North American tree squirrels and ground squirrels with overlapping ranges host different Cryptosporidium species and genotypes

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1. Introduction

Cryptosporidium is a genus of apicomplexan parasites that infects the gastrointestinal tract of a broad range of vertebrate species (Fayer, 2010), causing the diarrheal disease cryptosporidiosis. There is no effective drug treatment or vaccine for human cryptosporidiosis, and 2010), causing the diarrheal disease cryptosporidiosis. There is no effective drug treatment or vaccine for human cryptosporidiosis, and 2010), causing the diarrheal disease cryptosporidiosis. There is no effective drug treatment or vaccine for human cryptosporidiosis, and 2010), causing the diarrheal disease cryptosporidiosis. There is no effective drug treatment or vaccine for human cryptosporidiosis, and 2010), causing the diarrheal disease cryptosporidiosis. There is no effective drug treatment or vaccine for human cryptosporidiosis, and 2010), causing the diarrheal disease cryptosporidiosis. There is no effective drug treatment or vaccine for human cryptosporidiosis, and 2010), causing the diarrheal disease cryptosporidiosis. There is no effective drug treatment or vaccine for human cryptosporidiosis, and 2010), causing the diarrheal disease cryptosporidiosis. There is no effective drug treatment or vaccine for human cryptosporidiosis, and 2010), causing the diarrheal disease cryptosporidiosis. There is no effective drug treatment or vaccine for human cryptosporidiosis, and 2010), causing the diarrheal disease cryptosporidiosis. There is no effective drug treatment or vaccine for human cryptosporidiosis, and 2010), causing the diarrheal disease cryptosporidiosis. There is no effective drug treatment or vaccine for human cryptosporidiosis, and

Wildlife-associated Cryptosporidium are an emerging cause of cryptosporidiosis in humans. The present study was undertaken to determine the extent to which North American tree squirrels and ground squirrels host zoonotic Cryptosporidium species and genotypes. Fragments of the Cryptosporidium 18S rRNA and actin genes were amplified and sequenced from fecal samples obtained from three tree squirrel and three ground squirrel species. In tree squirrels, Cryptosporidium was identified in 40.5% (17/42) of American red squirrels (Tamiasciurus hudsonicus), 40.4% (55/136) of eastern gray squirrels (Sciurus carolinensis), and 28.6% (2/7) of fox squirrels (Sciurus niger). Human-pathogenic Cryptosporidium ubiquitum and Cryptosporidium skunk genotype were the most prevalent species/genotypes in tree squirrels. Because tree squirrels live in close proximity to humans and are frequently infected with potentially zoonotic Cryptosporidium species/genotypes, they may be a significant reservoir of infection in humans. In ground squirrels, Cryptosporidium was detected in 70.2% (33/47) of 13-lined ground squirrels (Ictidomys tridecemlineatus), 35.1% (27/77) of black-tailed prairie dogs (Cynomys ludovicianus), and the only golden-mantled ground squirrel (Callospermophilus lateralis) that was sampled. Cryptosporidium rubeyi and ground squirrel genotypes I, II, and III were identified in isolates from these ground squirrel species. In contrast to the Cryptosporidium infecting tree squirrels, these species/genotypes appear to be specific for ground squirrels and are not associated with human disease.

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Cryptosporidium in tree squirrels (Sciurini tribe) and ground squirrels (Marmotini tribe), whose ancestors diverged 30 million years ago (Mercer and Roth, 2003), have been conducted in the northeast (New York), upper midwest (Minnesota), and southwest (California) US. In New York, eastern gray squirrels (Sciurus carolinensis) and American red squirrels (Tamiasciurus hudsonicus), members of the tree squirrel tribe, hosted Cryptosporidium chipmunk genotype I, and Cryptosporidium skunk genotype (Feng et al., 2007; Ziegler et al., 2007). The skunk genotype, like C. ubiquitum and chipmunk genotype I, has been associated with disease in humans (Elwin et al., 2012; Robinson et al., 2008). At the same location, eastern chipmunks (Tamias striatus) and a woodchuck (Marmota monax), members of the ground squirrel tribe, also hosted C. ubiquitum and chipmunk genotype I. In contrast to the studies in New York, none of the ground squirrel species sampled in Minnesota or California hosted zoonotic Cryptosporidium species or genotypes (Atwill et al., 2004; Pereira et al., 2010; Stenger et al., 2015). Eastern chipmunks in Minnesota were primarily infected with Cryptosporidium chipmunk genotype II, a genotype for which no other host has been identified (Stenger et al., 2015). Golden-mantled ground squirrels (Callospermophilus lateralis), Belding’s ground squirrels (Urocitellus beldingi), and California ground squirrels (Otospermophilus beecheyi) in California hosted Cryptosporidium rubeyi and a number of genotypes that have been reported only in ground squirrels (Atwill et al., 2004; Li et al., 2015; Pereira et al., 2010).

If eastern gray squirrels and American red squirrels host zoonotic Cryptosporidium species/genotypes, and ground squirrels become infected when their ranges overlap those of tree squirrels, we hypothesized that differences in host range could explain the failure to detect zoonotic species/genotypes in ground squirrels in California. Eastern gray squirrels are found east of the 100th meridian (Fig. 1), apart from small, localized populations in California, and American red squirrels are rarely found west of the Rocky Mountains. We undertook the present study on Cryptosporidium infecting tree squirrels and ground squirrels in the upper midwest, an area encompassing the ranges of a number of tree and ground squirrel species. Despite their overlapping ranges, we found that tree squirrels and ground squirrels hosted different Cryptosporidium species and genotypes, and that only tree squirrels were a potential reservoir of infection in humans.

2. Materials and methods

2.1. Trapping and sample collection

Fecal samples were collected from eastern gray squirrels (Sca), fox squirrels (Sciurus niger; Scni), and American red squirrels (Tahu) in the tree squirrel tribe, and golden-mantled ground squirrel (CaLa), 13-lined ground squirrels (Ictidomys tridecemlineatus; Ictr), and black-tailed prairie dogs (Cynomys ludovicianus; Cylu) in the ground squirrel tribe. Samples were collected between July 2007 and May 2012. All squirrels, with the exception of black-tailed prairie dogs and the golden-mantled ground squirrel, were live-captured in Sherman box traps or Tomahawk cage traps baited with sunflower seeds and peanut butter. Captured animals were affixed with an ear-tag with a unique identification number used to identify recaptured animals and released in compliance with North Dakota State University Institutional Animal Care and Use policies. Fecal samples from trapped squirrels were collected directly from the trap or from the animal during handling. Fecal samples from black-tailed prairie dogs were collected from the

Fig. 1. Sample locations and the North American ranges of the tree squirrel and ground squirrel species sampled in this study.
considered statistically significant.

3. Results

3.1. Prevalence of Cryptosporidium in tree squirrels and ground squirrels

The overall prevalence of Cryptosporidium in tree squirrels (40.0%; 74/185) and ground squirrels (48.8%; 61/125) was similar ($X^2 = 2.35$, p-value > 0.05). In the tree squirrel tribe, Cryptosporidium was detected in 40.5% (17/42), 40.4% (55/136), and 28.6% (2/7) of American red squirrels, eastern gray squirrels, and fox squirrels, respectively. In ground squirrels, Cryptosporidium was detected in 70.2% (33/47) and 35.1% (27/77) of 13-lined ground squirrels and prairie dogs, respectively. The prevalence in 13-lined ground squirrels was significantly higher than that in prairie dogs ($X^2 = 14.44$, p < 0.001) or any of the tree squirrel species ($X^2 = 13.77$, p < 0.001). Cryptosporidium also was detected in the only golden-mantled ground squirrel sampled.

3.2. Cryptosporidium diversity in tree and ground squirrels

Isolates from 67/135 (49.6%) Cryptosporidium positive animals were genotyped by sequence analysis of the 18S rRNA and/or actin genes. Isolates from the remaining positive animals yielded sequences of insufficient quality to include in analyses. Genotyping was successful for 26/55 Cryptosporidium positive gray squirrels, 8/17 positive American red squirrels, 2/2 positive fox squirrels, 12/27 positive prairie dogs, 18/33 positive 13-lined ground squirrels, and 1/1 positive golden-mantled ground squirrels. NJ trees constructed from aligned 18S rRNA (Figs. 2 and S1) and actin (Figs. 3 and S2) sequences were consistent in showing seven major Cryptosporidium groups in squirrels. All sequenced isolates were included in phylogenies presented in Figs. S1 and S2; representative isolates from each group are included in Figs. 2 and 3.

Tree squirrel isolates clustered in three groups that were exclusive to tree squirrels in this study and corresponded to known Cryptosporidium species and genotypes. C. ubiquitum was identified in 19 eastern gray squirrels, six American red squirrels, and two fox squirrels at MN1 and MN2. One gray squirrel was positive for C. ubiquitum on three separate occasions during one week (1986-Scca-MN2, 1989-Scca-MN2, and 1996-Scca-MN2; accession nos. KT027448, KT027449, and KT027431, respectively).

Cryptosporidium skunk genotype was identified in four eastern gray squirrels, an American red squirrel, and a fox squirrel at MN1 and MN2. The fox squirrel (1992-Scci-MN2) showed evidence of a mixed infection with C. ubiquitum (identified from a sequence of the 18S rRNA gene; accession no. KT027438) and Cryptosporidium skunk genotype (identified from a sequence of the actin gene; accession no. KT027546). A gray squirrel (1994-Scca-MN2) also showed evidence of a mixed infection with C. ubiquitum (identified from a sequence of the 18S rRNA gene; accession no. KT027453) and Cryptosporidium skunk genotype (identified from a sequence of the actin gene; accession no. KT027458).

Cryptosporidium deer mouse genotype III was identified in five eastern gray squirrels and an American red squirrel at MN1. One of the gray squirrels (1929-Scca-MN1) was co-infected with Cryptosporidium deer mouse genotype III (identified from a sequence of the 18S rRNA gene; accession no. KT027461) and C. ubiquitum (identified from a sequence of the actin gene; accession no. KT027499).

Two C. ubiquitum subtypes were identified by gp60 sequence analysis (Figs. 4 and S3), and they were identical to subtypes XIIb and XIIc described by Li et al. (2014). Subtype XIIb was found in three eastern gray squirrels and four American red squirrels at MN1 and MN2. Subtype XIIc was identified in five eastern gray squirrels and a fox squirrel at MN2.

Sequences of the 18S rRNA and actin genes from ground squirrel isolates clustered in four groups. One of the groups included isolates of C. rubeyi, a recently described species reported in ground squirrels from California (Li et al., 2015). Cryptosporidium rubeyi was identified in 10 black-tailed prairie dogs at ND4 and a golden-mantled ground

entrance to burrows. One sample was collected per burrow opening. A fecal sample from a golden-mantled ground squirrel was collected from the ground. All fecal samples were stored at 4 °C prior to DNA extraction.

A map showing the sample locations and the range of each squirrel species sampled in the study is presented in Fig. 1. Gray squirrels and American red squirrels were sampled from two locations in western Minnesota (MN1 and MN2). Fox squirrels also were sampled from MN2. 13-lined ground squirrels were sampled from eastern (ND2) and north central (ND3) North Dakota. The golden-mantled ground squirrel was sampled from south central Montana (MT1), and prairie dogs were sampled from western North Dakota (ND4).

2.2. DNA extraction

DNA was extracted from fecal samples using an alkaline digestion and phenol-chloroform extraction method and purified using a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) (Feltus et al., 2006; Peng et al., 2003). DNA was stored at −20 °C until used in PCR assays.

2.3. Molecular analyses

A fragment of the Cryptosporidium 18S rRNA gene was amplified as described by Xiao et al. (2001), with the exception that a 0.5× buffer concentration was used (GoTaq Flexi DNA polymerase, Promega, Madison, WI). A fragment of the actin gene was amplified as previously described by Sulaiman et al. (2002). A fragment of the gp60 gene was amplified from C. ubiquitum isolates using the primers and amplification conditions described by Li et al. (2014). Secondary PCR products were separated on an agarose gel and visualized under UV illumination using SYBR Green or ethidium bromide staining.

Products were purified (Wizard SV, Promega, Madison, WI) and sequenced in both directions with secondary primers using a BigDye Terminator v3.1 cycle sequencing kit in an ABI Prism 3130 genetic analyzer (Applied Biosystems, Carlsbad, CA). Sequences were assembled using SeqMan (DNASTar, Madison, WI), and aligned using the MAFFT version 7 online server with automatic selection of alignment strategy ([http://mafft.cbrc.jp/alignment/server/](http://mafft.cbrc.jp/alignment/server/)) (Katoh and Standley, 2013). The alignment included published sequences from gastric Cryptosporidium species infecting mammals, birds, reptiles, and amphibians; sequences with a high similarity to study sequences using BLAST analysis; and sequences from Cryptosporidium genotypes previously reported in squirrels, other rodents, and hosts with a similar range to squirrels sampled in this study. Alignments were manually edited, including trimming at both ends, and phylogenetic analyses were performed using MEGA 6.0 (Tamura et al., 2013). Trees were inferred by the Neighbor-Joining (NJ) method and distance estimates were based on the Kimura 2-parameter distance model with pairwise deletions. Bootstrap values were determined from 1000 pseudoreplicates (Kimura, 1980; Saitou and Nei, 1987).

Sequences from this study have been deposited in GenBank under the accession numbers KT027431 to KT027549.

2.4. Statistical analyses

Prevalence was calculated by dividing the number of individuals that were PCR positive for the Cryptosporidium 18S rRNA gene by the total number of individuals caught. Recaptured squirrels were counted once in the prevalence calculations. Chi-square analysis was used to test for differences in Cryptosporidium prevalence between squirrel tribes and individual squirrel species. Statistical analyses were performed using the program R ([http://www.r-project.org/](http://www.r-project.org/)) (R Development Core Team, 2011) and a p-value ≤0.05 was considered statistically significant.
squirrel at MT1. At the 18S rRNA locus, C. rubeyi isolates from
the present study shared 99.6, 99.5, and 99.4% similarity, re-
spectively, with C. rubeyi Sbey11c from a California ground squirrel (accession no. KM010224), C. rubeyi Sltl05c from a golden-mantled ground squirrel (accession no. DQ295014), and C. rubeyi Sbld05c from a Belding's ground squirrel (accession no. DQ295013) (Table 1). At the actin locus, C. rubeyi isolates from the present study shared 99.6% similarity with C. rubeyi Sbey11c (accession no. KM010227) (Table 1). Actin sequences have not been reported for C. rubeyi isolates Sbld05c or Sltl05c.

The other groups infecting ground squirrels in the present study were named Cryptosporidium ground squirrel genotypes I, II, and III. Ground squirrel genotype I was identified in eight 13-lined ground squirrels at ND2 and ND3. One animal was positive for ground squirrel genotype I on two consecutive days (2278-lctr-ND3 and 2269-lctr-ND3; accession nos. KT027521 and KT027519, respectively), and another was positive for this genotype on three consecutive days (2484-lctr-ND2, 2482-lctr-ND2, and 2488-lctr-ND2; accession nos. KT027518, KT027522, and KT027523, respectively). Ground squirrel genotype I clustered with isolate Sbey03b (accession no. AY462232) from a California ground squirrel, with a mean sequence similarity of 99.5% at the 18S rRNA locus (Table 1).

Cryptosporidium ground squirrel genotype II was identified in a single black-tailed prairie dog at ND4, did not cluster with other ground squirrel genotypes, and was most similar to Cryptosporidium muskrat genotype II (accession no. EF641021), sharing 97.6% sequence similarity at the 18S rRNA locus (Table 1).

Ground squirrel genotype III formed a sister group with C. rubeyi, sharing 98.7% and 98.2% similarity, respectively, at the 18S rRNA and actin loci (Table 1). Ground squirrel genotype III also shared 99.2% similarity with C. rubeyi Sbld05c (accession no. DQ295013) at the 18S rRNA locus (Table 1). This genotype was exclusively found in 13-lined ground squirrels at ND2.
4. Discussion

This study on Cryptosporidium spp. infecting North American tree squirrels and ground squirrels has shown that most tree squirrels host zoonotic species and genotypes, and ground squirrels host species and genotypes that are tribe-specific and unlikely to cause human disease.

Tree squirrels were most frequently infected with two C. ubiquitum subtypes, XIIb and XIIc, which are known to cause human disease in the US (Li et al., 2014). Subtype XIIb was detected previously in a Verreaux's Sifaka and an eastern chipmunk, while subtype XIIc was isolated from a porcupine and was infectious for a goat under experimental conditions (Li et al., 2014). Although this is the first report of subtypes

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**Fig. 3.** Phylogenetic relationships of representative Cryptosporidium actin gene sequences. Isolates are identified by isolate number, abbreviation of host species name (Scca — Sciurius carolinensis; Tahu — Tamiasciurus hudsonicus; Ictr — Ictidomys tridecemlineatus; CyI — Cynomys ludovicianus; Cala — Callospermophilus lateralis), and sample location. Clades representing Cryptosporidium taxa are identified by shading and labeled. Trees were constructed using a neighbor-joining approach with bootstrapping based on 1000 pseudoreplicates.

**Fig. 4.** Phylogenetic relationships of representative Cryptosporidium ubiquitum gp60 sequences. Isolates are identified by isolate number, abbreviation of host species name (Scca — Sciurius carolinensis; Tahu — Tamiasciurus hudsonicus), and sample location. Trees were constructed using a neighbor-joining approach with bootstrapping based on 1000 pseudoreplicates.
Collectively, these data suggest that multiple Belding’s ground squirrel (Sbld05a) in California (Pereira et al., 2010). from 13-lined ground squirrels in the present study clustered with iso-
detected C. rubeyi relationship (Harrison et al., 2003; Herron et al., 2004). We also 13-lined ground squirrel, respectively, also share a close phylogenetic relationship, and ground squirrel genotype III were most similar, and American red squirrels at a single location in this study. This genotype with human disease, was found in eastern gray squirrels and

classified as a host of Cryptosporidium species/genotypes infecting tree squirrels and ground squirrels in New York (Feng et al., 2009; Elwin et al., 2012; Robinson et al., 2008).

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. isolates in group</th>
<th>Mean percent sequence similarity</th>
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<tbody>
<tr>
<td></td>
<td>Within group</td>
<td>Between group and other groups in this study</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>99.9</td>
<td>97.4 [Skunk]</td>
</tr>
<tr>
<td></td>
<td>Skunk</td>
<td>99.5 [Ground squirrel I]</td>
</tr>
<tr>
<td>Deer mouse III</td>
<td>99.3</td>
<td>97.3 [Skunk]</td>
</tr>
<tr>
<td>C. rubeyi</td>
<td>87.7 [Ground squirrel I]</td>
<td>95.9 [Ground squirrel III]</td>
</tr>
<tr>
<td></td>
<td>99.6 [C. rubeyi; DQ295014]</td>
<td>99.4 [C. rubeyi; DQ295013]</td>
</tr>
<tr>
<td>Ground squirrel I</td>
<td>99.9</td>
<td>97.3 [C. ubiquitum]</td>
</tr>
<tr>
<td>Ground squirrel II</td>
<td>ND</td>
<td>97.1 [Skunk]</td>
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<td>97.0 [C. ubiquitum]</td>
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<td>98.7 [C. rubeyi]</td>
<td>96.3 [C. ubiquitum]</td>
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<tr>
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<td>98.5 [Ground squirrel III]</td>
<td>92.7 [C. rubeyi]</td>
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<td>96.6 [C. rubeyi]</td>
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<td>98.2 [Ground squirrel III]</td>
<td>96.1 [C. rubeyi]</td>
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<tr>
<td>Ground squirrel III</td>
<td>100.0</td>
<td>93.1 [Ground squirrel III]</td>
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ND: Not determined because the group contained only one isolate.

XIIb and XIIc in tree squirrels, another wildlife-associated subtype, XIIId, was identified in eastern gray squirrels and American red squirrels in New York (Li et al., 2014). Similar to XIIb and XIIc, subtype XIIId has also been associated with human disease in the US (Li et al., 2014). Therefore, tree squirrels should be considered a potential source of C. ubiquitum infections in humans.

We found the skunk genotype in eastern gray, American red, and fox squirrels. This genotype was reported previously in eastern gray squirrels and river otters (Lontra canadensis) in New York (Feng et al., 2007), raccoons (Procyon lotor) in New York and Colorado, and striped skunks (Mephitis mephitis) in New York. Similar to C. ubiquitum, the skunk genotype is known to cause disease in humans (Davies et al., 2009; Elwin et al., 2012; Robinson et al., 2008).

Deer mouse genotype III, a genotype that has not been associated with human disease, was found in eastern gray squirrels and American red squirrels at a single location in this study. This genotype appears to be restricted to squirrels and deer mice (Feng et al., 2007; Stenger et al., Unpublished) and may be transmitted between sympatric populations of these hosts.

Out of the four ground squirrel associated species/genotypes, C. rubeyi and ground squirrel genotype III were most similar, and their predominant hosts in this study, the black-tailed prairie dog and 13-lined ground squirrel, respectively, also share a close phylogenetic relationship (Harrison et al., 2003; Herron et al., 2004). We also detected C. rubeyi in a golden-mantled ground squirrel, which has a range that overlaps that of the prairie dog in western Montana. Isolates clustering with C. rubeyi were previously reported in a golden-mantled ground squirrel (Stih05c), California ground squirrel (Sbeyl11c), and Belding’s ground squirrel (Stbd05c) in California (Atwill et al., 2004; Pereira et al., 2010). Similarly to C. rubeyi, ground squirrel genotype I from 13-lined ground squirrels in the present study clustered with isolates from California ground squirrels (Sbeyl03a and Sbeyl03b), Belding’s ground squirrel (Stbd05a) in California (Pereira et al., 2010). Collectively, these data suggest that multiple Cryptosporidium lineages have coevolved with ground squirrels and that specificity is not restricted to a single ground squirrel species. However, as these species and genotypes have been reported only in ground squirrels, they appear to be restricted to this squirrel tribe, and are therefore unlikely to cause human disease.

Despite the overlapping ranges of tree and ground squirrel species in the study area, we did not detect the same species/genotypes of Cryptosporidium in squirrels from different taxonomic tribes. Although the present study did not include eastern chipmunks, a ground squirrel species identified as a host of C. ubiquitum in New York (Feng et al., 2007; Ziegler et al., 2007), a previous comprehensive study on eastern chipmunks in Minnesota also failed to detect C. ubiquitum (Stenger et al., 2015), suggesting that this zoonotic species is not frequently transmitted between tree squirrels and ground squirrels in the upper midwest region of the US. Given its broad host specificity and infectivity for eastern chipmunks in New York, C. ubiquitum is likely to be infectious for the ground squirrels examined in this study, so why were they not infected? One possible explanation is the different ecologies and behaviors of tree squirrels and ground squirrels. Tree squirrel species usually remain active throughout the year, living in the trees of forests, riparian areas, and urban areas. In contrast, ground squirrel species seldom climb trees, living instead in underground burrow systems, most frequently in prairies or grasslands, where many species hibernate during the winter months. A second possible explanation is that oocysts of Cryptosporidium species/genotypes infecting tree squirrels require water for efficient transmission to ground squirrel hosts. Supporting this, the same C. ubiquitum subtypes were found in storm water, tree squirrels, and ground squirrels in New York (Li et al., 2014). Abundant precipitation in the eastern US transitions to drier conditions on either side of the 100th meridian, which bisects the study area in the upper midwest, and conditions become increasingly more arid from west of the 100th meridian to California. Black-tailed prairie dogs and other ground squirrel species that thrive in the arid conditions west of the 100th meridian rarely drink water and obtain moisture from plant material (Lehner et al., 2001). This would limit their contact with Cryptosporidium species and genotypes that require water for efficient transmission to ground squirrel hosts.
transmission between tree and ground squirrel hosts. A corollary, which could be tested experimentally, is that Cryptosporidium genotypes associated with western and midwestern ground squirrel species are adapted to transmission under arid conditions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2015.10.002.

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