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

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A B S T R A C T

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 analysed 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 × 3.69 μm) were significantly smaller than oocysts of C. tyzzeri-Mmm (mean: 4.49 × 3.90 μm). Mmm and Mmd were susceptible to experimental infection with both C. tyzzeri subtypes; however, the subtypes were not infective for the rodent species Meriones unguiculatus, Mastomys coucha, Apodemus flavicollis or Cavia porcellus. Overall, our results support the hypothesis that C. tyzzeri is coevolving with Mmm and Mmd.

1. Introduction

Cryptosporidium is the genus of apicomplexan protozoan parasites that causes cryptosporidiosis, a diarrhoeal disease that can become chronic and life-threatening in immunocompromised hosts (Anonymous, 1982; Soave et al., 1984). It is a significant cause of childhood diarrhoea and failure to thrive in non-industrialised 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; Kvác and Vitovec, 2003; Ziegler et al., 2007; Jirků 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 recognised based on additional genetic, morphometric and biological data (Fayer, 2010; Kvác et al., 2013). As a monoxenous, obligate and generally host-specific parasite, coevolution with the host is hypothesised 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. More recently, a number of studies have examined the role of coevolution and diversification in Cryptosporidium species.
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 standardised 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 Ila and Ilb 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, IlaA15G2R1 is a C. parvum subtype family (Ila) 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 (Geraldès 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; Guénét 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; Dureje et al., 2012). Due to the colonisation 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, 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; Dureje 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 intergress 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 characterised 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 centre, 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 centre. Two and four mice from east and west of the hybrid zone centre, 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 Da-cron 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 homogenised 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 characterisation 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 thrombospindin-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 visualised following agarose gel electrophoresis with ethidium bromide or Sybr Green dye. Products of expected size were purified (Wizard PCR Prep Kit, 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 analyser (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 TRAPC1 (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-labelled 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 1,000× 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 8-week-old adult SCID mice (Severe combined immunodeficiency strain, Charles River, Germany), BALB/c mice (Charles River), the wild-derived Mmd strain STUS (Pálek et al., 2008), and a wild-derived Mmm strain from Schwaben, 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...
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 detected by nested polymerase chain reaction (PCR) as described in Savic et al. (2007). Isolate DNA was subsequently amplified using Cryptosporidium oocyst wall protein; C1 (C 879) and C2 (A 879) differ by a nucleotide substitution at position 879 using sequence XM_627569 as a reference. Sequence XM_628162 was used as a ruler for calculating the distance from the hybrid zone center. A1 (T 78G1005), A2 (C 78G1005), and A3 (C78A 1005) differ by nucleotide substitutions at positions 78 and 1005 using sequence XM_627938 as a reference. Cryptosporidium oocyst wall protein; C1 (C 879) and C2 (A 879) differ by a nucleotide substitution at position 879 using sequence XM_627569 as a reference. Sequence XM_628162 as a ruler.  

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<th>Distance (km)</th>
<th>Hybrid index</th>
<th>Subspecies of mice</th>
<th>Cryptosporidium tyzzeri subtype</th>
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### Table 1

**Cryptosporidium tyzzeri subtype and host characteristics.**

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### Footnotes

a The distance from the hybrid zone centre is at 68.26 km.
b CR, Czech Republic; G, Germany; NZ, New Zealand.
c Hybrid index for each mouse based on 1401 single nucleotide polymorphism (SNP) loci (Baird et al., 2012).
d Mmm = *Mus musculus domesticus*, Mmd = *Mus musculus musculus.*
e gp60 sequences were grouped into one of two subtype families (IAxa and IAxb) in accordance with a nomenclature established previously (Sulaiman et al., 2005; Lv et al., 2009).
f Cryptosporidium oocyst wall protein; C1 (C 879) and C2 (A 879) differ by a nucleotide substitution at position 879 using sequence XM_627569 as a reference.
g Thrombospondin related adhesive protein of Cryptosporidium 1; T1 (C1923) and T2 (A1923) differ by a nucleotide substitution at position 1923 using C. parvum sequence XM_629162 as a ruler.
h *Mastomys coucha*; Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, (Czech Republic), yelow-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.
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 Kvác 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 Kvác and Vitovec (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, alcin 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 ($t_{\text{max}}$), maximum infection intensity concentration ($C_{\text{max}}$), and average number of excorated 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 analysed 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 centre 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 centre had a hybrid index ranging from 0.89–0.98, which is indicative of the Mmm subspecies (Table 1).

3.1. Molecular characterisation 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 Nos. DQ898158, AF112571, AF108863 and EU553589. COWP sequences formed two clades, labelled 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 centre whereas the C2 clade included all sequences from Mmd in the area west of the hybrid zone centre 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. AF266288) and sequences isolated from other rodent species, Clethrionomys glareolus (syn.: Myodes glareolus) (Accession No. AF266268), Microtus arvalis (Accession No. AJ489215) and A. 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 labelled 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 centre, A2 included sequences from Mmd in the area west of the hybrid zone centre and A3 contained sequences from New Zealand. A1 was characterised 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 centre 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 centre, 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 centre, 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 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: GGTACTGAAAATAATTCT) or four (G2135) or three (e.g. G2136 and all isolates from New Zealand), three (e.g. CR2163) or four (G2103) copies in IXb sequences. The consensus sequence of the 18 bp repeat differed between the two gp60 subtype families: all 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: ATTCTGTTACTGAGATA), and IXb sequences had two (G2136 and all isolates from New Zealand), three (e.g. G2135) or four (G2103) copies of the repeat (consensus: GGTACTGAAATAATTTCT).

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
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.

Table 2

<table>
<thead>
<tr>
<th>Geographic region</th>
<th>Average number of base substitutions per site ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>East hybrid&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>West hybrid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0284 ± 0.0060</td>
</tr>
<tr>
<td>New Zealand&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0253 ± 0.0057</td>
</tr>
<tr>
<td>United States&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.0264 ± 0.0059</td>
</tr>
<tr>
<td>China&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.0016 ± 0.0013</td>
</tr>
<tr>
<td>Kuwait&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.0035 ± 0.0022</td>
</tr>
</tbody>
</table>

<sup>a</sup> East of the hybrid zone centre (see Table 1).
<sup>b</sup> West of the hybrid zone centre (see Table 1).
<sup>c</sup> See Table 1.
<sup>d</sup> GenBank Accession No. HM234176.
<sup>e</sup> GenBank Accession Nos. GU951713, HM234177, HM234179 and HM234180.
<sup>f</sup> GenBank Accession No. AY738188.
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* = 0.3771–2.2672, *P* = 0.6035–0.1063; *F* = 0.6431–0.8917; *P* = 0.4116–0.5268). Oocysts of *C. tyzzeri*-Mmd (mean: 4.24 × 3.69 μm) were significantly smaller than oocysts of *C. tyzzeri*-Mmm (mean: 4.49 × 3.90 μm) (*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

Infecivity 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-Mmd 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 30 and 26 days p.i., respectively (Table 4, Fig. 5 Ca, Da). Cryptosporidium tyzzeri-Mmd DNA was detected in feces of Mmm STUS and Mmd SCHEST mice from 30 and 26 days p.i., respectively (\( P = 0.2316–0.8355 \); Fig. 5 Ca, 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 (\( t_{\text{max}} \) and \( c_{\text{max}} \)) occurred at 5–7 days p.i. and 12–15 days p.i. in immunocompetent and immunodeficient SCID mice, respectively (\( P = 0.0021 \)) (Fig. 5 Ab). In addition, the number of shed oocysts (AUC) was significantly higher in immunodeficient than in immunocompetent mice (\( P = 0.0021 \)). Infection intensity (AUC, \( t_{\text{max}} \) and \( c_{\text{max}} \)) 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 colonised 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 HMHZ differs from the pattern observed for microsporidia (Sak et al., 2011) and Helicobacter (Wasiuddin et al., 2012), which showed no subspecies specificity in the HMHZ. 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.

### Table 3

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Infection</th>
<th>Source</th>
<th>( n )</th>
<th>Length (µm)</th>
<th>Width (µm)</th>
<th>Shape index</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parvum</td>
<td>Natural</td>
<td>calf</td>
<td>100</td>
<td>4.42–6.44</td>
<td>5.51 ± 0.45</td>
<td>3.94–5.96</td>
</tr>
<tr>
<td>C. parvum</td>
<td>Experimental</td>
<td>SCID</td>
<td>100</td>
<td>4.42–6.35</td>
<td>5.50 ± 0.45</td>
<td>4.04–5.77</td>
</tr>
<tr>
<td>C. tyzzeri-Mmd</td>
<td>Natural</td>
<td>Wild Mmd</td>
<td>100</td>
<td>3.56–4.90</td>
<td>4.24 ± 0.26</td>
<td>3.37–4.33</td>
</tr>
<tr>
<td>C. tyzzeri-Mmd</td>
<td>Experimental</td>
<td>SCID</td>
<td>100</td>
<td>3.56–5.10</td>
<td>4.24 ± 0.27</td>
<td>3.17–4.33</td>
</tr>
<tr>
<td>C. tyzzeri-Mmd</td>
<td>Experimental</td>
<td>Mmm STUS</td>
<td>100</td>
<td>3.75–5.00</td>
<td>4.25 ± 0.24</td>
<td>3.19–4.29</td>
</tr>
<tr>
<td>C. tyzzeri-Mmm</td>
<td>Natural</td>
<td>Wild Mmm</td>
<td>100</td>
<td>3.89–5.00</td>
<td>4.47 ± 0.20</td>
<td>3.43–4.35</td>
</tr>
<tr>
<td>C. tyzzeri-Mmm</td>
<td>Experimental</td>
<td>SCID</td>
<td>100</td>
<td>3.89–4.98</td>
<td>4.53 ± 0.20</td>
<td>3.43–4.44</td>
</tr>
<tr>
<td>C. tyzzeri-Mmm</td>
<td>Experimental</td>
<td>Mmm STUS</td>
<td>100</td>
<td>3.98–4.91</td>
<td>4.49 ± 0.20</td>
<td>3.43–4.42</td>
</tr>
<tr>
<td>C. tyzzeri-Mmm</td>
<td>Experimental</td>
<td>Mmd SCHEST</td>
<td>100</td>
<td>3.89–4.95</td>
<td>4.49 ± 0.20</td>
<td>3.46–4.35</td>
</tr>
</tbody>
</table>

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 Mmd, 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.

<table>
<thead>
<tr>
<th>Host</th>
<th>Strain</th>
<th>Prepatent period (Mean days ± S.D.)</th>
<th>Patent period (Mean days ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mus musculus</td>
<td>SCID</td>
<td>C. tyzzeri-Mmd</td>
<td>C. tyzzeri-Mmm</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>BALB/c</td>
<td>C. tyzzeri-Mmd</td>
<td>C. tyzzeri-Mmm</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>STUS</td>
<td>C. tyzzeri-Mmd</td>
<td>C. tyzzeri-Mmm</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>DOMESTIC</td>
<td>C. tyzzeri-Mmd</td>
<td>C. tyzzeri-Mmm</td>
</tr>
</tbody>
</table>

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.
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 HMHZ, it is striking to find it in Poland, a country to the east of the HMHZ, 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 (Kváč, unpublished data). Secondly, are there genetic or biological differences between isolates from voles and field mice in Poland and isolates from Mmd in the HMHZ? 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 colonised 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 minisatelitte 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 gnotobiatic 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 (Froschke 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.

Acknowledgements

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References


Cryptosporidium parvum


