Diversity of *Cryptosporidium* spp. in *Apodemus* spp. in Europe

ondlová Šárka[a,b], **Hor i ková Michaela**[a,b], **Havrdová Nikola**[a,b], **Sak Bohumil**[b], **Hlásková Lenka**[b], **Perec-Matysiak Agnieszka**[c], **Kicia Marta**[d], **McEvoy John**[e], **Kvá Martin**[a,b,*]

[a]Faculty of Agriculture, University of South Bohemia in eské Bud jovice, Czech Republic
[b]Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, eské Bud jovice, Czech Republic [c]Department of Parasitology, Institute of Genetics and Microbiology, University of Wrocław, Poland [d]Department of Biology and Medical Parasitology, Wroclaw Medical University, Poland [e]Microbiological Sciences Department, North Dakota State University, Fargo, USA

Abstract

The genetic diversity of *Cryptosporidium* spp. in *Apodemus* spp. (striped field mouse, yellow-necked mouse and wood mouse) from 16 European countries was examined by PCR/sequencing of isolates from 437 animals. Overall, 13.7% (60/437) of animals were positive for *Cryptosporidium* by PCR. Phylogenetic analysis of small-subunit rRNA, *Cryptosporidium* oocyst wall protein and actin gene sequences showed the presence of *C. ditrichi* (22/60), *C. apodemi* (13/60), *Cryptosporidium* apodemus genotype I (8/60), *Cryptosporidium* apodemus genotype II (9/60), *C. parvum* (2/60), *C. microti* (2/60), *C. muris* (2/60) and *C. tyzzeri* (2/60). At the gp60 locus, novel gp60 families XVIIa and XVIIIa were identified in *Cryptosporidium* apodemus genotype I and II, respectively, subtype IIaA16G1R1b was identified in *C. parvum*, and subtypes IXaA8 and IXcA6 in *C. tyzzeri*. Only animals infected with *C. ditrichi*, *C. apodemi*, and *Cryptosporidium* apodemus genotypes shed oocysts that were detectable by microscopy, with the infection intensity ranging from 2,000 to 52,000 oocysts per gram of faeces. None of the faecal samples was diarrheic in the time of the sampling.
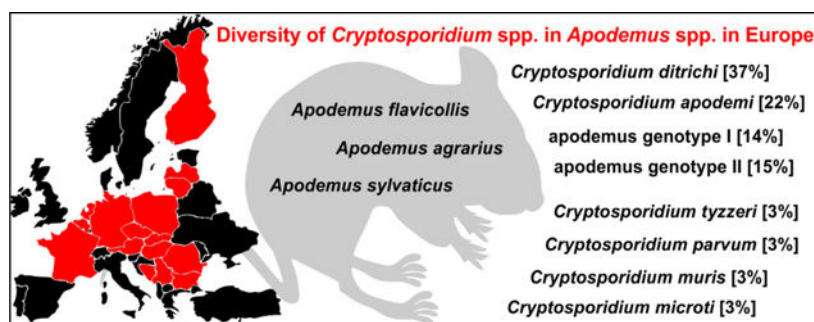
Graphical Abstract

[*]**Corresponding author:** Biology Centre of the Academy of Sciences of the Czech Republic, v.v.i., Branišovská 31, 370 05 eské Bud jovice, Czech Republic. Tel.: +420 387775419; fax: 420 385310388. kvac@paru.cas.cz (M. Kvá).

Diversity of *Cryptosporidium* spp. in *Apodemus* spp. in Europe

Apodemus flavicollis
Apodemus agrarius
Apodemus sylvaticus

Cryptosporidium ditrichi [37%]
Cryptosporidium apodemi [22%]
apodemus genotype I [14%]
apodemus genotype II [15%]
Cryptosporidium tyzzeri [3%]
Cryptosporidium parvum [3%]
Cryptosporidium muris [3%]
Cryptosporidium microti [3%]

Keywords

Epidemiology; Molecular analyses; Phylogeny; Rodentia

Introduction

Parasites of the genus *Cryptosporidium* (Apicomplexa), infect epithelial cells in the microvillus border of the gastrointestinal and respiratory tract of vertebrates (Cavalier-Smith 2014; Ryan and Xiao 2014; Striepen 2013). Studies on *Cryptosporidium* phylogenetics and biology have revealed extensive diversity and major differences in host specificity (Kvá et al. 2014b; Ryan and Xiao 2014). Species such as *C. parvum*, *C. baileyi*, *C. meleagridis* and *C. ubiquitum* exhibit relatively broad host specificity (Fayer 2007; Li et al. 2014; Nakamura and Meireles 2015; Stenger et al. 2015; Vetterling et al. 1971), while other species are specific to one or more closely related hosts. Determining the host range of *Cryptosporidium* can be complicated by the occurrence of mechanical passage, whereby low numbers of oocysts pass through the animal without causing an active infection and are detected in the faeces (Graczyk et al. 1998; Kvá et al. 2012). Mechanical passage is exemplified by reports of rodent-adapted *C. muris* in the faeces of snakes and lizards (Crawshaw and Mehren 1987; Graczyk et al. 1996; Xiao et al. 2004), which was due to the animals ingesting infected mice (Xiao et al., 2004). Similarly, *C. muris* and the house mouse-adapted *C. tyzzeri* have been detected in pig faeces and slurry (Kvá et al. 2012), probably as a result of the pigs ingesting infected mice or food contaminated by mouse faeces (Jenkins et al. 2010; Ren et al. 2012; Xiao et al. 2006), and *C. bovis*, a species that is specific for cattle (Fayer et al. 2005), was detected in a fully habituated western lowland gorilla, probably because the gorilla's environment was contaminated by grazing cattle (Sak et al. 2014).

Several *Cryptosporidium* spp. identified in murid rodents from the genus *Apodemus* are specific for other hosts (e.g. *C. hominis*, *C. muris*, *C. parvum*, *C. scrofarum*, *C. suis*, *C. ubiquitum*, *Cryptosporidium* chipmunk genotype I, *Cryptosporidium* muskrat genotype II and *Cryptosporidium* Naruko genotype) (Danišová 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). ondlová et al. (2018) recently described *C. apodemi* and *C. ditrichi* in species of *Apodemus* and presented evidence from experimental and field studies that these species are host specific. Given the limited scope of the original

study, the present study aimed at describing the occurrence of *C. apodemi* and *C. ditrichi* across 16 European countries.

Material and Methods

Ethics statement

Traps were checked frequently and handling time was minimized to reduce animal stress, and all applicable international, national and institutional guidelines for the care and use of animals were followed. The research was conducted under ethical protocols approved by the Institute of Parasitology, Biology Centre and Central Commission for Animal Welfare, the Czech Republic.

Sample collection and parasitological examination

From May to September in 2016 and 2017, wild *Apodemus* spp. were trapped in Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Czech Republic, Finland, France, Germany, Hungary, Latvia, Lithuania, Netherlands, Poland, Romania, Serbia and Slovakia (Table 1). Animals were captured with sterile live- or snap-traps baited with smoked cheese and were examined to determine the species. For live-trapped animals, faecal samples were collected directly from the trap or from the animal during handling and the animal was subsequently released. For snap-trapped animals, faecal samples were collected from the colon after animal dissection. Each faecal sample was stored in a separate screw-cap container, transported to the laboratory and screened for the presence of *Cryptosporidium* oocysts by brightfield microscopy (Olympus BX51, Tokyo, Japan), at 1,000× magnification, following aniline-carbol-methyl violet staining (Miláek and Vítovec 1985). The infection intensity was determined as the number of oocysts per gram (OPG) of faeces in accordance with Kvá et al. (2007).

Molecular characterisation

DNA was extracted from 100–200 mg of faeces by bead disruption for 60 s at 5.5 m/s using 0.5 mm glass beads in a Fast Prep 24 Instrument (MP Biomedicals, CA, USA), followed by isolation and purification using a commercially available kit in accordance with the manufactures instructions (Exgene™ Stool DNA mini, GeneAll Biotechnology Co. Ltd, Seoul, Korea). Purified DNA was stored at −20°C prior to amplification by PCR. A nested PCR approach was used to amplify a partial region of genes encoding the small ribosomal subunit rRNA (SSU; ~830 bp; Jiang et al. 2005; Xiao et al. 1999), actin (~1066 bp; Sulaiman et al. 2002), *Cryptosporidium* oocyst wall protein (COWP; ~550 bp; Spano et al. 1997) and 60 kDa glycoprotein (gp60; ~850 bp; Alves et al. 2003; Li et al. 2014). 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), 0.5× PCR buffer (SSU) or 1× PCR buffer (actin, COWP and gp60; Thermofisher Scientific), 6 mM $MgCl_2$ (SSU) or 3 mM $MgCl_2$ (actin, COWP and gp60), 200 μM each deoxynucleoside triphosphate (dNTP), 200 mM each primer and 2 μl nonacetylated bovine serum albumin (BSA; 10 mg/ml; New England Biolabs, Beverly, MA) in 50 μl reaction volume.

Phylogenetic analysis

Secondary PCR products were separated on an agarose gel and visualized under UV illumination using ethidium bromide staining. Products were purified (Gen Elute Gel Extraction Kit, Sigma, St. Louis, MO) and sequenced in both directions with secondary primers using a Big Dye Terminator v3.1 cycle sequencing kit in an ABI Prism 3130 genetic analyzer (Applied Biosystems, Carlsbad, CA). The nucleotide sequences of each gene obtained in this study were manually edited using the program Chromas Pro 2.1.4 (Technelysium, Pty, Ltd., South Brisbane, Australia), and aligned with previously published sequences using the MAFFT version 7 online server using the Q-INS-I algorithm for SSU, actin and COWP sequences and L-INS-I algorithm for gp60 sequences (http://mafft.cbrc.jp/alignment/server/). The alignment included published sequences from *Cryptosporidium* species and sequences with a high similarity to study sequences using BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). 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 gp60 alignments was done in the MEGA7 software. The Tamura 3-parameter model was selected for SSU, COWP and gp60 and the General Time Reversible model was used for actin alignment. All models were used under an assumption that rate variation among sites was gamma 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.) MH912926–MH912969 for COWP, MH912970–MH912990 for gp60, MH912991–MH913050 for SSU and MH913051–MH913110 actin gene.

Results

In total, 437 animals from the genus *Apodemus*, comprising 62 striped field mice (*Apodemus agrarius*), 325 yellow-necked mice (*Apodemus flavicollis*) and 50 wood mice (*Apodemus sylvaticus*), were sampled in 16 European countries (Table 1). Overall, 13.7% (60/437) of *Apodemus* spp. tested positive for *Cryptosporidium* by PCR (Table 1).

Out of 60 *Cryptosporidium* positive animals, 60, 60, 45 and 21 were genotyped by sequence analysis of SSU, actin, COWP and gp60 genes, respectively (Table 1). The remaining positive samples failed to amplify at COWP (n=15) and gp60 (n=39) loci. MP trees constructed from SSU, actin and COWP gene sequences in this study and representative sequences in GenBank showed the presence of eight *Cryptosporidium* spp. (Figs. 1–3). *Cryptosporidium ditrichi* (n=22) and *C. apodemi* (n=13), species that have been reported as *Apodemus* specific, were the most prevalent species in screened animals. In contrast, *C. parvum* (n=2), *C. muris* (n=2), *C. tyzzeri* (n=2) and *C. microti* (n=2), which are specific for other mammals, were detected rarely. Phylogenetic analysis of screened genes revealed the presence of two novel *Cryptosporidium* genotypes, which were named *Cryptosporidium* apodemus genotype I (n=8) and apodemus genotype II (n=9). SSU, actin and COWP sequences of these novel genotypes formed distinct groups that were closely related to each other and to *C. ubiquitum*. gp60 sequences of *Cryptosporidium* apodemus genotypes I and II clustered with isolates originally reported from *Apodemus* spp. and identified as *C.*

ubiquitum XIIe and XIIf, respectively (Fig. 4). In accordance with the gp60 nomenclature established by Sulaiman et al. (2005) we named the gp60 family of *Cryptosporidium* apodemus genotypes I and II as novel family XVII (previously known as *C. ubiquitum* XIIe) and XVIII (previously known as *C. ubiquitum* XIIf), respectively.

A ML tree constructed using gp60 sequences obtained in this study and sequences published in GenBank revealed that the *C. parvum* isolates from the Czech Republic and Germany belonged to family IIa subtype A16G1R1b, *C. tyzzeri* from the Czech Republic belonged to family IXa and *C. tyzzeri* from Serbia to novel family IXc (Fig. 4).

Cryptosporidium ditrichi, C. apodemi, *Cryptosporidium* apodemus genotype I and *Cryptosporidium* apodemus genotype II were detected in ten, five, three and five out of 16 countries, respectively (Table 1 and Fig. 1).

Out of the 60 animals that tested positive for *Cryptosporidium* by PCR, 31 (51.7%) shed oocysts that were detectable by microscopy. The infection intensity in microscopy positive animals ranged from 2,000 to 52,000 OPG (Table 1). Only animals that were PCR positive for *C. ditrichi*, *C. apodemi* or *Cryptosporidium* apodemus genotypes I and II shed detectable oocysts. Out of 22 animals positive for *C. ditrichi*, 16 (73%) shed detectable oocysts (2,000–52,000 OPG). Similarly, 75% (6/8) of animals infected with *Cryptosporidium* apodemus genotype I shed detectable oocysts (2,000–8,000 OPG). In contrast, only 38% (5/13) and 44% (4/9) of animals positive for *C. apodemi* and *Cryptosporidium* apodemus genotype II, respectively, shed detectable oocysts with an infection intensity ranging from 2,000 to 4,000 OPG. None of the faecal samples was diarrhoeic at the time of sampling.

Discussion

Microscopy-based tools used in early studies on *Cryptosporidium* from *Apodemus* were poor at distinguishing among species and genotypes. Hence, oocysts that were morphometrically similar to *C. parvum* were identified as *C. parvum* and those that were similar to *C. muris* were identified as that gastric species (Bednarska et al. 2007; Chalmers et al. 1997; Torres et al. 2000; Webster and Macdonald 1995a, b). When molecular tools were used in later studies, *C. parvum* was found to be an infrequent parasite of *Apodemus* (ondlová et al. 2018; Murakoshi et al. 2013; Perec-Matysiak et al. 2015; Song et al. 2015) and other rodents such as mice, rats, voles and squirrels (Hor i ková et al. 2018; Lv et al. 2009; Ng-Hublin et al. 2013; Prediger et al. 2017; Saki et al. 2016; Stenger et al. 2015). Consistent with those studies, we detected *C. parvum* in only two out of 60 animals that were positive for *Cryptosporidium*. The gp60 subtype of both isolates, IIaA16G1R1, and the subtype reported by Danišová et al. (2017) in *Apodemus*, IIaA18G3R1, are common in domesticated ruminants worldwide (Del Coco et al. 2014; Imre et al. 2013; Ng et al. 2012; Trotz-Williams et al. 2006; Wielinga et al. 2008). These findings suggest that *Apodemus* species are not major hosts of *C. parvum* and are probably only transiently infected following exposure to contaminated manure from ruminants. This is supported by our experimental studies, which showed that *C. parvum* is poorly infective for *A. sylvaticus*, infecting only one of three inoculated animals (unpublished data).

We found *C. muris* in only two animals (0.5%), consistent with the low prevalence reported in previous studies in the Czech Republic, Slovakia and Poland (ondlová et al. 2018; Danišová et al. 2017; Perec-Matysiak et al. 2015). A higher prevalence was reported in studies in the UK (5%; Chalmers et al. 1997), Spain (9.7%; Torres et al. 2000), Japan (13.3%; Murakoshi et al. 2013) and Korea (5.2–7.1%; Song et al. 2015). In Europe, all *C. muris* isolates that were genotyped were identified as RN66, a strain that is found predominantly in rats and house mice worldwide (Backhans et al. 2013; Iseki et al. 1989; Kvá et al. 2012; Rhee et al. 1995; Satoh et al. 2003; Xiao et al. 1999). In contrast, all isolates from Japan and Korea were identified as the Japanese field mouse genotype (Murakoshi et al. 2013; Song et al. 2015), which has only been found in *Apodemus* spp. in Japan and Korea.

This is the first report of *C. tyzzeri* in *Apodemus*. However, the low prevalence (0.5%) and the reported specificity of *C. tyzzeri* for the house mouse (Kvá et al. 2013), suggests that *Apodemus* spp. are minor hosts. Kvá et al. (2013) identified two major gp60 subtypes (IXa and IXb) of *C. tyzzeri* and showed that they had different natural host specificities – IXa was restricted to the house mouse subspecies *Mus musculus musculus* (Mmm) and IXb was restricted to *Mus musculus domesticus* (Mmd). In the present study, one of the *C. tyzzeri* isolates was the IXa subtype, and, consistent with the previous work by Kvá et al. (2013), it was recovered from an animal trapped in the Czech Republic, where only Mmm are found. The *C. tyzzeri* isolate from Serbia, an area that also only has Mmm (de Bellocq et al. 2015), had a novel gp60 subtype, IXc.

Cryptosporidium microti was detected in two animals in the present study. Given that this species is specific for voles and is not infectious for *Apodemus* spp. under experimental conditions (Hor i ková et al. 2018), its presence here may be the result of passive passage of oocysts ingested from a contaminated environment in an area of overlapping *Apodemus* and vole (*Microtus* spp.) habitats.

Cryptosporidium ditrichi and *C. apodemi* were the most prevalent species in this study and were found throughout Europe (12/16 countries). This is consistent with a previous study by ondlová et al. (2018), who found that these were the most prevalent species in *Apodemus* in the Czech Republic and Slovakia and showed that they were specifically infective for *Apodemus* under experimental conditions. Remarkably, only one other study of *Apodemus* reported a similar genotype. Song et al. (2015) identified *Cryptosporidium* sp. isolate KSFM, which shares 99.1% identity with *C. apodemi* at the SSU locus, from *A. agrarius* in South Korea. In the present study, *C. ditrichi* and *C. apodemi* were distributed in countries throughout Europe (12/16 countries), including Poland and Slovakia, where previous studies have failed to detect these species in *Apodemus* (Danišová et al. 2017; Perec-Matysiak et al. 2015). Although *Cryptosporidium* prevalence in host populations can fluctuate across locations, seasons and years (Bajer et al. 2002; Perec-Matysiak et al. 2015; Petersen et al. 2015), the absence of these species from previous studies is striking.

After *C. ditrichi* and *C. apodemi*, *Cryptosporidium* apodemus genotypes I and II were the next most frequently isolated *Cryptosporidium* from *Apodemus* spp. in the present study. *Cryptosporidium* apodemus genotype I was isolated previously from *A. flavicollis* in Poland

Perec-Matysiak et al. (2015) and identified as *C. ubiquitum* 4-O-10 [GenBank Acc. No. KC962124]. *Cryptosporidium* apodemus genotype II was detected previously in surface water in Japan and also identified as *C. ubiquitum* [GenBank Acc. No. AB694733; reported as the cervine genotype, which is the previous name for *C. ubiquitum*]. At the gp60 locus, *Cryptosporidium* apodemus genotypes I and II were identical to *C. ubiquitum* XIIe and XIIf, respectively, which were previously reported from *Apodemus agrarius* and *A. flavicollis* in Slovakia (Li et al. 2014). However, phylogenetic analysis based on SSU, actin and COWP gene sequences show that these genotypes are distinct from *C. ubiquitum*. Therefore, in accordance with the gp60 subtyping nomenclature (Lv et al. 2009; Sulaiman et al. 2005), we propose to rename the gp60 family XVIIa for *Cryptosporidium* apodemus genotype I and family XVIIIa for *Cryptosporidium* apodemus genotype II.

Consistent with most reports describing natural and experimental infections with *Cryptosporidium* spp. in wild animals (Castro-Hermida et al. 2011; ondlová et al. 2018; Holubová et al. 2016; Hor i ková et al. 2018; Kvá et al. 2014a; Li et al. 2015; Prediger et al. 2017), *Apodemus* spp. shed low numbers of oocysts, often below the detection limit of microscopy, and showed no clinical signs of cryptosporidiosis. This is in contrast to the high oocyst shedding and diarrhea caused by species such as *C. parvum* (in humans and neonatal livestock), *C. hominis* (in humans) and *C. meleagridis* (in humans and poultry) (Bouzid et al. 2013; Fayer et al. 1998; Jacobson et al. 2016; Papanikolopoulou et al. 2018; Pedraza-Diaz et al. 2001; T mová et al. 2002). The different outcomes of *Cryptosporidium*-host interactions are probably the result of coevolutionary adaptations, but the reason for the differences in infection intensity of *Cryptosporidium* in different hosts is not currently known.

Conclusions

Apodemus spp. in Europe are frequently infected with four *Cryptosporidium* species/genotypes – *C. apodemi*, *C. ditrichi*, *Cryptosporidium* apodemus genotype I and *Cryptosporidium* apodemus genotype II. Data from this and previous studies show that *Apodemus* spp. are minor hosts of *C. parvum*, *C. muris*, *C. tyzzeri*, *C. microti*, *C. ubiquitum*, *C. scrofarum*, *C. suis*, *C. hominis*, *Cryptosporidium* muskrat genotype II or *Cryptosporidium* chipmunk genotype I.

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Highlights

- Eight *Cryptosporidium* species detected in mice of the genus *Apodemus*

- *Apodemus* spp. in Europe are frequently infected with four *Cryptosporidium* spp.

- *Cryptosporidium apodemi* and *C. ditrichi* are most prevalent in *Apodemus* spp. Mice

- Novel *Cryptosporidium* apodemus genotype I and II were described in *Apodemus* spp.

- *Apodemus* spp. are minor hosts of *C. parvum*, *C. muris*, *C. tyzzeri* and *C. microti*
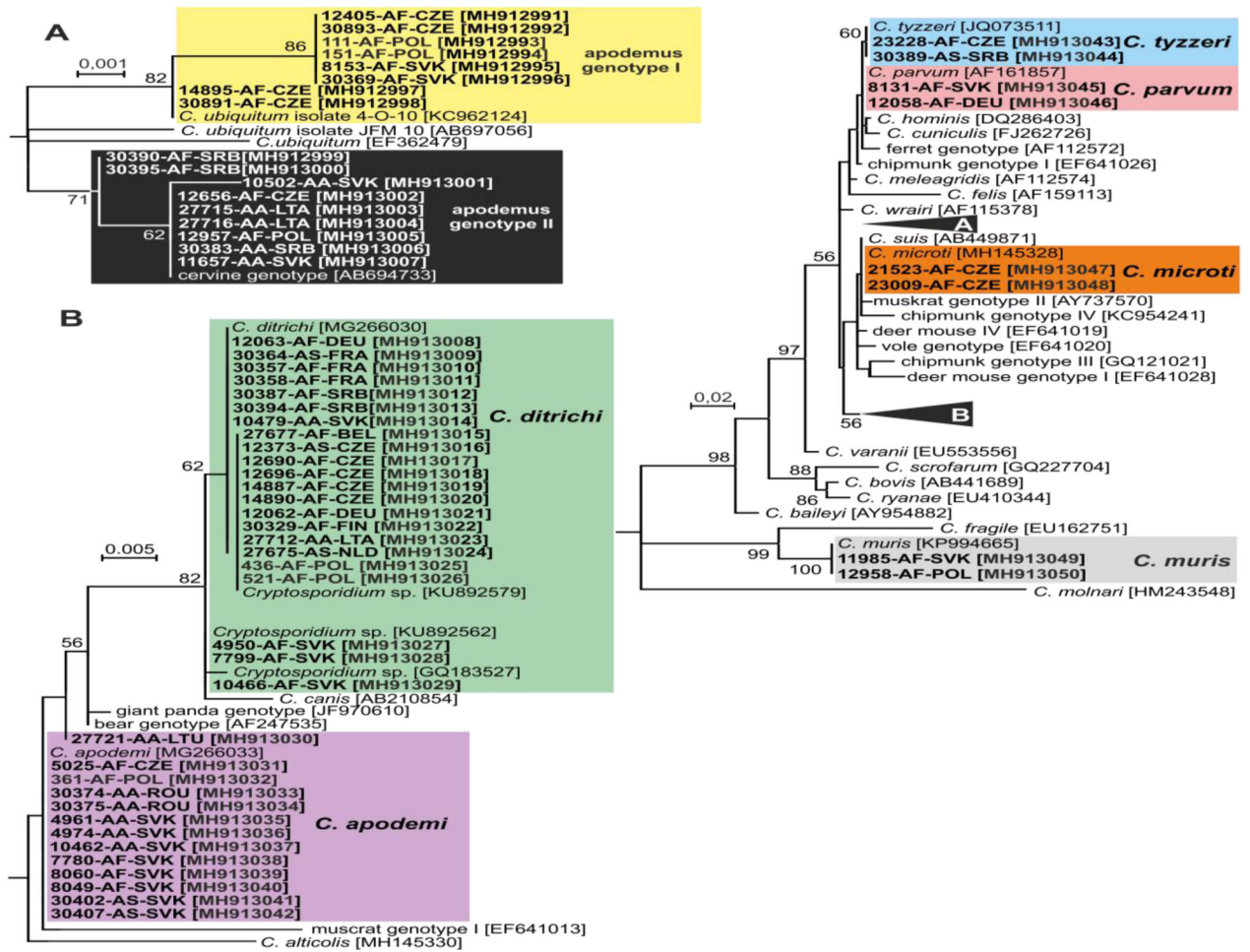
Fig. 1.

Maximum likelihood tree (−ln = 3040.40) based on partial small subunit ribosomal RNA gene sequences of *Cryptosporidium*, including sequences obtained in this study (highlighted and bolded). The alignment contained 780 base positions in the final dataset. Tamura's 3-parameter model was applied, using a discrete Gamma distribution and invariant sites. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates. The branch length scale bar, indicating the number of substitutions per site, is given in the tree. Sequences from this study are identified by isolate number (e.g. 8131), host species (AA for *Apodemus agrarius*, AF for *Apodemus flavicollis* and AS for *Apodemus sylvaticus*) and region (BEL for Belgium, CZE for Czech Republic, FIN for Finland, FRA for France, DEU for Germany, LTA for Latvia, LTU for Lithuania, NLD for Nederland, POL for Poland, ROU for Romania, SRB for Serbia and SVK for Slovakia).

Fig. 2.

Maximum likelihood tree (−ln = 7878.45) based on partial sequences of gene coding actin of *Cryptosporidium*, including sequences obtained in this study (highlighted and bolded). The alignment contained 695 base positions in the final dataset. The General Time Reversible model was applied, using a discrete Gamma distribution and invariant sites. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates. The branch length scale bar, indicating the number of substitutions per site, is given in the tree. Sequences from this study are identified by isolate number (e.g. 8131), host species (AA for *Apodemus agrarius*, AF for *Apodemus flavicollis* and AS for *Apodemus sylvaticus*) and region (BEL for Belgium, CZE for Czech Republic, FIN for Finland, FRA for France, DEU for Germany, LTA for Latvia, LTU for Lithuania, NLD for Nederland, POL for Poland, ROU for Romania, SRB for Serbia and SVK for Slovakia).
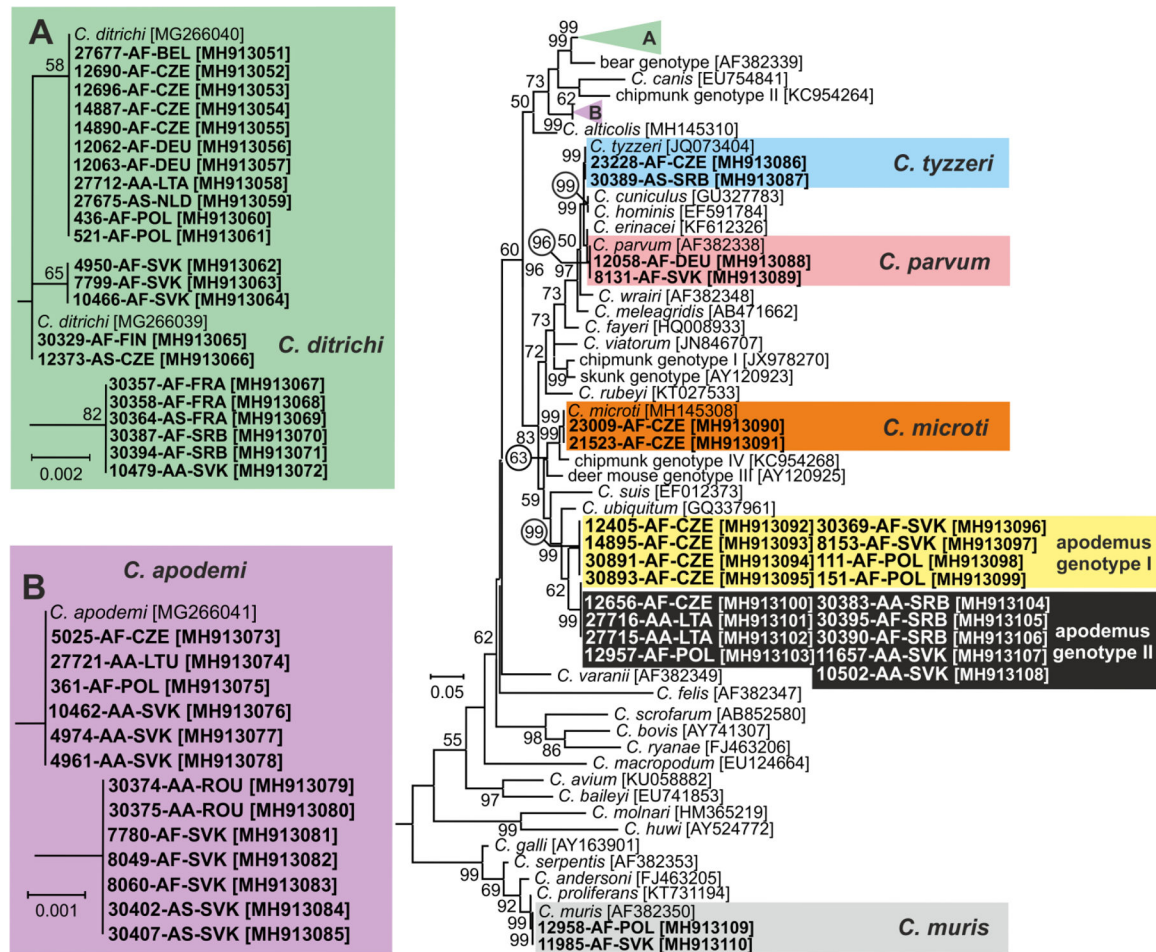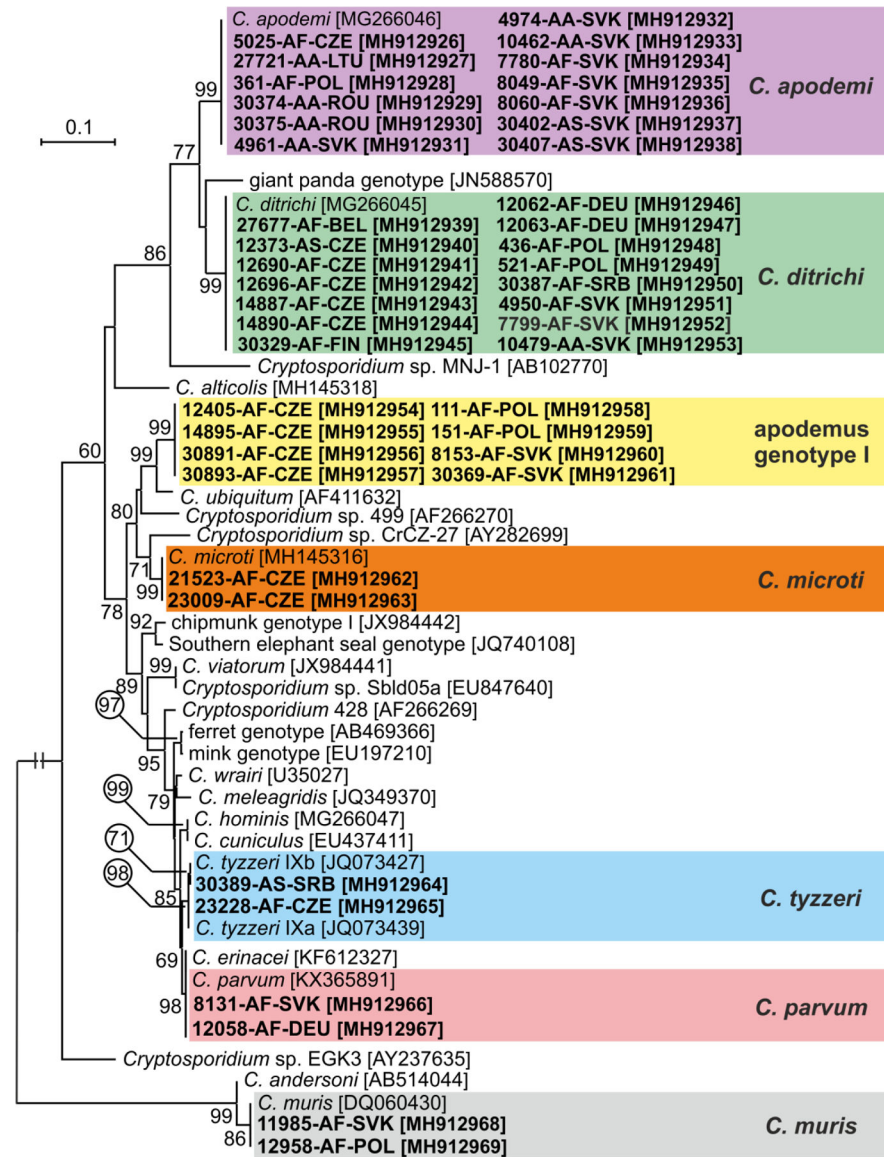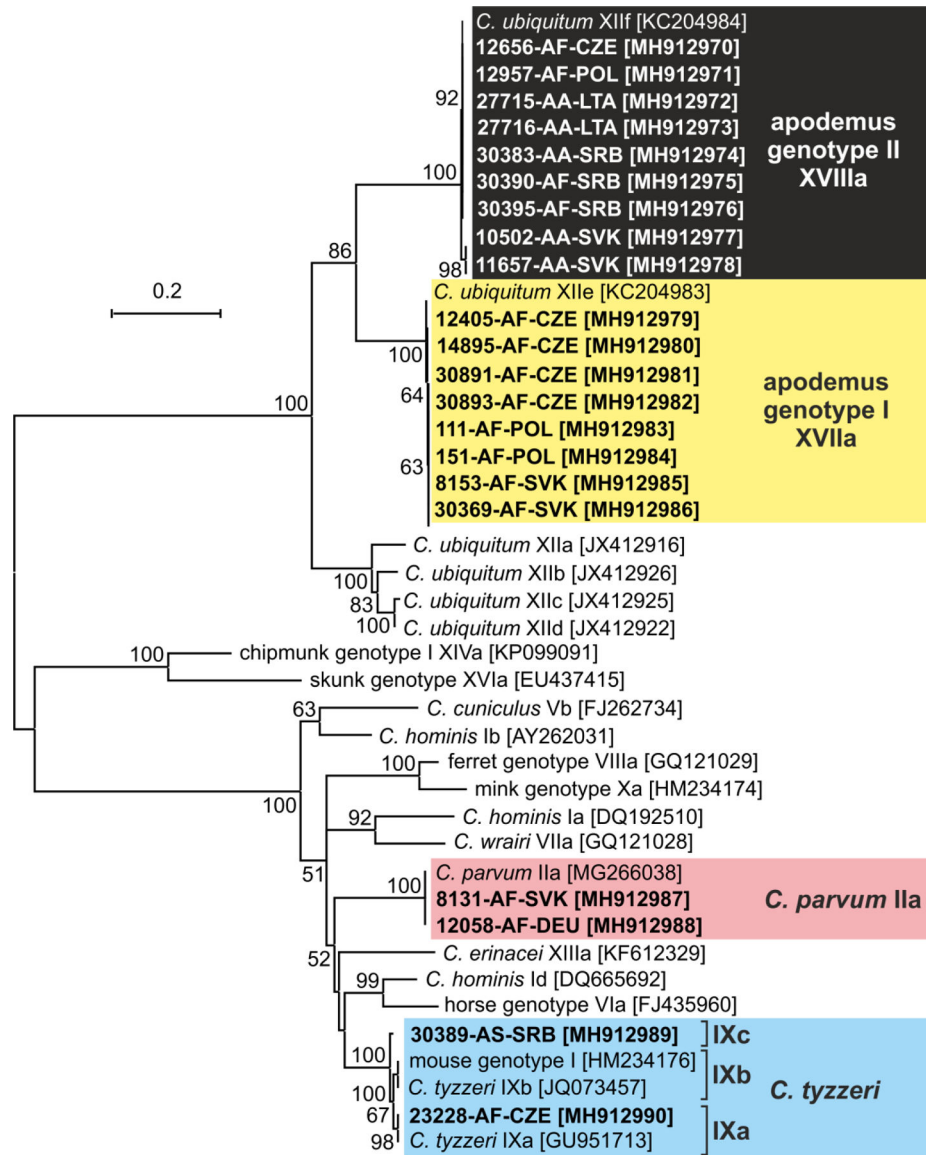
Fig. 3.

Maximum likelihood tree (-ln = 2652.86) based on partial sequences of gene coding *Cryptosporidium* oocyst wall protein (COWP), including sequences obtained in this study (highlighted and bolded). The alignment contained 455 base positions in the final dataset. Tamura's 3- parameter model was applied, using a discrete Gamma distribution and invariant sites. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates. The branch length scale bar, indicating the number of substitutions per site, is given in the tree. Sequences from this study are identified by isolate number (e.g. 8131), host species (AA for *Apodemus agrarius*, AF for *Apodemus flavicollis* and AS for *Apodemus sylvaticus*) and region (BEL for Belgium, CZE for Czech Republic, FIN for Finland, DEU for Germany, LTU for Lithuania, POL for Poland, ROU for Romania, SRB for Serbia and SVK for Slovakia).

Fig. 4.

Maximum likelihood tree (−ln = 6245.99) based on partial sequences of gene coding 60 kDa glycoprotein of *Cryptosporidium* (gp60), including sequences obtained in this study (highlighted and bolded). The alignment contained 947 base positions in the final dataset. Tamura's 3- parameter model was applied, using a discrete Gamma distribution and invariant sites. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates. The branch length scale bar, indicating the number of substitutions per site, is given in the tree. Sequences from this study are identified by isolate number (e.g. 8131), host species (AA for *Apodemus agrarius*, AF for *Apodemus flavicollis* and AS for *Apodemus sylvaticus*) and region (CZE for Czech Republic, DEU for Germany, LTA for Latvia, POL for Poland, SRB for Serbia and SVK for Slovakia).

Table 1.

Cryptosporidium species and genotypes based on amplification of the small subunit ribosomal rRNA (SSU), actin, *Cryptosporidium* oocyst wall protein (COWP) and 60kDa glycoprotein (gp60) genes by PCR in samples of striped field mice (*Apodemus agrarius*), yellow-necked mice (*Apodemus flavicollis*) and wood mice (*Apodemus sylvaticus*) from Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Czech Republic, Finland, France, Germany, Hungary, Latvia, Lithuania, Netherlands, Poland, Romania, Serbia and Slovakia.

Country	Host	N	Isolate ID	Microscopical positivity (OPG)	Genotyping at the loci			
					SSU	actin	COWP	gp60
Austria	*A. flavicollis*	5	-	-	-	-	-	-
	A. sylvaticus	3	-	-	-	-	-	-
Belgium	*A. flavicollis*	2	27677	Yes (4,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-
Bosnia and Herzegovina	*A. flavicollis*	1	-	-	-	-	-	-
Bulgaria	*A. agrarius*	5	-	-	-	-	-	-
	A. flavicollis	3	-	-	-	-	-	-
	A. sylvaticus	2	-	-	-	-	-	-
Czech Republic	*A. flavicollis*	153	14887	Yes (28,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-
			14890	Yes (2,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-
			12690	Yes (24,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-
			12696	Yes (52,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-
			5025	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	-
			12405	Yes (4,000)	apodemus genotype I	apodemus genotype I	apodemus genotype I	XVIIa
			30891	Yes (2,000)	apodemus genotype I	apodemus genotype I	apodemus genotype I	XVIIa
			30893	Yes (6,000)	apodemus genotype I	apodemus genotype I	apodemus genotype I	XVIIa
			23228	No	*C. tyzzeri*	*C. tyzzeri*	*C. tyzzeri*	IXaA8
			14895	Yes (2,000)	apodemus genotype I	apodemus genotype I	apodemus genotype I	XVIIa
			12656	No	apodemus genotype II	apodemus genotype II	-	XVIIIa
			21523	No	*C. microti*	*C. microti*	*C. microti*	-
			23009	No	*C. microti*	*C. microti*	*C. microti*	-
	A. sylvaticus	23	12373	Yes (8,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-
Finland	*A. flavicollis*	2	30329	Yes (2,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-

Country	Host	N	Isolate ID	Microscopical positivity (OPG)	Genotyping at the loci			
					SSU	actin	COWP	gp60
France	*A. flavicollis*	16	30357	Yes (6,000)	*C. ditrichi*	*C. ditrichi*	-	-
			30358	Yes (2,000)	*C. ditrichi*	*C. ditrichi*	-	-
	A. sylvaticus	4	30364	Yes (4,000)	*C. ditrichi*	*C. ditrichi*	-	-
Germany	*A. flavicollis*	10	12058	No	*C. parvum*	*C. parvum*	*C. parvum*	IIaA16GlRlb
			12062	No	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-
			12063	No	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-
Hungary	*A. agrarius*	4	-	-	-	-	-	-
Latvia	*A. agrarius*	11	27716	Yes (6,000)	apodemus genotype II	apodemus genotype II	-	XVIIIa
			27715	Yes (4,000)	apodemus genotype II	apodemus genotype II	-	XVIIIa
			27712	Yes (2,000)	*C. ditrichi*	*C. ditrichi*	-	-
Lithuania	*A. agrarius*	3	27721	Yes (2,000)	*C. apodemi*	*C. apodemi*	*C. apodemi*	-
Netherlands	*A. sylvaticus*	6	27675	Yes (6,000)	*C. ditrichi*	*C. ditrichi*	-	-
Poland	*A. flavicollis*	83	12958	No	*C. muris*	*C. muris*	*C. muris*	
			12957	No	apodemus genotype II	apodemus genotype II	-	XVIIIa
			151	No	apodemus genotype I	apodemus genotype I	apodemus genotype I	XVIIa
			111	Yes (2,000)	apodemus genotype I	apodemus genotype I	apodemus genotype I	XVIIa
			436	Yes (6,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-
			521	Yes (4,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-
			361	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	-
Romania	*A. agrarius*	2	30374	Yes (2,000)	*C. apodemi*	*C. apodemi*	*C. apodemi*	-
			30375	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	-
	A. flavicollis	1	-	-	-	-	-	-
	A. sylvaticus	1	-	-	-	-	-	-
Serbia	*A. agrarius*	4	30383	Yes (2,000)	*apodemus genotype II*	*apodemus genotype II*	-	XVIIIa

Country	Host	N	Isolate ID	Microscopical positivity (OPG)	Genotyping at the loci			
					SSU	actin	COWP	gp60
	A. flavicollis	14	30395	No	*apodemus genotype II*	*apodemus genotype II*	-	XVIIIa
			30390	Yes (2,000)	*apodemus genotype II*	*apodemus genotype II*	-	XVIIIa
			30387	Yes (22,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-
			30394	No	*C. ditrichi*	*C. ditrichi*	-	-
	A. sylvaticus	3	30389	No	*C. tyzzeri*	*C. tyzzeri*	*C. tyzzeri*	IXcA6
Slovakia	*A. agrarius*	33	10502	No	*apodemus genotype II*	*apodemus genotype II*	-	XVIIIa
			11657	No	*apodemus genotype II*	*apodemus genotype II*	-	XVIIIa
			10462	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	-
			4974	Yes (2,000)	*C. apodemi*	*C. apodemi*	*C. apodemi*	-
			4961	Yes (4,000)	*C. apodemi*	*C. apodemi*	*C. apodemi*	-
			10479	No	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-
	A. flavicollis	35	11985	No	*C. muris*	*C. muris*	*C. muris*	
			7799	No	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-
			4950	Yes (4,000)	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	-
			8131	No	*C. parvum*	*C. parvum*	*C. parvum*	IIaA16GlRlb
			10466	No	*C. ditrichi*	*C. ditrichi*	*C. ditrichi*	
			30369	Yes (8,000)	apodemus genotype I	apodemus genotype I	apodemus genotype I	XVIIa
			8153	No	apodemus genotype I	apodemus genotype I	apodemus genotype I	XVIIa
			8049	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	-
			8060	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	-
			7780	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	-
	A. sylvaticus	8	30402	Yes (4,000)	*C. apodemi*	*C. apodemi*	*C. apodemi*	-
			30407	No	*C. apodemi*	*C. apodemi*	*C. apodemi*	-

Oocysts were quantified by microscopy and reported per gram of faeces (OPG); **N** – Number of examined samples; **ID** – identification; **No** – microscopically negative; **Yes** – Microscopically positive