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ZEMĚDĚLSKÁ FAKULTA

Diverzita a biologie kryptosporidií hraboše polovitých (Arvicolinae)

Diversity and biology of *Cryptosporidium* in Arvicolinae rodents

disertační práce

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Horáková M., Čondlová Š., Holubová N., McEvoy J.M., Kváč M. 2016: Diversity of *Cryptosporidium* spp. infecting common voles (*Microtus arvalis*) – preliminary data. EMOP XII, University of Turku, Finsko, 20. – 24. 7. 2016 (poster).

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***Horáková M.**, Čondlová Š., Holubová N., McEvoy J.M., Kváč M. 2016: Diversity of *Cryptosporidium* spp. infecting common voles (*Microtus arvalis*) – preliminary data. 3rd International Forum on Medical and Veterinary Parasitology, Wroclaw, Polsko, 18. – 19. 11. 2016 (ústní prezentace).

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Předložená disertační práce zahrnuje výsledky týkající se diverzity a biologických vlastností kryptosporidií parazitujících u hrabošovitých. Ty byly získané na základě multidisciplinárního přístupu zahrnujícího molekulární biologii, parazitologii, zoologii, histologii a experimentální infekce a byly publikovány ve třech vědeckých impaktovaných časopisech.

ANOTACE

Kryptosporidie, parazitická protista patřící do kmene Apicomplexa, jsou velmi úspěšní paraziti, o čemž svědčí jejich široké spektrum hostitelů, celosvětové rozšíření a odolnost vůči i dezinfekčním prostředkům a lékům. Rozmanitost kryptosporidií je molekulárně studována již více než tři desetiletí. Zatímco kryptosporidie lidí a hospodářských zvířat jsou velmi dobře prostudovány, výzkum na jiných obratlovcích, včetně hlodavců, zaostává. Naše znalosti o biologických vlastnostech jednotlivých druhů a genotypů jsou vůči ginou nedostatečné nebo úplně chybějící. Tato práce je zaměřena na studium prevalence, rozmanitosti a biologických vlastností parazitů rodu *Cryptosporidium* parazitujících u hlodavců podčeledi Arvicolinae. Výsledky této práce povedou k lepšímu porozumění diverzity kryptosporidií a jejich hostitelské a tkáňové specifity.

ANNOTATION

Cryptosporidium, a parasitic protist in the phylum Apicomplexa, is hugely successful, as evidenced by its broad host range, global distribution, and resistance to disinfectants and drug treatments. Genetic diversity in the genus *Cryptosporidium* has been studied for more than three decades, with most research focused on isolates from humans and domestic animals, while research on other vertebrate hosts, including rodents, has lagged. Moreover, our knowledge about the biological characteristics of individual species and genotypes are mostly insufficient or missing. This thesis addresses the prevalence, diversity and biological characteristics of *Cryptosporidium* in Arvicolinae rodents. Addressing the gap in our knowledge will lead to better understanding of *Cryptosporidium* diversity and host/tissue specificity.

SOUHRN

Tato disertační práce se zabývá prevalencí a diverzitou kryptosporidií infikujících hlodavce podčeledi Arvicolinae. V letech 2014–2017 byly mikroskopickými a molekulárními metodami vyšetřeny vzorky trusu od hrabošů polních (*Microtus arvalis*) a norníků rudých (*Myodes glareolus*) z České republiky a Slovenska na přítomnost kryptosporidií. Genotypizace byla provedena pomocí nested PCR amplifikující gen kódující malou ribozomální podjednotku rRNA, aktin, COWP a HSP70. Specifická DNA kryptosporidií byla detekována u 74 hrabošů a 10 norníků. Fylogenetická analýza prokázala přítomnost dvou nových druhů (*C. alticolis* a *C. microti*) a šesti nových genotypů (*Cryptosporidium* vole genotyp II, *Cryptosporidium* vole genotyp III, *Cryptosporidium* vole genotyp IV, *Cryptosporidium* vole genotyp V, *Cryptosporidium* vole genotyp VI a *Cryptosporidium* vole genotyp VII) u hrabošů a čtyři genotypy kryptosporidií u norníků. Intenzita infekce se nelišila mezi samci a samicemi ani mezi juvenilními a dospělými jedinci. Žádné z přirozeně infikovaných zvířat nevykazovalo příznaky kryptosporidiózy. U izolátů *C. alticolis* a *C. microti* byla ověřena hostitelská specifita pomocí experimentálních infekcí. *Cryptosporidium alticolis* a *C. microti* byly infekční pro hraboše polní a hraboše pensylvánské (*Microtus pennsylvanicus*), ale nebyly infekční pro myšice lesní (*Apodemus flavicolis*), SCID, BALB/c a C57BL/6J myši (*Mus musculus*), potkany (*Rattus norvegicus*) nebo kuřata (*Gallus gallus* f. *domestica*). *Cryptosporidium alticolis* infikuje tenké střevo a má větší oocysty (5,4 × 4,9 μm) než *C. microti* (4,3 × 4,1 μm), které infikuje tlusté střevo. U žádného z experimentálně infikovaných hlodavců se neprojevily klinické příznaky infekce. Hraboši polní jsou vnímaví k infekci *C. parvum*, mikroskopicky však nebyly detekovány žádné oocysty a molekulárními metodami byla zjištěna patentní doba 7 dní. *Cryptosporidium apodemi* a *C. ditrichi*, druhy specifické pro hlodavce rodu *Apodemus*, nejsou infekční pro hraboše. Výsledky těchto studií ukazují hostitelskou specifitu kryptosporidií infikující hlodavce; severoameričtí a evropští hlodavci podčeledi Arvicolinae jsou hostiteli různorodých kryptosporidií, které, jak se zdá, koevolují se svými hostiteli. Genetické a biologické údaje podporují popis *C. alicolis* a *C. microti* jako samostatných druhů rodu *Cryptosporidium*.

SUMMARY

This thesis deals with the prevalence and diversity of *Cryptosporidium* parasitizing Arvicolinae rodents. Faecal samples from common (*Microtus arvalis*) and bank voles (*Myodes glareolus*), collected in the Czech Republic and Slovakia in 2014ï 2017, were screened for *Cryptosporidium* by microscopy and PCR/sequencing. Isolates were characterized by sequence and phylogenetic analyses of the small subunit ribosomal RNA, actin, *Cryptosporidium* Oocyst Wall Protein, and 70 kDa Head Shock Protein genes. Specific DNA of *Cryptosporidium* was detected in 74 common voles and 10 bank voles. Phylogenetic analysis revealed the presence of two new species (*C. alticolis* and *C. microti*) and six novel genotypes (*Cryptosporidium* vole genotype II, *Cryptosporidium* vole genotype III, *Cryptosporidium* vole genotype IV, *Cryptosporidium* vole genotype V, *Cryptosporidium* vole genotype VI and *Cryptosporidium* vole genotype VII) in common voles and four unnamed *Cryptosporidium* genotypes in bank voles. Rates of infection did not differ between males and females or between juveniles and adults. None of the animals that were naturally infected with *Cryptosporidium* had clinical cryptosporidiosis. Host specificity of *C. alticolis* a *C. microti* was examined experimentally. Oocysts of *C. alticolis* and *C. microti* were infectious for common (*Microtus arvalis*) and meadow voles (*M. pennsylvanicus*), but not for yellow necked mice (*Apodemus flavicolis*), SCID mice, BALB/c mice and C57BL/6J mice (*Mus musculus*), brown rats (*Rattus norvegicus*), or chickens (*Gallus gallus* f. *domestica*). *Cryptosporidium alticolis* infects the anterior small intestine and has larger oocysts (5.4 × 4.9 ε m) than *C. microti* (4.3 × 4.1 ε m), a species that infects the large intestine. None of experimentally infected rodents developed clinical signs of infection. Common voles are susceptible to *C. parvum* infection, but did not shed microscopically detectable oocysts, and the patent period was only 7 days. *Cryptosporidium apodemi* and *C. ditrichi*, species specific for rodent from genus *Apodemus*, are not infectious for common voles. Results of our studies show the host specificity of *Cryptosporidium* parasitizing voles; North American and European Arvicolinae host diverse *Cryptosporidium* spp., which in many cases appear to have coevolved with their hosts. Genetic and biological data support the establishment of *C. alticolis* and *C. microti* as separate species of the genus *Cryptosporidium*.

OBSAH

1. ÚVOD

Hlodavci obývají témĕř celý svět, kromě Antarktidy a některých izolovaných ostrovů (Wilson et Reeder 2005). Jsou to malí až středně velcí savci s krátkým reprodukčním cyklem a velkými vrhy, kteří jsou morfologicky a biologicky přizpůsobeni k různým životním stylům a životnímu prostředí. Právě tato vysoká adaptibilita činí z hlodavců jednoho z nejvhodnějších savců pro život na různých stanovištích (Krytufek et Vohralík 2005, Stefoff 2008). I přes prospěšné aktivity hlodavců, jako je provzdušňování půdy, minerální kolobĕh živin, zvýšení absorpce vody, mohou způsobovat významné ekonomické ztráty a zvyšovat riziko přenosu infekčních agens na člověka a jím chovaná zvířata (Khaghani 2007).

Rychlý rozvoj průmyslu a zemĕdĕlství, stejně jako změna klimatu po celém světě, vedly ke zvýšení výskytu chorob přenášených hlodavci. Patogenní agens přenášené hlodavci se dají rozdĕlit do dvou hlavních kategorií: a) přímo, b) nepřímo přenosné choroby. U první kategorie dochází k přenosu kousnutím či vdechováním zárodků infekčního agens z exkrementů, nepřímá cesta onemocnĕní bývá způsobena následkem konzumace potravin a vody kontaminované trusem či močí hlodavců (Buckle et Smith 2015). Hlodavci mohou být hostiteli řady původců zoonotických bakteriálních (leptospiróza, mor), parazitárních (leishmanióza, toxoplasmóza) a virových (hantavirové infekce, klíšťová encefalitida) onemocnĕní. Jsou také hostitelé celé řady druhů a genotypů parazitů patřících do rodu *Cryptosporidium* (Apicomplexa).

Kryptosporidie jsou celosvětově rozšíření paraziti, infikující převážně epiteliální buňky gastrointestinálního traktu hostitelů, kteří patří do všech tříd obratlovců, včetně člověka (Fayer et al. 2000, Dubey et al. 2002, Ziegler et al. 2007b, Ryan et Xiao 2014). Onemocnĕní vyvolané těmito protisty se nazývá kryptosporidióza a jeho průbĕh je závislý na řadě faktorů: na věku a imunitním stavu hostitele, ale i na koinfekci s jinými patogeny (paraziti, bakterie nebo viry) nebo na množství prodělaných kryptosporidiových infekcí (Pereira et al. 2002, Trotz-Williams et al. 2005, Geurden et al. 2006, Hong et al. 2007, Checkley et al. 2015, Baneth et al. 2016). Průbĕh infekce může být velmi variabilní od asymptomatického až po závažné gastrointestinální onemocnĕní, ohrožující život infikovaného jedince (Monis

et Thompson 2003, Hunter et al. 2007, Xiao 2010, Ryan et Power 2012, Kváļ et al. 2014b, Ryan et al. 2014).

Většina druhů a genotypů kryptosporidií se vyznačuje úzkou hostitelskou specifitou, například *C. canis*, *C. erinacei*, *C. felis*, *C. microti*, *C. rubeyi*, *C. scrofarum*, *C. suis* a *C. testudinis* (Iseki 1979, Fayer et al. 2001, Ryan et al. 2004, Němejc et al. 2013, Ng-Hublin et al. 2013, Kváļ et al. 2014a, Li et al. 2015, Ježková et al. 2016, Horčičková et al. 2018). U několika málo druhů, *C. baileyi*, *C. meleagridis*, *C. muris*, *C. parvum* a *C. ubiquitum*, byla popsána široká hostitelská specifita, přičemž *C. baileyi* a *C. parvum* jsou považovány za druhy s nejširší hostitelskou specifitou infikující pravděpodobně všechny savce i ptáky (Dubey et al. 2002, Ryan et al. 2003a, Ryan et al. 2003b, Ma et al. 2014, Nakamura et Meireles 2015).

V posledních 20 letech bylo v rámci řady molekulárně-epidemiologických studií prokázáno, že diverzita kryptosporidií, zvláště pak u volně žijících zvířat, je daleko větší než se předpokládalo. Zejména hlodavci, kteří představují asi 40 % diverzity všech savců, jsou parazitováni velkým množstvím kryptosporidií. V současné době je známo 9 druhů a kolem 50 genotypů parazitů patřících do rodu *Cryptosporidium*, kteří infikují hlodavce, z nichž některé mají zoonotický potenciál (Feng et al. 2007, Foo et al. 2007, Ziegler et al. 2007a, Kváļ et al. 2008a, 2013, Feng 2010, Ng-Hublin et al. 2013, Stenger et al. 2015a,b, 2018).

Přestože je kryptosporidiím hlodavců věnována relativně velká pozornost, o diverzitě, prevalenci a průběhu kryptosporidiových infekcí u zástupců podčeledi hrabošovitých (Arvicolinae) neexistuje mnoho údajů. Kryptosporidiemi u hrabošovitých se zabývá 20 relevantních publikací. Řada z nich se opírá výhradně o výsledky mikroskopických metod a o rozdělení nalezených kryptosporidií dle morfometrie oocyst na velké oválné oocysty - *C. muris* a malé sférické oocysty - *C. parvum* (Sinski et al. 1993, Laakkonen et al. 1994, Chalmers et al. 1997, Bull et al. 1998, Sinski et al. 1998, Torres et al. 2000, Bajer et al. 2002, Bednarska et al. 2007, Ziegler et al. 2007a). V nedávných molekulárně-genetických studiích však bylo prokázáno, že hrabošovití mohou být hostiteli celé řady druhů a genotypů kryptosporidií, o jejichž biologii dosud víme jen málo (Perz et Le Blancq 2001, Xiao et al. 2002, Bajer et al. 2003, Zhou et al. 2004, Ziegler et al. 2007b, Danišová et al. 2017, Stenger et al. 2017).

2. CÍL PRÁCE

Tato práce si klade za cíl rozšířit dosavadní poznatky o kryptosporidiích u hrabošovitých. Její význam spočívá v komplexním řešení problematiky týkající se biologie, diverzity a fylogeneze kryptosporidií u hrabošovitých pomocí mikroskopických, molekulárních, histologických a experimentálních metod.

Dílčí cíle

- Zdokumentovat výskyt a prevalenci kryptosporidií přirozeně infikujících hrabošovité (Arvicolinae).
- Popsat biologii jednotlivých druhů a genotypů kryptosporidií specifických pro zástupce podčeledi Arvicolinae.
- Vyhodnotit diverzitu a prevalenci kryptosporidií v závislosti na druhu, pohlaví a věku hostitele.
- Popsat lokalizaci infekce v gastrointestinálním traktu u přirozeně a experimentálně infikovaných hostitelů a charakterizovat průběh infekce.
- Ověřit hostitelskou specifitu jednotlivých genotypů.
- Vyhodnotit možný zoonotický potenciál nalezených druhů a genotypů.
- Na základě získaných výsledků popsat nalezené genotypy jako samostatné druhy.

3. OBECNÝ LITERÁRNÍ PŘEHLED

3.1. Kryptosporidie a kryptosporidióza

3.1.1. Historie

Před více než sto lety Ernest Edward Tyzzer detekoval asexuální a sexuální stádia parazita s nejasným taxonomickým postavením běžně infikujícího žaludeční žlázy laboratorních myší. Popsal, že každé vývojové stádium má organelu podobnou epimeritu gregarin, kterou je připojeno k hostitelské buňce a nazval tohoto parazita *Cryptosporidium muris* (Tyzzer 1907). V roce 1910, publikoval Tyzzer podrobnější popis životního cyklu včetně kreseb a fotografií parazita a navrhl název *Cryptosporidium* pro nový rod s typovým druhem *C. muris* (Tyzzer 1910). Podobné organismy také pozoroval na epitelu tenkého střeva u králíků a myší. Roku 1912 Tyzzer experimentálně infikoval laboratorní myši druhem kryptosporidií parazitujících v tenkém střevě myší a nazval ho *Cryptosporidium parvum* (Tyzzer 1912).

V roce 1929 Tyzzer popsal vývojová stádia podobná *C. parvum* ve slepém střevě kuřat (Tyzzer 1929). Tento druh pojmenovaný na jeho počest *C. tyzzeri* však nebyl z důvodů taxonomických nedostatků uznán za platný. V roce 1955 byl popsán v pořadí třetí druh kryptosporidií, *Cryptosporidium meleagridis*, parazitující u krůt a spojovaný s průjmovým onemocněním a smrtí infikovaných jedinců (Slavin 1955). Až do počátku 70. let 20. století, kdy byly popsány první případy kryptosporidiových infekcí u lidí a hospodářských zvířat (Panciera et al. 1971, Meisel et al. 1976, Nime et al. 1976, Lasser et al. 1979), nebyly kryptosporidie v popředí zájmu jak humánních, tak veterinárních lékařů (Fayer 2007).

V průběhu 70. až 90. let se předpokládalo, že diverzita druhů v rámci rodu *Cryptosporidium* je velmi malá. Původcem žaludeční kryptosporidiózy byl druh s velkými oválnými oocystami - *C. muris* a střevní kryptosporidiózy byl druh *C. parvum* s malými sférickými oocystami (Tzipori et al. 1980). V této době byla většina nálezů označována jako *C. parvum*-like nebo *C. muris*-like.

Na počátku 80. let byly kryptosporidie popsány jako jedna z hlavních příčin chronických průjmů u pacientů s AIDS (Current et al. 1983, Soave et al. 1984, D'antonio et al. 1985, Sallon et al. 1988). V roce 1993, v souvislosti s masivní epidemií v Milwaukee, USA, kdy bylo infikováno více než 400 000 osob, došlo

k výraznému posunu ve vnímání kryptosporidií jako významných lidských patogenů (Mackenzie et al. 1994). K zásadnímu posunu v poznání diverzity kryptosporidií došlo od druhé poloviny 90. let s rozvojem molekulárních metod (Xiao et al. 1999, Xiao et al. 2004, Fayer et Santín 2009).

3.1.2. Taxonomie

Rod *Cryptosporidium* řadíme do kmene Apicomplexa, který zahrnuje parazitická eukaryota, mající přítomný apikální komplex u některých stádií jejich vývojového cyklu. V rámci kmene Apicomplexa byly kryptosporidie, s ohledem na jejich vývojový cyklus, tradičně řazeny mezi kokcidie do řádu Eucoccidiorida (Levine 1984). Nicméně již Tyzzer v roce 1910 poznamenal, že se kryptosporidie od kokcidií výrazně liší, přestože je sám ke kokcidiím přiřadil (Tyzzer 1910). I přes podobnost s kokcidiemi se kryptosporidie od této skupiny parazitů liší v řadě znaků: **(i)** lokalizace vývojových stádií je omezena na apikální povrch hostitelské buňky, **(ii)** parazit je k buňce přichycen specializovanou organelou tzv. feeder organelou, **(iii)** produkují dva morfologicko-funkční typy oocyst, tenkostěnné oocysty excystují v těle hostitele, které zodpovídají za autoinfekci a silnostěnné, které slouží k infekci dalších hostitelů, **(iv)** oocysty postrádají sporocystu, mikropyle a polární granula, **(v)** jsou nevnímavé ke všem antikokcidikům **(vi)** přítomnost tzv. gamont-like extracelulárních vývojových stádií, která jsou podobná stádiím popsaným u gregarin (Tzipori et Widmer 2000, Hijjawi et al. 2002, Petry 2004, Rosales et al. 2005, Cabada et White 2010). Dříve byly kryptosporidie také zaměňovány s jinými druhy kokcidií, zejména se zástupci rodu *Sarcocystis* (Fayer et al. 1997).

S ohledem na podobnost s kokcidiemi a gregarinami se dlouhou dobu spekulovalo, že kryptosporidie představují „chybějící spojení" mezi těmito skupinami parazitů (Ryan et al. 2016). Na základě genomických a biochemických analýz bylo prokázáno, že kryptosporidie jsou evolučně odlišné od kokcidií a jsou více příbuzné gregarinám (Carreno et al. 1999, Zhu et al. 2000, Leander et al. 2003, Abrahamsen et al. 2004, Widmer et Sullivan 2012, Clode et al. 2015). Výsledky těchto studií byly podkladem pro formální přesunutí kryptosporidií z podtřídy Coccidia, třídy Coccidiomorphea do nové podtřídy Cryptogregarida, v rámci třídy Gregarinomorphea (Cavalier-Smith 2014).

3.1.3. Vývojový cyklus

Zástupci rodu *Cryptosporidium* mají složitý, monoxenní životní cyklus, který je primárně dokončen v gastrointestinálním traktu jednoho hostitele (Tzipori 1983, Current et Garcia 1991, Bouzid et al. 2013), v případě ptačích kryptosporidií i v plicích (Lindsay et Blagburn 1990). Životní cyklus kryptosporidií zahrnuje čtyři fáze: **(i)** excystace, **(ii)** merogonie, **(iii)** gametogonie a **(iv)** sporogonie (Thompson et al. 2005) (Obrázek 1).

(i) Vysporulované (infekční) oocysty se po pozření vnímavým hostitelem dostávají do gastrointestinálního traktu, kde dochází k procesu excystace a uvolnění čtyř infekčních pohyblivých sporozoitů (Reduker et Speer 1985). Excystace je spuštěna různými faktory, včetně redukčních podmínek, množstvím oxidu uhličitého, pankreatických enzymů, žlučových solí a teplotou (Fayer et Leek 1984, Reduker et Speer 1985, Blagburn et al. 1987, Robertson et al. 1993, O'donoghue 1995). Sporozoiti se pohybují klouzavým pohybem (Okhuysen et Chappell 2002) a aktivně napadají epiteliální hostitelské buňky (Wetzel et al. 2005). Sporozoiti a všechny následné endogenní sexuální a asexuální fáze se vyvíjejí uvnitř parazitoforní vakuoly, která je intracelulární, ale extracytoplasmatická.

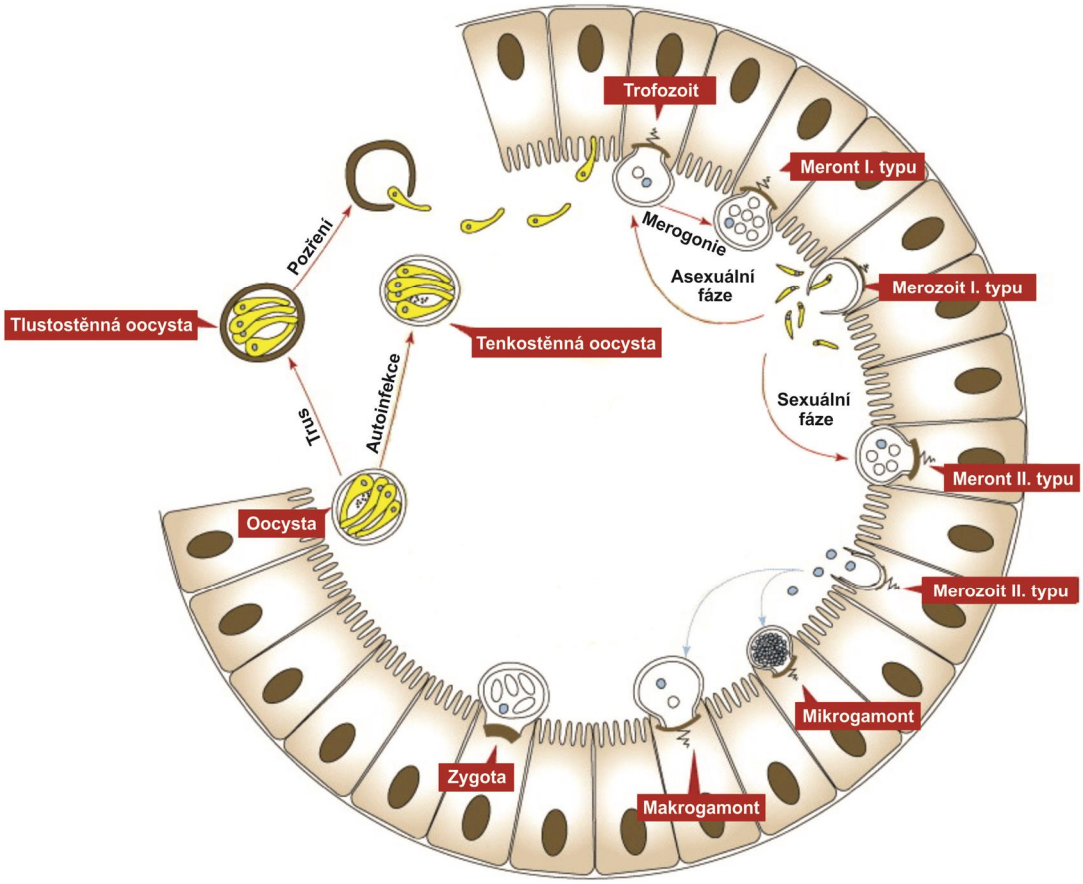
(ii) Po přichycení na buňku se sporozoiti diferencují do sférického trofozoitu a začíná první fáze nepohlavního množení (merogonie), charakterizovaná rozdělením jádra trofozoita, za vzniku metontu I. typu, obsahujícího šest až osm merozoitů I. typu (O'donoghue 1995). Merozoiti I. typu po uvolnění aktivně infikují další buňky, přičemž se vyvíjejí buď opakovaně v meront I. typu nebo meront II. typu. Jádro merontu II. typu se rozdělí na čtyři jádra a vznikají čtyři merozoiti II. typu.

(iii) Při pohlavní fázi (gametogonii) dochází ke vzniku mikrogamontů a makrogamontů z merozoitů II. typu (Goebel et Braendler 1982, Smith et Rose 1998). Jaderné dělení v mikrogamontu dává vzniknout 14 až 16 mikrogametám (ekvivalent samčí pohlavní buňky), zatímco jednojaderné makrogamonty se vyvíjejí v makrogamety (ekvivalent samičí pohlavní buňky) (O'donoghue 1995, Tzipori et Griffiths 1998).

(iv) Oplodněním makrogamety mikrogametou vzniká zygota. Následně se vytváří tři vrstvá stěna oocysty (tlustostěnné) a 2N oplodněné jádro zygoty. To se meioticky dělí (sporogonie) a vznikají čtyři haploidní sporozoiti. Plně sporulované oocysty se uvolňují do lumen střeva a vychází z těla ven stolicí či trusem, kde jsou okamžitě

infekční pro jiné vnímavé hostitele. Oocysty, které sporulují v respiračním traktu, byly nalezeny v nosních sekretech a sputu (Mor et al. 2010). Tlustostěnné oocysty mají stěnu, která je tvořena třemi vrstvami (vnější, střední, vnitřní). Tyto oocysty jsou vylučované z těla hostitele, mají vysokou odolnost vůči vnějšímu prostředí a jsou ihned schopné infekce (Smith et Rose 1998). Některé oocysty tzv. tenkostěnné mají jen dvouvrstvou stěnu (chybí střední vrstva). Během procesu sporulace oocysty dochází k degradaci křehké vnější vrstvy. Tyto oocysty mohou excystovat v těle hostitele a mohou způsobit autoinfekci uvolněním sporozoitů (Current et Reese 1986, Uni et al. 1987).

Obrázek 1. Schéma znázorňující životní cyklus kryptosporidií (Barta et Thompson 2006; upraveno)



Délka vývojového cyklu, tedy prepatentní perioda, je typická pro daný druh/genotyp kryptosporidie a daného hostitele. U řady popsaných druhů (např.

C. ducismarci, C. fragile, C. homai, C. nasorum nebo *C. rubeyi*) a vČtšiny genotypŧ není známá délka prepatentní periody pro vnímavé hostitele.

ObecnČ lze kryptosporidie rozdČlit dle doby potŕebné pro ukonļení vývojového cyklu na druhy a genotypy, jejichž prepatentní perioda se pohybuje od 4 do 10 dnŧ (vČtšina stŕevních druhŧ) a kryptosporidie jejichž vývoj trvá více než 10 dnŧ (žaludeļní druhy) (Kváļ et al. 2008b, Kváļ et al. 2016). Délka prepatentní periody vČtšiny stŕevních druhŧ kryptosporidií se pohybuje okolo 1 týdne, napŕíklad. *C. felis* u koļek 5ï6 dní (Iseki 1979), *C. parvum* u telat v rozmezí 2ï7 dní (Tzipori 1983), *C. scrofarum* 4ï6 dní u prasat (Kváļ et al. 2013a), *C. suis* u prasat 2ï9 dní (Enemark et al. 2003) nebo *C. xiaoi* u ovcí 7ï8 dní (Fayer et Santín 2009). NicménČ prŧbČh infekce je výsledkem interakce mezi parazitem a hostitelem. Výrazné rozdíly v prepatentní periodČ u nejvíce prozkoumaného druhu - *C. parvum* ukazují, že významnou roli hraje nejen imunitní stav jedince, ale i druh a vČk hostitele. Zatímco u juvenilních (7 dní starých) myší kmene BALB/c je vývojový cyklus dokonļen za 3ï4 dny, dospČlé BALB/c myši nejsou k infekci vnímavé (Mclaughlin et al. 2000). Naopak u všech vČkových kategorií imunodeficitních SCID myší dojde k ukonļení vývojového cyklu za 7ï10 dnŧ (Hikosaka et Nakai 2005, Benamrouz et al. 2012). JeštČ výraznČjší rozdíly v délce prepatentní periody byly popsány u žaludeļních kryptosporidií. Zatímco oba žaludeļní druhy *C. muris* a *C. proliferans* dokonļí vývojový cyklus v dospČlé BALB/c myši za 7ï10 dnŧ, prepatentní perioda *C. proliferans* u mastomyší je 18ï21 dnŧ a druhu *C. muris* pouze 7 dnŧ (Rhee et al. 1995, Rhee et al. 1999, Kváļ et al. 2016).

3.1.4. Pŕenosinfekce

PrimárnČ jsou kryptosporidie pŕenášeny fekálnČ-orální cestou buĎ: a) pŕímým kontaktem s infikovaným ļlovČkem ļi zvíŕetem, b) nepŕímo kontaminovanou potravovou ļi vodou (Xiao 2010). ZemČdČlské plodiny, živoļišné produkty a povrchové vody kontaminované výkaly infikovaných jedincŧ (osob a zvíŕat) jsou hlavními zdroji šíŕení kryptosporidiových infekcí v populacích vnímavých hostitelŧ (Nyachuba 2010, Budu-Amoako et al. 2011, Sponseller et al. 2014), pŕiļemž pŕenos kontaminovanou vodou je považován za hlavní zpŧsob pŕenosu kryptosporidií (Baldursson et Karanis 2011).

Následující vlastnosti kryptosporidií výrazným zpTsobem umogRují snadné gíSení tIchto parazitT. **(i)** Oocysty kryptosporidií jsou infekLní ihned po vylouLení z tIla stolicí Li trusem (Smith et Rose 1998). **(ii)** Hostitelé mohou vyluLovat infekLní oocysty do prostSedí i nIkolik mIsícT po odeznIní klinických pSíznakT (Jokipii et Jokipii 1986, Chappell et al. 1996). **(iii)** K vyvolání infekce je tSeba malá infekLní dávka (10 Li ménI oocyst) (Blagburn et Current 1983, Chappell et al. 1996, Okhuysen et al. 1999, Guerrant et al. 2008, Ghazy et al. 2016). **(iv)** Oocysty stSevních druhT kryptosporidií si zachovávají infekLnost po dobu více negG 6 mIsícT (Fayer et al. 1998a, Fayer et al. 1998b) a oocysty galudeLních druhT 5i 6 mIsícT (KváL et al. 2007) ve vhodných klimatických podmínkách. **(v)** AG na výjimky (*C. andersoni* nebo *C. proliferans*) mají kryptosporidie krátkou patentní periodu (4i 10 dnT) (Iseki 1979, Tzipori 1983, Enemark et al. 2003, KváL et al. 2013a, KváL et al. 2014a, KváL et al. 2016). **(vi)** Oocysty kryptosporidií jsou extrémnI odolné vTLi úLinkTm dezinfekLních preparátT na bázi chloru, které se bIGnI pouGívají pro dezinfekci vody (DolejG 2004, Fayer 2004, Domenéch-Sánches et al. 2008, Baldursson et Karanis 2011, Burnet et al. 2014).

3.1.5. Klinické pSíznaky

Kryptosporidióza je jedno z nejLastIjGích lidských stSevních onemocnIní ve vyspIlých a rozvojových zemích. Navzdory dTkazTm, Ge kryptosporidie jsou jedním ze LtyS patogenT zodpovIdných za vIGinu závaGných prTjmT u batolat a dItí (Kotloff et al. 2013), výzkumy léLby zaostávají za ostatními tSemi nejLastIjGími pTvodci prTjmových onemocnIní i rotaviry, *Shigella* a enterotoxigenní *Escherichia coli* (Striepen 2013).

ZávaGnost infekce je ovlivnIna vIkem, výGivou, druhem Li genotypem kryptosporidie a zejména imunitním stavem hostitele, který má zásadní vliv na prTbIh onemocnIní (Bjorneby et al. 1991a, Bjorneby et al. 1991b, Gentile et al. 1991, Cama et al. 2008).

U imunokompetentních jedincT mTGe mít kryptosporidiová infekce asymptomatický aG závaGný prTbIh (O'donoghue 1995, Chen et al. 2002, Blackburn et al. 2004, RaGková et al. 2013). Mezi hlavní pSíznaky patSí vodnatý prTjem, doprovázený bSiGními kSeLemi, únavou, nevolností a anorexií (Current et Garcia 1991, Chalmers et Davies 2010, Bouzid et al. 2013). MTGe se objevit také horeLka a

zvracení. Průjem přetrvává zpravidla po dobu 5–10 dní po nichž následuje proces samovyléčení, nicméně může docházet k relapsům. U imunosuprimovaných jedinců se často vyskytují velmi intenzivní průjmy, které mohou být v důsledku výrazné dehydratace organismu fatální (Current et Garcia 1991, Manabe et al. 1998). Mezi komplikace dokumentované u těžce deficitních pacientů patří zánět žlučových cest či slinivky břišní (Hunter et Nichols 2002, Denkinger et al. 2008). Respirační kryptosporidióza byla popsána nejčastěji u dětí (Mor et al. 2010). Tato infekce je charakteristická přítomností infiltrátů na plicích a dýchacími obtížemi, ale bývá často i asymptomatická.

Klinické projevy kryptosporidiové infekce však nejsou typické pro všechny druhy a genotypy kryptosporidií (Turkcapar et al. 2002, Houpt et al. 2005, Vítovec et al. 2006, Kváč et al. 2014b, Ryan et Xiao 2014, Segura et al. 2015). Střevní kryptosporidióza je popisována nejen u lidí infikovaných nejčastěji *C. hominis* a *C. parvum* (Moon et Bemrick 1981, Argenzio et al. 1990, Ebeid et al. 2003, Němejc et al. 2013, Kváč et al. 2014a), ale i řadou dalších druhů a genotypů kryptosporidií (např. *C. canis*, *C. felis*, *C. meleagridis* nebo *C. tyzzeri*) (Xiao et Ryan 2004, Fayer 2010). U hospodářských zvířat (skot, koně, prasata) a jejich juvenilních věkových skupin jsou klinické příznaky často spojovány s infekcí *C. parvum*, u ovcí s infekcí *C. xiaoi* (Diaz et al. 2015, Diaz et al. 2018, Majeed et al. 2018). Naopak většina kryptosporidií parazitujících u volně žijících zvířat nejsou spojována s žádnými příznaky onemocnění (Laakkonen et al. 1994, Chalmers et al. 1997, Song et al. 2015). Obdobně u *C. scrofarum* parazitujících u prasat nebo *C. bovis* a *C. ryanae* parazitujících u skotu nejsou popisovány žádné klinické příznaky infekce (Fayer et al. 2005, Fayer et al. 2008, Kváč et al. 2013a).

3.1.6. Diagnostika kryptosporidií

V klinických laboratořích je zapotřebí rychlých, citlivých a specifických diagnostických metod, které by mohly vést k rychlému návrhu vhodné terapie lidí i zvířat (Smith et al. 2006, Fletcher et al. 2012). Laboratorní metody, které jsou založené na mikroskopických vyšetřeních vzorků trusu k detekci oocyst kryptosporidií jsou nedostatečné v rozpoznání parazitů od jiných složek trusu podobného tvaru a velikosti, například kvasinky a řasy. Navíc při využití různých koncentračních metod musí být intenzita infekce relativně vysoká a citlivost

mezi rŢznými metodami se výraznŊ liąí (Kváļ et al. 2003, Mekaru et al. 2007). Proto byla vyvinuta Śada barvících technik (napŚ. cold Kinyoun, acid-fast staining, barvení dle Ziehl-Neelsen, barvení aniline-carbol-methyl violetí) pro detekci oocyst v roztŊru vzorku trusu (Miláļek et Vítovec 1985, O'donoghue 1995, Elliot et al. 1999, Jex et al. 2008, Chalmers et Katzer 2013). Morfologické a morfometrické rozdíly mezi jednotlivými druhy a genotypy nejsou natolik výrazné, aby mohly být pouąity pro spolehlivé odliąení jednotlivých zástupcŢ rodu *Cryptosporidium* (O'donoghue 1995, Fall et al. 2003, Checkley et al. 2015). Daląí metody pouąívané v klinických diagnostických laboratoŚích zahrnují pŚímou nebo nepŚímou imunofluorescenļní mikroskopii (napŚ. fluorescenļní barvení auraminem nebo fluorescenļní barvení komerļnŊ dostupnými sekundárnŊ znaļenými protilátkami proti stŊnŊ oocysty kryptosporidií) (Xiao et Herd 1993, Bialek et al. 2002, Mekaru et al. 2007). Diagnostika kryptosporidií se dále provádí na základŊ pŚítomnosti specifického antigenu kryptosporidií pomocí enzymatických imunitních testŢ (ELISA) nebo imunochromatografických testŢ (Morgan et al. 1998, Kaushik et al. 2008, Calderaro et al. 2011, Chalmers et al. 2011, Polage et al. 2011). Tyto metody dosahují vysoké úrovnŊ senzitivity a specificity a jsou snadno proveditelné (Kaushik et al. 2008, Chalmers et al. 2011, Christy et al. 2012). Vąechny výąe uvedené metody spojuje rychlost, relativnŊ nízká cena a nenároļnost na technické vybavení laboratoŚe. Na druhou stranu primárním nedostatkem tŊchto postupŢ je nemoąnost genotypizace, tedy urļení druhu nebo genotypu kryptosporidií (Jiang et Xiao 2003, Jothikumar et al. 2008, Ryan et Xiao 2014).

Pouze molekulárními metody, které jsou vysoce senzitivní a specifické - detekce velmi malého mnoąství kryptosporidií ve vzorku, umoąĎují genotypizaci (Smith et al. 2006, Thompson et Ash 2016). NejļastŊji pouąívaným genem pro charakterizaci druhŢ a genotypŢ kryptosporidií je malá ribozomální podjednotka rRNA (SSU) a gen kódující 60 kDA glykoprotein (gp60) (Alves et al. 2003, Xiao 2010). Pro genotypizaci kryptosporidií jsou dále pouąívány, i kdyą v daleko menąí míŚe, i daląí geny kódující 70 kDa heat shock protein (HSP70), *Cryptosporidium* oocyst wall protein (COWP) nebo aktin (Morgan-Ryan et al. 2001, Thompson et Ash 2016).

Citlivost jednotlivých metod se výraznŊ liąí, zatímco pro barvící, flotaļní a imunofluorescenļní metody je detekļní limit mezi 1 000ï 2 000 oocyst na gram trusu (OPG), imunoenzymatické metody jsou schopné detekovat mnoąství okolo 200ï 500

OPG a u molekulárních metod se detekční limit pohybuje v rozmezí 10ï 20 OPG (Weber et al. 1991, Tomanová 2017).

3.1.7. Léčba a prevence

Navzdory skutečnosti, že kryptosporidiové infekce jsou jednou z nejčastějších příčin průjmového onemocnění, je léčba pomocí antiparazitik nedostatečná. V průběhu posledních 20 let byly testovány stovky různých léčiv s různými účinnými látkami (Cabada et White 2010, Sparks et al. 2015). Například mnohé účinné látky, které se používají proti ostatním zástupcům kmene Apicomplexa, nejsou u kryptosporidií využitelné (Abrahamsen et al. 2004).

Jediným lékem, který je schválený pro léčbu kryptosporidiózy, je Nitazoxanid (Shirley et al. 2012, Checkley et al. 2015). Avšak u HIV pozitivních pacientů, stejně tak jako u podvyživených dětí není tento lék ani při použití vysokých dávek a dlouhodobé léčbě účinný (Amadi et al. 2009). Léčba HIV pozitivních jedinců závisí na obnově imunitního systému pomocí kombinované antiretrovirové terapie (Cabada et White 2010). Bylo zjištěno, že například inhibitor reduktázy 3-hydroxy-3-methylglutaryl-koenzym A (HMG-CoA) inhibuje vývoj parazitů a invazi hostitelských buněk kryptosporidiemi *in vitro* (Hommer et al. 2003, Bessoff et al. 2013, Debnath et al. 2013). Nicméně v počátku léčby dochází u této skupiny osob ke zvýšené mortalitě (Dillingham et al. 2009).

Hlavní překážkou pro vývoj léků je absence technik pro *in vitro* kultivaci kryptosporidií a tím i omezené možnosti geneticky manipulovat s geny parazita (Checkley et al. 2015, Miyamoto et Eckmann 2015, Ryan et Hijjawi 2015). Pokroky ve vývoji nových léků jsou také výrazně omezeny dostupností současných experimentálních zvířecích modelů (Kothavade 2011). Navíc zvířecí modely nejsou optimální pro některé lidské infekce, proto jsou jako alternativa používány lidské buněčné linie (Feng et al. 2006, Yang et al. 2010).

Další překážkou ve výzkumu a léčbě kryptosporidií je nedostatečné pochopení gastrointestinální a imunitní reakce na parazita. Tento pohled by mohl umožnit pokroky v preventivním výzkumu, usnadnit optimalizaci současných léčebných metod a stanovit specifické cíle pro preventivní opatření. Vzhledem k omezeným léčebným postupům je prevence považována za jedno z nejdůležitějších opatření proti kryptosporidióze. Za prevenci lze považovat základní hygienické návyky,

zejména ļasté mytí rukou, adekvátní oģetŚování pitné vody a dŢkladné omytí ovoce a zeleniny pŚed konzumací (Ramirez et al. 2004, Chalmers et Davies 2010, Sparks et al. 2015).

3.2. Hlodavci

Hlodavci jsou druhovņ nejpoļetnĻjģím Ŝádem savcŢ, kteŚí mají 28 ļeledí. Jednou z nich je ļeleŅ Cricetidae, která svojí rozmanitostí s 681 druhy ve 130 rodech a 6 podļeledích: hraboģovití (Arvicolinae), kŚeļci praví (Cricetinae), chlupáļi (Lophiomyinae), kŚeļkovití (Neotominae), kŚeļci ameriļtí (Sigmodontinae) a kŚeļci velkoocí (Tylomyinae) tvoŚí jednu z nejvņģích ļeledí savcŢ (Musser et Carleton 2005).

3.2.1. Hlodavci jako rezervoár pŢvodcŢ onemocnņní

Hlodavci s 2277 uznávanými druhy pŚedstavují asi 42 % celosvņtové biologické rozmanitosti savcŢ (Wilson et Reeder 2005). Aļkoliv lidé do velké míry kategorizovali hlodavce jako ģkŢdce v mņstském a zemņdņlském prostŚedí, vņģina druhŢ hlodavcŢ ģije ve volné pŚírodņ v malé interakci s lidmi. Menģina hlodavcŢ se vģak pŚizpŢsobila ģivotu v úzkém spojení s lidmi a s tím je spojené riziko pŚenosu váģných onemocnņní (Gratz 1994, Webster et Macdonald 1995). V pŚírodņ hrají divocí hlodavci dŢleģitou roli jako rezervoár mnoha patogenŢ, vļetnņ nņkterých, které mohou být pŚenáģeny na hospodáŚská zvíŚata a lidi (Pawelczyk et al. 2004, Buckle et Smith 2015). Vzhledem ke specifickému chování a biologii mohou hrát hlodavci roli mezihostitelŢ, definitivních a paratenických hostitelŢ (Hildebrand et al. 2009). Hlodavci jsou schopni využívat ģirokou ģkálu biotopŢ a prostŚedí na celém svņtņ. Tak se stávají ideálními hostiteli a vektory pro rŢzné patogeny vļetnņ parazitŢ a pŢvodcŢ zoonotických onemocnņní (Erhardová 1955, Zasukhin et al. 1958, Doby et al. 1965, Perryman 1990, Duszynski et Upton 2001, Svobodová et al. 2004, Appelbee et al. 2005, Duszynski et al. 2007). Mnozí hlodavci, vļetnņ hraboģovitých jsou hostitelé kryptosporidií.

3.2.2. Charakteristika podčeledi Arvicolinae (hrabošovití)

Podčeleď hrabošovití je tvořena skupinou malých hlodavců zahrnující lumíky, ondatry, pestrušky, hraboše, hryzce a slepušky. Jde o relativně novou evoluční skupinu s pravděpodobnou diverzifikací před 2 až 3 miliony lety v krátkém časovém rámci, která se vyvinula do jedné z nejvíce úspěšných skupin savců (Wilson et Reeder 2005, Martinkova et Moravec 2012). Tato skupina hlodavců má poměrně jednotný vzhled, ale liší se místem výskytu (biotopem). V této podčeledi je popsáno 151 druhů v 28 rodech (Nowak 1999, Musser et Carleton 2005).

Zástupci této podčeledi jsou rozšířeni v celé Holarktické oblasti (Conroy et Cook 2000) a obývají celou Severní Ameriku od Guatemaly na sever, Eurasii, Japonsko, Tchaj-wan, jihozápadní Čínu, severní Indii a Blízký východ (Carleton et Musser 1984). Tito hlodavci sídlí na široké škále stanovišť v mírných, boreálních, arktických a horských biomech. Mezi tyto přírodní stanoviště patří suché a vlhké listnaté a jehličnaté lesy, skalnaté horské svahy, alpské louky, prérie, stepi, zemědělská pole, polopouště, tundry, jezera, bažiny a rašeliniště (Fayer et al. 1998b, Musser et Carleton 2005).

Jedním z rodů patřících do podčeledi hrabošovití je rod *Microtus* (hraboš). Dle nejnovějších údajů bylo v rámci tohoto rodu rozpoznáno 65 druhů ve 14 podrodech, čímž se tento rod řadí mezi skupinu hlodavců s nejvíce popsanými druhy (Musser et Carleton 2005, Golenishchev et Malikov 2006, Lemskaya et al. 2010) a představuje tím téměř polovinu existujících druhů podčeledi hrabošovití (Shenbrot et Krasnov 2005). Přesný počet druhů rodu *Microtus* však zůstává nedefinovaný, neboť taxonomické postavení některých poddruhů, druhů a dokonce i skupin druhů je neustále revidován (Golenishchev et Sablina 1991, Krystufek et al. 1996, Gromov et Polyakov 1997, Musser et Carleton 2005, Golenishchev et Malikov 2006, Lemskaya et al. 2010).

Hrabоši rodu *Microtus* jsou ekologicky různorodí, většina druhů preferuje otevřené zatravněné plochy, jako jsou louky a pastviny, ale některé druhy využívají také lesy a vysočiny (Getz 1985, Hoffmann et Koeppl 1985, Mitchell-Jones et al. 1999, Nowak 1999). Mnoho druhů vykazuje pozoruhodnou distribuci v rozsáhlých oblastech, zatímco jiné zaujímají velmi omezené oblasti (Musser et Carleton 1993, Shenbrot et Krasnov 2005, Mitsainas et al. 2010). Tento rod je vynikajícím příkladem rychlého a rozsáhlého vření v evoluci savců, jehož výsledkem jsou

existující druhy rozmístěné v palearktických a nearktických oblastech (Reig 1989, Musser et Carleton 1993, Chaline et al. 1999, Nowak 1999, Jaarola et al. 2004).

V Evropě se hraboši rodu *Microtus* začali šířit na přelomu pliocénu a pleistocénu (De Garidel-Thoron 2007, Havlová 2012). Paleontologické údaje dokazují, že nejnovějším společným předkem rodu *Microtus* je *Allophaiomys pliocaenicus* (Brunet-Lecomte et Chaline 1992, Nadachowski et Zagorodnyuk 1996, Chaline et al. 1999), který zřejmě pochází z rodu *Mimomys* (Chaline et Graf 1988, Nadachowski et Zagorodnyuk 1996, Conroy et Cook 1999, Jaarola et al. 2004). Druh *A. pliocaenicus* se postupně rozšiřoval nezávisle v severní Eurasii, v centrální Asii a v Himálajích a v Severní Americe (Brunet-Lecomte et Chaline 1991, Chaline et al. 1999). Do nedávné doby byl výskyt *Allophaiomys* datován přibližně do doby před 2 miliony lety (Chaline et Graf 1988), ale nové nálezy tohoto druhu datují původ rodové linie do doby před 2,3ï 2,4 miliony let (Zheng et Zhang 2000).

Dalším z rodů patřících do podčeledi hrabošovití je rod *Myodes*, který spolu s dalšími rody lesních a alpských hlodavců (*Alticola*, *Caryomys*, *Eothenomys*, *Hyperacrius*) obývá celou Holarktickou oblast patří do tribu Myodini (Kohli et al. 2014). Ačkoliv je tribus Myodini monofyletický, řada vztahů mez jednotlivými zástupci zůstává nevyřešena a to jak uvnitř tribu, tak v rámci rodů (Buzan et al. 2008, Robovský et al. 2008). Diverzita uvnitř Myodini je spojována s glaciálními cykly (Cook et al. 2004) a geomorfními událostmi jako je vzestup tibetské plošiny v průběhu uplynulých 3,5 milionu let (Luo et al. 2004, Liu et al. 2012).

Myodes je jediný holarktický rod, který se vyskytuje po celé severní části Eurasie a Severní Ameriky (Carleton et Musser 2005). Rod *Myodes* zaznamenal během čtvrtohor složitý vývoj (Ledevin et al. 2010), prodělal opakované fáze izolace a expanze, což vyústilo ve složitou vnitrodruhovou genetickou diverzitu (Deffontaine et al. 2005, Kotlik et al. 2006, Deffontaine et al. 2009). Opakované izolace v ledovcových refugiích vedly k odlišení několika linií za méně než 300 000 let. Zástupci rodu *Myodes* jsou jedni z mála v rámci podčeledi Arvicolinae, kteří si zachovali kořeny zubů na rozdíl od mnoha jiných linií hrabošů, kterým neustále rostou moláry bez kořenů (Tesakov 1995).

Mezi populacemi norníka rudého (*Myodes glareolus*) byla dokázána alopatrická diferenciace populací ve Španělsku a Francii, zapříčiněna geografickou bariérou, Pyrenejemi (Deffontaine et al. 2005, Králová 2016). Norník rudý obývá širokou

gkálu stanovigŠ, vļ etnĐlesŢ, kŚovin, ģivých plotŢ, bŚehŢ a baģin (Bellamy et al. 2000, Macdonald 2001). UpŚednostŔuje listnaté, jehliļnaté a taigové lesy (Ostfeld 1985, Koskela et al. 1997, Prevot-Julliard et al. 1999, Yoccoz et al. 2001).

4. SHRNUTÍ VÝSLEDKŠ A DISKUZE

Vġechny dosaġené výsledky (obrázky, grafy, tabulky), pouġitý materiál a metody je moġno nalézt v pŚiloġených publikacích.

Z více neġ stovky studií, které jsou vĽnovány kryptosporidiím hlodavcĢ vyplývá, ġe jsou hostitelé 12 druhĢ a více jak 40 genotypĢ kryptosporidií (Fayer et al. 2005, Ryan et Hijjawi 2015, Kváļ et al. 2016, Ļondlová et al. 2018). ř ada z nich pŚedstavuje potenciální riziko infekce pro ļlovĽka (napŚ. *C. muris*, *C. parvum*, *C. ubiquitum*, *C. andersoni*, chipmunk genotyp I nebo skunk genotyp) (Katsumata et al. 2000, Guyot et al. 2001, Feltus et al. 2006, Elwin et al. 2012, Raġková et al. 2013, Guo et al. 2015).

Hlodavci jsou hostitelé jak kryptosporidií se ġirokou hostitelskou specifitou (napŚ. *C. muris*, *C. parvum* nebo *C. ubiquitum*), tak druhĢ ļi genotypĢ, které jsou úzce hostitelsky specifické (napŚ. *C. wrairi*, deer mouse genotyp Iï IV, rat genotyp Iï IV). Recentní a námi provedené studie ukazují, ġe kryptosporidie parazitující u hladovcĢ jsou hostitelsky adaptovány a speciovány na jednotlivé hostitele a ġe pŚenos kryptosporidií mezi taxonomicky pŚíbuznými, ale i vzdálenými skupinami je omezený (Morgan et al. 1999, Kváļ et al. 2013b, Zahedi et al. 2017).

NapŚíklad myġi (*Mus*) jsou hostitelé dvou specifických kryptosporidií, *C. tyzzeri* (dŚíve mouse genotyp I) a mouse genotyp II (Ren et al. 2012), kŚeļkové (Cricetinae) ļtyŚ genotypĢ - deer mouse genotyp I, II, III a IV (Xiao et al. 2002, Feng et al. 2007, Stenger et al. 2015b), ondatry (*Ondatra zibethicus*) dvou genotypĢ ï *Cryptosporidium* muskrat genotyp I a II (Perz et Le Blancq 2001, Xiao et al. 2002, Zhou et al. 2004, Feng et al. 2007), veverky (Sciuridae) jednoho druhu - *C. rubeyi* a nĽkolika genotypĢ - skunk genotyp, ferret genotyp a chipmunk genotyp I (Ziegler et al. 2007a, Lv et al. 2009, Li et al. 2015, Prediger et al. 2017) nebo morļata (*Cavia*) dvou druhĢ - *C. homai* a *C. wrairi* (Vetterling et al. 1971, Zahedi et al. 2017).

4.1. Diverzita kryptosporidií parazitujících u hraboġovitých

Do souļasné doby bylo publikováno 20 prací provedených v Ļeské republice, Finsku, Japonsku, Polsku, Slovensku, Ġpanġlsku, USA a Velké Británii, které se zabývají prevalencí a diverzitou kryptosporidií u hlodavcŢ patŚících do podļeledi hraboġovití.

Procento zvíŚat pozitivních na kryptosporidie se v jednotlivých studiích velmi liġí. Ve studiích, kde byla pouġita pouze mikroskopická technika, se prevalence pohybovala od 1 do 73 % (Sinski et al. 1993, Laakkonen et al. 1994, Chalmers et al. 1997, Sinski et al. 1998, Torres et al. 2000, Bajer et al. 2002, Bednarska et al. 2007). Podobný rozptyl (3ï 80 %) byl zaznamenán i ve studiích, kde byly pouġity molekulární metody. V souladu s pŚedeġlými studiemi jsme prokázali, ġe molekulární metody pro detekci kryptosporidií v trusu volnļ ġijících zvíŚat jsou senzitivnļjší neġ mikroskopická vyġetŚení (Ļondlová et al. 2018). Dále jsme ukázali, ġe rozdíly ve výsledcích získaných molekulárními a mikroskopickými metodami mohou být výraznļ ovlivnļny druhem/genotypem kryptosporidie. Zatímco 25 % hraboġŢ infikovaných druhem *C. microti* vyluļuje mikroskopicky detekovatelné mnoġství oocyst, v pŚípadļ infekce zpŢsobené *C. alticolis* je to jen 12 %. Obdobné rozdíly byly popsány napŚíklad u myġic, prasat nebo skotu. Zatímco u myġic pŚirozenļ infikovaných druhem *C. ditrichi* bylo 65 % zvíŚat mikroskopicky pozitivních, ġádné zvíŚe infikované *C. apodemi* nevyluļovalo detekovatelné mnoġství oocyst pomocí mikroskopických technik (Ļondlová et al. 2018). Selata infikovaná *C. scrofarum* a *C. suis* vyluļují více oocysty *C. scrofarum* (Jeníková et al. 2011, Kváļ et al. 2012). Obdobnļ skot infikovaný *C. parvum*, *C. andersoni*, *C. bovis* a *C. ryanae* vyluļuje více oocysty, to stejné bylo zaznamenáno u *C. parvum*, jedná-li se o tele nebo *C. andersoni*, jedná-li s o mladý a dospļlý skot (Kváļ et Vítovec 2003, Santín et al. 2004, Fayer et al. 2005, Kváļ et al. 2006).

Ze studií, které byly zaloġeny pouze na detekci oocyst pomocí svļtelné mikroskopie vyplývá, ġe hraboġovití jsou parazitovaní kryptosporidiemi *C. parvum* a *C. muris* (Sinski et al. 1993, Laakkonen et al. 1994, Chalmers et al. 1997, Sinski et al. 1998, Torres et al. 2000, Bajer et al. 2002, Bednarska et al. 2007). Výsledky této i pŚedchozích prací ukazují, ġe sekvence SSU genu kryptosporidií detekovaných z hraboġovitých jsou velmi heterogenní (Stenger et al. 2017). V souvislosti s tļmito výsledky a dŚívļjšími nálezy bylo konstatováno, ġe pouġití pouze sekvencí genu

kódujícího malou ribosomální podjednotku rRNA k vyvozování evolučních vztahů mezi jednotlivými druhy a genotypy může vést k chybným závěrům (El-Sherry et al. 2013, Stenger et al. 2015a, Stenger et al. 2015b).

Dříve publikované výsledky a námi provedená multilokusová analýza (SSU, aktin, HSP70 a COWP) ukázala, že hrabošovití jsou parazitovaní řadou druhů a genotypů kryptosporidií, které vytvářejí čtyři fylogeneticky příbuzné skupiny, přičemž většina detekovaných kryptosporidií klastrovala do blízkosti *Cryptosporidium* muskrat genotypu I a II (Xiao et al. 2002, Zhou et al. 2004, Feng et al. 2007, Ziegler et al. 2007b, Danišová et al. 2017, Stenger et al. 2017). Zjištěná diverzita kryptosporidií může být částečně výsledkem úzkého spojení s odlišnými hostitelskými druhy. Tento model evoluce byl již dříve popsán u *C. tyzzeri* (Kváč et al. 2013b).

Hrabošovití byli popsáni jako hostitelé *C. parvum*, *C. scrofarum*, *C. tyzzeri*, *Cryptosporidium* vole genotyp, *Cryptosporidium* muskrat genotyp I a II (Bajer et al. 2003, Zhou et al. 2004, Feng et al. 2007, Ziegler et al. 2007b, Perec-Matysiak et al. 2015, Danišová et al. 2017). Nicméně prevalence *C. parvum*, *C. scrofarum* a *C. tyzzeri* je velmi nízká, což může být v případě *C. scrofarum* a *C. tyzzeri* vysvětleno hostitelskou specifitou těchto druhů kryptosporidií a v případě *C. parvum* omezenou infektivitou pro dospělé hlodavce (Ren et al. 2012, Kváč et al. 2013b, Rašková et al. 2013). Nálezy hostitelsky nespecifických druhů a genotypů kryptosporidií u různých hostitelů nejsou ojedinělé. Ve většině případů se jedná o mechanickou pasáž oocyst zažívacím traktem hostitele, které se do hostitele dostaly buď s kontaminovanou vodou a potravou nebo v případě predátorů prostřednictvím přirozeně infikované kořisti. Například bylo prokázáno, že prasata nejsou vnímavá k infekci *C. muris* a *C. tyzzeri*, přestože jsou tyto druhy kryptosporidií, hostitelsky specifické pro hlodavce, často detekovány v trusu a kejdě prasat (Kváč et al. 2012, Němejc et al. 2013). Taktéž hadi nebo draví ptáci krmení hlodavci vylučovali oocysty *C. muris* a po změně potravy (hlodavci bez kryptosporidiové infekce) došlo k zastavení vylučování (Graczyk et Cranfield 1998, Ng et al. 2006).

V případě infekce *C. parvum* lze tedy konstatovat, že hrabošovití nejsou primárními hostiteli druhu *C. parvum* tak, jak bylo dlouhou dobu předpokládáno, nicméně jsou k infekci tímto druhem vnímaví. V rámci této práce jsme prokázali probíhající infekci *C. parvum* u jednoho ze tří experimentálně infikovaných

dospělých hrabošů polních (nepublikováno). První infekce (přítomnost oocyst v trusu pouze 3. a 7. DPI) a intenzita infekce (>2000 OPG) naznačují jen omezenou vnímavost těchto hostitelů k *C. parvum*.

V rozporu se studiemi založenými na mikroskopickém vyšetření nebyla potvrzena přítomnost *C. muris* v žádné práci, kde byly vzorky genotypizovány (Perz et Le Blancq 2001, Xiao et al. 2002, Bajer et al. 2003, Zhou et al. 2004, Feng et al. 2007, Ziegler et al. 2007b, Perec-Matysiak et al. 2015, Danišová et al. 2017, Stenger et al. 2017). Tyto výsledky, které experimentálně prokázali, že norník rudý (*Myodes glareolus*) a hraboši polní (*Microtus arvalis*) nejsou vnímaví k infekci žádným známým druhem žaludečních kryptosporidií parazitujících u savců (*C. andersoni, C. muris* a *C. proliferans*), jsou v souladu se zjištěním s Modrý et al. (2012). Jediný zástupce hrabošovitých vnímavý k *C. muris* a *C. proliferans* byl hraboš syslí (*Lasiopodomys brandtii*). Tyto rozdíly by bylo možné vysvětlit rozdílnou vnímavostí různých druhů hrabošovitých k infekci žaludečními kryptosporidiemi (Modrý et al. 2012). Zatímco norníci rudí a hraboši polní nejsou k infekci *C. muris* vnímaví a omezený výskyt oocyst (dvě zvířata od každého druhu) lze vysvětlit náhodnou infekcí při pasážích, u poddruhu norníka rudého (*Myodes glareolus skomerensis*), u kterého byla detekována téměř 50% prevalence žaludečními kryptosporidiemi (Bull et al. 1998), je možné, že tento poddruh norníků rudých s endemickým výskytem je vnímavý k infekci *C. muris*.

Sekvence izolátů kryptosporidií získaných v našich studiích, klastrují nejčastěji ke *Cryptosporidium* muskrat genotyp I a II a byly do současné doby zřídka detekovány u jiných hostitelů než u hrabošů (Robinson et al. 2011, Ruecker et al. 2012). Podobné je to i se sekvencemi klastrujícími ke genotypu W12 a ke *Cryptosporidium* vole genotyp I. Genotyp W12 byl do současné doby nalezen pouze ve vodách v New Yorku (Xiao et al. 2000) a nebyl dosud detekován v žádném hostiteli, *Cryptosporidium* vole genotyp byl nalezen u hrabošce pensylvánského a norníka rudohnědého (Feng et al. 2007, Ziegler et al. 2007a). *Cryptosporidium* deer mouse genotyp Iři IV, W29 genotyp a *C. ubiquitum* byly v našich studiích nalezeny výhradně u kšelovitých. *Cryptosporidium* deer mouse genotyp Iři IV nebyl dosud detekován u jiného hostitele než u kšelů rodu Peromyscus (Xiao et al. 2002, Feng et al. 2007, Stenger et al. 2015b). Přestože má druh *C. ubiquitum* širokou hostitelskou specifitu a byl nalezen například u činčily dlouhoocasé (*Chinchilla lanigera*), lemurů kata

(*Lemur catta*), myšice japonské (*Apodemus speciosus*) nebo veverky popelavé (*Sciurus carolinensis*) (Da Silva et al. 2010, Murakoshi et al. 2013, Qi et al. 2015, Stenger et al. 2015a), nebyl detekován u žádného ze zástupcŧ hrabošovitých (Perec-Matysiak et al. 2015, Stenger et al. 2017, Horňáková et al. 2018).

Hrabоši a křečkovití jsou z pohledu biotopŧ, které obývají, prostorově oddělení, což omezuje jejich mezidruhové interakce (Bowker et Pearson 1975) a nesdílejí shodné druhy a genotypy kryptosporidií. K obdobným závěrŧm dospěla i Ľondlová et al. (2018), která prokázala, že myšice sdílející stejné lokality s hrabоši jsou parazitováni odlišnými druhy a genotypy kryptosporidií. U hrabošovitých (*Microtus* spp. a *Myodes* spp.) byly naopak nalezeny shodné nebo příbuzné druhy a genotypy kryptosporidií bez ohledu na jejich geografické umístění (Zhou et al. 2004, Danišová et al. 2017, Stenger et al. 2017).

Hrabošovití jsou často infikováni kryptosporidiemi, ale žádný z dosud u nich popsaných druhŧ a genotypŧ, vyjma *C. parvum* a *C. scrofarum*, nebyl spojen s lidskou infekcí (Xiao et al. 2002, Zhou et al. 2004, Ziegler et al. 2007b). Lze tedy konstatovat, že hrabošovití nepředstavují riziko pro lidské zdraví.

4.2. Biologické vlastnosti kryptosporidií parazitujících u hrabošovitých a popis nových druhŧ

V současné době je uznáno téměř 40 druhŧ rodu *Cryptosporidium* a bylo popsáno více než 200 různých genotypŧ kryptosporidií (na základě odlišnosti SSU sekvencí). Vzhledem ke značné vnitrodruhové variabilitě SSU lokusu a faktu, že u většiny popsaných genotypŧ nejsou známy žádné jiné údaje, nelze spolehlivě říci, zda se jedná o samostatné druhy.

Znalosti o biologických vlastnostech zahrnujících hostitelskou specifitu, průběh infekce (prepatentní a patentní perioda, patogenita, intenzita infekce), lokalizaci v hostiteli nebo morfometrii oocyst se ukazují jako velmi významné nejen z pohledu odlišení jednotlivých druhŧ od sebe, ale zejména v porozumění interakce mezi parazitem a hostitelem napomáhající v boji proti těmto parazitŧm.

4.2.1. Morfometrie oocyst

V rámci rodu *Cryptosporidium* lze fylogeneticky odlišit dvě monofyletické skupiny kryptosporidií, které se zároveň od sebe liší lokalizací v hostiteli a velikostí a tvarem oocyst (Upton et Current 1985, Vítovec et al. 2006). Oocysty střevních druhů jsou sférického tvaru s velikostí od 4 do 6,5 µm (Tyzzer 1912, Fayer et al. 2008), oocysty žaludečních druhů jsou oválné o velikosti od 6,5 do 9 µm (Ryan et Xiao 2014). Velikost oocyst jednotlivých druhů jak střevních, tak žaludečních druhů se překrývá a není proto možné ve většině případů od sebe odlišit jednotlivé druhy/genotypy v rámci jednoho hostitele (Fayer 2010).

Oocysty *Cryptosporidium alticolis* (námi popsaný druh), které jsou z hostitele vylučovány plně vysporulované o velikosti 4,9–5,7 × 4,6–5,2 (*x* ± *SD* = 5,4 ± 0,2 × 4,9 ± 0,2 µm), poměr mezi délkou a šířkou 1,00–1,20 (*x* ± *SD* = 1,10 ± 0,05) jsou statisticky významně větší (P = 0,0001) než oocysty *Cryptosporidium microti* (námi popsaný druh), které měří 3,9–4,7 × 3,8–4,4 µm (*x* ± *SD* = 4,3 ± 0,1 × 4,1 ± 0,1 µm) poměr mezi délkou a šířkou 1,00–1,06 (*x* ± *SD* = 1,03 ± 0,02) a jsou také vylučovány plně vysporulované.

Přestože jsou morfometrické rozdíly mezi oocystami obou druhů výrazné, stejně jako například mezi *C. scrofarum* a *C. suis* u prasat (Ryan et al. 2004, Vítovec et al. 2006, Kváč et al. 2013a) nebo *C. apodemi* a *C. ditrichi* u myšic (Ježková et al. 2018), nelze doporučit využití tohoto znaku k diferenciální diagnostice.

Ostatní charakteristiky zahrnující tloušťku stěny oocysty, přítomnost reziduálního tělíska, počet sporozoitů, vnitřní struktura nebo detekce pomocí specifických protilátek proti *Cryptosporidium* spp. neumožňují odlišení jednotlivých druhů mezi sebou (Robinson et al. 2010, Kváč et al. 2014b, Kváč et al. 2016).

4.2.2. Hostitelská specifita

Hostitelská specifita je vedle genetických rozdílů jednou ze základních charakteristik pro odlišení jednotlivých druhů a genotypů kryptosporidií. Za hostitele jsou považováni jedinci, v nichž dochází k ukončení vývojového cyklu příslušné kryptosporidie a hostitel vylučuje do prostředí oocysty, které jsou geneticky totožné s těmi, které vyvolaly infekci (Fayer 2007). V rámci rodu *Cryptosporidium* rozlišujeme druhy a genotypy na druhy **i)** s úzkou hostitelskou specifitou, tedy

kryptosporidie parazitující jen u omezeného poļtu hostitelŢ (vŋtšina dosud popsaných druhŢ a genotypŢ kryptosporidií) a **ii)** se ģirokou hostitelskou specifitou, ty které parazitují u velkého poļtu hostitelŢ (*C. meleagridis*, *C. parvum* a *C. ubiquitum*). Obecnŋlze Śíci, ģe kryptosporidie parazitující u jedné tŚídy obratlovcŢ nejsou infekļní pro zástupce jiné tŚídy. Jedinými výjimkami jsou druhy *C. meleagridis* a *C. parvum*, které jsou infekļní jak pro savce, tak pro ptáky (Ditrich et al. 1991, Akiyoshi et al. 2002, Kimura et al. 2007, Zylan et al. 2008).

Naģe studie jednoznaļnŋ prokázala, ģe druhy *C. aticolis* a *C. microti* jsou hostitelsky specifické pro hraboģe a to nejenom pro pŢvodního hostitele hraboģe polního, ale i pro fylogeneticky pŚíbuzného hraboģe pensylvánského. Experimentálnŋ jsme potvrdili, ģe *C. alticolis* a *C. microti* nejsou schopny dokonļit svŢj vývojový cyklus v dalģích hostitelích nepatŚících mezi hraboģovité (Horļiļková et al. 2018). Dále jsme experimentálnŋ prokázali, ģe druhy *C. apodemi* a *C. ditrichi*, které jsou bŋžnými parazity hlodavcŢ rodu *Apodemus*, nejsou infekļní pro hraboģe polní (Ļondlová et al. 2018). Tyto výsledky potvrzují hypotézu, ģe vŋtšina kryptosporidií je úzce hostitelsky specifická (Dupont et al. 1995, Morgan-Ryan et al. 2002, Fayer 2004, Ifeonu et al. 2016).

4.2.3. PrŢbŋh infekce, lokalizace a patogenita

PrŢbŋh infekce vyvolaný kryptosporidiemi je ovlivnŋn Śadou faktorŢ, pŚiļemģ mezi nejdŢleģitŋjší patŚí **i)** interakce mezi hostitelem a druhem/genotypem kryptosporidií a **ii)** vŋk a imunitní stav hostitele (Lindsay et Blagburn 1990, Baishanbo et al. 2005).

Délka prepatentní periody *C. alticolis* a *C. microti* byla jak u hraboģŢ polních, tak i hraboģŢ pensylvánských shodná (4 DPI), coģ odpovídá dobŋ potŚebné k ukonļení vývojového cyklu vŋtšiny stŚevních druhŢ kryptosporidií u imunokompetentních hostitelŢ; *C. parvum* u myšího 3ï4 DPI, *C. parvum* u telat 2ï9 DPI, *C. occultus* u potkanŢ 4ï5 DPI, *C. scrofarum* u selat 4ï6 DPI, *C. suis* u selat 2ï9 DPI, nebo *C. tyzzeri* u myšího 4ï5 DPI (Iseki 1979, Tzipori 1983, Enemark et al. 2003, Fayer et Santín 2009, Kváļ et al. 2013a, Kváļ et al. 2013b, Kváļ et al. 2018).

V souladu s délkou patentní periody u druhŢ parazitujících v tenkém stŚevŋ jako je *C. meleagridis*, *C. parvum* nebo *C. tyzzeri* bylo pozorováno vyléļení hraboģŢ infikovaných *C. alticolis* do 15 DPI (Vítovec et Koudela 1992, Ren et al. 2012, Kváļ

et al. 2014a, Kváč et al. 2016). Naopak u druhu *C. microti*, jehož vývojový cyklus je lokalizovaný výhradně v tlustém střevě byla patentní perioda krátká (do 15 DPI), což je výrazně méně než u *C. occultus* (>30 DPI) a *C. suis* (>30 DPI) parazitujících také v tlustém střevě svých hostitelů (Ryan et al. 2004, Vítovec et al. 2006, Kváč et al. 2018).

Vývojový cyklus *C. microti* je shodně s *C. occultus* lokalizován na povrchovém epitelu tlustého střeva (Horčičková et al. 2018, Kváč et al. 2018) zatímco *C. suis* primárně infikuje lymfoglandulární komplexy tlustého střeva (Vítovec et al. 2006).

Infekce způsobená *C. alticolis* nebo *C. microti* nevyvolala žádné klinické příznaky u experimentálně infikovaných hrabošů. Tyto výsledky jsou v souladu se závěry většiny prací, které konstatují, že volně žijící savci velmi ojediněle trpí klinickou kryptosporidiózou a většina infekcí vyvolaná hostitelsky specifickými druhy a genotypy kryptosporidií probíhá bezpříznakově (Sturdee et al. 1999, Hikosaka et Nakai 2005, Castro-Hermida et al. 2011, Němejc et al. 2012, Ježková et al. 2018).

4.2.4. *Cryptosporidium alticolis* sp. n. a *Cryptosporidium microti* sp. n.

Cryptosporidium alticolis a *C. microti* jsou geneticky odlišné od dosud popsaných platných druhů rodu *Cryptosporidium*. *Cryptosporidium alticolis* sdílí na lokusu kódujícím SSU gen 95,2%, 94,7% a 94,3% sekvenční identity s *C. canis*, *C. suis*, respektive s *C. parvum*. Podobné rozdíly byly zjištěny i na ostatních lokusech. Na lokusu kódujícím aktin je tato shoda 87,9 %, 90,5 %, respektive 89,7 %, a na lokusu kódujícím HSP70 84,5 %, 91,2 %, respektive 90,5 %. Obdobně *C. microti* sdílí na lokusu kódujícím SSU gen 95,5%, 98,8%, respektive 96.4% sekvenční identity s *C. canis*, *C. suis*, respektive s *C. parvum*. Na lokusu kódujícím aktin je tato shoda 84,6 %, 93,1 %, respektive 90,5 % a na lokusu kódujícím HSP70 84,2 %, 93,1 %, respektive 92,6 %. Tyto rozdíly jsou v porovnání například s *C. hominis* a *C. parvum* sdílejících 98–99 % sekvenční identity nebo s *C. andersoni* a *C. muris* sdílejících 96–99 % identity na stejných lokusech výrazně vyšší (Horčičková et al. 2018).

Genetické a biologické údaje podpořily popsání dvou nových druhů v rámci rodu *Cryptosporidium*.

5. ZÁVĚRY

Pomocí multilokusových analýz bylo prokázáno, že hraboši mohou být parazitováni nejméně dosmi různými druhy a genotypy kryptosporidií.

- Bylo zjištěno, že hraboši nejsou přirozenými hostiteli *C. parvum*, druhu s nízkou hostitelskou specifitou, který byl běžně popisován u těchto hostitelů na základě mikroskopického vyšetření.
- Hraboši jsou parazitováni hostitelsky specifickými druhy a genotypy kryptosporidií, které nebyly dosud detekovány u žádných jiných hostitelů.

Výsledky práce prokazují, že kryptosporidie hostitelsky specifické pro hrabošovité nejsou věkově a pohlavně specifické.

- Statistické analýzy potvrdily, že není rozdíl v prevalenci detekovaných druhů a genotypů kryptosporidií v závislosti na věku nebo pohlaví.
- Experimentálně nebyl zjištěn rozdíl ve vnímavosti juvenilních a adultních jedinců hrabošů polních k infekci *C. microti* (nepublikovaná data).

Morfometrická, genetická a biologická data získaná při této práci vedla k popisu dvou nových druhů kryptosporidií v rámci rodu *Cryptosporidium*.

- Byly popsány dva nové druhy pojmenované *Cryptosporidium alticolis* sp. n. a *Cryptosporidium microti* sp. n.
- Oba druhy jsou hostitelsky specifické pro hraboše a neinfekční pro myši, myšice, potkany a kuřata.
- Druh *C. alticolis*, který infikuje tlusté střevo má menší, morfometricky odlišitelné oocysty od druhu *C. microti*, který infikuje tenké střevo.

Výsledky práce prokázaly, že zástupci rodu *Microtus* a *Myodes* obývající Severní Ameriku a Evropu jsou parazitováni odlišnými, ale fylogeneticky příbuznými druhy/genotypy kryptosporidií, které pravděpodobně koevoluvali spolu se svými hostiteli.

- Druhy *C. microti* a *C. alticolis* popsané v této práci a běžně se vyskytující v populaci hraboše polního jsou fylogeneticky blízce příbuzné ke kryptosporidiím parazitujícím u hrabošů pensylvánských.
- Experimentálně bylo prokázáno, že *C. microti* i *C. alticolis* z hraboše polního jsou infekční pro hraboše pensylvánského.

- Průběh a lokalizace infekce *C. microti* a *C. alticolis* u hraboše pensylvánských je obdobná s hraboši polními.
- Genotypy kryptosporidií parazitujících u hlodavců rodu *Myodes* získané z jedinců na území České republiky, Slovenska a USA jsou si fylogeneticky blízce příbuzné.

***Cryptosporidium ditrichi* a *C. apodemi* nejsou infekční pro hraboše polního.**

- Experimentálně bylo prokázáno, že *C. ditrichi* a *C. apodemi*, které jsou běžnými parazity hlodavců rodu *Apodemus*, nejsou infekční pro hraboše polního.

Bylo potvrzeno, že použití pouze sekvencí genu kódujícího malou ribosomální podjednotku rRNA k vyvozování evolučních vztahů mezi jednotlivými druhy a genotypy rodu *Cryptosporidium* může vést k chybným závěrům.

- Na základě výsledků práce doporučujeme pro fylogenetické analýzy používat jiné polymorfní lokusy, např. aktin, HSP70, TRAP‑C1 nebo COWP.

Výsledky získané při terénním sledování a v rámci experimentálních infekcí ukazují, že infekce druhy a genotypy kryptosporidií parazitujících u hrabošovitých nejsou spojeny s klinickými příznaky a patologickými změnami v infikovaných částech zažívacího traktu hostitele.

- Nebyla prokázána souvislost mezi průjmovými stavy a kryptosporidiovými infekcemi u odchycených volně žijících hrabošovitých.
- Hraboši experimentálně infikovaní *C. alticolis* nebo *C. microti* nevykazovali žádné klinické příznaky onemocnění v průběhu infekce.
- Nebyly pozorovány žádné makroskopické ani histopatologické změny v zažívacím traktu hostitelů infikovaných *C. alticolis* nebo *C. microti*.

Na základě vyšetření 1219 individuálních vzorků lze konstatovat, že hrabošovití nepředstavují potenciální riziko šíření zoonotických druhů kryptosporidií.

- Žádný z detekovaných druhů a genotypů kryptosporidií popsaných v této práci nebyl dosud spojen s lidskými případy kryptosporidiových infekcí.
- Experimentálně bylo prokázáno, že hraboši polní jsou vnímaví k infekci zoonotickým druhem *C. parvum* (nepublikováno).

6. PUBLIKACE, Z NICHŽ VYCHÁZÍ TATO PRÁCE

6.1. Diversity of *Cryptosporidium* in common voles and description of *Cryptosporidium alticolis* sp. n. and *Cryptosporidium microti* sp. n. (Apicomplexa: Cryptosporidiidae).

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Diversity of *Cryptosporidium* in common voles and description of *Cryptosporidium alticolis* sp. n. and *Cryptosporidium microti* sp. n. (Apicomplexa: Cryptosporidiidae)

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Abstract

Fecal samples from wild-caught common voles ($n = 328$) from 16 locations in the Czech Republic were screened for *Cryptosporidium* by microscopy and PCR/sequencing at loci coding small-subunit rRNA, *Cryptosporidium* oocyst wall protein, actin and 70 kDa heat shock protein. *Cryptosporidium* infections were detected in 74 voles (22.6%). Rates of infection did not differ between males and females nor between juveniles and adults. Phylogenetic analysis revealed the presence of eight *Cryptosporidium* species/genotypes including two new species, *C. alticolis* and *C. microti*. These species from wild-caught common voles were able to infect common and meadow voles under experimental conditions, with a prepatent period of 3–5 days post-infection (DPI), but they were not infectious for various other rodents or chickens. Meadow voles lost infection earlier than common voles (11–14 *vs* 13–16 DPI) and had significantly lower infection intensity. *Cryptosporidium alticolis* infects the anterior small intestine and has larger oocysts (5.4 × 4.9 μm), whereas *C. microti* infects the large intestine and has smaller oocysts (4.3 × 4.1 μm). None of the rodents developed clinical signs of infection. Genetic and biological data support the establishment of *C. alticolis* and *C. microti* as separate species of the genus *Cryptosporidium*.

Introduction

Cryptosporidium is an apicomplexan protist parasite that primarily infects the gastrointestinal epithelium of a broad range of vertebrate species including humans (Lv *et al*., 2009). Infections can be asymptomatic or can result in diarrhoea ranging from mild to severe. Disease severity depends mainly on the age and immune status of the host (Checkley *et al*., 2015; Baneth *et al*., 2016). Field studies have shown that genus *Cryptosporidium* is genetically diverse, with much of that diversity found in wildlife. Rodents are ubiquitous mammals comprising about 40% of mammalian diversity and occupying a wide range of habitats. Studies to date have shown that rodent species are predominantly parasitized with host-specific *Cryptosporidium* species and genotypes (Feng *et al*., 2007; Foo *et al*., 2007; Ziegler *et al*., 2007*a*; Kváč *et al*., 2008, 2013; Feng, 2010; Ng-Hublin *et al*., 2013; Stenger *et al*., 2015*a*, 2015*b*, 2018), although zoonotic species such as *C. parvum* and *C. ubiquitum* (Hajdušek *et al*., 2004; Rašková *et al*., 2013; Li *et al*., 2014; Perec-Matysiak *et al*., 2015) and livestock-specific species such as *C. scrofarum*, *C. andersoni* and *C. baileyi* (Ziegler *et al*., 2007*a*; Lv *et al*., 2009; Ng-Hublin *et al*., 2013; Danišová *et al*., 2017) have been reported. Despite a large number of studies, the diversity and biology of *Cryptosporidium* in several rodent hosts, including voles, have not been thoroughly characterized (Kváč *et al*., 2014; Stenger *et al*., 2018).

Early studies, relying on oocyst morphology to distinguish species, reported *C. parvum*, *C. muris* and *Cryptosporidium* sp. in voles (Chalmers *et al*., 1997; Torres *et al*., 2000; Sinski *et al*., 1993, 1998; Bull *et al*., 1998; Bajer *et al*., 2002; Bednarska *et al*., 2007). In more recent studies of voles, using more discriminatory genotyping tools to distinguish species, the prevalence of *C. parvum* was much lower than previously reported and *C. muris* was not detected. Additionally, common voles were not susceptible to *C. muris*, *C. proliferans* or *C. andersoni* under experimental conditions (Modrý *et al*., 2012). In contrast, *Cryptosporidium* muskrat genotypes I and II and *Cryptosporidium* isolates closely related to muskrat genotypes I and II have been reported frequently (online Supplementary Table S1). In the most recent study, the largest to date, Stenger *et al*. (2018) reported greater diversity of *Cryptosporidium* spp. infecting North American and European voles than previously known. They identified at least 18 different *Cryptosporidium* spp. by sequencing of the partial sequence of the small ribosomal subunit rRNA and actin genes in European and North American voles, and most of these were identified for the first time. Phylogenetic analyses indicated the *Cryptosporidium* spp.

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Fig. 1. Sampling locations across the study area in the Czech Republic. Sample site numbers indicate the following: (1) Dačice, (2) Výškovice, (3) Náměšť nad Oslavou, (4) Sedlečko u Tábora, (5) Dolní Třebonín, (6) Pelejovice, (7) Radimovice, (8) Budweiss, (9) Bavorovice, (10) Masákova Lhota, (11) Všechov u Tábora, (12) Opatovice, (13) Lovečkovice, (14) Soběslav, (15) Dubovice and (16) Zmišovice.

infecting voles from the different continents remained closely related (Stenger *et al.*, 2018). Collectively, data from studies on voles show that they are host to at least 20 *Cryptosporidium* species and genotypes (see online Supplementary Table S1). Most of the genotypes lack biological data such as course of infection and host range.

We undertook the present study to extend knowledge of the occurrence and diversity of *Cryptosporidium* spp. infecting the common vole (*Microtus arvalis*). We selected two isolates from wild-caught common voles and, in accordance with ICZN nomenclature rules and criteria established by the scientific community studying *Cryptosporidium* (Xiao *et al.*, 2004; Jirků *et al.*, 2008; Fayer, 2010), we describe the morphometry of oocysts, determine phylogenetic relatedness at multiple genetic loci and report on the infectivity for several hosts (voles, laboratory and yellow-necked mice, laboratory rats and chickens) under natural and experimental conditions. Outcomes from the study support the conclusion that the *Cryptosporidium* isolates are genetically and biologically distinct from previously described species. We therefore propose them as new species named *Cryptosporidium alticolis* sp. n. and *Cryptosporidium microti* sp. n.

Material and methods

Area and specimens studied

From 2014 to 2017 (May to September each year), wild-caught common voles were trapped using snap traps baited with apple and peanut at 16 locations in the Czech Republic (Fig. 1). After trapping, we identified the species, measured body mass (±1 g) and determined the sex of each individual. We estimated the age of each individual using body mass, such that an individual weighing <15 g was considered a juvenile and all other animals were considered adults. Following collection, we dissected each individual and collected a fecal sample from the colon. Fecal samples were stored at 4 °C without fixation. All fecal samples were screened for the presence of *Cryptosporidium* oocysts using the aniline–carbol–methyl violet (ACMV) staining (Miláček and Vítovec, 1985) followed by microscopic examination at 1000× magnification (light microscope Olympus BX51, Tokyo, Japan). During microscopic examination, we counted oocysts and we quantified the infection intensity as number of oocysts per gram of feces (OPG) according to Kváč *et al.* (2007).

Molecular characterization

DNA was extracted from 200 mg of feces by bead disruption for 60 s at 5.5 m s^{-1} using 0.5 mm glass beads in a Fast Prep 24 Instrument (MP Biomedicals, Santa Ana, CA, USA) followed by isolation and purification using a commercially available kit in accordance with the manufacturer's instructions (PSP spin stool DNA Kit, Invitek, Stratec, Berlin, Germany). Purified DNA was stored at −20 °C prior to amplification by PCR.

A nested PCR approach was used to amplify a partial region of the small ribosomal subunit rRNA (SSU; ~830 bp; Xiao *et al.*, 1999; Jiang *et al.*, 2005), actin (~1066 bp; Sulaiman *et al.*, 2002), *Cryptosporidium* oocyst wall protein (COWP) (~550 bp; Spano *et al.*, 1997) and 70 kilodalton heat shock protein genes (HSP70; ~1950 bp; Sulaiman *et al.*, 2000).

The primary PCR mixtures contained 2 μL of template DNA, 2.5 U of *Taq* DNA Polymerase (Dream Taq Green DNA Polymerase, Thermofisher Scientific, Waltham, MA, USA), 0.5× PCR buffer (SSU) or 1× PCR buffer (actin, COWP and HSP70; Thermofisher Scientific), 6 mM $MgCl_2$ (SSU) or 3 mM $MgCl_2$ (actin, COWP and HSP70), 200 μM each deoxynucleoside triphosphate, 100 mM each primer and 2 μL non-acetylated bovine serum albumin (BSA; 10 mg ml^{-1}; New England Biolabs, Beverly, MA, USA) in 50 μL reaction volume. The secondary PCR mixtures were similar to those described above for the primary PCR, with the exception that 2 μL of the primary PCR product was used as the template, the $MgCl_2$ concentration was 3 mM and no BSA was used. DNA of *C. parvum* and molecular grade water were used as positive and negative controls, respectively. Secondary PCR products were detected by 2% agarose gel electrophoresis, visualized by ethidium bromide staining and extracted using GenElute™ Gel Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA). Purified secondary products were sequenced in both directions with an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the secondary PCR primers and the BigDye1 Terminator v3.1 cycle sequencing kit (Applied Biosystems) in 10 μL reactions.

Table 1. Number of wild-caught common voles positive for *Cryptosporidium* by PCR and microscopy, by sex and age

Sex	Age	*n*	PCR positive	Microscopically positive
Female	J	29	9	3
	A	113	24	3
Male	J	45	10	3
	A	141	31	10
Total		328	74	19

J, juvenile; A, adult.

Phylogenetic analysis

The nucleotide sequences of each gene obtained in this study were edited using the ChromasPro 2.4.1. (Technelysium, Pty, Ltd, South Brisbane, Australia) and aligned with each other and with reference sequences from GenBank using MAFFT version 7 online server using the Q-INS-I algorithm (http://mafft.cbrc.jp/alignment/software/). Alignment adjustments were made manually to remove artificial gaps using BioEdit 7.0.5.3 (Hall, 1999). Phylogenetic analyses were performed and the best DNA/protein phylogeny models were selected using the MEGA7 software (Guindon and Gascuel, 2003; Tamura *et al.*, 2013) and Geneious v7.1.7 (http://www.geneious.com). Phylogenetic trees were inferred by maximum likelihood (ML) method, with the substitution model that best fits the alignment selected using the Bayesian information criterion. ML analysis of SSU, actin, COWP and HSP70 alignments was done in the MEGA7 software and concatenated SSU–actin–

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Table 2. *Cryptosporidium* spp. in wild common voles (*Microtus arvalis*)

Isolate ID	Location (number of screened samples/ positive)	Microscopical positivity (OPG)	Genotyping at the gene loci (GenBank Acc. No. used in the phylogenetic trees)			
			SSU	Actin	COWP	HSP70
19608[a]	Dačice (97/25)	Yes (4000)	*C. microti*	*C. microti*		
19612[a]		No	*C. microti*	*C. microti*		
19615		No	*C. microti*	*C. microti*		
19618[a]		No	*C. microti*	*C. microti*	*C. microti*	
20055[a]		No	*C. microti*	*C. microti*	*C. microti*	
20057		Yes (4000)	*C. microti*	*C. microti*		
20059		Yes (18 000)	vole VII	vole VII	vole VII	
20063		No	vole V	vole V	vole V	
20065[a]		Yes (6000)	*C. alticolis* (KY 644657)	*C. alticolis*	*C. alticolis*	*C. alticolis*
23407		No	*C. microti*	*C. microti*		
23408		No	*C. microti*	*C. microti*		
23409[b]		No	vole V (MH145331)	vole V (MH145311)	vole V (MH145319)	vole V (MH145325)
23410		No	*C. microti*	*C. microti*		
23390		No	vole V	vole V		
22731		Yes (8000)	*C. alticolis*	*C. alticolis*	*C. alticolis*	
23392[b]		No	vole VII (MH145333)	vole VII (MH145313)	vole VII (MH145321)	vole VII (MH145327)
23393		No	*C. microti*	*C. microti*		
23250		No	*C. microti*	*C. microti*	*C. microti*	
23251		No	*C. microti*	*C. microti*		
23231		No	*C. microti*	*C. microti*		
23111[b]		Yes (2000)	*C. alticolis* (MH145330)	*C. alticolis* (MH145310)	*C. alticolis* (MH145318)	*C. alticolis* (MH145324)
23112		No	*C. alticolis*	*C. alticolis*	*C. alticolis*	
23746[a]		Yes (30 000)	*C. microti*	*C. microti*	*C. microti*	*C. microti*
23747		No	*C. alticolis*	*C. alticolis*		
23748[a]		No	*C. microti*	*C. microti*	*C. microti*	*C. microti*
20062[a,b]	Výškovice (3/1)	No	vole III (MH145329, KY644593)	vole III (MH145309)	vole III (MH145317)	
23750[a]	Náměšť nad Oslavou (40/8)	No	*C. microti*	*C. microti*		
23400[a,b]		No	vole VI (MH 145332)	vole VI (MH 145312)	vole VI (MH 145320)	vole VI (MH 145326)
23405[a]		No	vole VI	vole VI		
28082[b]		Yes (16 000)	vole IV (MH145335)	vole IV (MH145315)		
30906		No	vole IV	vole IV		
30908		No	vole IV	vole IV		
30909[b]		No	vole II (MH145334)	vole II (MH145314)	vole II (MH145322)	
30928		No	*C. microti*	*C. microti*		
22339	Sedlečko u Tábora (35/2)	No	*C. microti*			
22336		No	*C. microti*			
21146	Dolní Třebonín (32/8)	Yes (36 000)	*C. microti*	*C. microti*		
22352		No	*C. microti*	*C. microti*	*C. microti*	
23115[a]		No	*C. microti*	*C. microti*	*C. microti*	

(*Continued*)

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Table 2. (*Continued.*)

Isolate ID	Location (number of screened samples/ positive)	Microscopical positivity (OPG)	Genotyping at the gene loci (GenBank Acc. No. used in the phylogenetic trees)			
			SSU	Actin	COWP	HSP70
23236[a]		No	*C. microti* (KY644604)	*C. microti* (KY657294)	*C. microti*	
23743[a]		No	*C. microti*	*C. microti*	*C. microti*	
24128[a]		Yes (24 000)	vole VI	vole VI	vole VI	
24129[a]		No	vole VI (KY644632)	vole VI	vole VI	
25643[a]		No	*C. microti*	*C. microti*	*C. microti*	
24514	Pelejovice (37/2)	No	*C. microti*	*C. microti*		*C. microti*
24916[a]		No	vole V (KY644670)	vole V		
24919[a]	Radimovice (18/7)	No	*C. microti*	*C. microti*		
24922[a]		No	*C. microti*	*C. microti*	*C. microti*	*C. microti*
24923[b]		No	*C. microti* (MH 145328)	*C. microti* (MH145308)	*C. microti* (MH145316)	*C. microti* (MH145323)
24924		No	*C. microti*	*C. microti*		
24926[a]		No	*C. microti*	*C. microti*	*C. microti*	*C. microti*
25163[a]		No	*C. microti*	*C. microti*	*C. microti*	*C. microti*
25164[a]		No	*C. microti*	*C. microti*	*C. microti*	*C. microti*
28061	České Budějovice (2/1)	No	vole V			
28315	Masákova Lhota (18/7)	No	*C. alticolis*	*C. alticolis*		*C. alticolis*
28317		Yes (10 000)	*C. microti*	*C. microti*	*C. microti*	
28566		Yes (4000)	*C. microti*	*C. microti*		
28567		No	vole VI	vole VI		
28665		No	*C. microti*	*C. microti*		
28667		No	*C. microti*	*C. microti*		
29936		No	*C. microti*	*C. microti*	*C. microti*	
28422	Všechov u Tábora (30/12)	No	vole VII	vole VII		
28423		Yes (42 000)	*C. microti*	*C. microti*	*C. microti*	
28425		No	*C. alticolis*	*C. alticolis*		
28428		No	vole VII	vole VII		
28429		Yes (8000)	*C. microti*	*C. microti*		
28539		Yes (14 000)	*C. microti*	*C. microti*	*C. microti*	
28540		Yes (32 000)	*C. microti*	*C. microti*		
28541		Yes (18 000)	vole VII	vole VII		
28543		No	*C. microti*	*C. microti*	*C. microti*	
28545		Yes (6000)	*C. microti*	*C. microti*		
28546		No	*C. microti*	*C. microti*		
28549		Yes (8000)	*C. microti*	*C. microti*		
30904	Opatovice (4/1)	No	*C. microti*	*C. microti*	*C. microti*	

Isolates were characterized by microscopy, including infection intensity expressed as number of oocyst per gram of feces (OPG), and PCR analysis of the small ribosomal subunit rRNA (SSU), actin, *Cryptosporidium* oocyst wall protein (COWP) and 70 kDa heat shock protein (HSP70) genes. Only localities where *Cryptosporidium*-positive animals were trapped are shown.
[a]Sequences of SSU and actin previously obtained in the study of Stenger *et al.* (2018).
[b]Sequence of isolates used in phylogeny trees.

COWP alignment was done in RAxML v7.2.8 implemented in Geneious. The General Time Reversible model was selected for SSU, actin, HSP70 and concatenated SSU–actin–COWP alignment and the Tamura 3-parameter model was used of COWP alignment. All models were used under an assumption that rate variation among sites was γ distributed with invariant sites.

Bootstrap support for branching was based on 1000 replications. Phylograms were edited for style using CorelDrawX7. Sequences have been deposited in GenBank under the accession numbers (Acc. nos.) MH145308–MH145335.

Origin of specimens for transmission studies

Isolates of *C. alticolis* sp. n. and *C. microti* sp. n. were obtained from wild-caught common voles trapped at Dačice and Radimovice, respectively, in the Czech Republic. Oocysts from

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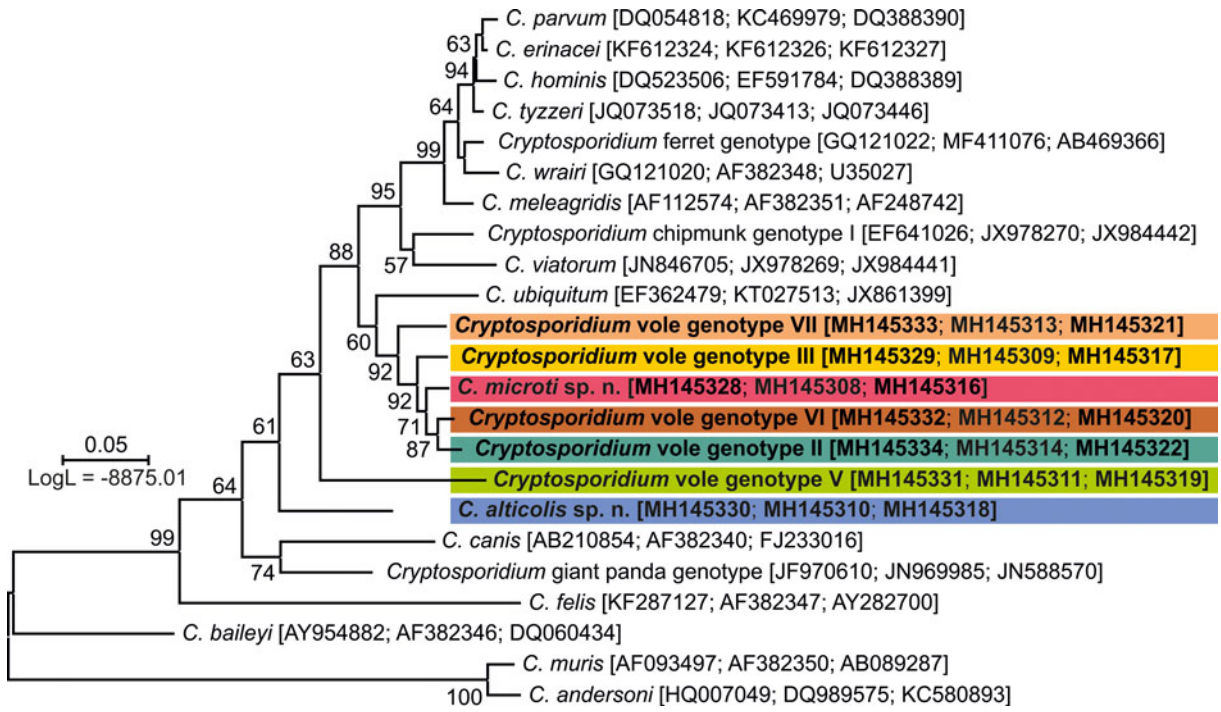

Fig. 2. A maximum likelihood (ML) tree based on concatenated small subunit rRNA (SSU), actin and *Cryptosporidium* oocyst wall protein (COWP) gene sequences. A representative of each SSU, actin and COWP species/genotype from wild-caught common voles from this study is highlighted in bold. GenBank accession numbers are shown in parenthesis after the isolate identifier. Numbers at the nodes represent the bootstrap values gaining more than 50% support. Branch length scale bar indicates the number of substitutions per site.

each species were used to infect a 6-month-old common vole (vole 0). Oocysts from vole 0 were purified using caesium chloride gradient centrifugation (Arrowood and Donaldson, 1996) and used for analysis of oocyst morphometry and to infect other animals (see below).

Transmission studies

We experimentally determined the infectivity and pathogenicity of *C. alticolis* sp. n. and *C. microti* sp. n. for 6-month-old common voles, meadow voles (*Microtus pennsylvanicus*) and yellow-necked mice (*Apodemus flavicollis*); 2-month-old SCID (severe combined immunodeficiency), BALB/c and C57BL/6J mice (*Mus musculus*) and brown rats (*Rattus norvegicus*); and 3-day-old chickens (*Gallus gallus* f. *domestica*). Common voles and yellow-necked mice used for infectivity studies were obtained from captive colonies maintained at the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic. Laboratory (i.e. house mouse) mice and rats were purchased from Charles River Laboratories, Sulzfeld, Germany. Chickens originated from International Testing of Poultry, Ústrašice, Tábor, Czech Republic. Meadow voles were obtained from a captive colony maintained at Smith College, Northampton, Massachusetts, USA and used in transmission studies at North Dakota State University, USA. All other experiments were performed at the Biology Centre of the Academy of Sciences of the Czech Republic. In determining infectivity and pathogenicity, we used five individuals from each species/group. A week prior to inoculation, fecal samples from all individuals were screened daily for the presence of *Cryptosporidium* oocysts and specific DNA of *Cryptosporidium* spp. using parasitological and molecular tools (SSU) as described above. Individuals were housed separately in plastic cages with sterilized bedding and supplied with a sterilized diet and water *ad libitum*. Each animal was inoculated orally by gavage with 100 000 purified oocysts suspended in 200 μL of distilled water. Fecal samples from each individual were screened daily for the presence of *Cryptosporidium* oocysts using ACMV staining and specific DNA using nested PCR targeting the SSU gene. At least three amplicons of each target gene were sequenced directly in both directions from each infected individual.

All experiments were terminated 30 days post-infection (DPI). Course of infection indicators, including fecal consistency, fecal colour and infection intensity, was examined.

Histopathological and scanning electron microscopy examinations

The gastrointestinal tract of one animal from each group was examined following necropsy at 6 DPI (this time was selected based on preliminary results; data not shown). The entire small and large intestine was divided into 1 cm sections and samples were processed for histology, scanning electron microscopy (SEM) and PCR/sequencing. Specimens for histology were fixed in 4% buffered formalin and processed by the usual paraffin method. Histological sections (5 μm) were stained with haematoxylin and eosin and periodic acid–Schiff stains. The specimens for SEM were fixed overnight at 4 °C in 2.5% glutaraldehyde in 0.1 M phosphate buffer, washed three times for 15 min in the same buffer, post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer for 2 h at room temperature and finally washed three times for 15 min in the same buffer. After dehydration in a graded acetone series, specimens were dried using the critical point technique, coated with gold and examined using a JEOL JSM-7401F-FE SEM.

Oocyst morphometry

Oocysts of *C. alticolis* sp. n. and *C. microti* sp. n. were examined using differential interference contrast (DIC) microscopy, ACMV staining and fluorescence microscopy (Olympus IX70, Tokyo, Japan) following labelling with genus-specific FITC-conjugated

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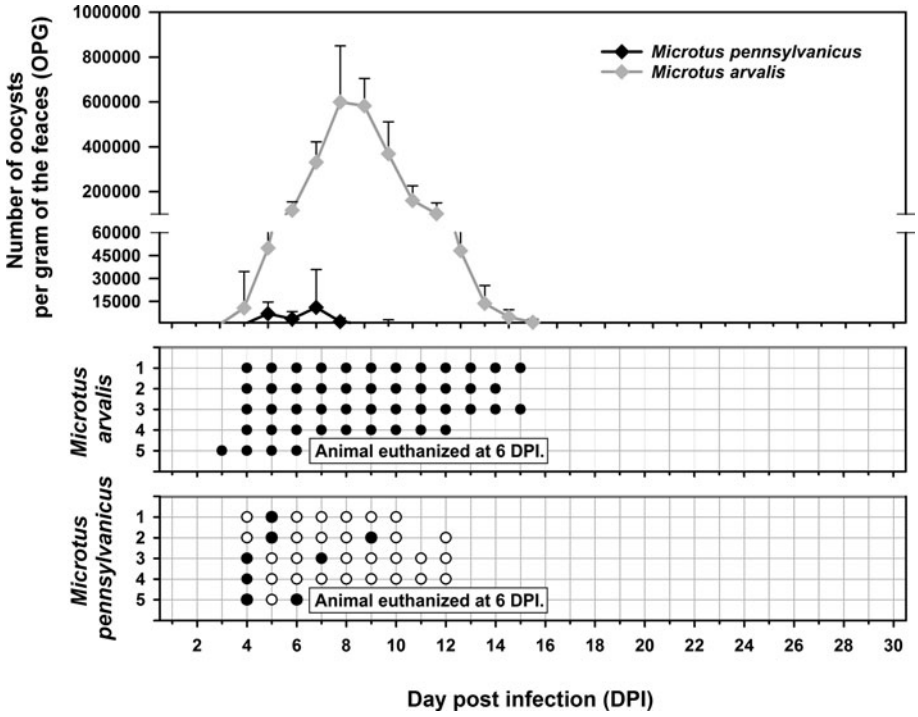

Fig. 3. Course of infection of *Cryptosporidium alticolis* sp. n. in experimentally infected common voles (*Microtus arvalis*) and in meadow voles (*Microtus pennsylvanicus*) based on coprological and molecular examination of feces. Any circles indicate detection of specific DNA, black circle indicates microscopic detection of oocysts.

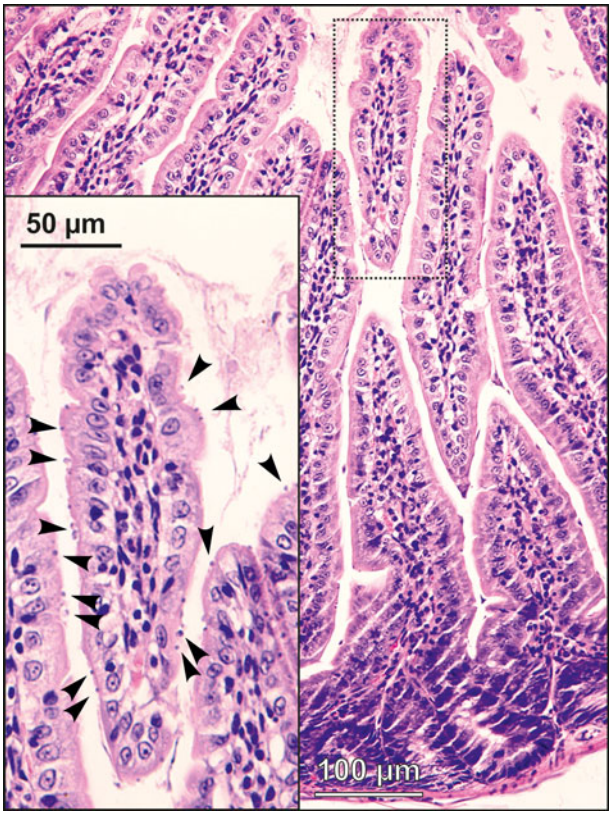

Fig. 4. Developmental stages (arrowheads) of *Cryptosporidium alticolis* sp. n. in mucosal glandular epithelium from the duodenum of an experimentally infected common vole (*Microtus arvalis*). Bar included in each picture.

antibodies (*Cryptosporidium* IF Test, Crypto Cell, Medac, Wedel, Germany). Morphometry of oocysts was determined 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 50 oocysts of each isolate were measured under DIC at 1000× magnification and the ratio of the length/width of each oocyst was calculated. The mean and standard deviation (S.D.) of length, width and ratio of the length/width of oocysts of each isolate were calculated.

Animal care

Animal caretakers wore disposable coveralls, shoe covers and gloves whenever entering the rooms where animals were housed. All wood-chip bedding, feces and disposable protective clothing were sealed in plastic bags, removed from the buildings and incinerated at the end of the study.

Statistical analysis

Prevalence was calculated by dividing the number of positive individuals by the total number of individuals sampled. Differences in *Cryptosporidium* prevalence were determined by χ^2 analysis using a 5% significance level. The hypothesis tested in the analysis of oocyst morphometry was that two-dimensional mean vectors of measurement are the same in the two populations being compared. Hotelling's T2 test was used to test the null hypothesis. Analyses were performed using program Epi Info (TM) 7.1.1.14 (Centers for Disease Control and Prevention, GA, USA) and R 3.5.0. (https://www.r-project.org/).

Results

Prevalence and infection intensity of Cryptosporidium

Out of 328 fecal samples from wild-caught common voles, 19 (5.8%) were microscopically positive for the presence of oocysts of *Cryptosporidium* sp. and 74 (22.6%) were positive for the presence of specific DNA of *Cryptosporidium* spp. (Table 1). All microscopically positive samples were also positive using PCR. Positive voles were trapped at 11 out of 16 localities (Table 2). There was no difference ($\chi^2 = 0.0153$; D.F. = 1; $P = 0.9016$) in the prevalence of *Cryptosporidium* spp. in males (22.0%; 41/186) and females (23.2%; 33/142). Similarly, the prevalence did not

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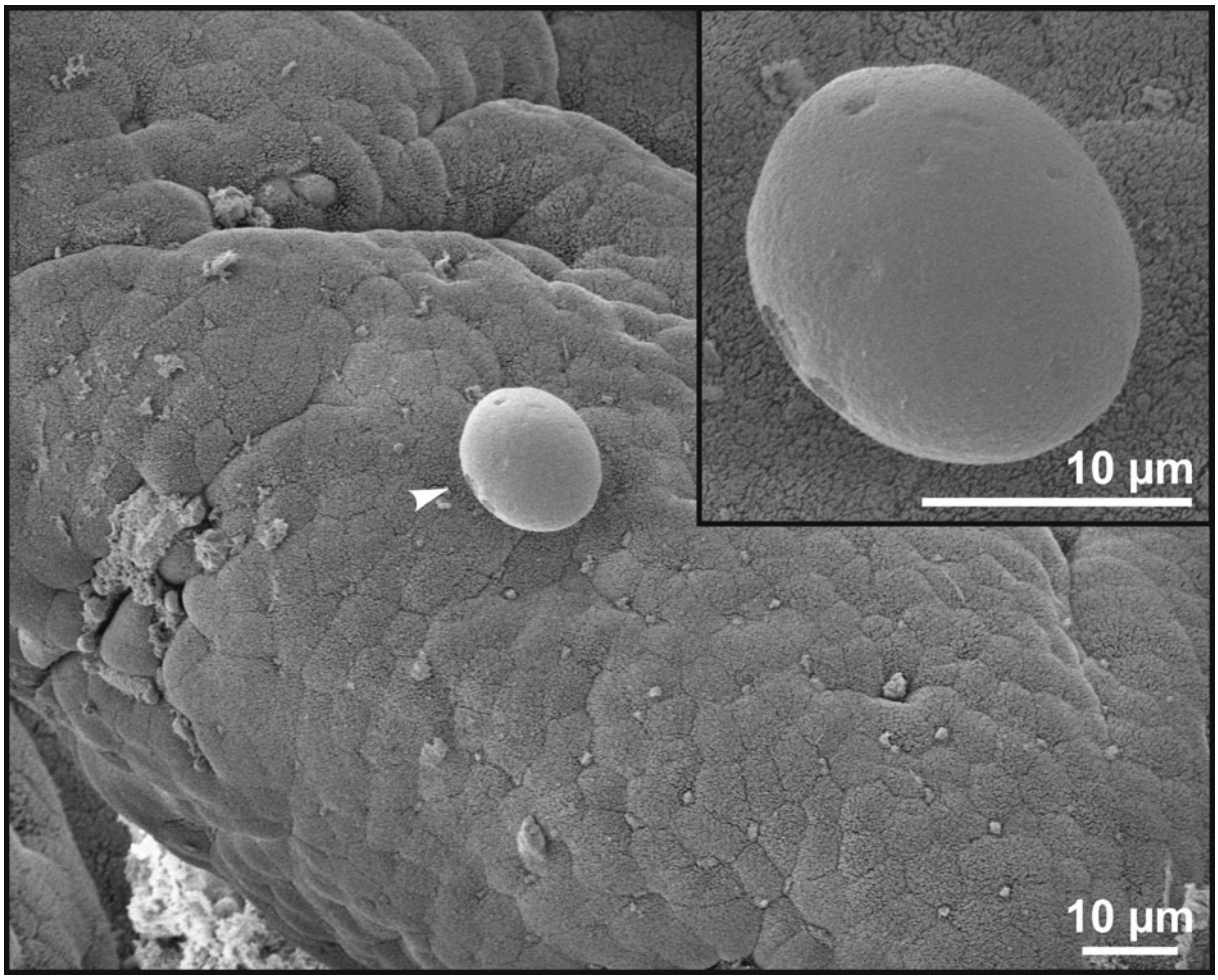

Fig. 5. Scanning electron photomicrograph of the jejunal epithelium of an experimentally infected common vole (*Microtus arvalis*). Attached developmental stage of *Cryptosporidium alticolis* sp. n. (arrowhead; detail in the upper right corner).

differ ($\chi^2 = 0.3254$; D.F. = 1; $P = 0.5684$) between juvenile (25.7%; 19/74) and adult voles (21.7%; 55/254; Table 2). Infection intensity, which ranged from 4000 to 42 000 OPG, did not differ ($P = 0.1773$) between males (2000–36 000 with mean 15 000 OPG) and females (4000–42 000 with mean 20 000 OPG). None of the trapped voles had diarrhoea.

Out of 74 voles positive for *Cryptosporidium*, 74, 71, 33 and 14 were genotyped by sequence analysis of SSU, actin, COWP and HSP70 genes, respectively (Table 2, Fig. 2 and online Supplementary Figs S1–S4). The remaining positive samples yielded sequences of insufficient quality to include in analyses (three actin sequences) or failed to amplify at COWP ($n = 41$) and HSP70 ($n = 60$) loci.

Sequence analysis revealed the presence of eight genotypes of *Cryptosporidium*, of which two are described here as new species (Table 2). ML trees inferred from sequences of SSU, actin, COWP and HSP70 genes individually or SSU, actin and COWP in concatenation formed three major phylogenetic groups (Fig. 2 and online Supplementary Figs S1–S4). Group 1 included *C. microti* sp. n. and *Cryptosporidium* vole genotypes II, III, VI and VII. *Cryptosporidium microti* ($n = 47$) was identical to *Cryptosporidium* sp. isolate 19608-Miar-EU previously recovered from a wild-caught common vole in the Czech Republic [Acc. No. KY657290] and was closely related to *Cryptosporidium* muskrat genotype II [Acc. No. AY737571], *Cryptosporidium* sp. isolate 1857-Mipe-NA from a wild-caught meadow vole [Acc. No. KY644574] and *Cryptosporidium* sp. isolate 1544-Pero-NA from a wild-caught *Peromyscus* mouse [Acc. No. KY644565] in the USA, sharing 99.2%, 98.8% and 98.6% sequence identity, respectively.

Cryptosporidium vole genotype III ($n = 1$) was identical to *Cryptosporidium* sp. isolate 20062-Miar-EU from a wild-caught common vole in the Czech Republic (Acc. No. KY644593) and clustered with *Cryptosporidium* sp. isolate 10482-Mygl-EU from a wild-caught bank vole (Acc. No. KY644595) and *Cryptosporidium* sp. isolate 2035-Myga-NA from a wild-caught Southern red-backed vole (Acc. No. KY644592) in Slovakia and the USA, respectively, sharing 99.8 and 99.5% sequence identity.

Cryptosporidium vole genotype VI ($n = 5$) was identical to *Cryptosporidium* sp. isolate 24129-Miar-EU from a wild-caught common vole in the Czech Republic (Acc. No. KY644632) and clustered with *Cryptosporidium* vole genotype II ($n = 1$) from the present study (Acc. No. MH145334), sharing 99.1% sequence identity. *Cryptosporidium* vole genotype VII ($n = 5$), a genotype that was first identified in this study, clustered with the *Cryptosporidium* vole genotype (Acc. No. EF641020) and *Cryptosporidium* sp. isolate 1947-Mipe-NA (Acc. No. KY644626), both from wild-caught meadow voles in the USA, sharing 98.9 and 98.5% sequence identity, respectively. *C. alticolis* sp. n. ($n = 7$), the only member of group 2, was identical to *Cryptosporidium* sp. isolate 20065-Miar-EU from a wild-caught common vole in the Czech Republic (Acc. No. KY644657), and clustered with *Cryptosporidium* sp. isolate 2333-Pero-NA from a wild-caught meadow vole in the USA (Acc. No. KY644655) and *Cryptosporidium* sp. isolate Mrb001 from a grey red-backed vole in Japan (Acc. No. AB477098), sharing 97.3 and 97.5% sequence identity, respectively.

Group 3 comprised *Cryptosporidium* vole genotype IV ($n = 3$) and vole genotype V ($n = 5$). *Cryptosporidium* genotype vole V was

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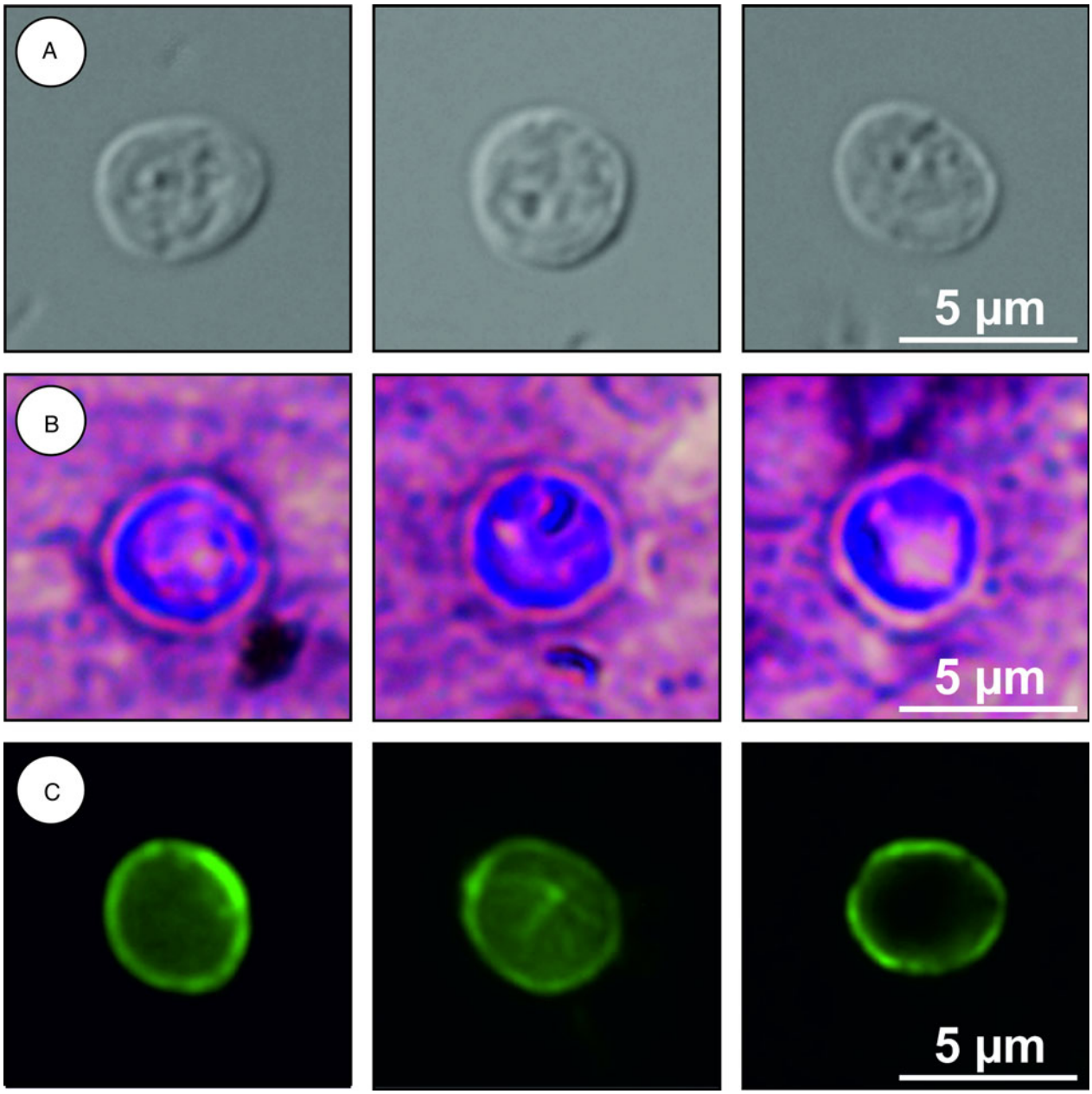

Fig. 6. *Cryptosporidium alticolis* sp. n. oocysts visualized in various preparations: (A) differential interference contrast microscopy and stained by (B) aniline–carbol–methyl violet and (C) anti-*Cryptosporidium* FITC-conjugated antibody. Bar included in each picture.

identical to *Cryptosporidium* sp. isolate 24916-Miar-EU from a wild-caught common vole in the Czech Republic (Acc. No. KY644670) and formed a sister group with muskrat genotype I (Acc. No. EF641013) and *Cryptosporidium* sp. isolate 1962-Mipe-NA from a wild-caught meadow vole (Acc. No. KY644685), both in the USA, sharing 98.1 and 98.0% sequence identity, respectively. *Cryptosporidium* vole genotype IV, which was reported for the first time in this study, clustered outside of this group.

Based on evidence that they are genetically and biologically distinct from known *Cryptosporidium* species, we describe *C. alticolis* sp. n. and *C. microti* sp. n. as new species of the genus *Cryptosporidium*. Descriptions of *C. alticolis* sp. n. and *C. microti* sp. n. follow.

Cryptosporidium alticolis *sp. n.*

Prevalence and infection intensity. Seven voles (2.1%) from three localities had DNA of *C. alticolis* sp. n. detectable by PCR, of which three had oocysts that were detectable by microscopy with an infection intensity of 2000–8000 OPG (Table 2).

Experimental transmission. Oocysts of *C. alticolis* sp. n. from naturally infected common voles were infectious for common and meadow voles, but not for yellow-necked mice, SCID mice, BALB/c mice, C57BL/6J mice, brown rats or chickens. The prepatent period of *C. alticolis* sp. n. in common and meadow voles was 3–4 DPI (Fig. 3). Whereas common voles shed oocysts of *C. alticolis* sp. n. continuously during the patent period (12–15 DPI), meadow voles shed oocysts sporadically up to 12 DPI (Fig. 3). The infection intensity of *C. alticolis* sp. n. in common voles (2000–1000 000 OPG) was higher than in meadow voles (2000–50 000 OPG). No macroscopical changes were observed in the gastrointestinal tract of common or meadow voles infected with *C. alticolis* sp. n. and the surface epithelium remained intact. DNA of *C. alticolis* sp. n. was detected throughout the small and large intestine of common and meadow voles; however, endogenous developmental stages were detected only in the jejunum and ileum by histology

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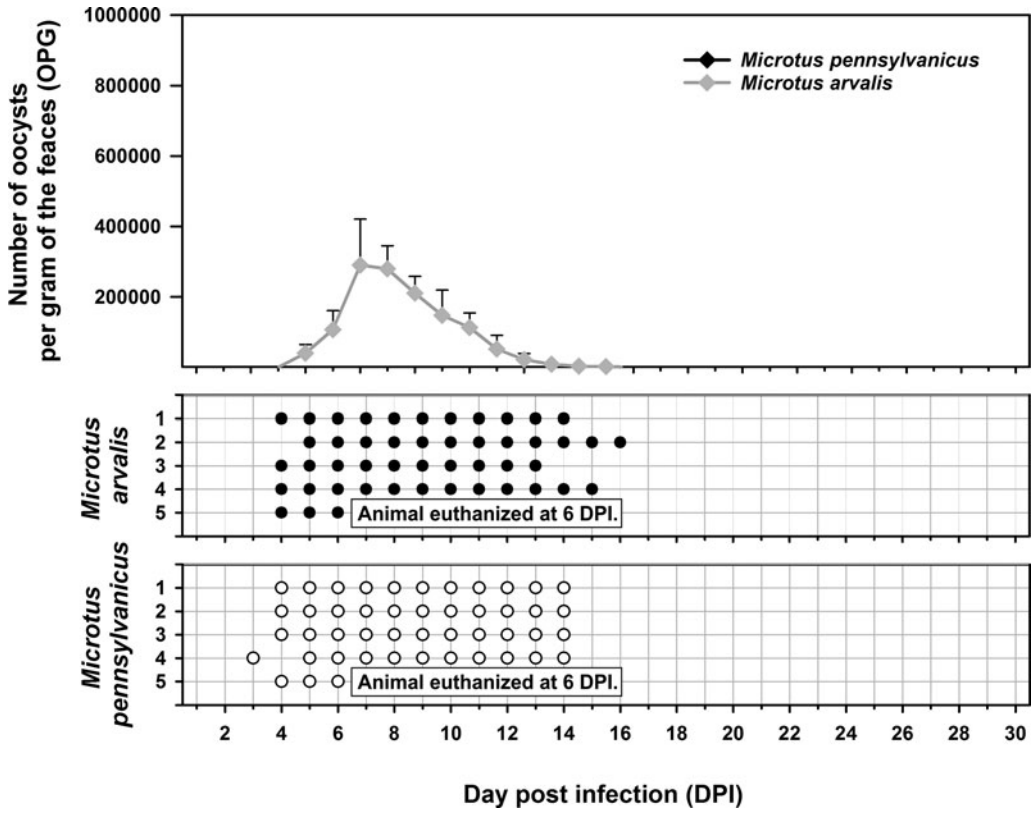

Fig. 7. Course of infection of *Cryptosporidium microti* sp. n. in experimentally infected common voles (*Microtus arvalis*) and in meadow voles (*Microtus pennsylvanicus*) based on coprological and molecular examination of feces. Any circles indicate detection of specific DNA, black circle indicates microscopic detection of oocysts.

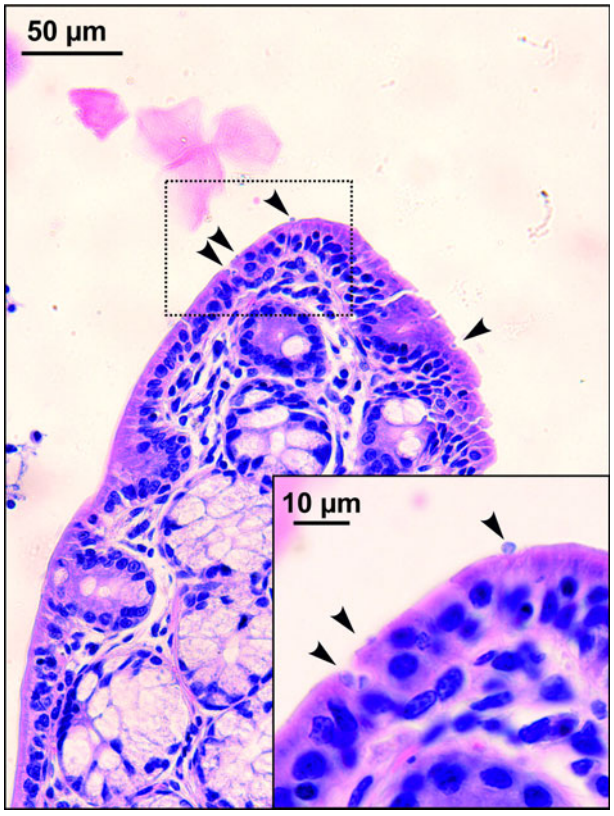

Fig. 8. Developmental stages (arrowheads) of *Cryptosporidium microti* sp. n. in mucosal glandular epithelium from the colon of an experimentally infected common vole (*Microtus arvalis*). Bar included in each picture.

and electron microscopy (Figs 4 and 5). *Cryptosporidium alticolis* sp. n. was not detected in the stomach and other organs (liver, pancreas, kidneys, lungs and spleen). None of the experimentally infected common or meadow voles were diarrhoeic. The *lamina propria* in the jejunum and ileum was slightly oedematous with occasional dilatation of lymphatic vessels (data not shown). Sequences of SSU, actin, COWP and HSP70 genes from experimentally infected hosts shared 100% identity with the isolate used in the inoculum.

Taxonomic summary

ZooBank number for species: urn:lsid:zoobank.org:act:D12C78AA-222E-4E07-A7CE-51AA6A747BC6
Description: Oocysts are shed fully sporulated with four sporozoites and an oocyst residuum. Sporulated oocysts ($n = 50$) measure 4.9–5.7 μm (mean ± S.D. = 5.4 ± 0.2 μm) × 4.6–5.2 μm (mean ± S.D. = 4.9 ± 0.2 μm) with a length/width ratio of 1.00–1.20 (mean ± S.D. = 1.10 ± 0.05) (Fig. 6). Morphology and morphometry of other developmental stages are unknown.
Type host: common vole (*M. arvalis*)
Type locality: Dačice (Czech Republic)
Other localities: Masákova Lhota and Všechov (Czech Republic)
Site of infection: jejunum and ileum (Figs 4 and 5)
Distribution: Czech Republic
Type material/hapanotype: Tissue samples in 10% formaldehyde and histological sections of infected jejunum (nos. 174/2016, 175/2016, 176/2016 and 177/2016) and ileum (nos. 178/2016 and 179/2016); genomic DNA isolated from fecal samples of naturally (isolation no. 23111) and experimentally (isolation no. 27124) infected *M. arvalis*; genomic DNA isolated from jejunal and ileal tissue of experimentally infected *M. arvalis* (isolation nos. 27035 and 27037, respectively); digital photomicrographs

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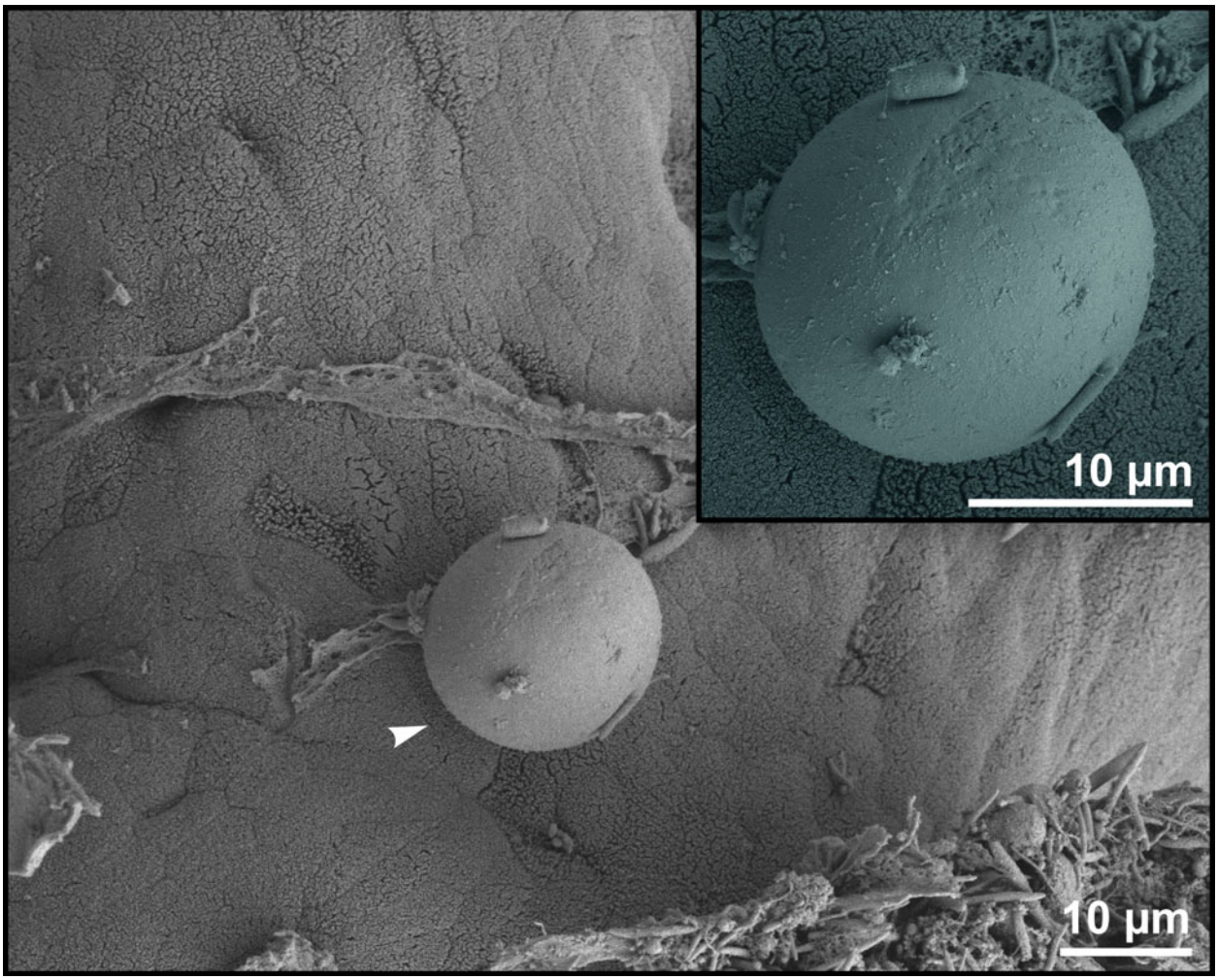

Fig. 9. Scanning electron photomicrograph of the colon epithelium of a common vole (*Microtus arvalis*). Attached developmental stage of *Cryptosporidium microti* sp. n. (arrowhead; detail in the upper right corner).

(nos. DIC 1-13/23111, MV 1-11/23111, IF 1-9/23111, HI 1-3/27124 and SEM 1-3/27124) and fecal smear slides with oocysts stained by ACMV staining from experimentally infected *M. arvalis* (nos. 27124/3, 27124/4, 27124/5 and 27124/6). Specimens deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

Reference sequences: Partial sequences of SSU, actin, COWP and HSP70 genes were deposited at GenBank under Acc. Nos. MH145330, MH145310, MH145318 and MH145324, respectively.

Etymology: The species name *alticolis* is derived from the Latin noun 'alticola' (meaning a vole).

Differential diagnosis: Oocysts of *C. alticolis* are larger than those of *C. microti* ($P = 0.001$), have similar ACMV staining to other species of *Cryptosporidium* and cross-react with antibodies developed primarily for *C. parvum* (Fig. 6). It can be differentiated genetically from other *Cryptosporidium* spp. based on sequences of SSU, actin, COWP and HSP70 genes. Endogenous development of *C. alticolis* sp. n. takes place in the small intestine, whereas *C. microti* develops in the large intestine.

Cryptosporidium microti *sp. n.*

Prevalence. Forty-seven wild-caught common voles (14.3%) from nine localities were positive for *C. microti* sp. n. by PCR, of which 12 had oocysts detectable by microscopy. The infection intensity ranged from 4000 to 42 000 OPG.

Experimental transmission. Oocysts of *C. microti* sp. n. from naturally infected common voles were infectious for common and meadow voles, but not for yellow-necked mice, SCID mice, BALB/c mice, C57BL/6J mice, brown rats or chickens. Common voles shed *C. microti* sp. n. from 4 to 16 DPI, with oocysts detectable by microscopy throughout this period. The infection intensity ranged from 2000 to 430 000 OPG with maximum shedding at 6–7 DPI (Fig. 7). In meadow voles, DNA of *C. microti* sp. n. was detected from 4 to 14 DPI; however, oocysts were not detectable by microscopy at any time during the patent period.

Sequences of SSU, actin, COWP and HSP70 genes from experimentally infected hosts shared 100% identity with the isolate used in the inoculum. Specific DNA of *C. microti* sp. n. was found exclusively in the caecum and colon of common and meadow voles. Endogenous developmental stages were detected in the caecum and colon of the common vole (Figs 8 and 9), but were not detected in the meadow vole. Infections were not associated with macroscopical or pathological changes in the digestive tract of common or meadow voles and these animals showed no signs of diarrhoea.

Taxonomic summary

ZooBank number for species: urn:lsid:zoobank.org:act:4FD6136C-3932-4881-BE49-4714A5AB488A

Description: Oocysts are shed fully sporulated with four sporozoites and an oocyst residuum. Sporulated oocysts ($n = 50$)

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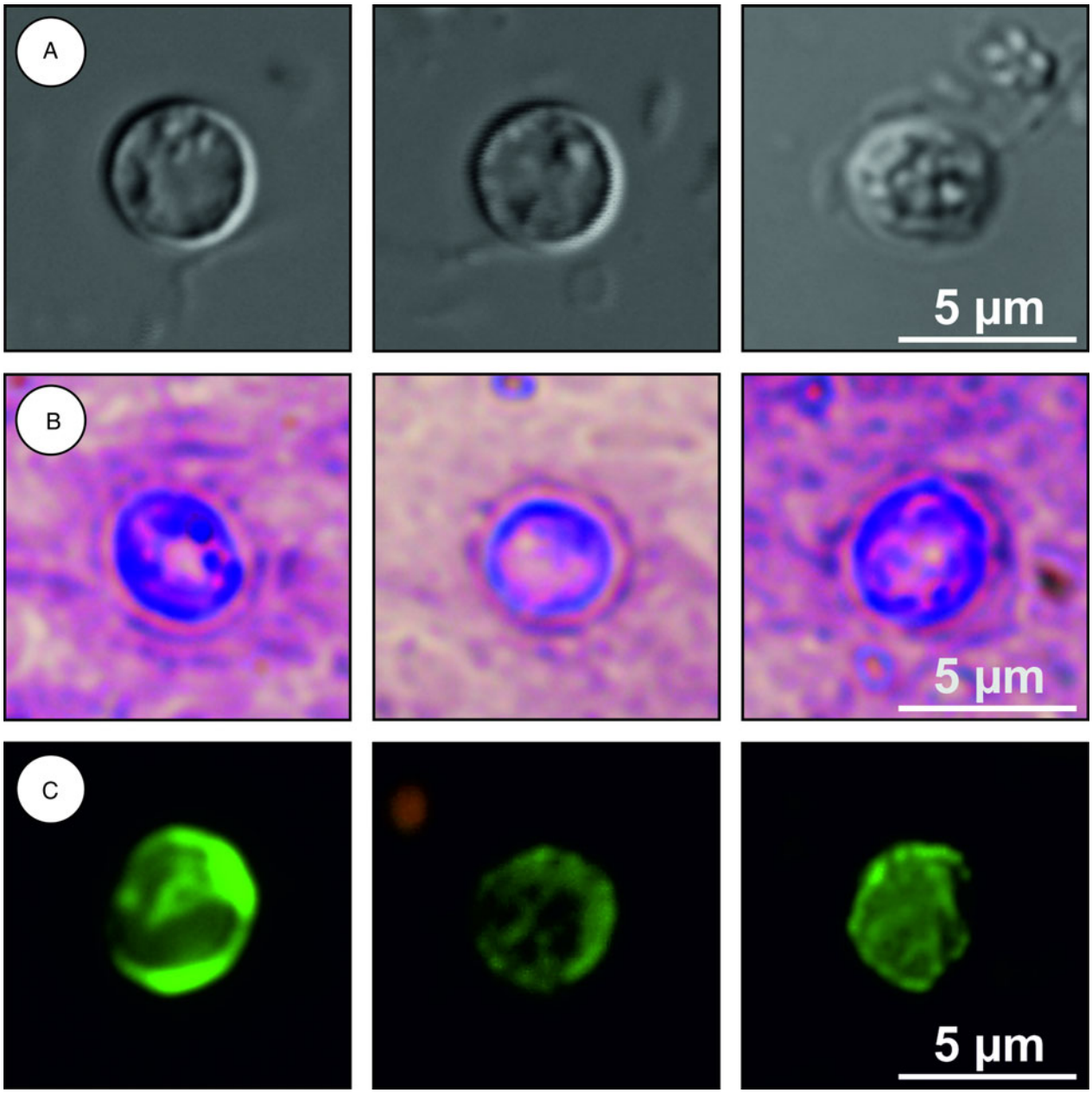

Fig. 10. *Cryptosporidium microti* sp. n. oocysts visualized in various preparations: (A) differential interference contrast microscopy and stained by (B) aniline–carbol–methyl violet and (C) anti-*Cryptosporidium* FITC-conjugated antibody. Bar included in each picture.

measure 3.9–4.7 μm (mean ± S.D. = 4.3 ± 0.1 μm) × 3.8–4.4 μm (mean ± S.D. = 4.1 ± 0.1 μm) with length/width ratio of 1.00–1.06 (mean ± S.D. = 1.03 ± 0.02) (Fig. 10). Morphology and morphometry of other developmental stages are unknown.

Type host: common vole (*M. arvalis*)

Type locality: Radimovice (Czech Republic)

Other localities: Dačice, Zňátky, Sedlečko, Dolní Třebonín, Pelejovice, Masákova Lhota, Všechov and Opatovice (Czech Republic)

Site of infection: caecum and colon (Figs 8 and 9)

Distribution: Czech Republic

Type material/hapanotype: Tissue samples in 10% formaldehyde and histological sections of infected caecum (nos. 97/2016 and 98/2016) and colon (nos. 99/2016 and 100/2016), genomic DNA isolated from fecal samples of naturally (isolation no. 24923) and experimentally (isolation no. 28063) infected *M. arvalis*; genomic DNA isolated from ceacal and colonical tissue of experimentally infected *M. arvalis* (isolation nos. 29751 and 29753, respectively); digital photomicrographs (nos. DIC 1-11/24923, MV 1-9/24923, IF 1-9/24923, HI 1-3/28063 and SEM 1-3/28063) and fecal smear slides with oocysts stained by ACMV staining from experimentally infected *M. arvalis* (nos. 28063/3, 28063/4, 28063/5 and 28063/6). Specimens deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

Reference sequences: Partial sequences of SSU, actin, COWP and HSP70 genes were deposited at GenBank under Acc. Nos. MH145328, MH145308, MH145316 and MH145323, respectively.

Etymology: The species name microti is derived from the Latin noun 'microtus' (meaning a vole).

Differential diagnosis: Oocysts of *C. microti* sp. n. are smaller than those of *C. alticolis* sp. n. ($P = 0.001$), have similar ACMV staining to other species of *Cryptosporidium* and cross-react with antibodies developed primarily for *C. parvum* (Fig. 10). It can be differentiated genetically from other *Cryptosporidium* spp. based on sequences of SSU, actin, COWP and HSP70 genes. Endogenous

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development of *C. microti* sp. n. takes place in the large intestine, whereas *C. alticolis* sp. n. develops in the small intestine.

Discussion

This and other genotyping studies have shown that voles host several *Cryptosporidium* species and genotypes that appear to be host specific and not infectious for humans, but they rarely host *C. parvum* (Feng *et al.*, 2007; Stenger *et al.*, 2018; Ziegler *et al.*, 2007*a*, 2007*b*). The finding that oocysts of *C. alticolis* sp. n. and *C. microti* sp. n. are indistinguishable from oocysts of *C. parvum* suggests that earlier detections of *C. parvum*, which were not supported by genotyping data, were misidentifications. Oocyst size is generally only useful for differentiating intestinal (smaller and rounder) and gastric (larger and more oval) species of *Cryptosporidium* (Ryan and Xiao, 2014).

Cryptosporidium microti sp. n. and *Cryptosporidium* vole genotypes II, III, VI and VII clustered as part of a large heterogeneous group in ML trees. This is generally consistent with the report by Stenger *et al.* (2018) that *Cryptosporidium* genotypes from voles in the Europe and North America formed between three and four phylogenetic groups in ML trees.

Cryptosporidium alticolis sp. n. and *C. microti* sp. n. are genetically distinct from other known species of *Cryptosporidium*. *Cryptosporidium alticolis* sp. n. shares 95.2, 94.7 and 94.3% sequence identity, respectively, with *C. canis*, *C. suis* and *C. parvum* at the SSU locus; 87.9, 90.5 and 89.7%, respectively, at the actin locus; and 84.5, 91.2 and 90.5%, respectively, at the HSP70 locus. At the COWP locus, *C. alticolis* sp. n. shared 88.1 and 89.9% sequence identity, respectively, with *C. canis* and *C. parvum*. *Cryptosporidium microti* sp. n. shared 95.5, 98.8 and 96.4% sequence identity, respectively, with *C. canis*, *C. suis* and *C. parvum* at the SSU locus; 85.6, 91.6 and 90.5%, respectively, at the actin locus; and 84.2, 93.1 and 92.6%, respectively, at the HSP70 locus. At the COWP locus, *C. microti* sp. n. shared 86.7 and 91.5% sequence identity, respectively, with *C. canis* and *C. parvum*. In comparison, *C. hominis* and *C. parvum* share 98–99% identity and *C. muris* and *C. andersoni* share 96–99% identity at these loci.

The prevalence of *Cryptosporidium* in voles ranges from 1 to 100% (Laakkonen *et al.*, 1994; Perz and Le Blancq, 2001; Bajer *et al.*, 2002, 2003; Zhou *et al.*, 2004). The prevalence in wild-caught common voles in the present study (23%) was greater than the 14% reported by Stenger *et al.* (2018) using similar detection methods, and much lower than the 62–73% reported by Bajer *et al.* (2002) and Bajer (2008) using microscopic detection, a method that is less sensitive than PCR. The prevalence of *Cryptosporidium* can be affected by factors such as age, season, population density, location, weather and climate, diet and water consumption (Nichols *et al.*, 2014).

Cryptosporidium microti sp. n. dominated at most locations in this study. Mixed infections were not detected, but they cannot be ruled out because the methods used were not effective at detecting multi-species infections. Microscopy cannot differentiate among species with similar sized oocysts and PCR preferentially amplifies DNA from the dominant species/genotype (Santín and Zarlenga, 2009; Jeníková *et al.*, 2011; Ma *et al.*, 2014; Qi *et al.*, 2015).

Cryptosporidium alticolis sp. n. infects the small intestine, which is similar to most intestinal *Cryptosporidium* spp. of mammals (Ryan and Xiao, 2014). In contrast, *C. microti* is only the third species, after *C. suis* in pigs and *C. oculltus* in rats, reported to infect the colon (Ryan *et al.*, 2004; Vítovec *et al.*, 2006; Kváč *et al.*, 2018). Similar to *C. oculltus* (Kváč et al., 2018), *C. microti* sp. n. localizes to the mucosal surface in the large intestine. In contrast, *C. suis* predominates in the glandular epithelium of the submucosal colonic lymphoglandular complexes in pigs (Vítovec *et al.*, 2006).

Neither *C. alticolis* sp. n. nor *C. microti* sp. n. developed clinical signs in common voles or meadow voles under experimental conditions in the present study. This is consistent with the reports that wild animals rarely display signs of clinical cryptosporidiosis (Sturdee *et al.*, 1999; Hikosaka and Nakai, 2005; Castro-Hermida *et al.*, 2011; Němejc *et al.*, 2012; Čondlová *et al.*, 2018).

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182018001142

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Conflicts of interest. None.

Ethical standards. The research was conducted under ethical protocols approved by the Institute of Parasitology, Biology Centre and Central Commission for Animal Welfare, Czech Republic (protocol nos. 071/2010 and 114/2013) and Institutional Animal Care and Use Committee North Dakota State University, ND, USA (protocol no. A18014).

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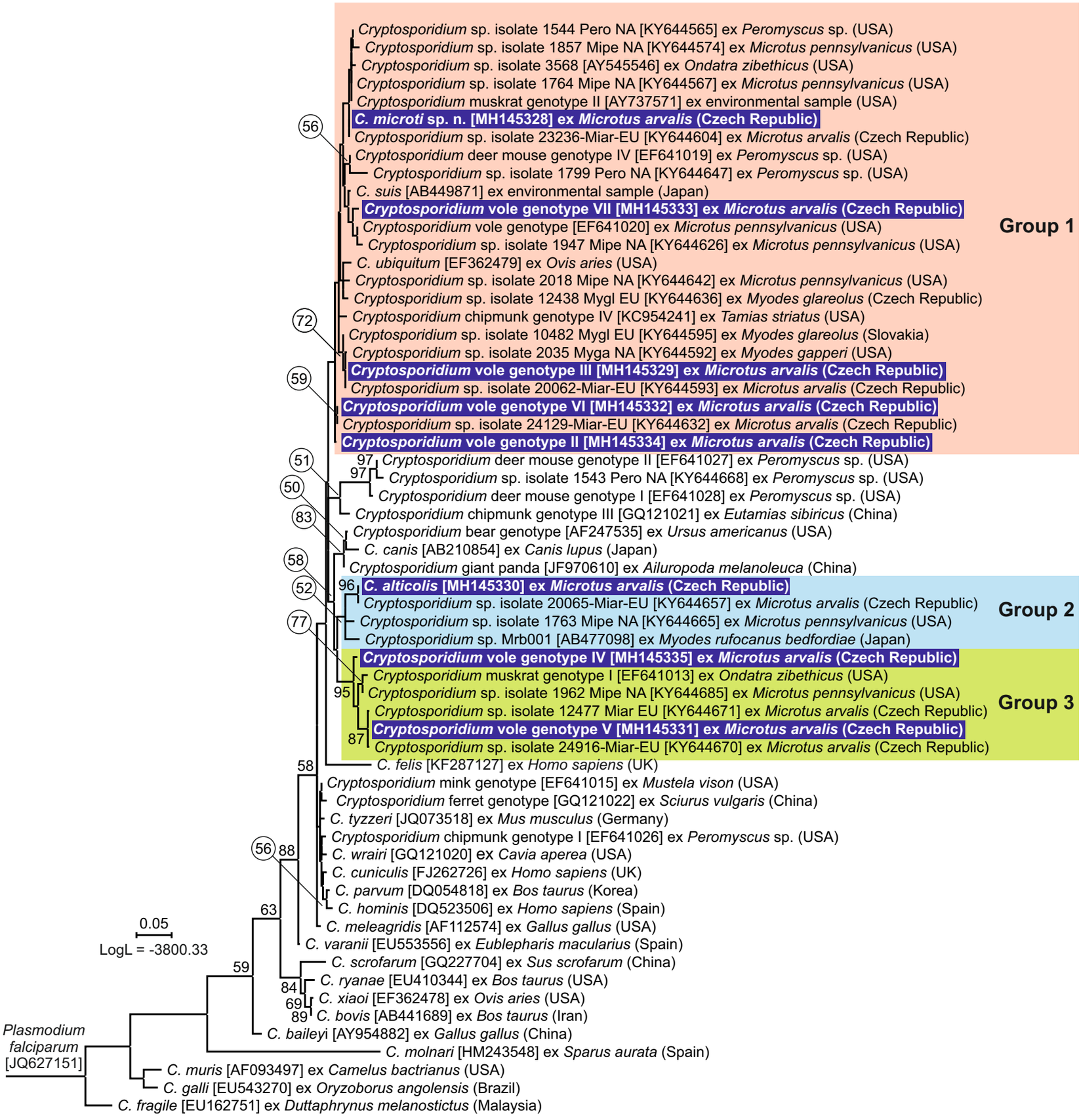
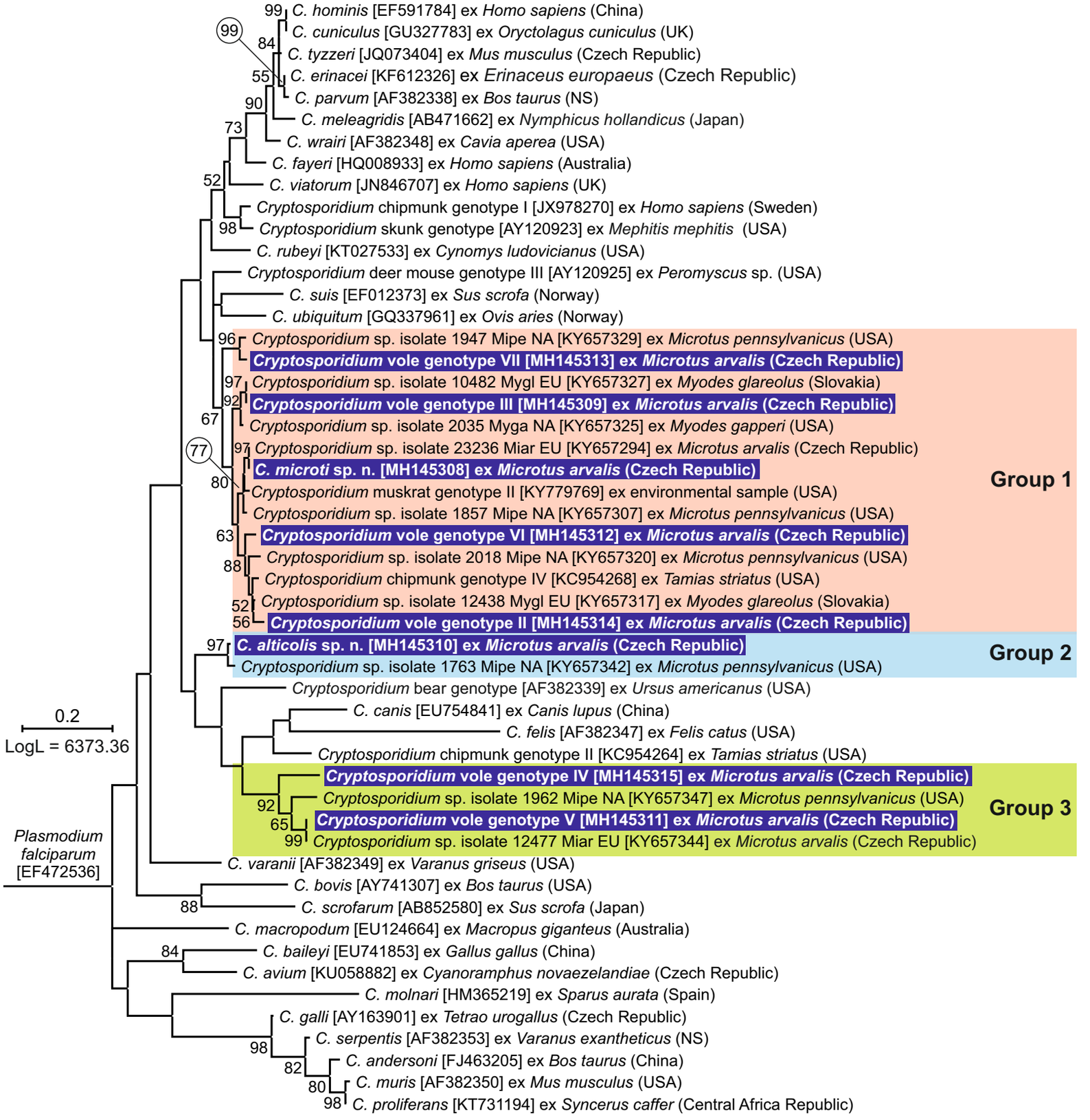
Supplementary Table S1. Occurrence of species of the genus *Cryptosporidium* infecting representatives of the subfamily Arvicolinae identified on the basis of microscopic[1] and molecular[2] tools amplifying partial sequences of small subunit ribosomal rRNA (SSU), *Cryptosporidium* oocyst wall protein (COWP), and 60 kDa glycoprotein (GP60) genes.

Host (common name)	**Country**	***Cryptosporidium* spp.**	**Loci for genotyping**	**No. of screened/ positive**	**References**
Myodes gapperi (southern red-backed vole)	USA	muskrat genotype I[2]	SSU	5/4	Feng et al. (2007)
		Cryptosporidium sp.[1]	–	301/19	Ziegler et al. (2007a)
		muskrat genotype II[2]		NS/1	
		Cryptosporidium sp.[2]	SSU	NS/6	Ziegler et al. (2007b)
		C. parvum[2]		NS/1	
		Cryptosporidium spp.[2]	SSU, actin	27/15	Stenger et al. (2018)
Myodes glareolus (bank vole)	Finland	*C. parvum*[1]	–	131/1	Laakkonen et al. (1994)
		C. tyzzeri[2]	COWP	12/5	Bajer et al. (2003)
	Poland	*C. parvum*[1]	–	459/324	Bajer et al. (2002)
				8/5	Bednarska et al. (2007)
				275/55	Sinski et al. (1998)
		Cryptosporidium sp.[1]		102/23	Sinski et al. (1993)
		Cryptosporidium spp.[1]		1523/819	Bajer (2008)
		Cryptosporidium spp.[2]		69/47	Perek-Matysiak et al. (2015)
	Slovakia	*C. parvum*[2]	SSU, gp60	75/3	Danišová et al. (2017)
		C. scrofarum[2]	SSU	75/4	
		environment isolate[2]	SSU	75/6	
		muskrat genotype I[2]	SSU	75/3	
	Spain	*C. parvum*[1]	–	49/10	Torres et al. (2000)
		C. muris[1]		49/2	
	UK	*C. muris*[1]	–	123/2	Chalmers et al. (1997)
		C. parvum[1]		123/11	
	USA	*Cryptosporidium* spp.[2]	SSU, actin	140/10	Stenger et al. (2018)
Myodes glareolus skomerensis (Skomer bank vole)	UK	*C. parvum*[1]	–	114/9	Bull et al. (1998)
		C. muris[1]		114/55	
Myodes rufocanus bedfordiae (red-backed vole)	Japan	*Cryptosporidium* sp. Mrb001[2]	SSU	NS	Unpublished (GenBank Acc. No. AB477098)
Microtus agrestis (field vole)	Finland	*Cryptosporidium* sp.[1]	–	131/1	Laakkonen et al (1994)
	Czech Republic	*Cryptosporidium* spp.[2]	SSU, actin	353/50	Stenger et al. (2018)
Microtus arvalis (common vole)	Poland	*C. tyzzeri*[2]	COWP, SSU	12/6	Bajer et al. (2003)
				274/200	Bajer et al. (2002)
		C. parvum[1]	–	7/5	Bednarska et al. (2007)
				19/4	Sinski et al. (1998)
		Cryptosporidium spp.[1]	–	419/261	Bajer (2008)
Microtus pennsylvanicus (meadow vole)	USA	vole genotype I[2]	SSU	10/1	Feng et al. (2007)
		muskrat genotype II[2]		10/2	
		Cryptosporidium sp.[1]	–	297/13	Ziegler et al. (2007a)
		muskrat genotype II[2]	SSU	NS/5	Ziegler et al. (2007b)
		Cryptosporidium sp.[2]		NS/4	
		Cryptosporidium spp.[2]	SSU, actin	311/163	Stenger et al. (2018)
Microtus pinetorum (woodland vole)	USA	*Cryptosporidium* spp.[2]	SSU, actin	41/21	Stenger et al. (2018)
Ondatra zibethicus (muskrat)	Poland	*C. parvum*[1]	–	9/5	Sinski et al. (1998)
	USA	*C. parvum*[2]	SSU	6/6	Perz and Le Blancq (2001)
		Cryptosporidium sp.[1]	–	149/1	Ziegler et al. (2007a)
		Cryptosporidium spp.[2]	SSU, actin	42/4	Stenger et al. (2018)
		muskrat genotype I[2]	SSU	237/24	Zhou et al. (2004)
		muskrat genotype II[2]		237/6	
		muskrat genotype I[2]	SSU	1/1	Feng et al. (2007)
		muskrat genotype I[2]	SSU	1/1	Xiao et al. (2002)

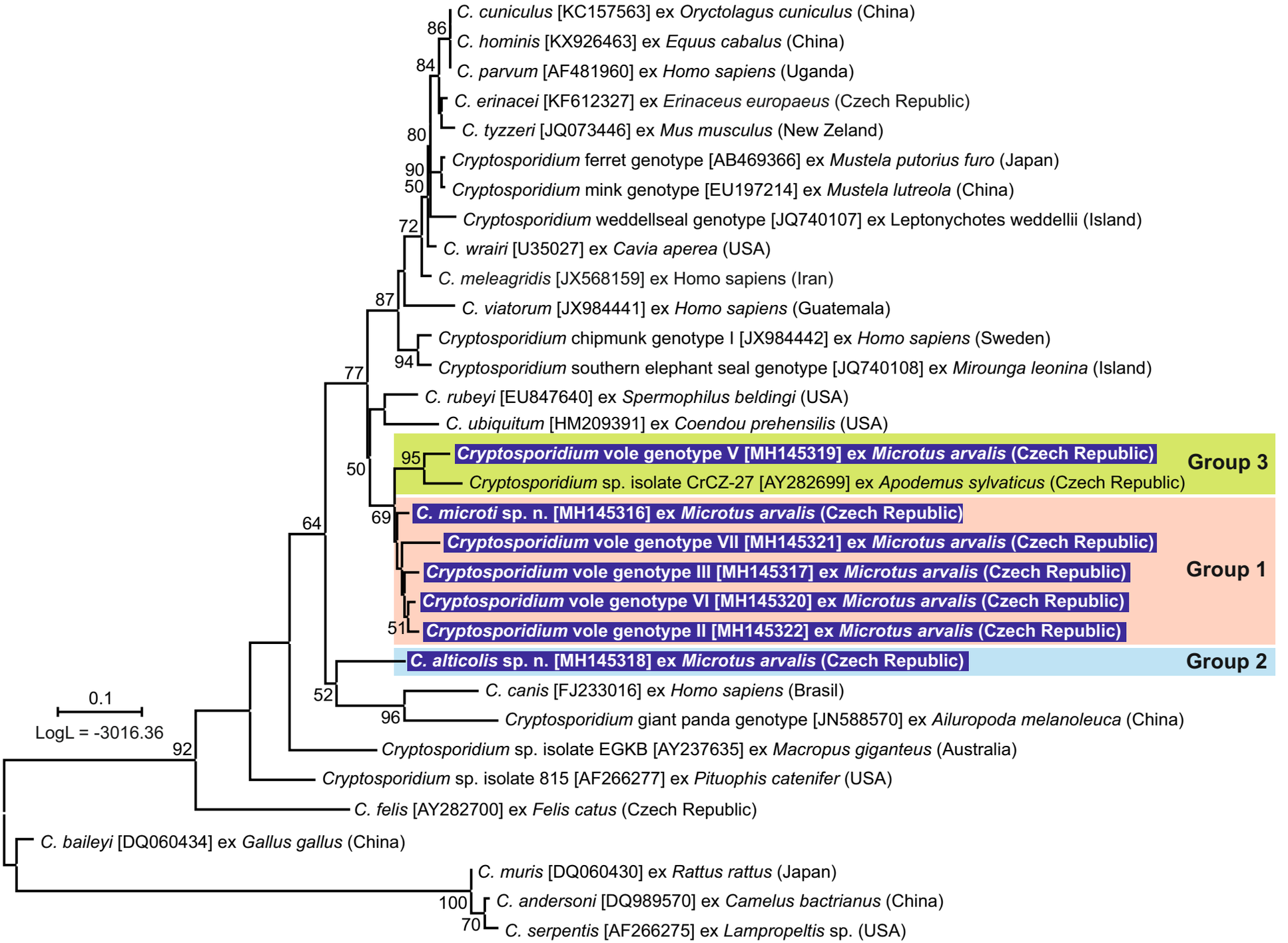
NS – not specified



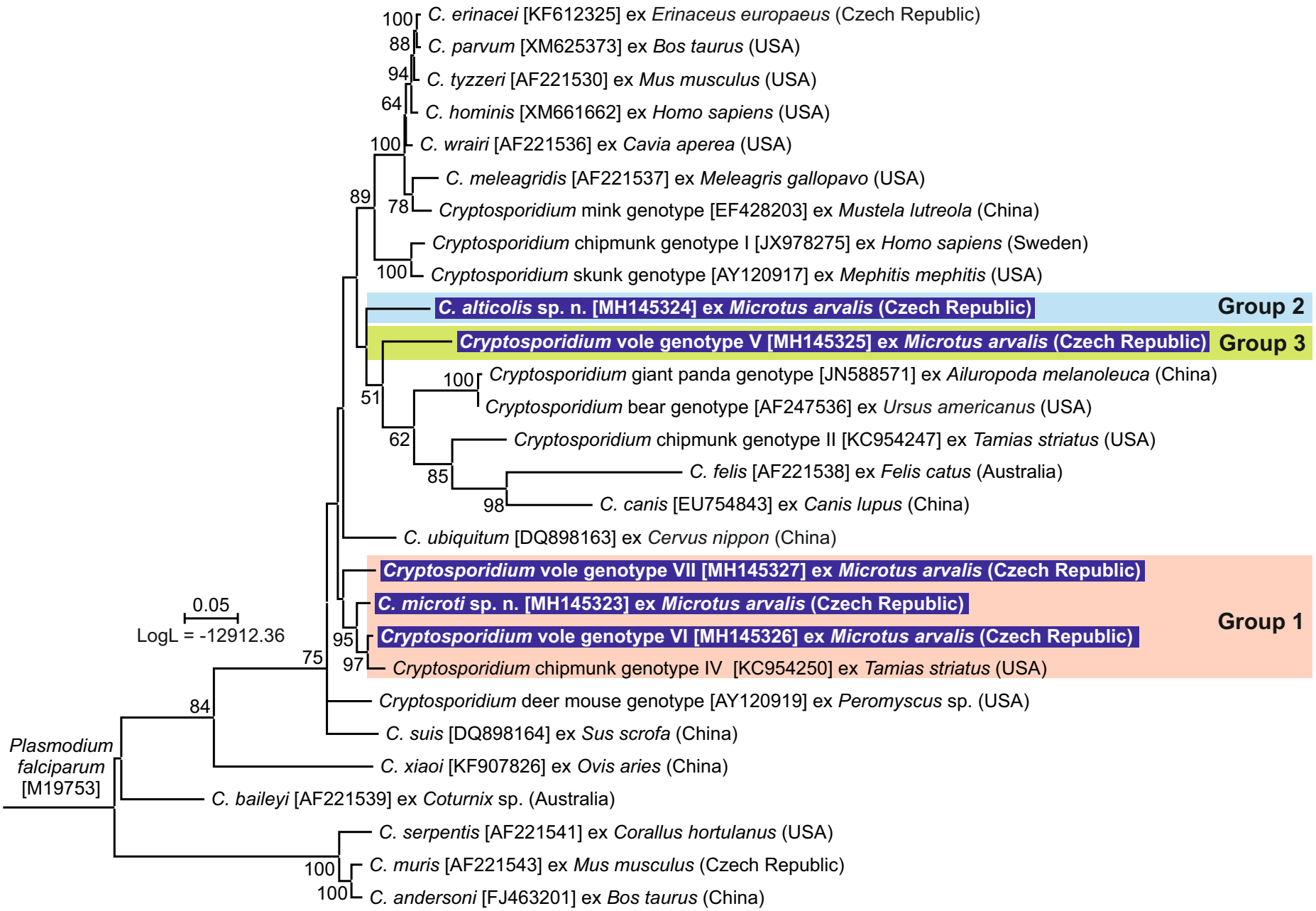
Supplementary Fig. S1. A maximum likelihood (ML) tree based on sequences of the gene encoding the small ribosomal subunit rRNA (SSU). A representative of each SSU species/genotype sequenced in this study is highlighted in bold and boxed. GenBank accession numbers, host species (Latin name) and country of isolate origin are shown in after the isolate identifier. The ML tree was rooted with a SSU sequence from *Plasmodium falciparum* [Acc. No.: EF472536]. Numbers at the nodes represent the bootstrap values gaining more than 50% support. Branch length scale bar indicates the number of substitutions per site.



Supplementary Fig. S2. A maximum likelihood (ML) tree based on actin gene sequences. A representative of each actin species/genotype from this study is highlighted in bold and boxed. GenBank accession numbers, host species (Latin name) and country of isolate origin are shown in after the isolate identifier. The ML tree was rooted with an actin sequence from *Plasmodium falciparum* [Acc. No.: EF472536]. Numbers at the nodes represent the bootstrap values gaining more than 50% support. Branch length scale bar indicates the number of substitutions per site.



Supplementary Fig. S3. A maximum likelihood (ML) tree based on *Cryptosporidium* oocyst wall protein (COWP) gene sequences. A representative of each COWP species/genotype from this study is highlighted in bold and boxed. GenBank accession numbers, host species (Latin name) and country of isolate origin are shown in after the isolate identifier. GenBank accession numbers are shown in parenthesis after the isolate identifier. The ML tree was rooted with COWP sequences of gastric *Cryptosporidium* spp. Numbers at the nodes represent the bootstrap values gaining more than 50% support. Branch length scale bar indicates the number of substitutions per site.



Supplementary Fig. S4. A maximum likelihood (ML) tree based on 70 kilodalton heat shock protein (HSP70) gene sequences. A representative of each HSP70 species/genotype from this study is highlighted in bold and boxed. GenBank accession numbers, host species (Latin name) and country of isolate origin are shown in after the isolate identifier. GenBank accession numbers are shown in parenthesis after the isolate identifier. The ML tree was rooted with a HSP70 sequence from *Plasmodium falciparum* [Acc. No.: M19753]. Numbers at the nodes represent the bootstrap values gaining more than 50% support. Branch length scale bar indicates the number of substitutions per site.

6.2. *Cryptosporidium* infecting wild cricetid rodents from the subfamilies Arvicolinae and Neotominae.

Stenger B.L.S*, **Horčičková M.***, Clark M.E., Kváč M., Ľondlová Ġ., Khan E., Widmer G., Xiao L., Giddings C.W., Pennil C., Stanko M., Sak B., McEvoy J.M. Parasitology, 2018, 145: 326ï 334.

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Cryptosporidium infecting wild cricetid rodents from the subfamilies Arvicolinae and Neotominae

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SUMMARY

We undertook a study on *Cryptosporidium* spp. in wild cricetid rodents. Fecal samples were collected from meadow voles (*Microtus pennsylvanicus*), southern red-backed voles (*Myodes gapperi*), woodland voles (*Microtus pinetorum*), muskrats (*Ondatra zibethicus*) and *Peromyscus* spp. mice in North America, and from bank voles (*Myodes glareolus*) and common voles (*Microtus arvalis*) in Europe. Isolates were characterized by sequence and phylogenetic analyses of the small subunit ribosomal RNA (SSU) and actin genes. Overall, 33·2% (362/1089) of cricetids tested positive for *Cryptosporidium*, with a greater prevalence in cricetids from North America (50·7%; 302/596) than Europe (12·1%; 60/493). Principal Coordinate analysis separated SSU sequences into three major groups (G1-G3), each represented by sequences from North American and European cricetids. A maximum likelihood tree of SSU sequences had low bootstrap support and showed G1 to be more heterogeneous than G2 or G3. Actin and concatenated actin-SSU trees, which were better resolved and had higher bootstrap support than the SSU phylogeny, showed that closely related cricetid hosts in Europe and North America are infected with closely related *Cryptosporidium* genotypes. Cricetids were not major reservoirs of human pathogenic *Cryptosporidium* spp.

Key words: *Cryptosporidium*, Cricetidae, phylogenetics, biogeography.

INTRODUCTION

Cryptosporidium is a genus of apicomplexan parasites with species that infect all major vertebrate groups (Fayer, 2010; Ryan, 2010; Kváč *et al*. 2014). Infections can result in the diarrhoeal disease cryptosporidiosis, which can be chronic and even fatal in the absence of a competent immune response (Checkley *et al*. 2015).

Early efforts to characterize *Cryptosporidium* – using descriptions of oocyst morphology, identification of surface antigens and isoenzyme analyses – lacked the resolution necessary to differentiate taxa infecting closely related hosts (Nichols *et al*. 1991; Nina *et al*. 1992; Ogunkolade *et al*. 1993; McLauchlin *et al*. 1998). Molecular tools have revealed tremendous genetic diversity in the genus *Cryptosporidium*, and more than 30 species and tens of genotypes have been described to date (Ryan *et al*. 2014; Holubová *et al*. 2016; Ježková *et al*. 2016; Kváč *et al*. 2016). One hypothesis holds that *Cryptosporidium* diversification is promoted by coevolutionary interactions with hosts, and this is supported by the findings that some closely related *Cryptosporidium* spp. infect a narrow range of closely related hosts. However, other species can infect a broad range of distantly related hosts, suggesting that coevolution is not the only driver of *Cryptosporidium* diversification.

Rodents are a useful model to study *Cryptosporidium* diversification. These ubiquitous mammals comprise about 40% of the mammalian diversity, with over 2200 species in 31 families and 481 genera, occupy a wide range of habitats, are extremely fecund and host diverse *Cryptosporidium* species and genotypes (Kváč *et al*. 2014). In addition to hosting species with a broad host specificity, including *Cryptosporidium muris*, *Cryptosporidium parvum*, and *Cryptosporidium ubiquitum*, rodents

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host more than 20 *Cryptosporidium* genotypes that appear to have a relatively narrow host range. For example, rats are commonly infected with *Cryptosporidium* rat genotypes I–IV, which have not been detected in other rodent species (Kimura *et al*. 2007; Paparini *et al*. 2012; Ng-Hublin *et al*. 2013; Zhao *et al*. 2015). Similarly, different species/genotypes of *Cryptosporidium* infect the squirrel tribes Marmotini and Sciurini (Stenger *et al*. 2015*b*). Narrowly specific *Cryptosporidium* species/genotypes may diverge as a consequence of host divergence, as was observed in the house mouse, where two subspecies (*Mus musculus musculus* and *M. m. domesticus*) that diverged 0·5 Mya (Bonhomme and Searle, 2012) hosted different subtypes of *C. tyzzeri* (Kváč *et al*. 2013).

The Cricetidae, at almost 600 species, is the second-largest family of mammals, comprising the subfamilies Cricetinae (hamsters), Sigmodontinae (including the cotton rat, climbing mice and water mice), Tylomyinae (including vesper rats and climbing rats), Neotominae (including deer mice and woodrats) and Arvicolinae (voles, muskrats and lemmings). The Cricetinae are exclusively Palearctic, being found in central and eastern Europe and parts of Asia. The Neotominae, Thylomyinae and Sigmodontinae are Nearctic/Neotropical, and are predominantly found in North, Central and South America, respectively. The Holarctic Arvicolinae underwent an explosive radiation, resulting in 151 extant species in 28 genera, as they dispersed from Asia to Europe and North America (NA) (Steppan *et al*. 2004; Wilson and Reeder, 2005).

Several *Cryptosporidium* genotypes appear to be specific to cricetids, and some may be specific for cricetid subfamilies. *Cryptosporidium* vole genotype and muskrat genotypes I and II have been reported only in arvicolines (voles and muskrats). Similarly, *Cryptosporidium* deer mouse genotypes I-IV appear mostly restricted to deer mice, in the subfamily Neotominae (Perz and Le Blancq, 2001; Xiao *et al*. 2002; Zhou *et al*. 2004; Feng *et al*. 2007; Ziegler *et al*. 2007; Lv *et al*. 2009; Robinson *et al*. 2011; Ruecker *et al*. 2012).

Here we report a study on *Cryptosporidium* infecting wild cricetid rodent populations in NA (at sites in North Dakota, Minnesota, South Dakota and Tennessee) and Europe (at sites in the Czech Republic and Slovakia). Data from the study contribute to the understanding of *Cryptosporidium* evolution in closely related hosts on different continents.

MATERIALS AND METHODS

Ethics statement

The research was conducted under ethical protocols approved by the Institute of Parasitology, Biology Centre and Central Commission for Animal Welfare, Czech Republic (protocol nos. 071/2010 and 114/2013) and North Dakota State University Institutional Animal Care and Use Committee (protocol A11060).

Sample collection – NA. Meadow voles (*Microtus pennsylvanicus*), southern red-backed voles (*Myodes gapperi*), muskrats (*Ondatra zibethicus*) and *Peromyscus* mice (deer mice, *Peromyscus maniculatus* and white-footed mice, *Peromyscus leucopus*, were not distinguished in this study) were sampled in North Dakota, South Dakota and Minnesota. Woodland voles (*Microtus pinetorum*) and *Peromyscus* mice were sampled in an area Tennessee. Except for muskrats, North American cricetids were live captured in Sherman box traps and fecal samples were collected from the trap or directly from the animal during handling. Captured animals were ear-tagged and released. Animals that died in traps were dissected and samples of intestinal contents were examined. Muskrats were sampled by collecting feces from muskrat mounds. All samples were stored at 4 °C prior to DNA extraction.

Sample collection – Europe (EU). Common voles (*Microtus arvalis*) and bank voles (*Myodes glareolus*) were captured in Sherman box traps in the Czech Republic and Slovakia. Trapped animals were euthanized and samples were collected from the intestines following dissection.

Polymerase chain reaction amplification and sequencing. For North American samples, DNA was isolated from samples by alkaline digestion, phenol-chloroform extraction and purified using a QIAmp DNA Stool Mini Kit (Qiagen, Valencia, CA) as previously described (Peng *et al*. 2003; Feltus *et al*. 2006). For European samples, 200 mg of feces was homogenized by bead disruption using FastPrep-24 (Biospec Products, Bartlesville, OK) for 60 s at a speed 5·5 m/s. Total DNA was extracted using the PSP Spin Stool DNA Kit (Invitek, Berlin, Germany).

DNA was stored at −20 °C until used in PCR assays. Fragments of the *Cryptosporidium* small subunit (SSU) and actin genes were amplified using nested PCR assays as described previously (Xiao *et al*. 2001; Sulaiman *et al*. 2002). Secondary products were visualized with SYBR Green or ethidium bromide following electrophoresis on an agarose gel.

PCR products were purified (Wizard SV, Promega, Madison, WI or GenElute™ Gel Extraction Kit, Sigma-Aldrich, St. Louis, MO) and sequenced in both directions with secondary primers using a BigDye Terminator v3·1 cycle sequencing kit in an ABI Prism 3130 genetic analyzer (Applied Biosystems, Carlsbad, CA). Sequences were assembled using SeqMan (DNAStar, Madison, WI).

Phylogenetic analysis. Sequences were aligned using the MAFFT version 7 online server with automatic selection of alignment strategy (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standley, 2013). Alignments were manually edited and phylogenetic analyses were performed using MEGA 6·0 (Tamura *et al*. 2013). The evolutionary history of aligned sequences was inferred using the maximum likelihood (ML) method (Saitou and Nei, 1987), with the substitution model that best fit the alignment selected using the Bayesian information criterion. The Hasegawa–Kishino–Yano model (Hasegawa *et al*. 1985) was selected for SSU alignments, and the general time reversible model (Tavaré, 1986) was selected for actin and concatenated actin-SSU alignments. Both models were used under an assumption that rate variation among sites was gamma distributed. A bootstrap consensus tree was inferred from 1000 pseudoreplicates. Phylogenetic analyses, including analysis of substitution model goodness of fit, were carried out using MEGA 6·0. Phylogenetic trees were edited for style using Adobe Illustrator CS5·1 (AdobeSystems, Inc., San Jose, CA).

Principal coordinate analysis. Sequences were aligned with ClustalW (Thompson *et al*. 1994) and manually trimmed to remove terminal nucleotides not present in all sequences. For each alignment (SSU, actin, and concatenated SSU-actin sequences), a matrix of pairwise distances between sequences was constructed using the program dist.seqs in mothur (Schloss *et al*. 2009). Distance matrices were imported into GenAlEx (Peakall and Smouse, 2012) and distances visualized by Principal Coordinate analysis (PCoA).

Statistical analysis. Prevalence was calculated by dividing the number of positive individuals by the total number of individuals sampled. Differences in *Cryptosporidium* prevalence were determined by Chi-square analysis using a 5% significance level. Analyses were performed using the statistical program R (R Core, 2013). The statistical significance of clusters visualized by PCoA was tested using ANOSIM in mothur (Clarke, 1993).

RESULTS

In total 1089 animals from the family Cricetidae were sampled at locations in NA (596 animals) and Europe (493 animals). A total of 681 samples were obtained from the 596 North American cricetids. The greater number of samples than animals was due to some animals from NA being sampled multiple times. All animals from Europe were sampled only once. Overall, 33·2% (362/1089) of cricetids tested positive for *Cryptosporidium*, with a greater prevalence in cricetids from NA (50·7%; 302/596) than Europe (12·1%; 60/493). Excluding repeat samples from the same animal, the prevalence in North American cricetids was 48·7% (290/596). In NA, the lowest prevalence was in muskrats (9·5%; 4/42) ($P < 0·05$). *Peromyscus* mice (56·6%; 99/175), southern red-backed voles (55·6%; 15/27), meadow voles (52·4%; 163/311) and woodland voles (51·2%; 21/41) had a similar prevalence. In Europe, the prevalence in common voles and bank voles was 14·2% (50/353) and 7·1% (10/140), respectively ($P < 0·05$).

Analysis of SSU sequences

Cryptosporidium SSU sequences were obtained from 126 animals and relationships among sequences were examined using PCoA and ML analysis (online Supplementary Fig. S1).

We used PCoA to visualize the matrix of pairwise genetic distances in a simplified, two-dimensional Euclidean space. Sixty-three percent of the SSU sequence variation was explained by two principal Coordinate, along which sequences separated into three groups that were statistically different from each other (G1–G3) (online Supplementary Fig. S1). These PCoA groups were overlaid on a ML tree constructed from *Cryptosporidium* SSU sequences (online Supplementary Fig. S1).

G1 included 97 sequences from all hosts and geographic locations examined in the study. Within G1, sequences from 28 meadow voles, 20 common voles, a muskrat and a *Peromyscus* mouse clustered with muskrat genotype II in the ML tree. G1 also included sequences clustering with *C. ubiquitum*, deer mouse genotypes I–IV, W29 genotype, fox genotype, vole genotype, chipmunk genotype IV and sequences that did not cluster with previously described species or genotypes.

Sequences from G2 formed a reasonably well-supported clade in the ML tree, within which sequences from meadow voles in NA and common voles in Europe formed separate clusters. This clade also included *Cryptosporidium* W12 genotype (AY007254), which was previously isolated from surface water in New York but has not been reported previously in an animal host. None of the sequences in the present study shared 100% identity with the W12 genotype.

Nested within a well-supported clade that included all sequences from G3, sequences from meadow voles and a muskrat in NA formed a sister group with sequences from common voles in Europe. The North American group included sequences previously identified as muskrat genotype I. A third group in this clade comprised sequences from bank voles in Europe, a sequence previously isolated from a yellow-necked mouse (*Apodemus flavicollis*) in Sweden (JN172968), and a sequence isolated from water in the UK (HM015876).

In some cases, divergent SSU gene sequences were obtained from different samples of the same animal. Sequences from three samples of the same *Peromyscus* mouse (1835-Pero-NA, 1851-Pero-NA, and 1852-Pero-NA) shared between 99·1 and 99·6% identity with each other and clustered with deer mouse genotype IV, which was previously isolated from a *Peromyscus* mouse in New York (EF641019). The samples were collected on 2 consecutive days: 1835-Pero-NA was obtained from the feces of the animal on the first day. The animal was released and was recaptured the next day, at which point the animal died in the trap, was dissected and 1851-Pero-NA and 1852-Pero-NA were obtained from the intestine. A fourth sequence (1848-Pero-NA) from the same animal, which was isolated from feces on the second day, clustered with the W29 genotype (JQ413356) as a sister group to deer mouse genotype IV, sharing between 98·1% and 98·5% sequence identity with 1835-Pero-NA, 1851-Pero-NA and 1852-Pero-NA.

Analysis of actin and concatenated actin-SSU gene sequences

Actin sequences were obtained from 70 samples and relationships among sequences were determined by PCoA and ML analysis. Sequences separated into five statistically different groups in the PCoA (G1-G5), and these groups were highlighted on the ML tree (Fig. 1 and online Supplementary Fig. S2).

Sequences in G1 formed three major clades in the ML tree (labelled A–C in Fig. 1 and online Supplementary Fig. S2). Clade A, which had 71% bootstrap support, comprised four closely-related subclades. One of the subclades comprised entirely of sequences from bank voles in Europe. Two subclades included sequences from North American meadow voles only, and one subclade contained sequences from five meadow voles and a *Peromyscus* mouse in this study and a sequence previously identified as muskrat genotype II. Clade B had 89% bootstrap support and included four subclades, two of which formed closely related sister groups. One of the sister groups included a sequence from a North American red-backed vole (2031-Myga-NA) and a sequence previously isolated from a North American eastern chipmunk. The other sister group comprised three identical sequences from bank voles in the Czech Republic. A third subclade comprised sequences from a meadow vole and woodland vole in NA. A fourth subclade included sequences from the common vole in Europe. Clade C, which had 94% bootstrap support, included identical sequences from a common vole and two bank voles in Europe, and a sequence from a red-backed vole in NA that clustered separately, sharing 99·0% identity with the sequences from European voles.

Sequences in G2 formed two clades. One of the clades included sequences from meadow voles that were identified as the vole genotype in the SSU phylogeny, a sequence from a woodland vole (2331-Mipi-NA) and a sequence from a red-backed vole (1937-Myga-NA). A second clade in G2 contained 1543-Pero-NA from a *Peromyscus* mouse and a sequence previously identified as deer mouse genotype II; this clade was more closely related to sequences from *Peromyscus* mice in G3 than sequences from voles in G2. The four sequences from *Peromyscus* mice in G3 included 1835-Pero-NA and 1848-Pero-NA, which were from a single animal and were identified as deer mouse genotype IV and W29 genotype, respectively, at the SSU locus (online Supplementary Fig. S1). G4 and G5 formed well-supported clades in the ML tree, and nested within each were sequences that clustered by host/geographic location.

PCoA and ML analysis of SSU and actin gene sequences in concatenation produced similar groupings to actin sequences. The exception was 1543-Pero-NA1, which was not part of a PCoA group in the analysis of concatenated sequences (Fig. 2 and online Supplementary Fig. S3).

DISCUSSION

Cryptosporidium diversity may result, in part, from a close association with diverging host species. This model of evolution is supported by evidence that *Cryptosporidium* has diverged with subspecies of the house mouse, *Mus musculus* (Kváč *et al*. 2013). Two subspecies, *Mus musculus musculus* and *M. m. domesticus*, which diverged after becoming geographically isolated about 0·5 Mya, host genetically and biologically distinct subtypes of *C. tyzzeri*, and the subtypes have remained host-specific despite the establishment of secondary contact between *M. m. musculus* and *M. m. domesticus*. The study by Kváč *et al*. (2013) demonstrated that knowledge of the timing of host divergence can be used to understand the dynamics of parasite divergence. Using a similar approach in the present study, we examined *Cryptosporidium* diversity in rodent species from the family Cricetidae.

Cryptosporidium from voles exhibited considerable SSU sequence heterogeneity, which is consistent with previous studies on *Cryptosporidium* from voles and muskrats. Most sequences clustered with previously named *Cryptosporidium* genotypes, including muskrat genotype I, muskrat genotype II, vole genotype and fox genotype. Sequences clustering with muskrat genotypes I and II were rarely detected in hosts other than voles, which is consistent with previous reports that these genotypes primarily infect voles, and are found less frequently in muskrats, *Peromyscus* mice and foxes (Zhou *et al*. 2004; Feng *et al*. 2007; Ziegler *et al*. 2007; Robinson

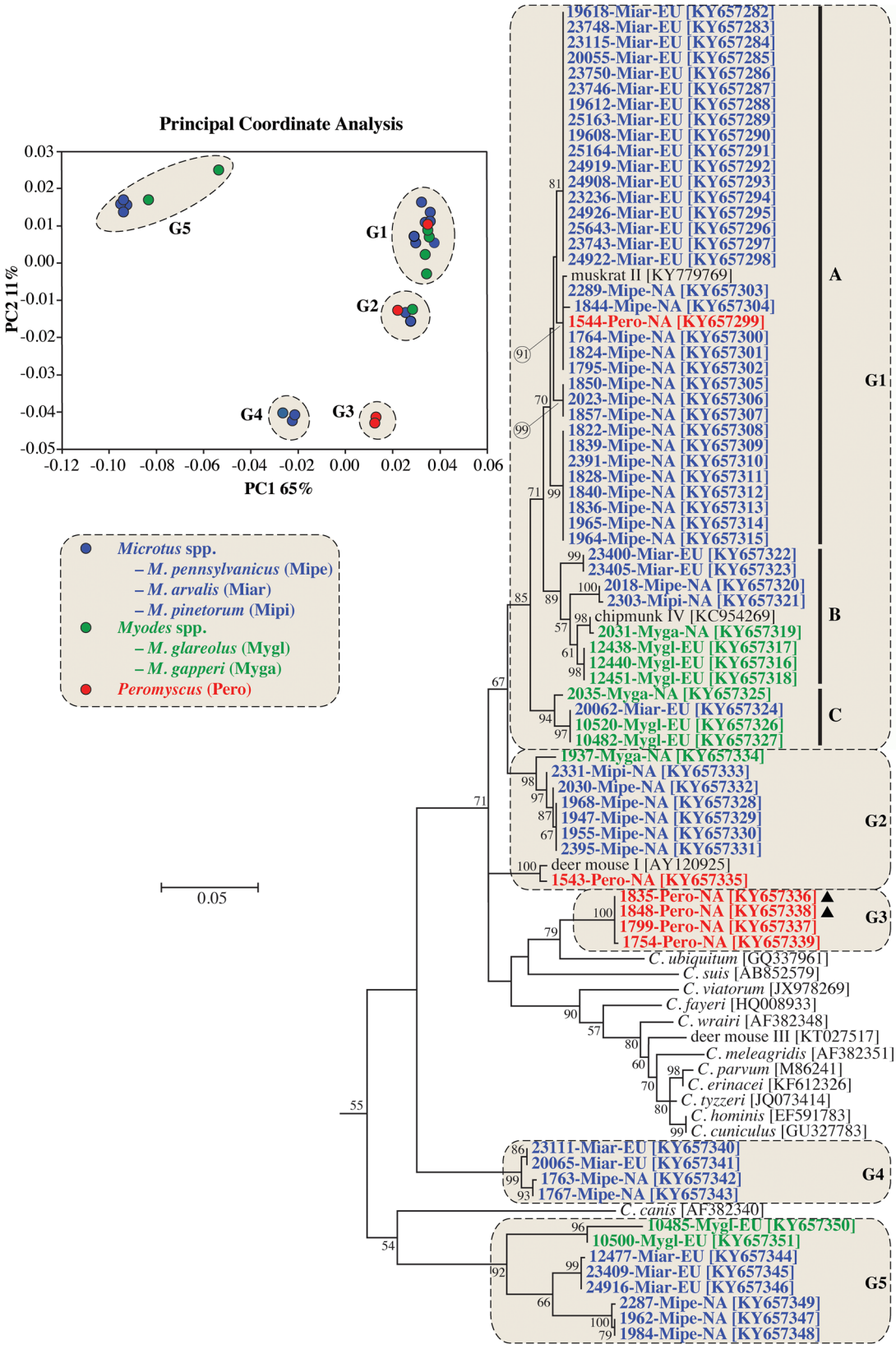

Fig. 1. Principle Coordinate Analysis (PCoA) and a maximum likelihood (ML) tree based on actin gene sequences. The five major PCoA groups (G1-G5) are highlighted against a cream background with dashed border on the ML tree. G1 is further broken down into three subgroups (A–C). Sequences from this study are identified by region (NA for NA and EU for Europe), and they are colour coded based on the genus of the host from which the sample was obtained (blue for *Microtus* spp., green for *Myodes* spp., and red for *Peromyscus* spp.). A solid black triangle (▲) identifies isolates from the same animal. The ML tree was rooted with an actin sequence from *Plasmodium falciparum* (accession number: EF472536). Due to limited space, the outgroup and some basal *Cryptosporidium* taxa are not shown. An expanded tree is shown in online Supplementary Fig. S2.

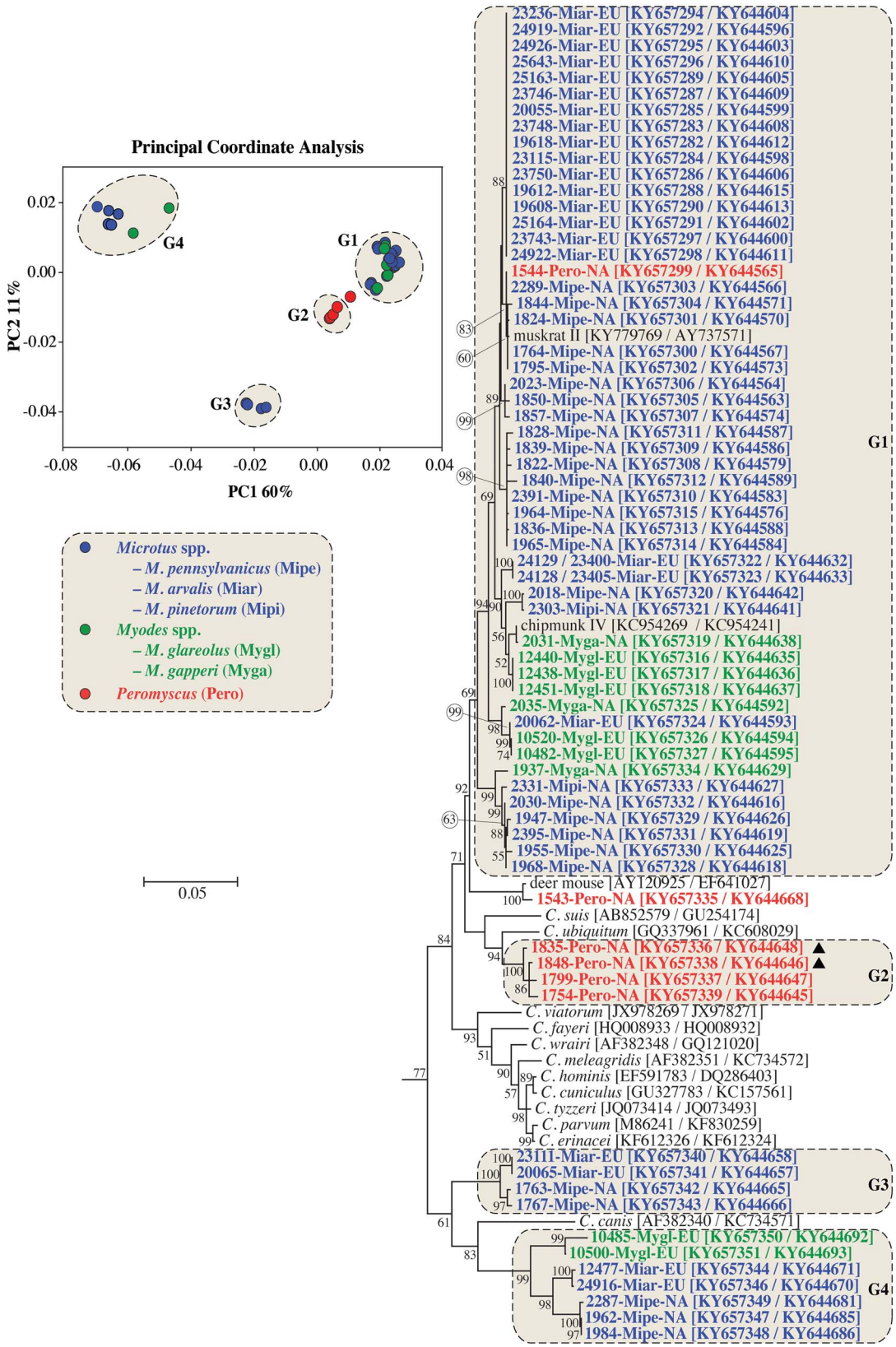

Fig. 2. Principle Coordinate Analysis (PCoA) and a maximum likelihood (ML) tree based on concatenated actin and small subunit rRNA (SSU) gene sequences. The four major PCoA groups (G1–G4) are highlighted against a cream background with dashed border on the ML tree. Sequences from this study are identified by region (NA for NA and EU for Europe), and they are colour coded based on the genus of the host from which the sample was obtained (blue for *Microtus* spp., green for *Myodes* spp. and red for *Peromyscus* spp.). A solid black triangle (▲) identifies isolates from the same animal. The ML tree was rooted with a concatenated actin/SSU sequence from *Plasmodium falciparum* (accession numbers: EF472536/JQ627149). Due to limited space, the outgroup and some basal *Cryptosporidium* taxa are not shown. An expanded tree is shown in online Supplementary Fig. S3.

et al. 2011; Ruecker *et al*. 2012). Therefore, despite the assigned genotype names, voles should be considered the major host for muskrat genotypes I and II. Similarly, we found that sequences clustering with the W12 and vole genotypes were exclusive to voles. The vole genotype has been identified previously in meadow voles (Feng *et al*. 2007; Ziegler *et al*. 2007), but this is the first report of a host for the W12 genotype, which was previously reported only in water (Feng *et al*. 2007; Ruecker *et al*. 2007).

The 102 variants detected among 134 SSU sequences examined suggests that cricetids host diverse *Cryptosporidium* taxa. The multiple-taxa hypothesis is predicated on the assumption that SSU sequences are orthologous, which is generally true; however, SSU sequences could also have a paralogous relationship. Some apicomplexans, including *Cryptosporidium*, can have divergent SSU paralogues that complicate the accurate reconstruction of evolutionary histories (Le Blancq *et al*. 1997; Xiao *et al*. 1999; Morgan *et al*. 2001; Kimura *et al*. 2007; Santín and Fayer, 2007; Lv *et al*. 2009; Sevá Ada *et al*. 2011; Ikarashi *et al*. 2013; Ng-Hublin *et al*. 2013; Stenger *et al*. 2015*a*). Ideally, paralogy should be tested in a single lineage, where it can be confirmed that the divergent SSU sequences are present in the same genome (Le Blancq *et al*. 1997). This is rarely possible in field studies on *Cryptosporidium* in complex fecal samples due to a lack of tools to propagate individual strains. Paralogy should be suspected when divergent SSU sequences co-occur in samples without the divergence of other polymorphic loci, such as actin and HSP70 (Stenger *et al*. 2015*a*). A limitation of this approach is the possibility that comparatively rare SSU and actin/HSP70 polymorphisms may not be detected by direct sequencing of PCR amplicons. In the present study, three isolates clustered with deer mouse genotype IV and three isolates clustered with the closely related W29 genotype at the SSU locus. All isolates clustering with deer mouse genotype IV and one of the W29 isolates were from a single animal and had identical sequences at the actin locus. Therefore, deer mouse genotype IV and W29 genotype could represent SSU paralogues rather than closely related taxa. Feng *et al*. (2007) similarly suggested that deer mouse genotypes I and II, which were detected in a single deer mouse, may be paralogues. Because paralogy is difficult to confirm in *Cryptosporidium*, when it is suspected, genes other than SSU should be used for phylogenetic reconstructions.

We found that, with few exceptions, the cricetid subfamilies Neotominae (*Peromyscus* mice) and Arvicolinae (voles and muskrats), which diverged about 19 Mya (Steppan *et al*. 2004), hosted phylogenetically distinct *Cryptosporidium* species and genotypes. Deer mouse genotypes I–IV, W29 genotype and *C. ubiquitum* were exclusively found in *Peromyscus* mice. *Cryptosporidium ubiquitum*, which was found in a single *Peromyscus* mouse, has a broad host specificity that includes many rodent and non-rodent mammals. We previously detected *C. ubiquitum* and deer mouse genotype III in squirrels from the same area as the *Peromyscus* mice sampled in the present study (Stenger *et al*. 2015*b*). Feng *et al*. (2007) also found *C. ubiquitum* and deer mouse genotype III in *Peromyscus* mice and squirrels in the eastern USA, suggesting frequent transmission between these different rodent families. This could be explained by the propensity of *Peromyscus* mice and squirrels to occupy the same habitat (Brunner *et al*. 2013). In contrast, voles and *Peromyscus* mice are known to spatially segregate within grassland habitats, limiting interspecific interactions (Bowker and Pearson, 1975).

Cryptosporidium genotypes infecting *Microtus* spp. and *Myodes* spp. generally clustered separately in actin and actin-SSU phylogenies, regardless of geographic location, suggesting that *Cryptosporidium* has coevolved with these cricetid genera. This is consistent with the *Myodes-Microtus* divergence time estimate of 5·76–9 Mya (Robinson *et al*. 1997; Conroy and Cook, 1999), before they colonized NA. Myodes likely colonized NA from Eurasia in the late Pliocene (3·6–2·58 Mya) to early Pleistocene (2·58–0·78 Mya) (Cook *et al*. 2004) and *Microtus* followed sometime later (Martin, 2003).

Although this study found that cricetids are frequently infected with *Cryptosporidium*, the species/genotypes pose little threat to human health. Only *C. ubiquitum*, which we detected in a single *Peromyscus* mouse, has been associated with human disease (Chalmers *et al*. 2011; Cieloszyk *et al*. 2012; Li *et al*. 2014).

In summary, North American and European cricetids host diverse *Cryptosporidium* spp., which in many cases appear to have coevolved with their hosts. Using only sequences of SSU to infer evolutionary relationships of *Cryptosporidium* may lead to erroneous conclusions, so it is recommended to use other polymorphic loci in phylogenetic analyses.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0031182017001524

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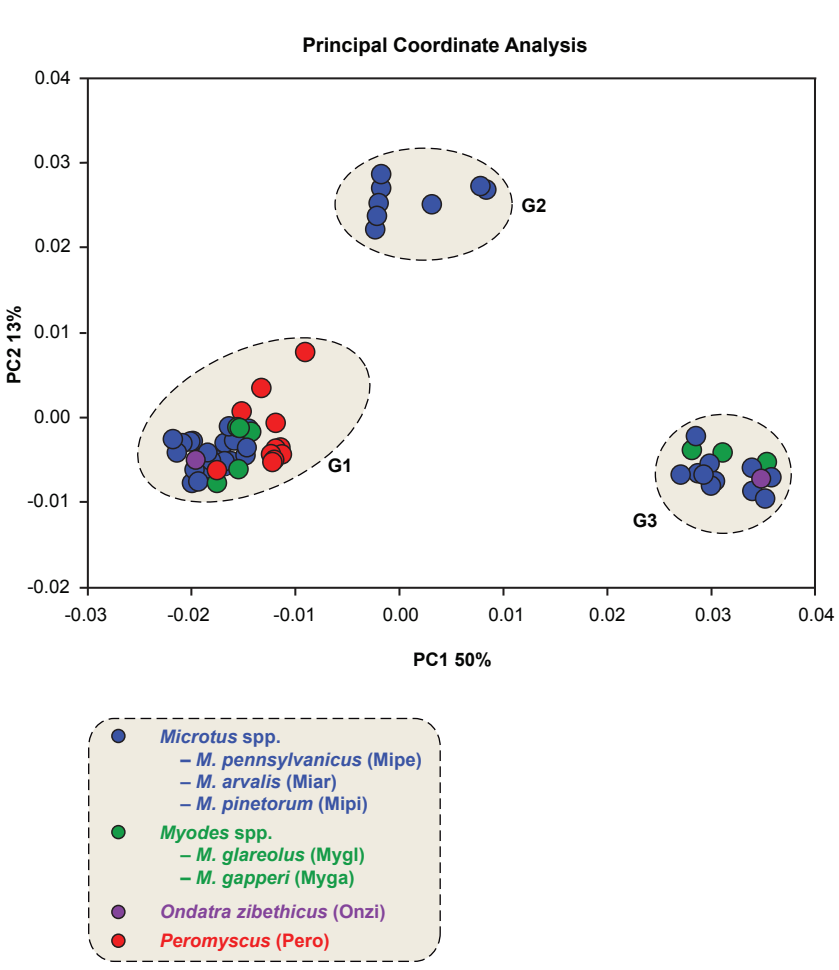
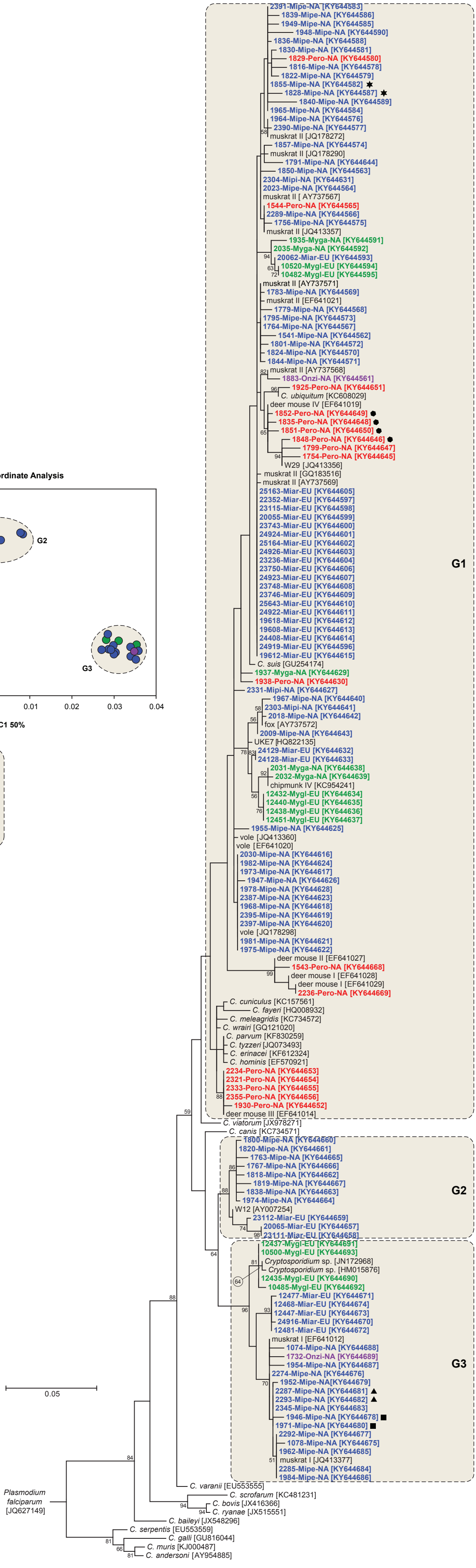
Fig. S1. Principle Coordinate Analysis (PCoA) and a maximum likelihood (ML) tree based on small subunit rRNA (SSU) gene sequences. The three major PCoA groups (G1-G3) are highlighted against cream background with dashed border on the ML tree. Sequences from this study are identified by region (NA for North America and EU for Europe), and they are color coded based on the genus or species of the host from which the sample was obtained (blue for *Microtus* spp., green for *Myodes* spp., red for *Peromyscus* spp., and purple for *Ondatra zibethicus*). Isolates obtained from the same animal are identified by a common symbol (▲, ■, or ●). The ML tree was rooted with an SSU sequence from *Plasmodium falciparum* (accession number: JQ627149).

Fig. S1


Principal Coordinate Analysis
PC2 13%
PC1 50%
0.04
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
-0.02
-0.01
0.00
0.01
0.02
0.03
0.04
G1
G2
G3
Microtus spp.
– M. pennsylvanicus (Mipe)
– M. arvalis (Miar)
– M. pinetorum (Mipi)
Myodes spp.
– M. glareolus (Mygl)
– M. gapperi (Myga)
Ondatra zibethicus (Onzi)
Peromyscus (Pero)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1949-Mipe-NA [KY644585]
1948-Mipe-NA [KY644590]
1836-Mipe-NA [KY644588]
1830-Mipe-NA [KY644581]
1829-Pero-NA [KY644580]
1816-Mipe-NA [KY644578]
1822-Mipe-NA [KY644579]
1855-Mipe-NA [KY644582]
1828-Mipe-NA [KY644587]
1840-Mipe-NA [KY644589]
1965-Mipe-NA [KY644584]
1964-Mipe-NA [KY644576]
2390-Mipe-NA [KY644577]
58
muskrat II [JQ178272]
1857-Mipe-NA [KY644574]
muskrat II [JQ178290]
1791-Mipe-NA [KY644644]
1850-Mipe-NA [KY644563]
2304-Mipi-NA [KY644631]
2023-Mipe-NA [KY644564]
muskrat II [AY737567]
1544-Pero-NA [KY644565]
2289-Mipe-NA [KY644566]
1756-Mipe-NA [KY644575]
muskrat II [JQ413357]
1935-Myga-NA [KY644591]
2035-Myga-NA [KY644592]
94
20062-Miar-EU [KY644593]
63
72
10520-Mygl-EU [KY644594]
10482-Mygl-EU [KY644595]
muskrat II [AY737571]
1783-Mipe-NA [KY644569]
muskrat II [EF641021]
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1795-Mipe-NA [KY644573]
1764-Mipe-NA [KY644567]
1541-Mipe-NA [KY644562]
1801-Mipe-NA [KY644572]
1824-Mipe-NA [KY644570]
1844-Mipe-NA [KY644571]
82
muskrat II [AY737568]
1883-Onzi-NA [KY644561]
96
1925-Pero-NA [KY644651]
C. ubiquitum [KC608029]
deer mouse IV [EF641019]
1852-Pero-NA [KY644649]
1835-Pero-NA [KY644648]
65
1851-Pero-NA [KY644650]
1848-Pero-NA [KY644646]
1799-Pero-NA [KY644647]
94
1754-Pero-NA [KY644645]
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muskrat II [GQ183516]
muskrat II [AY737569]
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22352-Miar-EU [KY644597]
23115-Miar-EU [KY644598]
20055-Miar-EU [KY644599]
23743-Miar-EU [KY644600]
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25164-Miar-EU [KY644602]
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23750-Miar-EU [KY644606]
24923-Miar-EU [KY644607]
23748-Miar-EU [KY644608]
23746-Miar-EU [KY644609]
25643-Miar-EU [KY644610]
24922-Miar-EU [KY644611]
19618-Miar-EU [KY644612]
19608-Miar-EU [KY644613]
24408-Miar-EU [KY644614]
24919-Miar-EU [KY644596]
19612-Miar-EU [KY644615]
C. suis [GU254174]
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1938-Pero-NA [KY644630]
2331-Mipi-NA [KY644627]
1967-Mipe-NA [KY644640]
58
2303-Mipi-NA [KY644641]
56
2018-Mipe-NA [KY644642]
fox [AY737572]
2009-Mipe-NA [KY644643]
UKE7 [HQ822135]
78
83
24129-Miar-EU [KY644632]
24128-Miar-EU [KY644633]
92
2031-Myga-NA [KY644638]
2032-Myga-NA [KY644639]
chipmunk IV [KC954241]
56
12432-Mygl-EU [KY644634]
12440-Mygl-EU [KY644635]
76
12438-Mygl-EU [KY644636]
12451-Mygl-EU [KY644637]
1955-Mipe-NA [KY644625]
vole [JQ413360]
vole [EF641020]
2030-Mipe-NA [KY644616]
1982-Mipe-NA [KY644624]
1973-Mipe-NA [KY644617]
1947-Mipe-NA [KY644626]
1978-Mipe-NA [KY644628]
2387-Mipe-NA [KY644623]
1968-Mipe-NA [KY644618]
2395-Mipe-NA [KY644619]
2397-Mipe-NA [KY644620]
vole [JQ178298]
1981-Mipe-NA [KY644621]
1975-Mipe-NA [KY644622]
deer mouse II [EF641027]
1543-Pero-NA [KY644668]
99
deer mouse I [EF641028]
deer mouse I [EF641029]
2236-Pero-NA [KY644669]
C. cuniculus [KC157561]
C. fayeri [HQ008932]
C. meleagridis [KC734572]
C. wrairi [GQ121020]
C. parvum [KF830259]
C. tyzzeri [JQ073493]
C. erinacei [KF612324]
C. hominis [EF570921]
2234-Pero-NA [KY644653]
2321-Pero-NA [KY644654]
2333-Pero-NA [KY644655]
88
2355-Pero-NA [KY644656]
1930-Pero-NA [KY644652]
deer mouse III [EF641014]
G1
59
C. viatorum [JX978271]
C. canis [KC734571]
1800-Mipe-NA [KY644660]
1820-Mipe-NA [KY644661]
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86
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1818-Mipe-NA [KY644662]
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88
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74
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98
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G2
64
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81
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Cryptosporidium sp. [HM015876]
64
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88
12477-Miar-EU [KY644671]
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96
93
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muskrat I [EF641012]
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1971-Mipe-NA [KY644680]
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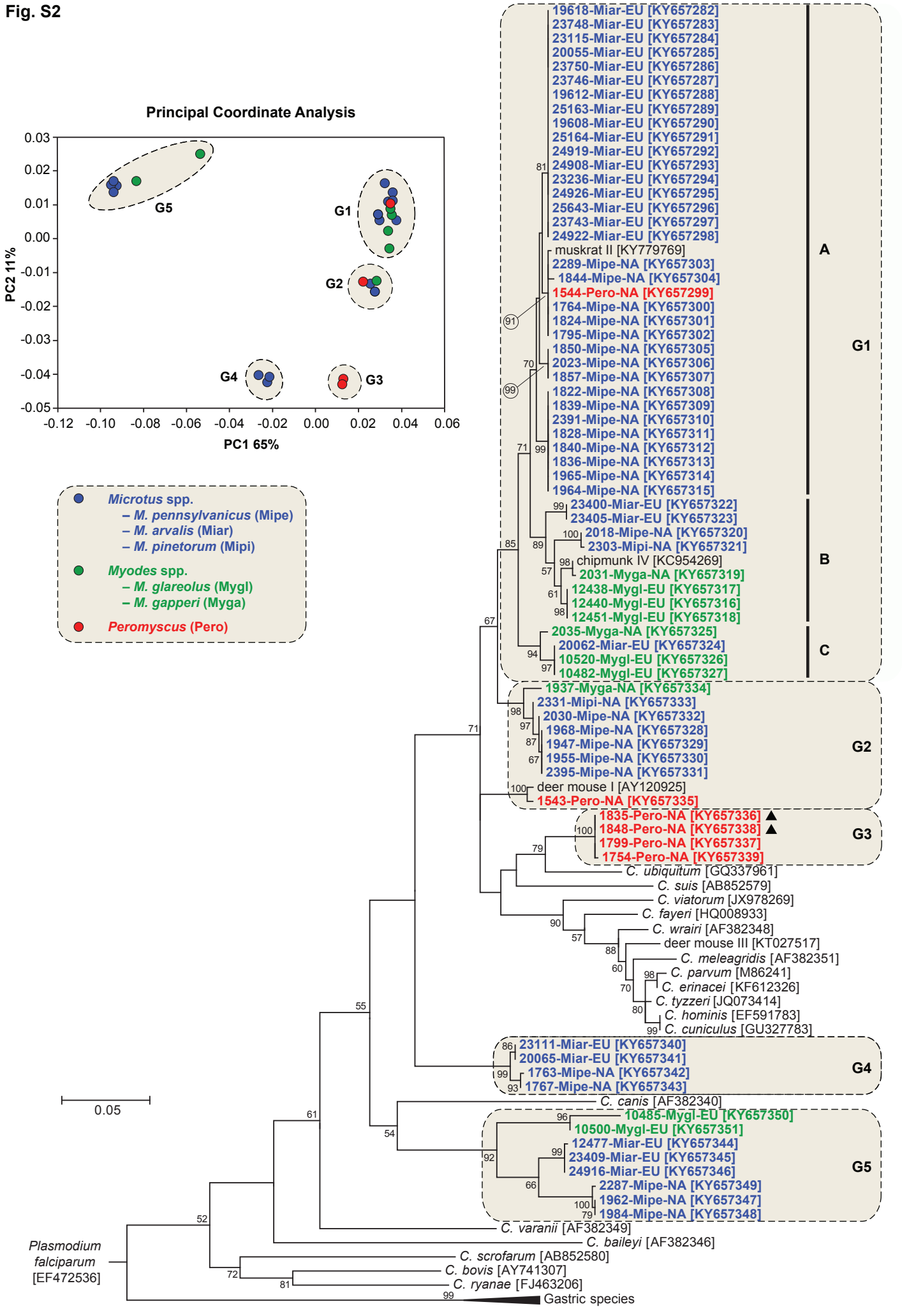
1984-Mipe-NA [KY644686]
G3
0.05
84
Plasmodium falciparum [JQ627149]
C. varanii [EU553555]
C. scrofarum [KC481231]
94
C. bovis [JX416366]
94
C. ryanae [JX515551]
C. baileyi [JX548296]
C. serpentis [EU553559]
81
C. galli [GU816044]
66
C. muris [KJ000487]
81
C. andersoni [AY954885]

Fig. S2. Principle Coordinate Analysis (PCoA) and expanded maximum likelihood (ML) tree based on actin gene sequences. Expansion of the tree in Fig. 1 that includes the phylogenetic positions of *C. varanii*, *C. baileyi*, *C. scrofarum*, *C. bovis*, *C. ryanae*, and gastric *Cryptosporidium* species (shown as a collapsed group). The five major PCoA groups (G1-G5) are highlighted against a cream background with dashed border on the ML tree. G1 is further broken down into three subgroups (A-C). Sequences from this study are identified by region (NA for North America and EU for Europe), and they are color coded based on the genus of the host from which the sample was obtained (blue for *Microtus* spp., green for *Myodes* spp., and red for *Peromyscus* spp.). A solid black triangle (▲) identifies isolates from the same animal. The ML tree was rooted with an actin sequence from *Plasmodium falciparum* (accession number: EF472536).

Fig. S2


Principal Coordinate Analysis
PC2 11%
PC1 65%
G1
G2
G3
G4
G5
Microtus spp.
– M. pennsylvanicus (Mipe)
– M. arvalis (Miar)
– M. pinetorum (Mipi)
Myodes spp.
– M. glareolus (Mygl)
– M. gapperi (Myga)
Peromyscus (Pero)
19618-Miar-EU [KY657282]
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23115-Miar-EU [KY657284]
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23750-Miar-EU [KY657286]
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19612-Miar-EU [KY657288]
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muskrat II [KY779769]
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1839-Mipe-NA [KY657309]
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chipmunk IV [KC954269]
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12438-Mygl-EU [KY657317]
12440-Mygl-EU [KY657316]
12451-Mygl-EU [KY657318]
2035-Myga-NA [KY657325]
20062-Miar-EU [KY657324]
10520-Mygl-EU [KY657326]
10482-Mygl-EU [KY657327]
A
B
C
G1
1937-Myga-NA [KY657334]
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1968-Mipe-NA [KY657328]
1947-Mipe-NA [KY657329]
1955-Mipe-NA [KY657330]
2395-Mipe-NA [KY657331]
deer mouse I [AY120925]
1543-Pero-NA [KY657335]
G2
1835-Pero-NA [KY657336]
1848-Pero-NA [KY657338]
1799-Pero-NA [KY657337]
1754-Pero-NA [KY657339]
G3
C. ubiquitum [GQ337961]
C. suis [AB852579]
C. viatorum [JX978269]
C. fayeri [HQ008933]
C. wrairi [AF382348]
deer mouse III [KT027517]
C. meleagridis [AF382351]
C. parvum [M86241]
C. erinacei [KF612326]
C. tyzzeri [JQ073414]
C. hominis [EF591783]
C. cuniculus [GU327783]
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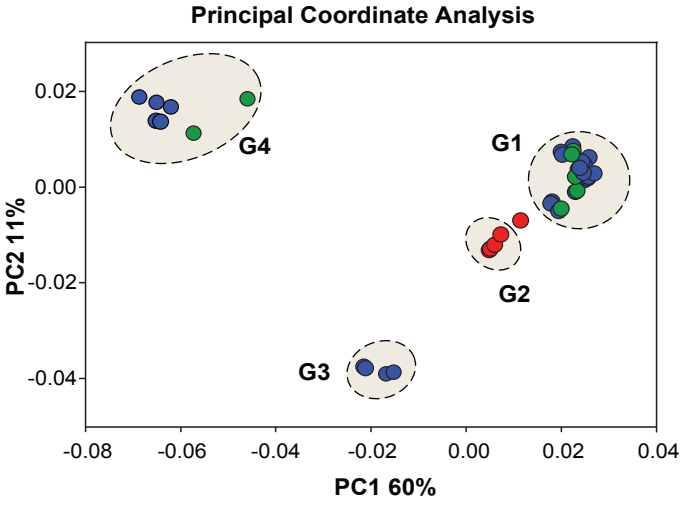
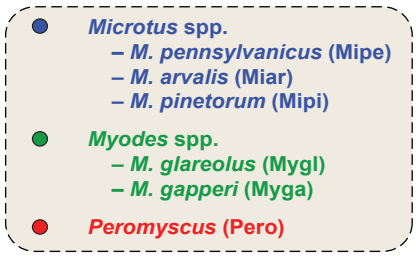
20065-Miar-EU [KY657341]
1763-Mipe-NA [KY657342]
1767-Mipe-NA [KY657343]
G4
C. canis [AF382340]
10485-Mygl-EU [KY657350]
10500-Mygl-EU [KY657351]
12477-Miar-EU [KY657344]
23409-Miar-EU [KY657345]
24916-Miar-EU [KY657346]
2287-Mipe-NA [KY657349]
1962-Mipe-NA [KY657347]
1984-Mipe-NA [KY657348]
G5
C. varanii [AF382349]
C. baileyi [AF382346]
C. scrofarum [AB852580]
C. bovis [AY741307]
C. ryanae [FJ463206]
Gastric species
Plasmodium falciparum [EF472536]
0.05

Fig. S3. Principle Coordinate Analysis (PCoA) and expanded maximum likelihood (ML) tree based on concatenated actin and small subunit rRNA (SSU) gene sequences. Expansion of the tree in Fig. 2 that includes the phylogenetic positions of *C. varanii*, *C. baileyi*, *C. scrofarum*, *C. bovis*, *C. ryanae*, and gastric *Cryptosporidium* species (shown as a collapsed group). The four major PCoA groups (G1-G4) are highlighted against a cream background with dashed border on the ML tree. Sequences from this study are identified by region (NA for North America and EU for Europe), and they are color coded based on the genus of the host from which the sample was obtained (blue for *Microtus* spp., green for *Myodes* spp., and red for *Peromyscus* spp.). A solid black triangle (▲) identifies isolates from the same animal. The ML tree was rooted with a concatenated actin / SSU sequence from *Plasmodium falciparum* (accession numbers: EF472536 / JQ627149).

Fig. S3


Principal Coordinate Analysis
PC2 11%
PC1 60%
0.02
0.00
-0.02
-0.04
-0.08
-0.06
-0.04
-0.02
0.00
0.02
0.04
G1
G2
G3
G4


Microtus spp.
– M. pennsylvanicus (Mipe)
– M. arvalis (Miar)
– M. pinetorum (Mipi)
Myodes spp.
– M. glareolus (Mygl)
– M. gapperi (Myga)
Peromyscus (Pero)

23236-Miar-EU [KY657294 / KY644604]
24919-Miar-EU [KY657292 / KY644596]
24926-Miar-EU [KY657295 / KY644603]
25643-Miar-EU [KY657296 / KY644610]
25163-Miar-EU [KY657289 / KY644605]
23746-Miar-EU [KY657287 / KY644609]
20055-Miar-EU [KY657285 / KY644599]
23748-Miar-EU [KY657283 / KY644608]
19618-Miar-EU [KY657282 / KY644612]
23115-Miar-EU [KY657284 / KY644598]
23750-Miar-EU [KY657286 / KY644606]
19612-Miar-EU [KY657288 / KY644615]
19608-Miar-EU [KY657290 / KY644613]
25164-Miar-EU [KY657291 / KY644602]
23743-Miar-EU [KY657297 / KY644600]
24922-Miar-EU [KY657298 / KY644611]
1544-Pero-NA [KY657299 / KY644565]
2289-Mipe-NA [KY657303 / KY644566]
1844-Mipe-NA [KY657304 / KY644571]
1824-Mipe-NA [KY657301 / KY644570]
muskrat II [KY779769 / AY737571]
1764-Mipe-NA [KY657300 / KY644567]
1795-Mipe-NA [KY657302 / KY644573]
2023-Mipe-NA [KY657306 / KY644564]
1850-Mipe-NA [KY657305 / KY644563]
1857-Mipe-NA [KY657307 / KY644574]
1828-Mipe-NA [KY657311 / KY644587]
1839-Mipe-NA [KY657309 / KY644586]
1822-Mipe-NA [KY657308 / KY644579]
1840-Mipe-NA [KY657312 / KY644589]
2391-Mipe-NA [KY657310 / KY644583]
1964-Mipe-NA [KY657315 / KY644576]
1836-Mipe-NA [KY657313 / KY644588]
1965-Mipe-NA [KY657314 / KY644584]
24129-Miar-EU / 23400-Miar-EU [KY657322 / KY644632]
24128-Miar-EU / 23405-Miar-EU [KY657323 / KY644633]
2018-Mipe-NA [KY657320 / KY644642]
2303-Mipi-NA [KY657321 / KY644641]
chipmunk IV [KC954269 / KC954241]
2031-Myga-NA [KY657319 / KY644638]
12440-Mygl-EU [KY657316 / KY644635]
12438-Mygl-EU [KY657317 / KY644636]
12451-Mygl-EU [KY657318 / KY644637]
2035-Myga-NA [KY657325 / KY644592]
20062-Miar-EU [KY657324 / KY644593]
10520-Mygl-EU [KY657326 / KY644594]
10482-Mygl-EU [KY657327 / KY644595]
1937-Myga-NA [KY657334 / KY644629]
2331-Mipi-NA [KY657333 / KY644627]
2030-Mipe-NA [KY657332 / KY644616]
1947-Mipe-NA [KY657329 / KY644626]
2395-Mipe-NA [KY657331 / KY644619]
1955-Mipe-NA [KY657330 / KY644625]
1968-Mipe-NA [KY657328 / KY644618]
G1
deer mouse I [AY120925] / [EF641027]
1543-Pero-NA [KY657335 / KY644668]
C. suis [AB852579 / GU254174]
C. ubiquitum [GQ337961 / KC608029]
1835-Pero-NA [KY657336 / KY644648]
1848-Pero-NA [KY657338 / KY644646]
1799-Pero-NA [KY657337 / KY644647]
1754-Pero-NA [KY657339 / KY644645]
G2
C. viatorum [JX978269 / JX978271]
C. fayeri [HQ008933 / HQ008932]
C. wrairi [AF382348 / GQ121020]
C. meleagridis [AF382351 / KC734572]
C. hominis [EF591783 / DQ286403]
C. cuniculus [GU327783 / KC157561]
C. tyzzeri [JQ073414 / JQ073493]
C. parvum [M86241 / KF830259]
C. erinacei [KF612326 / KF612324]
23111-Miar-EU [KY657340 / KY644658]
20065-Miar-EU [KY657341 / KY644657]
1763-Mipe-NA [KY657342 / KY644665]
1767-Mipe-NA [KY657343 / KY644666]
G3
C. canis [AF382340 / KC734571]
10485-Mygl-EU [KY657350 / KY644692]
10500-Mygl-EU [KY657351 / KY644693]
12477-Miar-EU [KY657344 / KY644671]
24916-Miar-EU [KY657346 / KY644670]
2287-Mipe-NA [KY657349 / KY644681]
1962-Mipe-NA [KY657347 / KY644685]
1984-Mipe-NA [KY657348 / KY644686]
G4
C. varanii [AF382349 / EU553555]
C. scrofarum [AB852580 / KC481231]
C. bovis [AY741307 / JX416366]
C. ryanae [FJ463206 / JX515551]
C. baileyi [AF382346 / JX548296]
Gastric species
Plasmodium falciparum
[EF472536 / JQ627149]
0.05

6.3. *Cryptosporidium apodemi* sp. n. and *Cryptosporidium ditrichi* sp. n. (Apicomplexa: Cryptosporidiidae) in *Apodemus* spp.

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Cryptosporidium apodemi sp. n. and *Cryptosporidium ditrichi* sp. n. (Apicomplexa: Cryptosporidiidae) in *Apodemus* spp.

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Abstract

Faecal samples from striped field mice (n = 72) and yellow-necked mice (n = 246) were screened for *Cryptosporidium* by microscopy and PCR/sequencing. Phylogenetic analysis of small-subunit rRNA, *Cryptosporidium* oocyst wall protein and actin gene sequences revealed the presence of *C. parvum*, *C. hominis*, *C. muris* and two new species, *C. apodemi* and *C. ditrichi*. Oocysts of *C. apodemi* are smaller than *C. ditrichi* and both are experimentally infectious for yellow-necked mice but not for common voles. Additionally, infection by *C. ditrichi* was established in one of three BALB/c mice. The prepatent period was 7–9 and 5–6 days post infection for *C. apodemi* and *C. ditrichi*, respectively. The patent period was greater than 30 days for both species. Infection intensity of *C. ditrichi* ranged from 4000–50,000 oocyst per gram of faeces and developmental stages of *C. ditrichi* were detected in the jejunum and ileum. In contrast, neither oocysts nor endogenous developmental stages of *C. apodemi* were detected in faecal or tissue samples, although *C. apodemi* DNA was detected in contents from the small and large intestine. Morphological, genetic, and biological data support the establishment of *C. apodemi* and *C. ditrichi* as a separate species of the genus *Cryptosporidium*.

Keywords: Europe; Experimental infection; Molecular analyses; Oocyst size; Phylogeny; Rodentia

Introduction

Cryptosporidium species are apicomplexans that infect the epithelial cells of the gastrointestinal, respiratory and urinary tract of vertebrates (Ryan and Xiao 2014). More than 35 species of *Cryptosporidium* have been formally described and are considered valid. Additionally, a large number of *Cryptosporidium* genotypes/isolates, which lack the biological and morphological data necessary for species designation, have been reported in vertebrates and the environment (Kváč et al. 2014; Robertson et al. 2014; Ryan and Xiao 2014). Molecular studies have shown that *Cryptosporidium* infecting humans and livestock represent a small fraction of the diversity in the genus (Nakamura and Meireles 2015; Stenger et al. 2015a; Yang et al. 2015). Rodents, an order that com-

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0932-4739/

prises about 40% of the mammalian diversity, host much of the described diversity in the genus *Cryptosporidium* (Kváč et al. 2014; Li et al. 2015; Ng-Hublin et al. 2013; Stenger et al. 2015b). *Apodemus*, in the rodent family Muridae, comprises approximately 20 Palearctic species, divided into four groups according to their evolution (Filippucci et al. 2002; Liu et al. 2004; Wojcik et al. 2004). *Cryptosporidium* was first reported in *Apodemus* in the late 1990s, and several of the earlier studies, which were based on descriptions of oocyst morphology, identified *C. parvum* and *C. muris* (Bednarska et al. 2007; Chalmers et al. 1997; Torres et al. 2000). It is now known that many *Cryptosporidium* species have morphologically indistinguishable oocysts and can only be distinguished by genotyping. Using genotyping, 12 *Cryptosporidium* species and genotypes have been identified in different species of *Apodemus*, including *C. ubiquitum*, *C. scrofarum*, *C. suis*, *C. hominis*, *C. muris*, *C. parvum*, *Cryptosporidium* cf. *parvum*, *Cryptosporidium* Naruko genotype, *C. muris* Japanese field mouse genotype, *Cryptosporidium* muskrat genotype II, *Cryptosporidium* chipmunk genotype I, and *Cryptosporidium* sp. KSFM (Danisova et al. 2017; Hajdušek et al. 2004; Hikosaka and Nakai 2005; Kulis-Malkowska 2007; Li et al. 2014; Murakoshi et al. 2013; Perec-Matysiak et al. 2015; Song et al. 2015). Most of these cryptosporidia occur rarely in *Apodemus* and are more typically found in other hosts, so they are not considered specific for *Apodemus*. We undertook the present study to describe the presence of *Cryptosporidium* spp. in the genus *Apodemus* in central Europe. Additionally, we described the experimental transmission, oocyst morphology and molecular characteristics of *Apodemus*-associated *Cryptosporidium* spp. Based on these data, we describe two new *Cryptosporidium* species that are specific for the genus *Apodemus* and we propose that they be named *Cryptosporidium apodemi* sp. n. and *Cryptosporidium ditrichi* sp. n.

Material and Methods

Specimens studied

The research was performed on rodents of the genus *Apodemus* in the Czech Republic and Slovakia. Animals were trapped with snap traps baited with smoked cheese. After identification of species and gender, the animal was dissected and a faecal sample was collected from the colon. Each sample was preserved in 2.5% potassium dichromate and stored at 4 °C. All faecal samples obtained from individual animals were monitored for the presence of *Cryptosporidium* oocysts using the aniline-carbol-methyl violet (ACMV) staining method (Miláček and Vítovec 1985) with microscopic examination at a magnification of ×1000. The infection intensity was determined from the microscopic examination as number of oocysts per gram (OPG) according to Kváč et al. (2007).

Molecular characterisation and phylogenetic analysis

Genomic DNA was extracted from 200 mg of faecal samples 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) by Sak et al. (2008). DNA was isolated by using an Exgene™ stool DNA mini kit (GeneAll®, Korea) in accordance with the manufacturer's instructions. DNA was stored at −20 °C until used in PCR assays. Nested-PCR protocols were used to amplify partial sequences of the *Cryptosporidium* small-subunit rRNA gene (SSU) according to Jiang et al. (2005), the *Cryptosporidium* 60-kDa glycoprotein gene (gp60) according to Alves et al. (2003), actin gene according to Sulaiman et al. (2002) and *Cryptosporidium* oocyst wall protein gene (COWP) according to Spano et al. (1997). Negative (molecular grade water) and positive controls (DNA of *C. hominis* subtype Id) were included in each PCR amplification. Secondary products were visualized with ethidium bromide following electrophoresis on an agarose gel. PCR products were purified with GenElute™ Gel Extraction Kit (Sigma–Aldrich, St. Louis, MO) and sequenced in both directions with secondary primers using a BigDye Terminator v3.1 cycle sequencing kit in an ABI Prism 3130 genetic analyser (Applied Biosystems, Carlsbad, CA). The nucleotide sequences were assembled using ChromasPro 2.1.4 (www.technelysium.com.au/ChromasPro.html), edited using BioEdit 7.04 (www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned with previously published sequences using the MAFFT version 7 online server using the Q-INS-i algorithm for SSU, actin, and COWP sequences and L-INS-i algorithm for gp60 sequences (http://mafft.cbrc.jp/alignment/server/).

Phylogenetic analyses were performed using MEGA 6.0 (www.megasoftware.net/). The evolutionary history of aligned sequences was inferred using the maximum likelihood (ML) method (Saitou and Nei 1987), with the substitution model that best fit the alignment selected using the Bayesian information criterion. The General Time Reversible model was selected for alignment of actin and gp60 alignments and the Tamura 3-parameter test was selected for the SSU and COWP alignments. All models were used under an assumption that rate variation among sites was gamma distributed. A bootstrap consensus tree was inferred from 1000 pseudoreplicates. Phylogenetic trees were edited for style using CorelDrawX7 (Corel Corporation, Ottawa, Ontario, Canada). Sequences have been deposited in GenBank under the accession numbers MG266030–MG266048.

Source of oocyst for morphometric and transmission studies

Oocysts of *Cryptosporidium ditrichi* sp. n. from five naturally infected yellow-necked mice (*Apodemus flavicollis*),

which were trapped at three localities in the Czech Republic, and oocysts of *Cryptosporidium apodemi* sp. n. from three naturally infected striped field mice (*Apodemus agrarius*), which were trapped at two localities in Slovakia, were purified using caesium chloride gradient centrifugation for morphometry analyses (Arrowood and Donaldson 1996). Oocyst of each taxon were pooled and used for experimental infection studies.

Morphological evaluation

Oocysts were examined using differential interference contrast (DIC) microscopy, following ACMV staining, and fluorescence microscopy, following labelling with genus-specific FITC-conjugated antibodies (IFA; Cryptosporidium IF Test, Crypto cel, Cellabs Pty Ltd., Brookvale, Australia). Morphometry was determined by 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 DP73 Digital Colour Camera. Length and width of oocysts (n = 50) were measured under DIC at 1000× magnification and these measurements were used to calculate the shape index. Oocysts were measured by the same person using the same microscope. Photomicrographs of oocysts observed by DIC, ACMV and IFA were deposited as a phototype at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

Experimental infection

To study the course of infection and host specificity, purified oocysts were used to infect 8-week-old yellow-necked mice, BALB/c mice (*Mus musculus*), and common voles (*Microtus arvalis*). 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. Each experimental animal was inoculated orally by stomach tube with 50,000 purified oocysts of appropriate taxa suspended in 200 μl of distilled water. Animals serving as negative controls were inoculated orally by stomach tube with 200 μl of distilled water. Faecal samples of all animals were screened daily for the presence of *Cryptosporidium* oocysts using ACMV staining, and the presence of *Cryptosporidium*-specific DNA was confirmed using nested PCR targeting the SSU gene. All experiments were terminated 30 days post infection (DPI). Infection intensity was reported as OPG, as previously described by Kváč et al. (2007). In addition, faecal consistency and general health status were examined daily. To study site of infection, a susceptible host was euthanized during the patent period and tissue specimens of the digestive tract (oesophagus, stomach, duodenum, jejunum, ileum, cecum, and colon) and other organs (liver, kidney, spleen and lungs) were processed for PCR detection, histology and electron microscopy. Animal caretakers wore new disposable coveralls, shoe covers, and gloves every time they entered the experimental room. All wood-chip bedding, faeces, and disposable protective clothing were sealed in plastic bags, removed from the experimental room, and incinerated.

Histopathological and scanning electron microscopy examinations

The complete examination of all gastrointestinal organs was conducted at necropsy. Tissue specimens were sampled and processed for histology according to Kváč and Vítovec (2003), scannig electron microscopy (SEM) according to Valigurová et al. (2008) and for PCR analyses. Histology sections were stained with hematoxylin and eosin (HE) and Periodic Acid–Schiff (PAS) stain, and genus-specific FITC-conjugated monoclonal antibodies targeting *Cryptosporidium* oocyst wall antigens (*Cryptosporidium* IF Test, Crypto Cel, Medac). All samples processed for SEM were examined by JEOL JSM-7401F.

Statistical analysis

Prevalence was calculated by dividing the number of positive individuals by the total number of individuals sampled. Differences in *Cryptosporidium* prevalence were determined by Chi-square analysis using a 5% significance level. Analyses were performed using the program Epi Info (TM) 7.1.1.14 (Centers for Disease Control and Prevention, GA, USA).

Ethics statement

The research was conducted under ethical protocols approved by the Institute of Parasitology, Biology Centre, and Central Commission for Animal Welfare, Czech Republic (protocol nos. 071/2010 and 114/2013).

Results

Out of 318 rodents, comprising 72 striped field mice and 246 yellow-necked mice, sampled at 11 locations in the Czech Republic and 9 in Slovakia, 17 and 41 were positive for *Cryptosporidium* by microscopy and PCR, respectively (Table 1). All microscopically positive animals were also PCR positive. The overall prevalence of *Cryptosporidium* spp. in *Apodemus* spp. was 12.9% (41/318). The *Cryptosporidium* prevalence in yellow-necked mice (13.4%; 33/246) and striped field mice (11.1%; 8/72) was similar ($\chi 2 = 0.098$, *d.f.* 1). Out of 41 *Cryptosporidium* positive animals, 40, 41 and 25 were genotyped by sequence analysis of SSU, actin and COWP genes, respectively (Table 1). The remaining positive samples yielded sequences of insufficient quality to include in analyses. Phylogenetic analysis of SSU, actin and COWP sequences using the ML method revealed the presence of *C. parvum*, *C. hominis* and *C. muris*, each in a single sample (Table 1, Fig. 1–3).

Table 1. *Cryptosporidium* species and genotypes in wild yellow-necked mice (*Apodemus flavicollis*) and striped field mice (*Apodemus agrarius*) in the Czech Republic (CZE) and Slovakia (SVK). Isolates were characterized by microscopy, including infection intensity expressed as number of oocyst per gram of faeces (OPG), and PCR analysis of the small ribosomal subunit rRNA (SSU), actin, *Cryptosporidium* oocyst wall protein (COWP) and 60 kDa glycoprotein (gp60) genes.

Isolate ID	Host species	Location (country)	Microscopical positivity (OPG)	Genotyping at the gene loci			
				SSU	Actin	COWP	gp60
12391	*A. flavicollis*	Opatovice 2 (CZE)	Yes (4000)	*C. ditrichi*	*C. ditrichi*		
12414	*A. flavicollis*	Opatovice 2 (CZE)	Yes (6500)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
12423	*A. flavicollis*	Opatovice 1 (CZE)	Yes (10,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
12426	*A. flavicollis*	Opatovice 1 (CZE)	No	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
12427	*A. flavicollis*	Opatovice 2 (CZE)	Yes (4000)	*C. ditrichi*	*C. ditrichi*		
12667	*A. flavicollis*	Opatovice 2 (CZE)	Yes (25,000)	*C. ditrichi*	*C. ditrichi*		
12668	*A. flavicollis*	Opatovice 2 (CZE)	No	*C. ditrichi*	*C. ditrichi*		
12679	*A. flavicollis*	Opatovice 2 (CZE)	Yes (15,000)	*C. ditrichi*	*C. ditrichi*		
12699	*A. flavicollis*	Opatovice 2 (CZE)	Yes (10,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
12710	*A. flavicollis*	Opatovice 2 (CZE)	No	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
24843	*A. flavicollis*	České Budějovice (CZE)	Yes (13,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
25372	*A. flavicollis*	Opatovice 1 (CZE)	Yes (13,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
25374	*A. flavicollis*	Opatovice 1 (CZE)	No	*C. ditrichi*	*C. ditrichi*		
25378	*A. flavicollis*	Dolní Třebonín (CZE)	Yes (10,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
28036	*A. flavicollis*	České Budějovice (CZE)	Yes (13,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
28060	*A. flavicollis*	Opatovice 1 (CZE)	Yes (4000)	*C. ditrichi*	*C. ditrichi*		
28531	*A. flavicollis*	Opatovice 1 (CZE)	No	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
28533	*A. flavicollis*	Opatovice 1 (CZE)	Yes (4000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
28534	*A. flavicollis*	Opatovice 1 (CZE)	Yes (13,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
28535	*A. flavicollis*	Opatovice 1 (CZE)	No	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
30890	*A. flavicollis*	Hůry (CZE)	Yes (25,000)	*C. ditrichi*	*C. ditrichi*		
4950	*A. flavicollis*	Rozhanovce (SVK)	No	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
8147	*A. flavicollis*	Hýľov (SVK)	No	*C. muris*	*C. muris*		
10466	*A. flavicollis*	Rozhanovce (SVK)	No	*C. ditrichi*	*C. ditrichi*		
11979	*A. flavicollis*	Rozhanovce (SVK)	Yes (22,000)	*C. ditrichi*	*C. ditrichi*		
21787	*A. flavicollis*	Košice 1(SVK)	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	
21931	*A. flavicollis*	Košice 1 (SVK)	No	*C. apodemi*	*C. apodemi*	–	
21993	*A. flavicollis*	Košice 2 (SVK)	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	
21999	*A. flavicollis*	Košice 2(SVK)	No	*C. apodemi*	*C. apodemi*		
27649	*A. flavicollis*	Rozhanovce (SVK)	Yes (13,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
30399	*A. flavicollis*	Komárno (SVK)	No		*C. ditrichi*	*C. ditrichi*	
30405	*A. flavicollis*	Komárno (SVK)	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	
30406	*A. flavicollis*	Komárno (SVK)	No	*C. apodemi*	*C. apodemi*		
4951	*A. agrarius*	Rozhanovce (SVK)	No	*C. parvum*	*C. parvum*	*C. parvum*	IIaA16G1R1b
10467	*A. agrarius*	Rozhanovce (SVK)	No	*C. hominis*	*C. hominis*	*C. hominis*	IbA10G2
10496	*A. agrarius*	Rozhanovce (SVK)	No	*C. apodemi*	*C. apodemi*		
10508	*A. agrarius*	Rozhanovce (SVK)	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	
10510	*A. agrarius*	Rozhanovce (SVK)	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	
10517	*A. agrarius*	Rozhanovce (SVK)	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	
11983	*A. agrarius*	Rozhanovce (SVK)	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	
30403	*A. agrarius*	Komárno (SVK)	No	*C. apodemi*	*C. apodemi*		

Subtyping of *C. parvum* and *C. hominis* at the gp60 locus revealed the presence of subtype families IIaA16G1R1 and IbA10G2, respectively (tree not shown). All remaining isolates clustered in one of two clades. Descriptions of oocyst morphology and experimental infectivity of isolates from these clades support a separate species designation, and a description of these novel species follows.

Cryptosporidium apodemi sp. n.

Prevalence and infection intensity

Out of 318 mice examined, 12 (3.8%) had DNA of *C. apodemi* detectable by PCR. None of these positive samples had oocysts detectable by microscopy (Table 1).

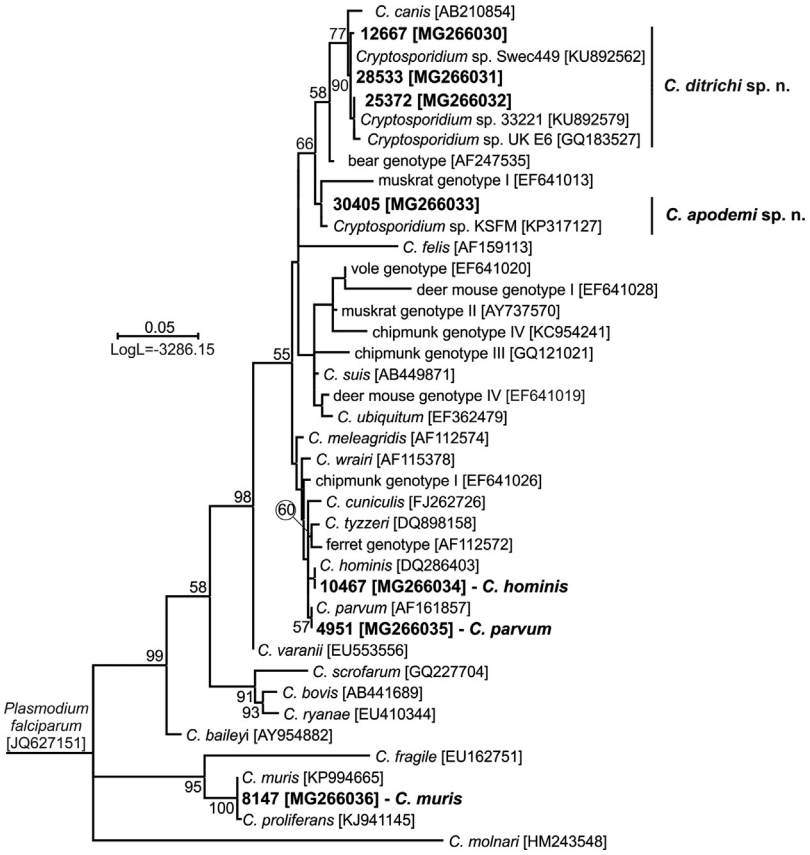

Fig. 1. Maximum likelihood tree based on partial small subunit ribosomal RNA gene sequences of *Cryptosporidium* (n = 40), including *Cryptosporidium ditrichi* sp. n. and *Cryptosporidium apodemi* sp. n. Sequences from this study are bolded. The alignment contained 550 base positions in the final dataset. The Tamura 3-parameter method modelled by using a discrete Gamma distribution was used. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates. Branch length scale bar indicate number of substitution per site.

Molecular characterization and phylogenetic analysis

All isolates of *C. apodemi* shared 100% identity at the SSU, actin and COWP loci, and phylogenetic analysis revealed *C. apodemi* to be a sister clade of muskrat genotype I (Figs. 1–3). At the SSU locus, *C. apodemi* shared 99.1% identity with a 480 bp sequence from isolate KSFM [Acc. No. KP317127], which was obtained from a striped field mouse in South Korea.

Experimental host transmissions

Experimental infection was established in yellow-necked mice but not in BALB/c mice or common voles. Specific DNA of *C. apodemi* was first detected in faeces 7–9 DPI. Occasional presence of specific DNA was detected up to 30 DPI (Fig. 4). No oocysts were detected by microscopy during the experimental infectivity studies. Sequences of SSU, actin and COWP genes from experimentally infected hosts shared 100% identity with the isolate used in the inoculum. No macroscopical changes were observed in infected mice and the surface epithelia were intact. An examination by histology and electron microscopy did not reveal the presence of developmental stages in any part of digestive tract or other organs (liver, pancreas, kidneys, lungs, and spleen). Specific DNA of *C. apodemi* was detected in the content of the small and large intestine. All experimentally infected yellow-necked mice exhibited growth that was typical of their size and weight. None of the faecal samples was diarrhoetic.

Taxonomic summary

Cryptosporidium apodemi sp. n.

Description. Oocysts are shed fully sporulated with 4 sporozoites and oocyst residuum inside. Sporulated oocysts (n = 50) measure 3.9–4.7 (mean = 4.2) × 3.8–4.4 (mean = 4.0) with a length to width ratio of 1.03 (1.0–1.06) (Fig. 5). Morphology and morphometry of other developmental stages is unknown.

Type host: striped field mouse (*Apodemus agrarius*)

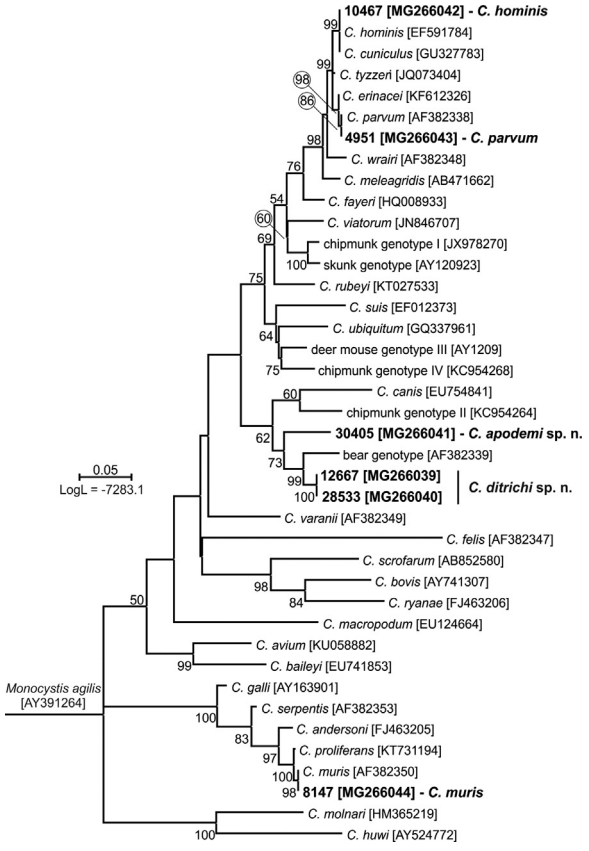

Fig. 2. Maximum likelihood tree based on partial actin gene sequences of *Cryptosporidium* (n = 40), including *Cryptosporidium ditrichi* sp. n. and *Cryptosporidium apodemi* sp. n. Sequences from this study are bolded. The alignment contained 696 base positions in the final dataset. The General Time Reversible method modelled by using a discrete Gamma distribution was used. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates. Branch length scale bar indicate number of substitution per site.

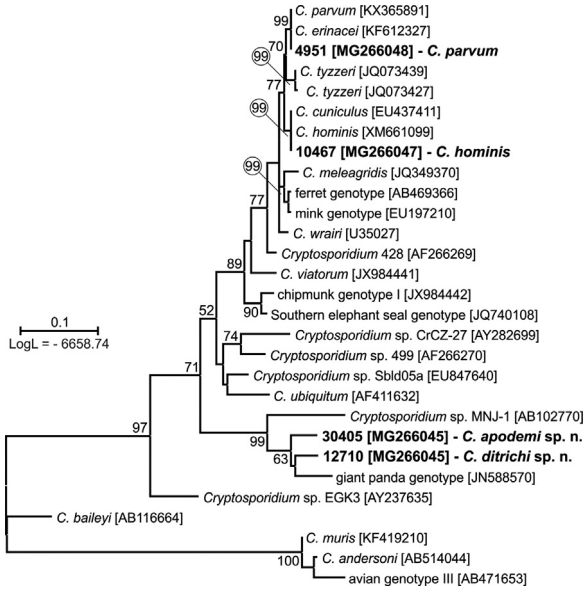

Fig. 3. Maximum likelihood tree based on partial sequences of *Cryptosporidium* (n = 29) coding *Cryptosporidium* oocyst wall protein gene, including *Cryptosporidium ditrichi* sp. n. and *Cryptosporidium apodemi* sp. n. Sequences from this study are bolded. The alignment contained 384 base positions in the final dataset. The Tamura 3-parameter method modelled by using a discrete Gamma distribution was used. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates. Branch length scale bar indicate number of substitution per site.

Other host: yellow-necked mouse (*Apodemus flavicollis*)

Type locality: Rozhanovce, Košice and Komárno (Slovakia)

Site of infection: intestine

Distribution: Slovakia

Material deposited: Slides with oocysts and DNA are deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic. Partial sequences of SSU, actin and COWP genes were

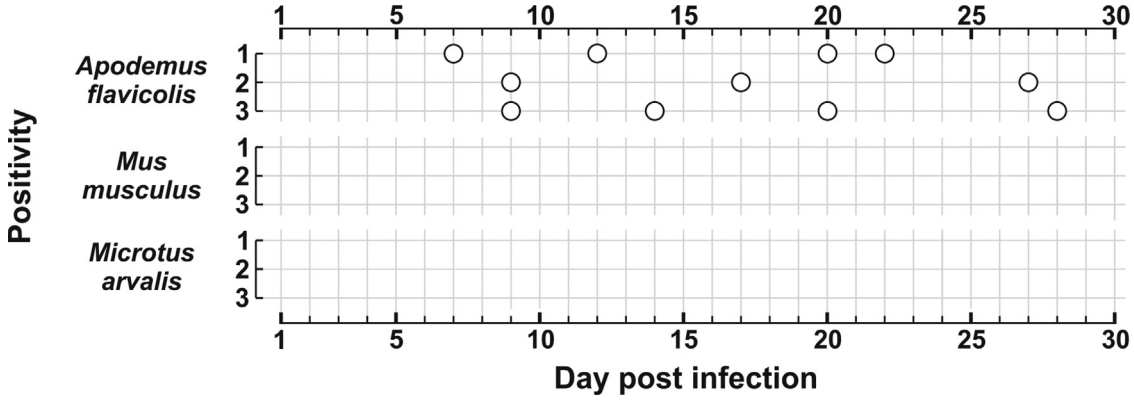

Fig. 4. Course of infection of *Cryptosporidium apodemi* sp. n. based on coprological and molecular examination of faeces. Circles indicate detection of specific DNA.

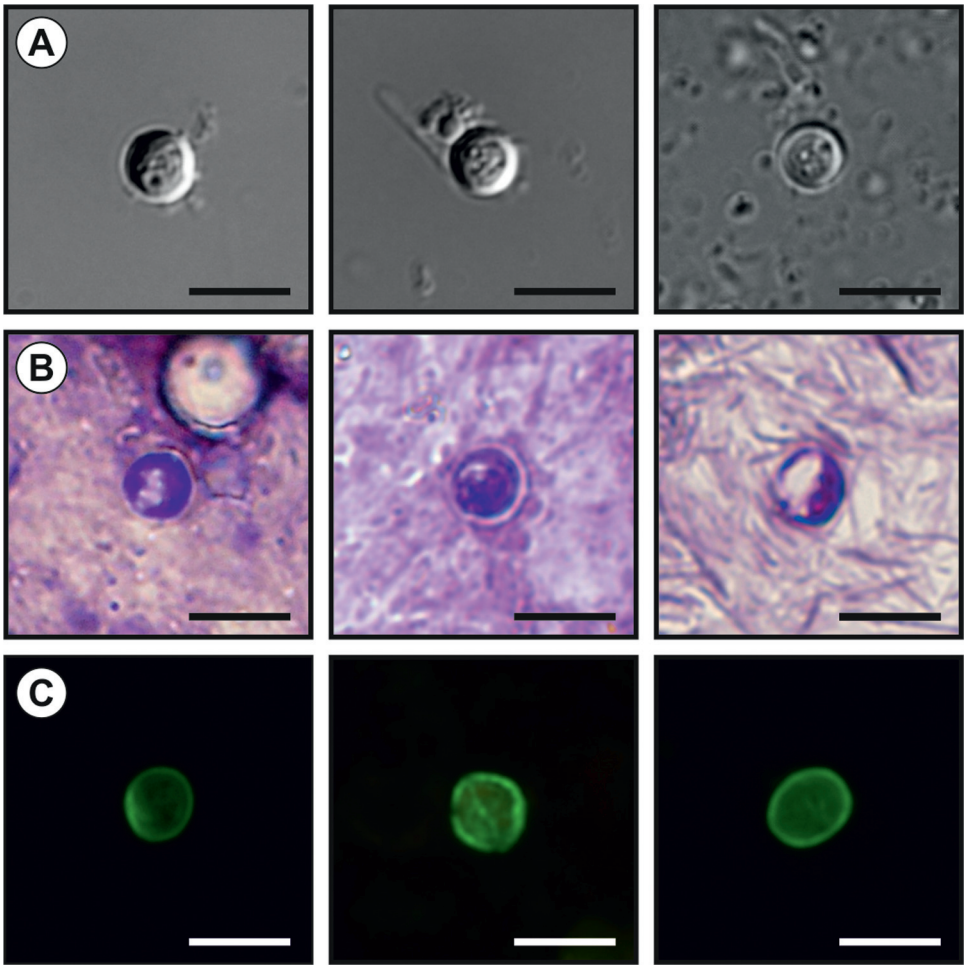

Fig. 5. *Cryptosporidium apodemi* sp. n. oocysts visualized in various preparations: (A) differential interference contrast microscopy and stained by (B) aniline–carbol–methyl violet and (C) anti-*Cryptosporidium* FITC-conjugated antibody. Bar = 5 μm.

deposited at GenBank (Acc. Nos. MG266033, MG266041 and MG266046).

Etymology: The species name apodemi is derived from the genus *Apodemus*, latin name for Eurasian field mice.

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

Cryptosporidium ditrichi sp. n.

Prevalence and infection intensity

Out of 318 examined mice, 26 (8.2%) were positive for DNA of *C. ditrichi*. Of these, 17 (65%) shed oocysts detectible by microscopy at the time of trapping. The infection intensity in microscopy positive animals ranged from 4000 to 25,000 OPG.

Molecular characterization and phylogenetic analysis

Sequences of *C. ditrichi* formed a well-supported clade that included *Cryptosporidium* SSU sequences from raw water and a human in Sweden and from raw water in the UK. Three variants of the *C. ditrichi* SSU gene shared 98.9–100% similarity with each other. All variants were detected in the Czech Republic (Acc. Nos. MG266030–MG266033), but only one was detected in Slovakia (Acc. No. MG266032; Fig. 1). Two variants of the *C. ditrichi* actin sequence differed by a single synonymous substitution. Both actin variants were detected in the Czech Republic (Acc. Nos. MG266039 and MG266040), but only one was found in Slovakia (Acc. No. MG266040; Fig. 2). COWP gene sequences did not differ among isolates of *C. ditrichi*. Phylogenetic analyses of

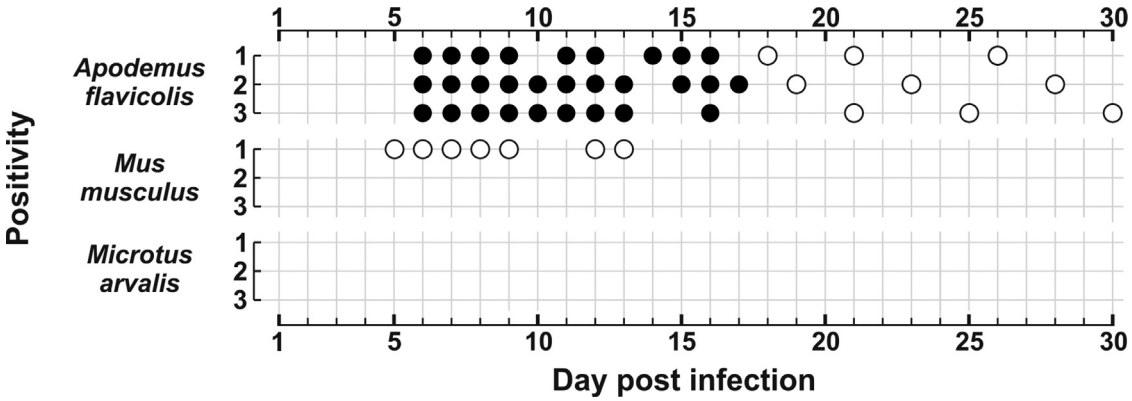

Fig. 6. Course of infection of *Cryptosporidium ditrichi* sp. n. based on coprological and molecular examination of faeces. Circles indicate detection of specific DNA, black circle indicates microscopic detection of oocysts.

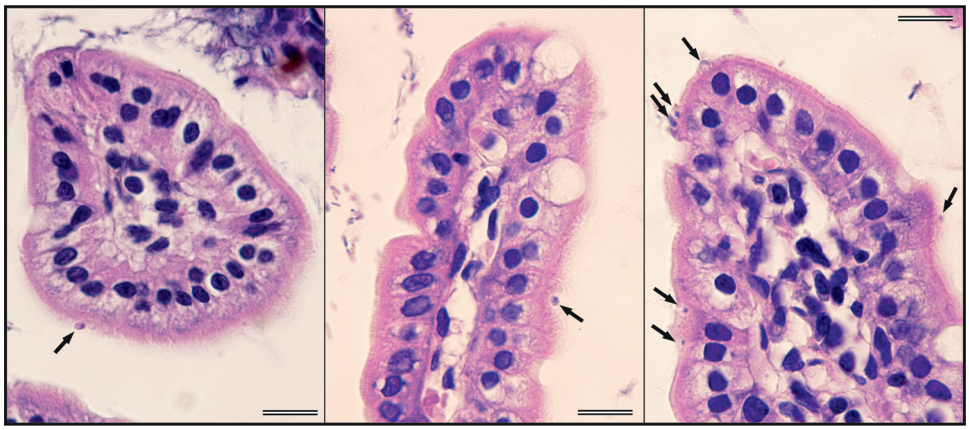

Fig. 7. *Cryptosporidium* developmental stages (arrows) in mucosal glandular epithelium from the ileum of experimentally infected yellow-necked mouse (*Apodemus flavicollis*) with dose 50,000 oocysts of *Cryptosporidium ditrichi* sp. n., sacrificed 10 DPI. Bar = 25 μm.

sequences of all genes confirmed the position of *C. ditrichi* as a separate taxon (Figs. 1–3).

Experimental host transmissions

Experimental infection was successful in a yellow-necked mouse but not in common voles. Specific DNA of *C. ditrichi* was first detected in faeces 6 DPI and intermittent shedding was detected in daily samples up to 30 DPI (Fig. 6). The SSU, actin and COWP sequences of *C. ditrichi* recovered from faecal samples of experimentally infected animals were identical to those in the inoculum. Oocysts were detected by microscopy only during the first 12 days of the patent period, with an infection intensity ranging from 5000 to 50,000 OPG. After 12 days, DNA of *C. ditrichi* was detected intermittently by PCR (Fig. 6). No macroscopical changes were observed in the gastrointestinal tract of yellow-necked mice positive for *C. ditrichi* and the surface epithelia were intact. Examination of the epithelium by histology and electron microscopy revealed the presence of developmental stages attached to the microvillar border in the posterior of the jejunum and the ileum (Figs. 7 and 8), and their absence from the first half of the small and large intestine. The *lamina propria* was slightly edematous with occasional dilatation of lymphatic vessels.

One of three BALB/c mice was susceptible to *C. ditrichi* infection. Specific DNA was detected from 5 to 13 DPI. All experimentally infected animals exhibited growth that was typical of their size and weight. None of the faecal samples was diarrhoeal.

Taxonomic summary

Cryptosporidium ditrichi sp. n.

Description. Oocysts are shed fully sporulated with 4 sporozoites and oocyst residuum inside. Sporulated oocysts (n = 50) measure 4.5–5.2 μm (mean = 4.7) × 4.0–4.6 μm (mean = 4.2) with a length to width ratio of 1.12 (1.0–1.2) (Fig. 9). Morphology and morphometry of other developmental stages is unknown.

Type host: yellow-necked mouse (*Apodemus flavicollis*).

Type locality: Branišov, Dolní Třebonín, Hluboká nad Vltavou, Opatovice and Vimperk (Czech Republic)

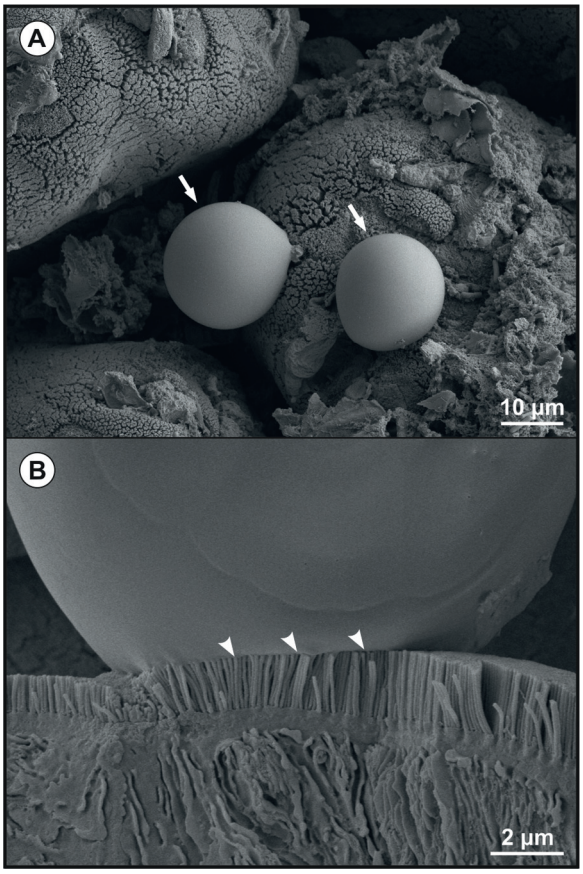

Fig. 8. Scanning electron photomicrograph of epithelium of jejunum of a yellow-necked mouse (*Apodemus flavicollis*) sacrificed 10 DPI. **(A)** Attached developmental stages (arrows) of *Cryptosporidium ditrichi* sp. n. **(B)** Detail of connection (arrow heads) between of parasitophorous sac and microvillous surface. Scale bar included in each picture.

Site of infection: small intestine – jejunum and ileum (Figs. 7 and 8)

Other hosts: mouse (*Mus musculus*), human (*Homo sapiens*)

Distribution: the Czech Republic and Slovakia

Material deposited: Slides with oocysts and DNA are deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic. Partial sequences of SSU, actin and COWP genes were deposited at GenBank (Acc. Nos. MG266030-MG266032, MG266039, MG266040 and MG266045).

Etymology: This species is named *Cryptosporidium ditrichi* sp. n. in honour of Dr. Oleg Ditrich, an accomplished teacher and parasitologist, and one of the pioneers of *Cryptosporidium* research in the Czech Republic.

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

Discussion

Rodents are naturally infected with several *Cryptosporidium* spp. (Feng 2010). Here, we report five different *Cryptosporidium* in *Apodemus* spp., including *C. parvum* and *C. muris*, species with a relatively broad host range, *C. hominis*, a human pathogen with a narrow host range, and two novel species, which we have named *C. apodemi* and *C. ditrichi*. *Cryptosporidium parvum*, *C. muris* and *C. hominis* have been reported in *Apodemus* species previously; however, consistent with our findings, the prevalence of *C. hominis* was very low (Danisova et al. 2017; Hajdušek et al. 2004; Perec-Matysiak et al. 2015; Song et al. 2015). The gp60 subtype family of the *C. hominis* isolate from *A. agrarius* in Slovakia in the present study was identical to that reported by Danisova et al. (2017) in the same species from the same country. Other *Cryptosporidium* spp. that have been reported previously in *Apodemus*, including *C. suis*, *C. scrofarum*, and muskrat genotypes I and II (Danisova et al. 2017; Hikosaka and Nakai 2005; Li et al. 2014; Murakoshi et al. 2013; Perec-Matysiak et al. 2015; Song et al. 2015), were not detected in the present study. The novel *Cryptosporidium* species reported in the present study have not been reported previously in *Apodemus* spp. However, *Cryptosporidium* sp. KSFM from *A. agrarius* in South Korea shares 99.1% identity with *C. apodemi* at the SSU locus Song et al. (2015). Other genotypes from *A. agrarius* and *A. chejuensis* in South Korea Song et al. (2015), which shared 92.9–98.6% similarity with the bear genotype, could have been similar to *C. ditrichi*, but the sequences were not published in GenBank so they could not be compared. *Cryptosporidium ditrichi* has been reported in raw water in Norway and the United Kington (Chalmers et al. 2010) and in a human infection in Sweden (Acc. No. KU892562; unpublished). *Apodemus* spp. are distributed throughout the Palearctic in Europe, and could have been the source of water contamination and human infection in these countries.

Cryptosporidium apodemi and *C. ditrichi* were not infectious for *Microtus arvalis* in experimental infections, which is consistent with the absence of these species from wild *Microtus* spp. sampled at the same location as *Apodemus* from the present study Stenger et al. (2017). The finding that *C. ditrichi* infected only one of three BALB/c mice under experimental conditions, and that the patent period was short and produced no detectable oocysts by microscopy, suggests that *M. musculus* is not a significant host. This is consistent with the absence of *C. ditrichi* from *M. musculus* in field studies (Kváč et al. 2014).

Phylogenetic analyses based on SSU, actin and COWP gene sequences showed that *Apodemus* spp. in this study

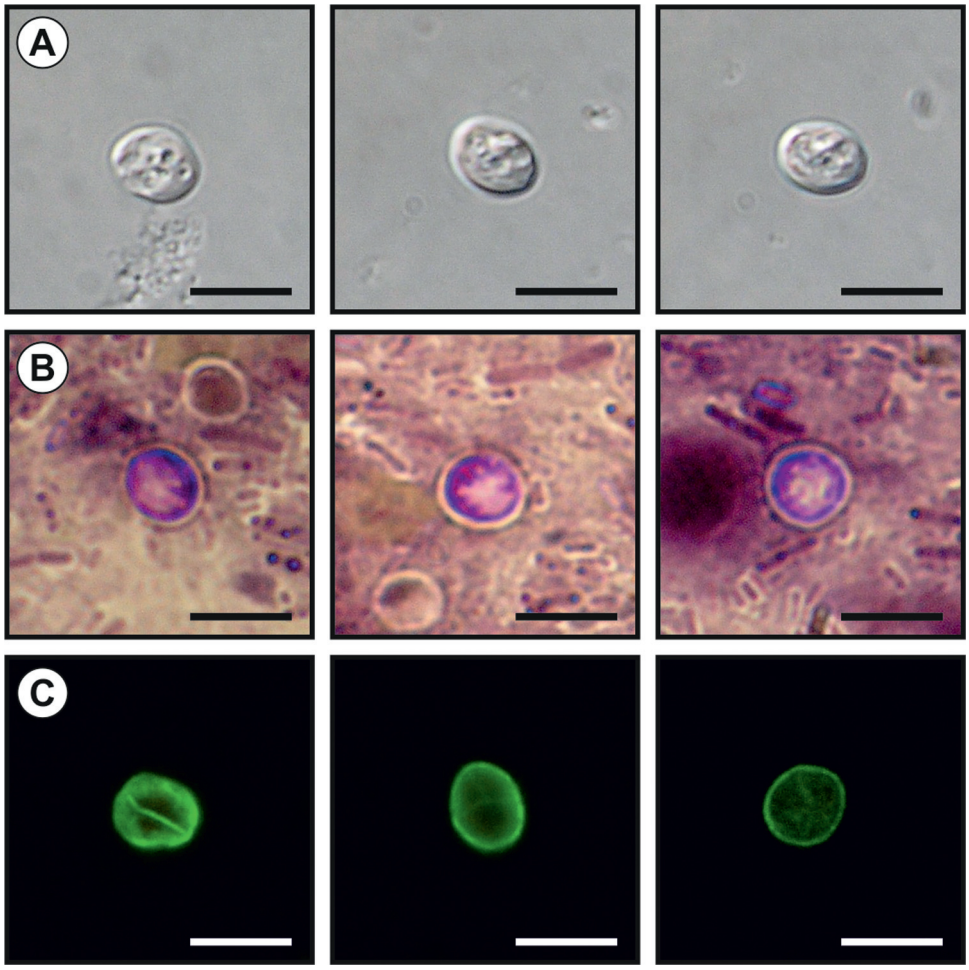

Fig. 9. *Cryptosporidium ditrichi* sp. n. oocysts visualized in various preparations: (A) differential interference contrast microscopy and stained by (B) aniline–carbol–methyl violet and (C) anti-*Cryptosporidium* FITC-conjugated antibody. Bar = 5 μm.

was frequently infected by two *Cryptosporidium* that are genetically distinct from previously described species. At the SSU locus, *C. apodemi* shared 97.0%, 95.1% and 93.0% sequence identity with *C. canis*, muskrat genotype I and *C. felis*, respectively. This is far greater than the identity of *C. hominis* and *C. cuniculus* (98.9%); *C. bovis* and *C. xiaoi* (99.5%). At the actin locus, *C. apodemi* shared 89.3% and 83.8% sequence identity with *C. canis* and *C. felis*, respectively, and at COWP locus, 93.9% and 87.2% sequence identity with the giant panda genotype and *C. ubiquitum*, respectively. SSU sequences of *C. ditrichi* clustered with SSU sequences reported from raw water, sharing 98.9–100% sequence identity. A sequence from a raw water sample was identical to one of the *C. ditrichi* variants. *Cryptosporidium* UK E6 [Acc. No. GQ183527] clusters within the *C. ditrichi* clade and should be considered a *C. ditrichi* variant. Intraspecific variability of SSU gene copies has been described in other *Cryptosporidium*, such as *C. parvum*, *C. hominis*, *C. andersoni* and *C. ubiquitum* (Fayer et al. 2010; Laatamna et al. 2015; Nagano et al. 2007; Xiao et al. 1999). Two actin sequence variants of *C. ditrichi* shared 99.8% identity. Similarly, actin variants were previously reported in *C. tyzzeri* Kváč et al. (2012). At the actin locus, *C. ditrichi* shared 95.5% and 91.9% sequence identity with the bear genotype and *C. canis*, respectively. At the COWP locus, *C. ditrichi* shared 93.9% and 85.4% sequence identity with the giant panda genotype and *C. ubiquitum*, respectively.

The morphology of oocysts of *C. apodemi* and *C. ditrichi* is typical of intestinal species of the genus *Cryptosporidium*. The size range of intestinal *Cryptosporidium* spp. mostly overlap (Fayer 2010), which is the case for *C. apodemi* and *C. ditrichi*. Although the mean size of *C. apodemi* is smaller than *C. ditrichi* it is not possible to distinguish these species microscopically in field samples.

Infections by *C. apodemi* and *C. ditrichi* produced no clinical signs in *Apodemus* spp. in the present study. This is consistent with the several studies, including studies on *Apodemus*, that have found wild animals to rarely develop clinical cryptosporidiosis (Bajer et al. 2003; Bednarska et al. 2007; Danisova et al. 2017; Hikosaka and Nakai 2005; Perec-Matysiak et al. 2015; Song et al. 2015; Torres et al. 2000).

Acknowledgements

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