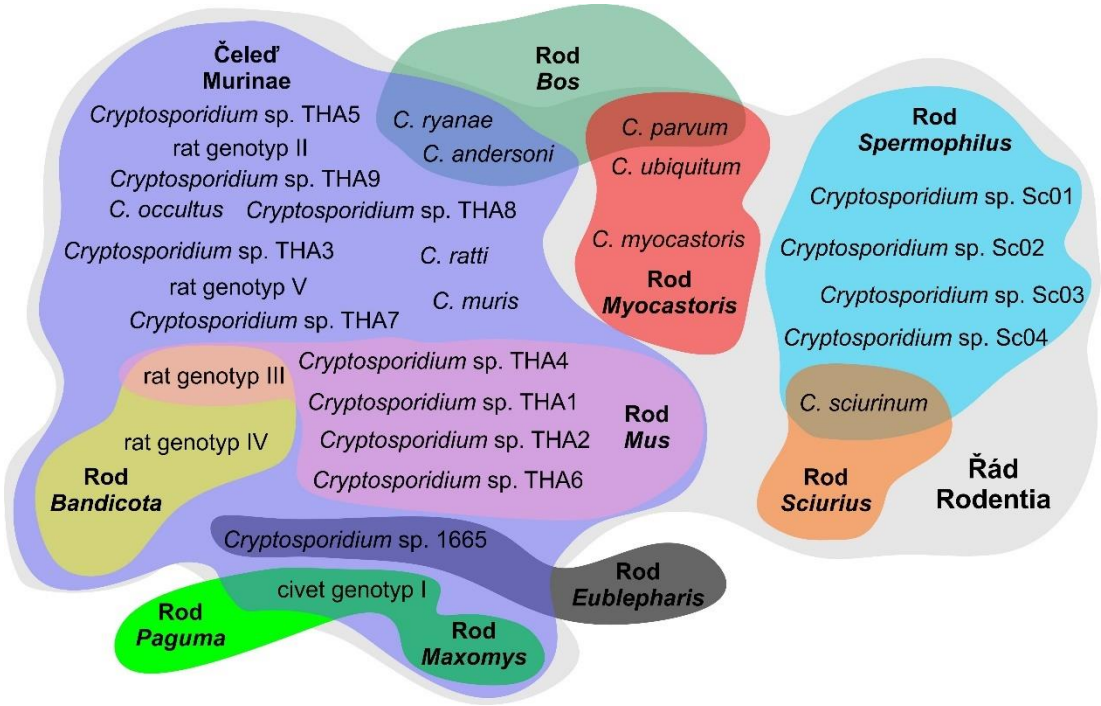
Dalšími významnými faktory, které ovlivňují prevalenci, jsou metody detekce (mikroskopická vs. molekulární detekce) a výběr studované populace. Řada prací je zaměřena pouze na zvířata s klinickými příznaky (Xiao et Feng 2008, Paparini et al. 2012, Wang et al. 2022b). Zvířata, která jsou v klinické fázi infekce, vylučují dostatečné množství oocyst, které je možné detekovat pomocí mikroskopických metod, jejichž spodní hranice senzitivity se pohybuje okolo 2000–5000 oocyst na 1 gram trusu (Hijjawi et al. 2023). Naše výsledky ukázaly, že průběh kryptosporidiové infekce u studovaných synantropních hlodavců byl chronický s velmi nízkou intenzitou infekce. Ze 176 molekulárně pozitivních zvířat vylučovalo oocysty pouze 13. Tyto výsledky zcela korespondují s dříve publikovanými nálezy u jiných skupin hlodavců, ale i dalších volně žijících obratlovců (Ježková et al. 2016, Čondlová et al. 2018, Horčičková et al. 2019, Kváč et al. 2021, Mensah et al. 2023).

3.2 Diverzita kryptosporidií synantropních hlodavců

V průběhu našich studií bylo detekováno u potkanů, krys, myší, veverek, nutrií a syslů devět druhů a 19 genotypů kryptosporidií (Tabulka 4, Obrázek 5). Výsledky našich prací potvrzují teorii o obrovské druhové rozmanitosti rodu *Cryptosporidium*. Bylo detekováno 13 nových genotypů kryptosporidií (Tabulka 4). Většina nalezených druhů a genotypů kryptosporidií byla popsána u dosud málo nebo vůbec studovaných skupin hostitelů. Devět nových genotypů (TH1–9) bylo nalezeno u hlodavců rodu *Rattus*, *Mus* a *Bandicota* v Thajsku a čtyři (Sc01–04) byly popsány ze syslů obecných v České republice. Výskyt jednotlivých druhů a genotypů kryptosporidií v závislosti na hostitelích a geografickém výskytu je uveden v tabulce 4 a schematicky znázorněn na obrázku 5. Výsledky našich prací (**přílohy I–V**) ukazují, že jak nově detekované, tak dříve publikované druhy a genotypy kryptosporidií parazitující u hlodavců se vyznačují úzkou hostitelskou specifitou (Obrázek 5). Výjimku tvoří druhy a genotypy, které jsou hostitelsky specifické

pro jiné obratlovce, jejichž přítomnost ve vyšetřovaných vzorcích lze vysvětlit kontaminací potravy a vody nebo predací přirozených hostitelů. Příkladem může být přítomnost specifické DNA *C. ryanae* a *C. andersoni* u potkanů odchycených na farmách skotu, který je přirozeným hostitelem těchto druhů kryptosporidií nebo *Cryptosporidium* sp. 1665 jehož přirozeným hostitelem jsou gekončíci, kteří se běžně vyskytují v místech odchytu studovaných hlodavců a mohli se stát kořistí asijské krysy domácí, ve které byla tato kryptosporidie detekována. Obdobné nálezy nejsou v literatuře ojedinělé. Společným znakem těchto záchytů bývá častá absence oocyst ve vyšetřovaném vzorku a nízká prevalence (Al-Abedi et al., Yimming et al. 2016, Myšková et al. 2019, Kváč et al. 2021, Guy et al. 2022).

Obrázek 5. Hostitelská specifita druhů a genotypů kryptosporidií detekovaných v rámci předložené disertační práce.



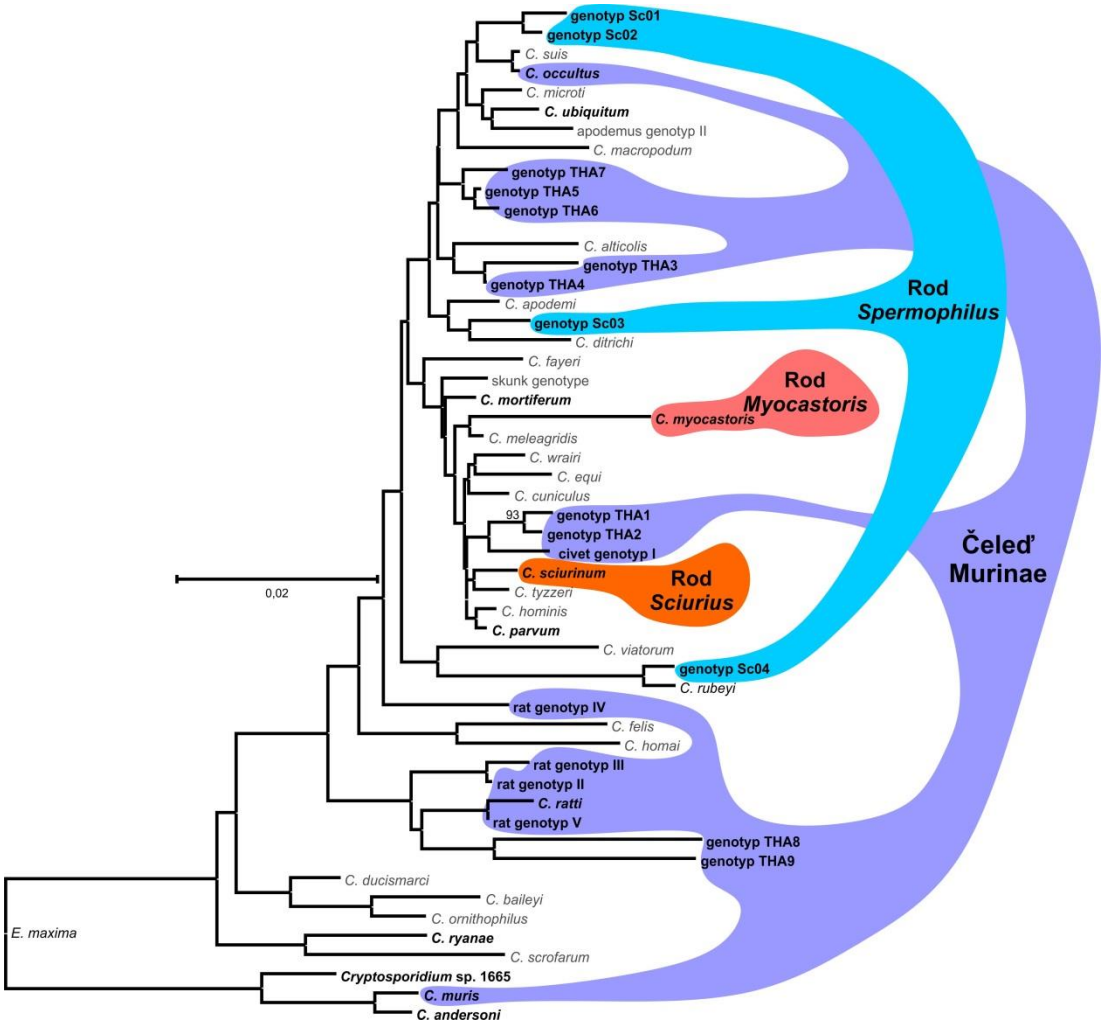
Tabulka 4. Přehled detekovaných druhů a genotypů kryptosporidií u vyšetřených synantropních hlodavců.

Hostitel (vědecké jméno)	**Země**	**Druh/genotyp (počet záchytů)**	**Publikace**
Potkan obecný (*Rattus norvegicus*)	CZ	*C. muris* (4), *C. andersoni* (3), *C. ryanae* (1), *C. occultus* (3), *C. ratti (*23), rat genotyp IV (16), rat genotyp V (5)	**Příloha II**
	TH	*C. ratti* (6), rat genotyp IV (15), *C. parvum* (1), *C. proliferans* (2), *C. occultus* (1), *C. muris* (3)	Dosud nepublikováno
Asijská krysa domácí (*Rattus tanezumi*)	TH	rat genotyp II (2), *C. occultus* (1), *C. muris* (1), *Cryptosporidium* sp. 1665 (1), *Cryptosporidium* sp. THA8 (1)	Dosud nepublikováno
Krysa ostrovní (*Rattus exulans*)	KH	*C. occultus* (1), rat genotyp III (1), rat genotyp IV (1)	Dosud nepublikováno
	TH	*Cryptosporidium* sp. THA8 (1)	Dosud nepublikováno
Krysa sikkimská (*Rattus andamanensis*)	TH	*C. occultus* (1)	Dosud nepublikováno
Krysa (*Rattus sp.*)	PH	rat genotyp II (2), rat genotyp III (1)	Dosud nepublikováno
Krysa ostnitá (*Maxomys surifer*)	TH	civet genotyp I (1)	Dosud nepublikováno
Hlodoun menší (*Cannomys badius*)	TH	*Cryptosporidium* sp. THA3 (1)	Dosud nepublikováno
Krysa kaštanová (*Niviventer fulvescens*)	TH	*Cryptosporidium* sp. THA7 (3), *Cryptosporidium* sp. THA5 (1), *Cryptosporidium* sp. THA9 (1), *C. muris* (1)	Dosud nepublikováno
Krysa bělozubá (*Berylmys bowersi*)	TH	*C. muris* (1)	Dosud nepublikováno
Bandikota indická (*Bandicota indica*)	TH	rat genotyp IV (2), rat genotyp III (1)	Dosud nepublikováno
Krysa (*Apomys sp.*)	PH	rat genotyp IV (1)	Dosud nepublikováno
Myš ryukyu (*Mus caroli*)	TH	*Cryptosporidium* sp. THA4 (4)	Dosud nepublikováno
Myš plavohnědá (*Mus cervicolor*)	TH	*Cryptosporidium* sp. THA1 (11), *Cryptosporidium* sp. THA4 (3), *Cryptosporidium* sp. THA2 (1), *Cryptosporidium* sp. THA6 (1), rat genotyp III (1)	Dosud nepublikováno
Nutrie říční (*Myocastor coypus*)	CZ	*C. parvum* (1), *C. myocastoris* (2)	**Příloha III**
	SK	*C. ubiquitum* (5), *C. myocastoris* (3)	**Příloha III**
Veverka obecná (*Sciurus vulgaris*)	CZ	*C. sciurinum* (20)	**Příloha IV**
	SK	*C. sciurinum* (6)	**Příloha IV**
Sysel obecný (*Spermophilus citellus*)	CZ	*C. sciurinum* (1), *Cryptosporidium* sp. Sc01 (2), *Cryptosporidium* sp. Sc02 (2), *Cryptosporidium* sp. Sc03 (2), *Cryptosporidium* sp. Sc04 (2)	**Příloha VI**

CZ: Česká republika; **KH:** Kambodža; **PH:** Filipíny; **SK:** Slovensko; **TH:** Thajsko

Jednotlivé genotypy popsané v této práci (**příloha VI**, nepublikováno) se od sebe fylogeneticky výrazně odlišují a na základě dosavadních znalostí se domníváme, že představují samostatné druhy (Obrázek 6).

Obrázek 6. Fylogenetické vztahy mezi druhy a genotypy *Cryptosporidium* spp. získanými v této práci (zvýrazněno tučně) na základě analýzy maximální věrohodnosti (ML) odvozených z konkatenovaných sekvencí vytvořených z částečné DNA sekvencí lokusů kódujících malou podjednotku rRNA a aktinu. Hostitelská specifita jednotlivých izolátů je barevně znázorněna.



V souladu s předešlými studiemi provedenými na savcích a ptácích jsme prokázali vysokou diverzitu kryptosporidií u synantropních hlodavců a geografickou distribuci některých druhů/genotypů kryptosporidií (Kimura et al. 2007, Lv et al. 2009, Feng et al. 2011, Stenger et al. 2017, Horčičková et al. 2019, Wei et al. 2019, Li et Atwill

2021, Liao et al. 2021). Studie (**přílohy II, VI,** dosud nepublikováno) zaměřené na hlodavce z čeledi myšovití a rodu *Spermophilus* ukázaly, že tito hlodavci jsou hostitelé nejméně 17 druhově-specifických kryptosporidií (Obrázek 5, **příloha II, VI** a dosud nepublikováno), z nichž některé se vyskytovaly napříč studovanými lokalitami (Tabulka 4), zatímco jiné byly detekovány pouze u hostitelů v rámci jedné geografické lokality. Nově popsané genotypy *Cryptosporidium* sp. THA1–THA9 byly popsány výhradně u hlodavců v Thajsku. *Cryptosporidium* sp. genotyp rat II a III byly dosud popsány u potkanů a krys v Asii, severní Austrálii, na Korsice a Kanárských ostrovech. V našem případě byl *Cryptosporidium* sp. genotyp rat III nalezen u bandikoty indické (Thajsko). V ostatních částech světa, kde byly obdobné studie prováděny, včetně naší práce, nebyly tyto genotypy detekovány (Paparini et al. 2012, Garcia-Livia et al. 2022). Obdobně bylo zjištěno, že syslové rodu *Spermophilus* v České republice jsou parazitováni odlišnými genotypy kryptosporidií než jejich příbuzní obývající Severní Ameriku (**příloha VI**). Takováto geografická diverzita v rámci rodu *Cryptosporidium* není neobvyklá a byla již u volně žijících hostitelů v minulosti popsána (Kváč et al. 2013b, Stenger et al. 2015b, Stenger et al. 2017).

3.3 Biologická charakterizace kryptosporidií synantropních hlodavců

3.3.1 Morfologie a morfometrie oocyst kryptosporidií synantropních hlodavců popsaných v rámci disertační práce

Morfologie a morfometrie je jednou ze základních podmínek popisu nového druhu. Nicméně vzhledem k malé variabilitě nelze tuto biologickou charakteristiku považovat za spolehlivý diagnostický znak pro identifikaci druhu či genotypu kryptosporidií (Tabulka 5, Obrázek 7). Za spolehlivé druhové určení je tedy považována molekulární charakterizace vybraných genů.

V souladu s předešlými studiemi jsme prokázali, že velikost a tvar oocyst jednotlivých druhů kryptosporidií je vlastností druhu bez vlivu hostitele (Ryan et al. 2003, Ryan et al. 2004, Holubová et al. 2016).

Tabulka 5. Velikost oocyst druhů kryptosporidií popsaných v rámci této práce.

Druh	Velikost oocyst (µm)			Hostitel	Citace
	Délka		Šířka		
C. occultus	5,20 (4,66–5,53)	×	4,94 (4,47–5,44)	Potkan obecný	Kváč et al. (2018)
C. ratti	4,90 (4,40–5,40)	×	4,60 (4,30–5,10)	Potkan obecný	Ježková et al. (2021b)
C. myocastoris	5,02 (4,80–5,2)	×	4,85 (4,70–5,00)	Nutrie říční	Ježková et al. (2021a)
C. sciurinum	5,54 (5,12–6,00)	×	5,22 (4,77–5,66)	Veverka obecná	Prediger et al. (2021)
C. mortiferum	5,64 (5,50–5,89)	×	5,37 (4,86–5,60)	Čipmank východní	Tůmová et al. (2023)

Cryptosporidium occultus

Oocysty *C. occultus* pocházející z přirozeně infikovaných potkanů (*Rattus norvegicus*) byly morfometricky shodné s oocystami, které byly získány z experimentálně infikovaných potkanů a které měřily 4,66–5,33 µm (průměr = 5,20 µm) × 4,47–5,44 µm (průměr = 4,94 µm) a indexu tvaru 1,05 (1,00–1,17). Oocysty *C. occultus* nelze morfometricky odlišit od oocyst dalších hlodavčích druhů a genotypů kryptosporidií jako například *C. alticolis* a *C. ditrichi* (Čondlová et al. 2018, Horčičková et al. 2019). Podrobný popis a fotodokumentace oocyst *C. occultus* je k dispozici v **příloze I**.

Cryptosporidium ratti

Oocysty *C. ratti* z přirozeně infikovaných potkanů (*Rattus norvegicus*) měřily 4,4–5,4 µm (průměr ± SD = 4,9 ± 0,2 µm) × 4,3–5,1 µm (průměr ± SD = 4,6 ± 0,2 µm) s poměrem délky k šířce 1,0–1,1 (průměr ± SD = 1,1 ± 0,1) a shodovaly se velikostí s oocystami získanými z experimentálně infikovaných potkanů. Oocysty *C. ratti* jsou menší než oocysty *C. parvum* nebo *C. occultus* (Ježková et al. 2021b). Podrobný popis a fotodokumentace oocyst *C. ratti* je k dispozici v **příloze II**.

Cryptosporidium myocastoris

Oocysty *C. myocastoris* pocházející z přirozeně infikovaných nutrií (*Myocastor coypus*) byly morfometricky shodné s oocystami, které byly získány

z experimentálně infikovaných nutrií a které měřily 4,8–5,2 μm (průměr = 5,02 μm) × 4,7–5,0 μm (průměr = 4,85 μm) a indexu tvaru 1,04 (1,00–1,08). Oocysty *C. myocastoris* jsou menší než oocysty *C. parvum* a *C. ratti*, nicméně tyto rozdíly nemají praktický význam pro identifikaci (Ježková et al. 2021a). Podrobný popis a fotodokumentace oocyst *C. occultus* je k dispozici v **příloze III**.

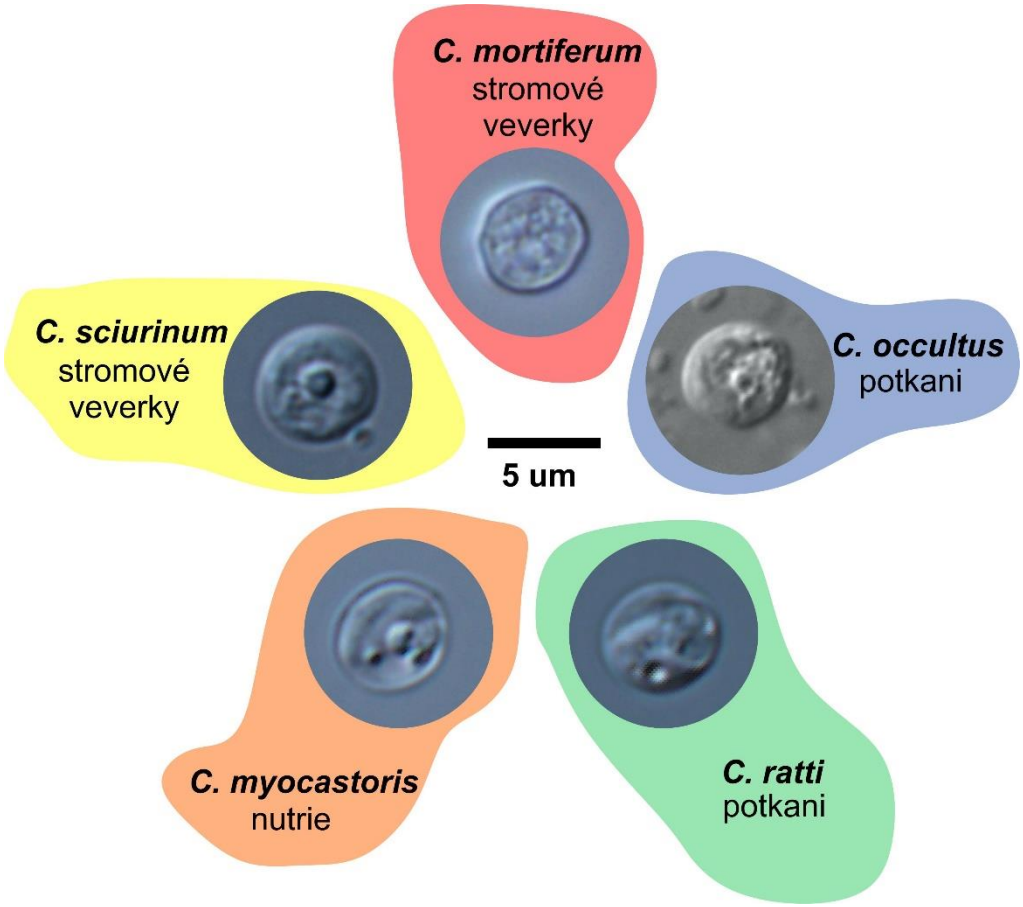
Cryptosporidium sciurinum

Ooocysty *C. sciurinum* získané z přirozeně infikované veverky (*Sciurus vulgaris*) (izolát 45901) měřily 5,12–6,00 μm (průměr ± SD = 5,54 ± 0,20 μm) × 4,77–5,66 μm (průměr ± SD = 5,22 ± 0,18 μm) s poměrem délky k šířce 1,00–1,26 (průměr ± SD = 1,07 ± 0,05). Podrobný popis a fotodokumentace oocyst *C. sciurinum* je k dispozici v **příloze IV**.

Cryptosporidium mortiferum

Oocysty *C. mortiferum* byly získány z přirozeně infikovaného člověka (*Homo sapiens*) a měřily 5,50–5,89 μm (průměr ± SD = 5,64 ± 0,19 μm) × 4,86–5,60 μm (průměr ± SD = 5,37 ± 0,17 μm) s poměrem délky k šířce 1,01–1,14 (průměr ± SD = 1,05 ± 0,05). Velikost oocyst *C. mortiferum* se nelišila od oocyst vylučovaných experimentálně infikovanými hostiteli (*Sciurus vulgaris*, *Sciurus carolinensis*, *Mus musculus* a *Mustela putorius furo*). Podrobný popis a fotodokumentace oocyst *C. mortiferum* je k dispozici v **příloze V**.

Obrázek 7. Tvar a morfologie *Cryptosporidium occultus*, *Cryptosporidium ratti*, *Cryptosporidium myocastoris*, *Cryptosporidium sciurinum* a *Cryptosporidium mortiferum*. Typický hostitel je uveden pod druhem kryptosporidie (**přílohy I–V**).

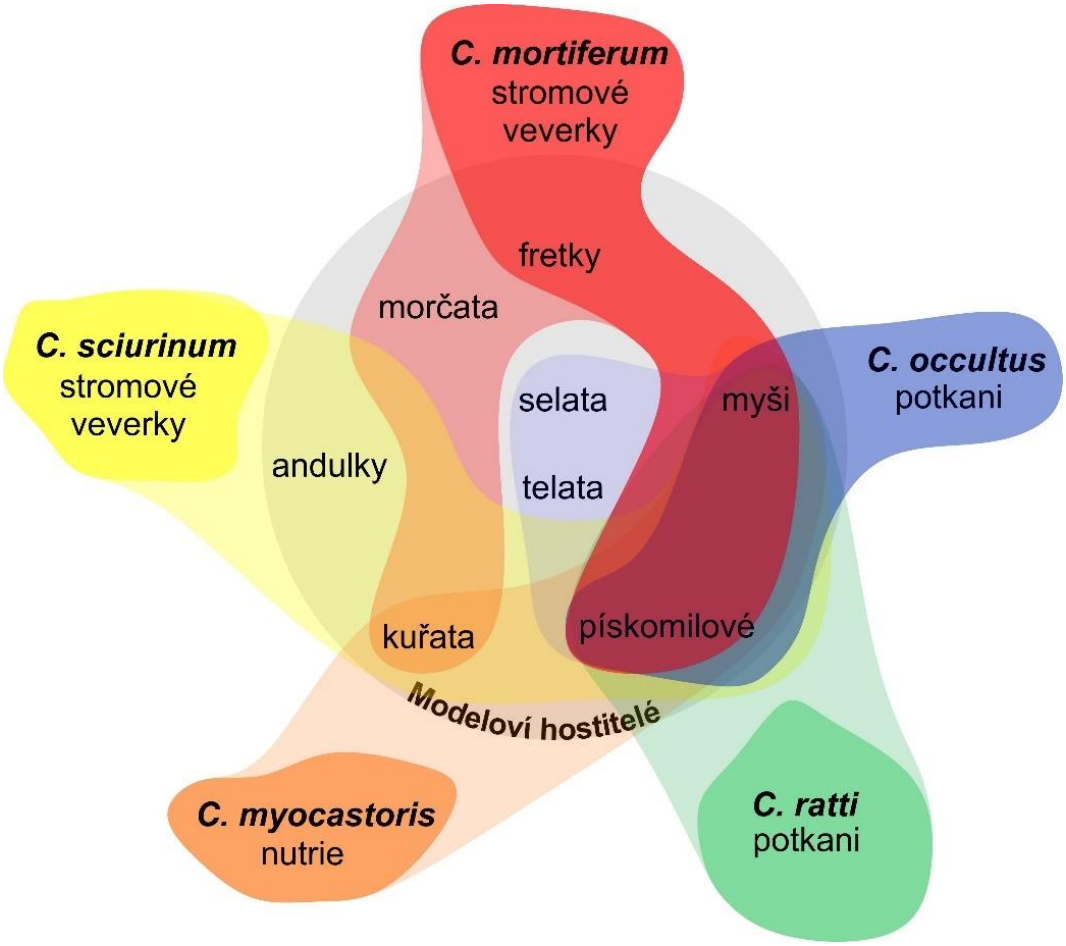


3.3.2 Hostitelská specifita kryptosporidií synantropních hlodavců

Na základě provedených experimentálních infekcí, byla prokazována hostitelská a tkáňová specifita. Jedná se o důležité biologické vlastnosti jednotlivých druhů a genotypů kryptosporidií. Experimentální infekce byly provedeny pro pět nově popsaných druhů kryptosporidií. Jednotlivé druhy byly testovány vždy na původním hostiteli vyjma druhu *C. sciurinum*, u kterého nebylo z logistických důvodů možné provést experimenty s tímto izolátem na veverkách obecných. Dále byla hostitelská specifita testována na modelových laboratorních, hospodářských a volně žijících zvířatech (myši, potkani, pískomilové, morčata, fretky, veverky, telata, selata, kuřata, andulky). Podrobnosti k jednotlivým experimentům jsou uvedeny v příslušných publikacích (**přílohy I–V**). Druh *C. occultus* byl infekční pro potkany, myši

a pískomily (**příloha I**). Druhy *C. ratti* a *C. myocastoris* se zdají být úzce hostitelsky specifické a byly infekční pouze pro potkany, respektive pro nutrie (**příloha II a III**). Obdobně druh *C. sciurinum* specifický pro veverky obecné nebyl infekční pro žádného jiného modelového hostitele (**příloha IV**). U druhu *C. mortiferum* byla prokázána nejširší hostitelská specifita, byl infekční pro myši, fretky, pískomily, veverky obecné a veverky popelavé (**příloha V**). Hostitelská specifita je znázorněna na obrázku 8.

Obrázek 8. Hostitelská specifita *Cryptosporidium occultus*, *Cryptosporidium ratti*, *Cryptosporidium myocastoris*, *Cryptosporidium sciurinum* a *Cryptosporidium mortiferum*. Typický hostitel je uveden pod druhem kryptosporidie. Tmavá barva přidělená druhu kryptosporidie znázorňuje vnímavé hostitele a světlá barva hostitele nevnímavé (**přílohy I–V**).



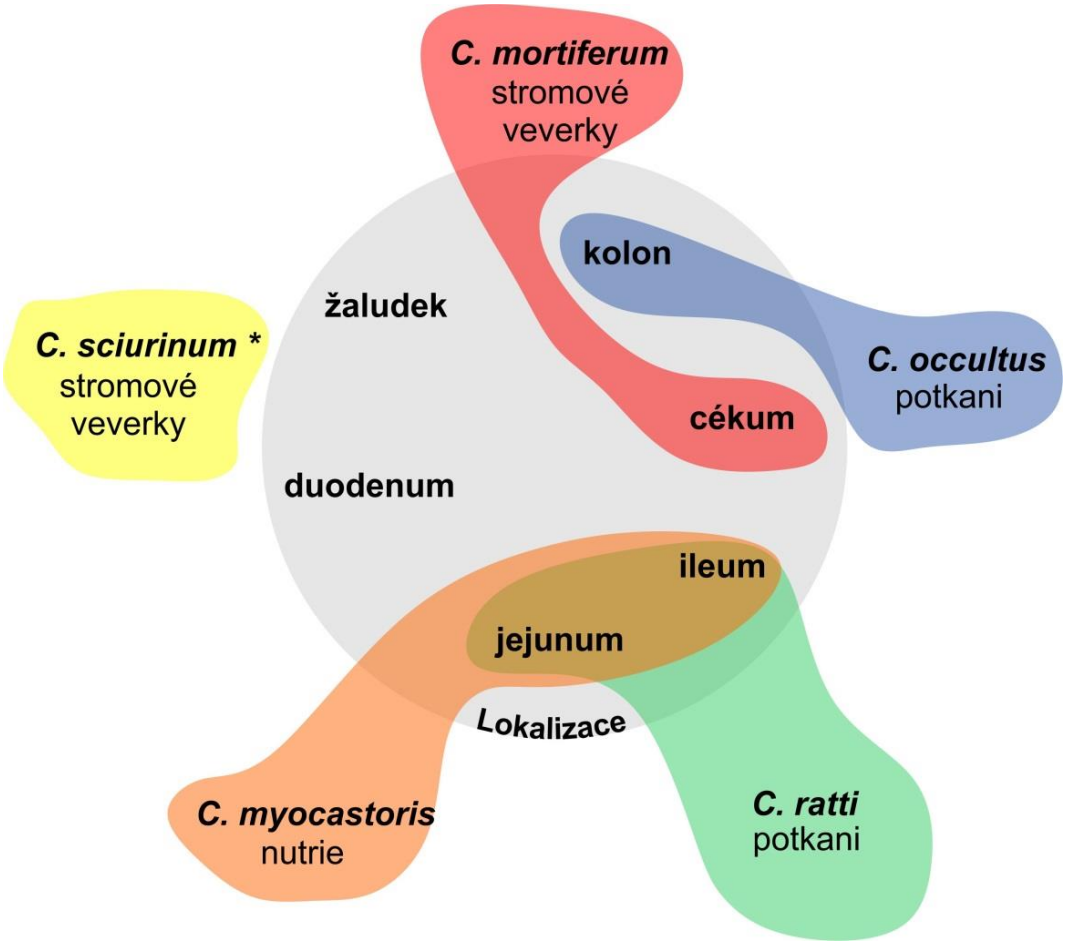
3.3.3 Tkáňová specifita kryptosporidií synantropních hlodavců

Detekce predilekčního místa infekce v gastrointestinálním traktu hostitelů, může napomoci při odlišení jednotlivých druhů kryptosporidií (Holubová et al. 2020, Ježková et al. 2021a). Nicméně je tento přístup značně náročný, zahrnuje podrobné zkoumání každého hostitele z hlediska infekce jednotlivých tkání. Z tohoto důvodu se jedná o časově i finančně náročnou studii zahrnující různé typy barvení, histologické zpracování jednotlivých tkání a případně i elektronovou mikroskopii. Při běžné diagnostice tyto metody nenachází uplatnění, nicméně pro popis nových druhů je tento postup žádoucí (Kváč et al. 2014a, Ježková et al. 2021b).

Řadou studií bylo prokázáno, že jednotlivé druhy, případně genotypy kryptosporidií jsou obvykle charakterizovány úzkou tkáňovou specifitou. Příslušný druh kryptosporidií se poutá k jednomu danému predilekčnímu místu infekce (Lindsay et al. 2000, Ryan et al. 2008, Kváč et al. 2018, Holubová et al. 2020). Lokalizace infekce společně s morfologickou charakterizací oocyst může napomoci při determinaci druhu či genotypu. Nicméně bylo již prokázáno několik případů, kdy se lokalizace a morfologie oocyst odlišných druhů může překrývat. Takovým příkladem mohou být druhy *C. alticolis* a *C. ditrichi* vyskytující se u myšic v jejunu a ileu (Čondlová et al. 2018, Horčičková et al. 2019). Obdobně se v tenkém střevě prasat vyskytují druhy *C. parvum* a *C. scrofarum* (Kváč et al. 2013a, Li et al. 2013). V tlustém střevě potkanů lze nalézt *C. occultus*, ale příležitostně může být osídleno i druhem *C. meleagridis* (Kimura et al. 2007, Kváč et al. 2018).

Na základě provedených experimentů byla prokázána tkáňová specifita u námi popsaných druhů. *C. oocultus*, jak již bylo zmíněno, se vyskytuje v tlustém střevě potkanů. *C. ratti* bylo detekováno u potkanů v jejunu a ileu. Predilekčním místem *C. myocastoris* parazitujícím u nutrií je posteriorní část jejuna a ileum. Lokalizace infekce *C. sciurinun* nebyla v naší studii prokázána a *C. mortiferum*, parazitující u veverek, bylo detekováno ve slepém a tlustém střevě (Obrázek 9).

Obrázek 9. Tkáňová specifita *Cryptosporidium occultus*, *Cryptosporidium ratti*, *Cryptosporidium myocastoris* a *Cryptosporidium mortiferum*. Typický hostitel je uveden pod druhem kryptosporidie. * Tkáňová specifita *Cryptosporidium sciurinum* nebyla zjištěna (**přílohy I–V**).



Pro průkaz hostitelské a tkáňové specifity je nezbytná infekční dávka životaschopnými oocystami. Při nesplnění této podmínky může být celý experiment negativně ovlivněn. Dále je třeba sledovat imunitní stav a věk hostitele, které v experimentech mohou sehrát významnou roli (Fayer et al. 2007, Kváč et al. 2013a, Holubová et al. 2016). Vhodné pro experimentální infekce je používat čerstvé, maximálně dva měsíce staré oocysty, které byly skladovány při chladničkové teplotě 4–8 °C (Kváč et al. 2018, Horčičková et al. 2019, Ježková et al. 2021a). U imunosuprimovaných a imunodeficitních jedinců, kteří jsou více vnímaví k infekci kryptosporidiemi, může být výrazně prodloužena prepatentní perioda (Kváč et al. 2011). Je nezbytné vzít tento fakt v potaz, aby nebyly

experimenty předčasně ukončeny (Kváč et al. 2008). Experimentální zvířata, která se již v minulosti setkala s kryptosporidiemi mohou být k opakované infekci méně vnímavá, případně zcela rezistentní (Jalovecká et al. 2010). Tuto problematiku je třeba vzít v potaz při využití volně žijících, případně konvenčně chovaných zvířat pro experimentální infekce.

3.3.4 Patogenita kryptosporidií synantropních hlodavců

Kryptosporidiové infekce jsou obecně spojovány se závažným klinickým onemocněním, kryptosporidiózou, s typickými průjmovými epizodami, které mohou mít fatální konec (Matsuura et al. 2017, Bartley et al. 2023, Ootawa et al. 2023). Nicméně ne všechny druhy kryptosporidií vyvolávají klinické onemocnění. Lze dokonce říci, že infekce způsobené většinou druhů a genotypů kryptosporidií probíhají asymptomaticky (**přílohy I–V**) (Ježková et al. 2016, Holubová et al. 2019, Horčičková et al. 2019, Holubová et al. 2020).

V rámci této práce byla popsána patogenita u všech námi studovaný/popsaných druhů. V případě druhů *C. occultus*, *C. ratti*, *C. myocastoris* a *C. sciurinum* nebyly pozorovány žádné klinické příznaky infekce u žádného z infikovaných hostitelů (**přílohy I–IV**). Histologické analýzy a vyšetření pomocí skenovací elektronové mikroskopie neprokázalo výrazné patologické změny u žádného ze studovaných hostitelů. Jediným společným prvkem všech infekcí bylo prodlužování klků mikrovilární vrstvy infikovaných epiteliálních buněk (**přílohy I–IV**). Pouze u druhu *C. mortiferum* byla pozorována kryptosporidióza s vysokou (fatální) virulencí pro veverky obecné (**příloha V**). Zatímco u veverek popelavých, které jsou přirozenými hostiteli *C. mortiferum,* byla pozorována v průběhu infekce jen mírná apatie, projevující se sníženým zájmem o okolí a potravu a se změnou konzistence trusu z pevné na pastovitou, infekce stejným druhem kryptosporidie způsobila u veverek obecných ztrátu kondice, zájem o potravu s nástupem průjmového onemocnění s fatálními následky. Veverky trávily většinu dne v úkrytu, byly letargické a nereagovaly na vnější stimuly jako potravu, vodu, manipulaci a čištění klece. Při výskytu závažných klinických příznaků byly humánně usmrceny (**příloha V**). Výsledky této práce ukazují, že stejný izolát kryptosporidie může mít různou patogenitu a virulenci pro různé druhy hostitelů.

3.3.5 Molekulární detekce a diferenciace kryptosporidií

Vzhledem k tomu, že dle morfologie oocyst nelze přesně určit o jaký druh kryptosporidií se jedná, k rozlišení jednotlivých druhů se obvykle využívá molekulárních metod. Citlivost těchto metod se pohybuje mezi 1 až 10 oocystami ve vyšetřovaném vzorku (Smith et al. 2006, Thompson et Ash 2016). Pro genotypizaci jsou používány rodově specifické primery amplifikující různé geny, nejčastěji gen kódující malou podjednotku rRNA (Sulaiman et al. 1999). Nicméně vyhodnocování evolučních vztahů pouze na základě rRNA může vést k chybným závěrům (Li et al. 2014b, Stenger et al. 2015a, Ježková et al. 2021a). Z tohoto důvodu bývají využívány i další lokusy jako gen kódující heat shock protein 70 (HSP70), aktin, *Cryptosporidium* oocyst wall protein (COWP), 60 kDa glykoprotein (gp60) a thrombospondin-related adhesive protein (TRAP–C1) (Morgan-Ryan et al. 2001, Tang et al. 2016). Při studiu vnitrodruhové variability, nebo v případě smíšených infekcí, je nezbyné opakované sekvenování PCR produktů, dále klonování PCR produktů s následným sekvenováním, případně sekvenování nové generace (Grinberg et al. 2013, Paparini et al. 2015, Stenger et al. 2015a).

V současné době masivně používané Sangerovo sekvenování přináší zásadní nevýhodu, kterou je nízná pravděpodobnost detekce smíšených infekcí. Z principu metody vyplývá, že ve vzorku je přednostně amplifikována DNA druhu, který je v hostiteli dominantně zastoupen (Grinberg et al. 2013). Výsledky našich i předchozích studií jednoznačně potvrzují, že většina obratlovců, synantropní hlodavce nevyjímaje, je parazitována celou řadou druhů a genotypů kryptosporidií (Wang et al. 2021, Hancke et Suarez 2022, Lu et al. 2022, Feng et al. 2023, Chen et al. 2023a). Jen u syslů sledovaných v rámci této práce byly detekovány čtyři nové genotypy kryptosporidií (**příloha VI**).

Při zpracovní práce jsme si byli vědomi limitů používaných molekulárních metod a proto u všech vyšetřovaných vzorků byla provedena genotypize rDNA lokusu v kombinaci s dalšími výše uvedenými geny. Pro popis nového druhu byly sekvenovány nejméně tři různé geny. Konkrétní popis jednotlivých metodik je podrobně popsán v přiložených publikacích (**přílohy I–VI**).

3.4 Synantropní hlodavci jako potenciální zdroj zoonotických kryptosporidií a zdroj infekce pro hospodářská zvířata

Přestože jsou synantropní hlodavci parazitováni převážně hostitelsky specifickými genotypy, mohou být hostiteli celé řady dalších, často zoonotických druhů kryptosporidií (Kimura et al. 2007, Bahrami et al. 2012, Ng-Hublin et al. 2013a). Vzhledem k tomu, že oocysty kryptosporidií si i po průchodu zažívacím traktem nevnímavého hostitele mohou zachovávat svou infektivitu (Graczyk et al. 1996), mohou představovat synantropní hlodavci s ohledem na jejich celosvětové rozšíření a způsob života ideální pasivní přenašeče pro šíření kryptosporidií (Zhao et al. 2018).

Z našich a dříve publikovaných dat lze vyvodit závěr, že přestože synantropní hlodavci sdílí prostředí s lidmi a jimi chovanými zvířaty, nedochází k zásadnímu přenosu kryptosporidií ve směru hlodavec→hospodářské zvíře, hospodářské zvíře→hlodavec, člověk→hlodavec (Kilonzo et al. 2013, Li et al. 2020a).

Z pohledu zoonotického přenosu kryptosporidií představují synantropní hlodavci relativně malé riziko a záleží vždy na konkrétním druhu a genotypu kryptosporidie. Z kryptosporidií specifických pro hlodavce přestavují potenciální zoonotické riziko druhy *C. occultus*, *C. muris*, *C. ditrichi*, *C. tyzzeri*, *C. viatorum* a *C. mortiferum*. Nicméně s ohledem na dosud zjištěný zanedbatelný podíl lidských infekcí způsobený druhy *C. occultus*, *C. muris*, *C. ditrichi*, *C. tyzzeri* je riziko přenosu zanedbatelné. U druhů *C. parvum* a *C. ubiquitum*, které jsou v rámci lidské populace zodpovědné za vysoké procento nákaz, jsou u synantropních hlodavců detekovány ojediněle a lze předpokládat, že studovaní synantropní hlodavci hrají minoritní roli v přenosu těchto druhů kryptosporidií. Poslední a současně rizikovou skupinu pro člověka tvoří druhy *C. viatorum* a *C. mortiferum*. Podíl lidských infekcí způsobených těmito dvěma druhy postupně vzrůstá. Zejména v případě druhu *C. mortiferum* dochází rychlému nárůstu počtu lidských infekcí. Příčinu lze hledat právě v kontaktu člověka s volně žijícími druhy adaptujícími se na synantropní způsob života (Guo et al. 2015, Wu et al. 2020, Xu et al. 2020, Bujila et al. 2021, Sardar et al. 2021, Alderisio et al. 2023).

4 ZÁVĚRY

- Byla prokázána obrovská druhová rozmanitost kryptosporidií infikujících studované synantropní hlodavce. Na základě multilokusové genotypizace bylo detekováno 9 druhů a 19 genotypů *Cryptosporidium* spp.
- Bylo popsáno pět nových genotypů rodu *Cryptosporidium*.
- Na základě studia genetických a biologických odlišností bylo ustanoveno pět nových druhů rodu *Cryptosporidium*, jmenovitě *C. occultus*, *C. ratti*, *C. myocastoris*, *C. sciurinum* a *C. mortiferum*.
- Rozšířili jsme znalosti o tkáňové specifitě kryptosporidií.
- Infekce způsobené většinou detekovaných druhů a genotypů kryptosporidií synantropních hlodavců nejsou provázeny klinickými příznaky.
- Velikost oocysty nelze použít jako diferenciální znak.
- Intenzita kryptosporidiových infekcí je u synantropních hlodavců většinou nízká, pod limitem mikroskopických metod.
- Experimentálně byla prokázána 100% letalita *C. mortiferum* pro veverky obecné, zatímco u veverek šedých byly pozorovány jen mírné klinické příznaky onemocnění.
- Syslové obecní (ČR) nesdílejí identické *Cryptosporidium* spp. se sysly obývajícími území Severní Ameriky.
- Kryptosporidie detekované u synantropních hlodavců nepředstavují významné riziko pro člověka a jím chovaná domácí a hospodářská zvířata.

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6 PŘÍLOHY

6.1 Příloha I

***Cryptosporidium occultus* sp. n. (Apicomplexa: Cryptosporidiidae) in rats.**

Kváč M., Vlnatá G., **Ježková J.**, Horčičková M., Konečný R., Hlásková L., McEvoy J., Sak B. 2018: European Journal of Protistology 63: 96–104.

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Cryptosporidium occultus sp. n. (Apicomplexa: Cryptosporidiidae) in rats

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Abstract

Cryptosporidium parvum VF383 has been reported in humans, domesticated ruminants, and wild rats worldwide and described under several names including *Cryptosporidium suis*-like, based on its close phylogenetic relationship to *C. suis*. Unlike *C. suis*, however, it has never been detected in pigs. In the present work, *C. parvum* VF383 originating from wild brown rats was not infectious for piglets or calves but was infectious for laboratory brown rats, BALB/c mice, and Mongolian gerbils. The prepatent period was 4–5 days for all rodents. The patent period was longer for rats (>30 days) than other rodents (<20 days). None of the rodents developed clinical signs of infection. In all rodents, life cycle stages were detected in the colon by histology and electron microscopy. Oocysts were morphometrically similar to those of *C. parvum* and smaller than those of *C. suis*, measuring 5.20 × 4.94 μm. Phylogenetic analyses of 18S rRNA, actin, and HSP70 gene sequences revealed *C. parvum* VF383 to be genetically distinct from, *C. suis*, and other described species of *Cryptosporidium*. Morphological, genetic, and biological data support the establishment of *C. parvum* VF383 as a new species, and we propose the name *Cryptosporidium occultus* sp. n.
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Keywords: Histology; Molecular phylogeny; Morphometry; New species; Transmission studies

Introduction

Protist parasites belonging to the genus *Cryptosporidium* primarily infect the gastrointestinal tract of their vertebrate hosts (Fayer 2010). There are currently about 35 valid species of *Cryptosporidium* in fish, amphibians, reptiles, birds, and mammals, most of which have been described from morphological, biological, and molecular data. Many more *Cryptosporidium* genotypes, which lack the biological and morphological data necessary for species designation, have been reported in vertebrates and the environment (Fayer 2010). In 2002, Ong et al. (2002) used a partial sequence of the small ribosomal subunit rRNA (18S rRNA) gene to identify a novel *Cryptosporidium* sp. in humans, and they named the genotype *C. parvum* VF383. In 2007, isolate K4515, which had an identical 18S rRNA sequence to *C. parvum* VF383, was reported from the faeces of cattle (*Bos taurus*) in Denmark. This isolate was named *C. suis*-like to reflect its close relationship to *Cryptosporidium suis* (Langkjær et al. 2007). Since 2010, genotypes identical to *C. parvum* VF383 have been reported from cattle, water buffalo (*Bubalus bubalis*), domestic yaks (*Bos grunniens*), humans (*Homo sapiens*), and wild brown rats (*Rattus tanezumi*) worldwide and published under different names including *C. suis*-like, *Cryptosporidium* sp. RTA368, *Cryptosporidium* sp. W20486 and *Cryptosporidium* sp. AQ7 (see

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Table 1. Identification of *Cryptosporidium occultus* sp. n., which has been described under different names in mammalian hosts worldwide. Identification was based on partial sequences of 18S rRNA, actin or HSP70 genes.

Country	Host (scientific name)	No. of positive	Age (year)	Sequences (GenBank association number; *100% sequence identity within locus)			Description (manuscript†, GenBank§)	Reference (#year of publishing in GenBank)
				18S rDNA	actin	HSP70		
Denmark	Cattle (*Bos taurus*)	3	0.3–1	DQ182599*	NA	DQ182598*	*C. suis*-like†, *Cryptosporidium* sp. K4515§	(Langkjær et al. 2007)
India		1	0.3–1	GQ345008*	NA	NA	*C. suis*-like†, *Cryptosporidium* cf. *suis*§	(Khan et al. 2010)
England		NA	NA	HQ822134*	NA	NA	*Cryptosporidium* sp. P156§	Unpublished (2012)#
Australia		9	<1	KC778530*	NA	NA	*Cryptosporidium* sp. genotype RTA368†,§	(Abeywardena et al. 2013)
China		2	43,132	KM110047*	NA	NA	*C. suis*-like†, *Cryptosporidium* cf. *suis*§	(Ma et al. 2015)
China	Yak (*Bos gunniens*)	2	>2	KU052809*	NA	NA	*C. suis*-like†, *Cryptosporidium* cf. *suis*§	(Li et al. 2016)
Brazil	Water buffalo (*Bubalus bubalis*)	NA	NA	JX559850*	JX559851*	NA	*Cryptosporidium* sp. AQ7§	Unpublished (2012)#
Philippines	Tanezumi rat (*Rattus tanezumi*)	14	NA	JX485388*	JX485409*	NA	*C. suis*-like†, *C. suis*§	(Ng-Hublin et al. 2013)
				JX485390	JX485412 JX485417 JX485418			
England	Human (*Homo sapiens*)	NA	NA	HQ822146*	HQ822148*	HQ822147*	*Cryptosporidium* sp. W20486§	Unpublished (2012)#
Canada		NA	NA	AY030084*	NA	NA	*C. parvum* VF383†,§	(Ong et al. 2002)

NA, not available.
*indicates 100% sequence identity within locus; †indicates the name of the isolate described in the manuscript; §indicates the name of the isolate reported in the GenBank; #indicates publication year of the sequence in the GenBank.

Table 1 for details). Respecting the principle of priority, we will hereafter refer to the genotype as *C. parvum* VF383. Biological properties of *C. parvum* VF383 are not yet known, but we hypothesized that its major host would be one of the previously reported hosts. In accordance with established rules for describing new species of the genus *Cryptosporidium* (Fayer 2010; Jirků et al. 2008; Xiao et al. 2004a) and ICZN nomenclature rules, we described the morphology of oocysts, natural and experimental infectivity, and multi-locus sequence data for *C. parvum* VF383. Outcomes from the study lead us to conclude that *C. parvum* VF383 is genetically and biologically distinct from previously described *Cryptosporidium* species and we therefore propose that it be named a new species, *Cryptosporidium occultus*.

Material and Methods

Specimens studied

Isolates of *C. parvum* VF383 were obtained from four wild brown rats (*Rattus norvegicus*) trapped at four locations in the Czech Republic. Locations were 60–100 km apart. Oocysts of *C. parvum* VF383 from these rats were pooled and used to infect a single 8-week-old laboratory rat (rat 0). Oocysts from rat 0 were purified using cesium chloride gradient centrifugation (Arrowood and Donaldson 1996) and used to infect other animals (see below). Faecal samples were examined for the presence of oocysts and specific DNA following aniline-carbol-methyl violet (ACMV) staining (Miláček and Vítovec 1985) and PCR (see below), respectively.

Molecular analysis

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 (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 region of the 18S rRNA (~830 bp; Jiang et al. 2005; Xiao et al. 1999), actin (~1066 bp; Sulaiman et al. 2002) and heat shock protein 70 genes (HSP70; ~1950 bp; Sulaiman et al. 2000). The primary PCR mixture contained 2 μl of template DNA, 10 μl of 2 × AmpONETM HS-Tag premix (GeneAll Biotechnology Co., Ltd.), 200 nM each primer and molecular grade water up to a volume of 20 μl. The reaction conditions for secondary PCR 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. A negative (molecular grade water) and positive control (DNA of *C. parvum* strain HA subtype IIa) were included in each PCR amplification. PCR cycling conditions were identical to original reports. Secondary PCR products were visualized by ethidium bromide staining (0.2 μg/ml) and extracted using GenElute Gel Extraction Kit (Sigma, St. Louis, MO). 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. The nucleotide sequences of each gene obtained in this study were manually edited using the program ChromasPro 2.1.4 (Technelysium, Pty, Ltd., South Brisbane, Australia), and aligned with previously published sequences using the MAFFT version 7 online server using the Q-INS-i algorithm. Phylogenetic analyses were performed and the best DNA/Protein phylogeny models were selected using MEGA6 (Tamura et al. 2013). Phylogenetic trees were inferred by the maximum likelihood (ML) method, with the substitution model that best fits the alignment selected using the Bayesian information criterion. The Tamura 3-parameter model (Tamura 1992) was selected for 18S rDNA and HSP70 alignments, and the general time reversible model (Tavaré 1986) was selected for the actin alignment. Bootstrap support for branching was based on 1000 replications. Phylograms were drawn using MEGA6 and were manually adjusted using CorelDraw X7. Sequences of 18S rDNA, actin, and HSP70 derived in this study have been deposited in GenBank under accession numbers MG699168–MG699179.

Morphological evaluation

Faecal smears were fixed with 100% methanol and oocysts of *C. parvum* VF383 were examined using brightfield microscopy, following ACMV staining in accordance with Miláček and Vítovec (1985), and fluorescence microscopy, following labeling with genus-specific FITC-conjugated antibodies in accordance with manufacturer's instructions (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 at 1000× magnification and the shape index of each oocyst was calculated. As a control, the morphometry of *C. parvum* strain HA (n = 30) from a naturally infected 25-day-old calf (*Bos taurus*), maintained in SCID mice at Institute of Parasitology of BC CAS, v.v.i., Czech Republic, was measured by the same person using the same microscope.

Transmission study

Six 8-week-old rats (*Rattus norvegicus*), including rat 0, five BALB/c mice (*Mus musculus*), five Mongolian gerbils (*Meriones unguiculatus*), five 4-week-old piglets (*Sus scrofa*), and five 12-week-old calves (*Bos taurus*) were used for experimental infection studies with *C. parvum* VF383. Two weeks prior to experimental infections, animals were

screened daily for the presence of specific DNA and oocysts of *Cryptosporidium*. To prevent environmental contamination with oocysts, laboratory rodents were housed in plastic cages and supplied with a sterilized diet and sterilized water *ad libitum*. Piglets and calves were kept in an experimental stable with sterilized bedding and were supplied with sterilized food and water *ad libitum*. Each animal was inoculated orally with 5000 purified oocysts suspended in 200 μl (rodents) or 20 ml (piglets and calves) of distilled water. Shedding of oocysts and presence of specific DNA were individually screened in each animal daily from 3 to 30 days post infection (DPI). Faecal consistency and colour and general health status of animals were examined daily. The infection intensity was determined from the microscopic examination as a number of oocysts per gram (OPG) according to Kváč et al. (2007). Animal caretakers wore disposable coveralls, shoe covers, and gloves every time they entered the facility rooms. All wood-chip bedding, faeces, and disposable protective clothing were sealed in plastic bags, removed from the buildings, and incinerated. All housing, feeding, and experimental procedures were conducted under protocols approved by the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic and Institute and National Committees (Protocol No. 114/2013 and 52/2014).

Histology and electron microscopy

The complete examination of all gastrointestinal organs of two animals from each group (at 6 and 15 DPI) was conducted at necropsy. Tissue specimens from the stomach, small intestine, and large intestine were sampled and processed

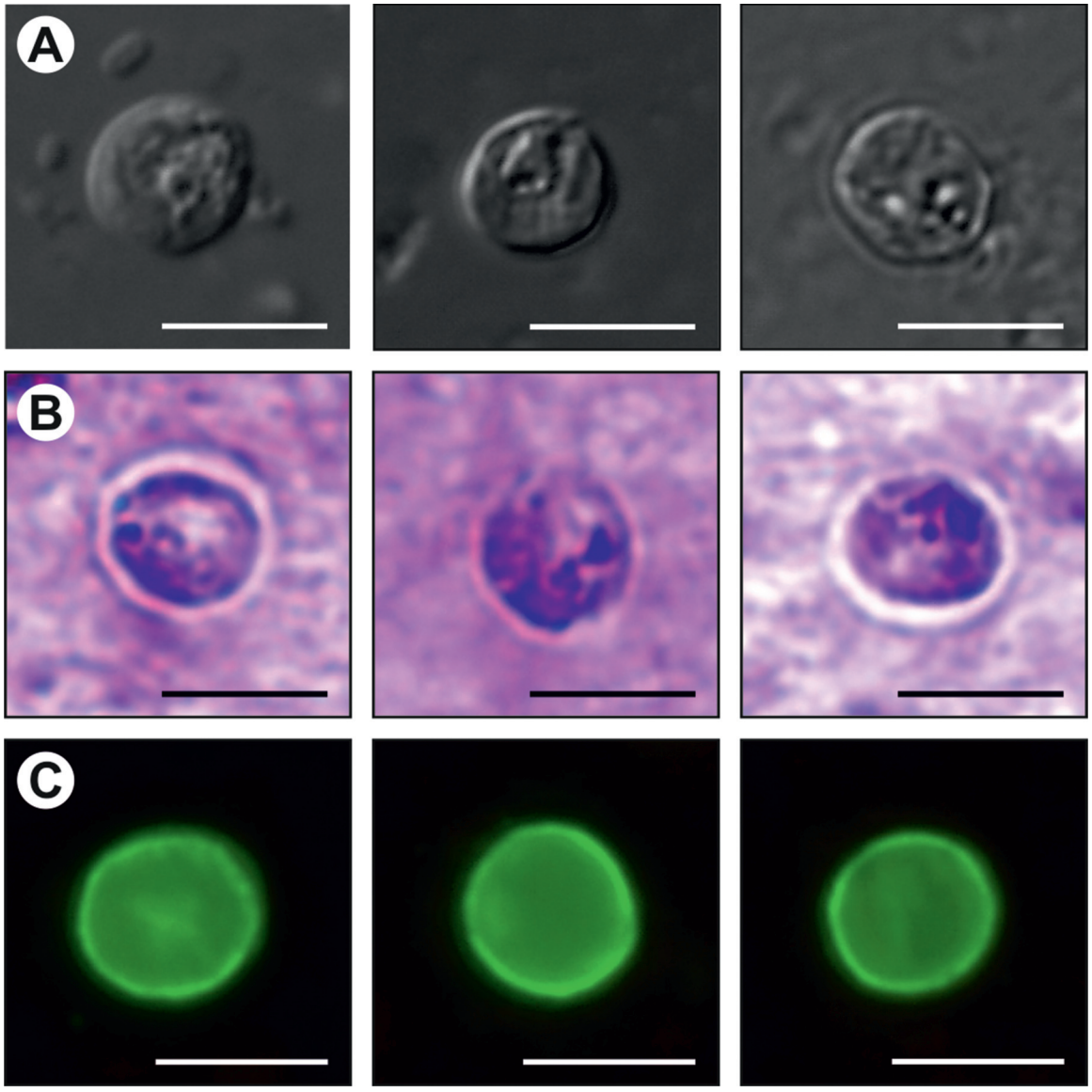

Fig. 1. Oocysts of *Cryptosporidium occultus* sp. n. visualized by microscopy **(A)** under differential interference contrast, **(B)** following aniline–carbol–methyl violet staining, and **(C)** following labeling with FITC-conjugated anti-*Cryptosporidium* antibody. Bar = 5 μm.

for histology and scanning electron microscopy (SEM). The specimens for histology were fixed in 4% buffered formalin and processed by the usual paraffin method. Histology sections (5 μm) were stained with hematoxylin and eosin (HE) and Periodic Acid–Schiff (PAS) stains. The specimens for SEM were fixed overnight at 4 °C in 2.5% glutaraldehyde in 0.1 M phosphate buffer, washed 3 times for 15 min in the same buffer, postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer for 2 h at room temperature, and finally washed 3 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.

Results

Oocysts of *C. parvum* VF383 from four naturally infected wild brown rats were morphometrically identical to those from experimentally infected rats and similar in size to *C. parvum* from a naturally infected 25-day-old calf. Microscopic examination of oocysts in faecal smears showed typical *Cryptosporidium* ACMV staining characteristics and typical apple green fluorescence following labeling with the FITC conjugated anti-*Cryptosporidium* oocyst wall antibody (see Fig. 1 for representative images of ACMV stained and FITC-labeled oocysts). At the 18S rDNA, actin and HSP70 loci, all four isolates from wild rats shared 100% identity with each other and with *C. parvum* VF383 [DQ182599, HQ822148, and DQ182598] from ruminants, rats, and humans (Table 1, Fig. 2). Maximum likelihood trees (Fig. 2) showed that *C. parvum* VF383 is most closely related to *C. suis*, sharing 99.7%, 98.0%, and 97.9% similarity at 18S rDNA (718 bp), actin (833 bp), and HSP70 (1847 bp) loci, respectively. Experimentally inoculated pigs and calves did not shed *Cryptosporidium* that was detectable by microscopy or PCR. These animals also had no endogenous stages detectable by histology or electron microscopy. Inoculated rats, gerbils and mice shed *C. parvum* VF383 that was detectable by PCR at 4–5 days post infection (DPI), but only rats shed oocysts detectable by microscopy (Fig. 3). Rats shed oocysts/specific DNA until the conclusion of the experiment (30 DPI). Gerbils and mice stopped shedding between 6 and 19 DPI (Fig. 3). Infection intensity in rats ranged from 2000 to 370,000 OPG, and ‘was greatest between 11 and 17 DPI, with a daily mean of 120,000 OPG. Examination of gastrointestinal tract tissue from susceptible rodents by

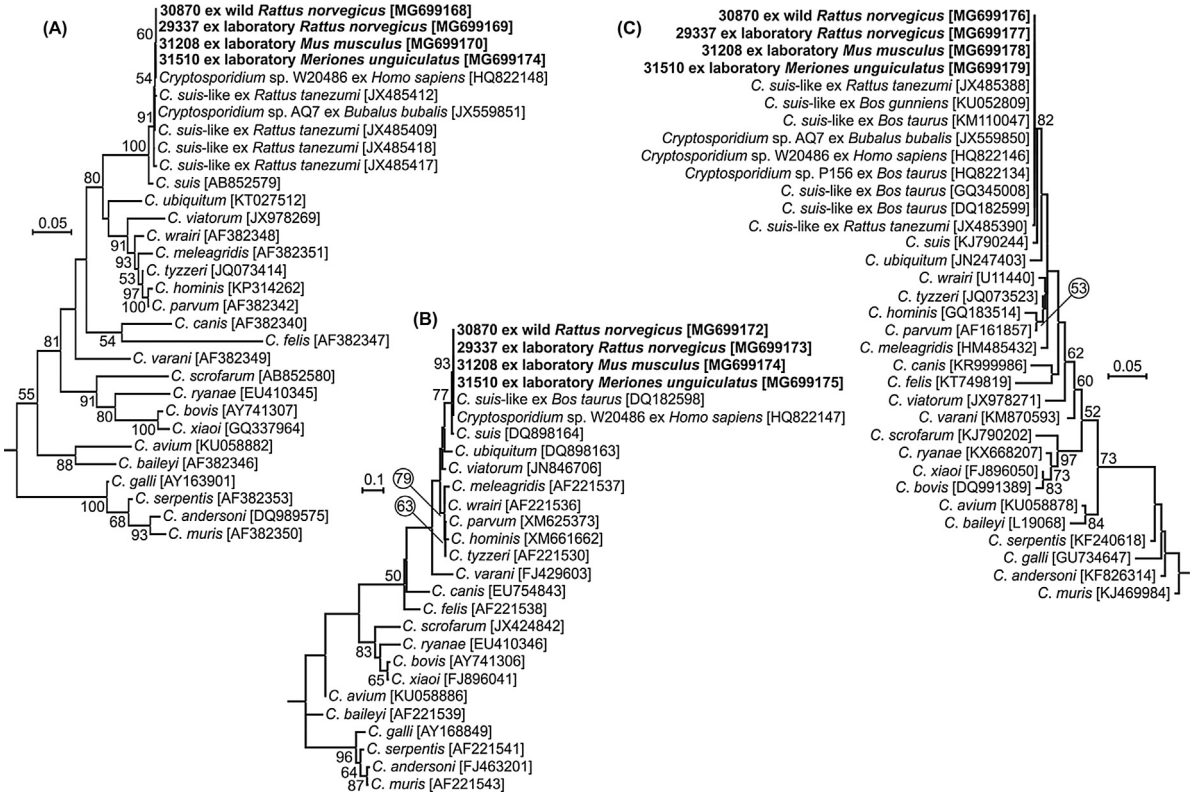

Fig. 2. Phylogenetic relationships among isolates of *Cryptosporidium occultus* sp. n. in this study (bolded) and other *Cryptosporidium* spp. by a maximum likelihood of **(A)** the partial actin gene (733 base positions in the final dataset, GTR + G + I model, ML LogL = −5132.48), and **(B)** the partial HSP70 gene (287 base positions in the final dataset, T92 + G model, ML LogL = −2061.88), and **(C)** the partial 18S rRNA gene (519 base positions in the final dataset, T92 + G model; ML LogL = −2114.91). Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates. ML trees were rooted with sequences from *Plasmodium falciparum* (actin [M22719], HSP70 [NC_004328.2], and 18S rDNA [JQ627151]).

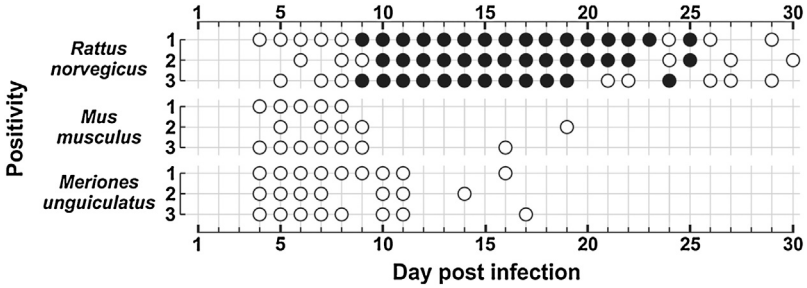

Fig. 3. Course of infection of *Cryptosporidium occultus* sp. n. in rats (*Rattus norvegicus*), BALB/c mice (*Mus musculus*) and Mongolian gerbils (*Meriones unguiculatus*) based on molecular and microscopical examination of faeces. An open circle indicates the detection of a specific DNA fragment; a closed circle indicates the detection of oocysts by microscopy.

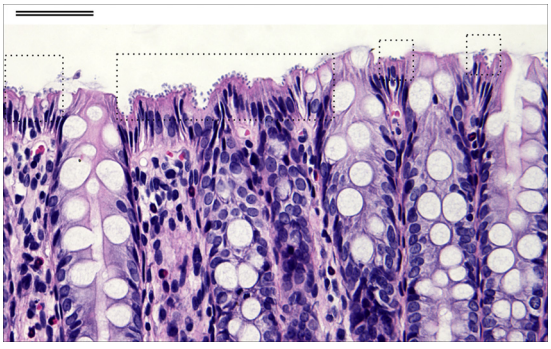

Fig. 4. *Cryptosporidium* life cycle stages (indicated by boxes with a dashed line) in the mucosal glandular epithelium from the colon of a laboratory rat (*Rattus norvegicus*) experimentally infected with 5000 oocysts of *Cryptosporidium occultus* sp. n., sacrificed 15 days post infection. The section was stained with hematoxylin and eosin. Bar = 50 μm.

histology, electron microscopy and PCR analysis revealed the presence of *Cryptosporidium* developmental stages only in the colon (Figs. 4 and 5). No macroscopic lesions were observed in internal organs. Compared to rats, where epithelial cell infection was massive (Fig. 5), only sporadic infection was observed in mice and gerbils. In rats, the luminal epithelium throughout the large intestine was densely covered with developmental stages (Fig. 5) and infected epithelial cells were slightly dilated. Inflammatory changes were not found in the *lamina propria* of the infected large intestine. None of the faecal samples were diarrheal. Sequences of individual genes obtained from susceptible hosts shared 100% identity with the inoculum (from rat 0) and original isolates from wild rats (Fig. 2). Morphological, experimental and molecular analyses support the description of *C. parvum* VF383 as a new *Cryptosporidium* species, which we name *Cryptosporidium occultus*. A description of *C. occultus* follows.

Taxonomic summary

Cryptosporidium occultus sp. n.

Description. Oocysts are shed fully sporulated with 4 sporozoites and oocyst residuum inside. Sporulated oocysts (n = 30) measure 4.66–5.53 μm

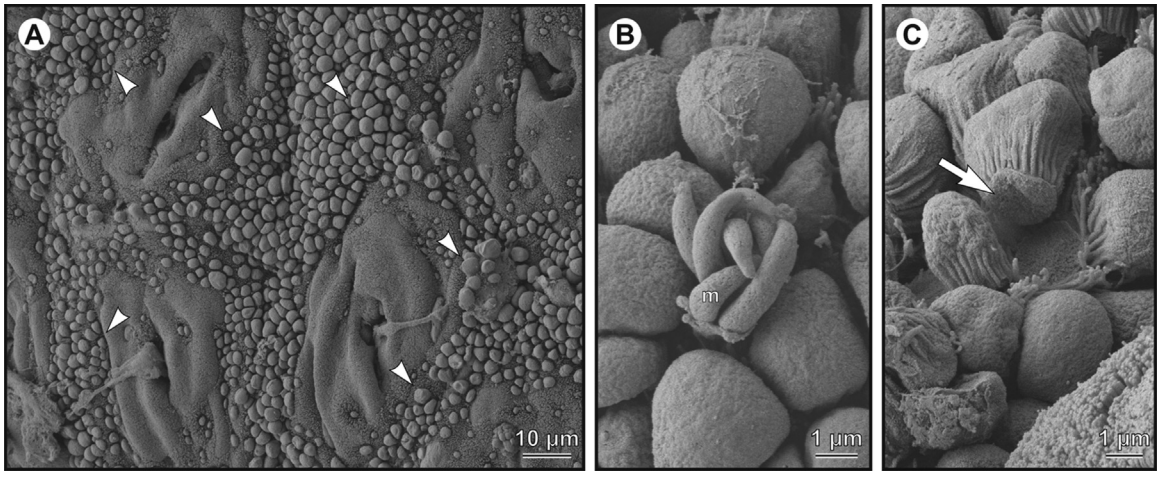

Fig. 5. Scanning electron photomicrograph of the colon epithelium of a laboratory rat (*Rattus norvegicus*) infected 5000 oocysts of *Cryptosporidium occultus* sp. n. and sacrificed 15 days post infection. **(A)** Colon epithelium covered by developmental stages of *Cryptosporidium occultus* sp. n. (arrowhead). **(B)** Ruptured parasitophorous sac releasing Type I merozoites (m). **(C)** Site of parasitophorous sac interaction with the host cell (arrow). Bar included in each figure.

(mean = 5.20 μm) × 4.47–5.44 μm (mean = 4.94 μm) with a length-to-width ratio of 1.05 (1.00–1.17) (Fig. 1). The morphology and morphometry of other developmental stages is unknown.

Type host: brown rat (*Rattus norvegicus*)

Type localities: České Budějovice, Cizkrajov, Lidéřovice, and Telč (Czech Republic)

Site of infection: colon

Other hosts: Tanezumi rat (*Rattus tanezumi*), domestic mouse strain BALB/c (*Mus musculus*), Mongolian gerbils (*Meriones unguiculatus*), cattle (*Bos taurus*), yak (*Bos grunniens*), water buffalo (*Bubalus bubalis*), and human (*Homo sapiens*)

Distribution: Australia, Brazil, Canada, China, Denmark, England, India, and Philippines

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 18S rRNA, actin, and HSP70 genes were deposited at GenBank (Acc. Nos. MG699168–MG699179).

Etymology: The species name is derived from the adjective *occultus*, in the nominative singular, meaning hidden, for its previously unclear host specificity.

Differential diagnosis. Vítovec et al. (2006) demonstrated that the size difference between *C. suis* and *C. parvum* oocysts is statistically significant ($P < 0.01$). Since *C. occultus* is very similar in size to *C. parvum*, oocysts of *C. occultus* are morphologically indistinguishable from those of *C. parvum* but distinguishable from those of *C. suis*. Oocysts of *C. occultus* have similar ACMV staining to other *Cryptosporidium* spp. and cross react with antibodies developed primarily for *C. parvum*. This species can be differentiated from other *Cryptosporidium* spp. based on sequences of 18S rRNA, actin, and HSP70 genes.

Discussion

This study has shown that *C. occultus*, which was named *C. suis*-like in some earlier reports, is not infectious for pigs under experimental conditions. These data, and the absence of reports on natural infections in pigs, lead us to conclude that *C. occultus* is not infectious for pigs, despite its close phylogenetic relationship to the pig-adapted species *C. suis.*

In contrast to previous reports describing the natural occurrence of *C. occultus* in bovids (16 cattle, 2 yaks, and 1 water buffalo) on four continents (Europe, Asia, Australia, and South America), we found that this species was not infectious for calves under experimental conditions. In contrast, *C. occultus* caused a prolonged (>30 days) infection in the colon of rats under experimental conditions. Consistent with the findings of a previous study on pigs infected with *C. suis*, a species that also infects the colon (Vítovec et al. 2006), rats infected with *C. occultus* shed fewer oocysts than would be predicted from the massive infection of the colonic epithelium. This contrasts with studies on *Cryptosporidium* species infecting the small intestine, where the intensity of oocyst shedding matched the intensity of developmental stages observed in the epithelium (Kváč et al. 2016; Kváč et al. 2014a; Ren et al. 2012; Vítovec and Koudela 1992). Evidence from studies on experimental and natural susceptibility to *C. occultus* (this study, Ng-Hublin et al. 2013) supports the conclusion that rats are a primary host.

The finding that mice and gerbils also are susceptible under experimental conditions suggests that *C. occultus* may infect a broad range of rodents, although there is no evidence of natural infections in rodents other than rats (Kváč et al. 2014b). Reports of *C. occultus* in cattle, yak and water buffalo could have been due to these bovids ingesting rat faeces in contaminated feed or water. Similarly, previous reports of *C. muris* and *C. tyzzeri* in the faeces and slurry of pigs (Chen and Huang 2007; Jenkins et al. 2010; Kváč et al. 2009; Xiao et al. 2006), despite the non-susceptibility of pigs under experimental conditions, was likely the result of wild rodents living in the close proximity to pigs on farms (Kváč et al. 2012). Mechanical passage of non-host-specific *Cryptosporidium* sp. has been reported in mammals, birds and reptiles (Crawshaw and Mehren 1987; Graczyk et al. 1996; Xiao et al. 2004b). We cannot rule out the possibility that *C. occultus* isolates reported previously in domesticated ruminants were a ruminant-adapted subtype. Those ruminant isolates were identical to isolates from the present study at the 18S rRNA and HSP70 loci; however, more discriminative loci, such as gp60, were not examined. The variability in host range of different *Cryptosporidium* subtypes within one taxon has been reported previously in *C. parvum* (most strains infect calves and humans but subtype IIc only infects humans), *C. andersoni* (only the Kawatabi strain is infectious for mice), and *C. ubiqutium* (subtype XIIa seems to be specific for ruminants while XIIb-e appears to be rodent specific) (Li et al. 2014; Matsubayashi et al. 2005; Nichols et al. 2014).

Although *C. occultus* has been reported from a case of clinical cryptosporidiosis in humans (Ong et al. 2002), extending the number of human-pathogenic *Cryptosporidium* species, infections in humans are likely to be rare.

The morphometry of *C. occultus* oocysts is typical of intestinal *Cryptosporidium* spp. (Fayer 2010), and oocysts cannot be distinguished microscopically from other species and genotypes infecting rats (Lv et al. 2009). However, *C. occultus* has smaller oocysts (5.2 μm × 4.9 μm) than the closely-related *C. suis* (6.2 μm × 5.5 μm; Vítovec et al. 2006).

Phylogenetic analyses show that *C. occultus* is genetically distinct from known *Cryptosporidium* species, including *C. suis*. At the 18S rDNA, actin, and HSP70 loci, *C. occultus* Exhibits 0.3%, 2.0%, and 2.1% sequence divergence,

respectively, from *C. suis*. These differences are similar to those between closely related *Cryptosporidium* species such as *C. tyzzeri* and *C. parvum* (0.6%, 1.3%, and 0.6%, respectively) and *C. cuniculus* and *C. hominis* (0.40%, 0.0%, and 0.6%, respectively). While sequences of 18S rDNA and HSP70 are monomorphic, 1–3 synonymous SNPs were found in actin sequences from some *C. occultus* isolates originating from the Philippines (Ng-Hublin et al. 2013). Similarly, actin polymorphisms have been observed in other *Cryptosporidium* species, such as *C. hominis* [KU892570 and KT948749] and *C. tyzzeri* [JQ073395, JQ073408, and JQ073413].

The results of this study demonstrate the necessity of using biological data as part of a multidisciplinary approach to understand the diversity of *Cryptosporidium.*

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6.2 Příloha II

Cryptosporidium ratti n. sp. (Apicomplexa: Cryptosporidiidae) and genetic diversity of _Cryptosporidium_ spp. in brown rats (_Rattus norvegicus_) in the Czech Republic.

Ježková J., Prediger J., Holubová N., Sak B., Konečný R., Feng Y., Xiao L., Rost M., McEvoy J., Kváč M. 2021: Parasitology 148: 84–97.

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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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Cryptosporidium ratti; infectivity; morphometric analysis; phylogeny; prevalence

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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

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.

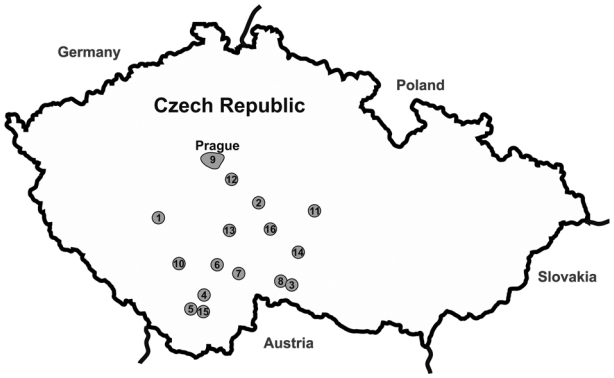

Fig. 1. Sampling locations across the study area in the Czech Republic. Sample site numbers indicate the name of locations and coordinates are in brackets: (1) Březnice (49.556628, 13.954390), (2) Chyšná (50.545694, 13.437376), (3) Cizkrajov (49.0303555, 15.390124), (4) České Budějovice (48.974749, 14.453704), (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.
aIsolate 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.

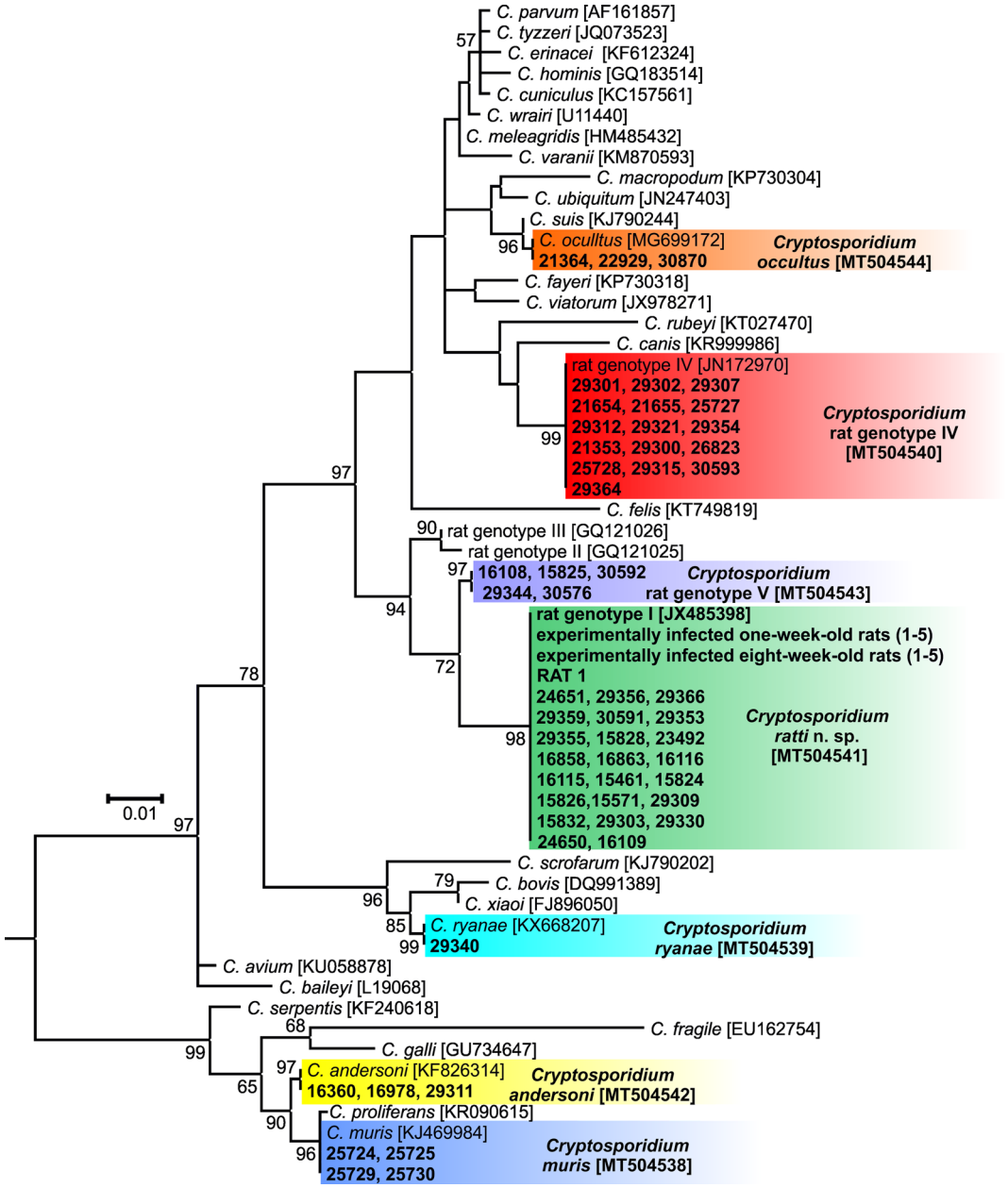

Fig. 2. Maximum likelihood tree based on partial sequences of the gene encoding the small subunit rRNA (*SSU*), including sequences obtained from naturally- and experimentally-infected hosts in this study. Tamura's 3-parameter model was applied, using a discrete Gamma distribution and invariant sites. 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

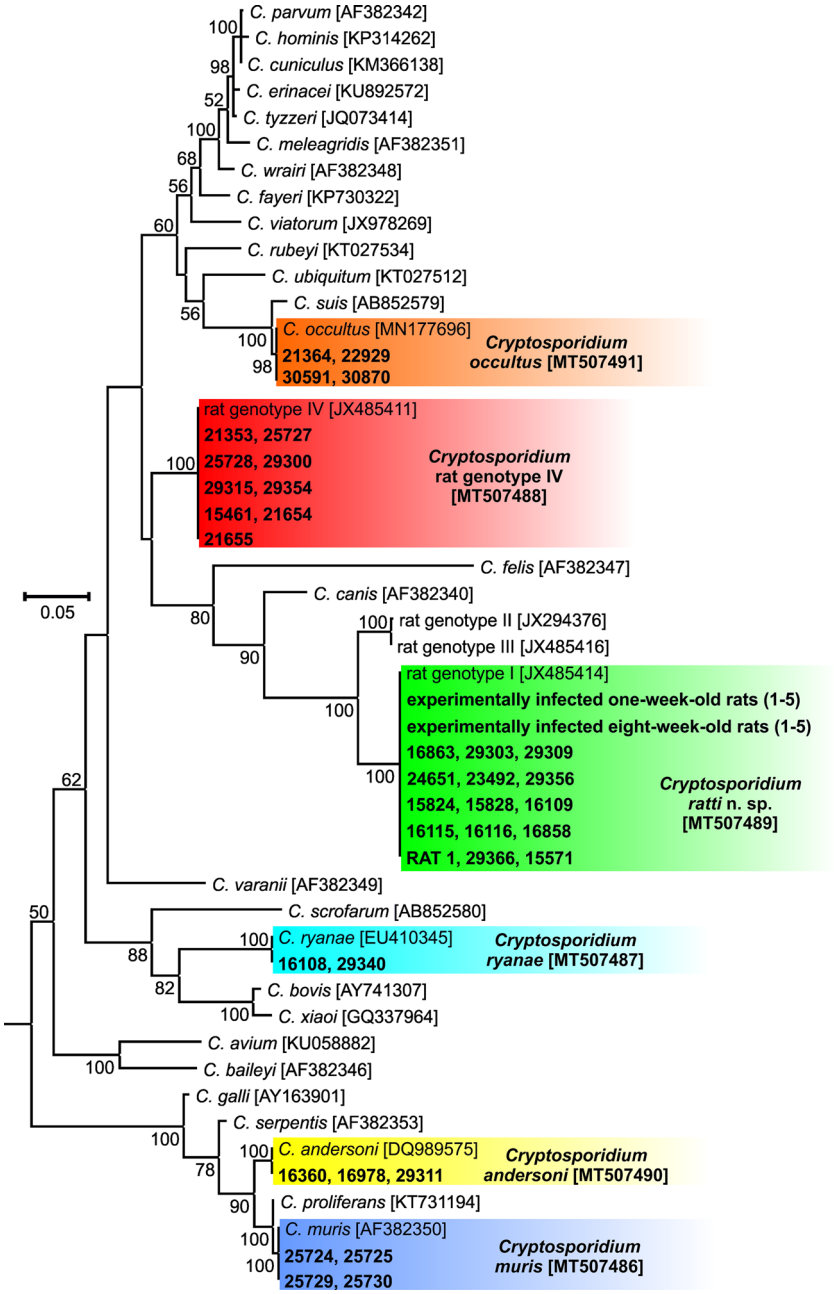

Fig. 3. Maximum likelihood tree based on partial sequences of the actin gene. The General Time Reversible model was applied, using a discrete Gamma distribution and invariant sites. The robustness of the phylogeny was tested with 1000 bootstraps and the numbers at the nodes represent the bootstrap *P* 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.

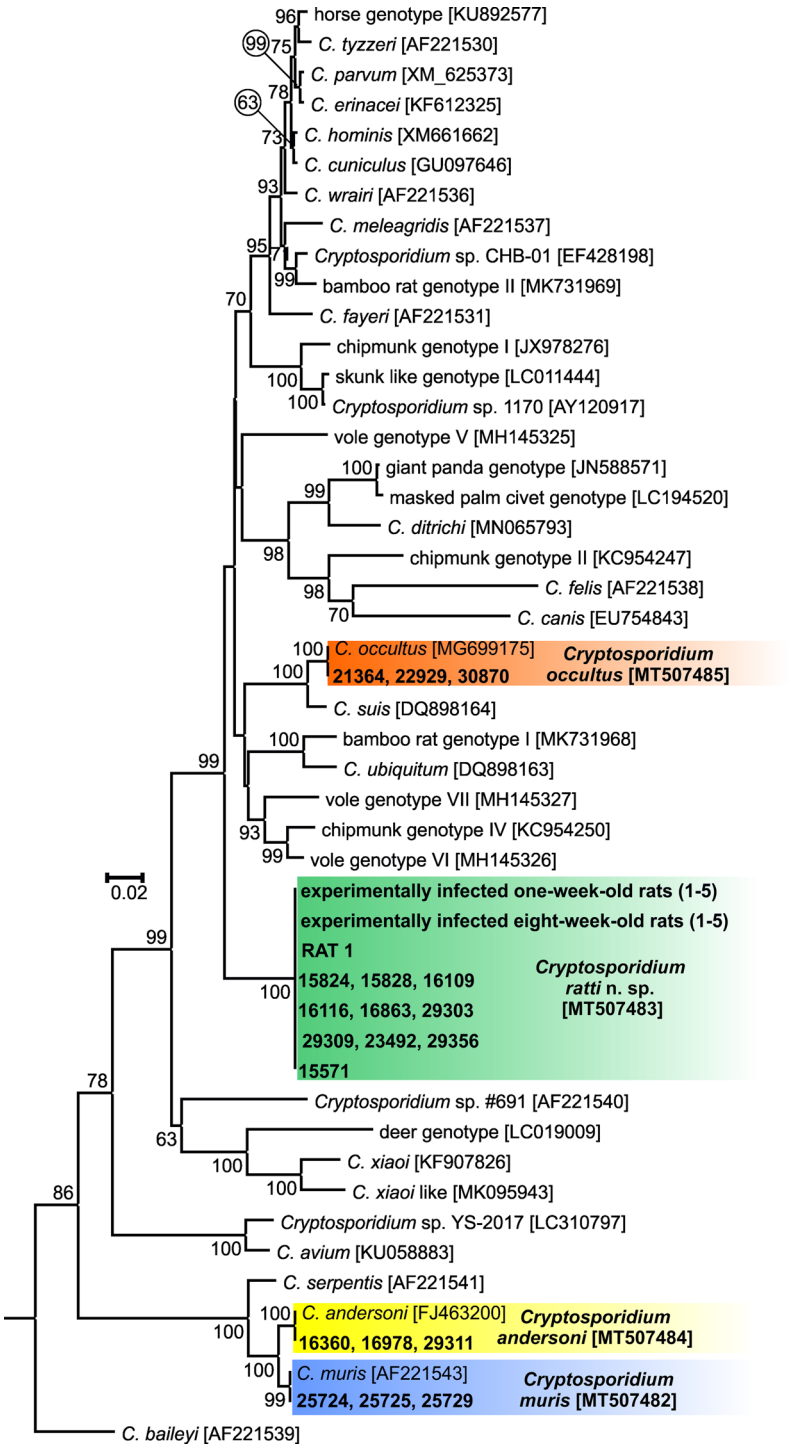

Fig. 4. Maximum likelihood tree based on partial sequences of the Heat Shock Protein 70 (*HSP70*) gene. The General Time Reversible model was applied, using a discrete Gamma distribution. The robustness of the phylogeny was tested with 1000 bootstraps and the numbers at the nodes represent the bootstrap *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

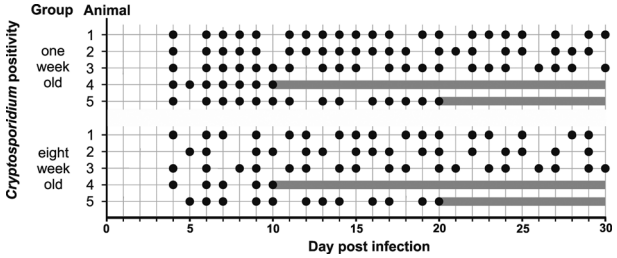

Fig. 5. Course of infection of *Cryptosporidium ratti* n. sp. in 1-week- and 8-week-old rats (*Rattus norvegicus*) based on microscopic and molecular (*SSU*) examination of feces. Grey circle indicates the detection of oocysts by microscopy, black circle indicates the detection of specific DNA by PCR.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

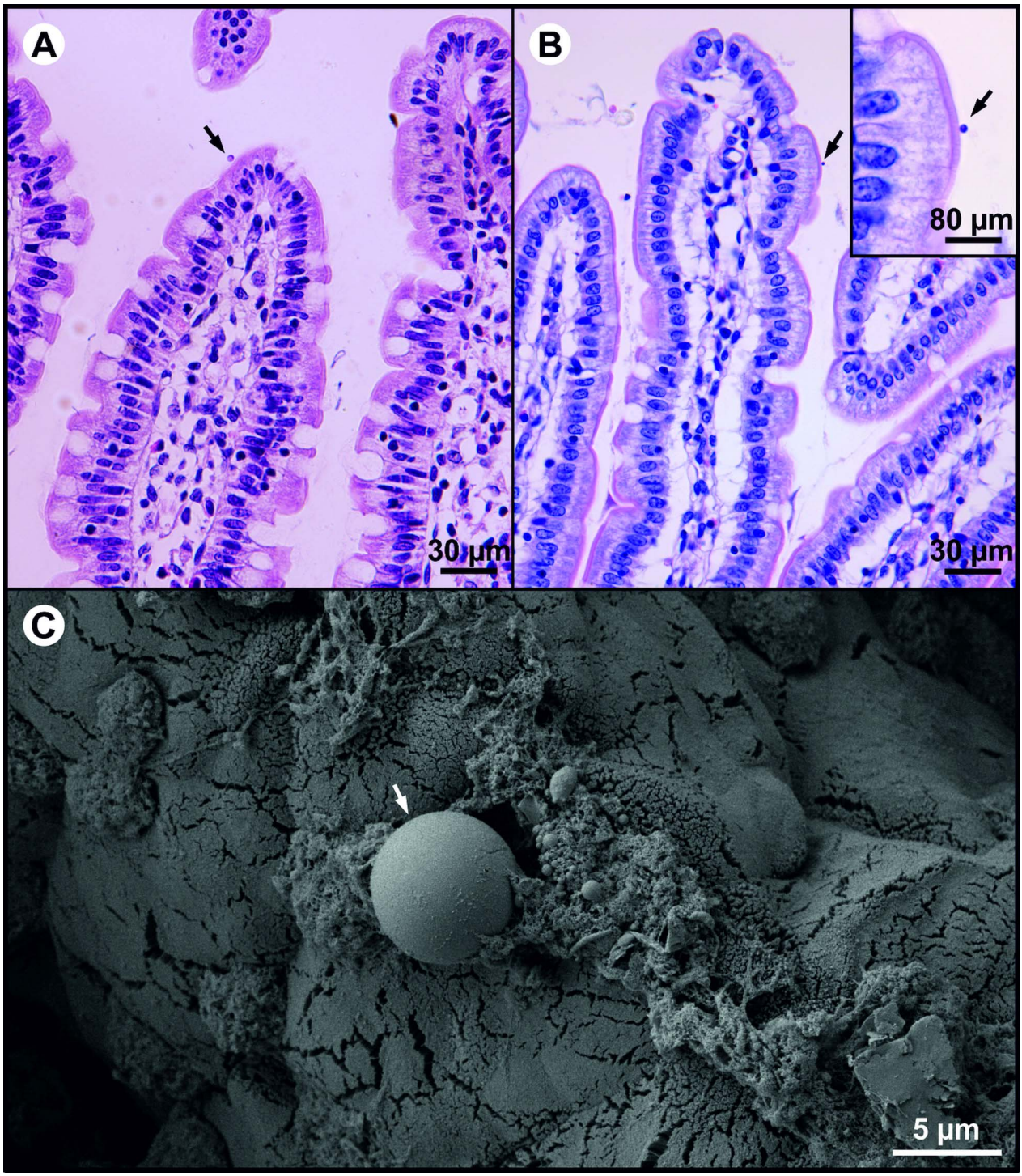

Fig. 6. Presence of developmental stages of *Cryptosporidium ratti* n. sp. (arrow) on jejunal mucosal epithelium in rat (*Rattus norvegicus*) infected at 1-week-old and sacrificed 10 days post infection. (A) and (B) histological sections stained by hematoxylin eosin, (C) scanning electron microphotograph.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

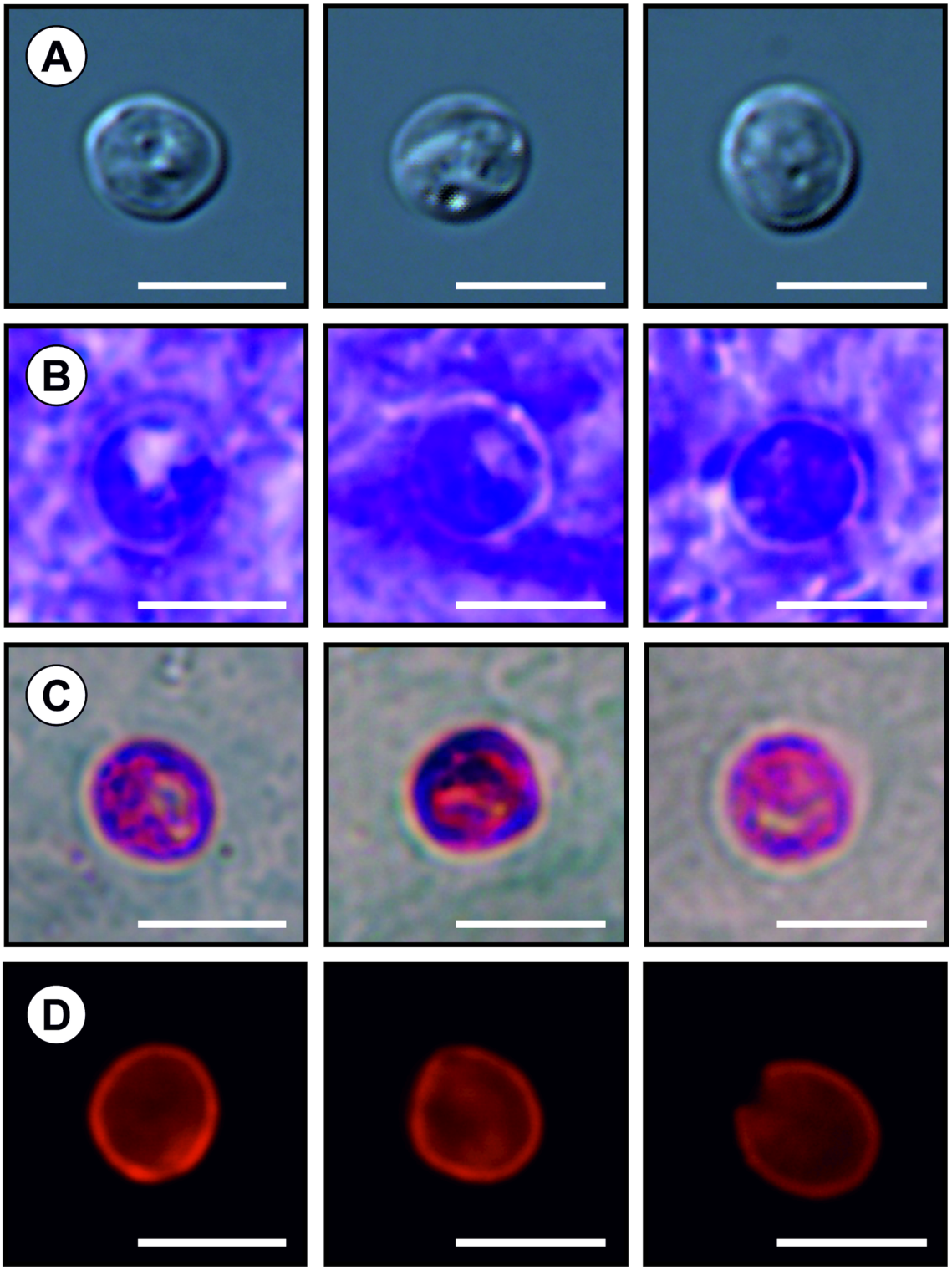

Fig. 7. Oocysts of *Cryptosporidium ratti* n. sp. (A) differential interference contrast microscopy, (B) aniline-carbol-methyl violet staining, (C) Ziehl-Nielsen staining, (D) labelling with antibody reagent consisting of a Cy3-labeled mouse monoclonal antibody made against *Cryptosporidium* oocyst outer 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 (*HSP*70) genes

	Gene locus		
Species/genotype	*SSU*	Actin	*HSP*70
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 *HSP*70 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 *HSP*70 locus. Analyses of the *HSP*70 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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6.3 Příloha III

Cryptosporidium myocastoris n. sp. (Apicomplexa: Cryptosporidiidae), the species adapted to the nutria (_Myocastor coypus_).

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Cryptosporidium myocastoris n. sp. (Apicomplexa: Cryptosporidiidae), the Species Adapted to the Nutria (*Myocastor coypus*)

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Abstract: *Cryptosporidium* spp., common parasites of vertebrates, remain poorly studied in wildlife. This study describes the novel *Cryptosporidium* species adapted to nutrias (*Myocastor coypus*). A total of 150 faecal samples of feral nutria were collected from locations in the Czech Republic and Slovakia and examined for *Cryptosporidium* spp. oocysts and specific DNA at the *SSU*, actin, *HSP*70, and *gp60* loci. Molecular analyses revealed the presence of *C. parvum* (n = 1), *C. ubiquitum* subtype family XIId (n = 5) and *Cryptosporidium myocastoris* n. sp. XXIIa (n = 2), and XXIIb (n = 3). Only nutrias positive for *C. myocastoris* shed microscopically detectable oocysts, which measured 4.8–5.2 × 4.7–5.0 µm, and oocysts were infectious for experimentally infected nutrias with a prepatent period of 5–6 days, although not for mice, gerbils, or chickens. The infection was localised in jejunum and ileum without observable macroscopic changes. The microvilli adjacent to attached stages responded by elongating. Clinical signs were not observed in naturally or experimentally infected nutrias. Phylogenetic analyses at *SSU*, actin, and *HSP*70 loci demonstrated that *C. myocastoris* n. sp. is distinct from other valid *Cryptosporidium* species.

Keywords: adaptation; prevalence; biology; course of infection; infectivity; oocyst size; phylogeny; parasite

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1. Introduction

Cryptosporidium is a protist genus that infects the gastrointestinal and respiratory tract of vertebrate hosts [1]. Cryptosporidiosis, the disease caused by members of this genus, frequently results in diarrhoea, which can be severe and fatal [2]. However, many species and genotypes of *Cryptosporidium*, particularly those infecting wild animals, do not cause clinical signs [3,4]. Genetic and biological studies have shown a high diversity within the genus *Cryptosporidium*, with much of this diversity observed in wildlife hosts [5–10]. To date, 47 valid species [11–13] and more than 100 genotypes, which are distinguished from valid species on the basis of molecular differences and probably represent separate species, have been described [2]. However, much remains to be discovered about the diversity of the genus *Cryptosporidium* and its host range.

Concerning host specificity, some species of *Cryptosporidium* have a broad host range (e.g., *C. parvum*, *C. meleagridis*, *C. baileyi* and *C. ubiquitum*), whereas others are restricted to a narrow group of hosts (e.g., *C. muris* and *C. andersoni*) or a single host (e.g., *C. wrairi*) [14].

The nutria (*Myocastor coypus*), also called coypu, is a native rodent of South America and has been introduced to several countries through meat and fur-farming [15]. In many of the regions where farming of nutria is popular, escaped individuals have established a local feral population. Feral nutrias occur on all continents, and they are included as one of the Top 100 Invasive Alien Species of Union Concern in Europe [16]. Parasites of wild, feral, and farmed nutrias are poorly studied [17–19], and there have been only four reports of *Cryptosporidium* infections.

Cryptosporidium sp., which based on oocyst size were reported as *C. parvum*, were first identified in the faeces of farmed nutrias in Poland [20]. The occurrence of *Cryptosporidium* sp. in wild nutrias was first described in Argentina in 2012 [15], but similarly to Pavlásek and Kozakiewicz [20], isolates were not genetically characterised and therefore the species remains unknown. *Cryptosporidium* copro-antigens were not detected in faeces from feral nutrias from Italy, and a study in the Czech Republic did not find *Cryptosporidium* DNA in faeces from farmed nutrias [18,21]. However, we later identified *C. ubiquitum* and the novel *Cryptosporidium* sp. coypu genotype in feral nutrias in Slovakia.

Building on our earlier findings, we performed a comprehensive study of *Cryptosporidium* in feral nutrias in the Czech Republic and Slovakia. We obtained an isolate of *Cryptosporidium* sp. coypu genotype and determined its biological properties, including oocyst size, host specificity, course and location of infection, and pathogenicity. Based on these data and data from our previous study, we conclude that the *Cryptosporidium* sp. coypu genotype is genetically and biologically distinct from valid *Cryptosporidium* species and is adapted to nutrias. We propose that it be named as a new species, *Cryptosporidium myocastoris* n. sp.

2. Materials and Methods

2.1. Area and Specimens Studied

Faecal samples from feral nutrias were collected from 7 and 11 localities in the Czech Republic and Slovakia, respectively, during the period 2016–2019 (Figure 1). Faecal samples were individually collected from the rectum of hunted nutria post-mortem or from the ground on riverbanks, placed into a sterile plastic tube, and delivered to the laboratory for processing. A faecal smear was prepared, stained with aniline–carbol–methyl violet (ACMV), and examined for the presence of *Cryptosporidium* spp. oocysts using light microscopy [22]. Infection intensity was expressed as the number of oocysts per gram of faeces (OPG) [23]. The OPG was estimated from the total number of oocysts on the slide and the mass of the faecal smear (approximately 0.015 g). Total genomic DNA (gDNA) was isolated and screened for the presence of *Cryptosporidium*-specific DNA by PCR/sequence analysis of the small subunit ribosomal RNA (*SSU*) gene (described below).

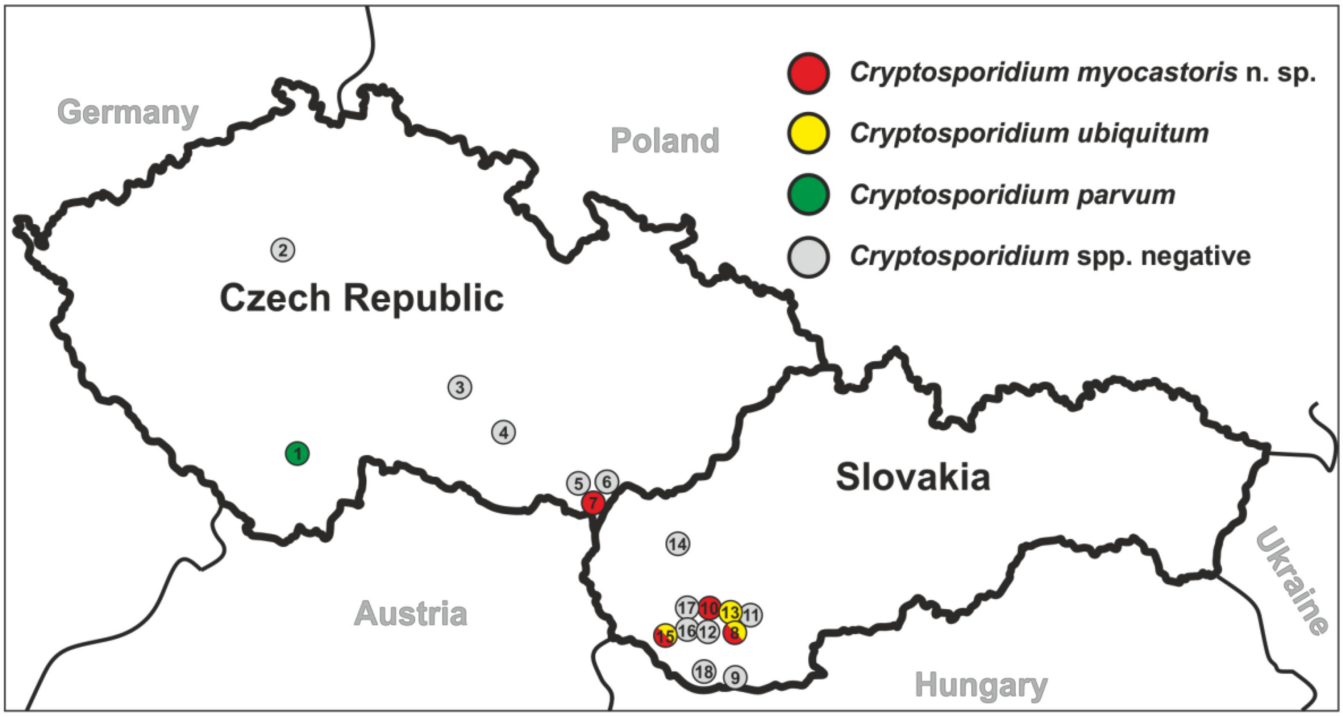

Figure 1. Sampling localities in the Czech Republic and Slovakia. For each site, the number indicates the name of locations (**1**) Planá nad Lužnicí (N 49°21.11527′, E 14°42.08325′); (**2**) Praha (N 50°4.78070′, E 14°24.81330′); (**3**) Jihlava (N 49°23.09785′, E 15°36.37422′); (**4**) Třebíč (N 49°12.58360′, E 15°52.28832′); (**5**) Břeclav (N 48°46.34147′, E 16°52.68835′); (**6**) Týnec (N 48°46.31570′, E 17°0.66778′); (**7**) Lanžhot (N 48°43.41558′, E 16°58.30782′); (**8**) Nové Zámky (N 48°0.76540′, E 18°11.75573′); (**9**) Komárno (N 47°45.04053′, E 18°8.98083′); (**10**) Šaľa (N 48°9.08273′, E 17°52.49152′); (**11**) Doný Ohaj (N 48°4.33237′, E 18°14.81102′); (**12**) Topolníky (N 47°57.61112′, E 17°45.37918′); (**13**) Palárikovo (N 48°2.15190′, E 18°2.70193′); (**14**) Nitrianský Hrádok (N 48°3.62700′, E 18°12.56517′); (**15**) Dunajská Streda (N 47°55.90470′, E 17°28.42662′); (**16**) Vlčny (N 48°2.69967′, E 17°57.72202′); (**17**) Diakovce (N 48°8.02725′, E 17°50.48138′); and (**18**) Lipové (N 47°50.41113′, E 17°51.33057′). The colour indicates the presence of *Cryptosporidium* spp.

2.2. Molecular Study

Total gDNA was extracted from 200 mg of faecal samples and 100 mg of tissue specimens using a GeneAll® Exgene™ Stool DNA mini Kit (GeneAll Biotechnology, co., Ltd.; Seoul, South Korea) and DNeasy Blood & Tissue kits (QIAGEN, Hilden, Germany), respectively, followed by bead disruption for 60 s at 5.5 m/s using 0.5 mm glass beads in a FastPrep®-24 Instrument (MP Biomedicals, Santa Ana, CA, USA). The acquired gDNA was stored at −20 °C.

Nested PCR protocols were used to amplify a partial sequence of the *SSU*, actin, 70 kDa heat shock protein (*HSP*70), and 60 kDa glycoprotein (*gp60*) genes using previously published protocols [24–29]. For the *SSU* fragment, the primers 5′TTCTAGAGCTAATACATG CG3′ and 5′CCCATTTCCTTCGAAACAGGA3′ were used in the primary reaction, and the primers 5′GGAAGGGTTGTATTTATTAGATAAAG3′ and 5′AAGGAGTAAGGAACAACC TCCA3′ were used in the secondary reaction. For the actin fragment, the primers 5′ATCRG WGAAGAAGWARYWCAAGC3′ and 5′AGAARCAYTTTCTGTGKACAAT3 were used in the primary reaction, and the primers 5′CAAGCWTTRGTTGTTGAYAA3′ and 5′TTTCTGT GKACAATWSWTGG3′ were used in the secondary reaction. For the HSP70 fragment, the primers 5′GCTCGTGGTCCTAAAGATAA3′ and 5′ACGGGTTGAACCACCTACTAAT3′ were used in the primary reaction, and the primers 5′ACAGTTCCTGCCTATTTC3′ and 5′GCTAATGTACCACGGAAATAATC3′ were used in the secondary reaction. For the *gp60* fragment, the primers 5′ATAGTCTCCGCTGTATTC3′ and 5′GGAAGGAACGATGTATCT3′ were used in the primary reaction and the primers 5′TCCGCTGTATTCTCAGCC3′ and 5′GCAGAGGAACCAGCATC3′ were used in the secondary reaction.

Some PCR conditions were modified from the original publications, as previously reported [11]. Molecular grade water and DNA of *C. occultus* were used as negative and positive controls, respectively, for the amplification of *SSU*, actin, and *HSP70* genes. DNA of *C. hominis* subtype family Ib was used as a positive control for amplification of the *gp60* fragment of *C. parvum* and *Cryptosporidium myocastoris* n. sp. DNA of *C. ubiquitum* subtype family XIIa was used as a positive control for the amplification of *C. ubiquitum*. The secondary PCR products were separated by electrophoresis on an agarose gel, stained with ethidium bromide and visualised under UV illumination. All amplicons were purified using the GenElute™ Gel Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA) and directly sequenced using the secondary PCR primers at Eurofins (Prague, Czech Republic).

Chromatogram analysis was performed using Chromas Pro 2.1.4 (Technelysium, Pty, Ltd., South Brisbane, Australia), and sequences were verified by BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed on 20 February 2021). The sequences obtained in this study and reference sequences obtained from GenBank were aligned using the MAFFT version 7 online server (http://mafft.cbrc.jp/alignment/server/; accessed on 20 February 2021) using the E-INS-i multiple alignment method. The alignments were manually trimmed and edited in BioEdit v.7.0.5 [30]. Phylogenetic analysis was performed using the maximum likelihood (ML) method, using evolutionary models selected by MEGAX [31]. Bootstrap supports were calculated from 1000 replications. Phylogenetic trees were produced by MEGAX and further edited for visualisation purposes with Corel DrawX7 (Corel Corporation, Ottawa, Ontario, Canada). Species-specific divergences were identified from proportional distances (%) which were calculated in the program Geneious v11.0.3 [32] based on the *SSU*, actin, and *HSP70* datasets of all sequences under study. All nucleotide sequence data were deposited in GenBank, Accession Numbers (Acc. nos.): MW274645-MW274661 for *SSU*, MW280959-MW280975 for actin, MW280976-MW280992 for *HSP*70, and MW280993-MW281009 for *gp60*.

2.3. Source of Oocysts of *Cryptosporidium myocastoris* n. sp.

Oocysts of *C. myocastoris* n. sp., recovered from naturally infected nutrias (isolate 31132) using sucrose [33] and caesium chloride gradient centrifugation [34], were used to experimentally infect a *Cryptosporidium*-negative nutria (nutria 0) and for morphometric study (described below).

2.4. Animals for Transmission Studies

Six adult nutrias (nutria 0 and nutrias 1–5; *Myocastor coypus*), five one-week- and eight-week-old gerbils (*Meriones unguiculatus*), five one-week- and eight-week-old SCID mice (*Mus musculus*; strain C.B-17), five one-week- and eight-week-old BALB/c mice, and five one-day-old chickens (*Gallus gallus* f. *domestica*) were used for transmission studies. Three animals from each group were used as negative controls. All nutrias used for the transmission study were screened daily for the presence of specific DNA and oocysts of *Cryptosporidium* for two weeks prior to experimental inoculation. Mice and gerbils, which were bred under laboratory conditions, were screened for the presence of specific DNA and oocysts of *Cryptosporidium* one week prior to experimental inoculation. One-day-old chickens, which were hatched under laboratory conditions, were screened on the day of hatching.

2.5. Animal Care

To prevent environmental contamination with oocysts, mice and gerbils were individually housed in ventilated cages (Tecniplast, Buguggiate, Italy), and chickens were housed in plastic boxes that were disinfected at 80 °C for one hour before being used. Nutrias were kept in boxes with secured walls. The individual boxes were disinfected with pressurized steam before use. Cages and boxes were sized in accordance with European and Czech Republic regulations on the protection of animals against cruelty. An external source of heat was used in the first five days for chickens. Each animal was supplied with a sterilized

diet and sterilized water ad libitum. Animal keepers wore disposable protective equipment during care of the animals. Woodchip and straw bedding and disposed protective clothing were removed from the experimental room and incinerated. All experimental procedures complied with the law of Czech Republic (Act No. 246/1992 Coll., on the protection of animals against cruelty). The study design was approved by ethical committees at the Biology Centre of CAS, the State Veterinary Administration, and the Central Commission for Animal Welfare under Protocol No. 35/2018 and 60/2019.

2.6. Design of Transmission Studies

Nutria 0 was inoculated orally with 5,000 purified oocysts of *C. myocastoris* n. sp. recovered from naturally infected nutria (described above) and suspended in 500 µL of distilled water. Oocysts of *C. myocastoris* n. sp., recovered from experimentally infected nutria 0, were molecularly characterised and used to infect other experimental animals (above). Other animals were inoculated with 20,000 purified oocysts of *C. myocastoris* n. sp. suspended in 500 µL (nutria), 200 µL (mice, gerbils, and chickens) of distilled water. Animals serving as negative controls were inoculated orally with 500 µL (nutria), 200 µL (mice and gerbils) or 20 µL (chickens) of distilled water. Faecal samples from each animal were screened daily for the presence of *Cryptosporidium* oocysts (ACMV staining) and *Cryptosporidium*-specific DNA (*SSU* gene amplification and sequencing). All experiments were terminated 30 days post-infection (DPI). The course of infection was evaluated based on the presence of *C. myocastoris* n. sp. specific DNA and the number of oocyst per gram of faeces as previously described by Kváč et al. [23]. Consistency and colour of faeces and health status were determined daily for each sample and animal, respectively. From each experimentally infected animal, PCR-positive samples from the beginning, middle, and end of the infection were additionally examined at the actin, *HSP70* and *gp60* genes to verify the identity of the *C. myocastoris* n. sp. with the inoculum and the original isolate.

2.7. Morphological Evaluation

The oocysts of *Cryptosporidium myocastoris* n. sp., which originated from naturally infected nutrias (isolates 31132 and 31459, Table 1) and experimentally infected nutrias (nutria N0 and nutria N1), were purified using sucrose and caesium chloride gradient centrifugation and examined using differential interference contrast (DIC) microscopy, bright field microscopy following ACMV and modified Ziehl-Neelsen (ZN) staining [35], and fluorescence microscopy following labelling of the *Cryptosporidium* oocyst wall with genus-specific FITC-conjugated antibodies (IFA; *Cryptosporidium* IF Test, Cryptocel, Cellabs Pty Ltd., Brookvale, Australia). Images of oocysts were collected using an Olympus Digital Colour camera (DP73) and Olympus cell SensEntry 2.1 software (Olympus Corporation, Shinjuku, Tokyo, Japan). Length and width of oocysts (n = 30) from naturally and experimentally infected nutrias were measured under DIC at 1000× magnification. The length-to-width ratio was calculated for each oocyst. As a control, the morphometry of *C. parvum* from naturally infected calfs (*Bos taurus*; n = 30) and *C. ratti* from experimentally infected rats (*Rattus novegicus*; n = 30) was used. Photomicrographs of *Cryptosporidium myocastoris* n. sp. oocysts under DIC, ACMV, ZN and IFA are part of this publication, and have been deposited as a phototype at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

Table 1. The occurrence and genetic diversity of *Cryptosporidium* spp. in the present study detected by the amplification of small subunit ribosomal rRNA (*SSU*), actin, 70 kDa heat-shock protein (*HSP70*) and 60 kDa glycoprotein (*gp60*) gene fragments in the wild coypus (*Myocastor coypus*) in the Czech Republic and Slovakia. Oocysts were quantified by microscopy and reported per gram of faeces (OPG).

Country	Locality #	No. of Positive/No. of Screened	ID of Positive Animal	Microscopical Positivity (OPG)	Genotyping at the Gene Loci			
					SSU	**Actin**	***HSP*70**	***GP*60**
Czech Republic	Planá nad Lužnicí (1)	1/11	41838	No	*C. parvum*	*C. parvum*	*C. parvum*	IIa
	Praha (2)	0/15	-	-	-	-	-	-
	Jihlava (3)	0/7	-	-	-	-	-	-
	Třebíč (4)	0/6	-	-	-	-	-	-
	Břeclav (5)	0/6	-	-	-	-	-	-
	Týnec (6)	0/15	-	-	-	-	-	-
	Lanžhot (7)	2/12	29639	No	*C. myocastoris*	*C. myocastoris*	*C. myocastoris*	XXIIb
			29370	No	*C. myocastoris*	*C. myocastoris*	*C. myocastoris*	XXIIa
	Nové Zámky (8)	2/12	31467	No	*C. ubiquitum*	*C. ubiquitum*	*C. ubiquitum*	XIId
			31472	No	*C. myocastoris*	*C. myocastoris*	*C. myocastoris*	XXIIb
Slovakia	Komárno (9)	0/3	-	-	-	-	-	-
	Šaľa (10)	1/5	31459	Yes (25,000)	*C. myocastoris*	*C. myocastoris*	*C. myocastoris*	XXIIa
	Dolný Ohaj (11)	0/7	-	-	-	-	-	-
	Topoľníky (12)	0/10	-	-	-	-	-	-
	Palárikovo (13)	1/6	-	-	-	-	-	-
	Nitrianský Hrádok (14)	0/5	-	-	-	-	-	-
	Dunajská Streda (15)	5/19	31123	No	*C. ubiquitum*	*C. ubiquitum*	*C. ubiquitum*	XIId
			31129	Yes (18,000)	*C. ubiquitum*	*C. ubiquitum*	*C. ubiquitum*	XIId
			31135	No	*C. ubiquitum*	*C. ubiquitum*	*C. ubiquitum*	XIId
			31136	No	*C. ubiquitum*	*C. ubiquitum*	*C. ubiquitum*	XIId
			31132	Yes (10,000)	*C. myocastoris*	*C. myocastoris*	*C. myocastoris*	XXIIa
	Vlčany (16)	0/6	-	-	-	-	-	-
	Diakovce (17)	0/1	-	-	-	-	-	-
	Lipové (18)	0/4	-	-	-	-	-	-

Numbers of localities correspond with numbers in Figure 1.

2.8. Clinical and Pathomorphological Examinations

A complete necropsy of two animals from each experimental group was performed at 10 and 20 DPI. Tissue specimens (oesophagus, stomach, duodenum, proximal, central and distal jejunum, ileum, caecum, colon, liver, spleen, kidney, bladder, and lung) from each animal were obtained using different sterile dissection tools for each location. Specimens from each organ were collected for PCR/sequencing, histology, and scanning electron microscopy (SEM). Histology sections were processed as reported by Kváč and Vítovec [36], and SEM sections were processed as described by Holubová et al. [11]. Histology sections were stained with haematoxylin and eosin (HE) and periodic acid–Schiff (PAS), examined at 100–400× magnification and documented using Olympus cell Sens Entry 2.1 (Olympus Corporation, Shinjuku, Tokyo, Japan) equipped with a digital camera (Olympus DP73). Specimens for SEM were examined using a JEOL JSM-7401F-FE scanning electron microscope equipped with a digital camera ETD Detector A PRED (Termo Fisher Scientific, Waltham, MA, USA).

2.9. Statistical Analysis

Differences in oocyst sizes were tested using Hotelling's multivariate version of the 2 sample *t*-test, *package ICSNP: Tools for Multivariate Nonparametrics* [37] in R 4.0.0. [38]. The hypothesis tested was that two-dimensional mean vectors of measurement are the same in the two populations being compared.

3. Results

In total, 72 and 78 faecal samples were examined from the Czech Republic and Slovakia, respectively (Table 1). None of the faecal samples had a consistency that would indicate diarrhoea. Examination of faecal smears revealed the presence of *Cryptosporidium* sp. oocysts in three samples, and infection intensity ranged from 10,000 to 25,000 OPG (Table 1). *Cryptosporidium*-specific DNA was detected in 11 samples by nested PCR targeting the *SSU* gene (Figure 2). From these positive samples, partial sequences of the genes encoding actin, *HSP*70 and *gp60* were amplified/sequenced. ML trees constructed from *SSU,* actin and *HSP70* sequences showed the presence of *C. parvum* (*n* = 1), *C. ubiquitum* (*n* = 5) and *Cryptosporidium myocastoris* n. sp. (*n* = 5). *Cryptosporidium myocastoris* n. sp., previously known as the *Cryptosporidium* coypu genotype, is described in detail as a new species later in this publication (Figures 2–4). There was no intraspecies variability in *SSU*, actin, and *HSP70* sequences from this study. Subsequent subtyping of *C. parvum* and *C. ubiquitum* at the *gp60* gene showed the presence of subtype families IIaA16G1R1 and XIId, respectively (Table 1, Figure 5). All *gp60* sequences from *C. ubiquitum* were identical (Figure 5). Two novel subtype families, which we have named XXIIa (*n* = 2) and XXIIb (*n* = 3), were detected within *C. myocastoris* n. sp. (Table 2, Figure 5).

Oocysts of *C. myocastoris* n. sp. recovered from naturally infected nutria (isolate 31132) were infectious for *Cryptosporidium*-free farmed nutria (nutria N0), which shed oocysts that were genetically and morphometrically identical to the inoculum, from five DPI (Table 2; Figures 2–5).

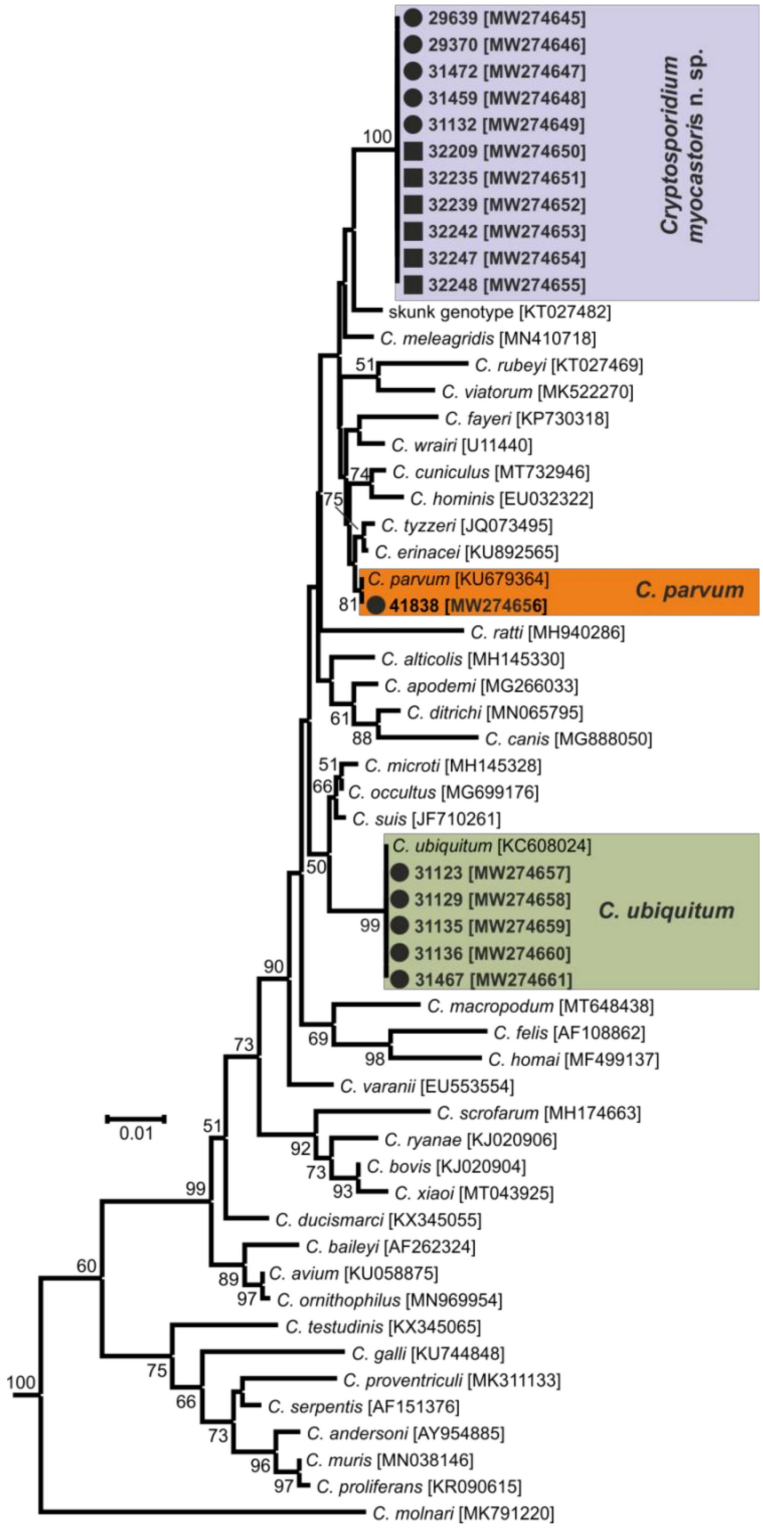

Figure 2. Maximum likelihood tree based on partial small subunit ribosomal RNA gene sequences of *Cryptosporidium* spp., including sequences obtained in this study (bolded and highlighted). The alignment contained 770 base positions in the final dataset. Numbers at the nodes represent the boot strap values with more than 50% boot strap support from 1000 pseudo replicates. The branch length scale bar, indicating the number of substitutions per site, is given in the tree. Sequences from this study are identified by an isolate number (e.g., 32247). Black circles and squares indicate natural and experimental infections, respectively.

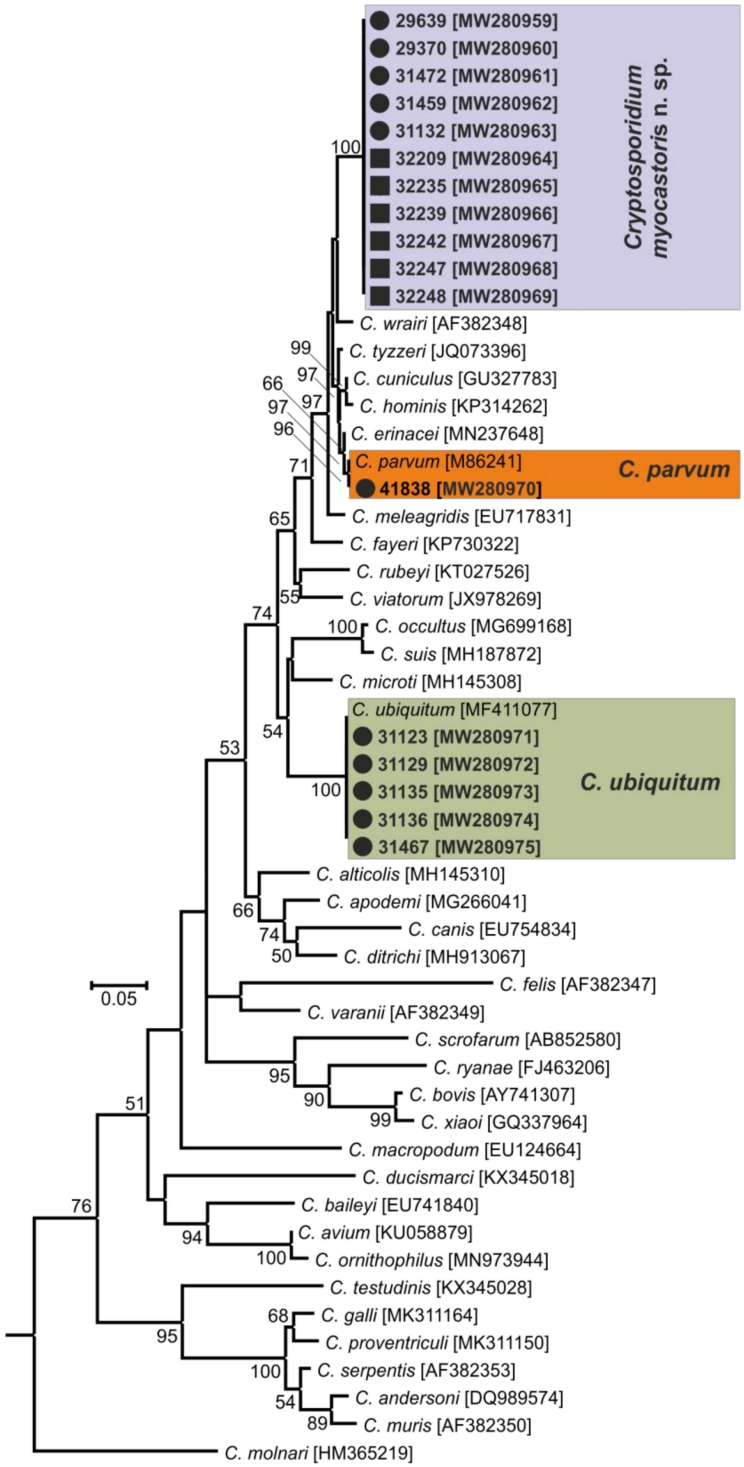

Figure 3. Maximum likelihood tree based on actin gene sequences of *Cryptosporidium* spp., including sequences obtained in this study (bolded and highlighted). The alignment contained 990 base positions in the final dataset. Numbers at the nodes represent the boot strap values with more than 50% boot strap support from 1000 pseudo replicates. The branch length scale bar, indicating the number of substitutions per site, is given in the tree. Sequences from this study are identified by an isolate number (e.g., 32247). Black circles and squares indicate natural and experimental infections, respectively.

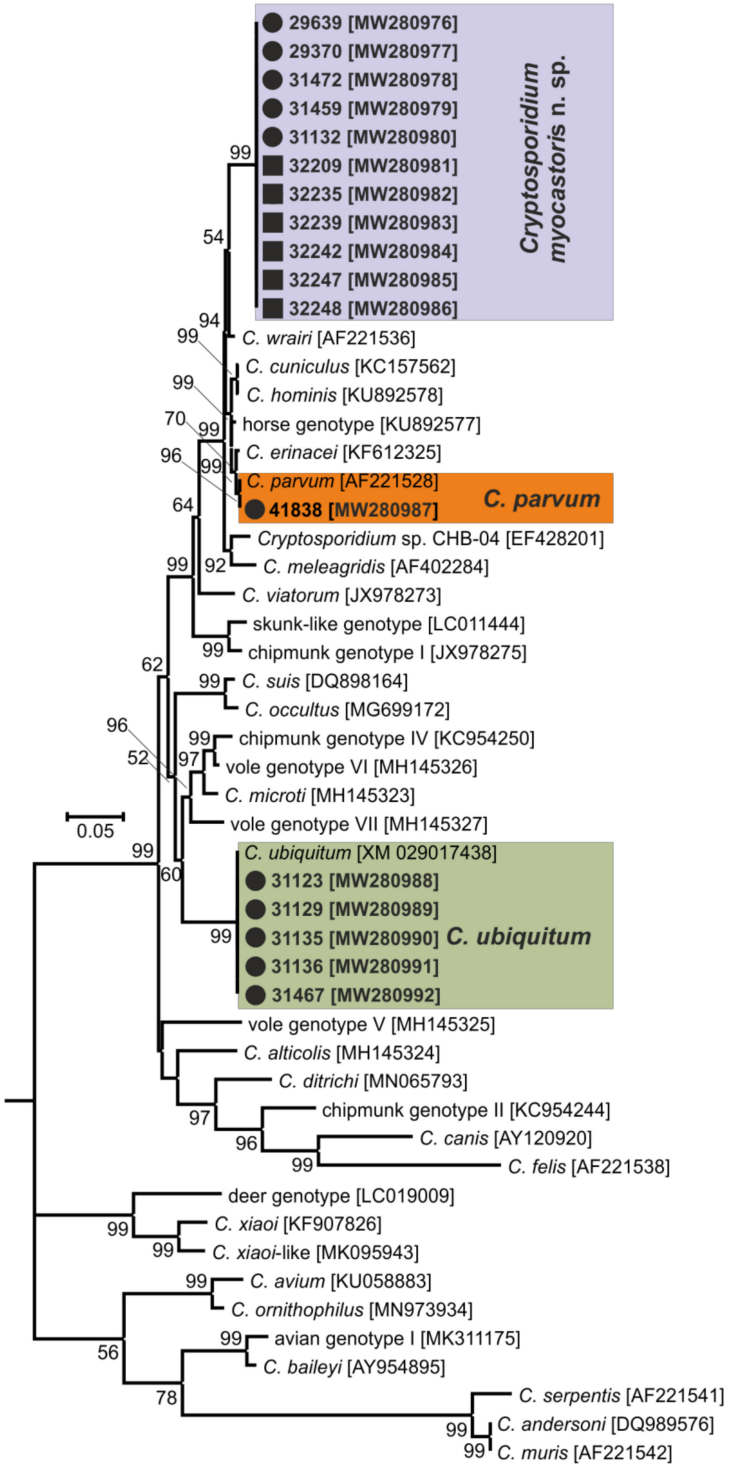

Figure 4. Maximum likelihood tree based on 70 kDa heat shock protein (*HSP70*) gene sequences of *Cryptosporidium* spp., including sequences obtained in this study (bolded and highlighted). The alignment contained 1172 base positions in the final dataset. Numbers at the nodes represent the boot strap values with more than 50% boot strap support from 1000 pseudo replicates. The branch length scale bar, indicating the number of substitutions per site, is given in the tree. Sequences from this study are identified by an isolate number (e.g., 32247). Black circles and squares indicate natural and experimental infections, respectively.

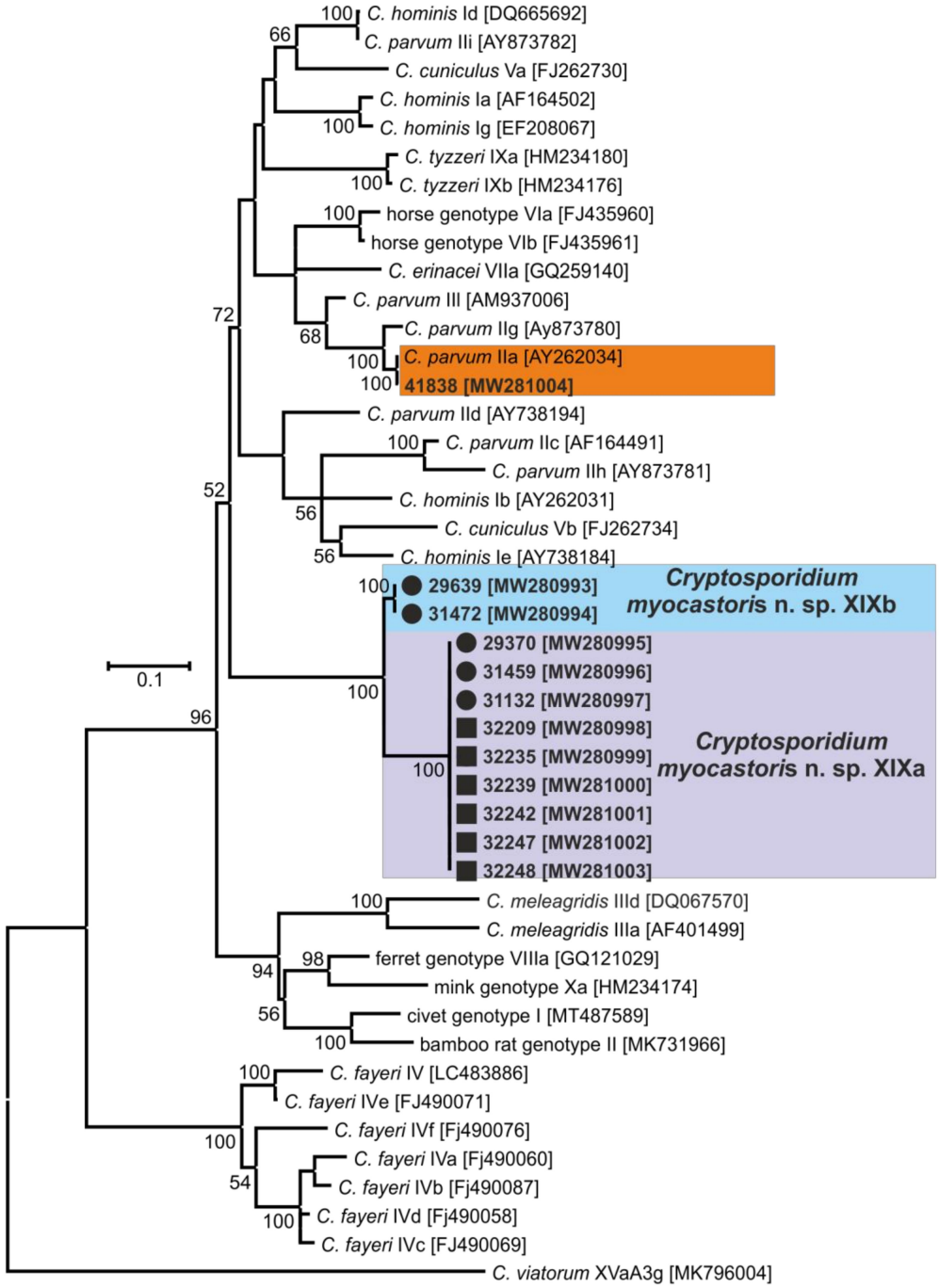

Figure 5. Maximum likelihood tree based on 60 kDa glycoprotein (*gp60*) gene sequences of *Cryptosporidium* spp., including sequences obtained in this study (bolded and highlighted). The alignment contained 1206 base positions in the final dataset. Numbers at the nodes represent the boot strap values with more than 50% boot strap support from 1000 pseudo replicates. The branch length scale bar, indicating the number of substitutions per site, is given in the tree. Sequences from this study are identified by an isolate number (e.g., 32247). Black circles and squares indicate natural and experimental infections, respectively.

Table 2. Size of *Cryptosporidium myocastoris* n. sp. oocysts recovered from naturally * and experimentally # infected nutrias (*Myocastor coypu*).

Isolate	Length (µm) Range (Mean ± SD)	Width (µm) Range (Mean ± SD)	Length/Width Ratio Range (Mean ± SD)
Nutria 31132 *	4.8–5.2 (5.02 ± 0.13)	4.7–5.0 (4.85 ± 0.10)	1.00–1.08 (1.04 ± 0.02)
Nutria 31459 *	4.8–5.3 (5.01 ± 0.14)	4.7–5.0 (4.81 ± 0.10)	1.00–1.06 (1.04 ± 0.01)
Nutria N0#	4.8–5.2 (5.00 ± 0.12)	4.7–5.0 (4.79 ± 0.09)	1.02–1.09 (1.04 ± 0.02)
Nutria N1#	4.8–5.3 (5.02 ± 0.14)	4.6–5.1 (4.85 ± 0.14)	1.02–1.07 (1.03 ± 0.01)

Note: Length and width of 30 oocysts from each isolate were measured under differential interference contrast at 1000× magnification, and out of these the length-to-width ratio of each oocyst was used to calculate.

Oocysts of *C. myocastoris* n. sp. from nutria N0 were infectious for five farmed nutrias (nutrias N1–N5), but not for one-week- and eight-week-old BALB/c and SCID mice, gerbils, or one-day-old chickens. All groups of nutrias, mice, gerbils, and chickens used as negative controls remained uninfected. Experimentally infected nutrias N1–N5 started to shed oocysts of *C. myocastoris* n. sp., detectable by light microscopy and PCR, at 5–6 DPI, and all animals remained infected until the end of the experiment (Figure 6). The infection intensity ranged from 2000 to 62,000 OPG. The highest infection intensity, 20,000–62,000 OPG, was observed from 6 to 11 DPI. Beginning on day 12 post-infection, all animals shed fewer than 10,000–15,000 OPG (Figure 6). None of the animals showed signs of cryptosporidiosis, and faecal consistency was appropriate to the age of the animal and the food intake. *Cryptosporidium myocastoris* n. sp. DNA and *Cryptosporidium* developmental stages were detected exclusively in posterior jejunum and ileum (Figures 7 and 8). Histology and SEM showed low infection intensity, with one or two developmental stages typically observed on an isolated villus in the posterior jejunum and ileum (Figures 7 and 8). This low infection intensity was consistent throughout the posterior jejunum and ileum. The brush border microvilli adjacent to attached developmental stages responded by elongation (Figure 8). The area of elongated microvilli increased with the size of the developmental stage, and the microvilli were elongated by up to 2 µm (Figure 8). The lamina propria in the jejunum and ileum was occasionally observed to be slightly oedematous, but these changes were probably not related to the *Cryptosporidium* infection.

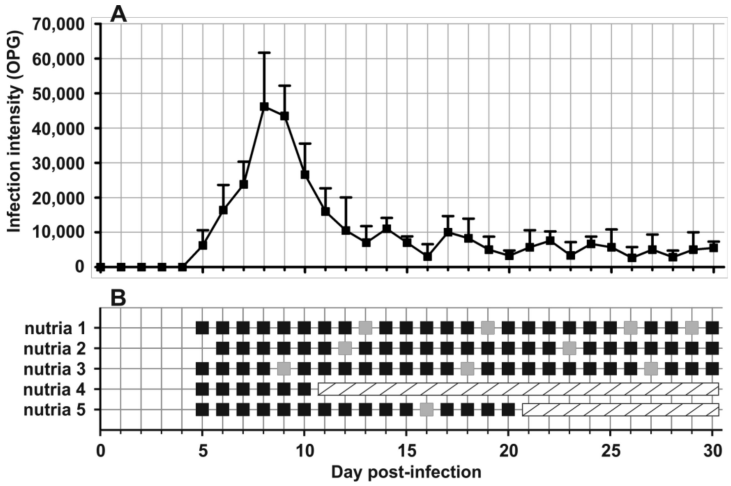

Figure 6. Course of infection of *Cryptosporidium myocastoris* n. sp. in experimentally inoculated nutria (*Myocastor coypu*). (**A**) Infection intensity expressed as number of oocysts per gram of faeces (OPG), and (**B**) detection of oocysts is based on molecular and microscopic examinations of faecal samples. Black squares indicate the presence of oocysts and specific *Cryptosporidium myocastoris* n. sp.; grey squares indicate the detection of specific DNA only without oocyst detection. Hatched rectangles indicate a missing animal due to sacrifice and dissection.

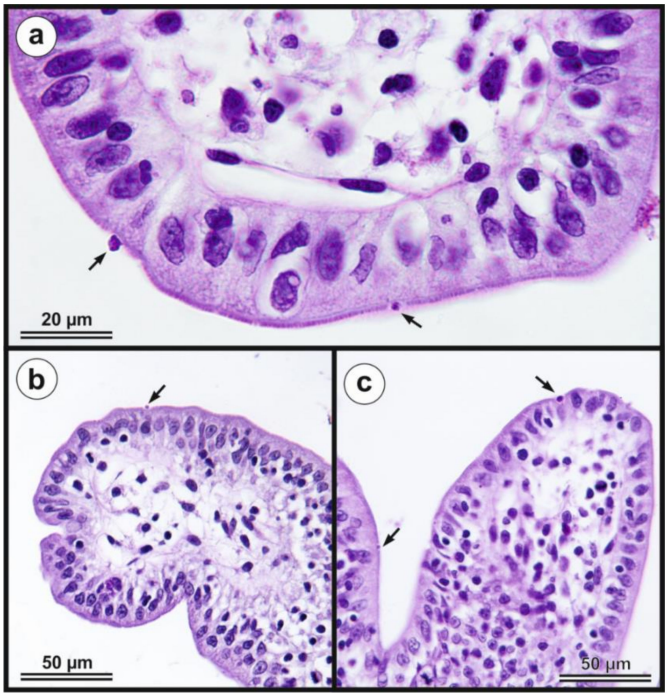

Figure 7. Histological sections stained by periodic acid–Schiff showing developmental stages of *Cryptosporidium myocastoris* n. sp. (arrow) on (**a**) jejunal and (**b,c**) ileal mucosal epithelium in experimentally infected adult nutria (*Myocastor coypu*) which was sacrificed 10 days post infection. Scale bar is included in each figure.

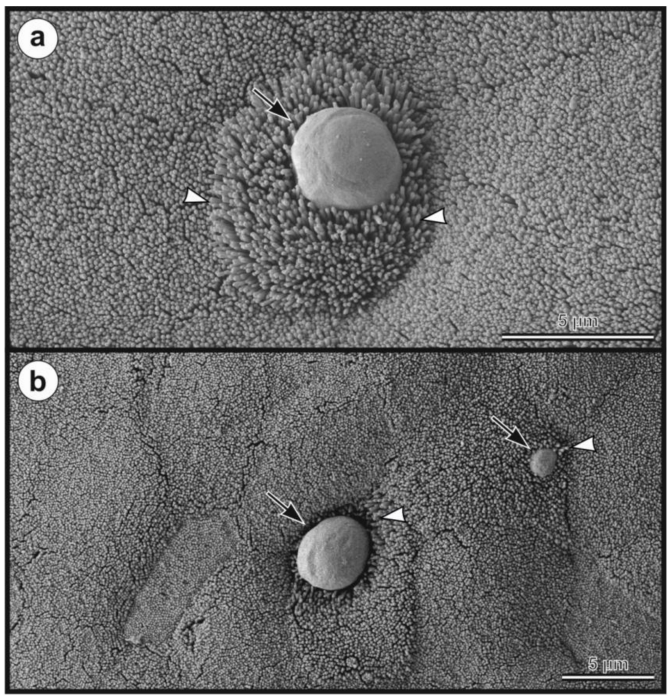

Figure 8. Scanning electron microphotograph showing developmental stages of *Cryptosporidium myocastoris* n. sp. (arrow) on (**a**) jejunal and (**b**) ileal mucosal epithelium in experimentally infected adult nutria (*Myocastor coypu*) which was sacrificed 10 days post infection. Elongation of the microvilli around attached developmental stage (arrowhead). Scale bar included in each figure.

Oocysts of *C. myocastoris* n. sp. recovered from experimentally infected nutrias (nutria N0 and N1) were morphometrically identical to those recovered from naturally infected nutrias (isolates 31132 and 31459; Table 2).

Taxonomic summary:
Family Cryptosporidiidae Léger, 1911
Genus *Cryptosporidium* Tyzzer, 1907
Cryptosporidium myocastoris n. sp.
Synonym: *Cryptosporidium* sp. coypu genotype ex *Myocastor coypus* of Kváč et al. [39], Slovakia.
Type-host: *Myocastor coypus* (Molina, 1782), nutria.
Other natural hosts: No other natural hosts are known.
Type-locality: Dunajská Streda (N 47°55.90470′, E 17°28.42662′), Slovakia.
Other localities: Lanžhot (N 48°43.41558′, E 16°58.30782′), Czech Republic; Šaľa (N 48°9.08273′, E 17°52.49152′), Slovakia.
Type-material: Faecal smear slides with oocysts stained by ACMV and ZN staining (nos. MV1/31132 and ZN2/31132); scanning electron microscopy specimens of infected jejunum (no. SEM23/2017) and ileum (no. SEM27/2017); histological sections of infected jejunum (no. H23/2017) and ileum (no. H27/2017); gDNA isolated from faecal samples of naturally (isolate 31132) and experimentally (isolate 32235) infected nutrias; gDNA isolated from jejunum and ileum of experimentally infected nutrias (isolates 32235 and 32236). All specimens are deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.
Site of infection: Posterior jejunum and ileum (present study, Figures 7 and 8).
Distribution: As *Cryptosporidium* sp. coypu genotype ex *Myocastor coypus*: Slovakia [39].
Prepatent period: *Myocastor coypus* 5–6 days (present study).
Patent period: At least 30 days in experimentally infected nutrias (*Myocastor coypus*; present study).
Representative DNA sequences: Representative nucleotide sequences of *SSU* [MW274649], actin [MW280963], *HSP*70 [MW280980] and *gp60* [MW280997 and MW280994] 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) [40], details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:FCAD0ED3-2DD0-4A79-93DD-D0C206EC6ACF. The LSID for the new name *Cryptosporidium myocastoris* n. sp. is urn:lsid:zoobank.org:act:E447F777-5495-4613-8447-D015339F6B32.
Etymology: The species name *myocastoris* is derived from the Latin noun myocastor, meaning nutria.
Description: Oocysts of *C. myocastoris* n. sp. (isolate 31132) are spherical, measuring 4.8–5.2 × 4.7–5.0 (5.02 ± 0.13 × 4.85 ± 0.10) with a length-to-width ratio of 1.00–1.08 (1.04 ± 0.02) (Figure 9). The oocyst wall is smooth and colourless (Figure 9a). The oocyst residuum is composed of numerous small granules and one spherical globule is clearly visible; a suture is not noticeable. Four sporozoites are clearly visible within oocysts. The morphology and morphometry of other developmental stages is unknown.
Remarks. Oocysts of *C. myocastoris* n. sp. are well stained by ACMV and ZN staining methods, similarly to other *Cryptosporidium* spp. (Figure 9b,c), and their oocyst walls cross-react with immunofluorescence reagents developed primarily for *C. parvum* (Figure 9d). Oocysts from naturally and experimentally infected nutrias did not differ in size ($T^2 = 0.16$, $df_1 = 2$, $df_2 = 121$, $p = 0.8506$; Table 2). Oocysts of *C. myocastoris* n. sp. are smaller than those of *C. parvum* ($T^2 = 33.11$, $df_1 = 2$, $df_2 = 48.15$, $p = p < 0.001$) and *C. ratti* ($T^2 = 33.22$, $df_1 = 2$, $df_2 = 45.96$, $p < 0.001$), but these differences are not of practical significance for identification (Table A1 in Appendix A). *Cryptosporidium myocastoris* n. sp. can be differentiated genetically from other *Cryptosporidium* species based on sequences of *SSU*, actin, and *HSP*70 genes. At the *gp60* locus, *C. myocastoris* n. sp. develops two well-supported clades. Pairwise distances between *Cryptosporidium myocastoris* n. sp. and the

selected closest and furthest *Cryptosporidium* species at *SSU*, actin, and *HSP*70 genes are shown in Table A2.

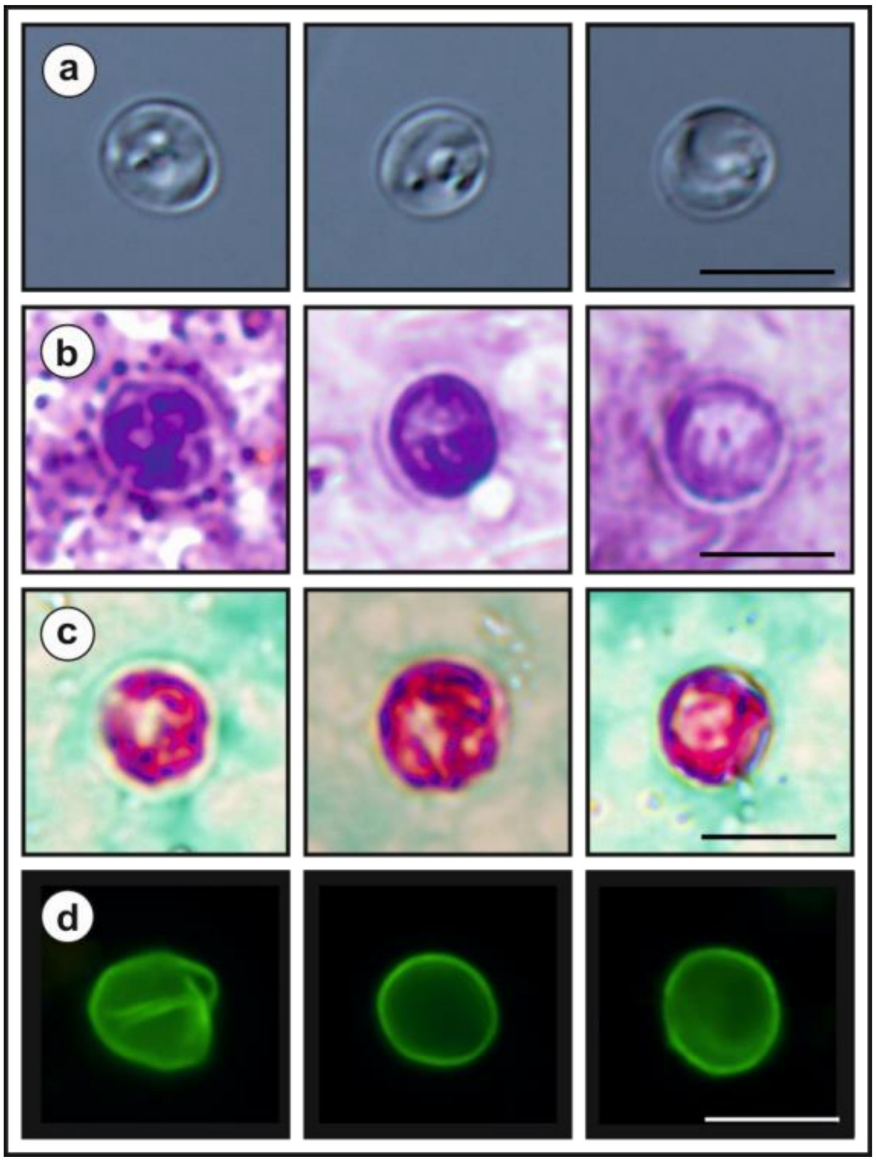

Figure 9. Oocysts of *Cryptosporidium myocastoris* n. sp. (**a**) in differential interference contrast microscopy, (**b**) stained by aniline–carbol–methyl violet staining, (**c**) stained by Ziehl–Nielsen staining, and (**d**) labelled with anti-*Cryptosporidium* FITC-conjugated antibody. Bar = 5 μm.

4. Discussion

The work presented here represents the most comprehensive study to date on *Cryptosporidium* infecting feral nutrias, and a novel, nutria-adapted *Cryptosporidium* species is described. The prevalence of *Cryptosporidium* in nutrias was relatively low (7.3%). In a study of 108 wild nutrias in Argentina, the prevalence of *Cryptosporidium* infection was similarly low (3.7%), although oocysts were detected by microscopy, which is less sensitive than PCR [15]. The prevalence of *Cryptosporidium* in other aquatic rodents also appears to be low. A study of 145 capybaras (*Hydrochoerus hydrochaeris*) in Brazil reported a prevalence of 5.5% [41]. Zhou et al. [42] did not detect *Cryptosporidium* in any of the 84 North American beavers (*Castor canadensis*) they tested in the United States, but they did report that almost 12% of 237 muskrats (*Ondatra zibethicus*) were positive. Paziewska et al. [43] reported that 7.7% of 22 European beavers (*Castor fiber*), sampled in Poland, had *Cryptosporidium* antigen in their faeces. Considering that *Cryptosporidium* infections are frequently associated with

transmission through contaminated water [44,45], the low prevalence in nutrias and other water rodents might be surprising. Future studies should address how *Cryptosporidium* spp. are transmitted in aquatic mammals: is waterborne transmission important, or is it some other route such as contaminated food or direct contact among individuals? In this context, it is noteworthy that *C. myocastoris* n. sp. has not been reported in any of the studies on the occurrence of *Cryptosporidium* spp. in surface water [46]. This absence may be explained by (i) the low prevalence within the population of nutrias together with the low infection intensity; or (ii) the limited number of studies reporting water contamination in the areas where nutrias occur.

This is the first study to genotype *Cryptosporidium* from nutrias. Martino et al. [15] found *Cryptosporidium* sp. oocysts (4 to 4.5 × 4.0 µm) in faecal samples from the colon and rectum of wild nutrias in Argentina. An earlier study reported *C. parvum* in nutrias based on oocyst morphology [15,20]. Given that *C. parvum* has a broad host specificity, is reported infrequently in wildlife species [5,6,47–50], and was found in a single nutria in the present study, it is possible that Pavlásek and Kozakiewicz [20] correctly identified the species. In support, they found that oocysts from naturally infected nutria were infectious for four-day-old laboratory mice under experimental conditions, a characteristic of *C. parvum* but not *C. myocastoris* (as we have shown in the present study). However, based on our current knowledge, oocyst morphology cannot reliably distinguish among intestinal species of *Cryptosporidium* [51], and the size of oocysts reported by Pavlásek and Kozakiewicz [20] (5.0 × 4.75 µm) is similar to *C. myocastoris* n. sp. (5.02 × 4.85 µm) and *C. ubiquitum* (5.04 × 4.66 µm), two species that we found to be more prevalent than *C. parvum* (5.19 × 4.9 µm) in nutrias.

The finding of *C. ubiquitum* and *C. myocastoris* n. sp. in several animals at different localities suggests that these species are common in feral nutrias. *Cryptosporidium ubiquitum*, a species with broad host specificity, has been reported in domestic and wild ruminants, rodents, carnivores, and human and non-human primates [52–56]. The *C. ubiquitum gp60* subtype family XIId in nutrias has been reported in humans, macaques, red deer, raccoons, woodchucks, chinchillas, and mink [18,29,56–59], suggesting that it is broadly specific. In contrast, *C. myocastoris* n. sp. appears to have a narrow host specificity. The origin of *C. myocastoris* n. sp. in nutria in central Europe is difficult to elucidate without further studies, but it may have been introduced into Europe with imported nutrias, similarly to the *Cryptosporidium* skunk genotype, which was likely introduced to Europe with eastern grey squirrels [60]. The specificity of *C. myocastoris* n. sp. for nutrias is supported by its presence in geographically isolated nutrias, its infectivity for nutrias under experimental conditions, and the absence of any record of this species in any of the thousands of molecular epidemiological studies published in last two decades [46,51,61,62]. For these reasons, it is most likely that nutrias are the major host of *C. myocastoris* n. sp., although we cannot exclude the possibility that other host species have a role as major or minor hosts.

Adaptation to one host species is not unique within the genus *Cryptosporidium*, and several mammalian *Cryptosporidium* spp. have been reported almost exclusively in a single host. Examples include *C. wrairi* and *C. homai* in guinea pigs (*Cavia porcellus*), *C. scrofarum* and *C. suis* in pigs (*Sus scrofa*), *C. bovis* in cattle (*Bos taurus*), *C. occultus* in rats (*Rattus* spp.), and *C. macropodum* in kangaroos [63–67]. The findings of these species in other hosts represent rare cases or mechanical passage rather than host adaptation [68–70].

The prepatent period (5–6 DPI) is consistent with other intestinal *Cryptosporidium* spp. that are specific for rodents, such as *C. ratti* (4–5 DPI) in rats, *C. alticolis* in voles (3–4 DPI), *C. tyzzeri* in mice (4–7 DPI), or other mammals, such *C. parvum* in calves (2–7 DPI) and *C. scrofarum* in pigs (4–6 DPI) [6,12,63,66,71,72].

Nutrias positive for *C. parvum* or *C. ubiquitum* did not have detectable oocysts in their faeces, suggesting a low level of infection, which is consistent with our previous finding [39]. Three out of five nutrias naturally infected with *C. myocastoris* n. sp. shed oocysts at levels between 10,000 and 20,000 OPG, and similar numbers were detected in experimentally infected nutrias. No macroscopic changes in the intestinal mucosa were observed at necropsy of *C. myocastoris* positive nutrias. The low level of oocyst shedding was consistent with the intensity of developmental stages detected in the intestinal epithelium and the absence of clinical symptoms. A similar relationship between intestinal involvement and oocyst shedding has been observed in other *Cryptosporidium* spp. infecting the small intestine [6,12,73–76]. Examination by scanning electron microscopy shows the elongation of the microvilli around the *C. myocastoris* developmental stages, which has also been previously observed in SCID mice infected with *C. parvum* [77]. Borowski et al. [78] reported the elongation of microvilli on the gliding trails of *C. parvum* sporozoites between an excysted oocyst and newly formed trophozoites. While the extending of microvilli in Borrowski's study was up to 15 µm, we observed much less extension. Clinical symptoms are rarely, if ever, observed in wild animals infected with host-specific *Cryptosporidium* species [79–81].

Equivalently to the host specificity, most *Cryptosporidium* species are characterized by adaptation to a different part of the digestive tract. Gastric *Cryptosporidium* of mammals exclusively infect the glandular part of the glandular stomach, whereas intestinal species are adapted to different parts of the small or large intestine. Similar organ adaptation has been reported in *Eimeria* spp. [82]. AS with *C. ratti* or *C. scrofarum*, the life cycle of *C. myocastoris* n. sp. is located in the posterior jejunum and ileum [12,66]. No developmental stages were found in the large intestine where the life cycle of, e.g., *C. occultus*, *C. suis* or *C. ornithophilus* is located [63,65,81].

For species-level differentiation of *Cryptosporidium*, the *SSU* marker has served well for more than 20 years [83]. However, *Cryptosporidium*, similarly to the related apicomplexans *Plasmodium* and *Eimeria,* has divergent paralogous copies of the SSU gene [84–88]. Our previous work has shown that only using sequences of SSU to infer evolutionary relationships of *Cryptosporidium* may lead to erroneous conclusions [5,29,89]. Therefore, it is necessary to use other polymorphic loci, such as *HSP70*, actin, or *COWP* genes, in phylogenetic analyses [5,85]. Although bootstrap support for the *SSU* tree was lower than for the actin and *HSP70* trees in this study, *C. myocastoris* n. sp. formed a separate clade in *SSU*, actin and *HSP70* ML trees, with the most closely related group comprising species such as *C. parvum*, *C. cuniculi*, and *C. wrairi*. At the *SSU* locus, the pairwise distance between *C. myocastoris* n. sp. and *C. parvum* (0.014) or *C. cuniculi* (0.018) is similar to that between *C. apodemi* and *C. occultus* (0.016) or *C. andersoni* and *C. serpentis* (0.019). At the actin locus, the distance between *C. myocastoris* n. sp. and *C. parvum*/*C. cuniculi* (0.039) is similar to that between *C. meleagridis* and *C. erinacei* (0.034). Comparably, at the *HSP70* locus, the distance between *C. myocastoris* n. sp. and *C. parvum*/*C. cuniculi* (0.035/0.031) is similar to the distance between *C. meleagridis* and *C. erinacei* (0.041) or *C. ornithophilus* and *C. avium* (0.035). These results support the genetic uniqueness of *C. myocastoris* n. sp. and their status as a separate species of genus *Cryptosporidium*.

5. Conclusions

In summary, the findings that *Cryptosporidium* sp. coypu genotype is genetically distinct from all described *Cryptosporidium* species and specific for nutrias under natural and experimental conditions support its description as a new species, and we propose that it be named *Cryptosporidium myocastoris* n. sp.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Biology Centre of CAS (protocol code 35/2018 and 60/2019 and approved 04/03/2018 and 10/05/2019, respectively).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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Conflicts of Interest: None of the authors has any competing interests in the manuscript.

Appendix A

Table A1. Oocyst sizes of valid species of the genus *Cryptosporidium*.

Species	Oocyst Size (μm)	Length/Width Ratio	Reference
Cryptosporidium myocastoris	4.8–5.2 × 4.7–5.0	1.00–1.08	This study
Cryptosporidium abrahamseni	3.82 ± 0.22 × 3.16 ± 0.18	1.22	[13]
Cryptosporidiumalticolis	4.9–5.7 × 4.6–5.2	1.00–1.20	[6]
Cryptosporidiumandersoni	6.0–8.1 × 5.0–6.5	1.07–1.50	[90]
Cryptosporidiumapodemi	3.9–4.7 × 3.8–4.4	1.00–1.06	[76]
Cryptosporidiumavium	5.3–6.9 × 4.3–5.5	1.14–1.47	[91]
Cryptosporidiumbaileyi	6.0–7.5 × 4.8–5.7	1.05–1.79	[92,93]
Cryptosporidium bollandi	2.82–3.11	unknown	[94]
Cryptosporidiumbovis	4.76–5.35 × 4.17–4.76	1.06	[64]
Cryptosporidiumcanis	3.68–5.88 × 3.68–5.88	1.04–1.06	[95]
Cryptosporidiumcichlidis	4.0–4.7 × 2.5–3.5	unknown	[96]
Cryptosporidiumcuniculus	5.55–6.40 × 5.02–5.92	1.11	[97]
Cryptosporidiumditrichi	4.5–5.2 × 4.0–4.6	1.0–1.2	[76]
Cryptosporidiumducismarci	4.4–5.4 × 4.3–5.3	1.1 ± 0.03	[98]
Cryptosporidiumerinacei	4.5–5.8 × 4.0–4.8	1.02–1.35	[73]
Cryptosporidiumfayeri	4.5–5.1 × 3.8–5.0	1.02–1.18	[99]
Cryptosporidium felis	5.0 × 4.5	unknown	[100]
Cryptosporidiumfragile	5.5–7.0 × 5.0–6.5	1.0–1.3	[101]
Cryptosporidiumgalli	8.0–8.5 × 6.2–6.4	1.3	[102]
Cryptosporidiumhominis	4.4–5.4 × 4.4–5.9	1.01–1.09	[103]
Cryptosporidiumhuwi	4.4–4.9 × 4.0–4.8	0.92–1.35	[9]
Cryptosporidiummacropodum	4.5–6.0 × 5.0–6.0	1.1	[67]
Cryptosporidiummeleagridis	4.5–6.0 × 4.2–5.3	1.00–1.33	[93,104]
Cryptosporidiummicroti	3.9–4.7 × 3.8–4.4	1.00–1.06	[6]
Cryptosporidiummolnari	3.23–5.45 × 3.02–5.04	1.00–1.17	[105]
Cryptosporidiummuris	6.6–7.9 × 5.3–6.5	1.1–1.5	[106]
Cryptosporidiumnasoris	3.6	unknown	[107]
Cryptosporidiumoccultus	4.66–5.53 × 4.47–5.44	1.00–1.17	[63]
Cryptosporidiumornithophilus	5.24–6.77 × 4.68–5.50	1.06–1.36	[81]

Table A1. *Cont.*

Species	Oocyst Size (μm)	Length/Width Ratio	Reference
Cryptosporidiumparvum	4.5–5.4 × 4.5–5.4	1.0–1.3	[108]
Cryptosporidiumproliferans	6.8–8.8 × 4.8–6.2	1.48	[109]
Cryptosporidiumproventriculi	6.70–8.40 × 5.10–6.3	1.08–1.41	[11]
Cryptosporidium ratti	4.4–5.4 × 4.3–5.1	1.0–1.1	[12]
Cryptosporidiumreichenbachklinkei	2.4–3.18 × 2.4–3.0	unknown	[96]
Cryptosporidiumrubeyi	4.4–5.0 × 4.0–5.0	1.08	[110]
Cryptosporidiumryanae	2.94–4.41 × 2.94–3.68	1.18	[111]
Cryptosporidiumscophthalmi	3.7–5.03 × 3.03–4.69	1.05–1.34	[112]
Cryptosporidiumscrofarum	4.81–5.96 × 4.23–5.29	1.07 ± 0.06	[66]
Cryptosporidiumserpentis	6.3 × 5.5	1.14 ± 0.11	[113]
Cryptosporidiumsuis	6.0–6.8 × 5.3–5.7	1.14	[65]
Cryptosporidiumtestudinis	5.8–6.9 × 5.3–6.5	1.1 ± 0.05	[98]
Cryptosporidium tyzzeri	4.64 ± 0.05 × 4.19 ± 0.06	1.11 ± 0.06	[71]
Cryptosporidiumubiquitum	4.71–5.32 × 4.33–4.98	1.08	[79]
Cryptosporidiumvaranii	4.8–5.1 × 4.4–4.8	1.03 ± 0.03	[114]
Cryptosporidiumviatorum	4.87–5.87 × 4.15–5.20	1.03–1.32	[115]
Cryptosporidiumwrairi	4.0–5.0 × 4.8–5.6	unknown	[61]
Cryptosporidiumxiaoi	2.94–4.41 × 2.94–4.41	1.15	[116]

Table A2. Pairwise distances between *Cryptosporidium myocastoris* n. sp. and selected closest and furthest *Cryptosporidium* species at small subunit ribosomal RNA (*SSU*), actin, and 70 kDa heat- shock protein (*HSP*70) genes.

Species/Genotype	Gene Locus		
	SSU	**Actin**	***HSP70***
C. andersoni	0.090	0.231	0.268
C. avium	0.045	0.175	0.215
C. baileyi	0.051	0.181	0.202
C. bovis	0.042	0.202	0.180
C. canis	0.043	0.167	0.213
C. cuniculi	0.018	0.039	0.031
C. felis	0.054	0.222	0.212
C. galli	0.094	0.206	0.252
C. hominis	0.023	0.044	0.031
C. muris	0.090	0.209	0.268
C. occultus	0.021	0.122	0.031
C. parvum	0.014	0.039	0.035
C. rubeyi	0.033	0.098	0.085
C. ryanae	0.045	0.222	0.186
C. suis	0.023	0.118	0.095
C. ubiquitum	0.035	0.108	0.109
C. xiaoi	0.048	0.205	0.201
C. wrairi	0.018	0.036	0.028

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6.4 Příloha IV

Cryptosporidium sciurinum n. sp. (Apicomplexa: Cryptosporidiidae) in Eurasian Red Squirrels (_Sciurus vulgaris_).

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Cryptosporidium sciurinum n. sp. (Apicomplexa: Cryptosporidiidae) in Eurasian Red Squirrels (*Sciurus vulgaris*)

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Abstract: *Cryptosporidium* spp. are common protozoan pathogens in mammals. The diversity and biology of *Cryptosporidium* in tree squirrels are not well studied. A total of 258 Eurasian red squirrels (*Sciurus vulgaris*) from 25 and 15 locations in the Czech Republic and Slovakia, respectively, were examined for *Cryptosporidium* spp. oocysts and specific DNA at the *SSU*, actin, *HSP70*, *TRAP-C1*, *COWP*, and *gp60* loci. Out of 26 positive animals, only juveniles (9/12) were microscopically positive (18,000 to 72,000 OPG), and molecular analyses revealed the presence of *Cryptosporidium* sp. ferret genotype in all specimens. Oocysts obtained from naturally-infected squirrels measured 5.54–5.22 µm and were not infectious for laboratory mice (BALB/c and SCID), Mongolian gerbils, Guinea pigs, Southern multimammate mice, chickens, or budgerigars. None of naturally infected squirrels showed clinical signs of disease. The frequency of occurrence of the ferret genotype in squirrels did not vary statistically based on host age, gender or country of capture. Phylogenetic analysis of sequences from six loci revealed that *Cryptosporidium* sp. ferret genotype is genetically distinct from the currently accepted *Cryptosporidium* species. Morphological and biological data from this and previous studies support the establishment of *Cryptosporidium* sp. ferret genotype as a new species, *Cryptosporidium sciurinum* n. sp.

Keywords: occurrence; biology; course of infection; infectivity; oocyst size; phylogeny; *Cryptosporidium* sp. ferret genotype

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1. Introduction

Cryptosporidium is a genus of single-cell protist parasites that infect the gastrointestinal, respiratory, and/or the urogenital tract of most vertebrates, including humans, causing the disease cryptosporidiosis [1]. The course of infection and severity of the disease depends on a host factors such as age, competence and maturity of the immune system, condition, the presence of secondary infections, and on the characteristics of the *Cryptosporidium* species or genotype [2]. Most of the species validly described so far (48) are narrowly host specific, and only a small number, including *C. parvum*, *C. ubiquitum*, and *C. baileyi*, have a broad host range [3,4]. In addition to the validly described species, dozens of genotypes have been described that lack sufficient data to justify a species designation. Genotypes are typically named for the host from which the novel *Cryptosporidium* DNA sequence is first identified. For example, *C. suis* was originally described as the *Cryptosporidium* pig genotype because it was first identified in a pig [5]. New genotypes are most frequently

described from partial sequences of the small subunit rRNA gene. The squirrel, family *Sciuridae*, is one of the most diverse and widely distributed families of mammals, and they host several species and genotypes of *Cryptosporidium*, some of which are infectious to humans and can cause severe and life-threatening diarrhoea [6].

Tree squirrels diverged from ground squirrels about 30 million years ago, and this polyphyletic tribe comprises hundreds of extant species. *Cryptosporidium* was first reported in a tree squirrel in 1982, when Sundberg et al. [7] isolated oocysts from an eastern grey squirrel (*Sciurus carolinensis*). Current [8] subsequently reported *Cryptosporidium* oocysts in a fox squirrel (*Sciurus niger*). Both authors identified the isolates as *C. parvum*, but this was likely incorrect, based on our present knowledge. We now know that several distinct *Cryptosporidium* species and genotypes with overlapping oocyst sizes infect tree squirrels. Four species and five genotypes of *Cryptosporidium* have been described in tree squirrels inhabiting North America: *C. ubiquitum*, *Cryptosporidium* sp. chipmunk genotype I, deer mouse genotype III, and skunk genotype in the American red squirrel (*Tamiasciurus hudsonicus*); *C. baileyi*, *C. muris*, *C. ubiquitum*, *Cryptosporidium* sp. chipmunk genotype I, deer mouse genotype III, and skunk genotype in the Eastern grey squirrel (*Sciurus carolinensis*); *C. ubiquitum* in the Fox squirrel (*Sciurus niger*) [7,9–12]. Fewer studies have reported on *Cryptosporidium* in tree squirrels outside of North America. In China, *C. parvum*, *C. wrairi*, and *Cryptosporidium* sp. rat genotype II have been detected in wild Pallas's squirrels (*Callosciurus erythraeus*), and *C. parvum*, *C. ratti*, *Cryptosporidium* sp. rat genotype II, ferret genotype, and chipmunk genotype III have been reported in Eurasian red squirrels [13–15]. In Europe, native Eurasian red squirrels are almost exclusively infected with *Cryptosporidium* sp. ferret genotype [16,17], although other *Cryptosporidium* species and genotypes, such as *C. ubiquitum*, *Cryptosporidium* sp. skunk genotype, and chipmunk genotype I, have been reported in areas where the Eurasian red squirrel is threatened by invasive, non-native species from Asia and North America [16,17]. It can be concluded from the literature that Eurasian red squirrels primarily host *Cryptosporidium* sp. ferret genotype, whereas the *C. ubiquitum* and *Cryptosporidium* skunk genotypes are most common in North American squirrels [7,9–17].

This study aimed to describe the biological, morphological, and genetic characteristics of *Cryptosporidium* sp. ferret genotype from Eurasian red squirrels in Central Europe. Our data show that the ferret genotype is the major *Cryptosporidium* species infecting Eurasian red squirrels in this region, and it is genetically and biologically distinct from valid *Cryptosporidium* species. We therefore propose that it be named *Cryptosporidium sciurinum* n. sp.

2. Materials and Methods

2.1. Ethics Statement

Traps were checked at least twice per day and handling time was minimized to reduce animal stress; all applicable international, national, and institutional guidelines for the care and use of animals were followed. Permits for trapping and handling squirrels and for the transmission study complied with the laws of the Czech Republic (Act No. 246/1992 Coll., on the protection of animals against cruelty). The study design was approved by the ethical committees at the Biology Centre of CAS, the State Veterinary Administration, and the Central Commission for Animal Welfare under protocol Nos. MZP/2019/603/1411 and 35/2018.

2.2. Trapping and Specimen Collection

Faecal samples were collected between June 2019 and June 2021 from Eurasian red squirrels at 25 locations, including seven rescue stations in the Czech Republic and 15 locations in Slovakia (Figure 1). A total of 265 faecal samples were obtained from 258 Eurasian red squirrels. Of these, 147 samples were from squirrels trapped in the wild and 111 were from animals housed at rescue stations. Two squirrels kept at a rescue station (nos. 45901 and 51489) were screened repeatedly. Wild squirrels were live-captured in a

Sherman box baited with a mixture of nuts and seeds. Each trapped squirrel was placed into a wire-mesh handling cone to minimize stress during handling and the sex, age, and body condition were recorded following [18]. The presence of any faecal material on the fur around the rectum was also recorded. After release of the trapped animal, faecal samples were collected from traps and individually placed in a sterile plastic tube, which was stored at 4–8 °C until subsequent processing. The consistency of faecal samples was recorded at the time of collection. A faecal smear was prepared from each sample, stained with aniline-carbol-methyl violet (ACMV) [19], and examined for the presence of *Cryptosporidium* spp. oocysts. Oocysts were quantified and the infection intensity was estimated using the method described by Kváč et al. [20]. Briefly, the slide was weighed to the nearest 0.001 g before and after preparation of the smear to determine the mass of faecal material added to the slide. After ACMV staining, all oocysts on the slide were counted and the number of oocysts per gram of faeces was calculated. Oocysts were enumerated from triplicate smears prepared from each sample. All samples were subsequently screened in triplicate for the presence of *Cryptosporidium*-specific DNA by PCR/sequencing of a fragment of the small subunit rRNA gene (SSU). If *Cryptosporidium*-specific DNA was detected, genotyping at other loci was performed as described below.

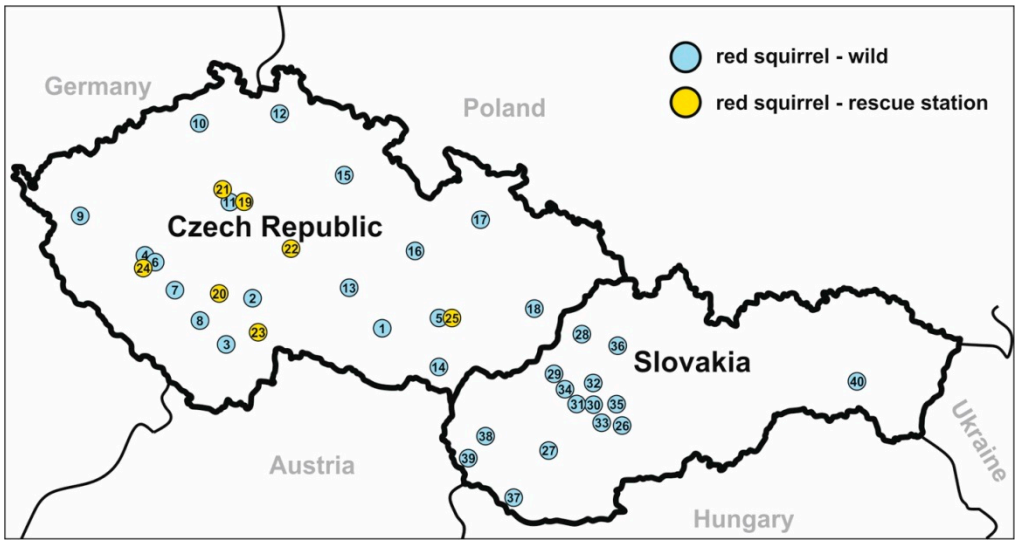

Figure 1. Sampling localities in the Czech Republic and Slovakia: For each site, the number indicates the name of locations (**1**) Třebíč; (**2**) Brandlín; (**3**) České Budějovice; (**4**) Plzeň; (**5**) Brno; (**6**) Starý Plzenec; (**7**) Chocenice; (**8**) Strakonice; (**9**) Mariánské Lázně; (**10**) Ústí nad Labem; (**11**) Praha; (**12**) Liberec; (**13**) Jihlava; (**14**) Břeclav; (**15**) Jaroměř; (**16**) Svitavy; (**17**) Bruntál; (**18**) Valašské Meziříčí; (**19**) Jinonice; (**20**) Makov; (**21**) Praha; (**22**) Vlašim; (**23**) Třeboň; (**24**) Plzeň; (**25**) Brno; (**26**) Zvolen; (**27**) Nitra; (**28**) Považská Bystrica; (**29**) Trenčín; (**30**) Handlová; (**31**) Prievidza; (**32**) Turčianské Teplice; (**33**) Žiar nad Hronom; (**34**) Bánovce nad Bebravou; (**35**) Kremnica; (**36**) Martin; (**37**) Gabčíkovo; (**38**) Pezinok; (**39**) Bratislava; (**40**) Košice.

2.3. *Source of Oocysts of Cryptosporidium* sp. *Ferret Genotype*

An isolate of *Cryptosporidium* sp. ferret genotype was obtained from a juvenile Eurasian red squirrel at a rescue station (isolate 45901, locality no. 23). Faecal samples from this squirrel were individually collected into sterile 50 mL vials with a few drops of dH_2O and stored at 4–8 °C. Oocysts were purified using caesium chloride gradient centrifugation [21] and used for morphometry and transmission studies (described below). Oocyst viability was examined following propidium iodide (PI) staining by a modified assay of Sauch et al. [22]. The oocysts were stored in PBS at 4–8 °C for a maximum of 4 weeks.

2.4. Molecular Characterization

Total genomic DNA (gDNA) was extracted from 5000 purified oocysts, 200 mg of faeces or 200 mg of tissue (see Clinical and Pathomorphological Examinations) by bead disruption (all sample types) 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 Exgene™ Stool DNA mini (GeneAll Biotechnology Co. Ltd., Seoul, Korea) or DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Purified DNA was stored at −20 °C prior to amplification by PCR. Nested-PCR protocols were used to amplify partial sequences of genes encoding *SSU*, actin, the 70 kDa heat shock protein (*HSP70*), the thrombospondin-related adhesive protein of *Cryptosporidium*-1 (*TRAP-C1*), *Cryptosporidium* oocyst wall protein (*COWP*), and the 60 kDa glycoprotein (*gp60*) using previously published protocols and primers [23–29]. Negative (molecular grade water) and positive controls (DNA of *C. tyzzeri* subtype family XIa) were included in each PCR amplification.

2.5. Sequence and Phylogenetic Analysis

Secondary PCR products were purified using Gen Elute Gel Extraction Kit (Sigma, St. Louis, MO, USA) and sequenced in both directions using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) using secondary PCR primers according to the manufacturer's instructions in a commercial laboratory (SEQme, Dobříš, Czech Republic). Sanger sequencing chromatogram files were edited using the ChromasPro 2.1.8 software (Technelysium, Pty, Ltd., South Brisbane, Australia), the obtained nucleotide sequences of each gene were aligned with each other and with reference sequences from GenBank (https://www.ncbi.nlm.nih.gov, accessed on 9 August 2021) using MAFFT version 7 online server (http://mafft.cbrc.jp/alignment/software/, accessed on 9 August 2021) and manually edited and trimmed using BioEdit v.7.0.5 [30]. Maximum likelihood (ML) and Neighbor-joining (NJ) trees were constructed using the Molecular Evolutionary Genetics Analysis (MEGAX) software after computing the most appropriate evolutionary models and values of all parameters for each model. Bootstrap support for branching was based on 1000 replications. Estimates of pairwise distances between species as the number of base substitutions per site from between sequences were calculated in MEGAX. Sequences have been deposited in GenBank under the accession numbers MZ726453–MZ726454 (*SSU*), MZ772035–MZ772036 (actin), MZ772046–MZ772047 (*HSP70*), MZ772037–MZ772038 (*TRAP-C1*), MZ772044–MZ772045 (*COWP*), and MZ772039–MZ772042 (*gp60*).

2.6. Oocyst Morphometry

The length and width of *Cryptosporidium* sp. ferret genotype oocysts (n = 50) was determined using a digital analysis of images (Olympus cellSens Entry 2.1 software, Olympus Corporation, Shinjuku, Tokyo, Japan) collected using an Olympus Digital Colour Camera DP73 (Olympus), using differential interference contrast (DIC) microscopy at 1000× magnification (Olympus IX70, Tokyo, Japan). These measurements were used to calculate the mean length, width, and the length-to-width ratio. Oocyst size was measured using the same microscope and by the same person. Faecal smears with oocysts of *Cryptosporidium* sp. ferret genotype were stained by modified ACMV, Ziehl–Neelsen (ZN; [31]) and labelled with a Cy3-labelled mouse monoclonal antibody targeting the Cryptosporidium oocyst outer wall antigenic sites (A400Cy2R-20X, Crypt-a-Glo, Waterborne, Inc., New Orleans, LA, USA) and with genus-specific, FITC-conjugated antibodies (IFA; *Cryptosporidium* IF Test, Crypto cel, Cellabs Pty Ltd., Brookvale, Australia). Type microphotographs of oocysts were taken from each staining/labelling.

2.7. Transmission Studies

All animals used in transmission studies were screened every day for the presence of oocysts of *Cryptosporidium* spp. and specific DNA (*SSU*) a week prior to transmission

studies. Five seven-day-and-eight-week-old SCID (strain C.B-17) and BALB/c mice (*Mus musculus*), five seven-day-and-eight-week-old Mongolian gerbils (*Meriones unguiculatus*), five seven-day-and-eight-week-old guinea pigs (*Cavia porcellus*), five seven-day-and-eight-week-old Southern multimammate mice (*Mastomys coucha*), five seven-day-old chickens (*Gallus gallus f. domestica*), and five adult budgerigars (*Melopsittacus undulatus*) were used for transmission studies. Three animals from each strain/species were used as a negative control. To prevent environmental contamination with *Cryptosporidium* spp., animals were housed in plastic cages/aviaries and supplied with a sterilized diet for appropriate host-species and sterilized water ad libitum. Seven-day-and-eight-week-old animals were each inoculated orally by stomach tube with 5000 purified viable oocysts suspended in 50 µL and 200 µL of distilled water, respectively. Faecal samples from all inoculated and control animals were collected daily for 20 days. All samples were screened for the presence of *Cryptosporidium* oocysts and specific DNA using microscopy (following ACMV staining) and PCR amplification of the *SSU* gene, respectively (methods described above). Animals were housed under conditions in accordance with Czech legislation (Act No 246/1992 Coll., on protection of animals against cruelty). Animal caretakers always wore sterile shoe covers, disposable coveralls, and disposable gloves when they entered the experimental room. Woodchip bedding and disposable protective clothing were removed from the experimental room and incinerated.

2.8. Clinical and Pathomorphological Examinations

All animals were sacrificed in accordance with Czech legislation (Act No 246/1992 Coll) at 20 days post-infection (DPI), and the complete examination of all gastrointestinal organs was conducted at necropsy. Tissue specimens from the oesophagus; stomach; duodenum; proximal, central and distal jejunum; ileum; caecum; colon; liver; spleen; kidney; bladder and lung were collected using different sterile dissection tools for each location and processed for histology [32], scanning electron microscopy (SEM) [33], and PCR amplification of the SSU gene. Histology sections (5 µm) were stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) and were examined at 100–400× magnification (Olympus IX70). Specimens for SEM were examined using a JEOL JSM-7401F-FE scanning electron microscope (Jeol, Tokyo, Japan).

2.9. Statistical Analysis

For the evaluation of difference in relative frequency in positivity between groups (country, age, gender), we used the two sample test for equality of proportions without continuity correction. Differences in oocyst sizes 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 4.1.0. [34]. The hypothesis tested was that two-dimensional mean vectors of measurement are the same in the two populations being compared.

3. Results

Cryptosporidium-specific DNA was identified in 26 squirrels by nested PCR targeting the *SSU* gene (Table 1). Of these 26 squirrels, nine (34.6%) were microscopically positive for the presence of *Cryptosporidium* oocysts, with an infection intensity ranging from 18,000 to 72,000 OPG. Microscopically detectable infection was observed exclusively in juvenile squirrels. However, the occurrence of infection detected by PCR did not differ between juveniles (8.9%) and adults (11.8%; $\chi^2 = 0.52991$, *d.f.* = 1, *p*-value = 0.4666). Similarly, the occurrence of infection was not affected by host gender ($\chi^2 = 0.2966$, *d.f.* = 1, *p*-value = 0.5860) or country of capture ($\chi^2 = 0.0028$, *d.f.* = 1, *p*-value = 0.9388; Table S1). Phylogenetic trees (ML, NJ) constructed from *SSU* sequences showed the presence of *Cryptosporidium* sp. ferret genotype in all samples (Figure 2). Out of the 26 *Cryptosporidium*-positive squirrels, 26, 26, 19, 16, 15, and 26 were genotyped by sequence analysis of the *SSU*, actin, *HSP70*, *TRAP-C1*, *COWP*, and *gp60* genes, respectively. For the remaining samples, either the PCR product was not amplified or the sequencing failed. For the actin, *HSP70*, *TRAP-C1*,

and *COWP* genes, isolates of *Cryptosporidium* sp. ferret genotype in this study shared 100% identity with each other and 99.8–100% identity with previously reported sequences (Figures S1–S4). Sequences of the *gp60* gene were obtained from all 26 isolates and were identified as *Cryptosporidium* sp. ferret genotype subtype families VIIIb (n = 12) and VIIIc (n = 14; Figure 3).

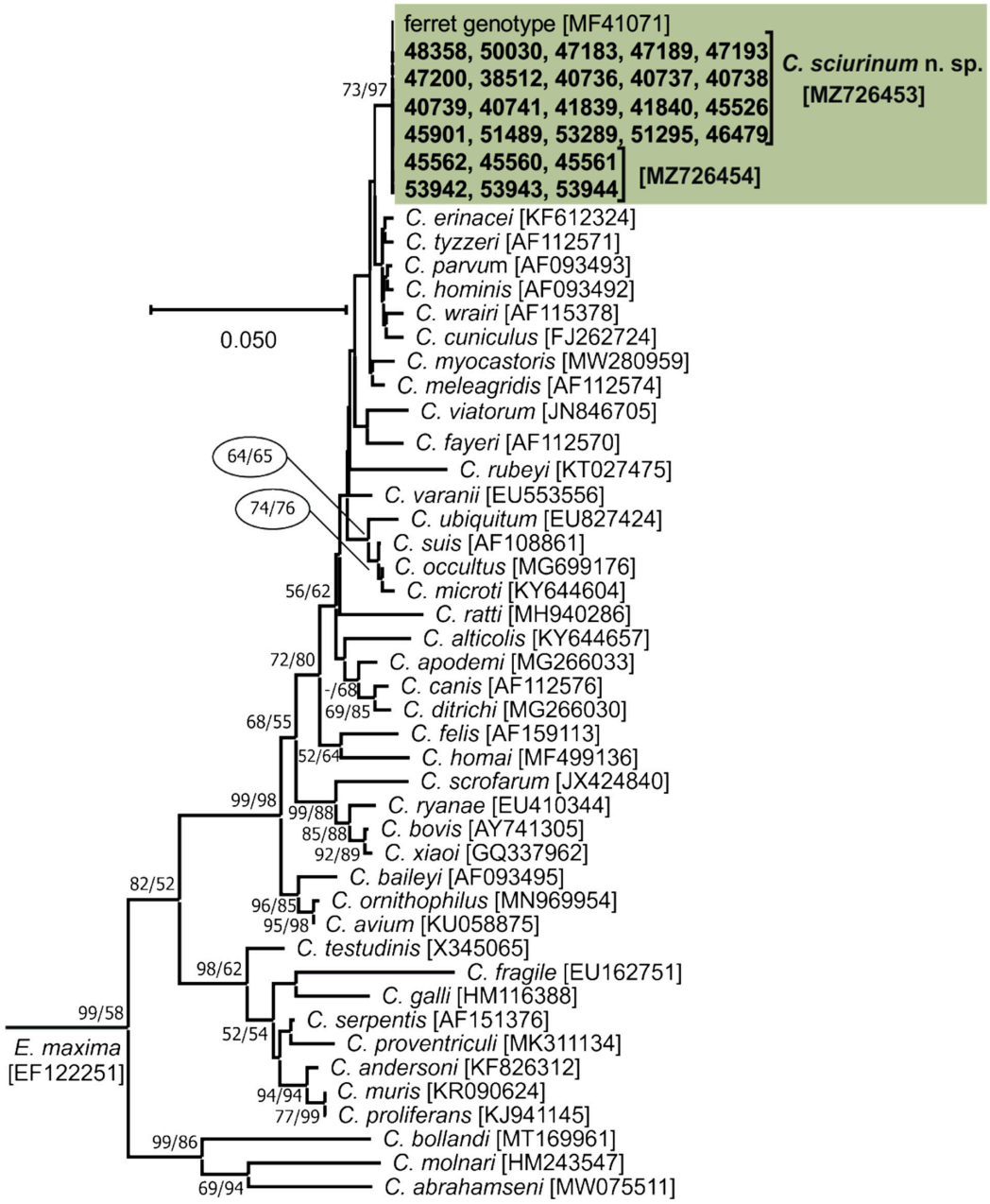

Figure 2. Evolutionary relationships of *Cryptosporidium* spp. at the small subunit rRNA (*SSU*) locus inferred by Maximum Likelihood (ML) and Neighbor-Joining (NJ) analyses using a General Time Reversible model with a gamma distribution: Percentage support (>50%) from 1000 pseudoreplicates from ML and NJ analysis, respectively, are indicated next to supported node. The '-' indicates support value <50%. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 70 nucleotide sequences and there were a total of 771 positions in the final dataset. The tree was rooted with the *SSU* sequence of *Eimeria maxima* [EF122251]. Sequences obtained in this study are identified by isolate number (e.g., 45901). The GenBank Accession number is in parenthesis. Isolates detected in this study are colour-coded.

Table 1. The occurrence and genetic diversity of *Cryptosporidium* spp. in Eurasian red squirrels (*Sciurus vulgaris*) trapped in the wild (W) and screened at rescue stations (RS) in the Czech Republic and Slovakia. Detected isolates were genotyped by sequence analysis of the small subunit ribosomal rRNA (*SSU*), actin, 70 kDa heat-shock protein (*HSP70*), thrombospondin-related adhesive protein of *Cryptosporidium*-1 (*TRAP-C1*), *Cryptosporidium* oocyst wall protein (*COWP*), and 60 kDa glycoprotein (*gp60*) gene fragments. Oocysts were quantified by microscopy and were reported per gram of faeces (OPG).

Country	Locality	Type	No. of Screened/No. of Positive	ID of Positive Animal	Microscopical Positivity	Sex/Age	Genotyping at the Gene Loci					
							SSU	Actin	*HSP70*	*COWP*	*TRAP-C1*	*gp60*
Czech Republic	(1)	W	2/0	-	-	-	-	-	-	-	-	-
	(2)	W	1/0	-	-	-	-	-	-	-	-	-
	(3)	W	5/2	48358	No	F/A	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	VIIIbA11G1R1
				50030	No	M/A	*C. sciurinum*	*C. sciurinum*	-	*C. sciurinum*	-	VIIIbA11G1R1
	(4)	W	2/0	-	-	-	-	-	-	-	-	-
	(5)	W	15/4	47183	No	M/J	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	-	*C. sciurinum*	VIIIbA11G1R1
				47189	No	F/A	*C. sciurinum*	*C. sciurinum*	-	ferret genotype	-	VIIIbA11G1R1
				47193	No	M/A	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	-	*C. sciurinum*	VIIIbA11G1R1
				47200	No	F/J	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	-	VIIIbA11G1R1
	(6)	W	1/0	-	-	-	-	-	-	-	-	-
	(7)	W	2/0	-	-	-	-	-	-	-	-	-
	(8)	W	4/0	-	-	-	-	-	-	-	-	-
	(9)	W	3/0	-	-	-	-	-	-	-	-	-
	(10)	W	3/0	-	-	-	-	-	-	-	-	-
	(11)	W	7/1	38512	No	F/A	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	-	-	VIIIcA10G2R1
	(12)	W	3/0	-	-	-	-	-	-	-	-	-
	(13)	W	6/0	-	-	-	-	-	-	-	-	-
	(14)	W	5/0	-	-	-	-	-	-	-	-	-
	(15)	W	5/0	-	-	-	-	-	-	-	-	-
	(16)	W	3/0	-	-	-	-	-	-	-	-	-
	(17)	W	4/0	-	-	-	-	-	-	-	-	-
	(18)	W	5/0	-	-	-	-	-	-	-	-	-
	(19) J	RC	14/0	-	-	-	-	-	-	-	-	-
	(20)	RC	4/0	-	-	-	-	-	-	-	-	-
	(21)	RC	37/5	40736	Yes	F/J	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	-	VIIIcA10G2R1
				40737	Yes	M/J	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	VIIIcA10G2R1
				40738	Yes	F/J	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	-	VIIIcA10G2R1
				40739	Yes	M/J	*C. sciurinum*	*C. sciurinum*	-	*C. sciurinum*	*C. sciurinum*	VIIIcA10G2R1
				40741	Yes	M/J	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	VIIIcA10G2R1
	(22)	RC	23/2	41839	Yes	F/J	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	VIIIcA10G2R1
				41840	No	M/A	*C. sciurinum*	*C. sciurinum*	-	-	*C. sciurinum*	VIIIcA10G2R1
				45526	No	M/A	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	-	*C. sciurinum*	VIIIbA11G1R1
	(23)	RC	21/5	45901	Yes	F/J	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	VIIIbA11G1R1
				51489	Yes	M/J	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	-	VIIIbA11G1R1
				53289	Yes	F/J	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	VIIIbA11G1R1
				51295	No	M/A	*C. sciurinum*	*C. sciurinum*	-	-	*C. sciurinum*	VIIIbA11G1R1
	(24)	RC	15/1	46479	No	F/A	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	-	-	VIIIbA11G1R1

Table 1. *Cont.*

Country	Locality	Type	No. of Screened/No. of Positive	ID of Positive Animal	Microscopical Positivity	Sex/Age	Genotyping at the Gene Loci					
							SSU	Actin	*HSP70*	*COWP*	*TRAP-C1*	*gp60*
	(25)	RC	15/0	-	-	-	-	-	-	-	-	-
Slovakia	(26)	W	8/0	-	-	-	-	-	-	-	-	-
	(27)	W	2/0	-	-	-	-	-	-	-	-	-
	(28)	W	5/1	45562	No	M/A	*C. sciurinum*	*C. sciurinum*	-	*C. sciurinum*	*C. sciurinum*	VIIIcA10G2R1
	(29)	W	2/0	-	-	-	-	-	-	-	-	-
	(30)	W	2/0	-	-	-	-	-	-	-	-	-
	(31)	W	5/1	45560	No	F/J	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	-	*C. sciurinum*	VIIIcA10G2R1
	(32)	W	4/0	-	-	-	-	-	-	-	-	-
	(33)	W	2/0	-	-	-	-	-	-	-	-	-
	(34)	W	6/0	-	-	-	-	-	-	-	-	-
	(35)	W	1/0	-	-	-	-	-	-	-	-	-
	(36)	W	4/0	-	-	-	-	-	-	-	-	-
	(37)	W	1/1	45561	No	F/A	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	-	VIIIcA10G2R1
	(38)	W	1/0	-	-	-	-	-	-	-	-	-
	(39)	W	7/2	53942	No	F/A	*C. sciurinum*	*C. sciurinum*	-	-	*C. sciurinum*	VIIIcA10G2R1
				53943	No	M/A	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	-	VIIIcA10G2R1
	(40)	W	8/1	53944	No	M/A	*C. sciurinum*	*C. sciurinum*	*C. sciurinum*	-	-	VIIIcA10G2R1

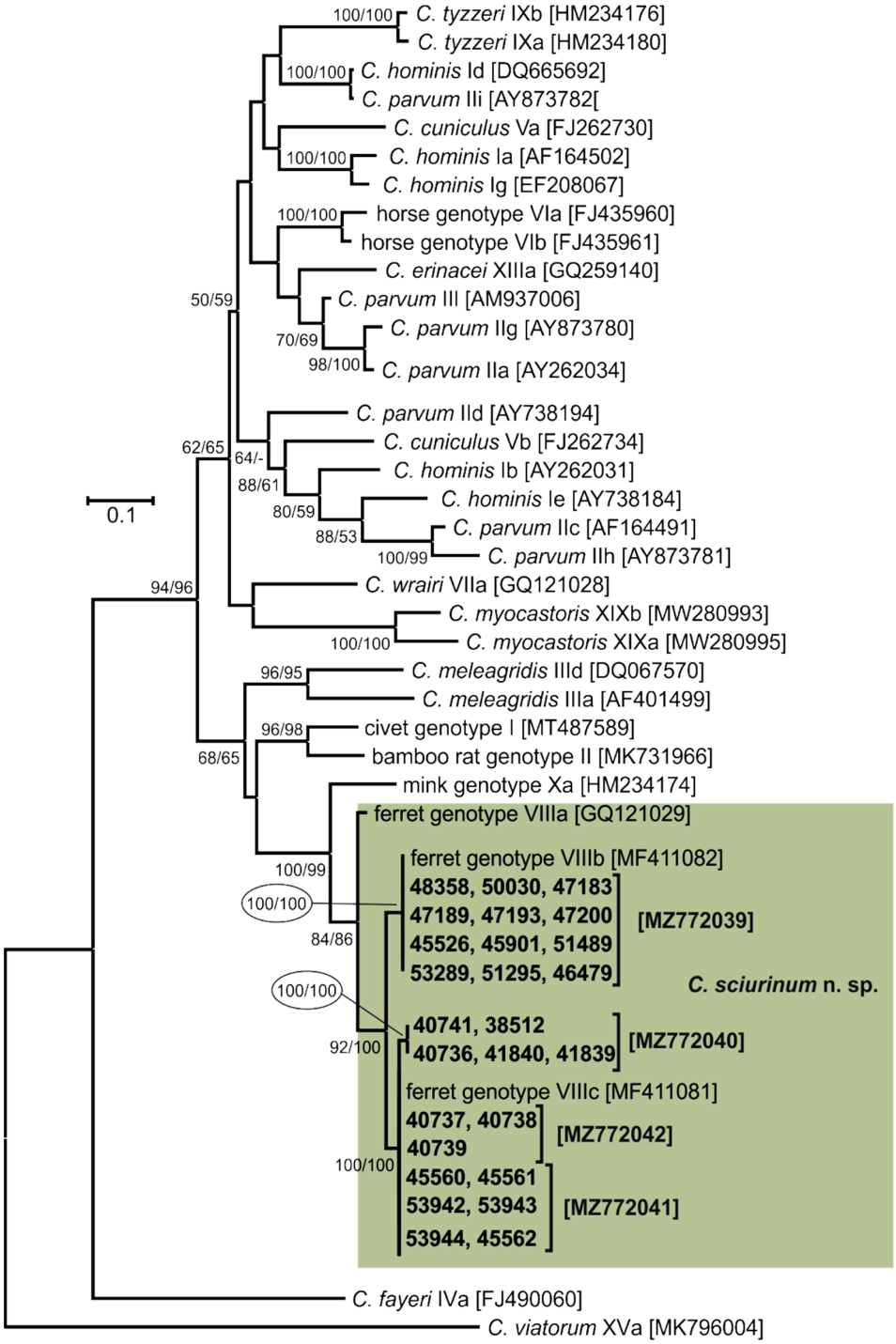

Figure 3. Evolutionary relationships of *Cryptosporidium* spp. at 60 kDa glycoprotein (*gp60*) locus inferred by Maximum Likelihood (ML) and Neighbor-Joining (NJ) analyses using a General Time Reversible model with a gamma distribution: Percentage support (>50%) from 1000 pseudoreplicates from ML and NJ analysis, respectively, are indicated next to supported node. The '-' indicates support value <50%. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 58 nucleotide sequences and there were a total of 1134 positions in the final dataset. The tree was rooted with the *gp60* sequence of *Cryptosporidium viatorum* [MK796004]. Sequences obtained in this study are identified by isolate number (e.g., 45901). The GenBank Accession number is in parenthesis. Isolates detected in this study are colour-coded.

Microscopic, molecular, and histological examination of inoculated animals showed no evidence of infection in juvenile or adult SCID and BALB/c mice, Mongolian gerbils, guinea pigs, Southern multimammate mice, juvenile chickens, and adult budgerigars. For logistical reasons, we were unable to determine the infectivity of *Cryptosporidium* sp. ferret genotype for squirrels under experimental conditions. We therefore determined the infection intensity in squirrels of different ages kept at rescue stations. Nine of the eleven juvenile animals infected with *Cryptosporidium* sp. ferret genotype shed microscopically detectable oocysts (18,000–74,000 OPG). In contrast, oocyst numbers were below the detection limit (2000 OPG) in animals older than 10 weeks of age. The highest infection intensity was observed in animals aged 6–7 weeks (mean 49,666 OPG) compared to animals aged 4–5 weeks (mean 22,666 OPG) and 8–9 weeks (mean 21,000 OPG) (Figure 4). The consistency of the faeces and lack of faeces in the fur surrounding the rectum indicated an absence of diarrhoeal disease. Oocysts of *Cryptosporidium* sp. ferret genotype were spherical, with a thick, clear, and smooth oocyst wall. Oocysts from isolate 45901, which were measured in suspension after purification, measured 5.54 × 5.22 µm, with a length to width ratio of 1.07 (Figure 5a). There was no significant difference in oocyst size between isolate 45901 and other isolates (40793, 51489, and 53289; Table S2). Oocysts of *Cryptosporidium* sp. ferret genotype in the faecal smears stained by AVMC and ZN showed typical *Cryptosporidium* staining characteristics (Figure 5b,c) and cross-reacted with immunofluorescence reagents developed originally for *C. parvum* (Figure 5d,e). Oocysts that were dried and fixed onto slides for staining and labelling were smaller than those measured in suspension (data not shown).

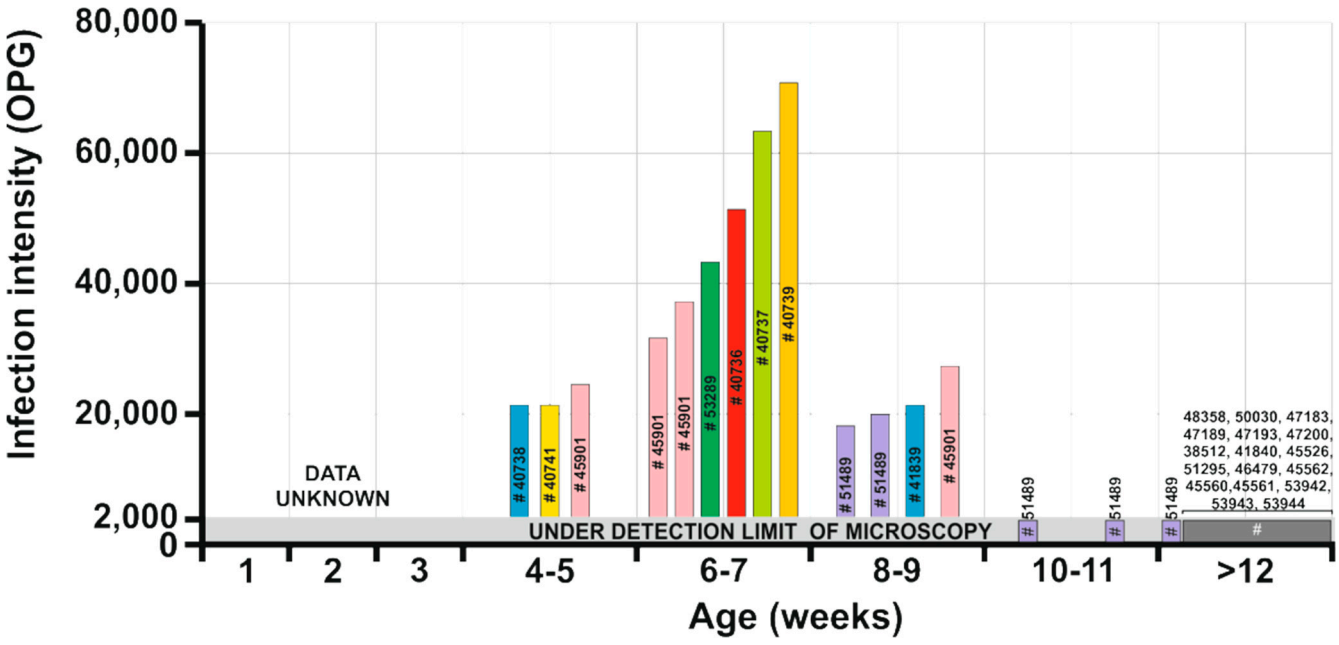

Figure 4. Infection intensity of *Cryptosporidium sciurinum* n. sp. in Eurasian red squirrels (*Sciurus vulgaris*) expressed as number of oocysts per gram of faeces (OPG). Data were collected from naturally infected Eurasian red squirrels. Animals aged 4–11 weeks old originated from rescue stations, and some of those animals were screened repeatedly (each animal is marked with a colour). The animal number (e.g., #45901) corresponds with the numbers in Table 1.

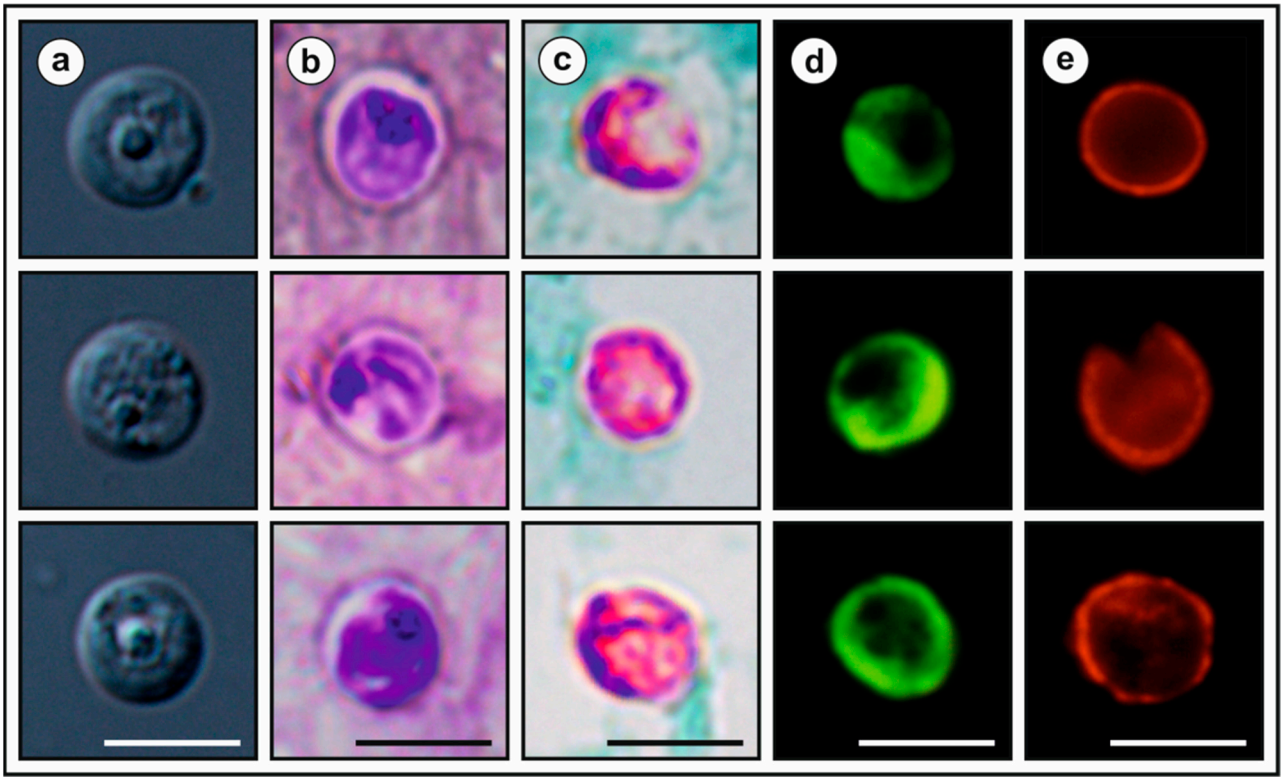

Figure 5. Oocysts of *Cryptosporidium sciurinum* n. sp. (**a**) under differential interference contrast microscopy, (**b**) stained by aniline-carbol-methyl violet staining, (**c**) stained by Ziehl–Nielsen staining, (**d**) labelled with anti-*Cryptosporidium* FITC-conjugated antibody, (**e**) labelled with anti-*Cryptosporidium* Cy3-conjugated antibody. Bar = 5 µm.

Based on the data presented here, we propose *Cryptosporidium* sp. ferret genotype as a new species—*Cryptosporidium sciurinum* n. sp.—, whose description is presented below.

Taxonomic summary
Family Cryptosporidiidae Léger, 1911
Genus *Cryptosporidium* Tyzzer, 1907
***Cryptosporidium sciurinum* n. sp.**
Syn: *Cryptosporidium parvum* ferret genotype ex black-footed ferret (*Mustela nigripes*) and *Cryptosporidium* sp. ferret genotype ex black-footed ferret (*Mustela nigripes*) of Xiao et al. [35].
Type-host: *Sciurus vulgaris* Linnaeus, 1758 (Rodentia: Muridae) Eurasian red squirrel.
Other natural hosts: black-footed ferret (*Mustela nigripes*), Siberian chipmunk (*Tamias sibiricus*), Eastern chipmunk (*Tamias striatus*), budgerigar (*Melopsittacus undulatus*).
Type-locality: Třeboň, Czech Republic.
Other localities: České Budějovice, Brno, Praha, Vlašim, whole Czech Republic; Považská Bystrica, Handlová, Gabčíkovo, Bratislava, Košice, whole Slovakia; Guangdong, China; Northern Italy; Georgia, USA.
Type-material: Faecal smear slides with oocysts stained by ACMV and ZN staining (nos. MV1-3/45901 and ZN1-2/45901) and gDNA isolated from faecal samples of a naturally infected Eurasian red squirrel (isolate 45901) are deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.
Site of infection: unknown.
Distribution: As *Cryptosporidium* sp. ferret genotype ex *Sciurus vulgaris*: China, Italy [13,16,17]; as *Cryptosporidium* sp. ferret genotype or *Cryptosporidium parvum* ferret genotype ex *Mustela nigripes*: USA [35], as *Cryptosporidium* sp. ferret genotype ex *Tamias striatus*, *T. sibiricus* and *Melopsittacus undulatus*: China [13,36].
Prepatent period: unknown.

Patent period: At least 5 weeks in naturally infected *Sciurus vulgaris* (isolate 51489 in the present study).
Representative DNA sequences: Representative nucleotide sequences of *SSU* [MZ726453], actin [MZ772035], *HSP70* [MZ772047], *TRAP-C1* [MZ772037], *COWP* [MZ772045], and *gp60* [MZ772039–MZ772042] genes are deposited 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) [37], details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:83ABAD68-07C6-4E51-8234- A2AC4C0EE72C. The LSID for the new name *Cryptosporidium sciurinum* n. sp. is urn:lsid:zoobank.org:act:E13F2B9F-D9C6-4ED9-9EA8-B94AEB6189A2.
Etymology: The species name *sciurinum* is derived from the Latin noun sciurus, meaning squirrel.

Description. Oocysts of *C. sciurinum* n. sp. (isolate 45901) are spherical, measuring 5.12–6.00 × 4.77–5.66 (5.54 ± 0.20 × 5.22 ± 0.18) with a length-to-width ratio of 1.00–1.26 (1.07 ± 0.05) (Figure 5). The oocyst wall is smooth and colourless (Figure 5a). The oocyst residuum is composed of numerous small granules and one spherical globule is clearly visible; a suture is not noticeable. Sporozoites are occasionally visible within oocysts. Morphology and morphometry of other developmental stages is unknown.

Remark 1. Oocysts of *C. sciurinum* n. sp. are stained by ACMV and ZN staining methods, similar to other *Cryptosporidium* spp. (Figure 5b,c), and their oocyst wall cross reacts with immunofluorescence reagents developed originally for *C. parvum* (Figure 5d,e). Oocysts from naturally infected squirrels did not differ significantly in size (Table S2). Oocysts of *C. sciurinum* n. sp. are larger than those of *C. parvum* (T^2 = 88.89, df_1 = 2, df_2 = 61.94, $p < 0.001$), *C. occultus* (T^2 = 45.84, df_1 = 2, df_2 = 65.83, $p < 0.001$) and *C. ratti* (T^2 = 88.22, df_1 = 2, df_2 = 24.75, $p < 0.001$), but these differences are not of practical significance for identification. *Cryptosporidium sciurinum* n. sp. can be differentiated genetically from other *Cryptosporidium* species based on sequences of *SSU*, actin, *HSP70*, *TRAP-C1*, *COWP*, and *gp60* genes. At *gp60* locus, *C. sciurinum* n. sp. is known to form three subtype families VIIIa, VIIIb, and VIIIc.

4. Discussion

Molecular studies conducted on three continents (North America, Europe, and Asia) have shown that tree squirrels host a variety of *Cryptosporidium* spp. [9–13,16,17]. While wild North American and Asian tree squirrels can be infected by a large number of species and genotypes of the genus *Cryptosporidium* [9–13], results from this and other studies suggest that wild Eurasian red squirrels are predominantly parasitized by *C. sciurinum* n. sp. [13,16,17]. The only exceptions are two cases of *Cryptosporidium* sp. chipmunk genotype I, which naturally infects grey squirrels, and two cases of *C. parvum* reported in Eurasian red squirrels in Italy [13,16,17]. In contrast to wild animals, Eurasian red squirrels kept as caged pets in China were found to be infected with *C. ratti* and *Cryptosporidium* sp. chipmunk genotype III, which are specific for other hosts [13]. In the present study, 10.1% (26/258) of Eurasian red squirrels were infected with *C. sciurinum* n. sp. Previous studies of *C. sciurinum* n. sp. in Eurasian red squirrels have reported prevalences of 10.6% (13/123) and 21.5% (15/70) in Italy and 26.3% in China [13,16,17]. The prevalence range of *Cryptosporidium* is similar with that found in other wild rodents, such as 14% in *Apodemus* spp. in Europe, 27% in *Apodemus speciosus* in Japan, 16% in brown rats (*Rattus norvegicus*) in Czech Republic, 12% in muskrats (*Ondatra zibethicus*) in USA, 30% in Chinese bamboo rats (*Rhizomys sinensis*) in China, or 7–14% in voles in Europe [38–42]. Consistent with most reports describing natural infections with *Cryptosporidium* spp. in wild rodents [16,17,38,42–44], Eurasian red squirrels infected with *C. sciurinum* n. sp. shed low numbers of oocysts, often below the detection limit of microscopy. A characteristic of *C. sciurinum* n. sp. infection in Eurasian red squirrels is that mostly juveniles shed oocysts at levels that are detectable

by microscopy [16,17]. This age-dependent variation in *Cryptosporidium* spp. infection intensity has been observed previously in several studies [44–49]. Juveniles are probably more susceptible to infection because of their naive and immature immune system, which permits a higher intensity of infection. The prepatent period of *C. sciurinum* n. sp. is unknown. From the results we have collected, it is clear that pups become infected at a very early age (as early as 4 weeks), and that the infection persists for more than a month. It is possible that asymptomatically infected adults are a source of infection for their young, but this would need to be investigated experimentally. The pathogenicity of *C. sciurinum* n. sp. is unknown; however, based on the observations of naturally infected squirrels in rescue stations, this *Cryptosporidium* species appears to have low pathogenicity for infected individuals. This finding is consistent with previously published results [16,17].

Cryptosporidium sciurinum n. sp. was firstly identified in three naturally infected ferrets (reported as *C. parvum* ferret genotype (AF112572)) [35]. Since then, this species has been found in five captive chipmunks (*T. sibiricus* and *T. striatus*), one budgerigar (*M. undulatus*), and dozens of wild and captive Eurasian red squirrels [13,15–17,35,36], suggesting that Eurasian red squirrels are a natural host of this parasite. This is supported by the finding in the present study that *C. sciurinum* n. sp. is not infective for SCID and BALB/c mice, Mongolian gerbils, guinea pigs, Southern multimammate mice, chickens, and budgerigars. The solitary infection reported in a budgerigar [36] could be the result of environmental contamination and the passage of oocysts or specific DNA through the digestive tract of a non-specific host, as has been described previously [50–53].

Oocysts of *C. sciurinum* n. sp. measured 5.54 × 5.22 µm, which is similar in size to a previously published isolate of *C. sciurinum* n. sp. (5.5 × 5.2 µm). *Cryptosporidium sciurinum* n. sp. is larger than *C. parvum* (5.2 × 4.9 µm) and *C. ratti* (4.9 × 4.6 µm), and is smaller than *Cryptosporidium* sp. chipmunk genotype I (5.8 × 5.4 µm), a species previously reported in Eurasian red squirrels [16,40]. However, the difference in size is not practically useful for distinguishing species by routine microscopy.

Although a description of oocyst morphology is a requirement for species description, molecular characterisation is necessary for species identification. Several previous studies have shown the presence of divergent copies of *SSU* gene within a *Cryptosporidium* species. Stenger et al. [54] found highly divergent SSU genotypes in *Cryptosporidium* sp. chipmunk genotype II. The co-occurrence of the genotypes in a host and the homogeneity of actin and *HSP70* sequences supported the conclusion that the divergent types are paralogs in a single *Cryptosporidium* lineage. Similarly, divergent types of *SSU* have been reported within, e.g., *C. andersoni*, *C. apodemi*, *C. ditrichi*, *C. parvum*, *C. ubiquitum*, *Cryptosporidium* sp. apodemus genotype I and II, or *Cryptosporidium* rat genotype II and III [15,42,43,55,56]. As inferring the evolutionary relationships of *Cryptosporidium* spp. using *SSU* sequences alone can lead to erroneous conclusions [54,57,58], we included other polymorphic loci for our analyses. Although some previous studies have shown polymorphism in actin, *HSP70*, and *TRAP-C1* genes in *Cryptosporidium* spp. [42,43,56], studies from our group and others have failed to find variants of these genes in *C. sciurinum* n. sp. [17,24,25]. All isolates of *C. sciurinum* n. sp. from the present study shared 100% identity at the COWP locus and differed from a sequence obtained from ferret (*Mustela putorius furo*) in Japan (AB469366) by a synonymous SNP (C/T) at position 387. A similar synonymous SNP at the COWP locus was observed in geographically distinct isolates of *C. tyzzeri* [59].

Phylogenetic analyses of *C. sciurinum* n. sp. at *SSU*, actin, *HSP70*, *TRAP-C1*, *COWP*, and *gp60* loci confirmed its status as a separate species from valid *Cryptosporidium* species. At all loci, *C. sciurinum* n. sp. formed a separate clade within the group of intestinal *Cryptosporidium* spp. that includes *C. meleagridis*, *C. parvum*, *C. hominis*, *C. wrairi*, and *C. tyzzeri*. At actin, *HSP70*, *TRAP-C1*, and *COWP* loci, the pairwise distances between *C. sciurinum* n. sp. and *C. meleagridis* (0.026, 0.036, 0.050, and 0.024, respectively) are similar to those between *C. andersoni* and *C. muris* (0.037, 0.018, 0.041, and 0.018, respectively) and *C. hominis* and *C. parvum* (0.018, 0.013, 0.018, and 0.016, respectively).

At the *gp60* locus, only subtype families VIIIb and VIIIc were detected in the present study. Both subtypes have been found exclusively in Europe. In contrast, subtype VIIIa was found exclusively in China [13]. Other studies have found that *gp60* subtype families differ in their geographic distribution. For example, *C. tyzzeri* subtype IXa has been detected only in house mice (*Mus musculus musculus*) inhabiting Eastern Europe, China, and Kuwait, while subtype IXb is found (*Mus musculus domesticus*) in Western Europe, USA, and New Zealand [59]. A similar difference in distribution has been reported for *C. parvum* and *C. ubiquitum gp60* subtypes [57,60]. It should be noted that gp60 subtype family VIIId was incorrectly reported in Eurasian red squirrels in Italy (the sequences actually belonged to *C. parvum* subtype IIa) and it should no longer be used for *C. sciurinum* n. sp. [17].

5. Conclusions

In summary, the present study confirms that *Cryptosporidium* sp. ferret genotype is genetically distinct from all currently accepted species of the genus *Cryptosporidium*, and its specificity for tree squirrels under natural conditions supports its description as a new species, which we propose be named *Cryptosporidium sciurinum* n. sp.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9102050/s1: **Figure S1.** Evolutionary relationships of *Cryptosporidium* spp. at the actin locus inferred by Maximum Likelihood (ML) and Neighbor-Joining (NJ) analyses using the Tamura-3-parameter model with a gamma distribution: Percentage support (>50%) from 1000 pseudoreplicates from ML and NJ analysis, respectively, are indicated next to supported node. The '-' indicates support value <50%. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 69 nucleotide sequences and there were a total of 727 positions in the final dataset. The tree was rooted with the actin sequence of *Plasmodium* sp. [KF159609]. Sequences obtained in this study are identified by isolate number (e.g., 45901). The GenBank Accession number is in parenthesis. Isolates detected in this study are colour-coded. **Figure S2.** Evolutionary relationships of *Cryptosporidium* spp. at 70 kDa heat-shock protein (*HSP70*) locus inferred by Maximum Likelihood (ML) and Neighbor-Joining (NJ) analyses using a General Time Reversible model with a gamma distribution: Percentage support (>50%) from 1000 pseudoreplicates from ML and NJ analysis, respectively, are indicated next to supported node. The '-' indicates support value <50%. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 69 nucleotide sequences and there were a total of 1728 positions in the final dataset. The tree was rooted with the *HSP70* sequence of *Eimeria maxima* [Z46964]. Sequences obtained in this study are identified by isolate number (e.g., 45901). The GenBank Accession number is in parenthesis. Isolates detected in this study are colour-coded. **Figure S3.** Evolutionary relationships of *Cryptosporidium* spp. at thrombospondin-related adhesive protein of *Cryptosporidium*-1 (*TRAP-C1*) locus inferred by Maximum Likelihood (ML) and Neighbor-Joining (NJ) analyses using a General Time Reversible model with a gamma distribution: Percentage support (>50%) from 1000 pseudoreplicates from ML and NJ analysis, respectively, are indicated next to supported node. The '-' indicates support value <50%. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 29 nucleotide sequences and there were a total of 470 positions in the final dataset. The tree was rooted with the *TRAP-C1* sequence of gastric *Cryptosporidium* spp. Sequences obtained in this study are identified by isolate number (e.g., 45901). The GenBank Accession number is in parenthesis. Isolates detected in this study are colour-coded. **Figure S4.** Evolutionary relationships of *Cryptosporidium* spp. at *Cryptosporidium* oocyst wall protein (*COWP*) locus inferred by Maximum Likelihood (ML) and Neighbor-Joining (NJ) analyses using a General Time Reversible model with a gamma distribution: Percentage support (>50%) from 1000 pseudoreplicates from ML and NJ analysis, respectively, are indicated next to supported node. The '-' indicates support value <50%. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 41 nucleotide sequences and there were a total of 477 positions in the final dataset. The tree was rooted with the *COWP* sequence of gastric *Cryptosporidium* spp. Sequences obtained in this study are identified by isolate number (e.g., 45901). The GenBank Accession number is in parenthesis. Isolates detected in this study are

colour-coded. **Table S1.** Occurrence of *Cryptosporidium sciurinum* n. sp. in faeces of Eurasian red squirrels (*Sciurus vulgaris*) based on microscopic and molecular examination. **Table S2.** Size of *Cryptosporidium sciurinum* n. sp. oocysts obtained from naturally infected Eurasian red squirrels (*Sciurus vulgaris*).

Author Contributions: Conceptualization, M.K., J.J. and J.P.; methodology, D.R. and B.S.; software, M.R.; validation, J.P., J.J., M.K. and B.S.; formal analysis, J.P., J.J., N.H., R.K. and B.S.; investigation, J.J., J.P., N.H., B.S., D.R. and R.K.; resources, D.R., J.J., J.P., N.H. and B.S.; data curation, J.P., J.J., J.M. and M.R.; writing—original draft preparation, J.P. and J.J.; writing—review and editing, M.K. and J.M.; visualization, B.S. and M.K.; supervision, M.K. and J.M.; project administration, M.K.; funding acquisition, M.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of the Biology Centre of CAS (MZP/2019/603/1411 approved 10 June 2019, and 35/2018 1 May 2018).

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Data Availability Statement: Data is contained within the article or supplementary material.

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Conflicts of Interest: None of the authors has any competing interest in the manuscript.

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6.5 Příloha V

Cryptosporidium mortiferum n. sp. (Apicomplexa: Cryptosporidiidae), the species causing lethal cryptosporidiosis in Eurasian red squirrels (_Sciurus vulgaris_).

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RESEARCH

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Cryptosporidium mortiferum n. sp. (Apicomplexa: Cryptosporidiidae), the species causing lethal cryptosporidiosis in Eurasian red squirrels (*Sciurus vulgaris*)

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Abstract

Background *Cryptosporidium* spp. are globally distributed parasites that infect epithelial cells in the microvillus border of the gastrointestinal tract of all classes of vertebrates. *Cryptosporidium* chipmunk genotype I is a common parasite in North American tree squirrels. It was introduced into Europe with eastern gray squirrels and poses an infection risk to native European squirrel species, for which infection is fatal. In this study, the biology and genetic variability of different isolates of chipmunk genotype I were investigated.

Methods The genetic diversity of *Cryptosporidium* chipmunk genotype I was analyzed by PCR/sequencing of the *SSU rRNA*, *actin*, *HSP70*, *COWP*, *TRAP-C1* and *gp60* genes. The biology of chipmunk genotype I, including oocyst size, localization of the life cycle stages and pathology, was examined by light and electron microscopy and histology. Infectivity to Eurasian red squirrels and eastern gray squirrels was verified experimentally.

Results Phylogenic analyses at studied genes revealed that chipmunk genotype I is genetically distinct from other *Cryptosporidium* spp. No detectable infection occurred in chickens and guinea pigs experimentally inoculated with chipmunk genotype I, while in laboratory mice, ferrets, gerbils, Eurasian red squirrels and eastern gray squirrels, oocyst shedding began between 4 and 11 days post infection. While infection in mice, gerbils, ferrets and eastern gray squirrels was asymptomatic or had mild clinical signs, Eurasian red squirrels developed severe cryptosporidiosis that resulted in host death. The rapid onset of clinical signs characterized by severe diarrhea, apathy, loss of appetite and subsequent death of the individual may explain the sporadic occurrence of this *Cryptosporidium* in field studies and its concurrent spread in the population of native European squirrels. Oocysts obtained from a naturally infected human, the original inoculum, were 5.64 × 5.37 µm and did not differ in size from oocysts obtained from experimentally infected hosts. *Cryptosporidium* chipmunk genotype I infection was localized exclusively in the cecum and anterior part of the colon.

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Conclusions Based on these differences in genetics, host specificity and pathogenicity, we propose the name *Cryptosporidium mortiferum* n. sp. for this parasite previously known as *Cryptosporidium* chipmunk genotype I.

Keywords Mortality, Biology, Course of infection, Cryptosporidiosis, Oocyst size, Phylogeny, Genetic diversity

Background

Cryptosporidium spp. are important causative agents of mild to severe diarrheal diseases in humans and various animals, with worldwide distribution [1]. The first descriptions of *Cryptosporidium* species were based on descriptions of oocyst morphology, localization of infection in the host or the occurrence in hosts [2–4]. For this reason, few species had been identified within the genus *Cryptosporidium* by the end of the twentieth century, with most species belonging to the *Cryptosporidium parvum*- and *C. muris*-like complexes [5, 6]. Developments in molecular biology and routine use of molecular techniques in research have contributed significantly to the discovery of great genetic diversity within the genus *Cryptosporidium* and supported the description of many new species and genotypes [7–10]. To date, there are 49 established species and dozens of genotypes that have been described based on molecular differences from valid species [7, 11]. Some species and genotypes, such as *C. parvum*, *C. hominis*, *C. meleagridis*, *C. felis* and *C. viatorum*, frequently cause diarrheal disease in humans, livestock and wildlife [3, 12–14], while others are not associated with clinical disease [15–17]. We have little knowledge about the biology and course of infection of most *Cryptosporidium* genotypes, but given their genetic divergence from named species, they likely represent distinct species.

Cryptosporidium chipmunk genotype I was first described in 2005 as *Cryptosporidium* isolate W17 in surface waters in the USA [18]. In 2007, the same genotype was identified in rodent fecal samples from the watershed of the New York City water supply, and it was renamed chipmunk genotype I [19]. Molecular epidemiological studies have shown that the natural hosts of chipmuk genotype I in North America are mainly eastern chipmunks (*Tamias striatus*) and eastern gray squirrels (*Sciurus carolinensis*) and occasionally rodents of the genus *Peromyscus* [19, 20]. In Europe, chipmunk genotype I was first reported in two Eurasian red squirrels (*Sciurius vulgaris*) in Italy [21]. Eastern gray squirrels and native Eurasian red squirrels are sympatric species in the UK and Italy [22, 23], most likely due to the introduction of the eastern gray squirrel into Europe during the last century. Prediger et al. [24] demonstrated that eastern gray squirrels introduced in northern Italy are parasitized with chipmunk genotype I. However, chipmunk genotype I was not detected in the Eurasian red squirrels in the same study, and therefore it was concluded that this genotype rarely infects them. In 2021, natural infections with chipmunk genotype I were reported in sick Eurasian red squirrels housed at a rehabilitation center in Sweden, one of which subsequently died of intestinal disease [25]. Based on these data, we hypothesized that chipmunk genotype I is highly pathogenic to Eurasian red squirrels, causing frequent mortality, which could explain why it was not detected in surveys of wild Eurasian red squirrels. The aim of this study was to describe the infectivity and pathogenicity of chipmunk genotype I in Eurasian red squirrels and eastern gray squirrels. Morphological and genetic characteristics of this *Cryptosporidium* genotype were studied. Findings demonstrated that chipmunk genotype I is highly pathogenic to the Eurasian red squirrel. Moreover, the data obtained confirmed that chipmunk genotype I is genetically and biologically distinct from valid *Cryptosporidium* species, and therefore we propose to name it *Cryptosporidium mortiferum* n. sp.

Methods

Source of chipmunk genotype I oocysts

The isolate (Chip_I) of chipmunk genotype I was obtained from a naturally infected, immunocompetent adult living in the USA. However, the patient's residency information is not available. The presence of oocysts in the diarrheal stool specimen was detected by direct immunofluorescence assay (Merifluor; Meridian Biosciences, Cincinnati, Ohio, USA), and the genotype was determined by PCR/sequencing. Oocysts from isolate Chip_I, purified by cesium chloride gradient centrifugation [26], were used for morphometric, molecular characterization and cross-transmission studies. In addition to isolate Chip_I, DNA from an additional five isolates (14762, 17064, 15003, SV33 and SV59), obtained previously from naturally infected eastern gray squirrels, Pallas squirrels (*Callosciurus erythraeus*) and Eurasian red squirrels in Italy, were used for molecular characterization [21, 24].

Oocyst morphometry

Oocysts of chipmunk genotype I (isolate Chip_I), from a naturally infected human and from experimentally infected hosts in the present study, were purified by cesium chloride [26] and used for morphological analyses. The length and width of 100 oocysts of each isolate

were measured, and a shape index was calculated. Pure oocysts from the *C. parvum* isolate HA ($n=100$), originally obtained from a naturally infected calf and maintained in SCID mice in the laboratories of the Biology Center of the Czech Academy of Science, Czech Republic (BC CAS), were used as controls. Oocysts were measured by the same worker using differential interference contrast (DIC) microscopy at 1000× magnification. Each slide was analyzed in a meandering pattern to avoid repeated measurement of an oocyst. Photographs of oocysts for morphometric analysis were analyzed using digital image analysis (Olympus CellSens Entry 2.1 software and Olympus Digital DP73 color camera, Olympus Corporation, Shinjuku, Tokyo, Japan). Various staining and labeling methods were also used to visualize the oocysts. Fecal samples containing oocysts of chipmunk genotype I were stained with aniline-carbol-methyl violet (ACMV) [27], modified Ziehl-Neelsen (ZN) [28] and phenol stain (AP) [29] and labeled with genus-specific FITC-conjugated antibodies (IFA; *Cryptosporidium* IF test, Cryptocell, Cellabs Pty Ltd., Brookvale, Australia) and with a Cy3-labeled mouse monoclonal antibody targeting the antigenic sites of the *Cryptosporidium* oocyst outer wall (A400Cy2R-20X, Crypt-a-Glo, Waterborne, Inc., New Orleans, LA, USA).

Molecular characterization and sequencing

Total genomic DNA (gDNA) was extracted from purified oocysts (100,000), feces (200 mg) or tissue samples (200 mg) using a GeneAll® Exgene™ Stool DNA Mini Kit (GeneAll Biotechnology Co., Ltd.; Seoul, South Korea) or a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, followed by bead disruption of the oocysts for 60 s at 5.5 m/s with 0.5-mm glass beads in a FastPrep®-24 Instrument (MP Biomedicals, Santa Ana, CA, USA). The acquired gDNA was stored at − 80 °C. The partial sequences of the genes for the small subunit of rRNA (*SSU*), actin, *Cryptosporidium* oocyst wall protein (*COWP*), 70-kDa heat shock protein (*HSP70*), thrombospondin-related adhesive protein of *Cryptosporidium*-1 (*TRAP-C1*) and 60-kDa glycoprotein (*gp60*) were amplified according to published nested PCR protocols and PCR primers [18, 30–34]. Primary and secondary reactions were performed in a 50 µl volume. The primary mixture consisted of 2 µl gDNA, 2.5 U Taq DNA polymerase (Dream Taq Green DNA Polymerase, Thermofisher Scientific, Waltham, MA, USA), 1× PCR buffer (Thermofisher Scientific), 200 nM of each primer, 6 mM $MgCl_2$ (*SSU*) or 3 mM $MgCl_2$ (actin, *COWP*, *HSP70*, *TRAP-C1* and *gp60*), 200 uM of each deoxynucleoside triphosphate and 2 ul of nonacetylated bovine serum albumin (BSA; 10 mg/ml; New England Biolabs, Beverly, MA, USA) and molecular grade water. The mixtures for secondary PCR were similar to those described above for primary PCR, except that 2 µl of the primary PCR product was used as the template, and the concentration of $MgCl_2$ for all amplified genes was 3 mM. Molecular water was used as a negative control, and DNA from *Cryptosporidium tyzzeri* (for *SSU*, actin, *COWP*, *HSP70* and *TRAP-C1*) and chipmunk genotype I (for *gp60*) were used as positive controls. Secondary PCR products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide. The amplicons were purified using the GenElute™ Gel Extraction Kit (Sigma, St. Louis, MO, USA) and sequenced using the secondary PCR primers by the commercial company SeqMe, s.r.o. (Dobříš, Czech Republic).

Phylogenetic analysis

The nucleotide sequences obtained were edited using Chromas Pro 2.4.1 software (Technelysium, Pty, Ltd., South Brisbane, Australia), verified by BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi), edited and aligned using BioEdit v.7.0.5 (Hall 1999). Final alignment of the obtained sequences with the reference sequences from GenBank was performed using the online server MAFFT version 7 (http://mafft.cbrc.jp/alignment/software/). The best model for the DNA/protein phylogeny was selected for each alignment using the Bayesian information criterion in MEGA 7. Neighbor-joining (NJ) and maximum likelihood (ML) approaches were computed in MEGA7 software [35, 36], using Tamura's three-parameter model + G + I [37] for the *SSU*, actin and *COWP* alignment and the general time-reversible model + G + I [38] for the *gp60*, *HSP70* and *TRAP-C1* alignment. Bootstrap support for branching was based on 1000 replications. Final trees were visualized using Corel Draw X7 software (https://www.corel.draw.com). The sequences obtained in this study were deposited in GenBank under the following accession numbers: OQ627025 to OQ627029 (*SSU*), OQ632461 to OQ632466 (actin), OQ632467 to OQ632474 (*COWP*), OQ632480 to OQ632487 (*HSP70*), OQ632488 to OQ632495 (*TRAP-C1*) and OQ632475 to OQ632479 (*gp60*).

Transmission studies

Fecal samples from all animals used in the transmission studies were examined daily for the presence of *Cryptosporidium* spp. oocysts and specific DNA (*SSU*) for 1 week prior to the transmission studies. A single 8-week-old SCID mouse (SCID 0) was infected with a dose of 100,000 oocysts of chipmunk genotype I isolate Chip_I. The oocysts of chipmunk genotype I obtained from SCID 0 were compared morphologically and molecularly with the original isolate Chip_I and used to infect other animals (see below). Three 8-week-old mice (*Mus musculus*)

of each strain (BALB/c, SCID, C57Bl6, $CD4^{-/-}$ and $CD8^{-/-}$), three 8-week-old gerbils (*Meriones unquiculatus*), three adult Guinea pigs (*Cavia aperea*), three adult ferrets (*Mustela putorius furo*), three adult Eurasian red squirrels, three adult eastern gray squirrels and three 7-day-old chickens (*Gallus gallus* f. *domestica*) were used for experimental transmission studies. Three animals from each group served as negative controls. Each experimental animal was orally administered a dose of 100,000 oocysts of chipmunk genotype I in a 200 µl volume. Control groups were orally inoculated with 200 µl sterile water. Beginning on the second day after inoculation, feces from all animals were collected individually and examined for the presence of oocysts (ACMV stain) and specific *Cryptosporidium* DNA (*SSU*). All animals were monitored for 30 days post-infection (DPI) or less if their health deteriorated because of infection and were therefore humanely killed. Oocysts were quantified, and the intensity of infection was estimated using the method described by Kváč et al. [20]. Briefly, the slide was weighed to the nearest 0.001 g before and after preparation of the smear to determine the mass of fecal material added to the slide. After ACMV staining, all oocysts on the slide were counted, and the number of oocysts per gram of fecal material was calculated. Oocysts were counted from triplicate smears of each sample. All experimental procedures complied with the laws of the Czech Republic (Act No. 246/1992 Coll., on the Protection of Animals against Cruelty, under protocols nos. MZP/2019/630/1411 and 35/2018). Rodents were housed individually in ventilated cages (Tecniplast, Buguggiate, Italy). Chickens were housed in boxes. Squirrels and ferrets were housed in separate cages. An external heat source was used for young birds during the first 5 days of life. Sterilized food and water were available ad libitum for all animals. Keepers wore sterile shoe covers, disposable coveralls and disposable gloves when entering the experimental room. Wood chip bedding and disposable protective clothing were removed from the experimental room and incinerated.

Clinical and pathomorphological examinations

All animals that shed oocysts or specific DNA after 30 DPI or that were killed early were necropsied. Tissue samples from the esophagus, stomach (mammalian only), proventriculus and ventriculus (chicken only), duodenum, proximal, central and distal jejunum; ileum, cecum, colon, cloaca and bursa of Fabricius (chicken only), liver, spleen, kidney, urinary bladder, trachea, lung, heart, eye and brain were collected using different sterile dissection tools for each site and processed for histology, scanning and transmission electron microscopy (SEM and TEM) and PCR genotyping (see above). Histological sections (5 µm) were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) and examined at 100–400× magnification (Olympus IX70) [39]. Samples for SEM were processed according to the protocol in Kváč et al. [15] and viewed using a JEOL JSM-7401F-FE SEM. Samples for TEM were processed according to the protocol in Valigurová et al. [40] and viewed using a JEOL JEM-2100F.

Staining of mucosal smears

Identification of developmental stages in the gastrointestinal tract of SCID mice was performed using Wright stained smears, which allow visualization of characteristic morphological structures [2]. Tissue samples from the cecum (selected based on the results of PCR, histology, SEM and TEM) were carefully washed with cold sterile PBS. The washed tissue was exposed to serum from *Cryptosporidium*-negative mice for 5 min to release developmental stages. The mucosa was carefully scraped with a scalpel and smeared on a glass. The wet smears were fixed in the vapor of 2% osmium tetroxide for 30 min and washed with methanol. The fixed smears were stained for 9 min with Wright diluted 1:1 with distilled water. Sporozoites released from excysted oocysts were stained with carbol-fuchsin [27]. Slides were viewed at 1000× magnification and documented using Olympus cell Sens Entry 2.1 (Olympus Corporation, Shinjuku, Tokyo, Japan) and a digital camera (Olympus DP73).

Statistical analysis

Differences in *Cryptosporidium* spp. oocyst size were tested using Hotelling's multivariate version of the two sample *t*-test, package ICSNP: Tools for Multivariate Nonparametrics [41] in R 4.2.2. [42]. The hypothesis tested was that the two-dimensional mean vectors of measurement are the same in the two populations being compared.

Results

Sequence and phylogenetic analysis

Partial amplicons of the genes encoding *SSU*, actin, *COWP*, *HSP70*, *TRAP-C1* and *gp60* were obtained from all chipmunk genotype I isolates included in this study from naturally infected Eurasian red squirrels (SV33 and SV59), eastern gray squirrels (14762), Pallas squirrels (15003 and 17064), a human (isolate CHIP_I) and experimentally infected laboratory mice, gerbils, ferrets, Eurasian red squirrels and eastern gray squirrels (isolate CHIP_I) (Figs. 1, 2, 3, 4, 5 and 6). The sequences of individual genes obtained from experimentally infected animals were not different from each other and were also identical to the original isolate CHIP_I. Phylogenetic relationships among

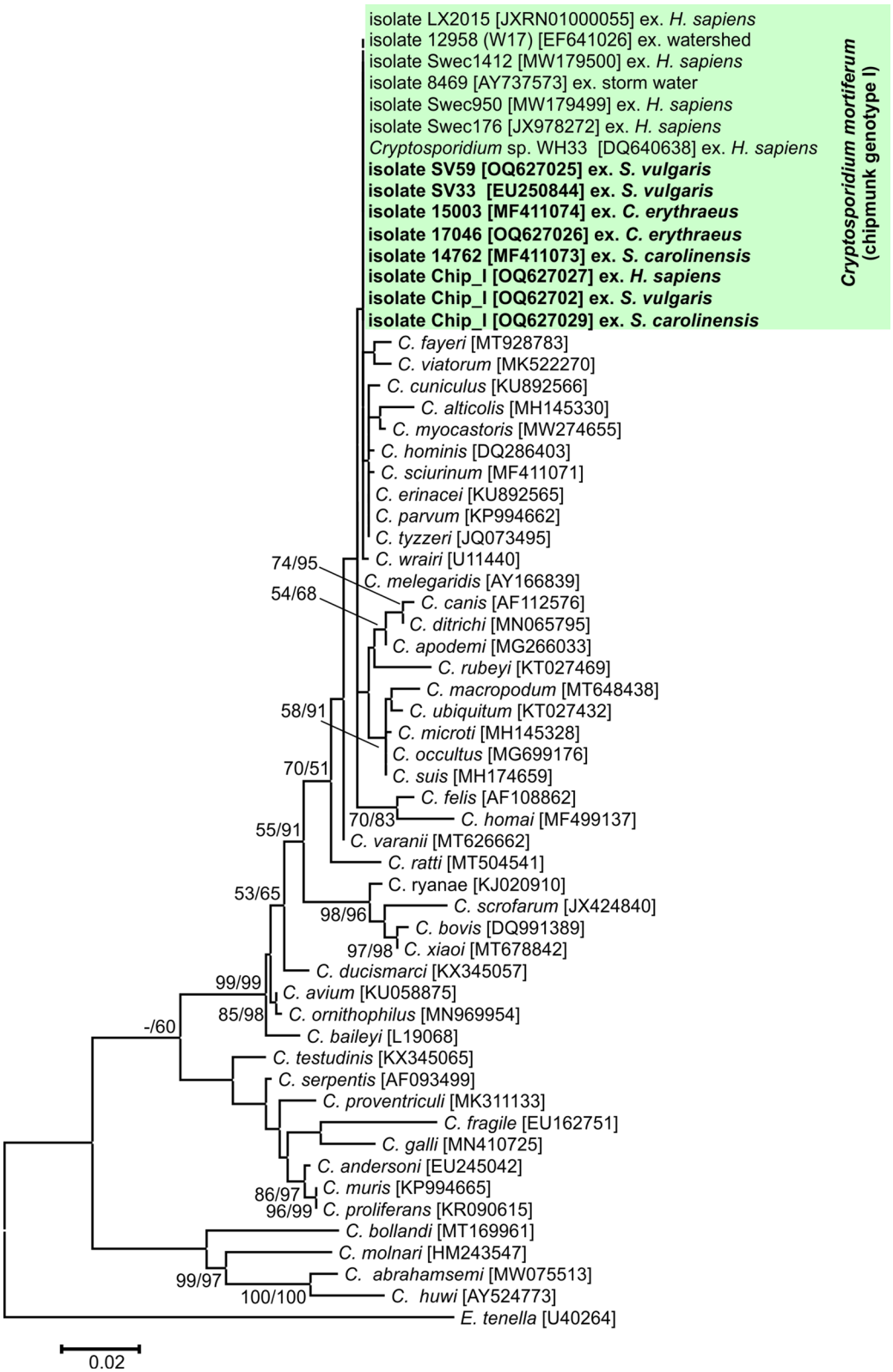

Fig. 1 Evolutionary relationships of *Cryptosporidium* spp. at the small subunit rRNA locus (*SSU*) inferred using the maximum likelihood (ML)/neighbor-joining (NJ) method. Percentage supports (> 50%) from 1000 pseudoreplicates from ML and NJ analysis, respectively, are indicated next to supported node. The GenBank accession number is in parentheses. Sequences obtained in this study are identified by isolate number (e.g. 14762) and highlighted

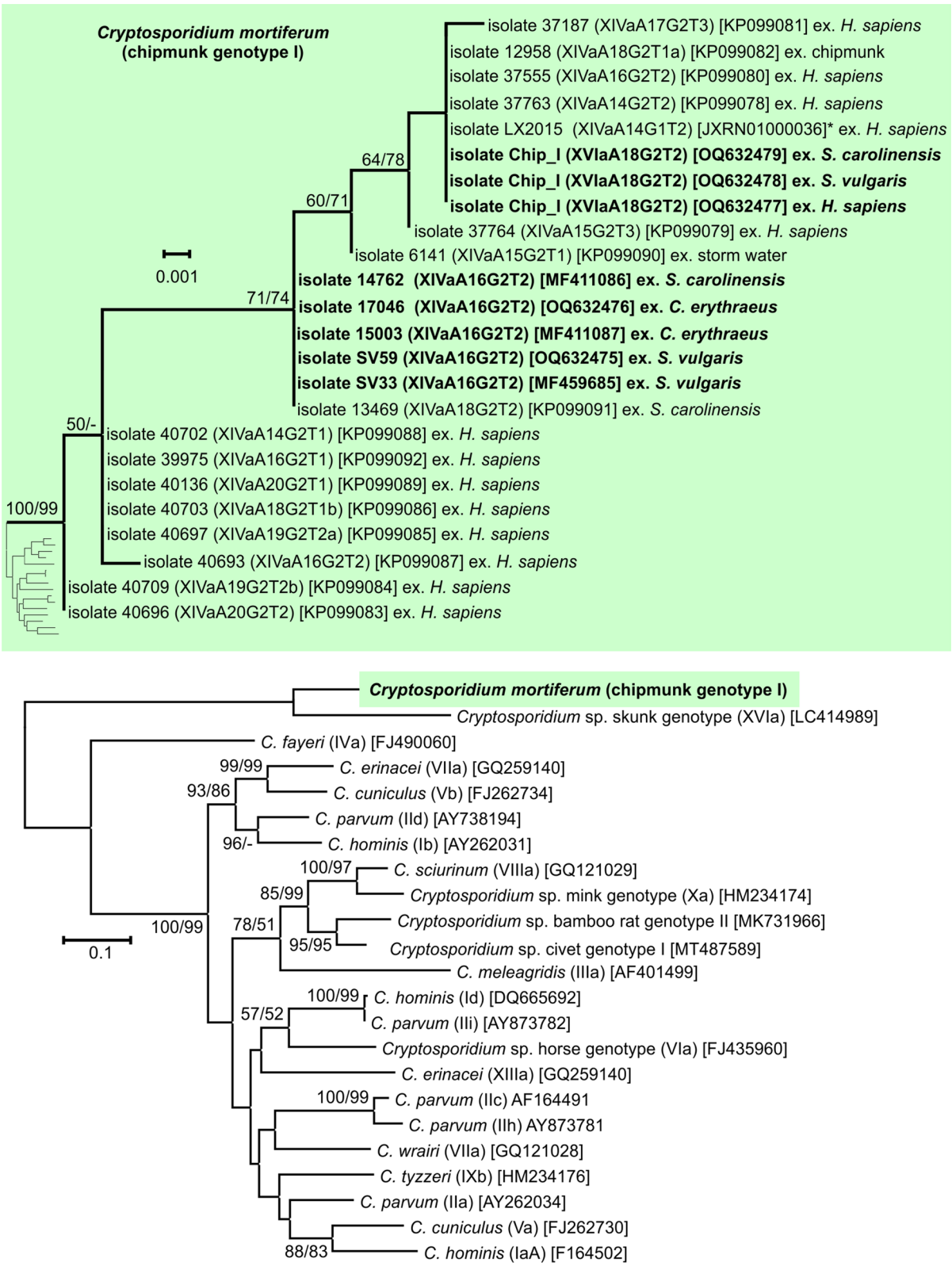

Fig. 2 Evolutionary relationships of *Cryptosporidium* spp. at the 60 kDa glycoprotein locus (gp60) inferred using the maximum likelihood (ML)/neighbor-joining (NJ) method. Percentage supports (> 50%) from 1000 pseudoreplicates from ML and NJ analysis, respectively, are indicated next to the supported node. The GenBank accession number is in parentheses. Sequences obtained in this study are identified by isolate number (e.g. 14762) and highlighted

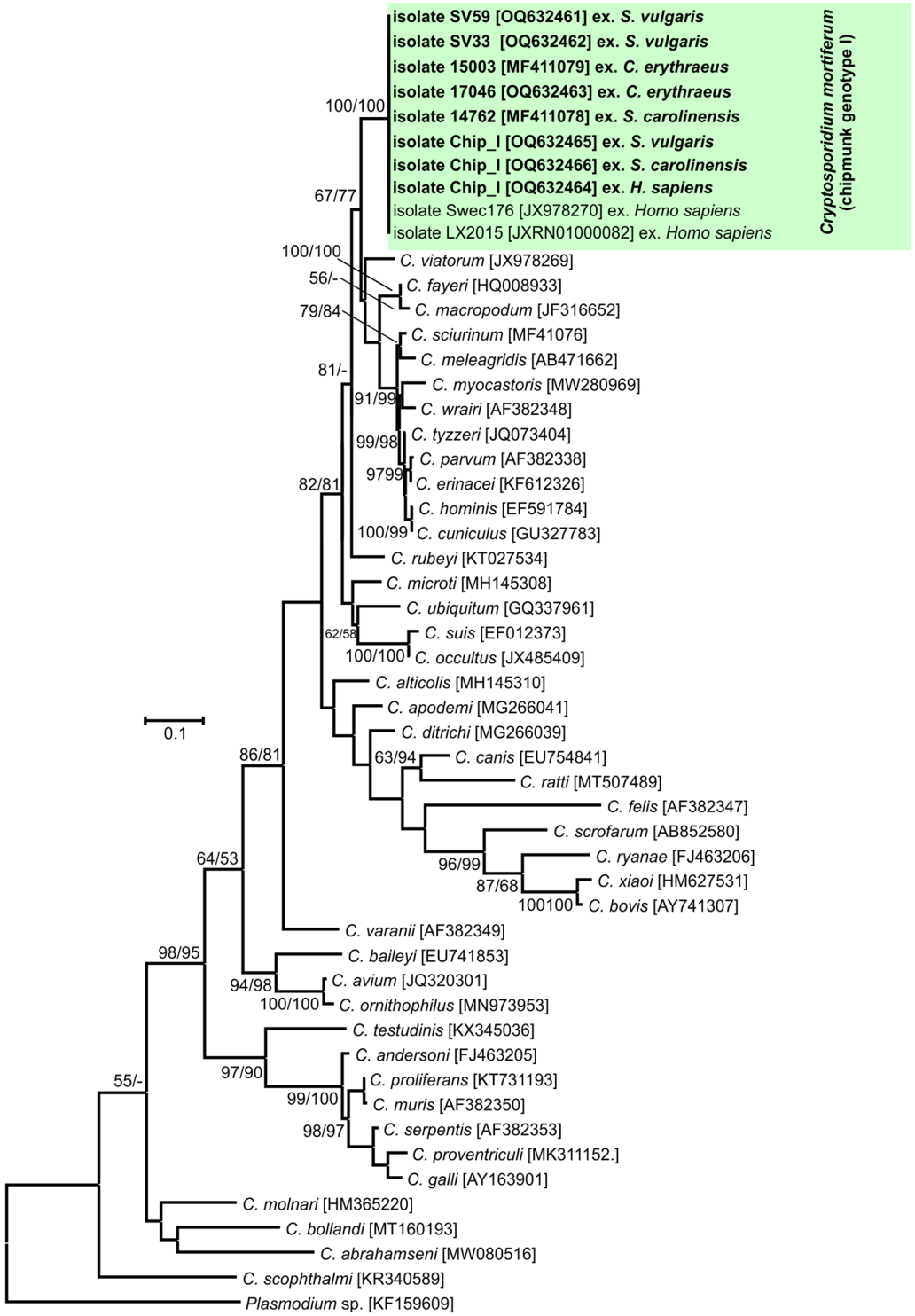

Fig. 3 Evolutionary relationships of *Cryptosporidium* spp. at the actin locus inferred using the maximum likelihood (ML)/neighbor-joining (NJ) method. Percentage supports (> 50%) from 1000 pseudoreplicates from ML and NJ analysis, respectively, are indicated next to supported node. The GenBank accession number is in parentheses. Sequences obtained in this study are identified by isolate number (e.g. 14762) and highlighted

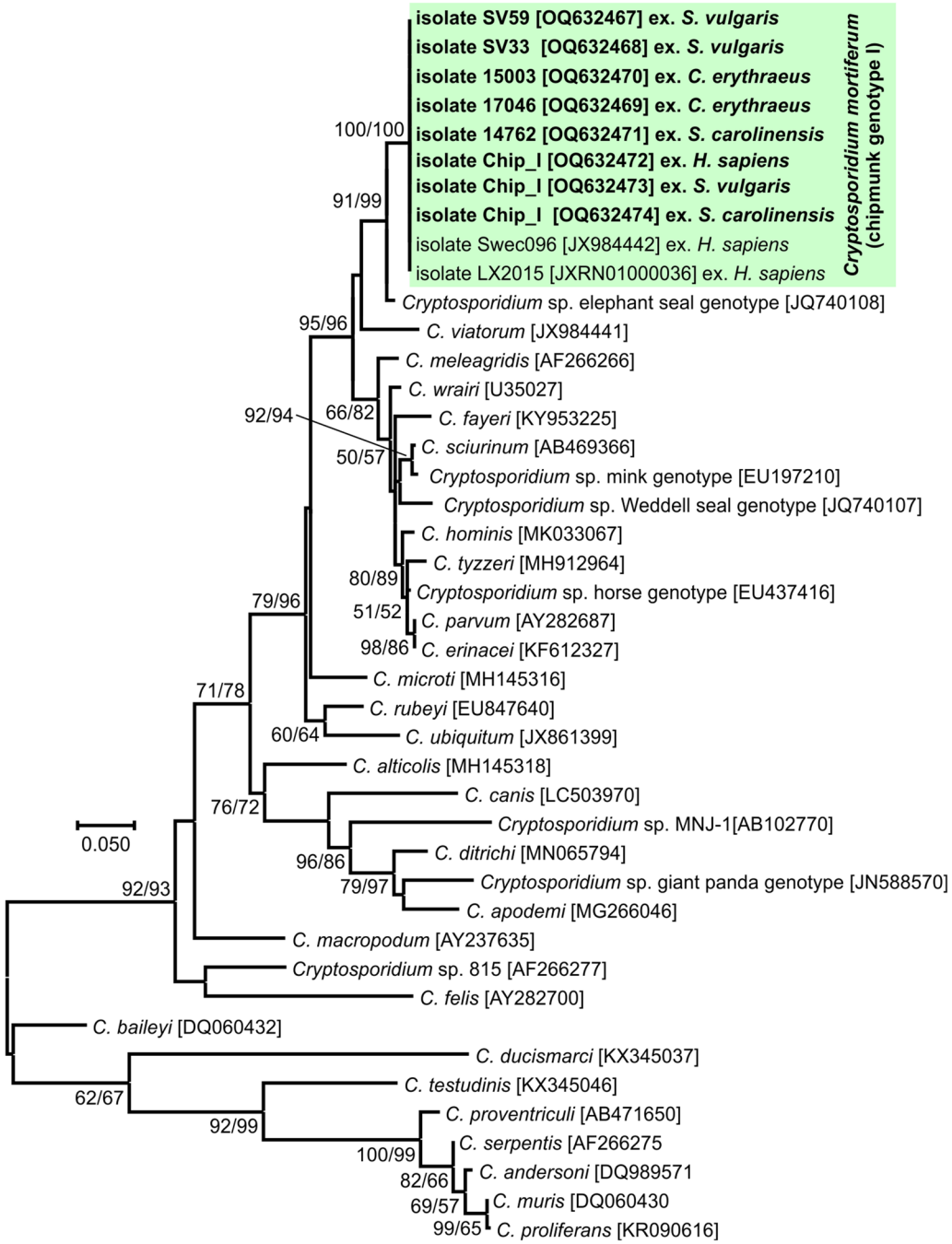

Fig. 4 Evolutionary relationships of *Cryptosporidium* spp. at the *Cryptosporidium* oocyst wall protein (*COWP*) locus inferred using the maximum likelihood (ML)/neighbor-joining (NJ) method. The evolutionary distances were computed using the General Time Reversible model with a gamma distribution. Percentage supports (> 50%) from 1000 pseudoreplicates from ML and NJ analysis, respectively, are indicated next to supported node. The GenBank Accession number is in parentheses. Sequences obtained in this study are identified by isolate number (e.g. 14762) and highlighted

chipmunk genotype I isolates and other *Cryptosporidium* spp. were inferred by ML and NJ analyses at all six loci and revealed similar tree topologies. All isolates of chipmunk genotype I used in this study were identical to each other and clustered with other previously reported chipmunk genotype I isolates (Figs. 1, 2, 3, 4, 5 and 6).

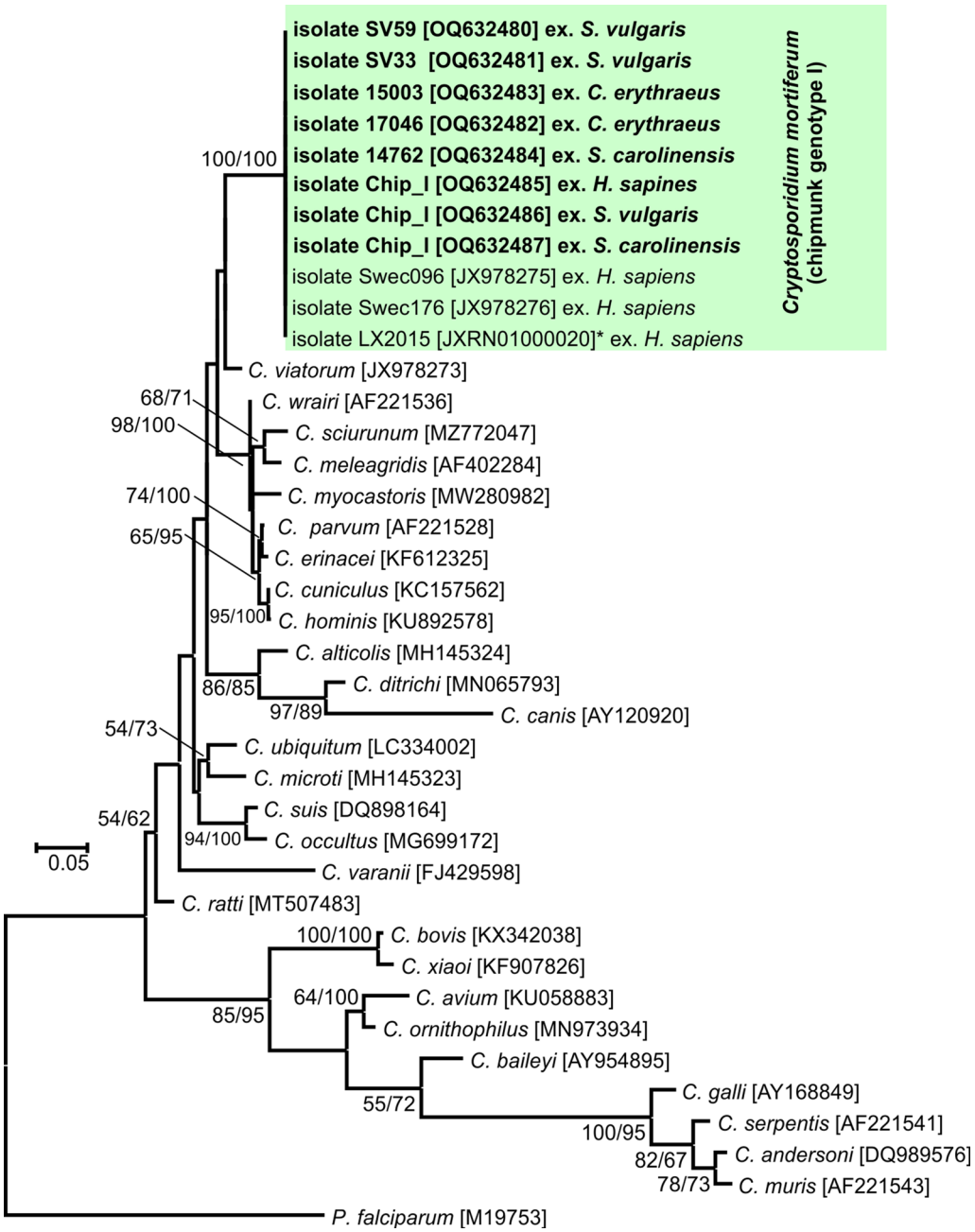

Fig. 5 Evolutionary relationships of *Cryptosporidium* spp. at the 70-kDa heat shock protein (*HSP70*) inferred using the maximum likelihood (ML)/neighbor-joining (NJ) method. Percentage supports (> 50%) from 1000 pseudoreplicates from ML and NJ analysis, respectively, are indicated next to supported node. The GenBank Accession number is in parentheses. Sequences obtained in this study are identified by isolate number (e.g. 14762) and highlighted

Host specificity and course of infection

No infection was detectable in chickens and Guinea pigs inoculated with 100,000 chipmunk genotype I oocysts (data not shown). Eurasian red squirrels, eastern gray squirrels, gerbils, ferrets and all mouse strains were susceptible to infection with chipmunk genotype I. In adult BALB/c and C57Bl6 mice, infection was not detectable microscopically, but molecular analyses revealed repeated presence of chipmunk genotype I DNA in fecal samples starting at 4–5 DPI (Fig. 7). $CD4^{-/-}$ and $CD8^{-/-}$ mice shed microscopically detectable oocysts with an intensity of 2000–6000 OPG starting at 4–5 DPI.

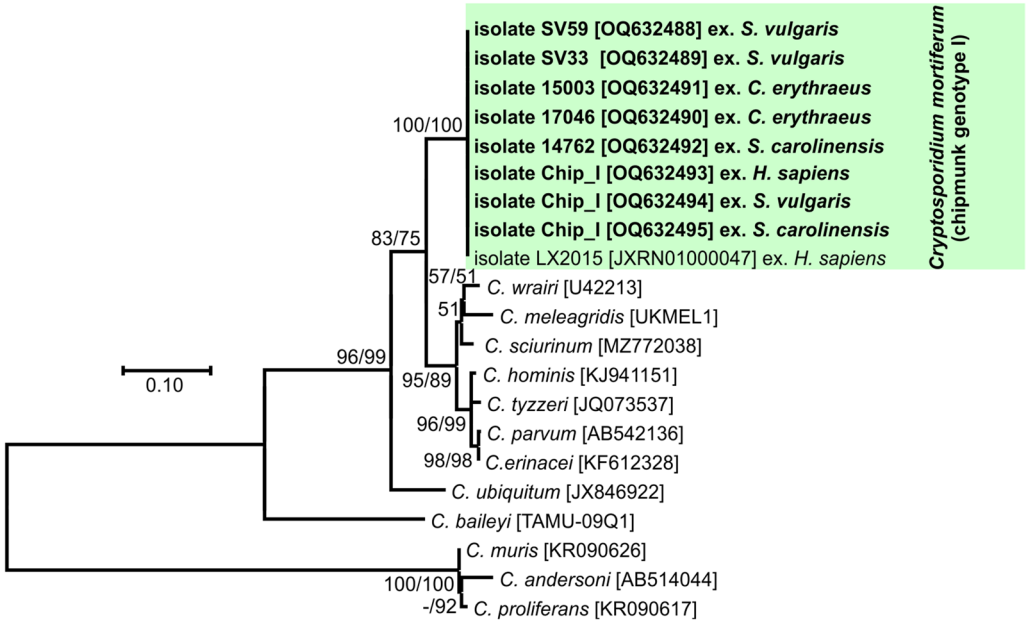

Fig. 6 Evolutionary relationships of *Cryptosporidium* spp. at the thrombospondin-related adhesive protein of *Cryptosporidium*-1 (*TRAP-C1*) locus inferred using the maximum likelihood (ML)/neighbor-joining (NJ) method. Percentage supports (> 50%) from 1000 pseudoreplicates from ML and NJ analysis, respectively, are indicated next to supported node. The GenBank accession number is in parentheses. Sequences obtained in this study are identified by isolate number (e.g. 14762) and highlighted

Infection in gerbils was detected by PCR only with the prepatent period was determined to be 11–12 DPI, and subsequently chipmunk genotype I DNA was detected intermittently until day 24 (Fig. 7b). Ferrets began shedding oocysts at 4 DPI. The intensity of infection ranged from 2000 to 20,000 OPG (Fig. 7a), and ferrets recovered spontaneously at 10–14 DPI. In SCID mice, the prepatent period was 9–10 DPI, and the animals shed many oocysts daily (10,000–150,000 OPG), without spontaneous recovery during the experiment (30 DPI). The prepatent period in eastern gray squirrels (7–8 DPI) was almost half that in Eurasian red squirrels (11–12 DPI). While eastern gray squirrels shed oocysts intermittently, with infection intensity ranging from 2000 to 30,000 OPG, infection in Eurasian red squirrels ranged from 10,000 to 1,500,000 OPG. In one of the eastern gray squirrels, mild apathy was observed at 7–8 DPI, manifested by decreased interest in the environment and food. The feces of this individual had a pasty consistency during this time. All eastern gray squirrels lost the infection within 9 days of the onset of shedding. Eurasian red squirrels rapidly lost their condition and appetite for food beginning on day 10 after infection. They spent most of the day in their shelter, were lethargic and did not respond to external stimuli such as feeding, watering, handling or cage cleaning. All Eurasian red squirrels with severe clinical signs were humanely killed (Fig. 7b).

Oocyst morphology

The size of chipmunk genotype I oocysts obtained from a naturally infected human was not statistically different from those obtained from experimentally infected mice, ferrets and squirrels, measuring 5.64 (5.50–5.89) × 5.37 (4.86–5.60) µm with an index of 1.05 (1.01–1.14) (Table 1). Chipmunk genotype I oocysts in the fecal smears stained with ACMV, ZN and AP showed the typical *Cryptosporidium* staining characteristics (Fig. 8b–d), and intensity of staining did not differ from *C. parvum* oocysts used as controls (data not shown). Immunofluorescent reagents originally developed for *C. parvum* oocysts also reacted with antigens of chipmunk genotype I oocysts, resulting in positive immunofluorescent labeling (Fig. 8e).

Infection site

Molecular, histological, SEM and TEM analyses showed the presence of chipmunk genotype I DNA and parasite developmental stages exclusively in the cecum of all susceptible hosts included in this study. Additionally, in SCID mice and Eurasian red squirrels, the proximal part of the colon was infected. The developmental stages covered almost the entire epithelial surface (Figs. 9 and 10).

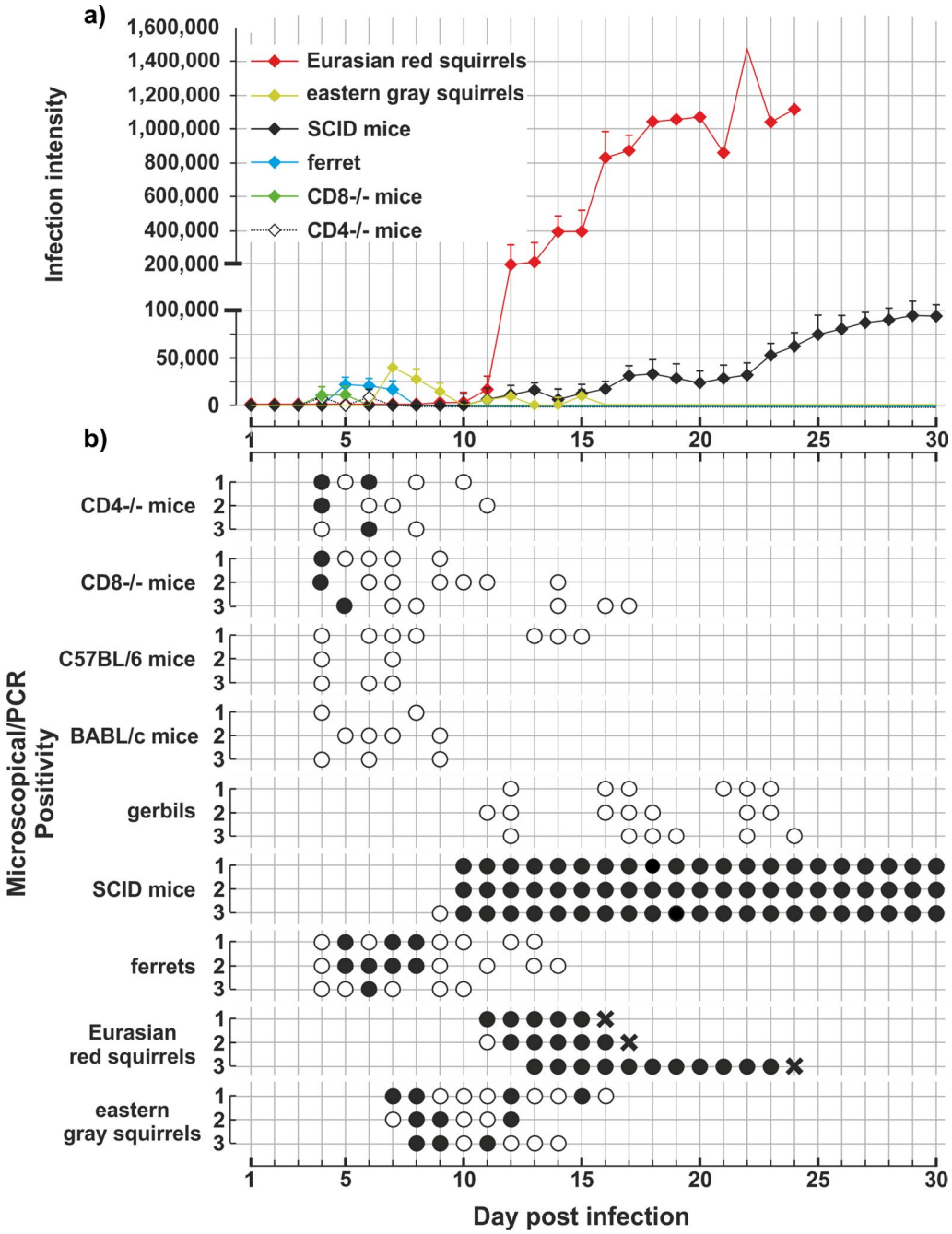

Fig. 7 Course of infection of *Cryptosporidium* chipmunk genotype I. in different strains of experimentally inoculated laboratory mice (*Mus musculus*), gerbils (*Meriones unquiculatus*), ferrets (*Mustela putorius furo*), Eurasian red squirrels (*Sciurus vulgaris*) and eastern gray squirrels (*Sciurus carolinensis*). **a** Infection intensity expressed as number of oocysts per gram of feces (OPG) and **b** detection of oocysts based on molecular and microscopic examination of fecal samples. Black circles indicate the presence of oocysts and specific DNA of *Cryptosporidium* chipmunk genotype I; white circles indicate detection of specific-DNA only without oocyst detection. Crosses indicate that an animal was killed because of poor health

Morphology and morphometry of endogenous developmental stages

It was almost impossible to identify developmental stages from SEM observations because most stages were covered with parasitophorous vacuoles. We were only able to identify stages with ruptured parasitophorous vacuoles. Early meronts with incompletely separated or fully developed merozoites and freely invading

Table 1 Size of *Cryptosporidium* chipmunk genotype I oocysts recovered from naturally (*) infected human (*Homo sapiens*) and experimentally (#) infected SCID (*Mus musculus*), Eurasian red squirrel (*Sciurus vulgaris*), eastern gray squirrel (*Sciurus carolinensis*) and domestic ferret (*Mustela putorius furo*)

Host (animal no.)	Length (μm) Range (mean ± SD)	Width (μm) Range (mean ± SD)	Length/width ratio Range (mean ± SD)
Human (CHIP_I)*	5.50–5.89 (5.64 ± 0.19)	4.86–5.60 (5.37 ± 0.17)	1.01–1.14 (1.05 ± 0.05)
SCID mouse (0)#	5.45–6.00 (5.62 ± 0.23)	4.82–5.64 (5.41 ± 0.24)	1.04–1.13 (1.06 ± 0.07)
SCID mouse (3)#	5.45–6.00 (5.62 ± 0.23)	4.82–5.64 (5.41 ± 0.24)	1.04–1.13 (1.06 ± 0.07)
Eurasian red squirrel (3)#	5.47–5.91 (5.57 ± 0.28)	4.89–5.63 (5.30 ± 0.19)	1.03–1.14 (1.04 ± 0.07)
Eastern gray squirrel (2)#	5.50–5.95 (5.61 ± 0.26)	4.90–5.60 (5.32 ± 0.22)	1.05–1.12 (1.04 ± 0.09)
Ferret (2)#	5.52–5.80 (5.60 ± 0.21)	4.81–5.69 (5.36 ± 0.23)	1.02–1.15 (1.05 ± 0.06)

Length and width of 100 oocysts from each isolate were measured under differential interference contrast at 1000× magnification, and these measurements were used to calculate the length-to-width ratio of each oocyst

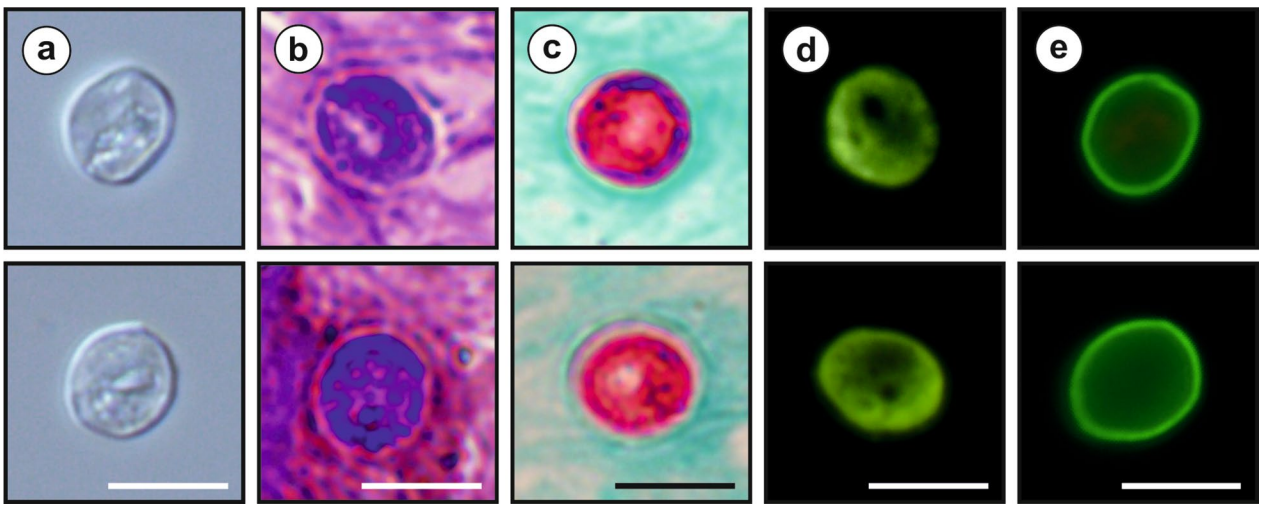

Fig. 8 Oocysts of *Cryptosporidium* chipmunk genotype I **a** in differential interference contrast microscopy, **b** stained by aniline-carbol-methyl violet staining, **c** stained by Ziehl-Nielsen staining, **d** stained by auramine-phenol staining and **e** labeled with anti-*Cryptosporidium* FITC-conjugated antibody. Bars = 5 μm

merozoites were observed. Using Wright-stained smears and analysis of TEM, we were able to determine all developmental stages. In the Wright-stained smears, the parasitophorous vacuoles surrounding the stages remained unstained, and the nuclei were stained pink (Fig. 11). Mononuclear trophozoites, transitional vegetative stages, were the most frequently observed stages, and their size was highly variable (Table 2).

Meronts with eight nuclei that became part of the forming merozoites were frequently observed, whereas meronts with four nuclei were rarely observed and differed from meronts with eight nuclei in that they had four nuclei from which four merozoites formed (Fig. 11e–h). Microgamonts were rarely observed and were easily recognized by 16 nuclei that became part of the forming microgametes (Fig. 11i–j). Macrogamonts were filled with oval amylopectin granules that gave the macrogamont a foamy appearance, making these stages easy to identify (Fig. 11k). A foamy structure was also observed in early zygotes, but it was less pronounced compared to the macrogamont stages (Fig. 11l). Zygotes and oocysts did not exhibit parasitophorous vacuoles. Oocysts were observed to be unstained and without significant structures. We were unable to distinguish between thin- and thick-walled oocysts. In the TEM analysis, the feeder organelle was clearly visible at most stages (Fig. 12). Early trophozoites were oval and became more spherical as they matured (Fig. 12b–c). Eight-nuclei meronts and four-nuclei meronts differed in the number of developing merozoites (Fig. 12d–g). TEM observation showed amylopectin granules not only in the macrogamont but also in the zygotes (Fig. 12k–l). Compared to the macrogamont, fewer amylopectin granules were present in the zygotes, and a new wall formation was visible

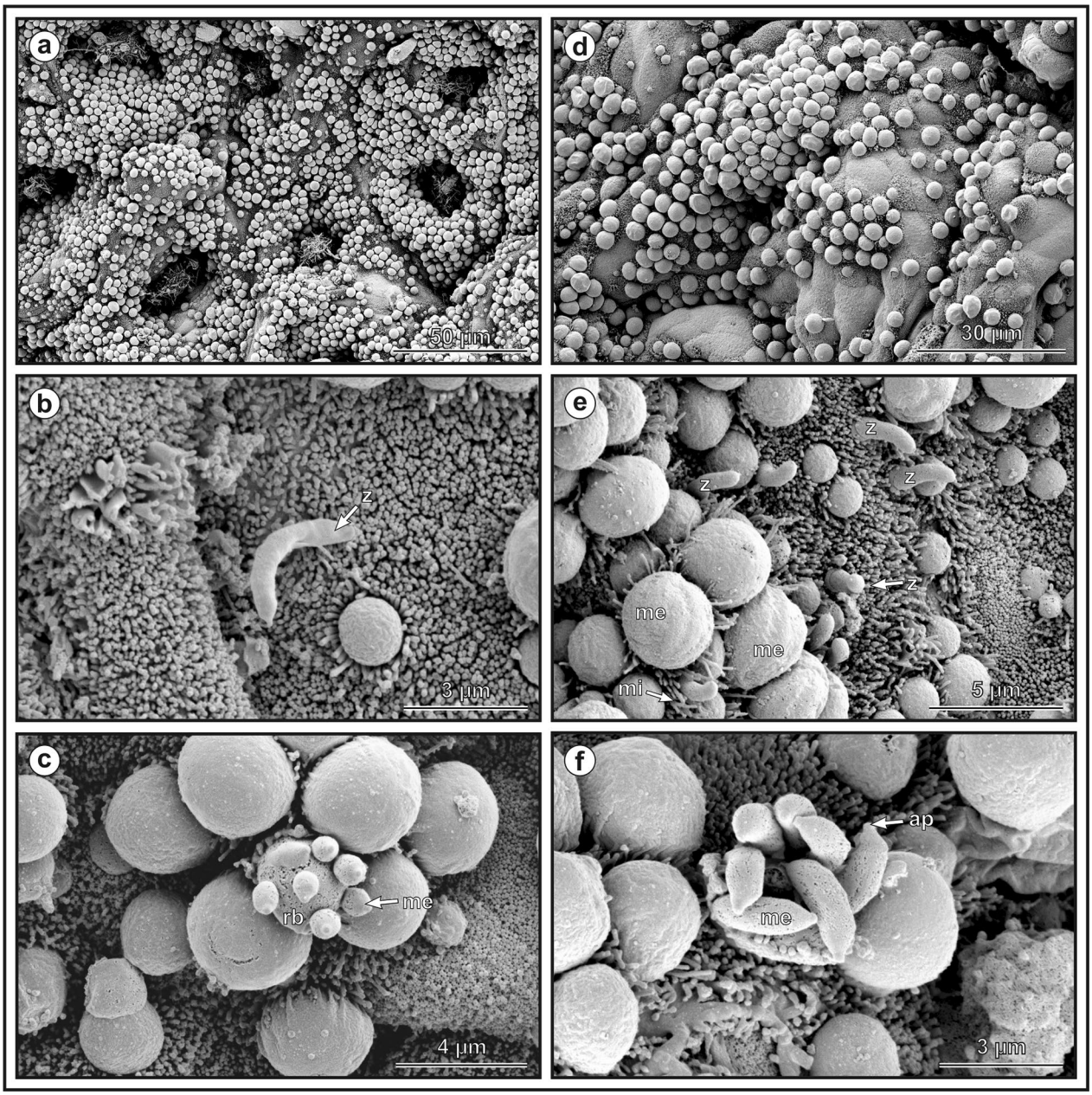

Fig. 9 Scanning electron microphotograph showing developmental stages of *Cryptosporidium* chipmunk genotype I on cecal mucosal epithelium in experimentally infected Eurasian red squirrel (*Sciurus vulgaris*) killed 16 days post infection (DPI) (**a**–**c**) and SCID mouse (*Mus musculus*) killed 30 DPI (**d**–**f**). **a** and **d** Surface of cecum covered with developmental stages, **b** released zoite (z); **c** merozoites (me) budding from residual body (rb); **e** zoites invading host tissue (z) with formation of merozoites covered with parasitophorous sac (me) and surrounded by elongated microvilli (mi), **f** mature meront with fully developed merozoites (me) with recognizable apical part (ap). Scale bars included in each figure

(Fig. 12l). Compared to the Wright stain, four sporozoites were visible inside the oocysts around a residual body formed from amylopectin granules (Fig. 12a).

Clinical signs and pathogenicity

In ferrets, clinical signs of cryptosporidiosis, vomiting and watery diarrhea occurred at 6–7 DPI. In infected SCID mice, a change in feces from solid globules to a paste-like consistency was observed from the 2nd week post infection, but no vomiting was observed. In one of the eastern gray squirrels, a slight apathy was observed at 7–8 DPI, manifested by reduced interest in the surroundings and food. The droppings in this individual had a pasty consistency during this period. Eurasian

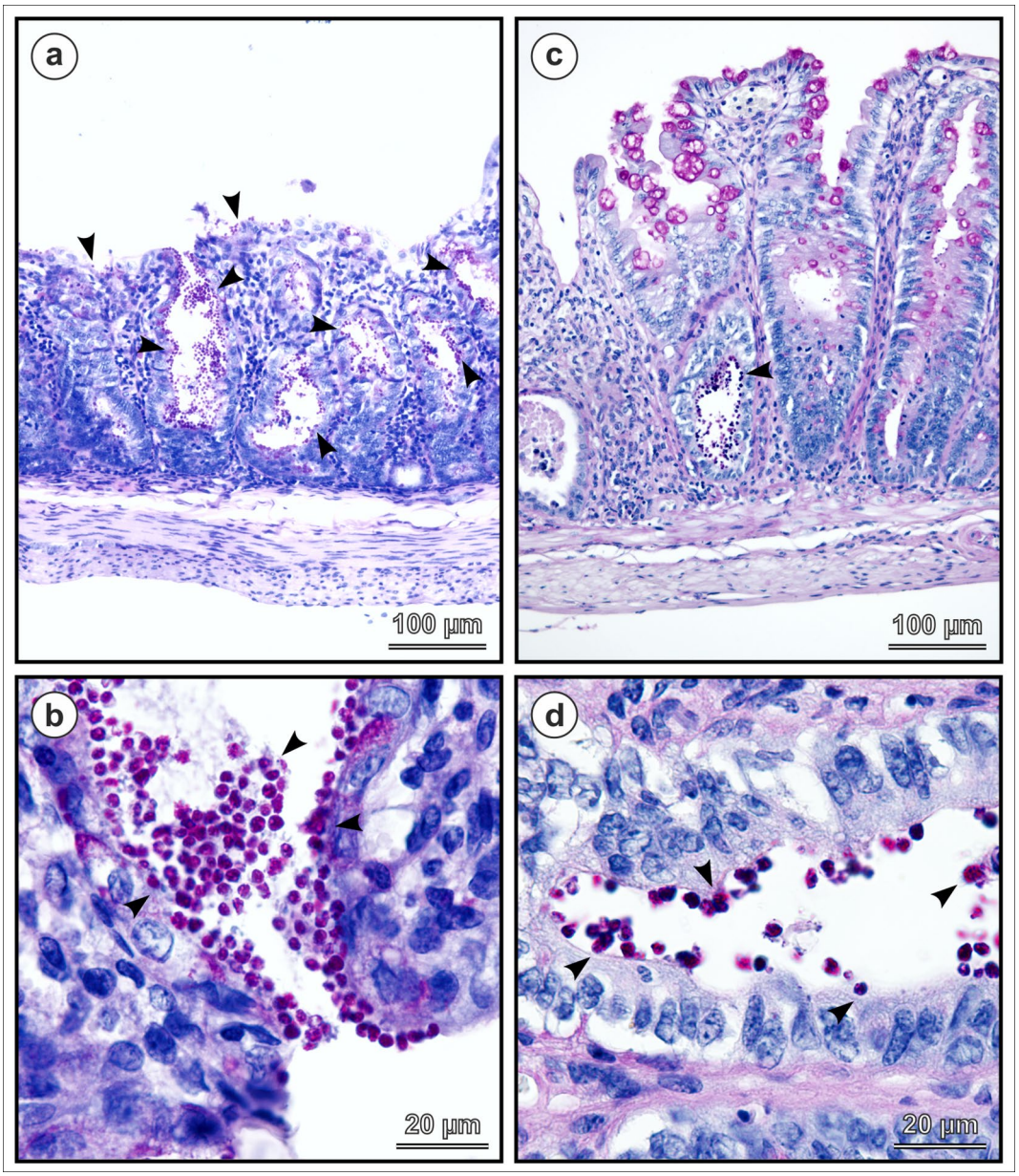

Fig. 10 Histology sections of the cecum of Eurasian red squirrel (*Sciurus vulgaris*) (**a**, **b**) and SCID mouse (*Mus musculus*) (**c**, **d**) experimentally infected with *Cryptosporidium* chipmunk genotype I and killed 16 and 30 days post infection, respectively. Attached developmental stages indicated by arrowhead. Periodic acid-Schiff (PAS) staining. Scale bar included in each figure

red squirrels quickly lost their condition and appetite for food from day 10 after infection. They spent most of the day in their shelter, were lethargic and did not respond to external stimuli-feeding, watering, handling or cage cleaning. Intestinal crypts were multifocally dilated with atrophy of epithelial and mucus cells. The formation of crypt microabscesses was locally observed. A multifocally to diffusely pronounced lymphoplasmocytic inflammatory infiltrate with admixture of smaller amounts of eosinophilic and neutrophilic granulocytes was present in the stroma of the intestinal mucosa. Reactive hyperplasia of submucosal lymphoid tissue/follicles was detected. Infected cells showed increased microcell elongation.

Based on the presented data, we propose *Cryptosporidium* chipmunk genotype I as a new species, with the species description presented below.

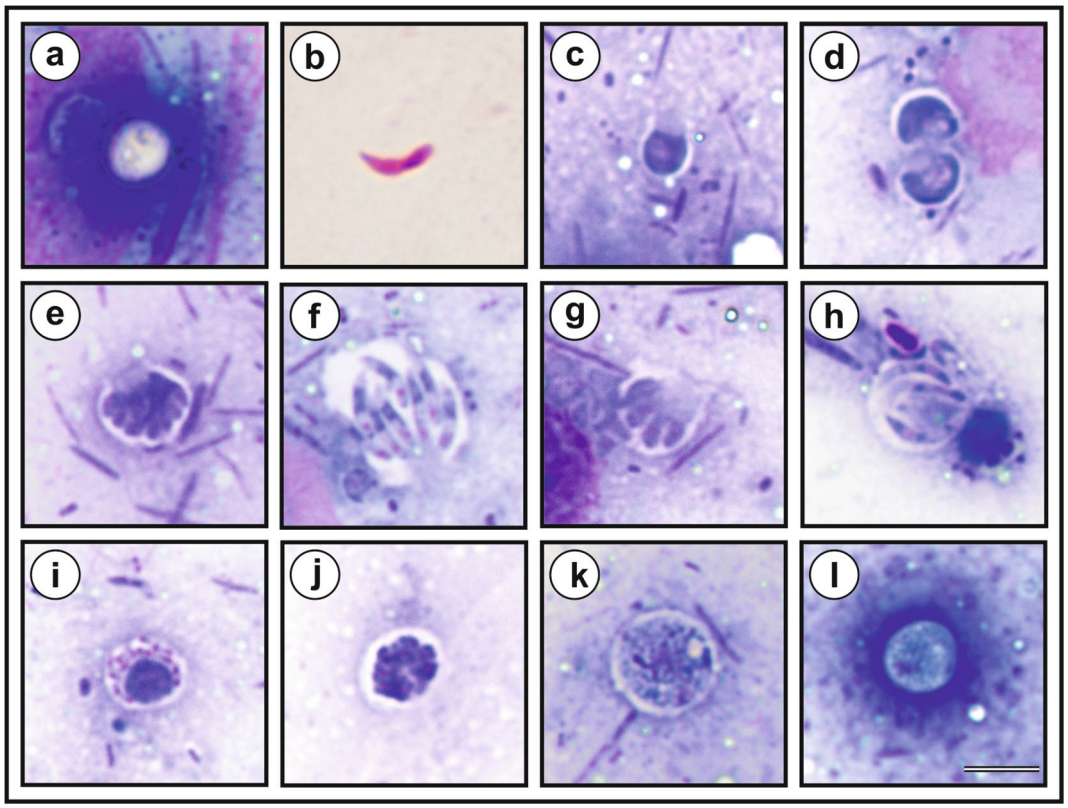

Fig. 11 Developmental stages of *Cryptosporidium* chipmunk genotype I in mucosal smears obtained from the cecum of SCID mouse (*Mus musculus*) experimentally infected with 100,000 oocysts and killed 30 days post infection. **a** Oocyst; **b** sporozoite; **c**, **d** mononuclear trophozoite; **e** eight-nuclei meront; **f** merozoites from eight-nuclei meront; **g** four-nuclei meront; **h** merozoites from four-nuclei meront; **i** and **j** microgamont; **k** macrogamont and **l** zygote. Bar = 10 µm

Table 2 Size of developmental stages of *Cryptosporidium* chipmunk genotype I obtained from the cecum of SCID mouse (*Mus musculus*) experimentally infected with 100,000 oocysts and killed 30 days post-infection

Developmental stages	Length (µm) Range (mean ± SD)	Width (µm) Range (mean ± SD)
Oocyst	5.50–5.89 (5.64 ± 0.19)	4.86–5.60 (5.37 ± 0.17)
Sporozoite	4.67–5.92 (5.42 ± 0.41)	0.55–0.64 (0.61 ± 0.03)
Trophozoite	1.95–5.27 (3.26 ± 0.76)	1.56–4.71 (2.75 ± 0.67)
Eight-nuclei meront	4.49–6.42 (5.54 ± 0.52)	4.07–6.04 (4.91 ± 0.53)
Merozoite from eight-nuclei meront	3.76–6.29 (5.22 ± 0.62)	0.51–0.98 (0.73 ± 0.12)
Four-nuclei meront	4.18–5.28 (4.65 ± 0.47)	3.86–5.21 (4.40 ± 0.58)
Merozoite from four-nuclei meront	4.88–5.63 (5.42 ± 0.32)	0.51–0.92 (0.73 ± 0.17)
Macrogamont	4.78–8.16 (5.93 ± 0.68)	4.37–6.46 (5.42 ± 0.62)
Microgamont	4.53–5.42 (4.91 ± 0.32)	4.14–4.42 (4.28 ± 0.10)
Zygote	4.91–5.53 (5.29 ± 0.22)	4.16–5.11 (4.79 ± 0.30)

Measurements were obtained via SEM

Taxonomic summary

Family Cryptosporidiidae Léger, 1911

Genus *Cryptosporidium* Tyzzer, 1907

***Cryptosporidium mortiferum* n. sp.**

Syn: *Cryptosporidium* chipmunk genotype (W17), *Cryptosporidium* genotype W17, *Cryptosporidium* chipmunk genotype I.

Type host: Eastern chipmunk (*Tamias striatus*) [19]

Other hosts: Deer mouse (*Peromyscus* sp.) [19], American red squirrel (*Tamiasciurus hudsonicus*) [20], Eurasian red squirrel (*Sciurus vulgaris*) [21], striped field mouse (*Apodemus agrarius*) [43], Ussuri white-toothed shrew (*Crocidura lasiura*) [43], Pallas's squirrel (*Callosciurus erythraeus*) [24], human (*Homo sapiens*) [44].

Type locality: New York, USA.

Type material: Fecal smear slides with oocysts stained by ACMV and ZN staining (nos. MV1-5/34351 and ZN1-5/34351); scanning electron microscopy specimens of infected cecum (nos. SEM220/2017, SEM221/2017, SEM43/2020 and SEM44/2020) and colon (nos. SEM222/2017, SEM223/2017, SEM52/2019 and SEM53/2019); transmission electron microscopy specimens of infected cecum (nos. TEM220/2017, TEM221/2017, TEM43/2020 and TEM44/2020) and colon (nos. TEM222/2017, TEM2023/2017, TEM52/2019 and TEM53/2019); histological sections of infected cecum (nos. H220/2017, H221/2017, H43/2020 and H44/2020) and colon (nos. H222/2017, H223/2017, H52/2019 and H53/2019); gDNA isolated from fecal samples of naturally infected human (isolate CHIP_I) and experimentally (isolate 34351) infected Eurasian red squirrel (*Sciurus vulgaris*); gDNA isolated from cecum and colon of experimentally infected Eurasian red squirrel (isolates 35039 and 35041). All specimens are deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

Site of infection: Cecum, colon (present study).

Prepatent period: Range from 4 to 12 DPI (this study), depending on the host species.

Patent period: Range from a few days to many weeks or a fatal infection (this study), depending on the host species and its immune response.

Representative DNA sequences: Representative nucleotide sequences of *SSU* [OQ627025–OQ627029], actin [OQ632461–OQ632466], *HSP*70 [OQ632480–OQ632487], *TRAP-C1* [OQ632488–OQ632495], *COWP* [OQ632467–OQ632474] and *gp60* [OQ632475–OQ632479] genes are deposited 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), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:1E90AC9B-DD71-4DF1-B554-F5AED-C82CD5A. The LSID for the new name *Cryptosporidium mortiferum* n. sp. is urn:lsid:zoobank.org:act:381F6044-C574-4F9D-8FC8-89BEABEB585B.

Description: The oocyst wall is smooth and colorless. The oocyst is composed of one spherical globule residuum, numerous small granules and four sporozoites. A suture is not noticeable. Sporulated oocysts measure 5.50–5.89 (5.64 ± 0.19) × 4.86–5.60 (5.37 ± 0.17) µm with a length-to-width ratio of 1.01–1.14 (1.05 ± 0.05) (Fig. 9).

Etymology: Since the infection caused by this species in Eurasian red squirrels is lethal, the species name *mortiferum* is derived from the Latin *mortifer*, meaning lethal.

Differential diagnosis: Oocysts of *C. mortiferum* are stained by ACMV and ZN staining methods and labeled with genus-specific antibodies targeting the *Cryptosporidium* oocyst outer wall antigenic sites, similar to other *Cryptosporidium* spp. (Fig. 9). Oocysts of *C. mortiferum* are larger than those of *C. parvum* ($T2 = 320.42$, $df1 = 2$, $df2 = 69.00$, $p < 0.001$) and *C. sciurinum* ($T2 = 67.32$, $df1 = 2$, $df2 = 84.60$, $p < 0.001$). The oocyst size cannot be used for species identification. *Cryptosporidium mortiferum* can be differentiated genetically from other *Cryptosporidium* spp. based on nucleotide sequences of *SSU*, actin, *HSP70*, *TRAP-C1*, *COWP* and *gp60* genes.

Discussion

Infection with *C. mortiferum* in eastern gray squirrels under experimental conditions is usually asymptomatic and is associated with a minor infection followed by self-healing. This is consistent with the finding by Prediger et al. of a low infection intensity in naturally infected eastern gray squirrels [24]. In agreement with the findings of Bujila et al. [25], we observed massive infections

(See figure on next page.)

Fig. 12 Developmental stages of *Cryptosporidium* chipmunk genotype I in transmission electron microscopy. **a** Oocyst with four sporozoites (s), residual body (rb) with amylopectin granules (ag), forming oocyst wall (ow) in parasitophorous sac (ps); **b** early trophozoite with one nucleus (n) inside parasitophorous sac (ps) and attached to microvilli border (mb) with feeding organelle (fo); **c** later trophozoite with one nucleus (n) inside parasitophorous sac (ps) and attached to microvilli border (mb); **d** early meront covered with parasitophorous sac (ps) with forming eight merozoites (me) connected to residual body (rb); **e** cross section of mature meront covered with parasitophorous sac (ps), fully developed eight merozoites (me) with visible nucleus (n) and connected to host cell by feeding organelle (fo); **f** cross section of early meront with forming four merozoites (me) with visible nucleus (n) and covered with parasitophorous sac (ps); **g** cross section of mature meront with fully developed four merozoites (me), covered with parasitophorous sac (ps) and attached to host cell by feeding organelle (fo); **h** empty parasitophorous sac (ps) attached to the host cell by feeding organelle (fo); **i** released merozoites (me) with visible nucleus (n); **j** early microgamont (mi) and attached to the host cell by feeding organelle (fo); **k** macrogamont covered with parasitophorous sac (ps) with foam-like appearance caused by amylopectin granules (ag) and visible nucleus (n); **l** zygotes with amylopectin granules (3), developing oocyst wall (1), parasitophorous sac (2). Bar = 1 um

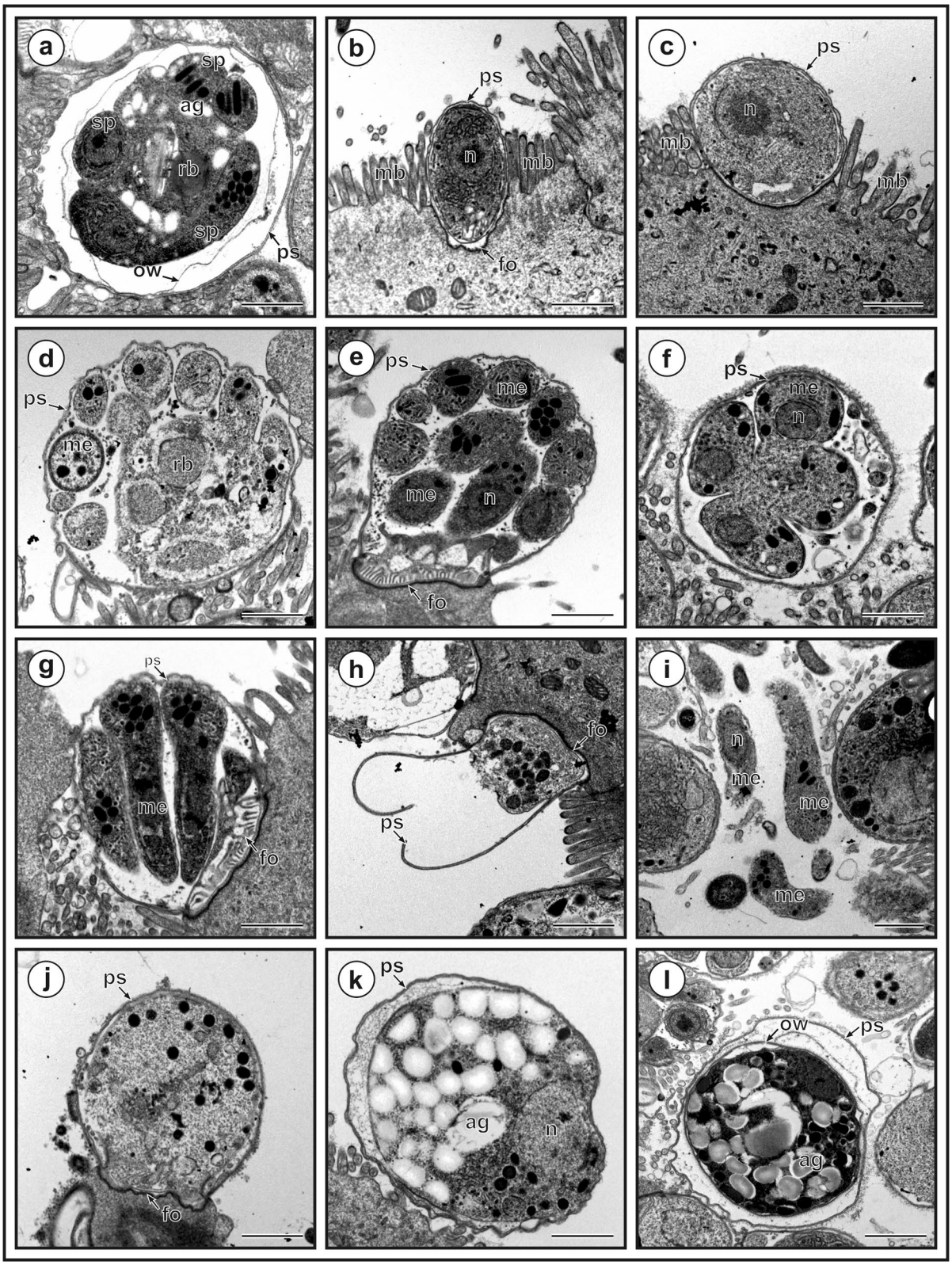

Fig. 12 (See legend on previous page.)

with *C. mortiferum* in Eurasian red squirrels, which were accompanied by severe clinical signs of cryptosporidiosis and resulted in death of the individuals. Because Eurasian red squirrels infected with *C. mortiferum* restrict their movement, lose interest in foraging and remain in their burrows most of the time, infected Eurasian red squirrels would be difficult to capture in live traps used in field research. It is likely that only a small percentage of individuals infected with *C. mortiferum*, such as those in the early stages of infection, would be captured in field studies.

This work clearly demonstrated that *C. mortiferum* pathogenicity varies among host species and that the course of infection is impacted by the host species and the immune status. The infection was asymptomatic in immunocompetent laboratory mice. Consistent with previous studies examining the host immune response to *Cryptosporidium* infection, mice deficient in CD4+ or CD8+ lymphocytes showed a longer course of infection with *C. mortiferum* than immunocompetent individuals [45, 46]. However, the absence of CD4 or CD8 lymphocytes did not prove critical, and animals with this deficiency recovered from infection. As with infections with *C. parvum*, *C. proliferans* and *C. tyzzeri*, self-curing did not occur in SCID mice infected with *C. mortiferum* [6, 45, 47]. SCID and BALB/c mice may serve as a suitable laboratory model for the study of cryptosporidiosis affecting the cecum. The prepatent period of *C. mortiferum* was found to be different in different host species/strains. While ferrets and mice, with the exception of SCID mice, began shedding oocysts/specific *C. mortiferum* DNA at 4–5 DPI, the prepatent period was longer in squirrels, SCID mice and gerbils (7–14 DPI). A similar difference in the length of the prepatent period, depending on the host species, was observed in *C. proliferans* [6]. The intensity of *C. mortiferum* infection also varied depending on the species and immune status of the host. Similar differences were observed in other *Cryptosporidium* species, e.g. *C. alticolis* and *C. microti* infecting various species of voles, *C. apodemi* and *C. ditrichi* parasitizing *Apodemus* spp., *C. proliferans* infecting various rodents or *C. ornithophilus* infecting geese, cockatiels and chickens [6, 7, 48, 49].

Most species of intestinal *Cryptosporidium* for which tissue specificity has been described parasitize the small intestine, e.g. *C. parvum*, *C. myocastoris*, *C. scrofarum*, *C. hominis*, *C. tyzzeri*, *C. ryanae* and *C. ditrichi* [14, 48, 50–54]. Only a small proportion of species parasitize the colon, or in birds, the bursa of Fabricius, e.g. *C. suis* or *C. baileyi* [55, 56]. The development of *C. mortiferum* occurs exclusively in the cecum and anterior colon. It is the first *Cryptosporidium* species in mammals to prefer this part of the intestine. A similar localization has already been described in *Cryptosporidium avium* and *C. ornithophilus* parasitizing birds [7, 17].

This study also described exogenous and endogenous developmental stages in *C. mortiferum*. The life cycle of *C. mortiferum* does not differ from that of previously described *Cryptosporidium* species. Consistent with studies that have examined the life cycle in vivo, we found no evidence for the occurrence of extracellular developmental stages described in cell-free cultures [57–59]. The oocysts of *C. mortiferum* measured 5.64×5.37 µm, which is similar in size to a previously published isolate of chipmunk genotype I (5.8×5.4 µm) found in Eurasian red squirrels [21]. The oocysts of this species are slightly larger than those of *C. sciurinum* (5.54×5.22 µm), *C. ubiquitum* (5.04×4.66 µm) and *C. parvum* (5.2×4.9 µm), which also have been detected in tree squirrels [8, 24, 60]. However, the differences in oocyst size are so small that they cannot be used for differential diagnosis among *Cryptosporidium* species by routine microscopy. Similar to previous studies, we were unable to distinguish between thin- and thick-walled oocysts, if present in this species [6, 61]. In agreement with the study by Holubová et al. [62], most of the observed developmental stages visualized by Wright staining were surrounded by a parasitophorous vesicle that appeared as an unstained aureole. In agreement with the same authors, mononuclear trophozoites and eight-nuclear merozoites were most frequently detected, while four-nuclei merozoites were rarely found with the Wright stain and TEM. In a recent in vitro study of *C. parvum*, English et al. [63] showed that the abundance of four-nuclear merozoites was low and that they were not required for microgamont and macrogamont formation. In addition, microgamonts were rarely found compared to macrogamonts [62, 63]. Examination of SEM showed elongation of microvilli of cells parasitized by developmental stages of *C. mortiferum*. Similar elongation was previously observed in coypu infected with *C. myocastoris* [53], SCID mice infected with *C. parvum* [64] and rats infected with *C. occultus* [15]. Borowski et al. [59] also reported the elongation of microvilli of cells infected with *C. parvum* in an in vitro model system.

Phylogenetic analyses at *SSU*, actin, *HSP70*, *TRAP-C1*, *COWP* and *gp60* loci confirmed previously published data showing that *C. mortiferum* is genetically distinct from other species within the genus *Cryptosporidium* and represent a separate species. At all loci, *C. mortiferum* formed a separate clade within the group of intestinal *Cryptosporidium* spp. and close to *C. viatorum*. At *SSU*, actin, *HSP70*, *TRAP-C1* and *COWP* loci, the pairwise distances between *C. mortiferum* and *C. viatorum* (0.005,

0.011, 0.007, ND and 0.013, respectively) were similar to those between *C. mortiferum* and *C. sciurinum*, which is a major *Cryptosporidium* species in Eurasian red squirrels (0.004, 0.012, 0.008, 0.016 and 0.013, respectively) and greater than those between *C. hominis* and *C. parvum* (0.002, 0.005, 0.003, 0.006 and 0.007, respectively) and between *C. hominis* and *C. cuniculus* (0.003, 0.001, 0.001, ND and ND, respectively).

To date, *C. mortiferum* has only been detected in the USA [44], Italy [21], Sweden [65] and South Korea [43], with most reported detections coming from the USA. The occurrence of *C. mortiferum* in Italy is related to the presence of introduced eastern gray squirrels, which are one of the natural hosts of this parasite. Sweden harbors only Eurasian red squirrels, and the authors suggest that these animals are natural hosts for *C. mortiferum* in Sweden [23]. Therefore, it is possible that *C. mortiferum* spread through the Eurasian red squirrel population and was introduced into northern Europe. However, studies in Central Europe have not shown that *C. mortiferum* is present in native squirrel populations in the Czech Republic and Slovakia, and we have no evidence of spread [8]. Therefore, the possibility that *C. mortiferum* was introduced to Sweden by other means cannot be excluded. This question needs further investigation. Similarly, we have no explanation for the isolated occurrence of *C. mortiferum* in the Ussuri white-toothed shrew in Korea. Guo et al. [34] showed two geographic clusters within the *C. mortiferum gp60* group (the XIVa subtype family). Samples from New York, Maine and Vermont, the three Northeastern US states, formed a common cluster, whereas samples from Minnesota and Wisconsin, the two Midwestern states, and Sweden formed another cluster [34]. All isolates obtained from naturally infected squirrels in Italy, as well as the CHIP_I isolate used in this study, belong to the Northeastern US group. These results show that, as in the Guo et al. [34] study, two separate geographic clusters within *C. mortiferum gp60* group also occur in Europe. Similarly, previous studies showed differences in geographic and host distribution of *gp60* families of *Cryptosporidium* spp. For example, *C. tyzzeri* family XIa exclusively infects *Mus musculus musculus* at the eastern part of the European mouse hybrid zone, while family XIb occurs only in the western part, infecting *M. m. domesticus* [47]. Similarly, *C. hominis* family Ib appears to predominate in most studies in Europe, North America and high-income countries in Oceania compared to other regions [66]. Due to the high pathogenicity of *C. mortiferum* to Eurasian red squirrels, which most likely complicates its detection in field research, and the lack of studies focusing on the occurrence of *Cryptosporidium* in squirrels in Europe, it is not possible to assess the occurrence and distribution of different *gp60* groups within the European red squirrel population.

Conclusions

This study confirms that *Cryptosporidium* chipmunk genotype I is biologically and genetically distinct from all currently recognized species of the genus *Cryptosporidium*. The results support the description of this *Cryptosporidium* as a separate species, which we propose to name *Cryptosporidium mortiferum* n. sp. Transmission studies demonstrate the high pathogenicity of this species to Eurasian red squirrels. The rapid progression of the infection, resulting in death of the infected individual, is one explanation why *C. mortiferum* is detected in Eurasian red squirrels at low prevalence, although it is infectious to them. In agreement with previous studies, Eurasian red squirrels are considered an important source of *C. mortiferum* infection for humans.

Abbreviations

ACMV	Aniline-carbol-methyl violet
AP	Auramine phenol
BSA	Bovine serum albumin
BC CAS	Biology Center of the Czech Academy of Science, Czech Republic
COWP	*Cryptosporidium* Oocyst wall protein
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
DPI	Days post-infection
FITC	Fluorescein isothiocyanate
gDNA	Genomic DNA
gp60	60-KDa glycoprotein gene
HE	Hematoxylin and eosin
HSP70	70-KDa heat-shock protein
ICZN	International Commission on Zoological Nomenclature
IFA	Immunofluorescence assay
LSID	Life Science Identifier
ND	Not defined
NJ	Neighbor-joining
ML	Maximum likelihood
OPG	Oocysts per gram
PAS	Periodic acid-Schiff
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
Taq polymerase	*Thermophilus aquaticus* Polymerase
TRAP-C1	Thrombospondin related adhesive protein of *Cryptosporidium*-1
SD	Standard deviation
SEM	Scanning electron microscopy
SCID	Severe combined immunodeficiency
SSU	Small subunit of rRNA
TEM	Transmission electron microscopy
ZN	Ziehl–Neelsen

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Author contributions
LT, JJ, BS, JM and MK designed the study. MK and LX performed study supervision. JJ, LT, JP, NH, DK, BS, LH and R.K. performed the experiments. LH, LT, JJ, JP and DK took care of experimental animals. LT, JJ, MR and MK analyzed the data. MK, LX, JM and MS contributed reagents/materials. JJ, JM and MK performed phylogenetic analysis. LT, RK, BS and MK performed histology, electron and light microscopy analysis. MR performed statistical analyses. LT, JJ, JM and MK wrote the first draft of the manuscript. NH and BS prepared the tables and the figures. LX, MS and MK undertook the final revision of the manuscript. LX and MK provided funding. All authors have read and agreed to the published version of the manuscript.

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Data availability
All type material and datasets on which the conclusions of the manuscript rely are stored at the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice, Czech Republic. Representative nucleotide sequences generated in this study were submitted to the GenBank database under the accession numbers OQ627025–OQ627029, OQ632461–OQ632495.

Declarations

Ethics approval and consent to participate
All experimental procedures complied with the laws of the Czech Republic (Act No. 246/1992 Coll., on the Protection of Animals against Cruelty). The study design was approved by the ethics committees of the BC CAS, the State Veterinary Administration and the Central Commission. Rodents were housed individually in ventilated cages (Tecniplast, Buguggiate, Italy). Chickens were housed in boxes. Squirrels and ferrets were housed in separate cages. The size of cages, boxes and aviaries was in accordance with Czech legislation (Act No. 246/1992 Coll. on the Protection of Animals from Cruelty) and European legislation.

Competing interests
The authors declare that they have no competing interests.

Connsent for publication
Not applicable.

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6.6 Příloha VI

European ground squirrels *Spermophilus citellus* (Linnaeus) do not share identical *Cryptosporidium* spp. with North American ground squirrels.

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European ground squirrels *Spermophilus citellus* (Linnaeus) do not share identical *Cryptosporidium* spp. with North American ground squirrels

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Abstract: *Cryptosporidium* Tyzzer, 1910 is one of the most common protistan parasites of vertebrates. The results of this study provide the first data on *Cryptosporidium* diversity in the European ground squirrel *Spermophilus citellus* (Linnaeus). A total of 128 faecal samples of European ground squirrels from 39 localities in the Czech Republic were analysed for the presence of *Cryptosporidium* spp. by microscopy and PCR/sequence analysis of small subunit ribosomal RNA (SSU) and the actin gene. While the microscopical examination did not reveal the presence of any *Cryptosporidium* oocysts, eight samples from six localities were PCR-positive. Phylogenetic analyses revealed the presence of five different *Cryptosporidium* spp. isolates. Four isolates, designated as *Cryptosporidium* sp. isolate Sc01–04, detected in wild populations and never recorded before, clustered closely to *Cryptosporidium* genotypes that have previously been found in North American ground squirrels' species. *Cryptosporidium sciurinum* Prediger, Ježková, Holubová, Sak, Konečný, Rost, McEvoy, Rajský et Kváč, 2021 was found in an animal sanctuary. Because *C. sciurinum* had previously been detected in Eurasian red squirrels *Sciurus vulgaris* Linnaeus at the same facility, it can be concluded that this *Cryptosporidium* was transmitted from tree squirrels to ground squirrels within the animal sanctuary. The results indicate that populations of European and North American ground squirrels are parasitised by different *Cryptosporidium* spp. At the same time, this is the first description of the occurrence of *C. sciurinum* in ground squirrels.

Keywords: genotyping, PCR, SSU, actin, Sciuridae, rodents

Cryptosporidium Tyzzer, 1910 (Apicomplexa: Cryptosporidiidae) is a genus of parasitic protists that commonly infect the gastrointestinal, respiratory, and/or urogenital tract of their hosts (Nader et al. 2019). They may cause a disease called cryptosporidiosis, characterised by various clinical symptoms including nausea, vomiting, and watery, non-bloody diarrhoea (Checkley et al. 2015). Whereas few species and genotypes of the genus *Cryptosporidium* had been described by the end of the 20th century, results of molecular and biological studies carried out in the past 20 years indicate a huge diversity within the genus, noting that most representatives have a narrow host specificity (Feng et al. 2018). To date, 51 species of *Cryptosporidium* and more than 120 genotypes have been reported in mammals, birds, reptiles, amphibians, and fish (Prediger et al. 2021, Ryan et al. 2021, Huang et al. 2023). The number of *Cryptosporidium* species, however, is probably much higher, as only a fraction of hosts has so far been examined for the presence of these parasites.

Rodents are a typical example of this – though they represent about 40% of mammalian diversity only 17 species and 44 genotypes of *Cryptosporidium* have been described from them to date (Ježková et al. 2021, Xu et al. 2022, Tůmová et al. 2023). The open habitats of Eurasia and North America are inhabited by 41 species of ground squirrels, formerly included in the genus *Spermophlilus* Cuvier (*sensu lato*). In the current concept, there are a total of six genera *Callospermophilus* Merriam, *Ictidomys* Allen, *Otospermophilus* Brandt, *Poliocitellus* Howell, *Spermophilus* Cuvier, *Urocitellus* Obolenskij and *Xerospermophilus* Merriam. Five genera occur in North America, of which *Urocitellus* is also found in Asia.

The genus *Spermophilus* (*sensu stricto*) is distributed only in Eurasia (Helgen et al. 2009). Atwill et al. (2001) used the PCR-RFLP of a partial product of the *Cryptosporidium* oocyst wall protein (COWP) gene to distinguish *Cryptosporidium* found in California ground squirrels *Otospermophilus beecheyi* (Richardson) in the USA from oth-

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er *Cryptosporidium* spp. They noted the presence of two distinct genotypes. Subsequent genotyping revealed the presence of *Cryptosporidium rubeyi* Li, Pereira, Larsen, Xiao, Phillips, Striby, McCowan et Atwill, 2015 (previously known as *Cryptosporidium* sp. "c" genotype), and *Cryptosporidium* sp. "a", "b", "d", and "e" genotypes in ground squirrels in the USA (Table 1). In addition, *Cryptosporidium* sp. ground squirrel I, II, and III were found in black-tailed prairie dogs *Cynomys ludovicianus* (Ord), and in thirteen-lined ground squirrels *Ictidomys tridecemlineatus* (Mitchill) (Stenger et al. 2015). The study by Li and Xiao (2019), who detected *Cryptosporidium* sp. in speckled ground squirrels *Spermophilus suslicus* (Güldenstaedt) by microscopy in Poland and Ukraine, is the only report documenting the occurrence of a *Cryptosporidium* sp. in ground squirrels in Europe. Xu et al. (2022) recently reported the presence of *Cryptosporidium equi* Huang, Chen, He, Chen, Huang, Li, Ryan, Kváč, Feng, Xiao et Guo, 2023 in Alashan ground squirrels (*Spermophilus alashanicus* Büchner) in China.

Given the limited scope and number of previously published studies, the objective of the present work was to describe the occurrence and genetic diversity of *Cryptosporidium* spp. in European ground squirrels *Spermophilus citellus* (Linnaeus) and to compare the results with data from North America.

MATERIALS AND METHODS

Faecal samples of European ground squirrels (EGS) were collected as "population samples", i.e., a sample consisting of approximately 7–8 fresh faecal pellets collected from different ground squirrel burrow entrances at a single locality. Ideally, each faecal pellet should originate from a different individual, so that their mixture represents the parasite diversity in the population of interest. Three samples were collected at each locality, except at localities 21, 22, 29, 36, 39 and 1, 2, 10, where four and five samples were collected, respectively (Fig. 1). Samples were collected from all known EGS localities in the Czech Republic from June 1 to August 31, 2019 (Fig. 1). Faecal smear was prepared from each sample, stained with aniline-carbol-methyl violet, and examined for the presence of *Cryptosporidium* spp. oocysts using light microscopy (Miláček and Vítovec 1985).

Total genomic DNA (gDNA) was extracted from 200 mg of each faecal sample using the Exgene Stool SV Minikit (GeneAll, Seoul, Korea) according to the manufacturer's instructions, followed by the homogenisation and disruption of oocysts in a FastPrep®-24 instrument (MP Biomedicals, Santa Ana, CA, USA). The obtained gDNA was stored at −20 °C. Sets of nested PCR primers were used to amplify the partial sequence of the small subunit rRNA gene (SSU) in all samples (Xiao et al. 1999). Subsequently, all *Cryptosporidium*-positive samples were used to amplify a partial sequence of the actin gene (Sulaiman et al. 2002).

The PCR conditions were modified as follows: the primary PCR mixtures contained 2 µl gDNA, 2.5 U Taq DNA polymerase (DreamTaq Green DNA Polymerase, Thermofisher Scientific, Waltham, MA, USA), 1 × PCR buffer (Thermofisher Scientific), 6 mM $MgCl_2$ (SSU), and 3 mM $MgCl_2$ (actin), 200 µl each of deoxynucleoside triphosphate, 100 mM of each primer, and 2 µl non-acetylated bovine serum albumin (BSA; 10 $mg.ml^{-1}$; New England Biolabs, Beverly, MA, USA) in 30 µl reaction volume. The secondary PCR mixtures were similar to those used for the primary PCR, except that 2 µl of the primary PCR product was used as the template. *Cryptosporidium baileyi* Current, Upton et Haynes, 1986 DNA and molecular grade water were included in each PCR amplification as positive and negative controls, respectively. All samples were analysed in triplicate. Secondary PCR products were purified using the Gen Elute Gel Extraction Kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions, and sequenced in both directions using the secondary PCR primers in a commercial laboratory (SeqMe s.r.o., Dobříš, Czech Republic).

Table 1. *Cryptosporidium* spp. reported from ground squirrels based on microscopic methods (1), PCR-RFLP of partial sequence of the oocyst wall protein gene of *Cryptosporidium* spp. (2), or sequencing of partial region of the small subunit ribosomal DNA (3).

Host species (scientific name)	Country	*Cryptosporidium* spp.	GenBank Accession No. (3)	Reference
California ground squirrel *Otospermophilus beecheyi* (Richardson)	USA	*Cryptosporidium rubeyi* (3)	KM010224	Li et al. (2015)
		Cryptosporidium sp. Sbey03a (3)	AY462231	Atwill et al. (2004)
		Cryptosporidium sp. Sbey03b (3)	AY462232	
		Cryptosporidium rubeyi (3)	AY462233	
		C. rubeyi (3)	DQ295012	Pereira et al. (2010)
		Cryptosporidium sp. Sbey05b (3)	NA	Pereira et al. (2010)
		C. parvum (2)	NA	Atwill et al. (2001)
		Cryptosporidium sp. Sbey11e (3)	KM010225	Li et al. (2015)
Belding's ground squirrel *Urocitellus beldingi* (Merriam)	USA	*Cryptosporidium* sp. Sbld05a (3)	DQ295017	Pereira et al. (2010)
		Cryptosporidium sp. Sbld05c (3)	DQ295013	
		Cryptosporidium sp. Sbld05d (3)	DQ295015	
Golden mantled ground squirrel *Callospermophilus lateralis* Say	USA	*Cryptosporidium* sp. Sltl05c (3)	DQ295014	Pereira et al. (2010)
		C. rubeyi (3)	KT027470	Stenger et al. (2015)
Black-tailed prairie dog *Cynomys ludovicianus* (Ord)	USA	*C. rubeyi* (3)	KT027469	Stenger et al. (2015)
		Cryptosporidium sp. ground squirrel II (3)	KT027480	
Thirteen-lined ground squirrel *Ictidomys tridecemlineatus* (Mitchill)	USA	*Cryptosporidium* sp. ground squirrel I (3)	KT027465	Stenger et al. (2015)
		Cryptosporidium sp. ground squirrel III (3)	KT027479	
Speckled ground squirrel *Spermophilus suslicus* (Güldenstaedt)	Ukraine	*Cryptosporidium* sp. (1)	NA	Li and Xiao (2019)
	Poland	*Cryptosporidium* sp. (1)	NA	Li and Xiao (2019)
Alashan ground squirrel *Spermophilus alashanicus* Büchner	China	*Cryptosporidium* sp. horse genotype (3)	ON384432	Xu et al. (2022)

NA - not available

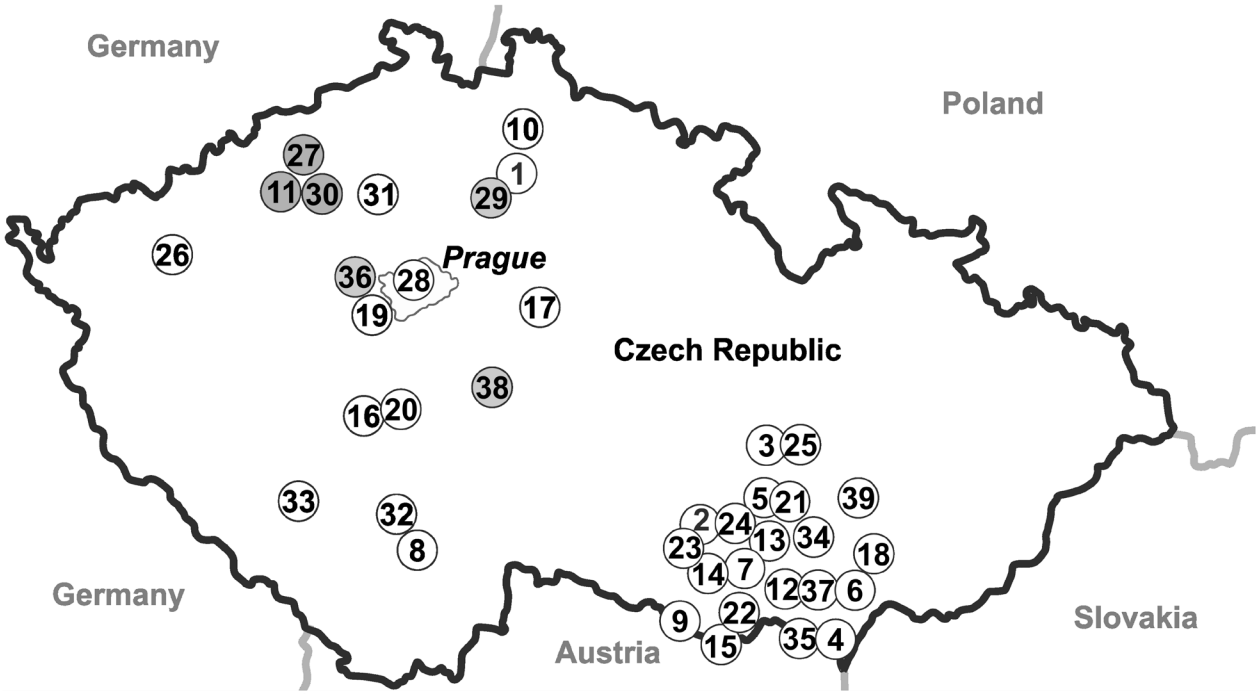

Fig. 1. Localities of European ground squirrels *Spermophilus citellus* (Linnaeus) in the Czech Republic, tagged with *Cryptosporidium* spp. detected in the present study (gray highlighted). (1) Bezděčín (50.39678N, 14.89578E), (2) Biskoupky (49.0941603N, 16.2877708E), (3) Bořitov (49.43611N, 16.59416E), (4) Břeclav (48.79083N, 16.8925E), (5) Brno* (49.23111N, 16.53475E), (6) Čejč (48.9573269N, 16.9670858E), (7) Černice (49.0794936N, 16.2768700E), (8) Hluboká nad Vltavou* (49.252595N, 14.2523050E), (9) Hnanice (48.7991011N, 15.9785106E), (10) Hodkovice (50.65722N, 15.07778E), (11) Hrádek (50.4056858N, 13.7549611E), (12) Hrušovany (49.03N, 16.58622E), (13) Ivančice (49.0940042N, 16.3767133E), (14) Jamolice (49.0820386N, 16.2526939E), (15) Jaroslavice (48.7511308N, 16.2343758E), (16) Bor (49.5825128N, 14.1774908E), (17) Kolín (50.00194N, 15.17333E), (18) Kyjov-Milotice (48.9807319N, 17.1243081E), (19) Loděnice (49.9849172N, 14.1638653E), (20) Loužek (49.5881256N, 14.1782872E), (21) Medlánky (49.2366000N, 16.5554264E), (22) Miroslav (48.93139N, 16.29861E), (23) Mohelno (49.1101433N, 16.1802542E), (24) Nature reserve Nad řekami (49.0941306N, 16.2936239E), (25) Obora (49.4496486N, 16.5907183E), (26) Olšová Vrata (50.2124686N, 12.9281961E), (27) Písečný vrch (50.4248033N, 13.7363736E), (28) Praha* (50.1212222N, 14.4030569E), (29) Radouč (50.4322797N, 14.9045978E), (30) Raná (50.4052894N, 13.7772233E), (31) Roudnice nad Labem (50.4081906N, 14.2340906E), (32) Rozovy* (49.2005147N, 14.3246867E), (33) Strakonice (49.2549969N, 13.8931092E), (34) Újezd u Brna (49.1099903N, 16.7623189E), (35) Valtice (48.7304497N, 16.7373322E), (36) Velká Dobrá (50.11278N, 14.08972E), (37) Velké Pavlovice (48.9084208N, 16.8039058E), (38) Vlašim* (49.7258478N, 14.9166669E), (39) Vyškov (49.2990500N, 17.0228883E). * breeding in captivity (zoo, animal sanctuary, rescue station)

The obtained sequences were verified by BLAST and edited using the ChromasPro 2.1.8 software (Technelysium, Pty, Ltd., South Brisbane, Australia). The sequences of each gene generated in this study were aligned with each other and with reference sequences from the GenBank database (https://www.ncbi.nlm.nih.gov) using the MAFFT v7 online server (http://mafft .cbrc.jp/align ment/software/) and Q-INS-i algorithm. The SSU sequences were aligned in nucleotide mode; the actin sequences were aligned in amino acid mode, then switched to nucleotide mode and used for analyses. Since the alignments contained sequences of different lengths, they were trimmed in BioEdit v7.0.5 (Hall 1999) to obtain the set of sufficient sequence lengths together with reasonable taxonomic representation.

The phylogeny was reconstructed using two approaches – maximum likelihood (ML) and Bayesian inference (BI). The best fitting evolutionary model was set by SMS: Smart Model Selection (http://www.atgc-montpellier.fr/phyml-sms/). The ML was computed in Phyml v2.4.3 (Guindon and Gascuel 2003), with the GTR + Γ + I model and non-parametric bootstrap analysis of 1,000 replicates. BI was performed in MrBayes v3.2.2 (Huelsenbeck and Ronquist 2001) under the GTR + Γ + I model, MCMC run for 10 million generations, and with tree sampling every 100 generations; the trees were summarised after removing 25% burn-in. The resulting trees were visualised in TreeView v1.6.6 (Page 1996), and graphically adjusted in Adobe Illustrator CC 2019 v.23.0.2 (Adobe Systems, Inc.). The obtained sequences were deposited in the GenBank database under the accession numbers OQ520104–OQ520112 (SSU) and OQ473495–OQ473499 (actin).

The study design was approved by the ethical committee of the Biology Centre, CAS. Since the collection of faecal samples was not invasive according to the laws of the Czech Republic (Act No. 246/1992 Coll. on the protection of animals against cruelty), resulting from international regulations, no permit was required.

RESULTS

A total of 128 faecal samples of EGS from 39 localities in the Czech Republic were examined by molecular analyses for the presence of parasites of the genus *Cryptosporidium*. No *Cryptosporidium* oocysts were microscopically detected in the faecal smears, while PCR analysis of the SSU revealed the presence of *Cryptosporidium*-spe-

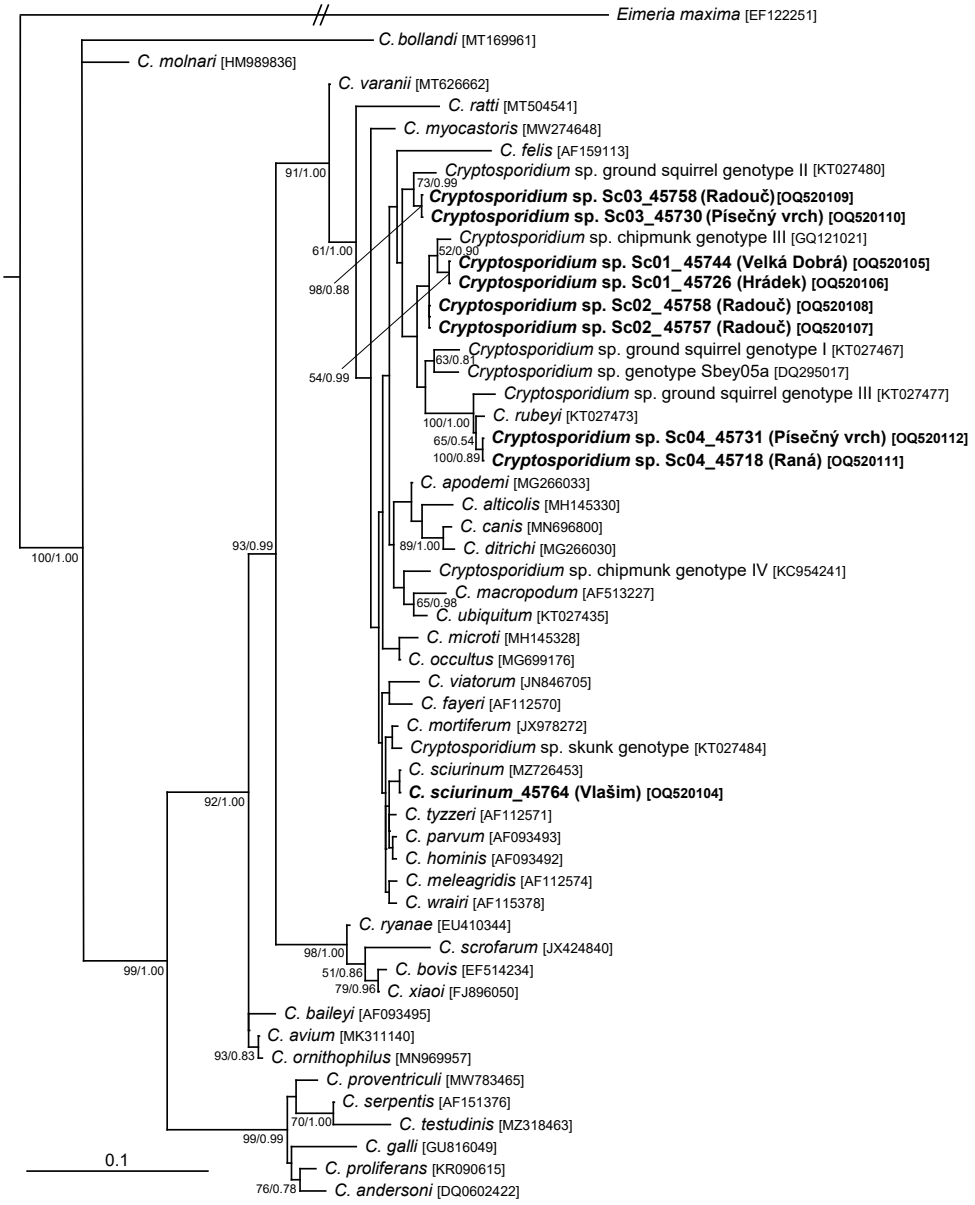

Fig. 2. Phylogenetic relationships of *Cryptosporidium* spp. from the European ground squirrel *Spermophilus citellus* (Linnaeus) in the Czech Republic inferred by the ML analysis of the partial SSU sequences. Numbers at the nodes show bootstrap values derived from ML analysis/posterior probabilities under BI analysis. Only bootstrap supports and posterior probabilities higher than 50% or 0.50, respectively, are shown.

cific DNA in nine samples (Fig. 1). Out of these samples, eight and five were genotyped by sequence analysis of the SSU and actin, respectively (Table 2). Three SSU-positive samples could not be successfully amplified or sequenced at the actin gene (Fig. 2).

The ML tree constructed from the alignment of SSU sequences revealed the presence of five different *Cryptosporidium* spp. isolates *Cryptosporidium sciurinum* Prediger, Ježková, Holubová, Sak, Konečný, Rost, McEvoy, Rajský et Kváč, 2021, the species previously found in Eurasian red squirrels *Sciurus vulgaris* Linnaeus, was repeatedly detected in samples of EGS from Vlašim (No. 38) at the SSU and actin genes (Figs. 2 and 3). The sequences of other four isolates were not identical to any sequences so far included in the GenBank database.

Cryptosporidium sp. isolate Sc01 was found in Velká Dobrá (No. 36) and Hrádek (No. 11) and formed a sister group to *Cryptosporidium* sp. ground squirrel genotype I in the SSU and actin trees. *Cryptosporidium* sp. isolate Sc02 was detected in Radouč (No. 29), and clustered together with isolate Sc01 and *Cryptosporidium* sp. ground squirrel genotype I in the SSU tree. In sample 45758, where the isolate Sc02 was detected, sequencing of one of the triplicates revealed the presence of another, different SSU sequence, designated as isolate Sc03. The same sequence was also detected in Písečný Vrch (No. 27, Table 2).

Actin sequences obtained from Radouč (No. 29) clustered to *Cryptosporidium* ground squirrel genotype II (Fig. 3) and were designated as isolate Sc03. *Cryptosporidium* sp. isolate Sc04, detected in Písečný Vrch (No. 27) and

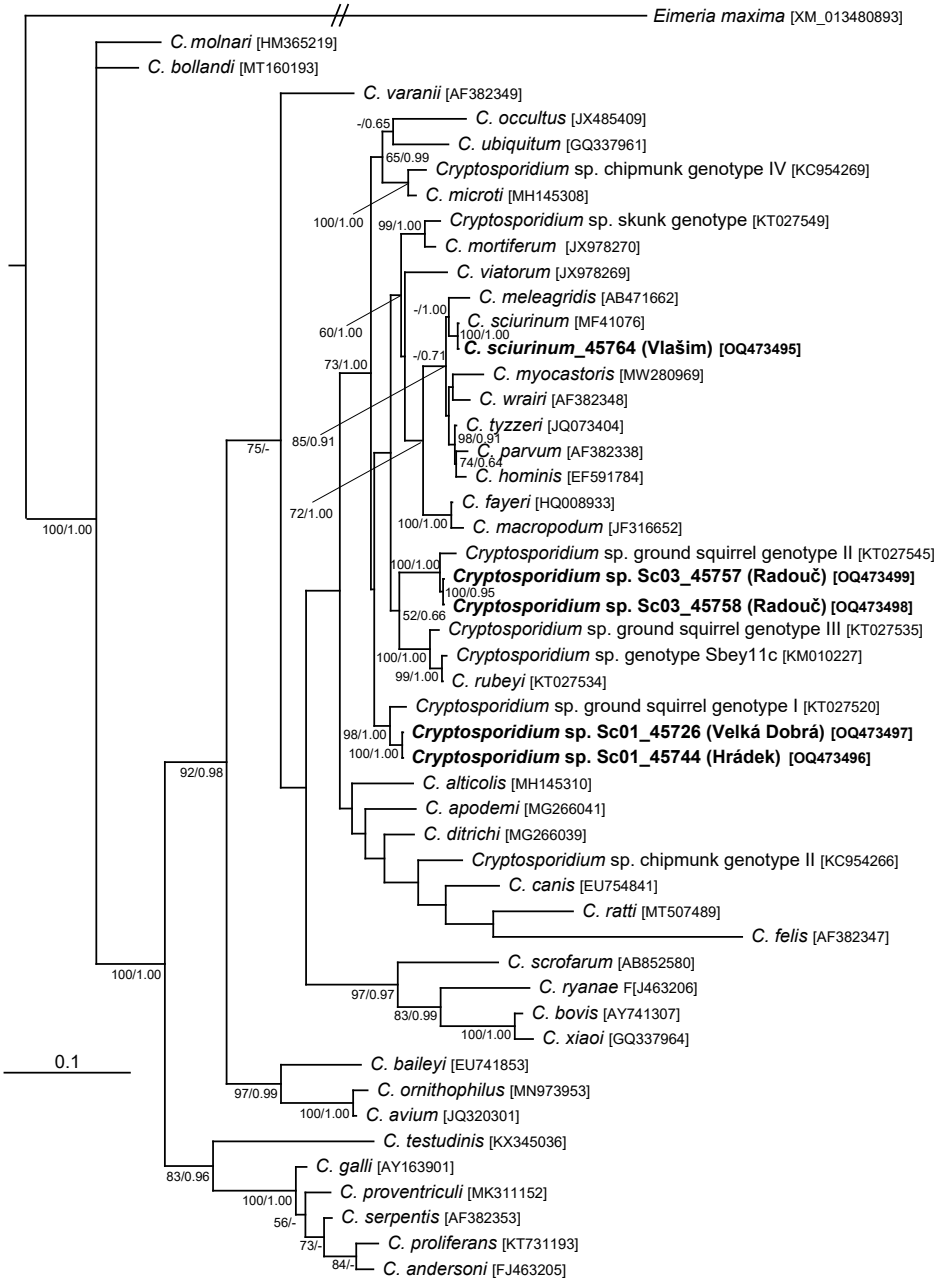

Fig. 3. Phylogenetic relationships of *Cryptosporidium* spp. from the European ground squirrel *Spermophilus citellus* (Linnaeus) in the Czech Republic inferred by the ML analysis of the partial actin sequences. Numbers at the nodes show bootstrap values derived from ML analysis/posterior probabilities under BI analysis. Only bootstrap supports and posterior probabilities higher than 50% or 0.50, respectively, are shown.

Raná (No. 30), formed a well-supported clade with *Cryptosporidium rubeyi* in the SSU tree (Fig. 2). Unfortunately, we were not able to obtain any actin sequences of adequate quality from Sc02 and Sc04 isolates (Table 2).

DISCUSSION

While *Cryptosporidium rubeyi* Li, Pereira, Larsen, Xiao, Phillips, Striby, McCowan et Atwill, 2015, *Cryptosporidium* sp. Sbey03a, Sbey05b, Sbld05a, and Sbld05d were reported as causative agents of cryptosporidial infection in ground squirrels in North America, none of these *Cryptosporidium* were detected in this study. Four (*Cryptosporidium* sp. isolate Sc01, Sc02, Sc03, and Sc04) of the five isolates recognised on the basis of the SSU sequences clustered closely to *Cryptosporidium* spp. that has been commonly found in ground squirrels of the genera *Otospermophilus* Brandt, *Ictidomys* Allen, and *Cynomys* Rafinesque in North America (Stenger et al. 2015). The occurrence of phylogenetically related *Cryptosporidium* in geographically distant populations of host species belonging to the same genus has been previously described.

It has been shown that parasite-host coevolution, host adaptation, and geographic segregation have led to the formation of subtype families with unique phenotypic traits within *Cryptosporidium parvum* Tyzzer, 1912, *C. hominis* Morgan-Ryan, Fall, Ward, Hijjawi, Sulaiman, Fayer,

Table 2. *Cryptosporidium* spp. detected in localities of European ground squirrel *Spermophilus citellus* (Linnaeus) in the Czech Republic. Numbers of localities correspond with numbers in Figure 1.

Locality (No.)	Isolate ID	Genotyping at loci	
		SSU	Actin
Velká Dobrá (36)	45744	*Cryptosporidium* sp. Sc01	*Cryptosporidium* sp. Sc01
Hrádek (11)	45726	*Cryptosporidium* sp. Sc01	*Cryptosporidium* sp. Sc01
	45757	*Cryptosporidium* sp. Sc02	*Cryptosporidium* sp. Sc03
Radouč (29)	45758	*Cryptosporidium* sp. Sc02 *Cryptosporidium* sp. Sc03	*Cryptosporidium* sp. Sc03
Raná (30)	45718	*Cryptosporidium* sp. Sc04	NA
Písečný Vrch (27)	45730	*Cryptosporidium* sp. Sc03	NA
	45731	*Cryptosporidium* sp. Sc04	NA
Vlašim (38)	45764	*C. sciurinum*	*C. sciurinum*

NA - indicates the failure of PCR amplification / sequencing

Thompson, Olson, Lal et Xiao, 2002, and *C. tyzzeri* Ren, Zhao, Zhang, Ning, Jian, Wang, Lv, Wang, Arrowood et Xiao, 2012 causing infection in most mammals, humans and mice, respectively (Kváč et al. 2013, Feng et al. 2018). Similarly, phylogenetically related genotypes of *Cryptosporidium* have been found to parasitise geographically separated populations of different vole species (Stenger et al. 2017, Horčičková et al. 2019).

Cryptosporidium sciurinum, which is commonly found in Eurasian red squirrels *Sciurus vulgaris* in Europe (Kváč et al. 2008, Prediger et al. 2017, 2021), was obtained only in Vlašim (No. 38), which is an artificially created animal rescue station. At this locality, EGS share the habitat with other small herbivorous mammals including Eurasian red squirrels. Given that *C. sciurinum* has previously been recorded in Eurasian red squirrels located in this station (Prediger et al. 2021), it is possible that transmission occurred between both hosts directly or via personnel or vehicles with whom the animals are in daily contact. This is the first description of the occurrence and transmission of *C. sciurinum* to ground squirrels.

The results of the multilocus genotyping and repeated sequencing of independent PCR products showed a mixed infection of *Cryptosporidium* spp. in the screened hosts. Given that many vertebrate hosts such as mice, rats, voles, cattle, or various bird groups are often parasitised by multiple species and genotypes of *Cryptosporidium*, such a finding is not surprising (Santín et al. 2008, Li et al. 2015, Nakamura and Meireles 2015, Horčičková et al. 2019, Ježková et al. 2021).

Compared to previous studies from the USA and Poland, we detected cryptosporidial infection in a small number of samples and localities (Stenger et al. 2015). Although in our study we detected 3.2% of positive samples in 6 out of the 39 localities, Kloch and Bajer (2012) reported 21–64% prevalence of *Cryptosporidium* spp. in *Spermophilus suslicus* populations in Poland and Ukraine, and Atwill et al. (2001, 2004) described 16–20% prevalence in California ground squirrels (*Otospermophilus beecheyi*). Moreover, the uniqueness of the presented results lies in the fact that almost all localities of EGS in the Czech Republic were examined.

The results indicate that only a small number of localities are infected, with some *Cryptosporidium* isolates found in several different localities. While the localities of Hrádek (No. 11), Raná (No. 30), and Radouč (No. 29), where all isolates except for the *C. sciurinum* (see above) were detected, are the natural localities of EGS in the Czech Republic, Velká Dobrá (No. 36) and Písečný Vrch (No. 27), though wild, artificially created. An explanation of the distribution of individual isolates of *Cryptosporidium* spp. across the localities may reside in the human-controlled movements of individuals between sites (reintroductions, repatriations), or the introduction of infection by workers/staff who perform the intentional trapping and sampling of individuals at the sites.

To conclude, the results of this work provide the first results on the diversity of *Cryptosporidium* spp. in EGS in the Czech Republic and show that the studied populations do not share the same *Cryptosporidium* species/genotypes with North American ground squirrels.

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