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Coevolution of *Cryptosporidium tyzzeri* and the house mouse (*Mus musculus*)★

Martin Kváček^{a,b}, John McEvoy^{c,*}, Martina Loudová^d, Brianna Stenger^c, Bohumil Sak^a, Dana Kváčová^a, Oleg Ditrich^a, Veronika Rašková^{a,b}, Elaine Moriarty^e, Michael Rost^f, Miloš Macholán^g, and Jaroslav Piálek^h

^aInstitute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, Brno, Czech Republic ^bFaculty of Agriculture, University of South Bohemia in České Budějovice, Czech Republic ^cDepartment of Veterinary and Microbiological Sciences, North Dakota State University, PO Box 6050, Dept. 7690, Fargo, ND 58108, USA ^dFaculty of Science, University of South Bohemia in České Budějovice, Czech Republic ^eChristchurch Science Centre, Institute of Environmental Science and Research Ltd, Christchurch, New Zealand ^fFaculty of Economics, University of South Bohemia in České Budějovice, Czech Republic ^gLaboratory of Mammalian Evolutionary Genetics, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Brno, Czech Republic ^hInstitute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Brno, Czech Republic

Abstract

Two house mouse subspecies occur in Europe, eastern and northern *Mus musculus musculus* (Mmm) and western and southern *Mus musculus domesticus* (Mmd). A secondary hybrid zone occurs where their ranges meet, running from Scandinavia to the Black Sea. In this paper, we tested a hypothesis that the apicomplexan protozoan species *Cryptosporidium tyzzeri* has coevolved with the house mouse. More specifically, we assessed to what extent the evolution of this parasite mirrors divergence of the two subspecies. In order to test this hypothesis, we analyzed sequence variation at five genes (ssrRNA, *Cryptosporidium* oocyst wall protein (COWP), thrombospondin-related adhesive protein of *Cryptosporidium* 1 (TRAP-C1), actin and gp60) in *C. tyzzeri* isolates from Mmd and Mmm sampled along a transect across the hybrid zone from the Czech Republic to Germany. Mmd samples were supplemented with mice from New Zealand. We found two distinct isolates of *C. tyzzeri*, each occurring exclusively in one of the mouse subspecies (*C. tyzzeri*-Mmm and *C. tyzzeri*-Mmd). In addition to genetic differentiation, oocysts of the *C. tyzzeri*-Mmd subtype (mean: $4.24 \times 3.69 \mu\text{m}$) were significantly smaller than oocysts of *C. tyzzeri*-Mmm (mean: $4.49 \times 3.90 \mu\text{m}$). Mmm and Mmd were susceptible to experimental infection with both *C. tyzzeri* subtypes; however, the subtypes were not infective for the rodent species *Meriones*

★Nucleotide sequence data reported in this paper are available in GenBank under accession numbers JQ073388–JQ073555.

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*Corresponding author. Tel.: +1 701 231 8530; fax: +1 701 231 9692. john.mcevoy@ndsu.edu.

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unguiculatus, *Mastomys coucha*, *Apodemus flavicollis* or *Cavia porcellus*. Overall, our results support the hypothesis that *C. tyzzeri* is coevolving with Mmm and Mmd.

Keywords

Cryptosporidium tyzzeri; *Mus musculus musculus*; *Mus musculus domesticus*; House mouse; Hybrid zone; Coevolution

1. Introduction

Cryptosporidium is the genus of apicomplexan protozoan parasites that causes cryptosporidiosis, a diarrheal disease that can become chronic and life-threatening in immunocompromised hosts (Anonymous, 1982; Soave et al., 1984). It is a significant cause of childhood diarrhea and failure to thrive in non-industrialized nations (Guerrant et al., 1999), and continues to be a major cause of waterborne disease worldwide (Hlavsa et al., 2005, 2011; Yoder and Beach, 2007; Reynolds et al., 2008; Chalmers and Giles, 2010; Chalmers et al., 2010; Yoder et al., 2010; Elwin et al., 2012). *Cryptosporidium* remains a significant health concern, in part, because drug treatments are limited and entirely ineffective in the absence of a robust T-cell mediated immune response (McDonald, 2011).

Cryptosporidium infects all major vertebrate groups including most mammalian species (Current et al., 1986; Kimbell et al., 1999; Kvá and Vítovec, 2003; Ziegler et al., 2007; Jirk et al., 2008; Gibson-Kueh et al., 2011). More than 50 genotypes have been identified, primarily from *ssrRNA* gene sequences, and at least 25 species have been recognized based on additional genetic, morphometric and biological data (Fayer, 2010; Kvá et al., 2013). As a monoxenous, obligate and generally host-specific parasite, coevolution with the host is hypothesized to drive diversification, this hypothesis being supported, in part, by the phylogenetic clustering of *Cryptosporidium* taxa from closely related host species (Xiao et al., 2002).

A number of studies have examined intraspecific diversity in *Cryptosporidium*, particularly in the major human pathogenic species *Cryptosporidium parvum* and *Cryptosporidium hominis* (Widmer et al., 2004; Gatei et al., 2007; Tanriverdi et al., 2008; Xiao, 2010). Among the reported single locus genotyping tools, those targeting the *gp60* gene appear particularly useful and are widely used (Jex and Gasser, 2010). This gene encodes a 60-kDa glycoprotein that is cleaved post-translationally to produce two glycoproteins, GP40 and GP15, which are expressed on the surface of sporozoites where they function during attachment to and invasion of host cells (Cevallos et al., 2000a, b; Strong et al., 2000). Most intraspecific variation in *gp60* is concentrated in a highly polymorphic microsatellite region that encodes a serine/threonine stretch in GP40. A standardized nomenclature has been established to represent sequence variation at the *gp60* locus (Sulaiman et al., 2005). The subtype family is identified by a roman numeral, which represents the *Cryptosporidium* sp./ genotype, and lower-case letter. For instance, Ia and Ib are subtype families in *C. hominis*, and IIa and IIb are subtype families in *C. parvum*. Depending on the species, subsequent uppercase letters and numbers represent the number of various tandem repeats in the microsatellite region. For example, IIaA15G2R1 is a *C. parvum* subtype family (IIa) with 15

TCA repeats (A), two TCG repeats (G), and one ACATCA repeat (R). Analysis of gp60 has contributed to the understanding of *Cryptosporidium* transmission and can serve as a marker for intraspecific biological differences, as illustrated by IIc, which is an apparently human-restricted subtype family in the generally zoonotic species *C. parvum* (Alves et al., 2003).

Cryptosporidium tyzzeri (previously mouse genotype I) is adapted to the house mouse (Ren et al., 2012), and has been infrequently isolated from other vertebrate species including the yellow-necked mouse, voles, snakes and rats (Morgan et al., 1998, 1999; Bajer et al., 2003; Xiao et al., 2004; Karanis et al., 2007). *Cryptosporidium tyzzeri* gp60 subtype families (IXa and IXb; Feng et al., 2011) appear to have a variable geographic distribution: IXa was identified in house mice from China and IXb in two mice from the United States (USA) (Lv et al., 2009; Feng et al., 2011; Ren et al., 2012). Although these data are limited, the gp60 subtype families could represent divergent *C. tyzzeri* populations, which have coevolved with geographically isolated subspecies of the house mouse.

House mice originated in south-central Asia or the Middle East some 1 million years ago (MYA) and subsequently diverged into several subspecies approximately 0.5 MYA (Geraldes et al., 2008; Duvaux et al., 2011; Auffray and Britton-Davidian, 2012; Bonhomme and Searle, 2012). One of these subspecies, *Mus musculus musculus* (hereafter abbreviated Mmm), has spread from this cradle to a vast area of northern Eurasia from central and northern Europe to the Far East. Another subspecies, *Mus musculus domesticus* (Mmd), expanded westward through Asia Minor to southern and western Europe and northern Africa, and later has spread worldwide (Boursot et al., 1993; Guénet and Bonhomme, 2003; Rajabi-Maham et al., 2008; Duvaux et al., 2011; Auffray and Britton-Davidian, 2012; Bonhomme and Searle, 2012; Cucchi et al., 2012). In the area of their secondary contact in Europe, the two subspecies have formed a hybrid zone over 2,500 km long, stretching from Norway to the Black Sea (Macholán et al., 2003; Jones et al., 2011; Úreje et al., 2012). Due to the colonization history, the house mouse hybrid zone (HMHZ) is older in the southeast than in the north; however, as argued by Baird and Macholán (2012), its age is old enough to settle into quasi-equilibrium allowing intermixing neutral variants. Traits with a negative effect on the fitness of hybrids will be prevented from crossing the zone and will display abrupt changes in frequencies between subspecies-specific variants (Barton and Hewitt, 1985; Payseur et al., 2004; Macholán et al., 2007; Macholán et al., 2011; Janoušek et al., 2012). Conversely, neutral variants will diffuse through the HMHZ freely with some delay due to linkage to counterselected loci (Barton, 1979). Finally, even slightly advantageous traits will cross the HMHZ quite rapidly and spread into the opposite genetic background as was demonstrated recently for the Y chromosome (Albrechtová et al., 2012; Úreje et al., 2012).

In this study, we test the hypothesis that *C. tyzzeri* is coevolving with its host. If so, we should observe higher divergence between parasites living in different mouse subspecies than between those living in the same subspecies. In the context of the HMHZ, an association between host and parasite genotypes would result in a steep transition of host-specific parasite genotypes from one side of the zone to the other whereas in the absence of the coevolution, the genotypes would freely introgress across the zone. Alternatively, some

C. tyzzeri genotypes could invade a novel, susceptible mouse genotype that has not coevolved with the parasite.

To test these evolutionary scenarios, we characterized *C. tyzzeri* isolates from naturally infected Mmd and Mmm in localities across the HMHZ. This sample was supplemented with Mmd individuals from New Zealand. We found that *C. tyzzeri* isolates from Mmm and Mmd differed genetically, morphometrically and biologically. Collectively, these data are evidence that *C. tyzzeri* is coevolving with the two *M. musculus* subspecies.

2. Materials and methods

2.1. Origin of *C. tyzzeri* isolates

Thirty-two *C. tyzzeri* isolates were recovered from *M. musculus* (17 males, 15 females) sampled at 14 localities scattered across the HMHZ (Fig. 1). Two-dimensional GPS coordinates from each sample site were collapsed into a one-dimensional axis with a perpendicular orientation to the zone as described in Macholán et al. (2007). The position of each locality along this axis was given as a distance from the westernmost point specified in Dufková et al. (2011) and Macholán et al. (2011). These distances ranged from 22 km at the westernmost site (Mmd range) to 136 km at the easternmost site (Mmm range) and the consensus zone center, estimated from 13 X-linked loci, was at 68.26 km (Dufková et al., 2011).

Mice were trapped with wooden and/or metal live traps baited with a mix of sardines in oil and oat flakes. Captured mice were transferred to a field laboratory where they were kept individually and provided with sterilized bedding material, pellets (VELAZ, Prague, Czech Republic) and tap water ad libitum. The proportions of males and females in the sampled population were similar in the areas east (six female and six male) and west (10 female and 11 male) of the hybrid zone center. Two and four mice from east and west of the hybrid zone center, respectively, were juveniles. All mice were dissected the day after capture. Fecal samples were collected from the colon and stored in 96% alcohol. A hybrid index (HI) was calculated for each mouse as the proportion of Mmm alleles across 1,401 subspecies-specific single nucleotide polymorphisms (SNPs) (Wang et al., 2011) (see Table S1 in Baird et al. (2012)); hence, HI values ranged from 0 (Mmd) to 1 (Mmm).

Twelve *C. tyzzeri* isolates were recovered from *M. musculus* sampled at a single location near Christchurch, New Zealand. The subspecies of the host was not determined; however, it was assumed to be *M. m. domesticus* (Mmd) based on a previous report (Searle et al., 2009). The mice were trapped using Elliott live traps (Elliott Scientific Equipment, Upwey, Vic, Australia), which contained Dacron for bedding/warmth and were baited with a mix of peanut butter and rolled oats. Mice were trapped from farmland surrounding the Landcare Research Animal facility in Lincoln, New Zealand. Captured mice were transferred to an indoor animal facility. Fecal samples were collected from the traps when the mice were first brought into the facility.

2.2. Sample collection and DNA extraction

In the Czech and German samples, 200 mg of feces were homogenized by bead disruption using FastPrep-24 (Biospec Products, Bartlesville, OK, USA) for 60 s at a speed 5.5 m.s⁻¹. Total DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) as described in Sak et al. (2011), and stored at -20°C until processed. DNA from fecal samples of New Zealand mice was extracted by alkaline digestion and phenol-chloroform extraction, and purified using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) as described in Peng et al. (2003). DNA was further purified according to manufacturer's instructions and stored at -20°C until processed.

2.3. PCR and sequence analysis

Molecular characterization was carried out using nested PCR at five loci: *ssrRNA* (ca. 830 bp; Xiao et al., 1999; Jiang et al., 2005), *Cryptosporidium* oocyst wall protein 1 (COWP; ca. 550 bp; Spano et al., 1997; Pedraza-Diaz et al., 2001), actin (ca. 1066 bp; Sulaiman et al., 2002), gp60 (830–870 bp; Alves et al., 2003), and thrombospondin-related adhesive protein of *Cryptosporidium*-1 (TRAP-C1; ca. 780 bp; Spano et al., 1998). Positive (*C. hominis* for *ssrRNA*, COWP, actin, gp60 and TRAP-C1) and negative controls were included in each analysis. Secondary PCR products were visualized following agarose gel electrophoresis with ethidium bromide or SYBR Green dye. Products of expected size were purified (Wizard SV, Promega, Madison, WI, USA or QIAquick, Qiagen, Hilden, Germany) and directly sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit with secondary PCR primers and an ABI Prism 3130 genetic analyzer (Applied Biosystems, Carlsbad, CA, USA). Sequences were assembled using SeqMan (DNASTar, Madison, WI, USA) and aligned using the ClustalW algorithm (Thompson et al., 1997).

2.4. Phylogenetic analyses

The evolutionary history of aligned sequences was inferred using the Neighbour-Joining method (NJ; (Saitou and Nei, 1987) based on Kimura 2-parameter (K2P) distances (Kimura, 1980). The bootstrap consensus tree was inferred from 1,000 pseudoreplicates. Trees were constructed using TREECON version 1.3b (Van de Peer and De Wachter, 1994).

Cryptosporidium tyzzeri gp60 sequences were grouped by geographic location and evolutionary divergence was determined using the K2P model to calculate the number of base substitutions per site from averaging over all sequence pairs between groups. All ambiguous positions were removed for each sequence pair. Analyses were carried out using MEGA5 (Tamura et al., 2011). Sequences of *ssrRNA* (JQ073483-JQ073504, JQ073506-JQ073515, JQ073517-JQ073523), COWP (JQ073415-JQ073446), actin (JQ073388-JQ073402, JQ073404-JQ073414), gp60 (JQ073447-JQ073469, JQ073471-JQ073479, JQ073481-JQ073482, JX575574-JX575581) and TRAPC-1 (JQ073524-JQ073539, JQ073541-JQ073544 and JQ073546-JQ073555) obtained in this study have been deposited in GenBank..

2.5. Morphometry and experimental transmission studies

Isolates CR2090 (*C. tyzzeri*-Mmd) and CR4293 (*C. tyzzeri*-Mmm) from Mmd and Mmm, respectively (see Table 1 for HIs), were used for oocyst morphometry and experimental transmission studies. The *C. parvum* isolate used for comparative studies originated from a naturally infected, 1 month old calf with diarrhoea that was bred outside the area from which isolates of *C. tyzzeri* were obtained.

Oocysts from each isolate were purified using a sucrose gradient (Arrowood and Sterling, 1987) and cesium chloride gradient centrifugation (Kilani and Sekla, 1987). Purified oocysts were stored for up to 4 weeks in darkness in distilled water with antimycotics and antibiotics at 4°C.

Cryptosporidium tyzzeri-Mmm, *C. tyzzeri*-Mmd and *C. parvum* oocysts were examined using differential interference contrast (DIC) and immunofluorescence (IF) microscopy. IF was carried out with genus-specific FITC-labeled antibodies targeting the *Cryptosporidium* oocyst wall (*Cryptosporidium* IF Test, Crypto Cel, Cellabs, Australia). Cell morphology was determined using digital analysis of images (M.I.C. Quick Photo Pro v.1.2 software; Optical Service, Czech Republic) collected at 1,000x magnification using an Olympus Camedia C 5060 WIDEZOOM 5.1 megapixel digital camera (Optical Service). Length and width were measured for oocysts of each isolate ($n = 100$) and a shape index was calculated. A 20 µl aliquot containing 100,000 purified oocysts was examined for each isolate.

Experimental infections were carried out using eight-week-old adult SCID mice (Severe combined immunodeficiency, strain C.B-17; Charles River, Germany), BALB/c mice (Charles River), the wild-derived Mmm strain STUS (Piálek et al., 2008); 24–26th generation of brother-sister mating; Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Czech Republic), and a wild-derived Mmd strain from Schweben, central Germany (8–10th generation of brother-sister mating; kept under the name SCHEST at the Institute of Vertebrate Biology). In addition to the house mouse models, we tested Mongolian gerbils (*Meriones unguiculatus*) (Charles River), southern multimammate mice (*Mastomys coucha*; Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, Czech Republic), yellow-necked mice (*Apodemus flavicollis*; Institute of Parasitology), and guinea pigs (*Cavia porcellus*; Institute of Parasitology).

Laboratory rodents were housed in plastic cages with sterilized wood-chip bedding placed in flexible film isolators (BEM, Znojmo, Czech Republic) with high-efficiency particulate air filters. Animals were supplied with a sterilized diet (VELAZ, Prague, Czech Republic) and sterilized water ad libitum.

Six animals from each host group were infected with *C. tyzzeri*-Mmd or *C. tyzzeri*-Mmm. In addition, one group of SCID mice ($n = 6$) was infected with *C. parvum*. Each animal was inoculated via a gastric tube with 1 million purified oocysts suspended in 200 µl of distilled water. Oocyst viability was >95% as determined by propidium iodide exclusion according to Sauch et al. (1991). Fecal samples from all experimental animals were collected daily starting on the third day p.i. Samples were stained with aniline-carbol-methyl violet and the presence of *Cryptosporidium*-specific DNA was confirmed using nested PCR amplification

of the gp60 gene (Alves et al., 2003). The infection intensity was determined as the number of oocysts per gram (OPG) of feces as described in Kvá et al. (2007). A susceptible host was one that, following inoculation with 1 million oocysts and an expected prepatent period (Ren et al., 2012), had *Cryptosporidium* oocysts or DNA in its feces, detectable by microscopy and PCR, respectively.

All experiments were terminated at 30 days p.i. A complete examination of all organs was conducted at necropsy. Tissue specimens of the gastrointestinal tract were sampled and processed for histology according to Kvá and Vítovec (2003) with a slight modification. The intestine was divided into 1 cm sections along its entire length and processed by paraffin embedding. Histological sections were stained with H&E, Wolbach's modification of Giemsa stain, alcian blue and FITC-conjugated antibodies targeting the *Cryptosporidium* oocyst wall (*Cryptosporidium* IF Test, Crypto Cel).

Animal caretakers wore disposable coveralls, shoe covers and gloves every time they entered the buildings. All wood-chip bedding, feces and disposable protective clothing were sealed in plastic bags, removed from the buildings and incinerated. All housing, feeding and experimental procedures were conducted under protocols approved by the Institute of Parasitology, Biology Centre and Institute of Vertebrate Biology of the Academy of Sciences of the Czech Republic and Central Commission for Animal Welfare, Czech Republic (protocol # 066/2010).

2.6. Statistical analyses

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 T^2 test was used to test the null hypothesis.

The course of infection was evaluated as the maximum infection intensity time ($t_{\max}$), maximum infection intensity concentration ($C_{\max}$), and average number of excreted oocysts during the patent period per mouse calculated as the area under curve (AUC) using the classical trapezoidal rule. Due to non-normality, the data were analyzed using the Kruskal-Wallis non-parametric test. The Wilcoxon test was used as a post-hoc test after a Dunn-Sidak adjustment. The Bartlett test was used to test homoscedasticity of differences in the prepatent and patent periods of different infections.

3. Results

Mice from west of the hybrid zone center had a hybrid index ranging from 0.02 to 0.14, indicating that most of their genomes represent the Mmd subspecies. Mice from east of the hybrid zone center had a hybrid index ranging from 0.89–0.98, which is indicative of the Mmm subspecies (Table 1).

3.1. Molecular characterization of *C. tyzzeri*

For the ssrRNA gene, all *Cryptosporidium* sequences were identical, irrespective of the host, and they were the same as *C. tyzzeri* sequences with GenBank accession numbers **DQ898158**, **AF112571**, **AF108863** and **EU553589**.

COWP sequences formed two clades, labeled C1 and C2, in the NJ tree (Fig. 2A). The C1 clade included all sequences from Mmm in the area east of the hybrid zone center whereas the C2 clade included all sequences from Mmd in the area west of the hybrid zone center and all sequences from Mmd in New Zealand (Table 1). The C2 clade also included a sequence from *C. tyzzeri* isolate 411 from *M. musculus* from the USA (accession no. **AF266268**) and sequences isolated from other rodent species, *Clethrionomys glareolus* (syn.: *Myodes glareolus*) (accession no. **AF266268**), *Microtus arvalis* (accession no. **AJ489215**) and *Apodemus flavicollis* (accession no. **AJ489217**) from Poland. Sequences in the C1 and C2 clades differed by a silent substitution at position 879 relative to a standard sequence (*C. parvum* COWP; accession no. **XM_627569**). The sequence from isolate 411 differed from all other sequences in C1 and C2 by a silent substitution at position 688.

Two clades of identical sequences were revealed in TRAP-C1: the T1 and T2 clades included all *C. tyzzeri* sequences from Mmm and Mmd, respectively (Table 1, Fig. 2B). The two groups differed by a silent substitution at position 1923 relative to a standard sequence (*C. parvum* TRAP-C1; accession no. **XM_628162**).

Actin sequences formed three clades in the NJ tree labeled A1, A2 and A3 in Fig. 2C. Sequences within these groups shared 100% identity. A1 included all sequences from Mmm in the area east of the hybrid zone center, A2 included sequences from Mmd in the area west of the hybrid zone center and A3 contained sequences from New Zealand. A1 was characterized by T at position 78 of a *C. parvum* actin sequence (accession no. **XM_627938**), whereas A2 and A3 had a C at this position. A3 differed from both A1 and A2 at position 1005 (A was present in the former and G in the latter two groups, respectively; Table 1).

Fig. 3 shows a NJ tree based on gp60 sequences. All *C. tyzzeri* formed a monophyletic group divided into two clades with 100% bootstrap support. The two clades correspond to the gp60 subtype families IXa and IXb (Feng et al., 2011). The IXa subtype family comprised all sequences from Mmm in the area east of the hybrid zone center and previously published sequences from *M. musculus* from China (accession no. **GU951713**) and a child from Kuwait (accession no. **AY738188**). The sequences from the Czech Republic were divided into two sister groups of identical sequences. The IXb subtype family included all sequences from Mmd in the area west of the hybrid zone center, all sequences from New Zealand Mmd mice, and a previously published sequence from *M. musculus* from the USA (accession no. **HM234176**). All sequences from New Zealand were identical and formed a separate clade within the IXb subtype family, whereas the *C. tyzzeri*-Mmd from the Czech Republic and Germany were more diversified and did not form a monophyletic group. The mean evolutionary divergence between *C. tyzzeri* gp60 sequences within the areas east and west of the hybrid zone center, estimated using the K2P model as the average number of base substitutions per site, was 0.0007 ± 0.0007 ($n = 12$) and 0.0022 ± 0.0010 ($n = 20$), respectively. Estimates of the evolutionary divergence between *C. tyzzeri* gp60 sequences in different geographic locations are presented in Table 2. These data show the lowest divergence among sequences from the same host subspecies, regardless of geographic location.

The number of serine coding TCA repeats in gp60 sequences varied between five and eight. All IXb sequences from New Zealand had five TCA repeats, three IXa sequences from the Czech Republic had eight repeats (CR2149, CR2085 and CR3175), and all other gp60 sequences had six TCA repeats. In addition to these repeats, 12 and 18 bp repeats were identified in all sequences using Tandem Repeats Finder (Benson, 1999). The 12 bp repeat (consensus: GGTACTCAAGGA) was present as two copies in IXa sequences and two (e.g. CR2163) or three (e.g. G2135) copies in IXb sequences. The consensus sequence of the 18 bp repeat differed between the two gp60 subtype families: all IXa sequences had two copies (consensus: ATTCTGGTACTGAAGATA), and IXb sequences had two (G2136 and all isolates from New Zealand), three (e.g. G2135) or four (G2103) copies of the repeat (consensus: GGTACTGAAAATAATTCT).

Using the NetNGlyc 1.0 server (<http://www.cbs.dtu.dk/services/NetNGlyc/>), gp60 sequences from *C. tyzzeri* isolates were predicted to encode one or more N-glycosylation sites (Fig. 4). IXa sequences encoded a single N-glycosylation site that was also present in IXb sequences. Two, three, and four copies of the 18 bp repeat in the IXb sequences coded for additional N-glycosylation sites.

3.2. Oocyst morphometry

Length, width, and a shape index (length/width) were calculated for oocysts of *C. tyzzeri*-Mmd and *C. tyzzeri*-Mmm from natural infections in Mmd and Mmm, respectively, and experimental infections in SCID mice, Mmm STUS and Mmd SCHEST. For comparative purposes, measurements were also taken from oocysts of *C. parvum* isolated from natural (calf) and experimental (SCID) infections. These data are presented in Table 3. Within *C. parvum*, *C. tyzzeri*-Mmd, and *C. tyzzeri*-Mmm, oocyst size did not differ significantly among hosts ($F = 0.3771\text{--}2.2672$, $P = 0.6035\text{--}0.1063$; $F = 0.6431\text{--}0.8917$; $P = 0.4116\text{--}0.5268$). Oocysts of *C. tyzzeri*-Mmd (mean: $4.24 \times 3.69 \mu\text{m}$) were significantly smaller than oocysts of *C. tyzzeri*-Mmm (mean: $4.49 \times 3.90 \mu\text{m}$) ($F = 224.9762$; $P < 0.001$). Oocysts of *C. tyzzeri*-Mmd and *C. tyzzeri*-Mmm were significantly smaller than *C. parvum* oocysts ($F = 1400.2950$ and $F = 985.4179$, respectively, $P < 0.001$ in both cases).

3.3. Experimental transmission studies

Infectivity was detected by examining feces for the presence of oocysts using microscopy and the presence of the *Cryptosporidium*-specific gp60 gene with PCR. *Cryptosporidium tyzzeri*-Mmd and *C. tyzzeri*-Mmm were found to be infective for immunocompetent (BALB/c, Mmm STUS and Mmd SCHEST) and immunodeficient (SCID) mice. In comparison, Mongolian gerbils, southern multimammate mice, yellow-necked mice and guinea pigs produced neither microscopically nor PCR detectable infection under the conditions of the study.

3.3.1. Course of infection—For each treatment, the method used to detect oocyst shedding (microscopy and PCR) did not affect estimation of the prepatent period. The mean of the prepatent period ranged from 4 days in SCID mice infected with *C. tyzzeri*-Mmd to 7 days in Mmd SCHEST mice infected with *C. tyzzeri*-Mmm (Table 4). The prepatent period

in Mmd SCHEST mice infected with *C. tyzzeri*-Mmm was longer than the prepatent periods in all other infections presented in Table 4 ($P = 0.0022$ – 0.0411).

Using microscopic detection, the patent period was significantly shorter in immunocompetent (mean 12.6 days) than immunodeficient SCID mice (> 26 days; $P = 0.0022$) (data not shown). In addition, *C. tyzzeri*-Mmm had a significantly longer patent period than *C. tyzzeri*-Mmd in the Mmd SCHEST strain ($P = 0.0108$) (Table 4). A comparatively different patent period was detected with PCR. *Cryptosporidium tyzzeri*-Mmd DNA was detected in feces of Mmm STUS and Mmd SCHEST mice from 4–30 and 4–26 days p.i., respectively (Table 4, Fig. 5Ca, Da). *Cryptosporidium tyzzeri*-Mmm DNA was detected in feces of Mmm STUS and Mmd SCHEST mice from 4–30 and 5–30 days p.i., respectively ($P = 0.2316$ – 0.8355 ; Fig. 5Ca, Da).

The infection intensity (OPG) varied among the susceptible hosts presented in Table 4 with the highest intensity observed in immunodeficient mice, regardless of the *C. tyzzeri* isolate used. Peak oocyst shedding ($t_{\max}$ and $C_{\max}$) occurred at 5–7 days p.i. and 12–15 days p.i. in immunocompetent and immunodeficient SCID mice, respectively ($P = 0.0021$) (Fig. 5Ab). In addition, the number of shed oocysts (AUC) was significantly higher in immunodeficient than immunocompetent mice ($P = 0.0021$). Infection intensity (AUC, $t_{\max}$ and $C_{\max}$) did not differ between *C. tyzzeri* infections in Mmd SCHEST and Mmm STUS mice, regardless of the isolate used in the infection ($W = 531.5$ – 549.0 , $P = 0.1910$ – 0.2633) (Fig. 5).

3.3.2. Pathological changes and clinical signs—There were no clinical signs or macroscopic changes associated with cryptosporidiosis in susceptible hosts autopsied at the peak of infection and 30 days p.i. Histological examination of the gastrointestinal tract of animals infected with *C. tyzzeri* revealed developmental stages primarily attached to the microvillar border of the duodenum, jejunum and ileum. Developmental stages were also present in the cecum of SCID mice. No pathological changes were detected in susceptible hosts. *Cryptosporidium* developmental stages were not found in the gastrointestinal tract of gerbils, multimammate mice, wood mice or guinea pigs.

4. Discussion

The 500,000-year separation of the subspecies Mmd and Mmm, followed by their secondary contact and establishment of a stable and narrow hybrid zone, affords a rare opportunity to study key questions of evolution and coevolution. The goal in this study was to determine the extent to which the intestinal parasite *C. tyzzeri* has coevolved with subspecies of the house mouse.

Four out of the five loci examined in *C. tyzzeri* were divergent between Mmd and Mmm populations. Sequences at the *ssrRNA* locus did not vary and, with the exception of *gp60*, sequence divergence between the two host subspecies at other loci was low, which suggests that the populations diverged relatively recently. There was a strong association between the mouse subspecies genomes and *C. tyzzeri* genotypes in the hybrid zone, and no evidence for introgression of either *C. tyzzeri* genotype to foreign mouse genomes (Table 1). The most parsimonious explanation is that either the *C. tyzzeri* subtypes are less fit in the opposite

mouse-subspecific genome or that the two subtypes can hybridize but the hybrids are selected against.

The Mmd COWP allele clustered with a COWP sequence from *C. tyzzeri* isolate 411 (accession no. **AF266268**) in a NJ tree (Fig. 2A). This isolate was reported from a house mouse captured in the eastern USA (Xiao et al., 2000), a region colonized by Mmd from western Europe. The gp60 sequence from isolate 411 clustered with the Mmd-associated IXb subtype family (Fig. 3). Collectively, these data show that the distribution of *C. tyzzeri*-Mmd matches the distribution of Mmd in Germany, the Czech Republic, New Zealand and the USA.

The host-subspecies restriction of *C. tyzzeri* genotypes in the HMMZ differs from the pattern observed for microsporidia (Sak et al., 2011) and *Helicobacter* (Wasimuddin et al., 2012), which showed no subspecies specificity in the HMMZ. However, as both of these studies used single-gene pathogen detection, they were more limited than the present study in their ability to detect pathogen differentiation. This fact also limits comparisons with *C. tyzzeri* from other non-*Mus* spp., as discussed later.

The Mmd COWP allele shared 100% identity with sequences from the bank vole (*C. glareolus*), the common vole (*M. arvalis*), and the yellow-necked mouse (*A. flavicollis*) in Poland (Bajer et al., 2003). Given that this allele was not detected in isolates from Mmm in the central European transect across the HMMZ, it is striking to find it in Poland, a country to the east of the HMMZ, well within the Mmm range. This finding raises two important questions that cannot be addressed satisfactorily using the current data. Firstly, is the Mmd COWP allele also prevalent in populations infecting Mmm or is it restricted to the hosts identified by Bajer et al. (2003)? Although data from Mmm in Poland are lacking, *C. tyzzeri* was not found in samples from *A. flavicollis* or *C. glareolus* in the Czech Republic and Slovakia, suggesting that *C. tyzzeri* is not prevalent in these animals across the two countries (Kvá, unpublished data). Secondly, are there genetic or biological differences between isolates from voles and field mice in Poland and isolates from Mmd in the HMMZ? The limitations of genetic comparisons using COWP alone are exemplified by the finding of Robinson et al. (2010) that two other species, *C. hominis* and *Cryptosporidium cuniculus*, are identical at this locus. The failure to experimentally infect *A. flavicollis* with isolates from Mmd or Mmm in this study suggests that these isolates are biologically different to those reported by Bajer et al. (2003). In a follow-up study, Bednarska et al. (2003) revealed that the *C. tyzzeri* isolates from voles and field mice from Poland were infective for C57BL/6 mice, an inbred strain with a prevailing genome derived from Mmd (Yang et al., 2011). However, mice were immunosuppressed prior to infection (Bednarska et al., 2003). In order to address the questions raised by these data, a more comprehensive molecular and biological examination of isolates from different hosts and geographic regions is required.

The gp60 locus was the most polymorphic of the five genes targeted, which is in accordance with high variability of this gene in other *Cryptosporidium* spp. (Alves et al., 2006; Feltus et al., 2006; Gatei et al., 2007). The gp60 sequences from Mmm and Mmd belonged to subtype families IXa and IXb, respectively. The IXa subtype was previously reported in *M. musculus* from China, a country colonized by Mmm in the north and another subspecies, *Mus*

musculus castaneus, in the south. The hybrid zone between Mmm and *M. m. castaneus* in the Far East is less clearly defined than the European HMHZ. However, these subspecies are mostly separated by the Yangtze River (Moriwaki et al., 1990). The reported location of the isolates from China (Linzhou and Kaifeng City, Henan Province) suggests that they were sampled from mice within the Mmm range (Tsuchiya et al., 1994). The IXb subtype was previously reported from *M. musculus* in the USA and, as discussed earlier, the host subspecies is assumed to be Mmd. The gp60 data from *M. musculus* subspecies in the USA, Czech Republic, Germany, New Zealand and China show that genetic variation in *C. tyzzeri* is explained by host-specific differences rather than geographic distance (Fig. 3).

The trinucleotide repeat region in *C. tyzzeri* was short relative to many other *Cryptosporidium* spp., including *C. parvum*. However, an 18 bp minisatellite region in the IXb subtype family encoded a variable number of N-glycosylation sites. The 18 bp repeat in IXa subtype family sequences did not encode N-glycosylation sites and to our knowledge this variable number of N-glycosylation sites is unique to the IXb subtype family. N-glycosylation is an important modifier of outer membrane proteins in the related apicomplexan genus *Toxoplasma* (Fauquenoy et al., 2008). A more detailed study is warranted to determine whether these sites have been lost by *C. tyzzeri* in Mmm (IXa subtype family) or have been gained by *C. tyzzeri* in Mmd (IXb subtype family).

In addition to congruent genetic differentiation, *C. tyzzeri* oocysts isolated from Mmd are significantly smaller than oocysts from Mmm. Oocysts from both mouse subspecies were smaller than those of *C. parvum* (Upton and Current, 1985), and similar to previous reports of *C. tyzzeri* from laboratory and wild mice, *C. glareolus*, *A. flavicollis* and *M. arvalis* (Bednarska et al., 2003; Lv et al., 2009; Ren et al., 2012). Moreover, isolates from the present study were morphologically similar to those in the original description of *C. parvum* by Tyzzer from laboratory mice originated from the USA (Tyzzer, 1912). Morphometry of both *C. tyzzeri* isolates and the *C. parvum* control was not significantly affected by host species.

Having shown that *C. tyzzeri* subtypes infecting Mmm and Mmd are genetically divergent and host-restricted in the HMHZ, the infectivity of divergent subtypes for each *M. musculus* subspecies was examined under experimental conditions. Similar to *C. parvum*, *C. tyzzeri* infects the small intestine of susceptible hosts and infectious stages have been identified in the jejunum and ileum of immunocompetent mice (Ren et al., 2012). We additionally found infectious stages in the duodenum of immunocompetent hosts and the duodenum and cecum of immunodeficient hosts infected with Mmm and Mmd *C. tyzzeri* subtypes.

Both Mmd and Mmm were susceptible to experimental infections with both *C. tyzzeri* subtypes, demonstrating that the subtypes are not strictly host-specific and neither subtype caused apparent disease. This observation seems to contradict the expectations derived from coevolutionary relationships and deserves some explanation. First, there were differences in the dynamics of the prepatent period (Table 4). If competition between the two *C. tyzzeri* subtypes for resources exists in nature then the subtype with a shorter prepatent period in a given subspecies eventually outcompetes the subtype with a longer prepatent period. The issue of competition could not be addressed in this study because the experimental design

controlled for *Cryptosporidium* co-infections. Nevertheless, elimination of one *Cryptosporidium* sp. by another has been demonstrated by Akiyoshi et al. (2003) who observed a rapid displacement of *C. hominis* by a closely related *C. parvum* in a gnotobiotic pig model of infection.

The inconsistent susceptibilities of house mouse subspecies to *C. tyzzeri* subtypes in natural and experimental settings also may be attributed, at least in part, to the use of inbred mice in experimental infections (Mmm STUS mice were fully inbred and Mmd SCHEST mice were moderately inbred). The removal of genetic variation through inbreeding, and particularly the reduced heterozygosity of genes in the major histocompatibility complex, can increase susceptibility to parasitism (Froeschke and Sommer, 2005; MacDougall-Shackleton et al., 2005; Meyer-Lucht and Sommer, 2005). Finally, mice in natural settings of the HMHZ tend to be parasitized with multiple parasite species (Sak et al., 2011; Baird et al., 2012; Wasimuddin et al., 2012), resulting in an array of parasite-parasite and parasite-host interactions that shape the immune response in ways that are difficult to replicate experimentally.

In summary, this study has shown that Mmm and Mmd are hosts of genetically and morphometrically divergent *C. tyzzeri* subtypes. The absence of introgression from either side of the HMHZ suggests that *C. tyzzeri* subtypes are relatively subspecies-specific in a natural setting, although Mmm and Mmd are susceptible to experimental infections with both subtypes. Collectively, these data support the conclusion that *C. tyzzeri* is coevolving with house mouse subspecies. More generally, the house mouse may be a useful model for understanding factors contributing to *Cryptosporidium* coevolution with hosts.

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Highlights

- Two house mouse subspecies are hosts of divergent *Cryptosporidium tyzzeri* subtypes
- *Cryptosporidium tyzzeri* subtypes are subspecies-specific in a house mouse hybrid zone
- Subspecies are susceptible to experimental infections with both *C. tyzzeri* subtypes

Fig. 1.

Sampling localities across the study area in Germany and the Czech Republic. The position of the *Mus musculus musculus*/*Mus musculus domesticus* hybrid zone is indicated with the dotted line. Positions of each locality along an axis perpendicular to the hybrid zone course are shown (see also Table 1); these positions are expressed relative to the westernmost locality presented in Dufková et al. (2011). Sampling site abbreviations: STR, Straas; LEHS, Lehsten; HEBA, Hebanz; BRAU, Braunersgrün; HOHE, Hohenberg; NEUE, Neuenreuth; HUR, H rka; WOHL, Wolfsbühl; KVET, Kv tná; KRAU, Krásné Údolí; PRGA, P ílezy; VER, Veruši ky; BUS, Buškovice; ZIST, Žihle.

Fig. 2. Neighbour-joining (NJ) trees depicting evolutionary relationships among *Cryptosporidium* spp. and genotypes inferred from a partial fragment of A) the *Cryptosporidium* oocyst wall protein 1 (COWP) gene, B) the thrombospondin related adhesive protein (TRAP-C1), and C) the actin gene. The Kimura 2-parameter (K2P) model was used in all three trees. The bootstrap support is based on 1,000 pseudoreplicates; branches with less than 50% support have been collapsed.

Fig. 3. Evolutionary relationships among *Cryptosporidium* spp. and genotypes inferred from a partial fragment of the gp60 gene. The neighbour-joining tree is based on the K2P model. The bootstrap consensus tree was inferred from 1,000 pseudoreplicates; only values greater than 50% are shown.

Fig. 4.

Alignment of a partial gp60 protein sequence from *Cryptosporidium parvum* subtype IIa (CAD98656) and *Cryptosporidium tyzzeri* sequences from this and other studies. N-glycosylation sites predicted by the NetNGlyc 1.0 server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) are bold, italicized and boxed. GenBank accession numbers are presented in parentheses.

Fig. 5.

Course of infection of *Cryptosporidium tyzzeri* CR4293 (Mmd subtype) and CR2090 (Mmm subtype) strains in A) SCID, B) BALB/c, C) STUS, D) SCHEST mice based on a) molecular and b) coprological examination. STUS, a wild-derived *Mus musculus musculus* strain in 24–26th generation of brother-sister mating; SCHEST, a wild-derived *Mus musculus domesticus* strain in 8–10th generation of brother-sister mating; OPG, oocyst per gram.

Table 1

Cryptosporidium tyzzeri subtype and host characteristics.

Isolate	Locality	Country ^b	Distance (km) ^a	Hybrid index ^c	Subspecies of mice ^d	Cryptosporidium tyzzeri subtype			
						GP60 ^e	Actin ^f	COWP ^g	TRAP-C1 ^h
East of the zone center ^a									
CR2149	Žihle	CR	135.32	0.97	Mmm	IXa	A1	C1	T1
CR2090	Buškovice	CR	126.58	0.97	Mmm	IXa	A1	C1	T1
CR2152	Buškovice	CR	126.58	0.97	Mmm	IXa	x ⁱ	x	T1
CR2206	V ruši ky	CR	117.18	0.97	Mmm	IXa	A1	C1	T1
CR2208	V ruši ky	CR	117.18	0.97	Mmm	IXa	A1	C1	T1
CR2125	P ílezy	CR	104.00	0.97	Mmm	IXa	A1	C1	T1
CR2127	P ílezy	CR	104.00	0.97	Mmm	IXa	x	x	x
CR2128	P ílezy	CR	104.00	0.97	Mmm	IXa	A1	C1	T1
CR2126	P ílezy	CR	104.00	0.97	Mmm	IXa	A1	C1	x
CR2084	Krásné Údolí	CR	103.91	0.98	Mmm	IXa	A1	C1	T1
CR2085	Krásné Údolí	CR	103.91	0.97	Mmm	IXa	A1	C1	T1
CR3175	Kv tná	CR	71.35	0.89	Mmm	IXa	x	x	x
West of the zone center ^a									
G2134	Wolfsbühl	G	62.30	0.06	Mmd	IXb	A2	C2	T2
CR4293	H rka	CR	57.77	0.14	Mmd	IXb	A2	C2	T2
CR2163	H rka	CR	57.77	0.14	Mmd	IXb	A2	C2	T2
G2103	Hohenberg	G	55.57	0.09	Mmd	IXb	A2	C2	T2
G2174	Neuenreuth	G	50.59	0.05	Mmd	IXb	x	C2	T2
G2160	Braunersgrün	G	48.81	0.05	Mmd	IXb	A2	C2	T2
G2194	Braunersgrün	G	48.81	0.05	Mmd	IXb	x	x	x
G2108	Hebanz	G	43.59	0.03	Mmd	IXb	A2	C2	T2
G2169	Hebanz	G	43.59	0.04	Mmd	IXb	A2	C2	x
G2110	Lehsten	G	35.29	0.02	Mmd	IXb	A2	C2	T2
G2181	Lehsten	G	35.29	0.03	Mmd	IXb	x	x	x
G3224	Lehsten	G	35.29	0.02	Mmd	IXb	A2	C2	T2
G2120	Lehsten	G	35.29	0.02	Mmd	IXb	A2	C2	x
G2117	Straas	G	22.54	0.02	Mmd	IXb	x	x	x
G2116	Straas	G	22.54	0.02	Mmd	IXb	x	x	x
G2136	Straas	G	22.54	0.02	Mmd	IXb	A2	C2	T2
G2177	Straas	G	22.54	0.02	Mmd	IXb	A2	C2	T2
G2099	Straas	G	22.54	0.02	Mmd	IXb	A2	C2	T2
G2135	Straas	G	22.54	0.02	Mmd	IXb	A2	C2	T2
G2179	Straas	G	22.54	0.02	Mmd	IXb	A2	C2	T2
New Zealand									
NZ1632	Christchurch	NZ	NA	NA	Mmd ^j	x	x	x	T2
NZ1633	Christchurch	NZ	NA	NA	Mmd	IXb	A3	C2	T2

Isolate	Locality	Country ^b	Distance (km) ^a	Hybrid index ^c	Subspecies of mice ^d	<i>Cryptosporidium tyzzeri</i> subtype			
						GP60 ^e	Actin ^f	COWP ^g	TRAP-C1 ^h
NZ1634	Christchurch	NZ	NA	NA	Mmd	IXb	x	C2	T2
NZ1635	Christchurch	NZ	NA	NA	Mmd	IXb	x	x	T2
NZ1636	Christchurch	NZ	NA	NA	Mmd	IXb	x	x	T2
NZ1637	Christchurch	NZ	NA	NA	Mmd	x	x	C2	x
NZ1638	Christchurch	NZ	NA	NA	Mmd	x	x	C2	x
NZ1639	Christchurch	NZ	NA	NA	Mmd	IXb	x	x	T2
NZ1640	Christchurch	NZ	NA	NA	Mmd	IXb	x	C2	T2
NZ1641	Christchurch	NZ	NA	NA	Mmd	IXb	x	C2	x
NZ1642	Christchurch	NZ	NA	NA	Mmd	IXb	x	C2	x
NZ1644	Christchurch	NZ	NA	NA	Mmd	IXb	A3	x	x

^aThe distance from the hybrid zone center is at 68.26 km.

^bCR, Czech Republic; G, Germany; NZ, New Zealand.

^cHybrid index for each mouse based on 1401 single nucleotide polymorphism (SNP) loci (Baird et al., 2012).

^dMmm = *Mus musculus musculus*, Mmd = *Mus musculus domesticus*.

^egp60 sequences were grouped into one of two subtype families (IXa and IXb) in accordance with a nomenclature established previously (Sulaiman et al., 2005; Lv et al., 2009).

^fA1 (T⁷⁸G¹⁰⁰⁵), A2 (C⁷⁸G¹⁰⁰⁵), and A3 (C⁷⁸A¹⁰⁰⁵) differ by nucleotide substitutions at positions 78 and 1005 using *C. parvum* sequence XM_627938 as a reference.

^g*Cryptosporidium* oocyst wall protein; C1 (C⁸⁷⁹) and C2 (A⁸⁷⁹) differ by a nucleotide substitution at position 879 using *C. parvum* sequence XM_627569 as a reference.

^hThrombospondin related adhesive protein of *Cryptosporidium* 1; T1 (G¹⁹²³) and T2 (A¹⁹²³) differ by a nucleotide substitution at position 1923 using *C. parvum* sequence XM_628162 as a ruler

ⁱx = not detected.

^jMice from New Zealand are probably *Mus musculus domesticus* based on Searle et al. (2009).

Table 2

Estimates of evolutionary divergence over gp60 sequence pairs between geographic regions.

Geographic region	Average number of base substitutions per site $\pm$ S.E.				
	East Hybrid ^a	West Hybrid ²	New Zealand ³	United States ⁴	China ⁵
West Hybrid ^b	0.0284 $\pm$ 0.0060				
New Zealand ^c	0.0253 $\pm$ 0.0057	0.0040 $\pm$ 0.0019			
United States ^d	0.0264 $\pm$ 0.0059	0.0017 $\pm$ 0.0009	0.0029 $\pm$ 0.0020		
China ^e	0.0016 $\pm$ 0.0013	0.0270 $\pm$ 0.0059	0.0233 $\pm$ 0.0054	0.0251 $\pm$ 0.0058	
Kuwait ^f	0.0035 $\pm$ 0.0022	0.0279 $\pm$ 0.0063	0.0243 $\pm$ 0.0059	0.0261 $\pm$ 0.0062	0.0019 $\pm$ 0.0015

^a East of the hybrid zone center (see Table 1).

^b West of the hybrid zone center (see Table 1)

^c See Table 1

^d GenBank accession number [HM234176](#)

^e GenBank accession numbers [GU951713](#), [HM234177](#), [HM234179](#) and [HM234180](#).

^f GenBank accession number [AY738188](#).

Table 3*Cryptosporidium tyzzeri* and *Cryptosporidium parvum* oocyst morphology.

Isolate	Infection	Source	n	Length (µm)		Width (µm)		Shape index
				Range	Mean ± S.D.	Range	Mean ± S.D.	Mean ± S.D.
<i>C. parvum</i>	natural	calf	100	4.42 – 6.44	5.51 ± 0.45	3.94 – 5.96	4.95 ± 0.49	1.12 ± 0.09
<i>C. parvum</i>	experimental	SCID	100	4.42 – 6.35	5.50 ± 0.45	4.04 – 5.77	4.95 ± 0.46	1.12 ± 0.09
<i>C. tyzzeri</i> -Mmd	natural	Wild Mmd	100	3.56 – 4.90	4.24 ± 0.26	3.37 – 4.33	3.73 ± 0.18	1.14 ± 0.08
<i>C. tyzzeri</i> -Mmd	experimental	SCID	100	3.56 – 5.10	4.24 ± 0.27	3.17 – 4.33	3.70 ± 0.19	1.15 ± 0.09
<i>C. tyzzeri</i> -Mmd	experimental	Mmm STUS	100	3.75 – 5.00	4.26 ± 0.24	3.19 – 4.29	3.69 ± 0.20	1.16 ± 0.09
<i>C. tyzzeri</i> -Mmd	experimental	Mmd SCHEST	100	3.65 – 4.62	4.24 ± 0.24	3.24 – 4.23	3.64 ± 0.16	1.17 ± 0.08
<i>C. tyzzeri</i> -Mmm	natural	Wild Mmm	100	3.89 – 5.00	4.47 ± 0.20	3.43 – 4.35	3.88 ± 0.21	1.16 ± 0.08
<i>C. tyzzeri</i> -Mmm	experimental	SCID	100	3.89 – 4.98	4.53 ± 0.20	3.43 – 4.44	3.91 ± 0.21	1.16 ± 0.08
<i>C. tyzzeri</i> -Mmm	experimental	Mmm STUS	100	3.98 – 4.91	4.49 ± 0.20	3.43 – 4.42	3.91 ± 0.20	1.15 ± 0.08
<i>C. tyzzeri</i> -Mmm	experimental	Mmd SCHEST	100	3.89 – 4.95	4.49 ± 0.20	3.46 – 4.35	3.90 ± 0.21	1.15 ± 0.08

Mmd, *Mus musculus domesticus*; Mmm, *Mus musculus musculus*; Calf, calf from commercial breed; SCID, severe combined immunodeficiency mice; Wild Mmm, wild eastern European house mice; Wild Mmm, wild western European house mice; Mmm STUS, strain of eastern European house mice, 24–26th generation held in captivity; Mmd SCHEST, strain of western European house mice, 8–10th generation held in captivity

Table 4

Prepatent and patent period of *Cryptosporidium tyzzeri*-Mmd (CR4293) and *C. tyzzeri*-Mmm (CR2090) in susceptible hosts based on microscopic examination of feces.

Host	Strain	<u>Prepatent period (Mean days $\pm$ S.D.)</u>		<u>Patent period (Mean days $\pm$ S.D.)</u>	
		<i>C. tyzzeri</i> -Mmd	<i>C. tyzzeri</i> -Mmm	<i>C. tyzzeri</i> -Mmd	<i>C. tyzzeri</i> -Mmm
<i>Mus musculus</i>	SCID	4.17 $\pm$ 0.41	4.00 $\pm$ 0.00	> 26	> 26
<i>Mus musculus</i>	BALB/c	4.00 $\pm$ 0.00	4.00 $\pm$ 0.00	6.17 $\pm$ 1.60	6.17 $\pm$ 3.37
<i>Mus m. musculus</i>	STUS	4.50 $\pm$ 0.84	4.00 $\pm$ 0.00	7.67 $\pm$ 2.16	7.17 $\pm$ 0.98
<i>Mus m. domesticus</i>	SCHEST	4.67 $\pm$ 0.82	6.17 $\pm$ 1.47	4.83 $\pm$ 4.71	14.17 $\pm$ 3.19

SCID, severe combined immunodeficiency mice; BALB/c, inbred immunocompetent laboratory mice; Mmm STUS, strain of *M. musculus musculus*, 24–26th generation held in captivity; Mmd SCHEST, strain of *M. musculus domesticus*, 8–10th generation held in captivity.