ECOLOGY OF *CRYPTOSPORIDIUM* PARASITES IN WILD RODENT POPULATIONS

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Brianna Leigh Schneck Stenger

In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Program:
Environmental and Conservation Sciences
Option:
Conservation Biology

November 2014

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Dr. John McEvoy
Co-Chair

Dr. Mark Clark
Co-Chair

Dr. Wendy Reed

Dr. Eakalak Khan

Approved:

04/13/2015
Date

Dr. Eakalak Khan
Department Chair

ABSTRACT

Cryptosporidium is a genus of ubiquitous parasites that have been detected worldwide in nearly 500 species of amphibians, birds, fish, mammals, and reptiles. Most research has focused on the *Cryptosporidium* species and genotypes infecting humans and livestock, because of the public health significance and economic importance of the diarrheal disease cryptosporidiosis. Relatively little is known about *Cryptosporidium*-host dynamics in wildlife hosts, even though a wide range of wildlife species are susceptible to *Cryptosporidium*. Insights into ecology and host-parasite dynamics in wild populations are necessary to understand the biology and evolution of *Cryptosporidium*; to predict the emergence of human and livestock pathogens; and to clarify *Cryptosporidium* taxonomy and systematics. The focus of this research was to study the ecology of *Cryptosporidium* in populations of cricetid (voles, *Peromyscus* mice, muskrats) and sciurid (squirrels and chipmunks) rodents, and characterize *Cryptosporidium* taxa by sequencing multiple genetic loci (18S rRNA and actin genes). Paralogous copies of the 18S rRNA gene in *Cryptosporidium* genotypes from wild rodents were common and affected phylogenetic inferences. Eastern chipmunks (*Tamias striatus*) were infected with *Cryptosporidium* chipmunk genotype II, which had 18S rRNA gene paralogs that shared ~93% similarity. The degree of divergence has not been previously described in any *Cryptosporidium* taxa, but is similar to the divergence described in *Plasmodium* species, which have functionally distinct 18S rRNA gene copies. Marmotini squirrels were mainly host to novel *Cryptosporidium* genotypes, and to the best of our knowledge, we provide the first molecular characterization of *Cryptosporidium* in black-tailed prairie dogs (*Cynomys ludovicianus*). *Cryptosporidium* host adaptation and specificity was not evident in in Sciurini rodents and they were host to two zoonotic taxa, *C. ubiquitum* and *Cryptosporidium* skunk genotype. In conclusion, *Cryptosporidium* was prevalent in cricetid and sciurid rodents, and the extent of host adaptation varied among *Cryptosporidium* taxa as they are likely shaped by differences in host-parasite ecology and evolution. The rodents sampled are not significant reservoirs of zoonotic *Cryptosporidium*, with the exception of tree squirrels. Sequencing multiple genetic loci helped identify the presence of paralogs and resolve cryptic *Cryptosporidium* taxa, which strengthened phylogenetic inferences leading to a better understanding of *Cryptosporidium* systematics.

ACKNOWLEDGEMENTS

This dissertation would not have been possible without the assistance, encouragement, and support of many people. I'd sincerely like to thank my advisors, Drs. John McEvoy and Mark Clark, for their continued guidance and allowing me to work on this interdisciplinary project. Also, for providing me with opportunities to teach and work on various other projects. I also want to thank Dr. Wendy Reed, Dr. Margaret Khaitsa, and Dr. Eakalak Khan for serving on my committee and for their continued support, patience, and encouragement. I also sincerely thank Dr. Martin Kváč for being an amazing collaborator and his willingness to help.

I cannot thank Cathy Giddings enough for all she has done for me (and other students) in and outside of the lab. I have learned so many things from her, which have helped me become a better scientist and person. She knew exactly when it was time for a Dairy Queen break or Redhawks game. Many thanks go out to all the McEvoy Lab grad students including Chris, Adam, and Tu for their friendship, willingness to help, and random brainstorming sessions. Also, thanks to the many undergraduate students that helped me both in the field and the lab, without them, I have no doubt I'd still be in the collection and PCR stage.

Thank you to the staff and faculty of the Dept. of Veterinary and Microbiological Sciences, Dept. of Biological Sciences, the Environmental & Conservation Sciences Program, and the Graduate School for their general assistance throughout the years I have been at NDSU. Mainly, Dr. Craig Stockwell, Dr. Will Bleier, Dr. Donna Jacob, Dr. Marinus Otte, Madonna Fitzgerald, Jerie Little, Phyllis Murray, Sandy Erickson, and Suzy Schmoll, who have kept normal day-to-day things moving, paperwork flowing, or allowed me to invade their property in my search for small mammals with *Cryptosporidium*.

Finally, I can't thank my family enough for their unending support, encouragement, guidance, help, and love. My parents, Bob and Cyndi, and brother, Nathan, for helping me get to this point in many different ways. Lastly, my husband John, for his continued love and support.

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GENERAL INTRODUCTION

Introduction

The genus *Cryptosporidium* consists of 26 species and more than 70 genotypes of ubiquitous, apicomplexan parasites that have been detected in hundreds of vertebrate species, including mammals, birds, reptiles, amphibians, and fish (Fayer, 2010; Kváč et al., 2014b). *Cryptosporidium* parasites infecting vertebrate hosts can cause the disease cryptosporidiosis, which is generally characterized by profuse, watery diarrhea; nausea; and vomiting (Current and Garcia, 1991). Cryptosporidiosis is primarily self-limiting in healthy individuals and supportive therapies are frequently adequate to treat the symptoms. However, with no universal effective drug treatment or vaccine, cryptosporidiosis can be fatal, particularly for hosts with a compromised or immature immune system (Garrido Davila and Ramirez Ronda, 1991; Quílez et al., 2008; Striepen, 2013). Driven by economic necessity and concerns for public and livestock health, most research has focused on disease in humans and domesticated animals (livestock, pets). However, wildlife are a major group of vertebrates susceptible to *Cryptosporidium* parasites, and we have a limited understanding of the *Cryptosporidium* parasites in these host species. Insights into ecology and host-parasite dynamics in wild populations are necessary to understand the biology and evolution of *Cryptosporidium*, to predict the emergence of human and livestock pathogens, and to clarify *Cryptosporidium* taxonomy and systematics.

Literature Review

Discovery and first description of *Cryptosporidium*. *Cryptosporidium* was first described by Ernest Tyzzer in the early 20th century (Tyzzer, 1907). He described the gastric species *Cryptosporidium muris* in 1910 and the intestinal species *Cryptosporidium parvum* in 1912 (Tyzzer, 1910, 1912), both from the house mouse (*Mus musculus*). Tyzzer classified *Cryptosporidium* as a coccidian, based on its life cycle, but also recognized how similar *Cryptosporidium muris* was to the gregarines, based on its localization at the epithelial cell surface and presence of extracellular stages in the lumen (Tyzzer, 1910). *Cryptosporidium* remained a coccidian for more than 80 years before phylogenetic studies of genes and genomes placed *Cryptosporidium* as an early branching Apicomplexan genus closely related to the gregarines (Figure 1) (Barta and Thompson, 2006; Carreno et al., 1999; Templeton et al., 2010; Zhu et

al., 2000). The phylum, Apicomplexa, includes the protozoan parasites with a structure known as the apical complex, which is used in host cell invasion.

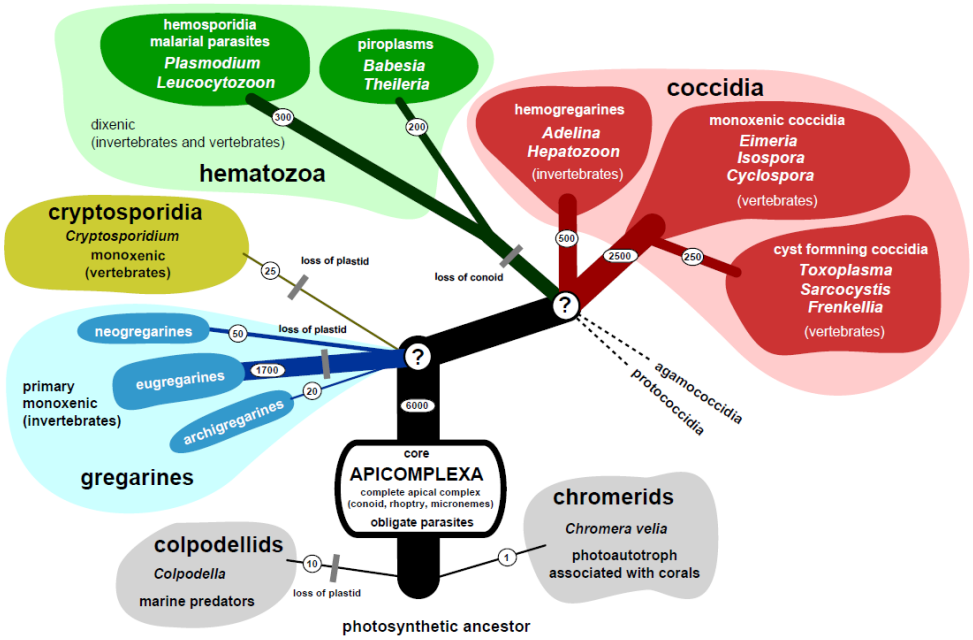

Figure 1. Hypothetical phylogenetic tree of Apicomplexan parasites and diversity indicated by branch thickness. (Slapeta, J., 2011, http://tolweb.org/Apicomplexa).

Cryptosporidium received little attention until the first reports of infections causing disease in humans were reported in 1976 (Meisel et al., 1976; Nime, 1976). Association of *Cryptosporidium* with chronic disease and death in AIDS patients, beginning in the early 1980s, and major waterborne outbreaks, including a massive outbreak in Milwaukee in 1993 (MacKenzie et al., 1994), elevated *Cryptosporidium* to its current status as a major human pathogen. Despite improvements to the drinking and recreational water infrastructure to combat this chlorine resistant parasite, numerous waterborne outbreaks are reported each year worldwide (Baldursson and Karanis, 2011; Karanis et al., 2007). Most recently, *Cryptosporidium* has been listed as the leading non-viral cause of diarrheal disease deaths in infants from developing countries (Kotloff et al., 2013; Striepen, 2013). The majority of human infections are caused by two species, *C. parvum* and *C. hominis*; however, there are 24 other *Cryptosporidium*

species and more than 70 unique *Cryptosporidium* genotypes that infect a wide range of vertebrate species (Fayer, 2010; Kváč et al., 2014b).

***Cryptosporidium* life cycle**. *The Cryptosporidium* life cycle, which is derived from work on the model species *C. parvum*, is monoxenous and has asexual and sexual stages (Figure 2). Oocysts excyst following ingestion to release four sporozoites through a suture opening in the oocyst wall (a) (Fayer, 2008). Each sporozoite invades a gastric or intestinal epithelial host cell (b), becomes rounded, and is enveloped by the host cell in a parasitophorous vacuole where the parasite, now a trophozoite (c), remains intracellular but extracytoplasmic (Huang et al., 2004; O'Donoghue, 1995).

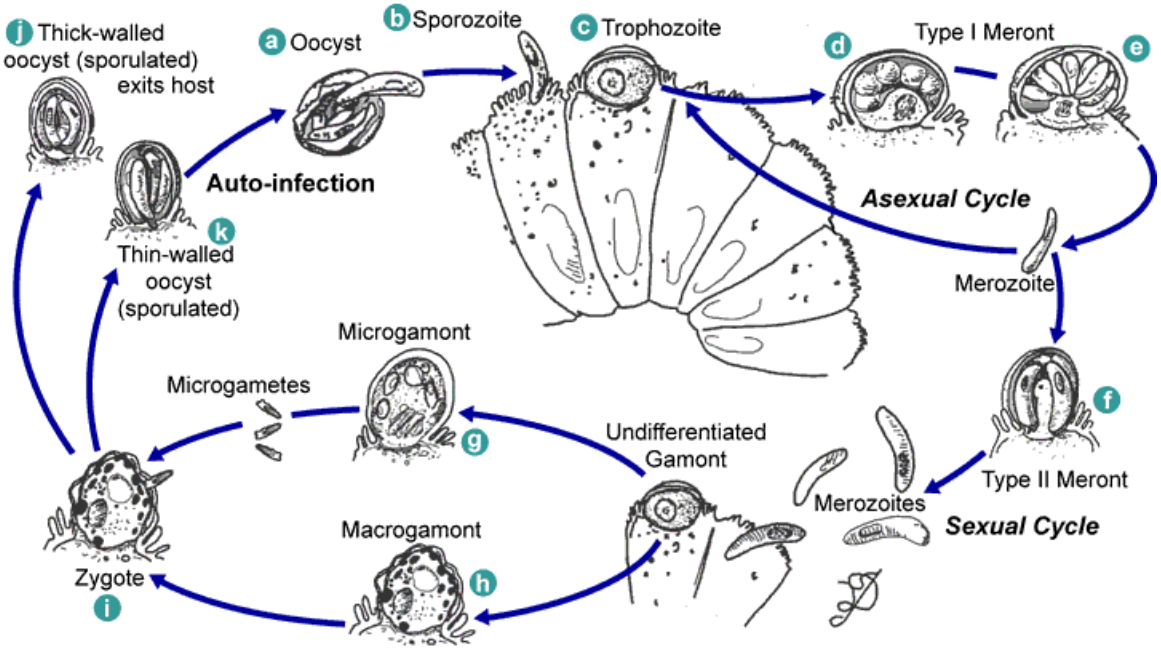

Figure 2. *Cryptosporidium* life cycle. (Centers for Disease Control and Prevention; http://www.cdc.gov/parasites/crypto/biology.html)

During the internalization process, a tunnel-like structure called the feeder organelle is formed, connecting the parasite to the host cell cytoplasm (Fayer, 2008; Huang et al., 2004). During asexual development, each trophozoite divides to produce eight (Type I meronts; d, e) or four (Type II meronts; f) merozoites in a process termed merogony (O'Donoghue, 1995). A third meront type is found in *Cryptosporidium baileyi* (Current et al., 1986). Merozoites released from mature meronts go on to infect other host cells where they develop into either Type I (d) or Type II meronts (f) (O'Donoghue, 1995).

Merozoites released from Type II meronts differentiate by gamogony into a microgamont (g) or macrogamont (h). The uninucleate macrogamont remains stationary while the microgamont releases up to 16 non-flagellated microgametes (O'Donoghue, 1995), which seek-out, penetrate, and fertilize the macrogamont. The mechanism by which the non-flagellated microgametes migrate to the macrogamonts remains unknown (Fayer, 2008). Following fertilization, a diploid zygote (i) is formed, undergoes sporogony to develop four sporozoites in a mature thick- or thinned-walled oocyst. Thick-walled (j) oocysts of both intestinal and gastric species usually leave the host in feces, but can also be expelled in respiratory/nasal secretions (Clavel et al., 1996; van Zeeland et al., 2008). Thinned-walled oocysts (k) contribute to an autoinfective cycle that maintains the infection in the host (O'Donoghue, 1995).

Thick-walled oocysts protect the four infectious sporozoites from harsh environmental conditions, including the chemicals used to treat drinking and recreational water (Betancourt and Rose, 2004; Carpenter et al., 1999). Oocysts can remain stable for long periods in cold, moist conditions. An exception is the amphibian-specific gastric species *Cryptosporidium fragile*, which is sensitive to hypertonic solutions and disintegrates after a month of storage in water (Jirků et al., 2008).

Transmission. Oocysts are generally transmitted from host to host via a fecal-to-oral route. Hosts include a number of vertebrate species, and transmission may be human-to-human (anthroponotic), animal to human and vice versa (zoonotic), and animal-to-animal. Transmission between hosts does not necessarily occur from direct contact with feces, but can occur through contaminated drinking and recreational water or fresh and prepared foods (Chalmers, 2012; Macarisin et al., 2010a; Macarisin et al., 2010b).

Most *Cryptosporidium* transmission studies have focused on identifying reservoirs and sources of human disease (Appelbee et al., 2005). Information on the sylvatic transmission dynamics (between wildlife species) is lacking. When examining individual fecal samples from wild animals, it can be difficult to differentiate infections from passive carriage resulting from ingestion of an infected animal (Feng et al., 2007; Ryan et al., 2003a; Xiao and Ryan, 2004); therefore, histological examinations and/or continuous monitoring are preferred when characterizing new host-parasite relationships. Sampling of wild animals can present challenges, including accessibility to animals and habitats, requirement for specialized equipment, requirement for permits, and human and wildlife safety concerns. These challenges are often

so prohibitive that researchers resort to opportunistic sampling or sampling captive animals, which do not accurately represent wild populations.

***Cryptosporidium* identification.** For much of the 20th century, *Cryptosporidium* was identified using microscopy to assess oocyst morphology, and *Cryptosporidium* was frequently confused with other apicomplexan parasites, such as *Sarcocystis* spp. (Xiao et al., 2004a). Identification methods improved with public health importance in the 1980s and 1990s, as researchers aimed to better understand taxonomy and identify sources of human and livestock cryptosporidiosis.

Although microscopy-based methods, including acid-fast staining and immunofluorescence assays, are useful for detecting the presence of *Cryptosporidium*, these methods cannot differentiate species (Casemore et al., 1985; Kehl et al., 1995). The delineation of species was initially based on strict host specificity and differences in oocyst morphology; however, this approach was brought into question when cross-transmission studies demonstrated parasites from one host species could infect other hosts. Also, morphology did not vary much among isolates (Fall et al., 2003; Xiao et al., 2004a). Consequently, several described species became invalid, including *C. anserinum* in geese and *C. rhesi* in monkeys (Barker and Carbonell, 1974; Fayer, 2008; Levine, 1980, 1984; Proctor and Kemp, 1974).

Many species were collapsed into a single species, *C. parvum*, when cross transmission studies failed to show strict host specificity and oocysts were similar in size (Xiao et al., 2004a). Other species including *C. felis, C. meleagridis*, and *C. wrairi,* were retained because they were well characterized (Iseki, 1979; Slavin, 1955; Vetterling et al., 1971). PCR and sequence analysis were increasingly adopted as a diagnostic tools in the 1990s, which led to greater sensitivity and an improved ability to distinguish among isolates (Morgan and Thompson, 1998). Restriction fragment length polymorphism analysis of PCR products led to the description of multiple taxa (Xiao et al., 2004b), and DNA sequencing further revolutionized taxonomic classification. The 18S rRNA gene is the most widely used target in molecular studies of *Cryptosporidium*, as evidenced by the large number of sequences published in GenBank. Molecular data is now a requirement for describing *Cryptosporidium* taxa and has led to a re-description of some *Cryptosporidium* taxa.

***Cryptosporidium* taxonomy.** The basic requirements for describing a *Cryptosporidium* species include: 1) description of oocyst morphology; 2) genetic characterization and comparison with published

sequences; 3) characterization of natural infections and, if possible, experimental infections; and 4) compliance with International Code of Zoological Nomenclature (ICZN) (Jirků et al., 2008; Xiao et al., 2004a). There are currently 26 recognized *Cryptosporidium* species (Table 1), which can be separated into two major biologically distinct groups: those infecting intestinal epithelial cells and those infecting gastric cells. One species, *C. baileyi*, which is considered an intestinal species, is also known to cause respiratory infections in birds (Cheadle et al., 1999; Molina-Lopez et al., 2010; van Zeeland et al., 2008). Although *C. baileyi* clusters with intestinal *Cryptosporidium* spp. in phylogenies inferred from sequences of the 18S rRNA gene and other loci, it clusters with gastric species at the HSP70 locus (Figure 3) (Xiao et al., 2002).

The widespread use of sequencing in the last decade has led to a large number of sequences deposited in GenBank from various host and environmental isolates (Xiao et al., 2004a). In addition to the 26 named species, more than 70 genotypes have been identified (Table 2), and this number is continually growing. Genotypes, which are frequently identified by unique 18S rDNA sequences, do not meet one or more of the requirements for species status, but may eventually be regarded as separate species once sufficient data are available (Fayer, 2010). The *Cryptosporidium* genotype designation system is also misleading, because a *Cryptosporidium* genotype may include multiple isolates with different 18S rDNA sequences; whereas in many other organisms, each unique DNA sequence is considered a different genotype (Xiao et al., 2002). For example, Ruecker et al. (Ruecker et al., 2013) reported 102 18S rDNA sequences (64 not previously reported) representing only 29 *Cryptosporidium* species and genotypes from water samples.

The lack of detailed information (e.g. host) for many of the *Cryptosporidium* genotypes and sequence variation within a taxon has contributed to the current confusion in *Cryptosporidium* taxonomy and systematics. When classifying and determining relationships between *Cryptosporidium* taxa, we need to be aware of the data going into the analyses (Morrison, 2006). More detailed descriptions of *Cryptosporidium* genotypes including characterizing multiple genetic loci such as the actin, hsp70, and *Cryptosporidium* oocyst wall protein (COWP) loci that will require the development of new primer sets, will help in resolving relationships between *Cryptosporidium* and determining species status (Power, 2010; Sulaiman et al., 2002; Sulaiman et al., 2000; Xiao et al., 2000b; Xiao et al., 2002).

Table 1. Valid *Cryptosporidium* species.

Species	Other names used in literature	Host	Location in Host	Reference
C. andersoni		Cattle	Gastric	(Lindsay et al., 2000)
C. baileyi	W10	Birds	Cloaca, Bursa, Trachea	(Current et al., 1986)
C. bovis	genotype bovine B	Cattle	Intestinal	(Fayer et al., 2005)
C. canis	dog genotype I, fox and coyote sub-genotypes	Canines	Intestinal	(Fayer et al., 2001)
C. cuniculus	rabbit genotype	Rabbits	Intestinal	(Robinson et al., 2010)
C. erinacei		Hedgehogs	Intestinal	(Kváč et al., 2014a)
C. fayeri	marsupial genotype I	Marsupials	Intestinal	(Ryan et al., 2008)
C. felis		Felines	Intestinal	(Iseki, 1979)
C. fragile		Amphibians	Gastric	(Jirků et al., 2008)
C. galli	*C. blagburni*, W22	Birds	Gastric	(Ryan et al., 2003b)
C. hominis	*C. parvum* genotype 1, W14, human genotype, genotype H,	Humans	Intestinal	(Morgan-Ryan et al., 2002)
C. macropodum	marsupial genotype II, EGK3	Marsupials	Intestinal	(Power and Ryan, 2008)
C. meleagradis		Birds, mammals	Intestinal	(Slavin, 1955)
C. molnari		Fish	Gastric and Intestinal	(Alvarez-Pellitero and Sitjà-Bobadilla, 2002)
C. muris		Rodents	Gastric	(Tyzzer, 1907, 1910)
C. parvum	*C. parvum* genotype II/2, bovine genotype, genotype B	Mammals	Intestinal	(Tyzzer, 1912)
C. ryanae	deer-like genotype	Cattle	Intestinal	(Fayer et al., 2008)
C. scrofarum	pig genotype II	Pigs	Intestinal	(Kváč et al., 2013)
C. serpentis		Reptiles	Gastric	(Levine, 1980)
C. suis	pig genotype I	Pigs	Intestinal	(Ryan et al., 2004b)
C. tyzzeri	mouse genotype I	Mice	Intestinal	(Ren et al., 2012)
C. ubiquitum	cervine genotype, W4, Sciuridae II, cervid, genotype 3	Mammals	Intestinal	(Fayer et al., 2010)
C. varanii	*C. saurophilum*	Reptiles	Intestinal and Cloaca	(Pavlásek et al., 1995)
C. viatorum		Humans	Intestinal	(Elwin et al., 2012b)
C. wrairi		Guinea pigs	Intestinal	(Vetterling et al., 1971)
C. xiaoi	*C. bovis*-like	Sheep	Intestinal	(Fayer and Santin, 2009)

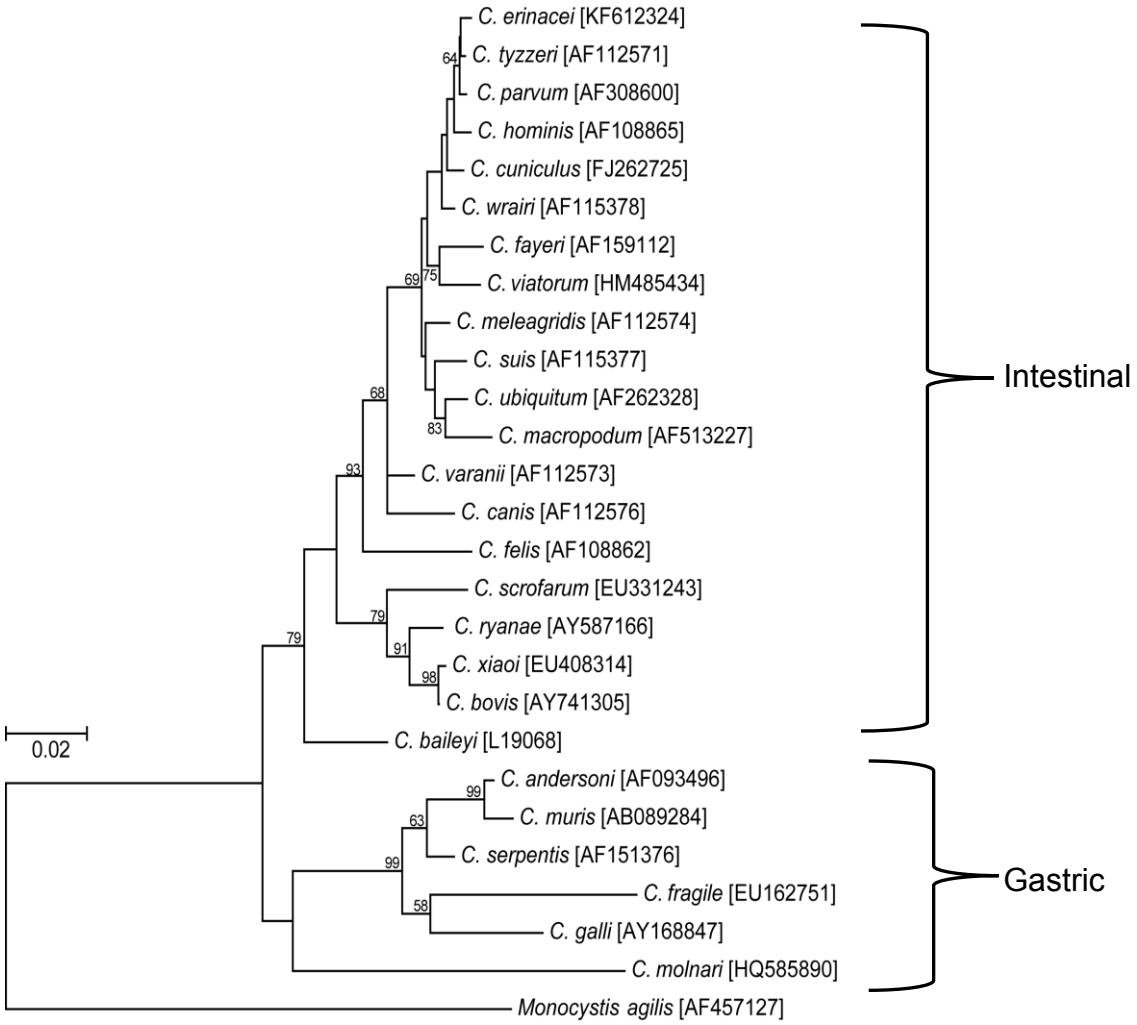

Figure 3. Neighbor-joining phylogenetic tree of the 26 *Cryptosporidium* species. Intestinal and gastric species are identified. Note the location of *C. baileyi* between the intestinal and gastric parasites.

Table 2. *Cryptosporidium* genotypes and alternative names.

Genotypes (other names)	Reference(s)
C. nasorum (*C. nasoris*)	(Hoover et al., 1981)
C. scophthalmi	(Alvarez-Pellitero et al., 2004)
Avian I-V	(Ng et al., 2006; Qi et al., 2011)
Bear	(Xiao et al., 2000c)
Beaver	(Feng et al., 2007)
Brushtail possum I-II	(Hill et al., 2008)
C. bovis-like	(Santín et al., 2007)
C. hominis-monkey	(Xiao et al., 1999b)
C. ryanae variant	(Feng et al., 2012)
C. suis-like	(Khan et al., 2010)
Chipmunk I (W17, Sciuridae I), II, III	(Feng et al., 2007)
Deer (W9)	(Xiao et al., 1999b)
Deer mouse I, II, III (W1), IV (W3, *Peromyscus* cluster II)	(Feng et al., 2007)
Duck	(Morgan et al., 2001)
Elephant seal (skunk-like)	(Rengifo-Herrera et al., 2011)
Eurasian woodcock	(Ryan et al., 2003a)
Ferret	(Xiao et al., 1999b)
Fox (W24)	(Xiao et al., 1999b)
Giant panda	(Liu et al., 2013)
Goose I-V	(Jellison et al., 2004; Xiao et al., 2002; Zhou et al., 2004b)
Guinea pig	(Huber et al., 2007)
Hamster	(Lv et al., 2009)
Horse	(Ryan et al., 2003a)
Kangaroo I	(Yang et al., 2011)
Lizard (*C. serpentis*-like)	(Xiao et al., 2002)
Mink	(Feng et al., 2007)
Mongoose	(Abe et al., 2004)
Mouse II	(Foo et al., 2007)
Muskrat I (W7)	(Xiao et al., 2002)
Muskrat II (W16)	(Zhou et al., 2004a)
Opossum I (W2, *C. fayeri*-opossum), II (W8)	(Xiao et al., 1999b; Xiao et al., 2002)
Piscine 1-8	(Koinari et al., 2013; Morine et al., 2012; Murphy et al., 2009; Reid et al., 2010; Ryan et al., 2004a; Zanguee et al., 2010)
Rat I, II, III, IV (W19)	(Ng-Hublin et al., 2013; Paparini et al., 2012)
Sbey/Sbld A	(Atwill et al., 2004; Pereira et al., 2010)
Sbey B	(Atwill et al., 2004; Pereira et al., 2010)
Sbey/Sbld/Sltl C	(Atwill et al., 2004; Pereira et al., 2010)
Sbld D	(Pereira et al., 2010)
Seal 1-4	(Bass et al., 2012; Rengifo-Herrera et al., 2013; Santín et al., 2005)
Shrew (W5)	(Feng et al., 2007)
Skunk (W13)	(Xiao et al., 1999b)
Snake I	(Xiao et al., 2002)
Snake II	(Xiao et al., 2004c)
Tortoise I, II (*C. ducismarci*)	(Traversa, 2010; Traversa et al., 2008; Xiao et al., 2002)
Vole (W15)	(Feng et al., 2007)
W12	(Xiao et al., 2000a)
W18	(Xiao et al., 2000a)
Wildebeest	(Alves et al., 2005)

18S rRNA gene. The 18S rRNA gene has been a useful target for phylogenetic studies of eukaryotic organisms. All eukaryotes have the gene and it tends to be present at a high copy number, which increases sensitivity of detection. 18S rRNA gene copies tend to evolve together (concerted evolution), due to their close association in tandem arrays on chromosomes, and this maintains the sequence homogeneity that is essential for phylogenetic studies (Nei and Rooney, 2005).

Although homogeneity of the 18S rRNA gene copies is generally assumed, exceptions have been found in some species, including apicomplexans such as *Plasmodium* spp. and *Eimeria* spp. in which paralogous 18S rRNA gene copies are known to exist and can be dispersed throughout the genome where they can be under different selective pressures (Barthelemy et al., 2007; El-Sherry et al., 2013; Gunderson et al., 1987; Krieger and Fuerst, 2002; McCutchan et al., 1995; van Wormhoudt et al., 2011). *Plasmodium* has relatively few 18S rRNA gene copies and they are dispersed across multiple chromosomes, which greatly limits the potential for unequal crossover and gene conversion, which are the drivers of homogenization in concerted evolution It has been proposed that, in contrast to concerted evolution, these dispersed 18S rRNA gene copies evolve by a birth-and-death model, whereby they evolve independently and can develop new functions, become non-functional, or be deleted (Nei and Rooney, 2005; Rooney, 2004). The divergent evolution of 18S rRNA genes could have major consequences for studies of phylogeny because paralogs are related by duplication, not descent (Buckler et al., 1997).

The copy number of the *C. parvum* 18S rRNA gene is low, copies are dispersed across multiple chromosomes, and divergent copies have been detected (Le Blancq et al., 1997), suggesting that they also could evolve by a birth-and-death model (Rooney, 2004). Heterogeneous 18S rRNA sequences, which could be divergent paralogs, have been detected in a number of *Cryptosporidium* taxa including *C. andersoni* (Ikarashi et al., 2013), *C. galli* (Morgan et al., 2001), *C. felis* (Xiao et al., 1999a), and the common brushtail possum genotype (Hill et al., 2008). This heterogeneity has been limited and is unlikely to have a major effect on phylogenetic inferences, but the potential for sequences to diverge together with a lack of tools to differentiate paralogs (genes related by duplication) and orthologs (genes related by descent) is a major concern.

***Cryptosporidium* in vertebrate animals.** Many *Cryptosporidium* spp. exhibit characteristics of host-adaptation, infecting or causing disease in a specific host species (e.g. humans) or groups of related hosts (Xiao et al., 2002). This knowledge has been used to assess disease risk and determine source(s) of infection. However, the host range of *Cryptosporidium* spp. can vary from narrow to broad (Cacciò et al., 2014; Elwin et al., 2012a; Feltus et al., 2006; Leoni et al., 2006; Morgan et al., 2000; Rašková et al., 2013; Robinson et al., 2008). *C. parvum* and *C. ubiquitum* each have a broad host range that includes humans, livestock, and wild mammals (Fayer, 2010; Fayer et al., 2010). In contrast, *C. wrairi* is has been reported only in guinea pigs (Vetterling et al., 1971). *Cryptosporidium* species associated with host(s) from one taxonomic class generally do not naturally infect a host from another class, with the exceptions of *C. parvum* and *C. meleagridis* (Fayer, 2010). Some cross-class host detections, such as *C. muris* (rodent adapted) in snakes, are likely to be the result of ingesting infected prey (Xiao et al., 2004a).

Cryptosporidium in fish. At approximately 30,000 species, fish make up more than half the total number of vertebrate species. This diverse group includes 3 extant classes of fish, Agnatha (hagfish and lampreys), Chondrichthyes (cartilaginous fishes), and Osteichthyes (bony fish). *Cryptosporidium* has been reported in a little over 20 of the approximately 28,000 species of bony fish.

Cryptosporidium has been found in wild, captive, freshwater, and marine fish, and can cause clinical signs of anorexia, emaciation, and mortality (Koinari et al., 2013; Murphy et al., 2009; Reid et al., 2010). *Cryptosporidium molnari*, the only recognized fish-adapted species, was first described in a gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) (Alvarez-Pellitero and Sitjà-Bobadilla, 2002). Since then, at least eight fish-adapted *Cryptosporidium* genotypes, piscine 1-8 (and variants), have been reported (Koinari et al., 2013; Morine et al., 2012; Murphy et al., 2009; Reid et al., 2010; Ryan et al., 2004a). *Cryptosporidium* species associated with humans and livestock also have been found in fish, including: *C. parvum*, *C. xiaoi*, and *C. scrofarum* in whiting (*Sillago vittata*) (Reid et al., 2010); *C. parvum* in Nile tilapias (*Oreochromis niloticus*), Silver barb (*Puntius gonionotus*), and mackerel scad (*Decapterus macarellus*) (Koinari et al., 2013); *C. hominis* in mackerel scad (Koinari et al., 2013); and *Cryptosporidium* rat genotype III-like isolate in a goldfish (*Carassius auratus*) (Morine et al., 2012). Some reports include *Cryptosporidium nasorum* and *C. scophthalmi* as species infecting fish because they are recognized as distinct parasites based on the physical description of oocysts and site of infection

(intestines) in fish; however, the lack of genetic information on these species renders them *nomen nudem*, as they have not met the basic requirements for species status (Alvarez-Pellitero et al., 2004; Hoover et al., 1981; Xiao et al., 2004a)

Cryptosporidium in amphibians. The vertebrate class Amphibia consists of more than 7000 species of ectothermic animals divided into three orders. *Cryptosporidium* has been described in frogs and toads of the order Anura, but not in salamanders or newts of the order Caudata, or caecillians of the order Gymnophiona. Although Anura comprises more than 85% of the known amphibians, and species in this order spend at least part of their lives in or near water, *Cryptosporidium* has only been reported in a captive Bell's horned frog (*Ceratophrys ornate*) (Crawshaw and Mehren, 1987), a captive South African clawed frog (*Xenopus laevis*) (Green et al., 2003), and doubtful/black-spined toads (*Duttaphrynus melanostictus*) (Jirků et al., 2008). The only species or genotype of *Cryptosporidium* identified was *C. fragile*, which infects the gastric epithelium of doubtful toads (Jirků et al., 2008).

Cryptosporidium in reptiles. The class Reptilia consists of approximately 10,000 species separated into four orders, Crocodilia, Rhynchocephalia, Squamata, and Testudines. *Cryptosporidium* has been detected in more than 55 species of the approximately 3,400 snake species (including pythons, boas, vipers, rattlesnakes, and other nonvenomous species) and approximately 25 species of the estimated 6,000 lizard species of the order Squamata (including, chameleons, geckos, iguana, monitors, and skinks). *Cryptosporidium* also has been detected in at least five species of tortoise in the order Testudine which consists of over 300 species of turtles and tortoises (reviewed by Kvac et al. (Kváč et al., 2014b)). Many of the infected animals have been pets or captive animals. *Cryptosporidium* has yet to be detected in alligators, crocodiles, caimens, and relatives in the order Crocodilia, or the two species of tuatara in the order Rhynchocephalia.

C. serpentis, *C. varanii* (syn. *C. saurophilum*), *Cryptosporidium* lizard genotype/*C. serpentis*-like genotype, *Cryptosporidium* tortoise genotype I, *Cryptosporidium* tortoise genotype II/*C. ducismarci* (*nomen nudem* because it lacks a morphological description), and *Cryptosporidium* snake genotypes I and II are the major taxa of *Cryptosporidium* adapted to and infecting snakes, lizards, and tortoises (Morgan et al., 1999; Richter et al., 2011; Richter et al., 2012; Rinaldi et al., 2012; Xiao et al., 2004c). *C. serpentis* is a gastric parasite known to cause gastritis, anorexia, lethargy and death in snakes. However,

some snakes and most lizards remain asymptomatic when infected with *C. serpentis* (Brownstein et al., 1977; Kimbell et al., 1999; Pedraza-Diaz et al., 2009). *C. varanii* typically infects the intestine of lizards and can cause disease and mortality in juveniles (Koudela and Modrý, 1998; Pavlásek and Ryan, 2008). As the names imply, *Cryptosporidium* tortoise genotype I and II (*C. ducismarci*) primarily infect tortoise species and can cause diarrhea and signs of disease (Griffin et al., 2010; Richter et al., 2012; Traversa, 2010; Traversa et al., 2008). These genotypes also have been detected in snakes and lizards (Alves et al., 2005; Pedraza-Diaz et al., 2009; Richter et al., 2012).

C. muris, *C. tyzzeri*, and *Cryptosporidium* rat genotype I have been detected in snakes, but experimental infections with avian and mammalian *Cryptosporidium* (*C. andersoni*, *C. baileyi, C. meleagridis, C. muris, C. muris*-like, and *C. wrairi*) have generally been unsuccessful (Pedraza-Diaz et al., 2009; Xiao et al., 2004c). *C. parvum*, *C. muris* and *Cryptosporidium* avian genotype V have been detected in lizards (Kik et al., 2011; Ryan et al., 2003a; Xiao et al., 2004c). The detection of mammalian and avian *Cryptosporidium* in reptiles could be attributed to the reptile ingesting infected prey (Xiao et al., 2004c).

Cryptosporidium in birds. The class Aves consists of approximately 10,000 species of egg-laying, winged, warm-blooded organisms. *Cryptosporidium* has been detected in approximately 130 avian species from 17 orders: Anseriformes (waterfowl), Caprimulgiformes (frogmouth), Charadriiformes (gulls, woodcock), Ciconiiformes (storks), Columbiformes (doves, pigeons), Coraciiformes (hornbills), Falconiformes (falcons), Galliformes (chicken, grouse, quail, partridge, pheasants), Gruiformes (cranes and coots), Passeriformes (perching birds), Pelecaniformes (cormorants), Piciformes (toucans), Psittaciformes (cockatoos, cockatiels, parrots), Phoenicopteriformes (flamingos), Sphenisciformes (penguins), Strigiformes (owls), Struthioniformes (ostriches and rheas) (reviewed by Kvac et al. (Kváč et al., 2014b)). Of the avian species sampled, cockatiels (*Nymphicus hollandicus*) host the greatest number of *Cryptosporidium* taxa including *C. baileyi*, *C. galli*, *C. meleagridis*, *C. parvum*, and avian genotype II, III and V (Abe and Iseki, 2004; Abe and Makino, 2010; Antunes et al., 2008; Gomes et al., 2012; Nakamura et al., 2009; Ng et al., 2006; Qi et al., 2011). Many identified hosts have been captive birds as pets or in pet shops, farms, zoological parks, or similar facilities. *Cryptosporidium* also has been reported in a number of wild birds including waterfowl, gulls, pigeons, Eurasian coot, cormorants, penguins, and a

handful of passerine species (Abreu-Acosta et al., 2009; Bogomolni et al., 2008; Fredes et al., 2008; Fredes et al., 2007; Jellison et al., 2009; Kuhn et al., 2002; Plutzer and Tomor, 2009; Ryan et al., 2003a; Sevá Ada et al., 2011; Smith et al., 1993)

Three species (*C. baileyi, C. galli,* and *C. meleagridis*) and at least 12 genotypes (*Cryptosporidium* avian genotypes I-V, *Cryptosporidium* goose genotypes I-V, *Cryptosporidium* Eurasian woodcock genotype, and *Cryptosporidium* duck genotype) are primarily found in avian hosts. Of these, *C. meleagridis* has been increasingly reported in mammals and may have originated in mammals and later adapted to birds (Xiao, 2010; Xiao et al., 2004a; Xiao et al., 2002). *C. parvum* is not uncommon in avian species and has been detected in Bengalese finch (*Lonchura striata domestica*), Canada geese (*Branta canadensis*), Pekin ducks (*Anas platyrhynchos*), white storks (*Ciconia ciconia*), gyrfalcon (*Falco rusticolus*), Eurasian coot (*Fulica atra*), and cockatiels (*Nymphicus hollandicus*) (Barbon and Forbes, 2007; Gomes et al., 2012; Graczyk et al., 1996; Graczyk et al., 1997; Majewska et al., 2009; Nakamura et al., 2009; Plutzer and Tomor, 2009). Frequently, these hosts may be serving as mechanical vectors, but *C. parvum* infection has been associated with disease in a gyrfalcon, which was successfully treated with paromomycin (Barbon and Forbes, 2007). Other mammal-adapted *Cryptosporidium* spp., including *C. hominis*, have been detected in, but are probably not infective for, rock pigeons (*Columba livia*) and Canada geese (Abreu-Acosta et al., 2009; Jellison et al., 2009; Zhou et al., 2004b). *Cryptosporidium* muskrat genotype I, *C. andersoni*, and *C. muris* have also been detected in Canada geese, quail-crested wood partridge (*Rolulus roul roul*), and tawny frogmouth (*Podargus strigoides*), respectively (Jellison et al., 2009; Ng et al., 2006).

Avians can be asymptomatic or exhibit symptoms such as a prolapsed cloaca, diarrhea, anorexia, and respiratory and ocular distress. These symptoms reflect infection sites that include the proventriculus (*C. galli* and *Cryptosporidium* avian genotype III), the intestine (*C. meleagridis* and *Cryptosporidium* avian genotype V), conjunctiva, trachea, air sacs, kidneys, urinary tract, bursa of Fabricius, and cloaca (*C. baileyi*) (Chvala et al., 2006; Lindsay et al., 1986; Ryan, 2010; Santos et al., 2005).

Cryptosporidium in mammals. Mammalia is the vertebrate class with fewest species (5,500), but *Cryptosporidium* has been detected in more than 240 mammalian species in 19 orders, which is more than in any other vertebrate class. Almost 200 of the mammalian species are found in only 4 orders of

placental mammals: Artiodactyla (65 species), Carnivora (37 species), human and non-human primates (48 species), and Rodentia (45 species).

The greatest number of *Cryptosporidium* taxa (species and genotypes) is found in the class Mammalia and includes: *C. andersoni, C. bovis, C. canis, C. cuniculus, C. erinacei, C. fayeri, C. felis, C. hominis, C. macropodum, C. muris, C. parvum, C. ryanae, C. scrofarum, C. suis, C. tyzzeri, C. ubiquitum, C. viatorum, C. wrairi, C. xiaoi*, and more than 40 genotypes (Tables 1 and 3). *C. baileyi* also has been detected in rodents and humans, but is predominantly associated with infections in birds (Ditrich et al., 1991; Ziegler et al., 2007).

Like the other vertebrate animals, mammals (including humans) may show symptoms of an infection or be asymptomatic. Generally, younger mammals and individuals with compromised immune systems are most susceptible and exhibit the diarrheal disease that in some cases can lead to death (de Graaf et al., 1999; Fayer et al., 1998; Kotloff et al., 2013). However, signs and symptoms such as abdominal discomfort, nausea, fever, weight loss, and dehydration are associated with other diseases so the incidence of *Cryptosporidium* infection in humans and other animals is likely to be underreported (Yoder et al., 2010).

Cryptosporidium has been detected in humans, livestock (cattle, pigs, and sheep), laboratory animals (mainly rodents), pets, and both captive and wild (non-captive) mammalian wildlife species (Table 3). However, many of the mammals identified as hosts for *Cryptosporidium* have been domesticated farm animals or captive animals at facilities such as zoos (reviewed in (Kváč et al., 2014b)). Host-parasite dynamics can be very different in captive versus wild animals, as they have different behaviors, community interactions, densities, stressors, and life cycles. Therefore, infections and evolution of *Cryptosporidium* in wild animals may be very different to those in captive animals.

Table 3. Mammalian species (excluding humans) positive for *Cryptosporidium* and the species or genotypes if known.

Order	Common name	Species name	*Cryptosporidium* spp.
Placental			
Artiodactyla			
	Addax	*Addax nasomaculatus*	
	Addra gazelle	*Gazella dama*	
	African buffalo	*Syncerus caffer*	*C. bovis, C. ubiquitum*
	Alpaca	*Lama pacos*	*C. parvum*
	Alpine ibex	*Capra ibex*	*C. ubiquitum*
	American bison	*Bison bison*	*C. tyzzeri*
	Armenian mouflon	*Ovis orientalis gmelini*	
	Axis deer	*Axis axis*	
	Bactrian camel	*Camelus bactrianus*	*C. andersoni, C. muris*
	Barasingha deer (swamp deer)	*Cervus duvauceli*	*C. parvum, C. ubiquitum*
	Barbary sheep	*Ammotragus lervia*	*C. tyzzeri*
	Black wildebeest	*Connochaetes gnou*	similar to shrew genotype(W5)
	Blackbuck	*Antilope cervicapra*	
	Blesbok	*Damaliscus dorcas philipsi*	*C. ubiquitum*
	Blue wildebeest (Blue-eared gnu)	*Connochaetes taurinus*	*C. parvum*
	Bongo	*Tragelaphus eurycerus*	*C. parvum*
	Caribou	*Rangifer tarandus*	
	Collared peccary	*Tayassu tajacu*	
	Cow	*Bos primigenius*	*C. andersoni, C. bovis, C. parvum, C. ryanae, C. ubiquitum, C. hominis, C. suis, C. canis*
	Dorca's gazelle	*Gazella dorcas*	
	Dromedary camels	*Camelus dromedaries*	
	Eland	*Taurotragus oryx*	*C. parvum*

(Continued)

Table 3. Mammalian species (excluding humans) positive for *Cryptosporidium* and the species or genotypes if known (continued).

Order	Common name	Species name	*Cryptosporidium* spp.
	Eld's deer	*Cervus eldi*	
	Elk (red deer or wapiti)	*Cervus elaphus*	*C. parvum, C. ubiquitum*
	Ellipsen waterbuck	*Kobus ellipsiprymmus*	
	European bison	*Bison bonasus*	*C. andersoni*
	Fallow deer	*Dama dama*	
	Fringe-eared oryx	*Oryx gazella callotys*	
	Giraffe	*Giraffa camelopardalis*	*C. muris*
	Goat	*Capra hircus*	*C. parvum, C. xiaoi, C. hominis*
	Guanaco	*Lama guanicoe*	
	Impala	*Aepyceros melampus*	*C. ubiquitum*
	Llama	*Lama glama*	
	Lowland anoa	*Bubalus depressicornis*	
	Moose	*Alces alces*	
	Mouflon	*Ovis musimon*	*C. ubiquitum*
	Mountain gazelle	*Gazella cuvieri*	
	Mule deer	*Odocoileus hemionus*	
	Muntjac deer	*Muntiacus reevesi*	
	Nile lechwe	*Kobus megaceros*	
	Nilgai	*Boselaphus tragocamelus*	
	Nyala	*Tragelaphus angasi*	*C. ubiquitum*
	Ox	*Bos taurus*	
	Pere David's deer	*Elaphurus davidianus*	
	Persian gazelle	*Gazella subgutterosa*	
	Pig	*Sus scrofa*	*C. parvum, C. suis, C. scrofarum, C. felis, C. hominis, C. meleagridis, C. muris, C. tyzzeri,* Eirew65.5, rat genotype, *C. suis*-like
	Pygmy hippopotamus	*Hexaprotodom liberiensis*	

(Continued)

Table 3. Mammalian species (excluding humans) positive for *Cryptosporidium* and the species or genotypes if known (continued).

Order	Common name	Species name	*Cryptosporidium* spp.
	Roe deer	*Capreolus capreolus*	*C. parvum, C. ubiquitum*
	Sable antelope	*Hippotragus niger*	*C. parvum*
	Sambar	*Cervus unicolor*	
	Scimitar horned oryx	*Oryx gazella dammah*	
	Sheep	*Ovis aries*	*C. parvum, C. ubiquitum, C. xiaoi, C. hominis, C. suis, C. andersoni*
	Sika deer	*Cervus nippon*	*C. parvum, C. ubiquitum*
	Slender-horned gazelle	*Gazella leptoceros*	*C. parvum*
	Springbok	*Antidorcas marsupialis*	
	Takin	*Budorcas taxicolor*	*C. tyzzeri*
	Thomson's gazelle	*Gazella thomsoni*	
	Thorold's deer	*Cervus albirostris*	
	Tule elk	*Cervus canadensis* ssp. *nannodes*	
	Turkomen markhor	*Capra falconeri*	
	Urial	*Ovis orientalis*	
	Water buffalo	*Bubalus bubalis*	*C. ryanae, C. ryanae* variant
	White-tailed deer	*Odocoileus virginianus*	deer genotype
	Yak	*Bos muttus*	*C. andersoni, C. bovis, C. parvum, C. ryanae, C. ubiquitum, C. xiaoi*
	Zebu	*Bos indicus*	*C. ryanae* variant
Carnivora			
	American mink	*Mustela vison*	mink genotype
	Arctic fox	*Vulpes lagopus*	
	Badger	*Meles meles*	
	Banded mongoose	*Mungos mungo*	
	Beech marten	*Martes foina*	
	Black bear	*Ursus americanus*	bear genotype

(Continued)

Table 3. Mammalian species (excluding humans) positive for *Cryptosporidium* and the species or genotypes if known (continued).

Order	Common name	Species name	*Cryptosporidium* spp.
	Black-footed ferret	*Mustela nigripes*	ferret genotype
	Bobcat	*Lynx rufus*	
	Brown bear	*Ursus arctos*	
	California sea lion	*Zalophus californianus*	
	Cat	*Felis catus*	*C. felis*
	Cheetah	*Acironyx jubatus*	
	Coyote	*Canis latrans*	*C. canis* coyote genotype
	Dog	*Canis familiaris*	*C. canis, C. parvum, C. meleagridis, C. muris*
	Ermine	*Mustela erminea*	shrew genotype (W5), W18
	European otter	*Lutra lutra*	
	Ferret	*Mustela putorius*	ferret genotype
	Giant panda	*Ailuropoda melanoleuca*	giant panda genotype
	Gray wolves	*Canis lupus*	*C. parvum*
	Grey fox	*Urocyon cinereoargenteus*	
	Grey seal	Halichoerus grypus	
	Harbor seal	*Phoca vitulina*	seal genotype 1 and 2
	Harp seal	*Pagophilus groenlandicus*	seal genotype 3
	Hooded seal	*Cystophora cristata*	seal genotype 1 and 2
	Leopard	*Panthera pardus*	*C. tyzzeri*
	Lesser panda	*Aliurus fulgens*	*C. tyzzeri*
	Malayan bear	*Helarctos malayanus*	
	Polar bear	*Ursus Thalarctos maritimus*	
	Raccoon	*Procyon lotor*	*C. parvum,* skunk genotype
	Raccoon dog	*Byctereutes procyonoides viverrinus*	*C. parvum*
	Red fox	*Vulpes vulpes*	*C. parvum*, muskrat genotype II

(Continued)

Table 3. Mammalian species (excluding humans) positive for *Cryptosporidium* and the species or genotypes if known (continued).

Order	Common name	Species name	*Cryptosporidium* spp.
	Ringed seal	*Phoca hispida*	*C. muris,* seal genotypes 1 and 2
	River otter	*Lontra canadensis*	mink genotype, skunk genotype
	Sea otter	*Enhydra lutris nereis*	
	Southern elephant seal	*Mirounga leonina*	elephant seal genotype
	Striped skunk	*Mephitis mephitis*	skunk genotype
	Weddell seal	*Leptonychotes weddellii*	seal genotype 4
Cetacea			
	Bowhead whales	*Balaena mysticetus*	
	Common dolphin	*Delphinus delphis*	
	North Atlantic right whales	*Eubalaena glacialis*	
Chiroptera			
	Big brown bat	*Eptesicus fuscus*	*C. parvum*
	Chinese rufous horseshoe bat	*Rhinolophus sinicus*	
	Fulvus roundleaf bat	*Hipposideros fulvus*	
	Large-footed mouse-eared bat	*Myotis adversus*	*C. tyzzeri*
	Leschenault's rousette	*Rousettus leschenaulti*	
	Little brown bat	*Myotis lucifugus*	
	Pomona roundleaf bat	*Hipposideros pomona*	
	Stoliczka's trident bat	*Aselliscus stoliczkanus*	
Erinaceomorpha			
	African hedgehog	*Ateletrix albiventris*	
	European hedgehog	*Erinaceus europaeus*	*C. erinaceai*
Hyracoidea			
	Rock hyrax	*Procavia capensis*	*C. muris*

(Continued)

Table 3. Mammalian species (excluding humans) positive for *Cryptosporidium* and the species or genotypes if known (continued).

Order	Common name	Species name	*Cryptosporidium* spp.
Lagomorpha			
	Cottontail	*Sylvilagus floridanus*	
	European rabbit	*Oryctolagus cuniculus*	*C. cuniculus*
Perissodactyla			
	Brazilian tapir	*Tapirus terrestris*	
	Horse	*Equus caballus*	*C. parvum*, horse genotype, hedgehog genotype
	Miniature horse	*Equus przewalski*	
	Przewalski's horse	*Equus ferus*	horse genotype
	Rhinoceros	*Rhinoceros unicornis*	
	Southern white rhinoceros	*Ceratotherium simum*	
	Zebra	*Equus zebra*	
Primates			
	Baboon	*Papio cynocephalus*	
	Black lemur	*Eulemur macaco*	
	Black-and-white colobus	*Colobus guereza*	
	Black-and-white ruffed lemur	*Lemur variegatus*	
	Black-capped squirrel monkey	*Saimiri sciureus boliviensis*	
	Bonnet macaque	*Macaca radiata*	
	Brown lemur	*Lemur macacomayottensis*	
	Brown spider monkey	*Ateles belzebuth hybridus*	
	Campbell's mona	*Cercopithecus campbelli*	
	Common marmoset	*Calithrix jacchus*	
	Coquerel's sifaka	*Propithecus verreauxi coquereli*	
	Cotton-tipped/pigtail macaque	*Macaca nemestrina*	
	Cotton-topped tamarin	*Saguinus oedipus*	
	Drill	*Mandrillus leucophaeus*	

(Continued)

Table 3. Mammalian species (excluding humans) positive for *Cryptosporidium* and the species or genotypes if known (continued).

Order	Common name	Species name	*Cryptosporidium* spp.
	Eastern rufous mouse lemur	*Microcebus rufus*	
	Gray langur	*Semnopithecus entellus thersites*	*C. parvum*
	Greater bamboo lemur	*Prolemur simus*	
	Japanese macaque	*Macaca fuscata*	
	Lesser slow loris	*Nycticebus pygmaeus*	
	Long-tailed macaque (Crab eating)	*Macaca fascicularis*	
	Mangabey	*Cercocebus albigena*	
	Marimonda spider monkey	*Ateles belzebuth*	
	Mountain gorilla	*Gorilla beringei beringei*	*C. parvum*
	Moustached tamarin	*Saguinus mystax*	
	Olive baboon	*Papio anubis*	*C. hominis*
	Orangutan	*Pongo pygmaeus*	
	Patas monkey	*Erythrocebus patas*	
	Pig-tailed macaque	*Macaca leonina*	
	Purple-faced lagur	*Trachypithecus vetulus philbricki*	*C. parvum*
	Red colobus	*Procolobus rufomitratus*	
	Red ruffed lemur	*Vaecia variegate rubra*	
	Red-tailed guenon	*Cercopithecus ascanius*	
	Rhesus macaque	*Macaca mulatta*	*C. hominis* monkey genotype, *C. hominis, C. felis, C. parvum*
	Ring-tailed lemur	*Lemur catta*	
	Savanna chimpanzee	*Pan troglodytes schweinfurthii*	
	Siamang	*Hylobates syndactylus syndactylus*	
	Squirrel monkey	*Saimiri sciureus*	

(Continued)

Table 3. Mammalian species (excluding humans) positive for *Cryptosporidium* and the species or genotypes if known (continued).

Order	Common name	Species name	*Cryptosporidium* spp.
	Talapoin monkey	*Miopithecus talapoin*	
	Tibetan macaque (Pere David's)	*Macaca thibetana*	
	Toque macaque	*Macaca sinica sinica*	*C. parvum*
	Velvet monkey	*Cercopithecus aethiops*	
	Vervet monkey	*Chlorocebus pygerythrus*	
	Western gorilla	*Gorilla gorilla*	
	White-collared monkey	*Cercocebus torquatus*	
	White-crowned mangabeys	*Cercocebus torquatus lunulatus*	
	White-faced saki	*Pithecia pithecia*	
Proboscidea			
	African elephant	*Loxodonta africana*	
	Indian elephant	*Elephas maximus*	
Rodentia			
	American red squirrel	*Tamiasciurus hudsonicus*	*C. ubiquitum,* chipmunk genotype I
	Bank or Red-backed vole	*Myodes glareolus*	
	Beaver	*Castor canadensis*	*C. ubiquitum,* beaver genotype
	Belding's ground squirrel	*Spermophilus beldingi*	Sbey/Sbld A, Sbey/Sbld/Stltl C, and Sbld D genotypes
	Black or House rat	*Rattus rattus*	*C. parvum,* rat genotypes II and III
	Bobak marmot	*Marmota bobac*	*C. andersoni*
	Brazilian porcupine	*Coendou prehensiles*	
	Brown or Norwegian rat	*Rattus norvegicus*	*C. muris, C. scrofarum, C. tyzzeri,* rat genotypes I-IV
	California ground squirrel	*Spermophilus beecheyi*	Sbey/Sbld A, Sbey B, Sbey/Sbld/Stltl C
	Campbell hamster	*Phodopus campbelli*	*C. andersoni, C. muris, C. parvum*
	Capybara	*Hydrochoerus hydrachaeris*	*C. parvum*

(Continued)

Table 3. Mammalian species (excluding humans) positive for *Cryptosporidium* and the species or genotypes if known (continued).

Order	Common name	Species name	*Cryptosporidium* spp.
	Chinchilla	*Chinchilla laniger*	
	Common vole (Orkney)	*Microtus arvalis*	
	Cotton rat	*Sigmodon hispidus*	
	Coypu	*Myocastor coypus*	
	Deer mouse or white footed mice	*Peromyscus* spp.	*C. parvum, C. ubiquitum,* chipmunk genotype I, muskrat genotype II, deer mouse genotypes I-IV
	East African mole rat	*Tachyoryctes splendens*	
	Eastern chipmunk	*Tamias striatus*	*C. andersoni, C. baileyi, C. ubiquitum,* chipmunk genotypes I and II
	Eastern gray squirrel	*Sciurus carolinensis*	*C. baileyi, C. muris, C. parvum, C. ubiquitum,* chipmunk genotype I, deer mouse genotype III, skunk genotype
	Eurasian red squirrel	*Sciurus vulgaris*	*C. ubiquitum,* ferret genotype, chipmunk genotype I
	European beaver	*Castor fiber*	
	Field vole	*Microtus agrestis*	
	Fox squirrel	*Sciurus niger*	
	Golden hamster	*Mesocricetus auratus*	*C. andersoni, C. muris, C. parvum*
	Golden-mantled ground squirrel	*Spermophilus lateralis*	Sbey/Sbld/Sltl C genotype
	Guinea pig	*Cavia porcellus*	*C. wrairi,* guinea pig genotype
	House mouse	*Mus musculus*	*C. muris, C. tyzzeri,* mouse genotype II
	Indian porcupine	*Hystrix indica*	
	Japanese field mouse	*Apodemus speciosus*	
	Meadow vole	*Microtus pennsylvanicus*	muskrat genotype II, vole genotype
	Muskrat	*Ondatra zibethicus*	muskrat genotype I and II
	North American porcupine	*Erethizon dorsatum*	

(Continued)

Table 3. Mammalian species (excluding humans) positive for *Cryptosporidium* and the species or genotypes if known (continued).

Order	Common name	Species name	*Cryptosporidium* spp.
	Pocket gopher	*Geomys bursarius*	
	Red-backed vole	*Clethrionomys rufocanus bedfordiae*	
	Siberian chipmunk	*Tamias sibiricus*	*C. muris, C. parvum,* chipmunk genotype III, ferret genotype
	Siberian hamster	*Phodopus sungorus*	*C. andersoni, C. muris, C. parvum,* hamster genotype
	Southern flying squirrel	*Glaucomys volans*	
	Southern red-backed vole (Boreal vole)	*Myodes gapperi*	*C. parvum,* vole cluster, W12, muskrat genotype I and II
	Striped field mouse	*Apodemus agrarius*	
	Tanezumi rat (Asian house rat)	*Rattus tanezumi*	*C. scrofarum, C. suis-like, C. tyzzeri,* rat genotypes II-I*V*
	Thirteen-lined ground squirrel	*Ictidomys tridecemlineatus*	
	Western Mediterranean mouse (Algerian mouse)	*Mus spretus*	*C. muris, C. parvum*
	Wood mouse	*Apodemus sylvaticus*	*C. muris, C. parvum*
	Woodchuck	*Marmota monax*	*C. ubiquitum*
	Yellow-necked mouse	*Apodemus flavicollis*	*C. parvum, C. tyzzeri*
Sirenia			
	Dugong	*Dugong dugon*	*C. hominis*
Soricomorpha	Brewer's mole	*Parascalops brewer*	
	Common shrew (Long-tailed shrew)	*Sorex araneus*	
	Greater white-toothed shrew	*Crocidura russula*	
	Masked shrew	*Sorex cinereus*	
	Northern short-tailed shrew	*Blarina brevicauda*	
	Pygmy shrew	*Sorex minutus*	
	White-toothed shrew	*Crocidura russula*	

(Continued)

Table 3. Mammalian species (excluding humans) positive for *Cryptosporidium* and the species or genotypes if known (continued).

	Order	Common name	Species name	*Cryptosporidium* spp.
Marsupials				
	Dasyuromorphia			
		Brown antechinus	*Antechinus stuartii*	
	Didelphimorphia			
		Virginia opossum	*Didelphis virginiana*	*C. fayeri, C. fayeri* opossum genotype (opossum genotype I), opossum genotype II
		White-eared opossum	*Didelphis albiventris*	
	Diprotodontia			
		Brushtail possum	*Trichosurus vulpecula*	brushtail possum genotype I and II
		Eastern grey kangaroo	*Macropus giganteus*	*C. fayeri, C. macropodum*
		Koala	*Phascolarctos cinereus*	*C. fayeri*
		Pademelon	*Thylogale billardierii*	
		Red kangaroo	*Macropus rufus*	*C. fayeri, C. macropodum*
		Red neck wallaby	*Macropus rufogriseus*	
		Swamp wallaby	*Wallabia bicolor*	*C. macropodum*
		Western grey kangaroo	*Macropus fuliginosus*	*C. macropodum,* kangaroo genotype I
		Yellow-footed rock wallaby	*Petrogale xanthopus*	*C. fayeri*
	Peramelemorphia			
		Greater bilbies	*Macrotis lagotis*	*C. muris*
		Southern brown bandicoot	*Isodon obesulus*	
		Western-barred bandicoot	*Peremeles bougainville*	*C. fayeri*
Monotremes				
	Monotremata			
		Short-beaked echidna	*Tacyglossus aculeatus*	

Dissertation Objectives

This research aims to better understand the ecology and evolution of *Cryptosporidium* in wildlife by using an interdisciplinary approach to study host and parasite population dynamics of wild small mammals. Outcomes will be used to assess the public health impact of the changing boundaries between wildlife and humans, and will help to clarify *Cryptosporidium* taxonomy and systematics.

This research focused on wild small mammals of the order Rodentia because: 1) *Cryptosporidium* prevalence can be quite high in some rodent species; 2) rodents are ubiquitous with populations and communities varying over short distances and with time; 3) rodents often live in close proximity to humans, which could increase the likelihood of zoonotic or anthroponotic transmission.

Objective 1. To investigate the *Cryptosporidium* parasites in populations of wild, North American rodent species. Wild rodents will be used as a model to understand factors affecting host-adaptation and host-parasite coevolution, and assess the role of wild rodents in the transmission of human- and livestock-pathogenic *Cryptosporidium.*

Objective 2. Use a multiple loci approach (18S rRNA and actin genes) to identify *Cryptosporidium* taxa and understand *Cryptosporidium* taxonomy and systematics. *Cryptosporidium* taxonomy and systematics is poorly understood, and confusion could be compounded by inferences made using a single genetic locus, the 18S rRNA gene, that is known to be heterogeneous in some taxa.

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PAPER 1. HIGHLY DIVERGENT 18S RRNA GENE PARALOGS IN A *CRYPTOSPORIDIUM* GENOTYPE FROM EASTERN CHIPMUNKS (*TAMIAS STRIATUS*)[1]

Abstract

Cryptosporidium is an apicomplexan parasite that causes the disease cryptosporidiosis in humans, livestock, and other vertebrates. Much of the knowledge on *Cryptosporidium* diversity is derived from 18S rRNA gene (18S rDNA) phylogenies. Eukaryote genomes generally have multiple 18S rDNA copies that evolve in concert, which is necessary for the accurate inference of phylogenetic relationships. However, 18S rDNA copies in some genomes evolve by a birth-and-death process that can result in sequence divergence among copies. Most notably, divergent 18S rDNA paralogs in the apicomplexan *Plasmodium* share only 89-95% sequence similarity, encode structurally distinct rRNA molecules, and are expressed at different life cycle stages. In the present study, *Cryptosporidium* 18S rDNA was amplified from 28/72 (38.9%) eastern chipmunks (*Tamias striatus*). Phylogenetic analyses showed the co-occurrence of two 18S rDNA types, Type A and Type B, in 26 chipmunks, and Type B clustered with a sequence previously identified as *Cryptosporidium* chipmunk genotype II. Types A and B had a sister group relationship but shared less than 93% sequence similarity. In contrast, actin and heat shock protein 70 gene sequences were homogeneous in samples with both Types A and B present. It was therefore concluded that Types A and B are divergent 18S rDNA paralogs in *Cryptosporidium* chipmunk genotype II. Substitution patterns in Types A and B were consistent with functionally constrained evolution; however, Type B evolved more rapidly than Type A and had a higher G+C content (46.3% versus 41.0%). Oocysts of *Cryptosporidium* chipmunk genotype II measured 4.17 µm (3.73–5.04 µm) × 3.94 µm (3.50-4.98 µm) with a length-to-width ratio of 1.06 ± 0.06 µm, and infection occurred naturally in the jejunum, cecum, and colon of eastern chipmunks. The findings of this study have implications for the use of 18S rDNA sequences to infer phylogenetic relationships.

Introduction

The apicomplexan *Cryptosporidium* parasitizes the major vertebrate groups (Kváč et al., 2014) and is a major cause of diarrheal disease in humans and livestock. Twenty-six species are currently

[1] The material in this chapter has been published in *Infection, Genetics, and Evolution*, an Elsevier journal publication. This chapter was co-authored by Brianna L.S. Stenger, Mark E. Clark, Martin Kváč, Catherine W. Giddings, Neil W. Dyer, Jessie L. Schultz and John M. McEvoy. Brianna L.S. Stenger had the primary responsibility for collecting data, analysis, interpretation, and writing of this manuscript.

recognized (Ryan et al., 2014), but these represent just a fraction of *Cryptosporidium* diversity. In addition the named species, more than 70 genotypes have been described from sequences of rRNA and protein coding genes. Genotypes are incomplete and temporary descriptors of *Cryptosporidium* that lack the morphological, biological, and genetic data to support full classification as species. However, despite their taxonomic uncertainty, genotype descriptions greatly enhance the understanding of *Cryptosporidium* diversity.

Phylogenetic relationships among eukaryotes are frequently inferred from sequences of the 18S rRNA gene (18S rDNA). Most genomes have multiple 18S rDNA copies arranged in tandem on a single chromosome, which evolve in concert because of frequent gene conversion and unequal crossover events. The sequence homogeneity among 18S rDNA copies that results from concerted evolution is necessary to accurately infer phylogenetic relationships. Some genomes have divergent 18S rDNA copies distributed across multiple chromosomes (Barthelemy et al., 2007; Gunderson et al., 1987; Krieger and Fuerst, 2002). Most notably, divergent 18S rDNA copies in the apicomplexan Plasmodium share only 89-95% sequence similarity (Qari et al., 1994). These paralogs encode structurally distinct rRNA molecules that are expressed at different life cycle stages (McCutchan et al., 1988; McCutchan et al., 1995). Rooney (2004) proposed that, in contrast to concerted evolution, these divergent 18S rDNA paralogs evolve by a birth-and-death process whereby new genes arise by duplication, evolve independently, and can acquire new functions, become non-functional, or be deleted. Although there have been reports of paralogous 18S rDNA copies in other apicomplexans, including *Cryptosporidium, Babesia*, *Theileria*, and *Eimeria*, the extent of their divergence has been considerably lower than that in Plasmodium (Bhoora et al., 2009; El-Sherry et al., 2013; Hill et al., 2008; Ikarashi et al., 2013; Le Blancq et al., 1997; Morgan et al., 2001; Santín and Fayer, 2007; Sevá Ada et al., 2011; Xiao et al., 1999).

We report the frequent co-occurrence of two highly divergent *Cryptosporidium* 18S rDNA types in eastern chipmunks (*Tamias striatus*) and present evidence that they are paralogs in a single lineage that infects the intestinal epithelium. The identification of highly divergent 18S rDNA paralogs in *Cryptosporidium* has implications for the use of this gene to infer phylogenetic relationships.

Materials and Methods

Sample collection. Samples were collected from seventy-two eastern chipmunks (*Tamias striatus*) from five locations in Minnesota, USA, during a three-year period. Chipmunks were live-captured in Sherman box traps, ear-tagged, and released in compliance with North Dakota State University Institutional Animal Care and Use policies.

Traps were baited with sunflower seeds or rolled oats mixed with peanut butter. Voided feces were collected from the trap of each captured chipmunk. Recaptured chipmunks, identified by an ear-tag, were counted only once in prevalence analysis. In addition to voided feces, samples were collected from the rectum of two chipmunks (Table 4; Tast-1 and Tast-5), and from the stomach, small intestine, large intestine, and cecum of one chipmunk (Table 4; Tast-14). Samples were stored at 4°C without fixatives prior to DNA isolation.

DNA isolation and PCR. DNA was isolated from samples by alkaline digestion and phenol-chloroform extraction, and purified using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) as described previously (Peng et al., 2003). DNA was stored at -20°C until use in PCR assays.

A genus-specific nested PCR assay was used to amplify ca. 830 bp of the *Cryptosporidium* 18S rRNA gene (Xiao et al., 2001). Based on a finding in the study that most 18S rDNA sequences belonged to one of two clades, which were named Type A and Type B, type-specific, semi-nested PCR assays were developed. Type A 18S rDNA was amplified using the primers TastIITAF1 (5'-GTG ACA TAC CAT TCA AGT TTC TGA CC) and TastIITAR (5'-GGG AAG GCT AAC AAC ATG TAA AGG C) in a primary reaction, and TastIITAF2 (5'-TCC TAA TAC AGG GAG GTA GTG AC) and TastIITAR in a secondary reaction. Type B 18S rDNA was amplified using primers TastIITBF1 (5'-ACG GAT CAC ACA CTT TCT CTG TG) and TastIITBR (5'-CTA AAG CCG TGC AGG AGC AAG AAA) in a primary reaction, and TastIITBF2 (5'-CGC GCA AAT TAC CCA ATC CTG ACA) and TastIITBR in a secondary reaction. Primary and secondary reactions for each target were prepared in a total volume of 50 µL, which included 1 µL of DNA template or 2.5 µL of primary product (secondary reactions), 200 µM dNTP, 1.5 mM MgCl2, 1× PCR buffer, 2.5U of Taq (Promega GoTaq Flexi), and 0.4 µM of each primer. Reaction conditions for the Type A primary and secondary PCR were an initial denaturation at 94°C for 3 min; 35 cycles of 94°C for 45 s, 54°C for 45 s, and 72°C for 30 s; and a final elongation step of 72°C for 7 min. Type B primary

and secondary PCR reaction conditions were as described for Type A, with the exception that the annealing temperatures were 55°C and 57°C in the primary and secondary reactions, respectively.

Table 4. Chipmunk samples positive for *Cryptosporidium* spp. 18S rDNA. Samples positive for chipmunk genotype II Type A, chipmunk genotype II Type B, chipmunk genotype IV, and *C. andersoni* are indicated.

Chipmunk ID	Sample ID	chipmunk genotype II Type A	chipmunk genotype II Type B	chipmunk genotype IV	*C. andersoni*
Tast-1	ID-1985	+	+	-	-
Tast-2	FL-2033	+	+	-	-
Tast-3	HR-2038	+	+	-	-
Tast-4	HR-2039	+	+	-	-
Tast-5	ID-2165	+	+	-	-
Tast-6	GM-2688	+	+	-	-
Tast-7	GM-2689	+	+	-	-
Tast-8	GM-2690	+	+	-	-
Tast-9	GM-2691	+	+	-	-
Tast-10	H-3117 [a]	-	+	+	-
	H-3130 [b]	+	+	+	-
	H-3214 [c]	+	+	-	-
Tast-11	H-3181	+	-	-	-
Tast-12	ID-3134	+	-	-	-
Tast-13	ID-3135	+	+	-	-
Tast-14	ID-3169 [d]	+	-	-	-
	ID-3153 [e]	+	+	-	+
	ID-3154 [f]	+	+	-	+
	ID-3155 [g]	+	+	-	-
	ID-3156 [h]	+	+	-	-
Tast-15	ID-3166	+	+	-	-
Tast-16	ID-3168	+	+	-	-
Tast-17	ID-3177 [i]	+	-	-	-
	ID-3175 [j]	+	+	-	-
Tast-18	ID-3344	+	+	-	-
Tast-19	ID-3347	+	+	-	-
Tast-20	ID-3348	+	+	-	-
Tast-21	ID-3349	+	+	-	-
Tast-22	ID-3350	+	+	-	-
Tast-23	ID-3351	+	+	-	-
Tast-24	ID-3353	+	+	-	-
Tast-25	ID-3356	+	+	-	-
Tast-26	ID-3358	+	+	-	-
Tast-27	ID-3359	+	+	-	-
Tast-28	ID-3362	+	+	-	-

Samples were of feces unless indicated otherwise. A sample was obtained from Tast-10 on [a] June 29, [b] June 30, and [c] September 1; A fecal sample was obtained from Tast-14 on [d] August 23 and samples of the [e] stomach, [f] small intestine, [g] large intestine, and [h] cecum were obtained following the animal's death on August 26; Samples were obtained from Tast-17 on [i] August 24 and [j] August 26.
+ Positive; **-** Negative.

Nested PCR assays were used to amplify fragments of actin (ca. 1066 bp; Sulaiman et al., 2002) and heat shock protein 70 (HSP70; ca. 1800 bp; Sulaiman et al., 2000) genes.

DNA from *C. hominis* and *C. parvum* was included as positive controls in the genus-specific 18S rDNA, actin, and HSP70 PCR assays. Water was included instead of DNA template as a negative control in all PCR assays. Secondary PCR products were visualized using SYBR Green dye or ethidium bromide staining following agarose gel electrophoresis. Products of expected size were purified (Wizard SV, Promega, Madison, WI) and stored at 4°C until required for sequencing.

Sequencing and phylogenetic analyses. Purified amplicons of 18S rDNA, actin, and HSP70 were sequenced directly in both directions using a BigDye Terminator v3.1 cycle sequencing kit with secondary PCR primers in an ABI Prism 3130 genetic analyzer (Applied Biosystems, Carlsbad, CA). An additional internal primer (5' GCT GCT GCC ATT GCT TAT GGT CTT 3') was used to sequence HSP70 amplicons.

Sequences were assembled using SeqMan (DNAStar, Madison, WI), aligned with previously published sequences using the MAFFT version 7 online server with automatic selection of alignment mode (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standley, 2013). Sequences from this study have been deposited in GenBank under the accession numbers KC954211 to KC954269 and KJ816864 to KJ816871. The evolutionary history of aligned sequences was inferred using the Maximum Likelihood (ML) method (Saitou and Nei, 1987), with the substitution model that best fit the alignment selected using the Bayesian information criterion. The Tamura 3-parameter model (Tamura, 1992) was selected for 18S rDNA alignments, and the general time reversible model (Tavaré, 1986) was selected for actin and HSP70 alignments. Both models were used under an assumption that rate variation among sites was gamma distributed. A bootstrap consensus tree was inferred from 1000 pseudoreplicates. Phylogenetic analyses, including analysis of substitution model goodness of fit, were carried out using MEGA 6.0 (Tamura et al., 2013). Phylogenetic trees were edited for style using Adobe Illustrator CS5.1 (Adobe Systems, Inc., San Jose, CA).

MAFFT aligned 18S rDNA sequences were examined for recombination using the phylogeny-based Probabilistic Divergence Measures (PDM) method in TOPALi v2.5 (Husmeier et al., 2005; Milne et al., 2009). A Markov chain Monte Carlo simulation was used to examine differences in the marginal

posterior distribution of tree topologies in a fixed size window of 200 moving in a step size of 10 along the length of aligned sequences. Bootstrap support was determined from 100 pseudoreplicates. Recombination breakpoints were predicted from the PDM analysis and ML trees were constructed from partitioned sequences using MEGA 6.0.

A structure-based alignment and secondary structure diagrams of 18S rDNA sequences were produced using SSU-ALIGN and SSU-DRAW, respectively (Nawrocki, 2009). SSU-ALIGN uses a eukaryotic 18S rRNA structure model inferred from available RNA sequence and crystal structure information (http://www.rna.ccbb.utexas.edu). Secondary structure was not predicted for the V4 region. Expansions of helix 11 in the V2 region model (helix nembering based on Wuyts et al., 2002) were handled poorly by the eukaryote secondary structure, so the stem-loop structure predicted by RNAfold (Hofacker et al., 1994) was used instead.

Nucleotide composition analysis and Tajima's relative rate test (Tajima, 1993) were carried out using MEGA 6.0.

Oocyst purification and morphometric analysis. Oocysts were isolated from the feces of two chipmunks, Tast-24 and Tast-25. Larger particles were removed by sieving through a US #35 mesh screen (opening 0.5 mm) followed by a US #325 mesh screen (opening 0.044 mm). Oocysts were subsequently purified using cesium chloride gradient centrifugation (Arrowood and Donaldson, 1996), and examined under differential interference contrast (DIC) and epifluorescence microscopy (Olympus, Center Valley, PA), following labeling with a *Cryptosporidium* genus-specific, FITC-conjugated antibody (Crypt-a-Glo, Waterborne Inc., LA).

Cell morphology was determined using digital analysis of images (MicroSuite™Five; Olympus Soft Imaging Solution GmbH, Lakewood, CO) collected at 1,000x magnification using a 12-megapixel digital microscope camera (DP10; Olympus). Length and width were measured for nineteen oocysts and a shape index was calculated. Twenty oocysts of *C. parvum* (Waterborne Inc., New Orleans, LA) measured by the same person under the same conditions served as a control.

Tissue sample collection and histology. Tast-25 was euthanized immediately after capture, and gastrointestinal tissue sections were excised from the stomach, duodenum, proximal jejunum, middle jejunum, distal jejunum, ileum, cecum, colon, and rectum. Tissue samples were stored in 10% formalin at

room temperature before embedding in paraffin. Five-micron cross-sections were prepared and deparaffinized from each tissue section, and stained with hematoxylin and eosin.

Results

***Cryptosporidium* prevalence in chipmunks.** Out of seventy-two chipmunks sampled, 28 (38.9%) tested positive for *Cryptosporidium* on at least one occasion during the study. In total, 35 positive samples were obtained from the 28 positive chipmunks: a single positive sample was obtained from 25 chipmunks; three positive samples were obtained from one chipmunk, Tast-10, on different days; five positive samples were obtained from the stomach, small intestine, large intestine, cecum, and rectum of one chipmunk, Tast-14; and two positive samples were obtained from one chipmunk, Tast-17, on different days (Table 4).

Two *Cryptosporidium* 18S rDNA types occurred frequently in chipmunks. Sequences of 18S rDNA obtained from 28 out of 35 positive samples were used to construct a ML tree (Figure 4). Amplicons from the remaining seven samples were refractory to sequencing.

Sequences from 24 out of 28 samples clustered in two sister groups, which were named Type A and Type B (Figure 4). Types A and B shared 92.7 ± 1.0% sequence identity and were both identified in five samples using the *Cryptosporidium* genus-specific PCR assay. Sequence identity within the Type A group was 99.8 ± 0.1% (range: 99.1 to 100%). Within the Type B group, which included *Cryptosporidium* chipmunk genotype II from an eastern chipmunk in New York State (accession no. EU096238), sequence identity was 99.2 ± 0.2% (range: 97.6 to 100%). The W21 genotype (accession no. AY737589), which was previously isolated from storm water in New York (Jiang et al., 2005), was intermediate between the types, but was more similar to Type B (96.2 ± 0.7% identity) than Type A (94.6 ± 0.9% identity).

Four samples from two chipmunks had sequences other than Types A and B (Figure 4). Sequences from ID-3153 and ID-3154, which were obtained from the stomach and small intestine, respectively, of Tast-14, shared 100% identity with a *C. andersoni* isolate (accession no. EU825734) from the Potomac River Watershed (Yang et al., 2008). A novel genotype was identified in H-3117 and H-3130, which were obtained from Tast-10 on consecutive days in June 2011 (Figure 4). This genotype, which was named *Cryptosporidium* chipmunk genotype IV, clustered with sequences from an environmental isolate in the UK (accession no. HQ822143), *Cryptosporidium* muskrat genotype II

(accession no. AY737570), *Cryptosporidium* deer mouse genotype IV (accession no. EF641019), *C. ubiquitum* (accession no. EF362479), and *C. suis* (accession no. AB449871) (Figure 4).

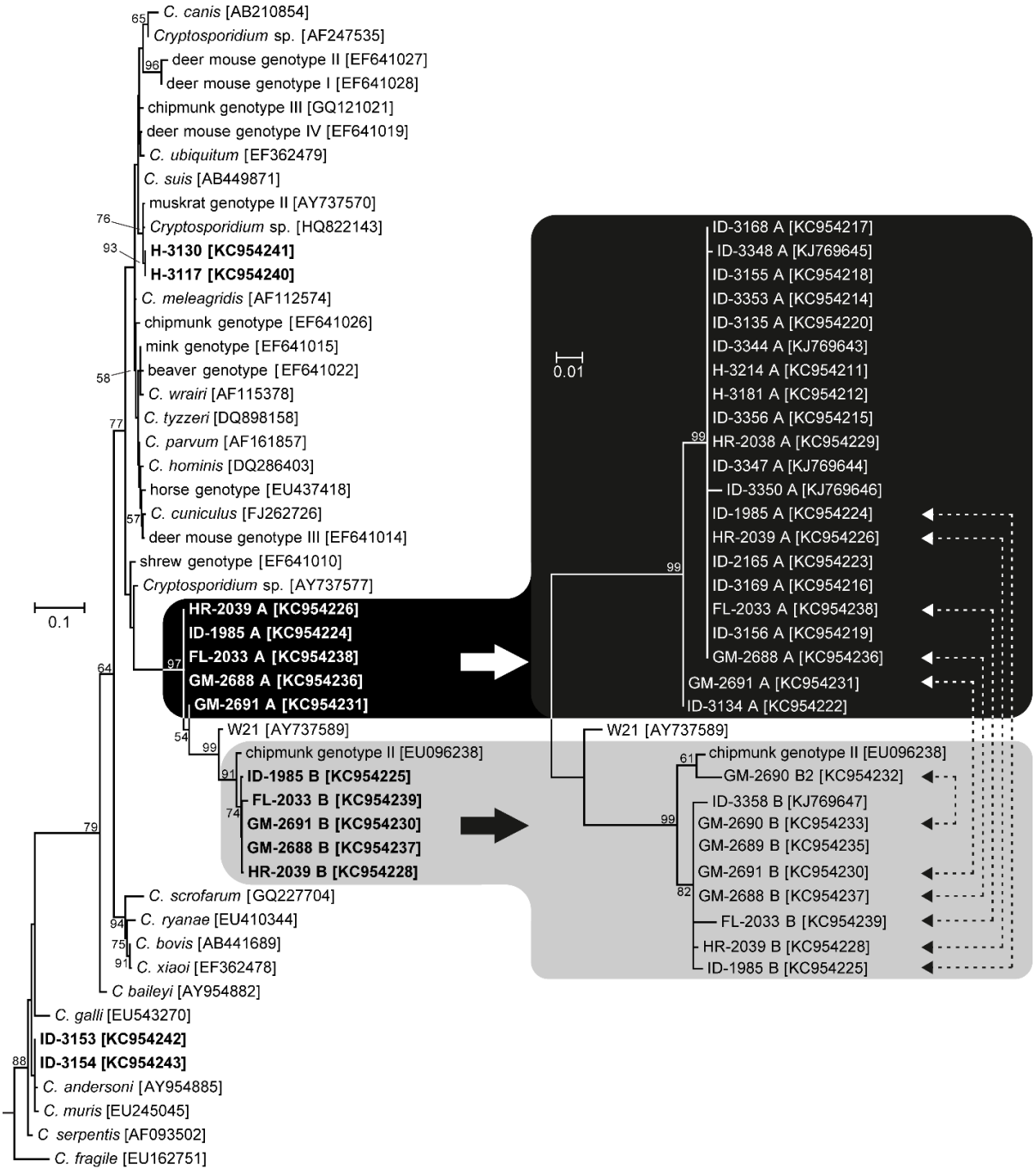

Figure 4. Maximum likelihood tree of 18S rDNA sequences. The Tamura-3-parameter substitution model was used. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Only bootstrap values >50% are shown. The tree on the left was rooted with 18S rDNA from *Monocystis agilis* (accession no. AF457127). Sequences from this study are bolded. Type A and Type B sequences are highlighted with black and gray backgrounds, respectively. Dashed lines with arrows link sequences from the same sample.

Types A and B are divergent 18S rDNA paralogs in a single lineage. Based on their sister group relationship and co-occurrence in five out of 24 samples, we hypothesized that Types A and B are divergent 18S rDNA paralogs in *Cryptosporidium* chipmunk genotype II. It was not feasible to test for paralogy by examining haploid genomes from single cells or clonally propagated isolates, so the approach taken was to determine a] how frequently Types A and B co-occurred in positive samples and b] to what extent other loci were divergent.

If Types A and B are paralogs in *Cryptosporidium* chipmunk genotype II, they would be expected to co-occur. The genus-specific 18S rDNA PCR assay is not useful for detecting individual members in mixed populations, so type-specific primers were designed. Combining data from the genus- and type-specific PCR assays, at least one of the types was detected in all 35 samples, and co-occurrence of Types A and B was detected in 30 out of 35 samples from 26 out of 28 *Cryptosporidium* positive chipmunks (Table 4). Type B was not detected in the only samples obtained from Tast-11 and Tast-12. Each of three chipmunks (Tast-10, Tast-14, and Tast-17) sampled on multiple occasions had a single sample from which only one type was detected. One of the three fecal samples (H-3117) obtained from Tast-10 on separate occasions was negative for Type A; the remaining samples, including a sample collected three days later than H-3117, had both types present. Only Type A was detected in a fecal sample from Tast-14 (ID-3169); however, both types were detected in samples obtained from the stomach (ID-3153), small intestine (ID-3154), large intestine (ID-3155), and cecum (ID-3156) of this animal three days later. One sample from Tast-17 (H-3177) was negative for Type B, but both types were detected in sample ID-3175 collected from Tast-17 two days later. Based on their frequent co-occurrence, we concluded that Types A and B are either paralogs in a single *Cryptosporidium* lineage or are from divergent taxa that co-infect chipmunks.

Actin and HSP70 genes in *Cryptosporidium* tend to be more divergent than 18S rDNA (Table 5). Therefore, if the *Cryptosporidium* 18S rDNA types in eastern chipmunks diverged because of speciation (orthology) rather than gene duplication (paralogy), it would be expected that actin and HSP70 genes should exhibit similar or greater divergence.

Table 5. Percent sequence identity at the 18S rDNA, actin, and heat shock protein-70 (HSP70) loci. Comparisons between *C.* parvum (accession nos. XM_625373, XM_001388245, and NC006986 for 18S rDNA, actin, and HSP70 loci, respectively), *C. hominis* (accession nos. XM_661662, XM_661095, and NW667442 for 18S rDNA, actin, and HSP70 loci, respectively), *C. muris* [accession nos. XM_002140816, XM_002140165, and EU245045 for 18S rDNA, actin, and HSP70 loci, respectively) and *Cryptosporidium* chipmunk genotype II Type A and Type B using the Kimura 2-parameter distance model.

Comparison	% sequence identity ± standard error		
	18S rDNA	Actin	HSP70
C. parvum and *C. hominis*	99.3 ± 0.3	98.5 ± 0.4	98.5 ± 0.3
C. parvum and *C. muris*	91.0 ± 1.1	81.4 ± 1.6	79.0 ± 1.2
Type A and Type B	92.7 ± 1.0	99.9 ± 0.1	99.9 ± 0.1
Type B and *C. parvum*	87.0 ± 1.5	88.7 ± 1.2	87.5 ± 0.9
Type A and *C. parvum*	93.6 ± 1.1	88.7 ± 1.2	87.5 ± 0.9
Type B and *C. muris*	85.6 ± 1.5	79.2 ± 1.7	73.5 ± 1.4
Type A and *C. muris*	89.4 ± 1.3	79.2 ± 1.7	73.5 ± 1.4

Thirty-four actin amplicons were sequenced from 16 samples: single amplicons were sequenced from eight samples and two to five amplicons were sequenced from eight samples. Sequences from H-3117 and H-3130 clustered with *Cryptosporidium* deer mouse genotype (accession no. AY120925), *C. ubiquitum* (accession no. GQ337961), and *C. suis* (accession no. EF012373) (Figure 5), which is consistent with the clustering of 18S rDNA sequences from *Cryptosporidium* chipmunk genotype IV. All other sequences clustered as a single group that was most similar to *C. canis* (accession no. AY120926) and a bear genotype (accession no. AF382339). Sequences within this group shared 99.9 ± 0.1% identity (Table 5).

Nineteen HSP70 sequences from nine samples clustered in two groups (Figure 6). Consistent with the clustering of 18S rDNA and actin sequences, the H-3130 HSP70 sequence clustered with *C. ubiquitum* (accession no. DQ898163), *C. suis* (accession no. DQ898164), and a deer mouse genotype (accession no. AY120919). All other sequences formed a separate group that was most similar to sequences from *C. canis* (accession no. AY120920), a bear genotype (accession no. AF247536), and a giant panda genotype (accession no. JN588571). Sequences in this group shared 99.9 ± 0.1% identity (Table 5).

With the exception of *Cryptosporidium* chipmunk genotype IV sequences obtained from H-3117 and H-3130, actin and HSP70 sequences from chipmunks formed a single homogeneous group, which is inconsistent with Types A and B being from divergent taxa. A more parsimonious explanation is that Types A and B are divergent paralogs in *Cryptosporidium* chipmunk genotype II.

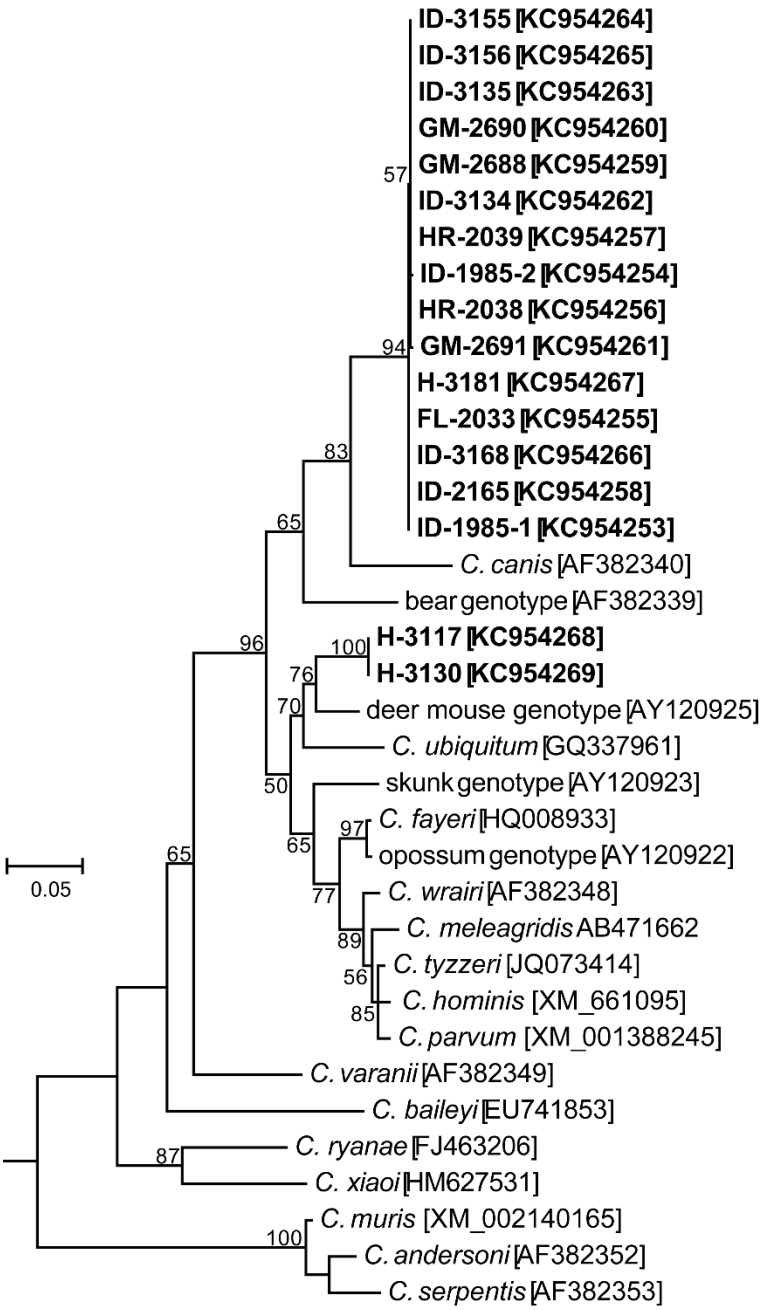

Figure 5. Maximum likelihood tree of actin gene sequences. The general time reversible substitution model was used. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Only bootstrap values >50% are shown. Sequences from this study are bolded. The tree was rooted with an actin gene sequence from *Plasmodium falciparum* (accession no. EF472536).

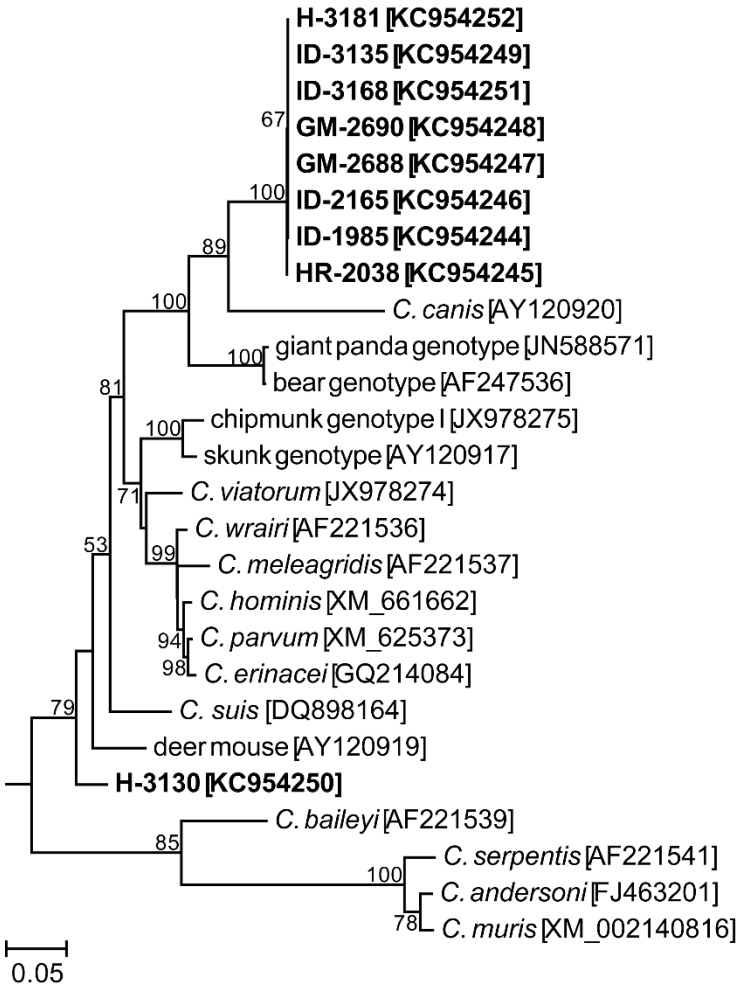

Figure 6. Maximum likelihood tree of heat shock protein 70 gene sequences. The general time reversible substitution model was used. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Only bootstrap values >50% are shown. Sequences from this study are bolded. The tree was rooted with a heat shock protein 70 gene sequence from *Plasmodium falciparum* (accession no. M19753).

The pattern of nucleotide substitutions suggests functionally constrained evolution of Types A and B. The pattern of nucleotide substitutions in Types A and B was determined to assess the likelihood that one or both types are non-functional pseudogenes. The evolution of functional 18S rDNA is constrained by the conserved secondary and tertiary structure of the 18S rRNA molecule, which is maintained by the coordinated evolution of base pairs (co-variation). In contrast, non-functional pseudogenes are expected to accumulate mutations randomly. The amplified *Cryptosporidium* 18S rDNA from HR-2039 Type A and HR-2039 Type B included three hypervariable stem-loop regions: part of V2 and the complete V3 and V4 regions (Figure 7). Sequence identity between Type A and Type B in Figure

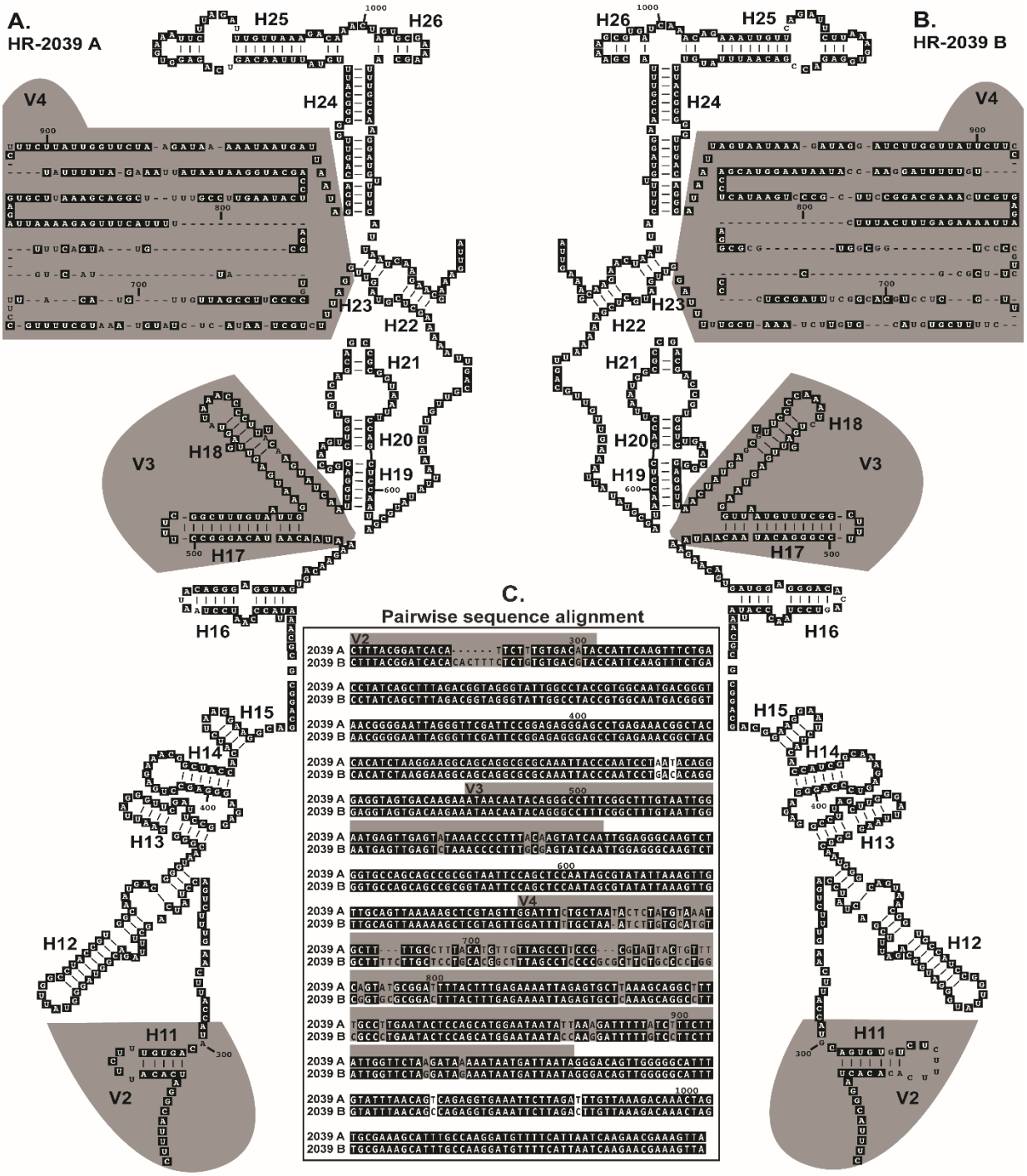

Figure 7. Structure and pairwise sequence alignments of HR-2039 Type A and HR-2039 Type B 18S rDNA. Structure alignments of HR-2039 Type A (A) and HR-2039 Type B (B) were constructed using SSU-ALIGN and SSU-DRAW (Feng et al., 2007). Helix numbering (H11-H26) is based on a system described previously (Nawrocki, 2009). The inset (C) shows a MAFFT pairwise sequence alignment of HR-2039 Type A and HR-2039 Type B, with sequence identity indicated by white lettering against a black background. Identical sequences from the pairwise alignment are also indicated in the structure alignment by white lettering against a black background. Hypervariable V2, V3, and V4 regions are highlighted in gray in the structure and pairwise alignments.

7 is indicated by white font against a black background in the pairwise and structure alignments. Comparing secondary structure alignments, the majority of substitutions occurred in the V2, V3, and V4 regions. In the V2 region, a six-nucleotide insertion and two substitutions in Type B expanded the stem and loop of helix 11. A third substitution occurred in a single-stranded region immediately downstream of helix 11. Helix 16 had two substitutions: one in the single-stranded loop and one in the stem. The substitution in the stem resulted in a non-canonical C-A pairing in Type A, which is conserved in intestinal *Cryptosporidium* species (data not shown). The canonical C-G pairing in Type B is conserved in gastric *Cryptosporidium* species (data not shown). Helix 18, in the V3 region, had three substitutions: one in the loop; one at a covarying site in the stem, resulting in an A-U pairing in Type A and a G-U pairing in Type B; and one at a non-covarying site in the stem. Helix 25 had two substitutions, both occurring in a single stranded bulge. This pattern of nucleotide substitutions is consistent with the evolution of Types A and B under structural constraints, suggesting they are not pseudogenes.

Type B has evolved more rapidly than Type A. Tajima's relative rate test (Tajima, 1993) was carried out to test the hypothesis that Types A and B have undergone different rates of evolution (Table 6). The null hypothesis of equal evolution rates was rejected with a high level of significance in tests conducted using three different outgroups. Type B sequences exhibited significantly greater rates of evolution than Type A.

Table 6. Tajima's relative rate test. Type A and Type B sequences from three samples were included in the analysis. Sequences from each sample were tested against three outgroups: *C. canis* (accession no. AB210854), *C. parvum* (accession no. AF161857), and *C. muris* (accession no. EU245045).

Outgroup	Sample	Number of unique sequence differences		Chi-squared statistic	P value
		Type A	Type B		
C. canis	ID-1985	2	36	30.4	<0.0001
	HR-2039	3	37	28.9	<0.0001
	GM-2688	3	37	28.9	<0.0001
C. parvum	ID-1985	1	37	34.1	<0.0001
	HR-2039	1	39	36.1	<0.0001
	GM-2688	1	39	36.1	<0.0001
C. muris	ID-1985	8	25	8.8	<0.01
	HR-2039	9	26	8.3	<0.01
	GM-2688	8	27	10.3	<0.01

Types A and B show evidence of recombination. Divergent 18S rDNA sequences were examined for evidence of recombination using the PDM method in TOPALi v2.5. A recombination breakpoint was predicted in the conservative core region between the hypervariable V3 and V4 regions. Phylogenies inferred from nucleotides 1–297 (Figure 8A) and 298–629 (Figure 8B) are discordant because of the chimeric sequences: FL-2033 B (accession no. KC954239) clustered with Type A in Figure 8A and Type B in Figure 8B. Similarly, ID-3134 A (accession no. KC954222), and GM-2691 A (accession no. KC954231) clustered with Type B in Figure 8A, sharing 100% identity with W21, and Type A in Figure 8B. The likelihood that the chimeric sequences were artifacts was considered low because a] amplicons were not cloned prior to sequencing, b] the same chimeric sequence was obtained from separate amplicons of ID-3134 A and GM-2691 A, and c] GM-2691 A was detected using both genus- and type-specific primers.

Types A and B have differing G+C contents. Type B sequences of *Cryptosporidium* chipmunk genotype II had a higher overall G+C content (46.3%) than Type A sequences (41.0%) (Table 7). The difference between the types was greatest in the V3 (3.6% difference) and V4 (13.1% difference) regions. The G+C content of the conservative 18S rDNA core, i.e. outside of the hypervariable regions, differed by 0.7%. The G+C content of chimeric sequences (GM-2691 A, ID-3134 A, and FL-2033 B) matched that of the opposite type in the V3 region (boxed cells in Table 7).

***Cryptosporidium* chipmunk genotype II infects the intestinal epithelium of chipmunks.** *Cryptosporidium* developmental stages were observed in the gastrointestinal epithelium of Tast-25, which was infected with *Cryptosporidium* chipmunk genotype II Types A and B. Endogenous stages were identified in the jejunum, cecum, and colon (Figure 9).

Oocysts of *Cryptosporidium* chipmunk genotype II are smaller than *C. parvum*. Oocysts of *Cryptosporidium* chipmunk genotype II from eastern chipmunks measured 4.17 µm (3.73–5.04 µm) × 3.94 µm (3.50-4.98 µm) with a length-to-width ratio of 1.06 ± 0.06 µm. Oocysts were smaller than *C. parvum*, which measured 5.06 µm (4.49–5.41 µm) × 4.75 µm (4.36–5.35 µm) with a length-to-width ratio was 1.07 ± 0.06 µm. Oocysts labeled with the FITC-conjugated antibody and examined by epifluorescence microscopy had a typical apple green, halo-like fluorescence (Figure 10).

Table 7. G+C content of 18S rRNA gene sequences. *Cryptosporidium* 18S rRNA gene sequences are from this study and published sequences in GenBank. *Plasmodium vivax* A, S, and O-type 18S rRNA gene sequences from GenBank also are included.

Species/genotype	Type	Isolate [accession no.]		Percent G+C			
				All [a]	Core [b]	V3 [c]	V4 [d]
chipmunk genotype II	A	FL-2033 A	[KC954238]	40.8	48.1	36.2	31.5
		GM-2688 A	[KC954236]	40.9	48.2	36.2	31.5
		GM-2691 A	[KC954231]	41.6	48.7	40.6	31.5
		H-3181 A	[KC954212]	40.8	48.1	36.2	31.5
		H-3214 A	[KC954211]	40.8	48.1	36.2	31.5
		HR-2038 A	[KC954229]	40.8	48.1	36.2	31.5
		HR-2039 A	[KC954226]	40.8	48.1	36.2	31.5
		ID-1985 A	[KC954224]	40.8	48.1	36.2	31.5
		ID-2165 A	[KC954223]	40.8	48.1	36.2	31.5
		ID-3134 A	[KC954222]	41.8	48.7	40.6	32.0
		ID-3135 A	[KC954220]	41.0	48.1	36.2	32.0
		ID-3155 A	[KC954218]	40.9	48.1	36.2	31.7
		ID-3156 A	[KC954219]	40.9	48.1	36.2	31.7
		ID-3168 A	[KC954217]	40.9	48.1	36.2	31.7
		ID-3169 A	[KC954216]	41.0	48.1	36.2	32.0
		ID-3344 A	[KJ769643]	40.8	48.1	36.2	31.5
		ID-3347 A	[KJ769644]	41.0	48.1	36.2	32.0
		ID-3348 A	[KJ769645]	40.7	48.1	36.2	31.0
		ID-3353 A	[KC954214]	41.0	48.1	36.2	32.0
		ID-3356 A	[KC954215]	41.0	48.1	36.2	32.0
		AVERAGE		**41.0**	**48.1**	**36.6**	**31.7**
chipmunk genotype II	B	Ec-14887	[EU096238]	45.6	48.7	40.6	43.1
		W21	[AY737589]	46.0	49.0	40.6	44.4
		FL-2033 B	[KC954239]	45.9	48.4	36.2	45.5
		GM-2688 B	[KC954237]	46.8	49.0	40.6	45.7
		GM-2689 B	[KC954235]	46.8	49.0	40.6	45.7
		GM-2690 B	[KC954233]	46.8	49.0	40.6	45.7
		GM-2690 B2	[KC954232]	45.1	48.4	40.6	42.6
		GM-2691 B	[KC954230]	46.8	49.0	40.6	45.7
		HR-2039 B	[KC954228]	46.6	49.0	40.6	45.2
		ID-1985 B	[KC954225]	46.5	48.7	40.6	45.2
		ID-3358 B	[KJ769647]	46.6	49.0	40.6	44.1
		AVERAGE		**46.3**	**48.8**	**40.2**	**44.8**
chipmunk genotype I (W17)			[EF641026]	35.9	48.1	29.0	20.7
chipmunk genotype III			[GQ121021]	36.0	48.1	28.2	21.6
chipmunk genotype IV		ID-3130	[KC954241]	36.0	47.8	27.5	21.1
C. parvum KSU-1	A		[AF015772]	35.8	48.1	29.0	20.7
	B		[AF308600]	36.1	47.8	29.0	21.0
C. baileyi			[AY954882]	39.1	48.7	36.2	26.2
C. serpentis			[AF093502]	40.0	49.0	36.2	26.9
C. fragile			[EU162751]	43.7	49.0	37.7	36.5
Plasmodium vivax	A		[U07367]	38.2	45.4	34.8	31.8
	S		[U03080]	37.7	44.4	30.0	32.7
	O		[U93095]	39.9	44.6	43.2	35.2

G + C content was determined from nucleotides 1-629 [a]; 32-215, 285-372, and 583-629 [b]; 216-284 [c]; and 373-582 [d]. Boxed cells highlight differences in G+C content of chimeric sequences

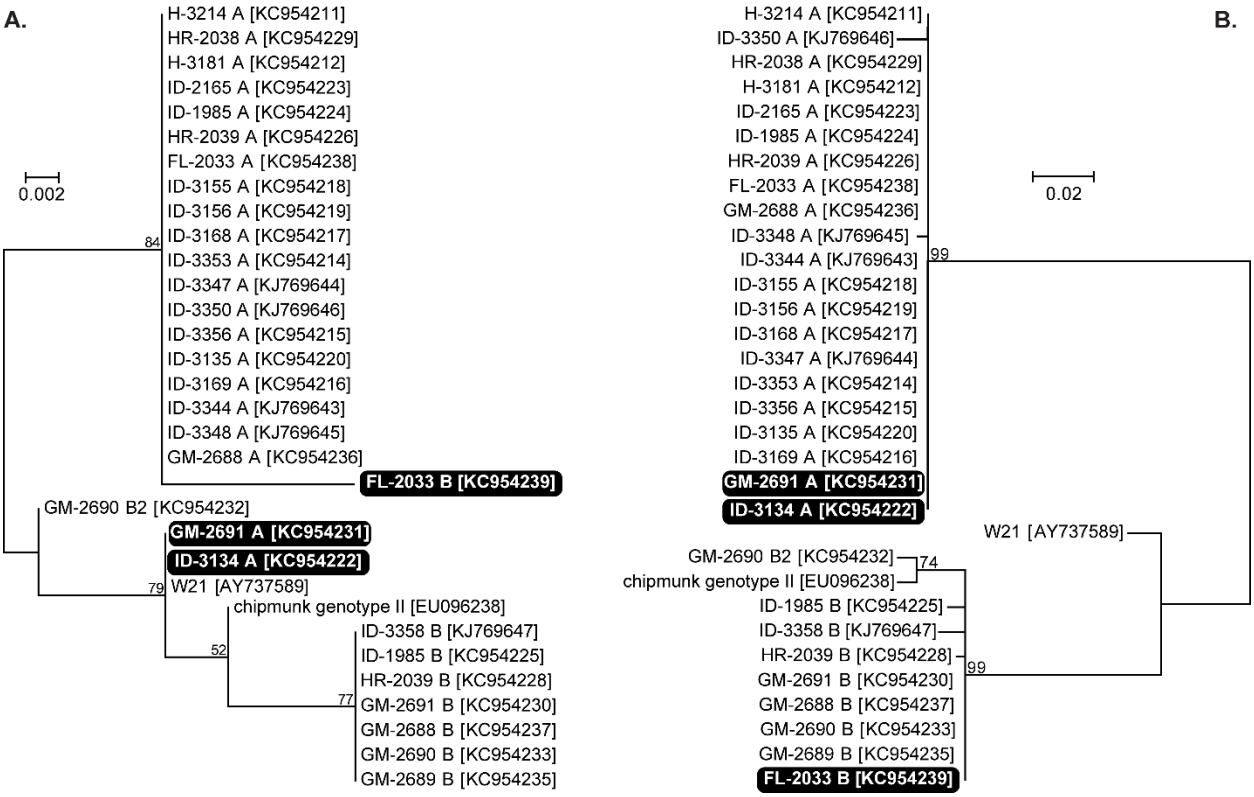

Figure 8. Phylogenetic evidence of chimeric Type A and Type B sequences. Maximum likelihood trees were constructed from nucleotides 1-297 (A) and 298-629 (B) of Type A and Type B 18S rRNA gene sequences. Nucleotide 297 represents the recombination breakpoint determined using the Probabilistic Divergence Measures method in TOPALi v2.5. Based on goodness of fit, Jukes-Cantor with invariant rates and Tamura 3-parameter with gamma-distributed rates were selected as substitution models for alignments of nucleotides 1-297 (A) and 298-629 (B), respectively (Tamura et al., 2013). Chimeric sequences resulting in discordance in the tree topologies are highlighted with white text against a black background. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Only bootstrap values >50% are shown.

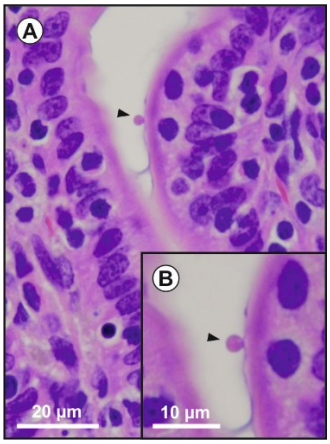

Figure 9. Endogenous *Cryptosporidium* chipmunk genotype II developmental stage (arrowhead). Hematoxylin and eosin stained *Cryptosporidium* developmental stage in the jejunum of an eastern chipmunk under A) 600× and B) 1000× total magnification.

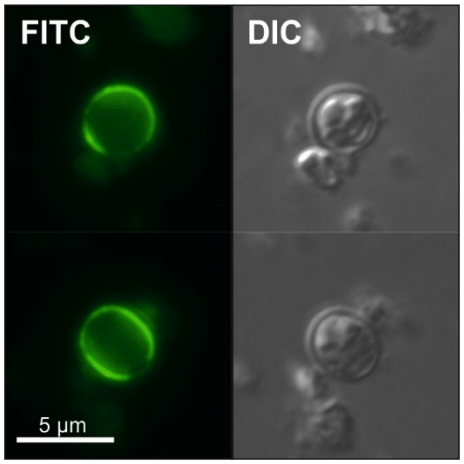

Figure 10. *Cryptosporidium* chipmunk genotype II oocysts. Oocysts were stained with anti-*Cryptosporidium* FITC-conjugated antibody (FITC) and viewed under epifluorescence and differential interference contrast (DIC) microscopy.

Discussion

We found that highly divergent *Cryptosporidium* 18S rDNA types with a sister group relationship co-occurred in 26/28 chipmunks. This frequent co-occurrence together with the homogeneity of actin and HSP70 sequences support the conclusion that the divergent types are paralogs in a single *Cryptosporidium* lineage. Because Type B clusters with the previously described *Cryptosporidium* chipmunk genotype II, it is proposed that the types be named *Cryptosporidium* chipmunk genotype II Type A and Type B.

The less than 93% sequence similarity shared by Types A and B is comparable to that shared by orthologous 18S rDNA sequences from intestinal and gastric *Cryptosporidium* species. Intestinal and gastric species branched early during *Cryptosporidium* evolution and, in addition to being considerably different at the genomic level, they have different oocyst morphologies, biochemical characteristics, and gastrointestinal niches (Widmer and Sullivan, 2012).

The divergence of 18S rDNA paralogs in chipmunk genotype II is considerably greater than that reported in other *Cryptosporidium* spp. including *C. parvum* (99% similarity; Le Blancq et al., 1997), *C. andersoni* (99% similarity; Ikarashi et al., 2013), and *C. galli* (98.6-99.1% similarity; Morgan et al., 2001). Types A and B also are more divergent than the 18S rDNA paralogs in *Babesia bigemina* (99% similarity; Reddy et al., 1991), *Eimeria mitis* (98% similarity; Vrba et al., 2011), *Eimeria meleagrimitis* (97% similarity; El-Sherry et al., 2013), and *Theileria equi* (96% similarity; Bhoora et al., 2009). *Plasmodium* species have the most divergent 18S rDNA paralogs in the phylum Apicomplexa, and the paralogs are

differentially expressed during parasite development: the A-type is expressed during asexual development in the vertebrate host and the S-type is expressed in sporozoites developing in the mosquito host (Gunderson et al., 1987; McCutchan et al., 1995). The divergence of Types A and B in the present study is higher than that of A- and S-types in *P. berghei* (95% similarity; Qari et al., 1994), similar to that of A- and S-types in *P. vivax* (92% similarity; Qari et al., 1994), and lower than that of A- and S-types in *P. falciparum* (89% similarity; Qari et al., 1994).

Given the extent to which sequences of Types A and B have diverged in chipmunk genotype II, it is reasonable to ask if they have evolved different functions, similar to the A- and S-types in *Plasmodium* species. Addressing this question will require the development of a suitable infection model to study the development of *Cryptosporidium* chipmunk genotype II. Without these biological studies, we cannot exclude the possibility that one or both of the types is a non-functional pseudogene; however, the pattern of nucleotide substitutions observed in the present study suggests that both types have evolved under functional constraints. If we assume that the types are functional, one possibility is that they have adapted to different conditions encountered by the parasite in its hibernating host. During hibernation, chipmunks alternate between periods of torpor, which is characterized by no food consumption, low metabolism, and a body temperature below 10°C, and brief periods of activity, during which individuals eat from a food cache, experience an elevated metabolism, and reach normothermic body temperatures of 36-41°C (Careau et al., 2012; Landry-Cuerrier et al., 2008; Munro et al., 2005; Snyder, 1982; Wang and Hudson, 1971). Adaptation of the 18S rRNA molecule to different body temperatures could allow *Cryptosporidium* chipmunk genotype II to survive during chipmunk hibernation. The difference in G+C content of the types could support this hypothesis, as a correlation has been show between the G+C content of 18S rRNA molecules and body temperature (Varriale et al., 2008).

The separation of 18S rDNA copies on different chromosomes limits the extent to which recombination can homogenize nucleotide sequences among copies. It has been proposed that apicomplexan rDNA paralogs evolve independently by a birth-and-death model, and similarity among copies is maintained by purifying selection rather than recombination (Rooney, 2004). However, our identification of chimeric sequences in chipmunk genotype II suggests that recombination could play a role in the evolution of *Cryptosporidium* 18S rDNA. El-Sherry et al. (2013) similarly detected chimeric 18S

rDNA sequences in *Eimeria*, but concluded that these were PCR artifacts. While artifactual recombination cannot be ruled out in the present study, the consistent amplification of chimeras in different samples and using different amplification primers supports the conclusion that recombination occurred naturally.

The remarkable divergence of Types A and B in chipmunk genotype II suggests that one or both types have undergone accelerated evolution. Ohno (1970) proposed that redundancy frees duplicated genes from natural selection, allowing them to rapidly acquire mutations and, potentially, new functions. Using Tajima's relative rate test, Type B was shown to have evolved more rapidly than Type A, suggesting that Type B is a duplicated (paralogous) copy. In a *Cryptosporidium* phylogeny constructed using Type A but not Type B (data not shown), Type A clusters with intestinal *Cryptosporidium* species, which is consistent with the intestinal localization of isolates from this study. In contrast, Type B clusters outside the intestinal and gastric groups.

Studies on *Cryptosporidium* in wild populations can provide insight into the evolution and biology of *Cryptosporidium* parasites. This study has shown that highly divergent 18S rDNA paralogs can occur in *Cryptosporidium*. Identifying such occurrences will be important for the accurate inference of species relationships from 18S rDNA sequences. Future studies should aim to identify the factors contributing to 18S rDNA divergence in *Cryptosporidium* and determine the functions of divergent paralogs.

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PAPER 2. *CRYPTOSPORIDIUM* IN WILD CRICETID RODENTS[1]

Abstract

We undertook a study of *Cryptosporidium* infecting populations of wild cricetid rodents in order to better understand the ecology and evolution of this intestinal parasite. Fecal samples were collected from meadow voles (*Microtus pennsylvanicus*), southern red-backed voles (*Myodes gapperi*), woodland voles (*Microtus pinetorum*), muskrats (*Ondatra zibethicus*), deer mice and white-footed mice (*Peromyscus* spp.) in North America, and from bank voles (*Myodes glareolus*) in Europe. *Cryptosporidium* isolates were characterized by sequence analysis of the 18S rRNA and actin genes and phylogenetic relationships were inferred. In total, 50.7% (302/596) of cricetids in North America were infected with *Cryptosporidium*. The prevalence was 9.5% (4/42) in muskrats, 51.2% (21/41) in woodland voles, 52.4% (163/311) in meadow voles, 55.6% (15/27) in southern red-backed voles, and 56.6% (99/175) in *Peromyscus* mice. Parasite prevalence was not correlated with host population density. A high degree of sequence heterogeneity was found at the 18S rDNA locus. Sequences from this study clustered with muskrat genotypes I and II, deer mouse genotypes I-IV, W12, chipmunk genotype IV, fox genotype, and vole genotype; however, sequence heterogeneity within each of these clusters was high and bootstrap support for some clusters was low. A concatenated 18S rDNA-actin phylogeny had better bootstrap support and fourteen clades were distinguished. Voles and *Peromyscus* mice generally hosted different clades, suggesting that *Cryptosporidium* spp. may have adapted differently to hosts of the cricetid subfamilies Arvicolinae (voles and muskrats) and Neotominae (*Peromyscus* mice). Evidence supporting coevolution (closely related parasites in closely related hosts) was found by the presence of closely related *Cryptosporidium* spp. in the related but geographically isolated southern red-backed voles (*Myodes gapperi*) in the United States and bank voles (*Myodes glareolus*) from Slovakia. Although cricetids are ubiquitous and frequently infected with a variety of *Cryptosporidium*, they do not appear to be important reservoirs of *Cryptosporidium* associated with disease in humans or livestock.

[1] The material in this chapter was co-authored by Brianna L.S. Stenger, Mark E. Clark, Martin Kváč, Catherine W. Giddings, and John M. McEvoy. Brianna L.S. Stenger had the primary responsibility for collecting data, analysis, interpretation, and writing of this manuscript.

Introduction

Cryptosporidium is a genus of ubiquitous apicomplexan parasites infecting more than 480 species of vertebrate hosts, including amphibians, birds, fish, mammals, and reptiles (Kváč et al., 2014b). Infections are frequently asymptomatic but can result in the disease cryptosporidiosis, which primarily manifests as diarrhea (Xiao et al., 2004). There are currently 26 recognized *Cryptosporidium* species, and many more genotypes have been described based on unique sequences at one or more loci (Kváč et al., 2014a). Wildlife are host to a number of *Cryptosporidium* taxa, including species and genotypes that cause disease in humans and livestock (Appelbee et al., 2005). Studies of *Cryptosporidium* ecology and evolution in wildlife are necessary to determine the extent of host-parasite coevolution and to assess the potential for novel human-pathogenic taxa to emerge.

Rodents, one of the largest vertebrate groups, frequently thrive in close proximity to humans (Honeycutt et al., 2007). More than 30 *Cryptosporidium s*pecies and genotypes have been reported in rodents, many of which can cause disease in humans and livestock. These include rodent-adapted *C. muris* and *C. tyzzeri* and a number of other species and genotypes, including *C. parvum*, *C. ubiquitum*, *C. meleagridis*, *C. scrofarum*, *C. andersoni*, skunk genotype, and chipmunk genotype I (Feltus et al., 2006; Feng et al., 2007; Kváč et al., 2009; Leoni et al., 2006; Lv et al., 2009; Ng-Hublin et al., 2013; Palmer et al., 2003; Rašková et al., 2013; Robinson et al., 2008; Ziegler et al., 2007a). Of these, *C. parvum* is the primary cause of cryptosporidiosis in humans and livestock (Xiao, 2010).

Most studies aimed at understanding *Cryptosporidium* in rodents have focused on Old World rats and mice from the family Muridae (Foo et al., 2007; Kimura et al., 2007; Kváč et al., 2013; Ng-Hublin et al., 2013; Paparini et al., 2012). However, rodents in the family Cricetidae, which includes voles, muskrats, and New World rats and mice, are known to host *C. muris*, *C. andersoni*, *C. parvum*, *C. ubiquitum*, *C. meleagridis*, and a number of apparently host-adapted genotypes (see Kváč et al., 2014b for a review). A heterogeneous group of closely related genotypes, collectively referred to as *Cryptosporidium* muskrat genotype II or muskrat genotype II variants, have been reported in meadow voles (*Microtus pennsylvanicus*), southern red-backed voles (*Myodes gapperi*), muskrats (*Ondatra zibethicus*), deer mice (*Peromyscus* spp.), a red fox (*Vulpes vulpes*), and surface water (Feng et al., 2007; Perz and Le Blancq, 2001; Robinson et al., 2011; Ruecker et al., 2012; Xiao et al., 2002; Zhou et

al., 2004; Ziegler et al., 2007a). *Cryptosporidium* muskrat genotype I, vole genotype, hamster genotype, deer mouse genotypes I-IV, W12 genotype, and chipmunk genotype I also have been reported in cricetid rodents (Feng et al., 2007; Lv et al., 2009; Zhou et al., 2004; Ziegler et al., 2007a).

Cricetid rodent population densities can vary considerably both spatially and temporally (Parmenter et al., 1999), providing an opportunity to study the relationship between host population density and parasite prevalence (Anderson and May, 1978; Arneberg et al., 1998; Esch et al., 1990). *Cryptosporidium* prevalence has previously been positively correlated to host population densities in wild Soay sheep (*Ovis aries*) (Craig et al., 2007), Eurasian red squirrels (*Sciurus vulgaris*) (Bertolino et al., 2003), and feral pigs (*Sus scrofa*) (Atwill et al., 1997). Therefore, we hypothesized that *Cryptosporidium* prevalence in cricetids rodents would be positively correlated to the host population density.

We report a study of *Cryptosporidium* infecting wild, cricetid rodent populations in the United States (at sites in North Dakota, Minnesota, South Dakota, and Tennessee) and Europe (at a site in Slovakia). Isolates were sequenced at the 18S rDNA and actin loci, and phylogenetic relationships were inferred from sequences of each locus and concatenated 18S rDNA and actin sequences. Cricetids hosted a number of closely related *Cryptosporidium* genotypes, which exhibited high 18S rDNA sequence variability. We found evidence of host specificity and coevolution among *Cryptosporidium* infecting voles and *Peromyscus* mice, including *Cryptosporidium* isolates with a sister group relationship infecting genotypes in closely related *Myodes* vole species from the United States and Europe. The *Cryptosporidium* genotypes detected in cricetid rodents were not associated with human and livestock disease.

Materials and Methods

Host species and sample collection. Meadow voles (*Microtus pennsylvanicus*), southern red-backed voles (*Myodes gapperi*), woodland voles (*Microtus pinetorum*), deer mice and white-footed mice (*Peromyscus* spp.) were live captured in Sherman box traps at sites in North Dakota, Minnesota, South Dakota and Tennessee from 2007-2012. Traps were baited with sunflower seeds or rolled oats and checked daily. Vole and mouse fecal samples were collected from the trap or directly from the animal during handling. Captured animals were ear-tagged and released in compliance with North Dakota State University Institutional Animal Care and Use policies. Bank voles (*Myodes glareolus*) were captured at a

site in Slovakia in 2011 and samples were collected from the intestines during dissection. Muskrat (*Ondatra zibethicus*) fecal samples were collected from May-July in 2009 from muskrat mounds at two sites in North Dakota. All samples were stored at 4°C prior to DNA extraction. Animals that died in traps were dissected and samples of intestinal contents were examined.

DNA extraction and PCR. DNA was recovered from samples by alkaline digestion, phenol-chloroform extraction, and purification using a QIAmp DNA Stool Mini Kit as previously described (Qiagen, Valencia, CA) (Feltus et al., 2006; Peng et al., 2003). DNA was stored at -20°C until used in PCR assays. A genus-specific nested PCR assay (Xiao et al., 2001) was used to amplify a fragment of the *Cryptosporidium* 18S rRNA gene using GoTaq® Flexi DNA polymerase (Promega, Madison, WI) with a buffer concentration of 0.5×. All samples that tested positive for the *Cryptosporidium* 18S rRNA gene were subsequently examined for the actin gene using a nested PCR approach (Sulaiman et al., 2002). Secondary products were visualized with SYBR Green or ethidium bromide following electrophoresis on agarose gels. Products of the expected size were purified using the Wizard Gel purification system (Promega, Madison, WI) and were sequenced directly or following cloning into a pGEM-T Easy Vector (Promega, Madison, WI). Cloned products were purified using the Zyppy Plasmid Miniprep kit (Zymo Research, Irvine, CA) before sequencing.

Sequencing and phylogenetic analyses. Purified 18S rDNA and actin amplicons were sequenced in both directions using a BigDye Terminator v3.1 cycle sequencing kit with primers from secondary PCR reactions or T7 and SP6 primers in the case of cloned products. Sequencing was carried out by Macrogen USA (Rockville, MD). Sequences were assembled using SeqMan (DNAStar, Madison, WI) and aligned with *Cryptosporidium* sequences in GenBank using the MAFFT online server (version 7) using the auto alignment strategy (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standley, 2013). Phylogenetic relationships of aligned sequences were inferred using a neighbor-joining method based on the Kimura 2-parameter distances model with pairwise deletions (Kimura, 1980; Saitou and Nei, 1987). A bootstrap consensus tree was inferred from 1000 pseudoreplicates. Trees were constructed using MEGA5 (Tamura et al., 2011).

Estimation of host density and statistical analyses. A trapping grid was established at sampling sites with a minimum of 30 traps set for 3 to 4 nights. An ear tag with a unique identification

number affixed to the animal upon first capture enabled identification of recaptures and tracking of samples from individual animals. Abundance estimates were obtained for hosts at specific sites and dates using a closed system mark-recapture method and calculated with the program MARK (White and Burnham, 1999). Abundance estimates were used to calculate population density for a $100m^2$ area. Prevalence was calculated as the number of positive individuals divided by the total number of individuals caught.

Chi-squares were used to determine if *Cryptosporidium* prevalence differed between host species, and correlation coefficients were calculated to assess the relationship between *Cryptosporidium* prevalence and host population density using the statistical program R (Team, 2013). A p-value of ≤ 0.05 was considered statistically significant.

Results

Cricetids were frequently infected with *Cryptosporidium*. Six hundred and eighty one samples were examined from 596 individual animals in North Dakota, Minnesota, South Dakota, and Tennessee. The overall prevalence of *Cryptosporidium* in cricetids was 50.7% (302/596). The lowest prevalence was in muskrats (9.5%; 4/42), followed by woodland voles (51.2%; 21/41), meadow voles (52.4%; 163/311), southern red-backed voles (55.6%; 15/27), and *Peromyscus* mice (56.6%; 99/175).

Prevalence of infection was not correlated with cricetid population density. Host density (number of hosts per $100m^2$) data was collected at some locations (Table 8). No correlations (p-values > 0.05) were found between host density and *Cryptosporidium* prevalence for meadow voles ($r = 0.34$, d.f. = 5, p-value > 0.05), vole species combined ($r = 0.18$, d.f = 9, p-value > 0.05), or all hosts combined ($r = 0.15$, d.f. = 12, p-value > 0.05).

Prevalence varied among different species at a single location and the same species at different locations. *Cryptosporidium* prevalence was 69% (47/68) in *Peromyscus* mice at ND1, which differed significantly ($X^2=8.201$, d.f.=1, p-value < 0.05) from the prevalence in meadow voles, 45% (33/73), at the same location. The prevalence in meadow voles at ND1 (45%) and ND2 (68%, 79/117) also differed significantly ($X^2=9.2508$, d.f.=1, p-value < 0.05).

Table 8. Host population density and *Cryptosporidium* prevalence for different hosts, locations, and sampling periods.

Host	Location	Sampling period	Density (no. per 100m^2)	Prevalence % (positive/ sampled)
Meadow vole	ND1	June 16–19, 2009	1.05	22% (9/41)
	ND1	August 9–12, 2011	0.09	43% (3/7)
	ND2	August 25–28, 2009	7.15	77% (23/30)
	ND2	August 25–28, 2010	3.00	88% (22/25)*
	ND2	September 13-16, 2011	1.65	40% (4/10)*
	MN2	June 6–10, 2010	3.37	18% (2/11)
	ND3	May 26–28, 2010	1.31	86% (6/7)
Woodland vole	TN1	February 26–March 1, 2010	1.57	64% (9/14)
	TN1	November 9–12, 2010	7.34	46% (12/26)*
Southern red-backed vole	MN1	August 15–18, 2009	0.26	50% (2/4)
	ND1	September 29–October 1, 2010	0.06	50% (2/4)*
Peromyscus spp.	MN1	April 3–6, 2010	0.30	40% 2/5
	MN1	October 7–11, 2010	2.35	66% 19/29*
	ND1	September 29–October 1, 2010	1.05	82% 40/49*

*Indicates location where only prevalence was calculated.

18S rDNA sequences from cricetids were heterogeneous and an 18S rDNA sequence tree had poor bootstrap support. Sequences of *Cryptosporidium* 18S rDNA were obtained from 96 isolates from 65 meadow voles (Mipe), three woodland voles (Mipi), five southern red-backed voles (Myga), 17 *Peromyscus* mice (Pema), two muskrats (Onzi), and four bank voles (Mygl). Most isolates clustered with previously described genotypes in a neighbor-joining tree, but sequence heterogeneity was high and the tree topology had low bootstrap support (Table 9, Figure 11).

Nine of the 96 (9.4%) isolates shared 100% sequence identity with previously reported *Cryptosporidium* genotypes. Seven of the nine genotypes were reported previously only in water, while the remaining two, *Cryptosporidium* deer mouse genotype III (accession no. EF641014) and *Cryptosporidium* chipmunk genotype IV (accession no. KC954240), were isolated from a deer mouse and an eastern chipmunk (*Tamias striatus*), respectively. 1964-Mipe-ND2 was identical to *Cryptosporidium* muskrat genotype II (accession no. JQ178272), and 1544-Pema-MN2 and 2289-Mipe-ND3 were identical to a *Cryptosporidium* muskrat genotype II variant (accession no. AY737570). 2395-Mipe-ND2 and 2397-Mipe-MN5 were identical to *Cryptosporidium* vole genotype (accession no. JQ178298). 2321-PemaTN1 and 2333-Pema-TN1 were identical to *Cryptosporidium* deer mouse genotype III (accession nos. EU825738, AY737557, EF641014). 2031-Myga-MN4 was identical to *Cryptosporidium* chipmunk genotype IV (accession no. KC954240), and 2303-Mipi-TN1 was identical to Cry_147, (accession no. EF061289). Isolates clustering with *Cryptosporidium* deer mouse genotypes I–IV were found exclusively in *Peromyscus* mice. Other isolates from *Peromyscus* spp. were similar to *Cryptosporidium* vole genotype (1938-Pema-MN1 shared 99.4% similarity with accession no. AY737563), *Cryptosporidium* muskrat (AY737570 and AY737569, respectively), *C. ubiquitum* (1925-Pema-MN1 shared 99.6% similarity with GenBank no. KC608029), and *Cryptosporidium* W29 genotype from water (1754-Pema-ND1, 1799-Pema-ND1, and 1848-Pema-ND1 shared 99.3, 99.3, and 99.7% similarity, respectively, with accession no. JQ413356).

Isolates from meadow voles clustered with *Cryptosporidium* muskrat genotypes I and II, *Cryptosporidium* vole genotype, *Cryptosporidium* fox genotype, and *Cryptosporidium* W12 genotype. Sequence heterogeneity was found in each cluster and was particularly high in the muskrat genotype II cluster.

Table 9. Estimates of evolutionary divergence over sequence pairs within each cricetid group. The average percent substitutions between sequence pairs within each group are shown with standard error (SE) estimates. Analyses were conducted using the Kimura 2-parameter model. The analysis involved actin and 18S rDNA sequences from 42 isolates.

Groups	Percent substitutions ± SE	
	Actin	18S rDNA
Cricetid 1	0.1 ± 0.1	0.2 ± 0.1
Cricetid 2	0.0 ± 0.0	0.7 ± 0.3
Cricetid 3	0.0 ± 0.0	0.8 ± 0.2
Cricetid 4	ND	ND
Cricetid 5	0.2 ± 0.2	0.2 ± 0.1
Cricetid 6	ND	ND
Cricetid 7	0.0	0.0
Cricetid 8	ND	ND
Cricetid 9	0.2 ± 0.1	0.5 ± 0.2
Cricetid 10	ND	ND
Cricetid 11	0.1 ± 0.1	1.4 ± 0.3
Cricetid 12	0.1 ± 0.1	0.7 ± 0.3
Cricetid 13	0.2 ± 0.1	0.1 ± 0.1
Cricetid 14	2.5 ± 0.6	0.3 ± 0.2

ND: Not determined because there was only one sequence in the group

Cryptosporidium infecting woodland voles in Tennessee and meadow voles in North Dakota and Minnesota were similar. 2303-Mipi-TN1 clustered with 2018-Mipe-ND2, and these genotypes also clustered with the fox genotype (accession no. AY737572). 2304-Mipi-TN1 clustered with sequences from meadow voles and a single *Peromyscus* mouse as part of a relatively large group that was similar to muskrat genotype II. 2331-Mipi-TN1 clustered with the vole genotype.

Similar genotypes were found in different species of *Myodes* from North American southern red-backed voles (*Myodes gapperi*) and European bank voles (*Myodes glareolus*): M992-Mygl-SL and M1000-Mygl-SL from bank voles clustered with 2035-Myga-MN4 and 1935-Myga-MN1 from southern red-backed voles. These genotypes were closely related to *Cryptosporidium* muskrat genotype II. 1937-Myga-MN1 clustered with 1938-Pema-MN1 and shared 99.4% similarity with *Cryptosporidium* vole genotype (accession no. AY737563). 2031-Myga-MN4 and 2032-Myga-MN3 formed a separate group that was most closely related to a cluster containing the *Cryptosporidium* fox genotype (GenBank no. AY737572) and *Cryptosporidium* sp. UK E7 (accession no. HQ822135).

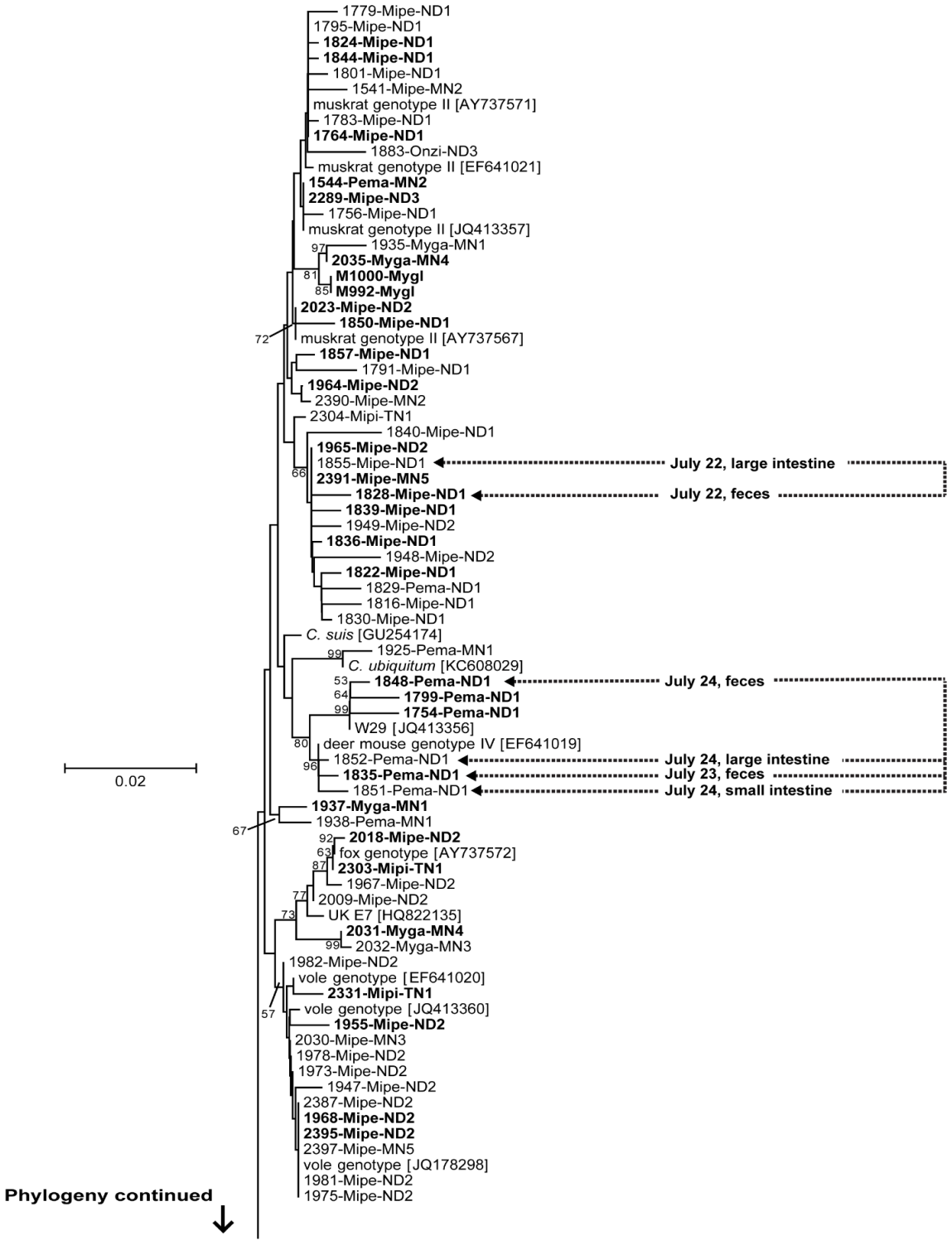

Figure 11. Phylogenetic relationships among *Cryptosporidium* 18S rRNA gene sequences. Isolates that also have an actin sequence are bolded. Trees were constructed using a neighbor-joining approach with bootstrapping based on 1,000 pseudoreplicates. The tree is rooted with *Plasmodium falciparum* (accession no. JQ627149).

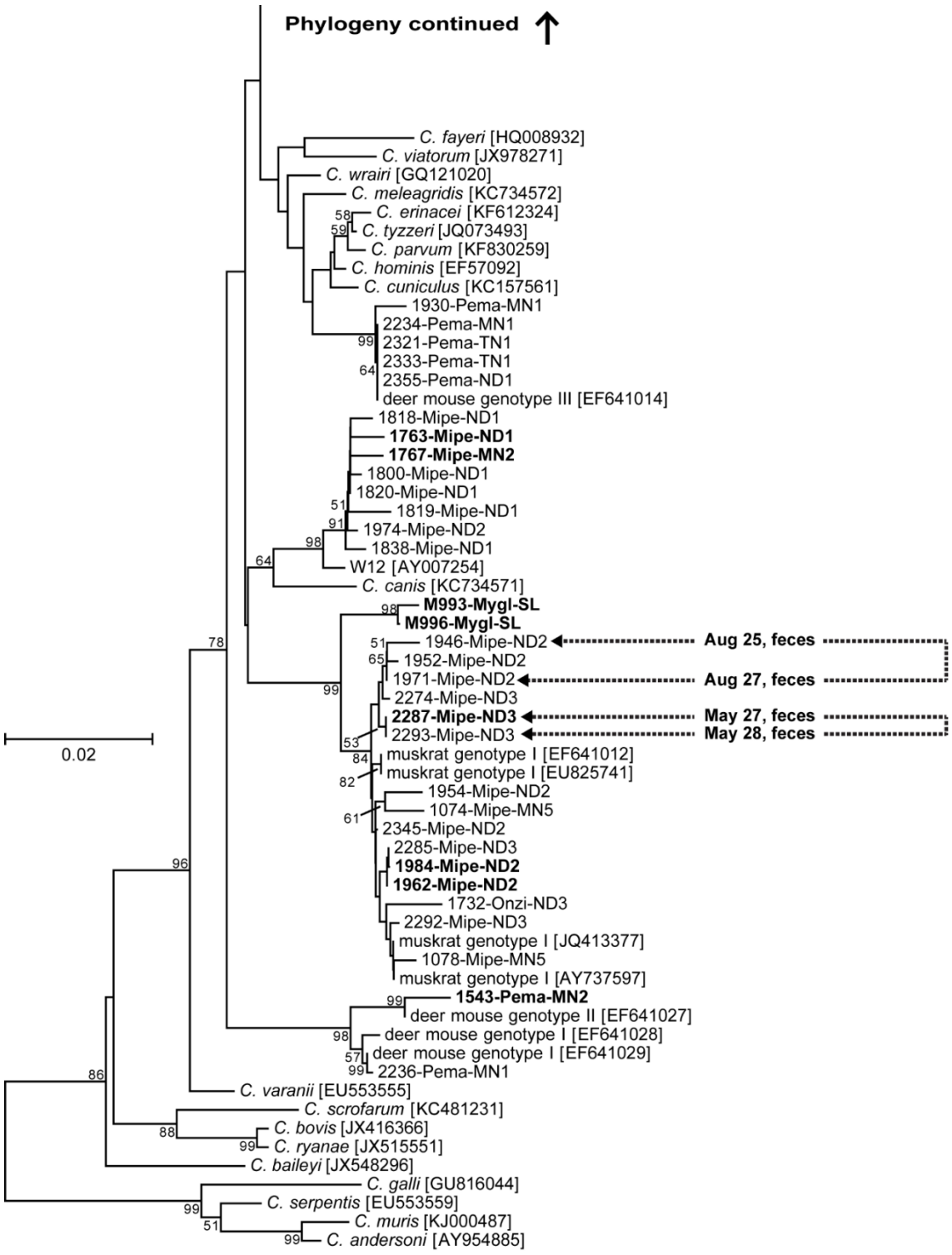

Figure 11. Phylogenetic relationships among *Cryptosporidium* 18S rRNA gene sequences (continued). Isolates that also have an actin sequence are bolded. Trees were constructed using a neighbor-joining approach with bootstrapping based on 1,000 pseudoreplicates. The tree is rooted with *Plasmodium falciparum* (accession no. JQ627149).

Two isolates from muskrats clustered with *Cryptosporidium* muskrat genotype I (1732-Onzi-ND3) and muskrat genotype II (1883-Onzi-ND3). A number of meadow vole isolates also clustered with muskrat genotype I, and two European bank vole isolates (M993-Mygl-SL and M996-Mygl-SL) had strong support as a sister clade to this group.

A tree produced from actin sequences had greater stability than an 18S rDNA tree. Actin sequences were obtained from 42 of the 96 samples (bolded isolates in Figure 11). Sequences clustered into fourteen major clades, named Cricetid 1 to Cricetid 14, in a neighbor-joining tree (Figure 12), and sequences within each clade were relatively homogenous (Table 9).

Cricetids 1–7 formed a well-supported (97% bootstrap value) group, within which Cricetids 1–3 clustered together with 87% bootstrap support and shared 98.3 ± 0.4% to 98.7 ± 0.4% sequence similarity. Isolates from Cricetids 1–3 clustered with *Cryptosporidium* muskrat genotype II in the 18S rDNA tree, but with poor bootstrap support.

Isolates comprising Cricetids 4 and 5 clustered with *Cryptosporidium* fox genotype and UK E7 in the 18S rDNA tree.

Similar to 18S rDNA sequences, 2035-Myga-MN4, representing cluster 6 in the actin tree, shared a 99 ± 0.4% similarity and clustered with M992-Mygl-SL and M1000-Mygl-SL, representing cluster 7 in the actin tree, supporting the close relationship of *Cryptosporidium* isolates from *M. gapperi* and *M. glareolus*.

Cricetids 8 and 9 shared 98.5 ± 0.4% sequence similarity and formed a well-supported group. Isolates comprising Cricetid 9 clustered with the vole genotype at the 18S rDNA locus.

Cricetids 10 and 11 were associated with *Peromyscus* mice. At the 18S rDNA locus, Cricetid 10 isolates clustered with *Cryptosporidium* deer mouse genotype II; Cricetid 11 isolates clustered with *Cryptosporidium* deer mouse genotype IV (1835-Pema-ND1) and *Cryptosporidium* W29 genotype (1754-Pema-ND1, 1799-Pema-ND1, and 1848-Pema-ND1).

Cricetid 12 isolates, which clustered with *Cryptosporidium* W12 genotype at the 18S rDNA locus, formed a separate clade, with relatively poor bootstrap support.

Cricetids 13 and 14 from US *Microtus pennsylvanicus* and European *Myodes glareolus*, respectively, were sister groups that shared 90.9-93.0% similarity. This is consistent with the sister group relationship inferred from the 18S rDNA tree.

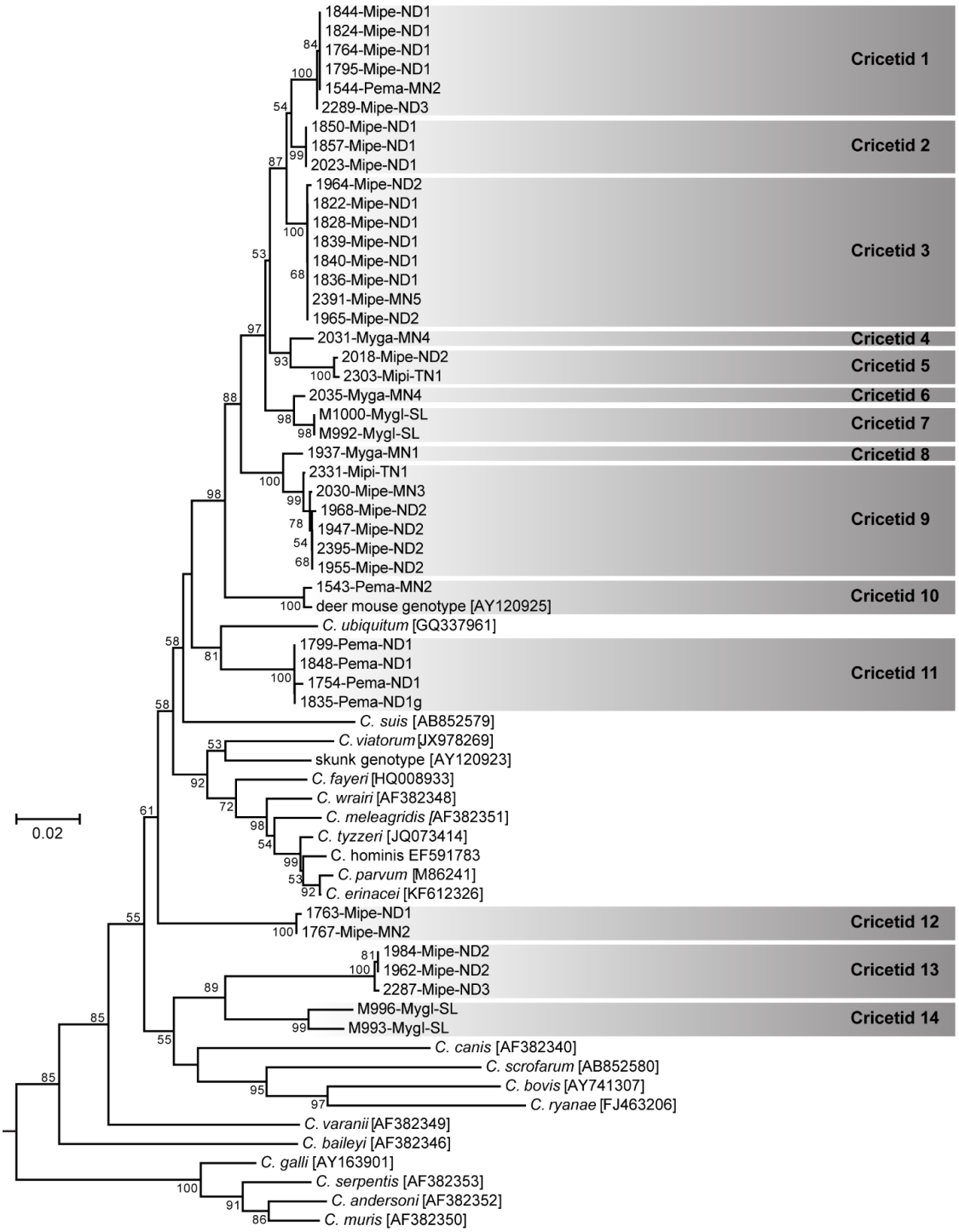

Figure 12. Phylogenetic relationships among *Cryptosporidium* actin gene sequences. Isolates that also have an 18S rRNA gene sequence are bolded. Fourteen major clades, representing *Cryptosporidium* taxa, are shaded. Trees were constructed using a neighbor-joining approach with bootstrapping based on 1,000 pseudoreplicates. The tree is rooted with *Plasmodium falciparum* (accession no. EF472536).

A concatenated 18S rDNA and actin sequence tree was similar to the actin sequence tree. A neighbor-joining tree constructed from concatenated 18S rDNA and actin sequences had the same topology as the actin sequence tree, with 14 clades, and bootstrap support for branching was generally greater. *Cryptosporidium* genotypes based on the concatenated 18S rDNA and actin sequence tree are shown by host species and location in Table 10.

Multiple isolates from a single animal and multiple amplicons from a single isolate exhibited 18S rDNA sequence heterogeneity. The 18S rDNA sequences of multiple isolates from meadow voles and a *Peromyscus* mouse were heterogeneous (Figure 11). Fecal isolates 1946-Mipe-ND2 and 1971-Mipe-ND2 collected from a single meadow vole on the 25th and 27th of August, 2009, had four single nucleotide polymorphisms. 2287-Mipe-ND3 (27th of May, 2010) differed from 2293-Mipe-ND3, which was isolated from the same meadow vole on the previous day, by a single deletion. 1855-Mipe-ND1 and 1828-Mipe-ND1 from the large intestine and feces, respectively, of a single meadow vole captured on the 22nd of July, 2009 differed by five single nucleotide polymorphisms. Four isolates from a single *Peromyscus* mouse shared 98.2-99.4% similarities (1848-Pema-ND1, 1835-Pema-ND1, 1851-Pema-ND1, 1852-Pema-ND1); of these, 1848-Pema-ND1 formed a separate clade. In contrast, actin sequences from these four isolates were homogeneous, forming the Cricetid 11 clade. In addition to 18S rDNA sequence heterogeneity among isolates from a single animal, multiple amplicons from a single isolate were heterogeneous. Two 18S rDNA amplicons from 1968-Mipe-ND2 differed by a single nucleotide polymorphism and a deletion (99.7% similarity).

Discussion

Genetic and biological diversity in the genus *Cryptosporidium* has been revealed by studies of isolates from divergent hosts in the major vertebrate classes. However, the more difficult task of discovering the drivers of parasite diversification, including the contributions of host and geographic determinants, requires ecological studies. The present study investigated *Cryptosporidium* in wild rodents of the family Cricetidae in North America and Europe. We show that voles and muskrats of the subfamily Arvicolinae and New World mice (*Peromyscus* spp.) of the subfamily Neotominae host different *Cryptosporidium* genotypes. *Peromyscus* mice host genotypes that cluster with *Cryptosporidium* deer

Table 10. *Cryptosporidium* genotypes in host species by location. Genotypes are based on concatenated sequences of 18S rDNA and actin. At the 18S rDNA locus, Cricetids 1-3, 6, and 7 cluster with muskrat genotype II, Cricetid 4 clusters with chipmunk genotype IV, Cricetid 5 clusters with the fox genotype, Cricetid 9 clusters with the vole genotype, Cricetid 10 clusters with deer mouse genotype II, Cricetid 11 clusters with deer mouse genotype IV and W29, Cricetid 12 clusters with W12 genotype, Cricetid 13 & 14 cluster with muskrat genotype I, and Cricetid 8 represents a novel genotype.

Location	Host				
	Meadow vole	**Woodland vole**	**Southern red-backed vole**	***Peromyscus* mice**	**Bank vole**
ND-1	Cricetids 1, 2, 3, 12			Cricetid 11	
ND-2	Cricetids 1, 2, 5, 9, 13				
ND-3	Cricetids 1, 13				
MN-1			Cricetid 8		
MN-2	Cricetid 12			Cricetids 1,10	
MN-3	Cricetid 9				
MN-4			Cricetids 4, 6		
TN-1		Cricetids 5, 9			
SL					Cricetids 7, 14

mouse genotypes I-IV and the *Cryptosporidium* W29 genotype from water. A heterogeneous group of closely related genotypes, which included multiple clusters of *Cryptosporidium* muskrat genotype II that were distinguished based on the actin sequences, were detected in multiple populations of meadow voles. Muskrats were host to *Cryptosporidium* muskrat genotypes I and II, which were also detected in voles. The finding of genotypes with a sister-group relationship in closely related North American southern red-backed voles and European bank voles is evidence supporting host-parasite coevolution.

Cryptosporidium was detected in more than half (50.7%) of the cricetid hosts sampled. The prevalence in *Peromyscus* mice (56.6%; 99/175) was higher than previously reported in *Peromyscus* mice in the New York City watershed (32.2%; 57/177 (Feng et al., 2007) and 6.5%; 165/2529 (Ziegler et al., 2007b)). Similarly, the prevalence in meadow voles in this study (52.4%; 163/311) was higher than the 30% (3/10) and 4.4 (13/297) reported in the New York watershed (Feng et al., 2007; Ziegler et al., 2007b). The prevalence in southern red-backed voles, 56.6% (15/27), was greater than reported by Ziegler et al. (Ziegler et al., 2007b) (6.3%; 19/301), but less than that reported by Feng et al. (Feng et al., 2007) (80%; 4/5). Future studies run concurrently could be used to determine if differences in prevalence are due to temporal or spatial effects because wild small mammal populations can vary widely between years, seasons, and locations.

We detected *Cryptosporidium* in 9.5% (4/42) of muskrats in North Dakota, which was similar to the 11.8% (28/237) prevalence reported previously in Maryland (Zhou et al., 2004), and higher than the 0.6% (1/149) prevalence reported the New York City watershed (Ziegler et al., 2007b). Although a separate study from lower New York State found 100% of muskrats were positive for *Cryptosporidium*, the sample size in that study was small (n=6) (Perz and Le Blancq, 2001), and it appears that, relative to other cricetids, muskrats have a low prevalence of *Cryptosporidium*.

We found no support for the hypothesis that *Cryptosporidium* prevalence is positively correlated with host population density. This is consistent with a previous study by Laakkonen et al. (Laakkonen et al., 1994), which found a low prevalence of *Cryptosporidium* by microscopy in bank voles (*Myodes glareolus*; 1/41 positive), field voles (*Microtus agrestis*; 1/131 positive), and root voles (*Microtus oeconomus*; 0/43 positive), despite a high host population density. However, the low prevalence could have been due, in part, to the poor sensitivity of microscopy relative to PCR (Morgan et al., 1998; Xiao et

al., 2006). Previous studies on other hosts, including wild Soay sheep (*Ovis aries*) (Craig et al., 2007), Eurasian red squirrels (*Sciurus vulgaris*) (Bertolino et al., 2003), and feral pigs (*Sus scrofa*) (Atwill et al., 1997), have found *Cryptosporidium* prevalence to be a positively correlated to host population density. However, within the Soay sheep study, the prevalence of *Giardia duodenalis* and *Eimeria* species were not positively correlated to sheep population density (Craig et al., 2007), suggesting that correlations vary among parasite species. Examining correlations at the *Cryptosporidium* genus level, which was necessary in the present study to obtain sufficient numbers for statistical analysis, could mask positive correlations between specific host and parasite taxa. For example, the prevalence of *Cryptosporidium* spp. with a narrow host range may be positively correlated to the population density of its specific host, and *Cryptosporidium* spp. with a broad host range may be positively correlated with the host community density and not that of a single host species (Seville et al., 1996).

Cryptosporidium deer mouse genotypes I-IV were found only in *Peromyscus* spp. despite the presence of voles in the same area, which suggests that the host range of these genotypes does not extend to voles. This is consistent with previous studies that have shown *Peromyscus* to be the only cricetid hosts of *Cryptosporidium* deer mouse genotypes I-IV (Feng et al., 2007; Xiao et al., 2002; Ziegler et al., 2007a). However, deer mouse genotype III has been reported in another rodent species, the eastern gray squirrel (*Sciurus carolinensis*) (Feng et al., 2007), which can be sympatric with *Peromyscus* mice (Brunner et al., 2013). One hypothesis to explain this observation is that *Peromyscus* mice are not the major host for *Cryptosporidium* deer mouse genotype III. Although they were the first identified host (Feng et al., 2007), they may be only minor hosts that are infrequently infected because of their shared habitat with the major host, gray squirrels. In support of this hypothesis, *Cryptosporidium* deer mouse genotype III was found only in a single *Peromyscus* mouse captured in the same area as meadow voles (Chapter 4). Also, meadow voles and *Peromyscus* are known to spatially segregate within a grassland, limiting interspecific interactions (Bowker and Pearson, 1975). The specificity of deer mouse genotype III for gray squirrels, *Peromyscus* mice, and voles should be examined experimentally. Combining experimental infectivity studies with quantitative studies under natural conditions and knowledge of host ecology will be necessary to fully understand the factors impacting the host range of *Cryptosporidium* deer mouse genotype III and other *Cryptosporidium* spp. in wildlife.

Cryptosporidium from voles exhibited considerable 18S rDNA sequence heterogeneity, which is consistent with previous studies on murid rats (*Cryptosporidium* rat genotypes II-IV) (Kimura et al., 2007; Lv et al., 2009; Ng-Hublin et al., 2013). Most sequences clustered with previously named *Cryptosporidium* genotypes, including muskrat genotype I, muskrat genotype II, vole genotype, and fox genotype. *Cryptosporidium* muskrat genotypes I and II were rarely detected in hosts other than voles, and *Cryptosporidium* W12 and vole genotypes were exclusive to voles. *Cryptosporidium* muskrat genotype II has been reported in meadow voles, red-backed voles, *Peromyscus* mice, red fox, muskrats, and multiple water samples (Feng et al., 2007; Robinson et al., 2011; Ruecker et al., 2012; Zhou et al., 2004; Ziegler et al., 2007a), *Cryptosporidium* vole genotype has been identified previously in meadow voles (Feng et al., 2007; Ziegler et al., 2007a). The association of *Cryptosporidium* W12 genotype with voles in this and other studies (Ziegler et al., 2007a), suggests that voles are the major hosts of this genotype. *Cryptosporidium* muskrat genotype I has been reported previously in meadow voles, red-backed voles, a fox, and muskrats (Feng et al., 2007; Zhou et al., 2004; Ziegler et al., 2007b). *Cryptosporidium* muskrat genotype I is the most frequent *Cryptosporidium* spp. identified in muskrats. In contrast, *Cryptosporidium* muskrat genotype II has been infrequently identified in muskrats, and based on the prevalence of muskrat genotype II in voles from this study, voles may be the major host. Muskrats and voles sharing very similar *Cryptosporidium* spp. could be explained by the closer evolutionary relationship of voles and muskrats, than voles and *Peromysus* mice.

Voles in the genus *Myodes* (southern red-backed voles in North America and bank voles in Slovakia) hosted *Cryptosporidium* genotypes that were distinct from, but closely related to, those infecting voles in the genus *Microtus* (meadow and woodland voles). One explanation for this finding is that *Myodes* and *Microtus* voles we trapped were rarely found at the same locations. The geographic ranges of meadow voles and southern red-backed voles overlap, but meadow voles are associated with prairie or grassland habitats and southern red-backed voles are associated with coniferous and deciduous forest habitats (Merritt, 1981; Reich, 1981). The habitat preferences of the vole species could limit the opportunity of *Cryptosporidium* being transmitted between host species and allow for lineages of *Cryptosporidium* to adapt to and evolve with the different host species (coevolution). This hypothesis could be tested by examining the *Cryptosporidium* in areas where both genera of voles can be found

together, which would include edge or riparian habitats. Although we did trap meadow voles and southern red-backed voles at a single location, MN5, a riparian habitat, no sequence data was not available for the red-backed voles.

Given the polyphyletic relationship of *Myodes*-associated genotypes, they appear to have diverged after the split of a number of *Microtus*-associated genotypes including *Cryptosporidium* muskrat genotype I, muskrat genotype II, and fox genotype. Trees constructed from actin and concatenated actin and 18S rDNA sequences show the polyphyletic relationship of *Myodes*-associated *Cryptosporidium* more clearly than those constructed from 18S rDNA sequence alone: 2031-Myga-MN4 (Cricetid 4) and 1937-Myga-MN1 (Cricetid 8) clustered with the *Cryptosporidium* fox genotype (Cricetid 5) and a novel genotype (Cricetid 9), respectively. The Cricetid 4-Cricetid 5 and Cricetid 8-Cricetid 9 clusters support coevolution of the *Cryptosporidium* with the hosts as more closely related genotypes were found in the meadow and woodland voles (Cricetid 5 and 9). Although we had limited sample sizes and locations, these genotypes could be endemic to North American voles and the divergence within each cluster has occurred since the host species have inhabited North America. The *Myodes*-*Microtus* divergence is estimated to have occurred between 5.76 to 9 million years ago (Conroy and Cook, 1999; Robinson et al., 1997). However, the invasion of North America was later. Southern red-backed vole ancestors likely colonized North America from Eurasia in the late Pliocene (3.6 to 2.58 million years ago) to early Pleistocene (2.58 million to 780,000 years ago) (Cook et al., 2004) and *Microtus* since at least the beginning of the Pleistocene (Martin, 2003).

The finding that the 2035-Myga-MN4 (Cricetid 6) genotype from a southern red-backed vole (*Myodes gapperi*) in Minnesota was more closely related to M1000-Mygl-SL and M992-Mygl-SL (Cricetid 7) genotypes from bank voles (*Myodes glareolus*) in Slovakia, rather than genotypes from sympatric *Microtus* species in Minnesota and North Dakota, suggests that the divergence of these genotypes is a result of coevolution with the hosts. Southern red-backed voles and bank voles very closely related (separated by at least 20,000 generations), able to interbreed in captivity, nearly indistinguishable, but are found on different continents (Cook et al., 2004; Grant, 1974). Continued and expanded sampling of these host species in addition to cross infections may provide further support for coevolution. In addition, *Myodes*-*Cryptosporidium* relationships could prove to be a useful model system to gather more detailed

information on *Cryptosporidium* evolution and diversification similar to Kvac et al.'s (Kváč et al., 2013) study of *C. tyzzeri* in house mouse (*Mus musculus*) subspecies. Sampling should include northern red-backed voles (*Myodes rutilus*), which are found throughout the Holarctic region. They are sympatric to bank voles in Eurasia and parapatric to southern red-backed voles in North America. This vole species, which likely expanded to North America around the late Pleistocene (5,000 to 126,000 years ago) (Cook et al., 2004), effectively bridges bank vole and southern red-backed vole distributions as northern red-backed-bank vole hybrids as well as hybrid red backed voles can be found in the wild (Melnikova (Rodchenkova) et al., 2012; Runck and Cook, 2005). Because *Cryptosporidium* are highly dependent on hosts for survival and transmission, host biogeography and phylogeography can be used to gain a better understanding of *Cryptosporidium* evolution and diversification.

The heterogeneity in 18S rDNA sequences from cricetid rodents led to a poorly supported phylogenetic tree, which may be the result of paralogous relationships among 18S rRNA gene copies in some *Cryptosporidium* lineages. Only nine (9.4%) of the 96 18S rDNA sequences were identical to previously described *Cryptosporidium* genotypes. However, the variation of 18S rDNA sequences is not unusual. We detected highly divergent copies of the 18S rRNA gene in *Cryptosporidium* chipmunk genotype II from eastern chipmunk hosts (Chapter 3) and multiple studies report a high level of 18S rDNA sequence heterogeneity in *Cryptosporidium* rat genotypes from murid rodents (Kimura et al., 2007; Lv et al., 2009; Ng-Hublin et al., 2013). Reports of heterogeneous 18S rDNA copies (paralogs) with low levels of divergence have been described in other *Cryptosporidium* species including *C. andersoni, C. felis, C. galli, C. hominis, C. parvum,* and *C. ubiquitum* (Ikarashi et al., 2013; Le Blancq et al., 1997; Morgan et al., 2001; Santín and Fayer, 2007; Sevá Ada et al., 2011; Xiao et al., 1999). 1835-Pema-ND1 (*Cryptosporidium* deer mouse genotype IV) and 1848-Pema-ND1 (*Cryptosporidium* W29 genotype) were from the same individual, clustered as sister taxa in the 18S rDNA sequence tree, and had identical sequences at the actin locus, suggesting that they may have a paralogous rather than an orthologous relationship. Feng et al. (10) reached a similar conclusion about *Cryptosporidium* deer mouse genotypes I and II following the detection of both genotypes in a single animal. Although, examining multiple amplicons from multiple loci can help to identify paralogs, extracting DNA from an individual *Cryptosporidium* sporozoite should be the goal in order to confirm that divergent 18S rRNA gene copies

are from a single genome. Achieving this goal is technically challenging, particularly from natural infections, and it has been successful only in *C. andersoni* (Ikarashi et al., 2013). Identification of paralogs is critical to prevent incorrect inference of phylogenetic relationships (Buckler et al., 1997; Rich et al., 1997).

Although prevalence was high in most cricetid host species, most *Cryptosporidium* spp. in these hosts pose little threat to human and livestock health. We found one occurrence of a *C. ubiquitum*-like genotype (1925-Pema-MN1) in a *Peromyscus* mouse: *C. ubiquitum* is a known cause of human cryptosporidiosis. 1925-Pema-MN1 differed from a *C. ubiquitum* isolate from a sheep (accession no. EU827398) by four single nucleotide substitutions. The single occurrence of *C. ubiquitum* in a cricetid rodent and the frequent occurrence of this species in squirrels at the same location (Chapter 4), suggests that cricetids are not major hosts of *C. ubiquitum*.

This study has shown that use of the 18S rRNA gene alone may lead to erroneous conclusions about relationships among *Cryptosporidium* infecting cricetid rodents. We suggest that future studies of these hosts should use multiple genetic loci to identify *Cryptosporidium* genotypes. Using this method, we found that the *Cryptosporidium* genotype muskrat II and its variants are actually multiple distinct taxonomic groupings. We also were able to detected three genotype clusters (Cricetid 4-Cricetid 5, Cricetid 6-Cricetid 7, Cricetid 8-Cricetid 9) where coevolution likely played a role in the diversification because more closely related hosts had more closely related *Cryptosporidium*. However more sampling and experimental studies are needed to help determine to what extent host relatedness and interactions or proximity between host species affects *Cryptosporidium* evolution.

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PAPER 3. CHARACTERIZATION OF *CRYPTOSPORIDIUM* IN NORTH AMERICAN TREE AND GROUND SQUIRRELS[1]

Abstract

A number of wildlife-associated *Cryptosporidium* spp. are infrequent causes of cryptosporidiosis in humans. In order to assess the potential for new human-pathogenic species to emerge with changing boundaries between humans and wildlife, it is necessary to understand the ecology of wildlife-associated *Cryptosporidium*. In the present study, *Cryptosporidium* infecting squirrels from Marmotini (ground squirrels) and Sciurini (tree squirrels) tribes in the family Sciuridae was characterized at the 18S rRNA and actin genetic loci. In the tree squirrel tribe, *Cryptosporidium* was identified in 40% (18/45) of American red squirrels (*Tamiasciurus hudsonicus*), 38.4% (56/146) of eastern gray squirrels (*Sciurus carolinensis*), and 28.6% (2/7) of fox squirrels (*Sciurus niger*). In ground squirrels, *Cryptosporidium* was identified in 64.8% (35/54) of 13-lined ground squirrels (*Ictidomys tridecemlineatus*), 50% (1/2) in golden-mantled ground squirrels (*Callospermophilus lateralis*), and 35.1% (27/77) in black-tailed prairie dogs (*Cynomys ludovicianus*). Phylogenetic analyses showed that tree squirrels and ground squirrels were host to different *Cryptosporidium* taxa. Ground squirrels were host to four genotypes, which we have named *Cryptosporidium* ground squirrel genotype I, II, III, and IV. *Cryptosporidium* ground squirrel genotypes I and II were similar to sequences previously reported in ground squirrels in California, and *Cryptosporidium* ground squirrel genotypes II and III were sister taxa found only in ground squirrel hosts. Tree squirrels were host to *Cryptosporidium* deer mouse genotype III, skunk genotype, and *C. ubiquitum*. Subsequent subtyping of *C. ubiquitum* isolates at the gp60 locus showed isolates belonged to either the XIIb or XIIc subtype family. Skunk genotype and both *C. ubiquitum* subtypes have been associated with disease in humans. Because tree squirrels are frequently infected with *C. ubiquitum* (69.2%; 27/39) and live in close proximity with humans, they may be a significant source of human cryptosporidiosis.

Introduction

Cryptosporidium is a genus of gastrointestinal parasites that affect a wide range of vertebrate species, including amphibians, birds, fish, mammals, and reptiles (Fayer, 2010). *Cryptosporidium* taxa

[1] The material in this chapter was co-authored by Brianna L.S. Stenger, Mark E. Clark, Martin Kváč, Catherine W. Giddings, and John M. McEvoy. Brianna L.S. Stenger had the primary responsibility for collecting data, analysis, interpretation, and writing of this manuscript.

that cause diarrheal disease in humans and livestock are a major concern, and a goal of research is to identify the reservoirs and transmission dynamics of these pathogenic parasites (Appelbee et al., 2005; Xiao et al., 2002). Studies of *Cryptosporidium* in wild mammalian host populations will be necessary to understand the public health impact of changing boundaries between humans and wildlife and, more fundamentally, will contribute to the understanding of *Cryptosporidium* biology and evolution.

Rodentia, the largest mammalian order, host more than 30 *Cryptosporidium* species and genotypes (reviewed by Kváč et al., 2014). Many rodents have the potential to transmit human- and livestock-pathogenic *Cryptosporidium* because their habitats overlap with human dwellings and farms. Squirrels, members of the rodent family Sciuridae, are known hosts of human-pathogenic *Cryptosporidium*: eastern gray squirrels (*Sciurus carolinensis*) from the Unites States host *C. parvum*, *C. ubiquitum*, skunk genotype, and chipmunk genotype I (Feng et al., 2007; Ziegler et al., 2007); American red squirrels (*Tamiasciurus hudsonicus*) host *C. ubiquitum* and chipmunk genotype I (Ziegler et al., 2007); eastern chipmunks (*Tamias striatus*) host *C. ubiquitum* and chipmunk genotype I (Feng et al., 2007; Ziegler et al., 2007); groundhogs (*Marmota monax*) host *C. ubiquitum* (Feng et al., 2007; Ziegler et al., 2007); yellow-bellied marmots (*Marmota flaviventris*) host *C. parvum*; Eurasian red squirrels (*Sciurus vulgaris*) host chipmunk genotype I (Kváč et al., 2008); and Siberian chipmunks (*Eutamias sibiricus*) host *C. parvum* (Lv et al., 2009). In addition, livestock-adapted *C. andersoni* has been reported in Bobak marmots (*Marmota bobac*) (Ryan et al., 2003) and an eastern chipmunk (chapter 2). *Cryptosporidium* also has been detected in southern flying squirrels (*Glaucomys volans*), fox squirrels (*Sciurus niger*), 13-lined ground squirrels (*Ictidomys tridecemlineatus*), and spotted souslik (*Spermophilus suslicus*), but the *Cryptosporidium* spp. was not identified from these hosts (Current, 1989; Kloch and Bajer, 2012). Novel *Cryptosporidium* genotypes also have been reported in golden-mantled ground squirrels (*Callospermophilus lateralis*), Belding's ground squirrels (*Urocitellus beldingi*), and California ground squirrels (*Otospermophilus beecheyi*) in California (Atwill et al., 2004; Pereira et al., 2010).

Few studies have investigated the relationship between *Cryptosporidium* prevalence in squirrels and dynamics of squirrel populations. Bertolino et al. (Bertolino et al., 2003) found a higher prevalence of *Cryptosporidium* in higher population densities of the Eurasian red squirrel hosts, suggesting a positive relationship between population density and parasite prevalence. *Cryptosporidium* prevalence also

differed between two Eurasian red squirrel populations within a year and varied at a location over time (Bertolino et al., 2003). Prevalence and intensity of *Cryptosporidium* shedding from California ground squirrels peaked in summer months, following the emergence of juvenile ground squirrels, and shedding averaged two- to four-fold higher in juvenile squirrels than adults (Atwill et al., 2001; Atwill et al., 2004). Determining the relationship between squirrel population dynamics and *Cryptosporidium* prevalence will inform our understanding of the role of squirrels as reservoirs of *Cryptosporidium* spp. and assess the risk to human health. More fundamentally, biological and ecological differences among squirrel species make them a useful model to study dynamics of host-parasite interactions that will lead to a better understanding of parasite biology and evolution.

Squirrel species reported as *Cryptosporidium* hosts are members of Sciuridae tribes Sciurini (subfamily Sciurinae) or Marmotini (subfamily Xerinae), commonly referred to as tree squirrels and ground squirrels, respectively (Steppan et al., 2004). Ground squirrels seldom climb trees and are typically associated with prairies or grasslands, living on the ground or in burrow systems below the ground where many species will hibernate during the winter months. In contrast, tree squirrels remain active throughout the year and will build nests in trees in forests, riparian and urbanized areas.

The goal of this study was to better understand the ecology of *Cryptosporidium* in wild populations of North American ground squirrels and tree squirrels. Because of differences in their biology, ecology, and behavior, we expected tree and ground squirrels to host different *Cryptosporidium* taxa. We were also interested in how prevalent human-pathogenic *Cryptosporidium* was in squirrels from the central United States, because previous reports identified human-pathogenic *Cryptosporidium* in squirrels from the eastern and western United States. Fragments of the 18S rRNA and actin genes were amplified and sequenced, and phylogenetic relationships were inferred. We also identified subtypes of *C. ubiquitum* isolates from tree squirrels using the gp60 gene to determine whether the subtype has been associated with human infections. In addition, we investigated whether *Cryptosporidium* prevalence was correlated with squirrel population density.

Materials and Methods

Trapping and sample collection. Fecal samples were collected from six species of squirrels. Three species – eastern gray squirrels (*S. carolinensis*), eastern fox squirrels (*S. niger*), and American

red squirrels (*T. hudsonicus*) – were from the sciuridae tribe Sciurini (tree squirrels). Golden-mantled ground squirrels (*C. lateralis*), 13-lined ground squirrels (*I. tridecemlineatus*), and black-tailed prairie dogs (*Cynomys ludovicianus*) were sampled from the tribe Marmontini (ground squirrels).

Squirrels, with the exception of black-tailed prairie dogs and golden-mantled ground squirrels, were live-captured in Sherman box traps or Tomahawk cage traps baited with sunflower seeds mixed with peanut butter, affixed with an ear-tag with a unique identification number, 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 entrance to burrows. One sample was collected per burrow opening. Fecal samples from golden-mantled ground squirrels were collected from the ground. All fecal samples were stored at 4°C prior to DNA extraction.

Sample locations are shown in Figure 13. Gray squirrels were sampled from western Minnesota and eastern North Dakota. Red squirrels and fox squirrels were sampled from western Minnesota. 13-lined ground squirrels were sampled from north central and eastern North Dakota. Golden-mantled ground squirrels were sampled from south central Montana and prairie dogs were sampled from western North Dakota.

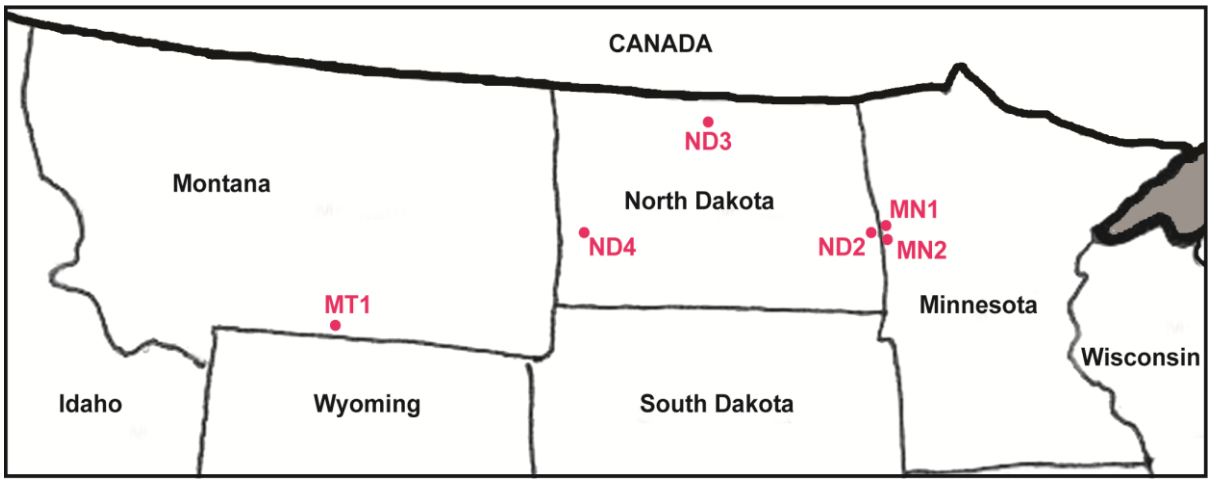

Figure 13. Squirrel fecal sample collection sites.

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.

Amplification of 18S rRNA, actin, and gp60 genes. A fragment of the *Cryptosporidium* 18S rRNA gene was amplified as described by Xiao et al. (Xiao et al., 2001), with the exception that a 0.5× buffer concentration was used (GoTaq Flexi DNA polymerase, Promega, Madison, WI), and a fragment of the actin gene was amplified as previously described by Sulaiman et al. (Sulaiman et al., 2002). Any isolates identified as *C. ubquitum* at the 18S rDNA or actin loci were subsequently to determine the gp60 subtype as previously described by Li et al. (Li et al., 2014). Secondary PCR products were visualized on an agarose gel under UV light with a SYBR Green dye or following ethidium bromide staining. Products were purified (Wizard SV, Promega, Madison, WI) and stored at 4°C.

Sequencing and phylogenetic analyses. Purified PCR products were sequenced in both directions with secondary primer sets 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/) (Katoh and Standley, 2013). Alignments were manually edited and phylogenetic analyses were performed using MEGA 6.0 (Tamura et al., 2013). Phylogenetic trees were inferred by the neighbor-joining method and distance estimates were based on the Kimura 2-parameter distance model with pairwise deletions. Bootstrapping values were determined from 1000 pseudoreplicates (Kimura, 1980; Saitou and Nei, 1987).

Host density. A mark-recapture method, with the assumption of a closed population, was used to estimate host abundance using Program MARK (http://www.phidot.org/software/mark/), and host density was estimated for 100 m^2 area (White and Burnham, 1999). A grid of 15 to 30 traps was set for 3 to 5 nights and checked daily at each trapping location. Each captured animal was marked with an ear tag, if they were not already tagged, and feces were collected from the trap or directly from the animal. Fecal samples were also collected from recaptured squirrels.

Statistical analyses. Prevalence was calculated by dividing the number of *Cryptosporidium*-positive individuals by the total number of individuals caught. Chi-squares were used to test for

differences in *Cryptosporidium* prevalence between individual squirrel species, *Cryptosporidium* prevalence by season in tree and ground squirrels, and human pathogenic *Cryptosporidium* species and genotypes in tree squirrels. A correlation coefficient was calculated to assess the relationship between *Cryptosporidium* prevalence and host population density of tree squirrels. Statistical analyses were performed using the program R (http://www.r-project.org./) (Team, 2013) and a p-value ≤ 0.05 was considered statistically significant.

Results

Prevalence of *Cryptosporidium* in tree and ground squirrels. *Cryptosporidium* was more prevalent in 13-lined ground squirrels (64.8%; 35/54) than in red squirrels (40.0%; 18/45), gray squirrels (38.4%; 56/146), and prairie dogs (35.1%; 27/77) (p-values < 0.05). *Cryptosporidium* also was found in 2/7 (28.6%) fox squirrels and 1/2 (50%) golden-mantled ground squirrels. The overall prevalence of *Cryptosporidium* in tree squirrels (38.4%; 76/198) and ground squirrels (47.4%; 63/133) was not significantly different ($X^2 = 2.64$, p-value > 0.05).

Samples were not obtained from tree squirrels in November; tree and ground squirrels in December; or ground squirrels in January, February, and March. *Cryptosporidium* was more prevalent in tree squirrels during late summer/fall (July–October; 52.4%; 43/82) than during spring/early summer (March–June; 24.1%; 26/108) ($X^2 = 16.21$, p-value < 0.05). There was no difference between *Cryptosporidium* prevalence in ground squirrels during late summer/fall (July-October; 66.7%; 26/39) than during spring/early summer (April-June; 63.2%; 12/19) ($X^2 = 0.07$, p-value > 0.05).

Cryptosporidium prevalence was not correlated with the population density of gray squirrels (r = 0.21, d.f. = 9, p-value > 0.05) or red and gray squirrels combined (r = 0.01 d.f.= 12, p-value > 0.05) (Table 11). Ground squirrel population density data were insufficient to examine correlations with *Cryptosporidium* prevalence.

***Cryptosporidium* diversity in tree and ground squirrels.** Seven major clades, representing seven *Cryptosporidium* taxa (species or genotype), were evident in phylogenies inferred from 18S rDNA (Figure 14) and actin sequences (Figure 15). Two subtypes of *C. ubiquitum* in tree squirrels were identified at the gp60 locus.

Table 11. Squirrel population density and *Cryptosporidium* prevalence.

Host	Location	Sampling period	Density (no. per 100m^2)	Prevalence % (positive/sampled)
	MN-1	August 15–19, 2009	1.350	64.3 (9/14)
	MN-1	February 2–18, 2010	0.505	0 (0/10)
	MN-1	June 23–25, 2010	0.266	33.3 (2/6)
	MN-1	October 8–11, 2010	0.541	70* (7/10)
Eastern	MN-1	May 26–June 10, 2011	0.772	50* (2/4)
gray	MN-1	May 16–24, 2012	0.323	33.3* (4/12)
squirrel	MN-2	September 2–5, 2009	0.369	85.7 (6/7)
	MN-2	April 5–8, 2010	0.875	38.4 (5/13)
	MN-2	September 7–13, 2010	0.482	33.3* (2/6)
	MN-2	June 13–16, 2010	0.674	22.2* (2/9)
	MN-2	February 13–16, 2012	0.956	50* (6/12)
American	MN-1	August 15–18, 2009	0.350	85.7 (6/7)
red	MN-1	May 16–24, 2012	0.140	66.7* (4/6)
squirrel	MN-2	June 23–25, 2010	0.471	0 (0/10)

*Indicates location where *Cryptosporidium* taxa information was not available.

Three taxa were exclusively found in tree squirrels in this study. *C. ubiquitum* was found in 19 gray squirrels, six red squirrels, and two fox squirrels at two locations in Minnesota (MN1 and MN2). One gray squirrel was positive for *C. ubiquitum* on three separate occasions (1986-Scca-MN2, 1989-Scca-MN2, and 1996-Scca-MN2 on September 2, 3, and 5, respectively, in 2009). *Cryptosporidium* skunk genotype was identified in three gray squirrels, one red squirrel, and one fox squirrel at MN1 and MN2.

The fox squirrel (1992-Scni-MN2) showed evidence of a mixed infection with *C. ubiquitum* (identified at the 18S rDNA locus) and *Cryptosporidium* skunk genotype (identified at the actin locus). *Cryptosporidium* deer mouse genotype III was identified in six gray squirrels and one red squirrel at a single location in Minnesota (MN1). One of the gray squirrels (1929-Scca-MN1) had a mixed infection with *Cryptosporidium* deer mouse genotype III (identified at the 18S rDNA locus) and *C. ubiquitum* (identified at the actin locus). In total, *C. ubiquitum* was found in 27 of the 39 (69.2%) tree squirrels for which sequence data was available.

Two *C. ubiquitum* gp60 subtypes were identified in tree squirrels (Figure 16). Sequences shared a 100% similarity with *C. ubiquitum* subtype XIIb (accession no. JX412926) or subtype XIIc (accession no. JX412925). *C. ubiquitum* subtype XIIb was identified in four isolates from three gray squirrels (1989-Scca-MN2, 1996-Scca-MN2, 2247-Scca-MN2, 2399-Scca-MN1) and four red squirrels (1905-Tahu-MN1, 1915-Tahu-MN1, 1916-Tahu-MN1, 2224-Tahu-MN2) from two locations in Minnesota, MN1 and MN2.

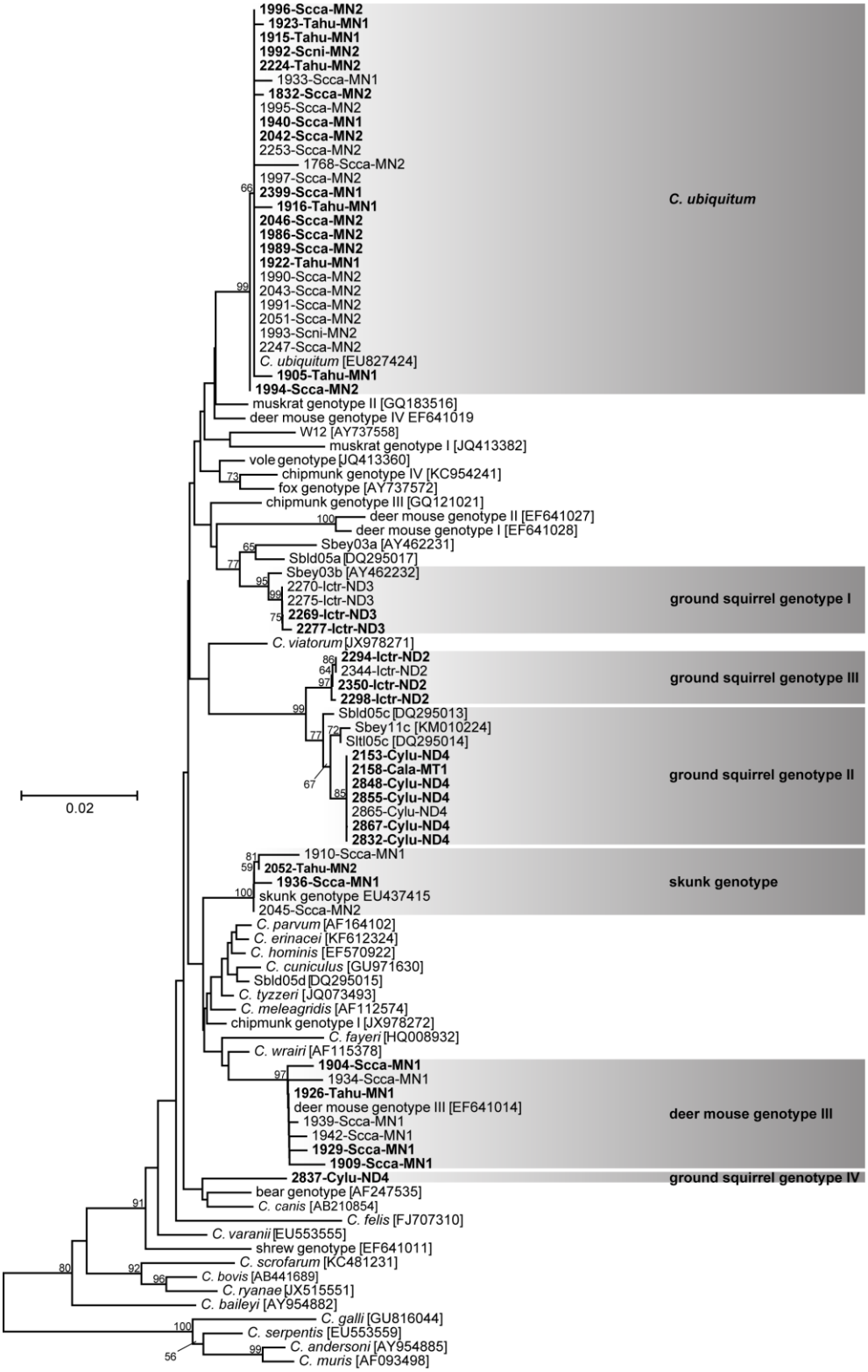

Figure 14. Phylogenetic relationships of *Cryptosporidium* 18S rRNA gene sequences. Isolates that also have an actin sequence are bolded. Seven major clades, representing *Cryptosporidium* taxa, are shaded. Trees were constructed using a neighbor-joining approach with bootstrapping based on 1,000 pseudoreplicates. The tree is rooted with *Plasmodium falciparum* (accession no. JQ627149).

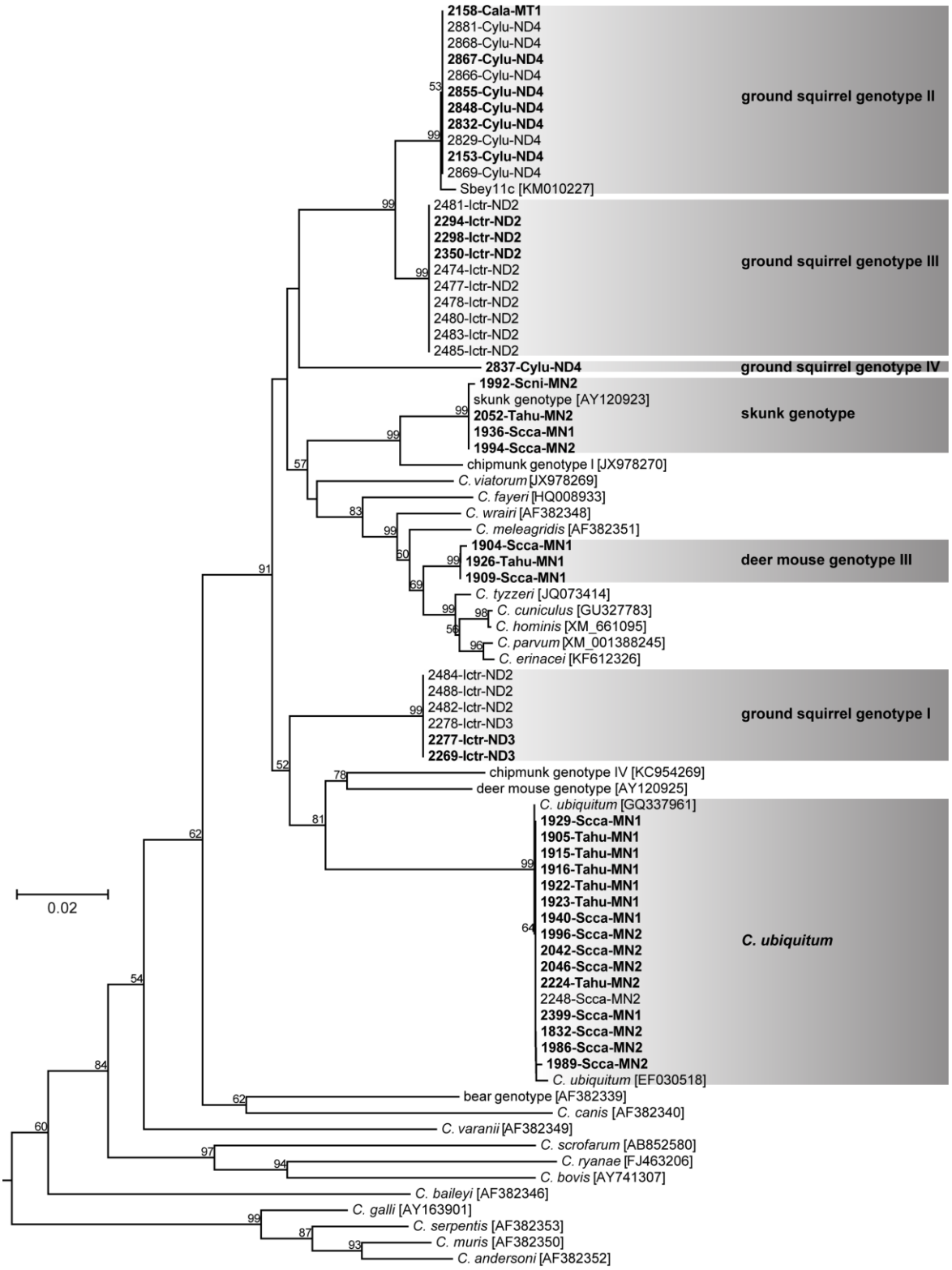

Figure 15. Phylogenetic relationships of *Cryptosporidium* actin gene sequences. Isolates that also have an 18S rRNA gene sequence are bolded. Seven major clades, representing *Cryptosporidium* taxa, are shaded. Trees were constructed using a neighbor-joining approach with bootstrapping based on 1,000 pseudoreplicates. The tree is rooted with *Plasmodium falciparum* (accession no. EF472536).

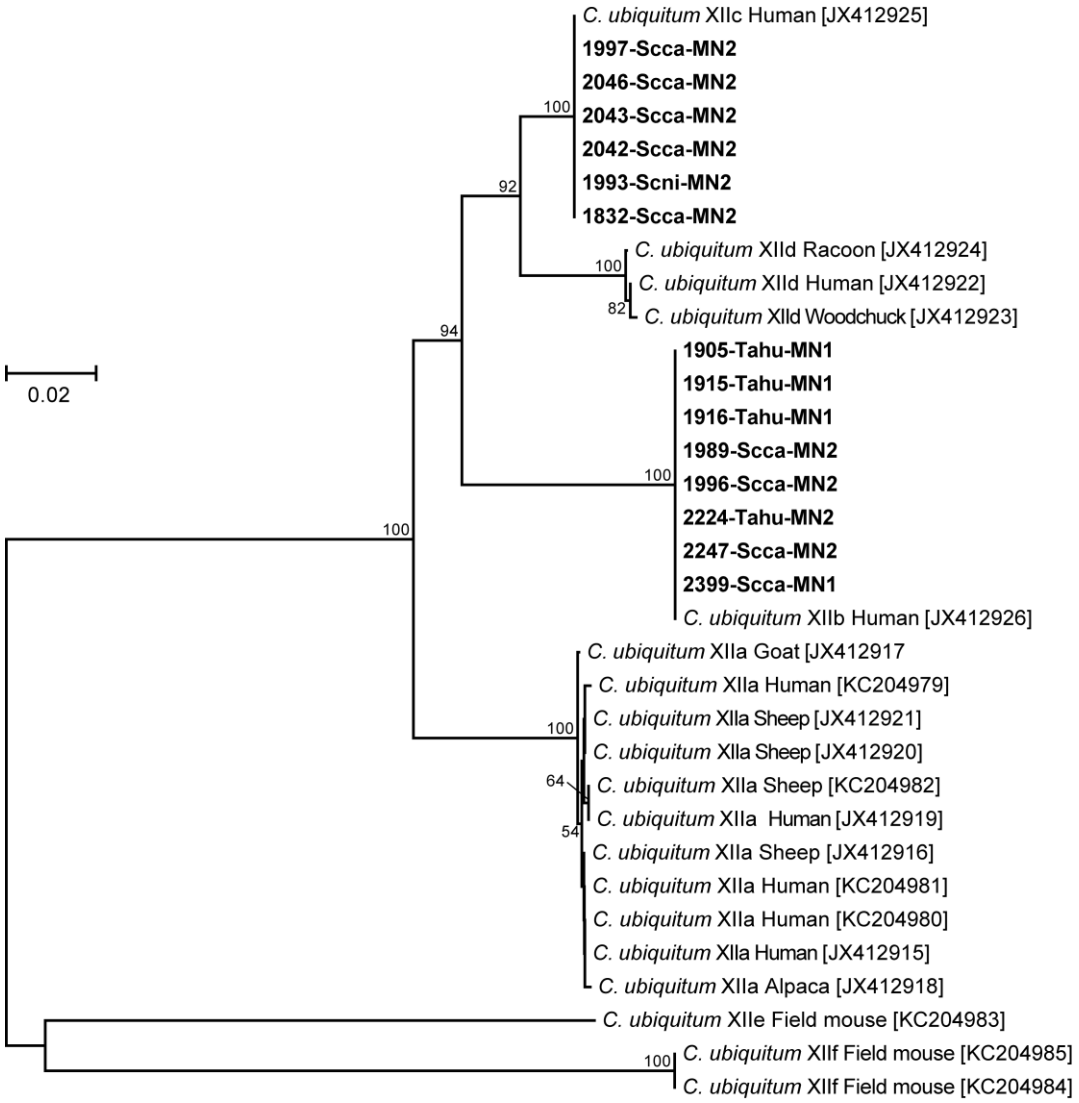

Figure 16. Phylogenetic relationships among *Cryptosporidium ubiquitum* gp60 sequences. Trees were constructed using a neighbor-joining approach with bootstrapping based on 1,000 pseudorelicates. The tree is rooted with C. parvum (accession no. FJ861304).

Subtype XIIc was detected in five gray squirrels (1832-Scca-MN2, 1997-Scca-MN2, 2042-Scca-MN2, 2043-Scca-MN2, 2046-Scca-MN2) and one fox squirrel (1993-Scni-MN2) from a single location in Minnesota, MN2.

Ground squirrels hosted four *Cryptosporidium* genotypes, which we have named *Cryptosporidium* ground squirrel genotypes I–IV. *Cryptosporidium* ground squirrel genotype I was identified in eight 13-lined ground squirrels at two locations in North Dakota (ND2 and ND3). Two animals were positive for *Cryptosporidium* ground squirrel genotype I on consecutive days in 2010: the first animal was positive on two consecutive days in May (2278-Ictr-ND3 and 2269-Ictr-ND3), and the second was positive on three

consecutive days in August (2484-Ictr-ND2, 2482-Ictr-ND2, and 2488-Ictr-ND2). At the 18S rDNA locus, *Cryptosporidium* ground squirrel genotype I was most similar to Sbey03b (accession no. AY462232) from a California ground squirrel (Table 12). *Cryptosporidium* ground squirrel genotype II was identified in 10 black-tailed prairie dogs in North Dakota (ND4) and a golden-mantled ground squirrel in Montana (MT1). At the 18S rDNA locus, this genotype shared 99.1, 99.0, and 98.8% similarity, respectively, with Sbld05c from a Belding's ground squirrel (accession no. DQ295013), Sltl05c from a golden-mantled ground squirrel (accession no. DQ295014), and Sbey11c from a California ground squirrel (accession no. KM010224). *Cryptosporidium* ground squirrel genotype III was exclusively found in four 13-lined ground squirrels at a single location in North Dakota (ND2). *Cryptosporidium* ground squirrel genotype IV, which was identified in a single black-tailed prairie dog in North Dakota (ND4), did not cluster with other ground squirrel genotypes, and was most similar to *Cryptosporidium* muskrat genotype II (Table 12).

Discussion

Tree squirrels and ground squirrels were host do different *Cryptosporidium* taxa. These findings suggest that *Cryptosporidium* species and genotypes have followed different evolutionary paths with these squirrel tribes, which may be influenced by the evolutionary, ecological, and/or biological differences between the Sciuridae tribes following their divergence in the early Oligocene (~30mya) (Mercer and Roth, 2003). In addition, *C. ubiquitum* was the most common *Cryptosporidium* identified in tree squirrels. Based on sequencing of the gp60 gene, the two *C. ubiquitum* subtype families, XIIb and XIIc, have been identified in humans and XIIB has been prevalent in storm and source water (Li et al., 2014). These data suggest tree squirrels are a significant source or reservoir of human pathogenic *Cryptosporidium*.

Cryptosporidium spp. infecting tree squirrels are not specific for this tribe. *C. ubiquitum* has additionally been identified in bovids, cervids, other rodents, carnivores, opossums, humans and other primates (Fayer et al., 2010). *Cryptosporidium* skunk genotype also has been identified in skunks (*Mephitis mephitis*), raccoons (*Procyon lotor*), river otters (*Lontra canadensis*), and humans (Feng et al., 2007; Robinson et al., 2008; Xiao et al., 2002; Zhou et al., 2004); and *Cryptosporidium* deer mouse genotype III has also been identified in deer mice (*Peromyscus* spp.)(Feng et al., 2007 and Chapter 3). Feng et al. (Feng et al., 2007) reported *C. ubiquitum*, *Cryptosporidium* deer mouse genotypes III, and

Table 12. *Cryptosporidium* taxa and the most similar 18S rDNA and actin sequences in GenBank.

Taxa (present study)	Host (present study)	18S rDNA Taxa; accession no.; similarity	Actin Taxa; accession no.; similarity
C. ubiquitum	Gray squirrel Red squirrel Fox squirrel	*C. ubiquitum*; EU827424; 99.2-100%	*C. ubiquitum*; GQ337961 99.4-99.9%
deer mouse genotype III	Gray squirrel Red squirrel	deer mouse genotype III; EU8825738; 99.3-100%	*C. tyzzeri*; JQ073414; 98.2-98.3%
skunk genotype	Gray squirrel Red squirrel	skunk genotype; AY737559; 99.0-99.7%	skunk genotype; AY120923; 99.9%
ground squirrel genotype I	13-lined ground squirrel	Sbey03b; AY462232; 98.5-99.5%	deer mouse genotype; AY120925; 92.5-93.2%
ground squirrel genotype II	Black-tailed prairie dog Golden-mantled squirrel	Sbld05c; DQ295013; 98.8-98.9%	chipmunk genotype I; JX978270; 93.2-94.0%
ground squirrel genotype III	13-lined ground squirrel	Sbld05c; DQ295013; 98.0-98.3%	deer mouse genotype; AY120925; 92.4-92.6%
ground squirrel genotype IV	Black-tailed prairie dog	muskrat genotype II; EF641021; 98.0%	chipmunk genotype I; JX978270; 92.6%

Cryptosporidium skunk genotype in not only eastern gray squirrels located in the New York City Department of Environmental Protection watershed, but in multiple wildlife species. The generalist nature of the *Cryptosporidium* spp. and habitats of tree squirrels that overlap with humans, livestock, and other wildlife species, could explain why *C. ubiquitum*, *Cryptosporidium* deer mouse genotype III, and skunk genotype were detected in tree squirrels.

Experimental host infection studies, continued sampling of wild hosts, and characterization of multiple genetic loci of the *Cryptosporidium* deer mouse genotype III and skunk genotype are also needed to determine whether transmission between host species is due to shared localities (overlapping habitats), and if tree squirrels could be the principal host of either or both genotypes. These data, leading to species descriptions, would help clarify the taxonomy as the current genotype names can be misleading as they do not accurately represent host specificity (Xiao et al., 2002).

C. ubiquitum is of particular public health concern because it has not only been associated with human disease and surface water contamination, but it has the largest host range and is one of the most widely distributed *Cryptosporidium* species (Chalmers et al., 2011; Cieloszyk et al., 2012; Elwin et al., 2012; Fayer et al., 2010; Jellison et al., 2009; Jiang et al., 2005; Li et al., 2014; Robinson et al., 2008). Recent genotyping of the highly polymorphic gp60 locus has revealed host-adapted structuring of *C. ubiquitum* in multiple host species, with the same subtype families detected in rodents, humans, and water (Li et al., 2014). We did not detect the XIId subtype family in any squirrels, the predominant subtype detected in sciurids (two gray squirrels, one eastern chipmunk, one red squirrel, one woodchuck) by Li et al. (Li et al., 2014), which was also detected in humans from the United States and the United Kingdom (Li et al., 2014). However, we did find the XIIb and XIIc subfamilies in seven and six isolates, respectively. The XIIc subtype was previously detected in captive prehensile-tailed porcupines and subsequently passaged through goats. Both subtype families were reported in humans from the United States and subtype XIIb in humans from the United Kingdom (Li et al., 2014). Previously, the only non-human hosts of the XIIb subtype family were an eastern chipmunk and a Verreaux's sifaka (Li et al., 2014). The subtype family XIIb was also detected in a majority of the storm water and source water isolates from the United States (Li et al., 2014). The high occurrence of *C. ubiquitum* in tree squirrels, the finding of the XIIb and XIIc subtype families that also infects humans, and a high incidence of the XIIb subtype in storm

water and source water, suggests tree squirrels could be a significant source of human-pathogenic *C. ubiquitum*.

Cryptosporidium spp. in ground squirrels from this study and previous studies from the western United States are specific for this tribe (Atwill et al., 2001; Atwill et al., 2004; Pereira et al., 2010). The sequence similarity of *Cryptosporidium* ground squirrel genotypes II and III mirrors the close genetic relationship of their primary hosts, black-tailed prairie dogs and 13-lined ground squirrels, respectively (Harrison et al., 2003; Herron et al., 2004), suggesting a coevolutionary relationship because related host species carried related parasites. *Cryptosporidium* ground squirrel genotype II was detected in a golden-mantled ground squirrel in this study, and highly similar sequences were reported from another golden-mantled ground squirrel (Sltl05c, 13), a California ground squirrel (Sbey11c), and a Belding's ground squirrel (Sbld05c, Pereira et al., 2010) from the western United States. The finding of *Cryptosporidium* ground squirrel genotype II in multiple ground squirrel species could, in part, be due to the overlap in geographic ranges, or sympatric nature, of the host species. Studies, based on oocyst morphology of *Eimeria* parasites, have found the same species or similar guilds of *Eimeria* spp. in sympatric white-tailed prairie dogs (*C. leucurus*), Wyoming ground squirrels (*U. elegans*), and 13-lined ground squirrels (Seville et al., 1992; Seville et al., 1996; Shults et al., 1990; Stanton et al., 1992). Sympatric host species and the relatedness of the host species (all ground squirrels within the tribe Marmotini) could not only explain the similarity of *Eimeria* parasites in ground squirrel species (Seville et al., 1992), but also the *Cryptosporidium*. Black-tailed prairie dogs are found in the Great Plains region of the United States and the western fringe of their range overlaps with the range of golden-mantled ground squirrels, which are found in parts of the Rocky Mountains. The range of golden-mantled ground squirrels also overlaps with the range of Belding's ground squirrels and further west, with California ground squirrels. Therefore, *Cryptosporidium* ground squirrel genotype II may not be specific to a single host species, but the Marmotini tribe, and be spread through the connection of ground squirrel ranges.

The range of 13-lined ground squirrels overlaps with black-tailed prairie dogs in addition to fox squirrels, eastern gray squirrels, and American red squirrels. We detected *Cryptosporidium* ground squirrel genotypes I and III, in 13-lined ground squirrels. While we did not sample multiple species of ground squirrels at the same location or both tree and ground squirrels at a location, we did sample other

small mammals where tree squirrels and 13-lined ground squirrels were trapped (Chapter 4). However, we did not find any of the *Cryptosporidium* ground squirrel genotypes in other small mammals including *Peromyscus* mice, meadow voles (*Microtus pennsylvanicus*), and northern short-tailed shrews (*Blarina brevicauda*). Nor did we find the *Cryptosporidium* spp. from *Peromyscus* mice, meadow voles, and northern short-tailed shrews in any 13-lined ground squirrels. Although, *C. ubiquitum* and *Cryptosporidium* deer mouse genotype III were detected in tree squirrels and *Peromyscus* mice at the same location. These data further support the hypothesis that the ground squirrel genotypes have adapted to hosts within the Marmotini tribe. Experimental infections of ground squirrel genotypes in tree squirrels and other hosts, as well as attempts to infect ground squirrels with other *Cryptosporidium* spp. are needed to better understand the host range and how host phylogenetics (relatedness) and ecology affect *Cryptosporidium* transmission.

It remains unclear why North American tree squirrels in this and other studies do not appear to carry host-specific *Cryptosporidium*, which is in contrast to North American grounds squirrels, with the exception of eastern chipmunks and groundhogs (Atwill et al., 2001; Atwill et al., 2004; Feng et al., 2007; Pereira et al., 2010; Ziegler et al., 2007). However, a trend can be seen in the geographic ranges of the squirrel species. Eastern gray squirrels, eastern fox squirrels, groundhogs, and eastern chipmunks are generally found east of the Rocky Mountains. American red squirrels are found mainly to the east, but also in parts of the Rocky Mountains, however, the Rocky Mountain populations have not been sampled for *Cryptosporidium*. The Rocky Mountain range could provide a barrier in altitude and habitat, but there are also climate and precipitation differences between the eastern and western United States separated by the 100th meridian. Environmental factors such as precipitation can influence the transmission of infectious and waterborne diseases (Curriero et al., 2001; Patz et al., 2008). The climate to the west of the 100th meridian is arid and rainfall if generally less than 20 inches per year, whereas yearly rainfall is usually greater than 20 inches in areas east of the 100th meridian (Schlenker et al., 2006). *Cryptosporidium* spp. are often transmitted through fecal contaminated water, and studies have reported higher concentrations of oocysts or incidence of cryptosporidiosis with season and higher precipitation (Atherholt et al., 1998; Britton et al., 2010; Naumova et al., 2005). The *Cryptosporidium* ground squirrel genotypes I-IV may be better suited to the more arid environment than other *Cryptosporidium* species.

Especially considering black-tailed prairie dogs rarely drink water, getting their water requirements from ingesting plant material (Lehmer et al., 2001). *Cryptosporidium* found in black-tailed prairie dogs is more likely transmitted through direct contact with feces, which is extremely probable because prairie dogs are highly social animals, and the *Cryptosporidium* spp. not as dependent on waterborne transmission as other *Cryptosporidium*.

A high density of hosts may result in a high prevalence of parasites (Arneberg, 2001; Arneberg et al., 1998); however, we found no correlation between tree squirrel population density and *Cryptosporidium* prevalence. This may be due to the generalist nature of *C. ubiquitum*, *Cryptosporidium* skunk genotype, and *Cryptosporidium* deer mouse genotype III. When a parasite infects a number of sympatric host species, the density of susceptible hosts essentially increases (Seville et al., 1996). Therefore, the prevalence of *Cryptosporidium* spp. found in a broad range of hosts may be better correlated with the combined density of susceptible host species within the vertebrate host community rather than with the density of squirrels alone.

A post-hoc analysis revealed a difference in the prevalence of the three tree squirrel host species combined with time of year. Prevalence increased from 24.1% in the early trapping season (March-June) to 52.4% in the late season (July-October). Others have noted seasonal changes in prevalence of *Cryptosporidium* in other wild North American rodents. Atwill et al. (da Silva et al., 2010) found the prevalence of *Cryptosporidium* in California ground squirrels peaked in June and July, and then sharply declined, which the authors suggested was due to the ground squirrel colonies developing immunity. Kilonzo et al. (Atwill et al., 2004) also noted an increase in *Cryptosporidium* prevalence in the fall in wild rodents (mainly cricetid rodents) from produce and cattle farms. The increase in prevalence in August in the present study could be due to dispersal of young tree squirrels born in the spring. Determining the relationship between *Cryptosporidium* prevalence and season will be helpful to understand *Cryptosporidium* transmission, such as assessing when *C. ubiquitum* is most prevalent in squirrels and how it corresponds to *C. ubiquitum* prevalence in water, livestock, and humans.

This study shows that zoonotic *C. ubiquitum* is common in tree squirrels, and to a lesser extent the zoonotic *Cryptosporidium* skunk genotype, from west central Minnesota and pose a potential public health risk. Host adaptation and host-parasite coevolution is evident in the *Cryptosporidium* ground

squirrel genotypes as only ground squirrel species (tribe Marmotini) were host to these genotypes. This information can be used to assess the role tree squirrels and ground squirrels play in disseminating human-pathogenic parasites and to clarify *Cryptosporidium* systematics and taxonomy.

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GENERAL DISCUSSION

Cryptosporidium is found on every continent and in the major vertebrate groups: amphibians, birds, fish, mammals, and reptiles. As obligate parasites, host biology, behavior, and ecology are likely to affect *Cryptosporidium* evolution and diversification. In addition, *Cryptosporidium* transmission will be influenced by the continuously changing boundaries between humans, livestock, and wildlife. However, the factors affecting *Cryptosporidium* evolution and host-parasite dynamics remain poorly understood. Including how the host range of *Cryptosporidium* taxa is influenced by opportunities to encounter new hosts and the relatedness of new hosts to the major host. These ecological and evolutionary effects have implications for human, livestock and wildlife health (Appelbee et al., 2005; Kloch and Bajer, 2012). To address these gaps, we undertook studies on *Cryptosporidium* ecology in wild populations of rodents in the Cricetidae (voles, muskrats, and *Peromyscus* mice) and Sciuridae (tree and ground squirrels) families. The rodent hosts were chosen as they are ubiquitous, populations and communities can change over relatively short distances and time, host to a number of *Cryptosporidium* taxa, but they are also found in close proximity to humans that may influence the likelihood of cross-species transmission.

Conclusions

The 18S rRNA gene is the most commonly used gene to detect the presence of *Cryptosporidium* and the only gene that has been sequenced for all *Cryptosporidium* species and genotypes. For many genotypes, it is the only gene for which any sequence has been reported. The finding that some 18S rDNA sequences from *Cryptosporidium* are paralogs has serious implications for the use of 18S rDNA to infer species relationships, as paralogs are related by duplication not descent (Buckler et al., 1997; Gabaldón and Koonin, 2013; Koonin, 2005; Sanderson and Shaffer, 2002). Distinguishing paralogous and orthologous relationships in *Cryptosporidium* 18S rDNA is complicated by the lack of tools to study genomes in isolation. Isolating DNA from single sporozoites, which have a haploid genome, can be used to confirm whether heterogeneous 18S rDNA sequences are from paralogs or orthologs (Chapters 2-4; Hill et al., 2008; Le Blancq et al., 1997; Morgan et al., 2001; Xiao et al., 1999). However, this has only recently been successful for *C. andersoni* (Ikarashi et al., 2013). Our approach to detecting paralogs was to: (1) sequencing multiple genetic loci in addition to the 18S rRNA gene; (2) sequencing multiple isolates from individual hosts; and (3) sequencing multiple amplicons from isolates. Using this approach, we found

that paralogs were common in *Cryptosporidium* taxa in cricetid and sciurid hosts, but that the extent of 18S rDNA heterogeneity varied among taxa (Chapters 2-4). The example of *Cryptosporidium* chipmunk genotype II, in particular, illustrates the potential for paralogs to be misidentified as different taxa. Similarly, *Eimeria mivati* and *E. mitis* were thought to be two separate species until both 18S rRNA and mitochondrial cytochrome c oxidase subunit I (COI) genes were sequenced (Vrba et al., 2011).

Studies have shown that parasite communities and host-parasite relationships are affected by host evolutionary and ecological factors including habitat use, population density, species diversity and relatedness. Hwang et al. (Hwang et al., 2010) found that endoparasite communities of red-backed voles (*Myodes gapperi*) varied between forested and burned habitats within a boreal ecosystem. Similarly, helminth communities varied in sympatric striped field mice (*Apodemus agrarius*) and yellow-necked mice (*A. flavicollis*) due to differences in host diet and habitat use (Ondrikova et al., 2010). Both mice species were found in forested areas, but striped field mice also utilized agricultural fields where, the authors suggested, they could be exposed to different parasites (Ondrikova et al., 2010). Cooper et al. (Cooper et al., 2012), found that primate ecology and geographic distribution are important determinants of initial contact between generalist parasites and hosts, and host phylogenetics are important after initial contact, illustrating that without the opportunity to infect another host species, the susceptibility or relatedness of the host is not an important factor. However, once host species boundaries change, opportunity and/or host phylogenetics can affect the host range of parasites.

Bertolino et al. (Bertolino et al., 2003) found that the probability of Eurasian red squirrels (*Sciurus vulgaris*) to be infected with *Cryptosporidium* increased with Eurasian red squirrel density. However, we found *Cryptosporidium* prevalence was not correlated with tree squirrel or vole densities (Chapter 3 & 4). A possible explanation could be related to the taxa of *Cryptosporidium* present in the ecosystem. *Cryptosporidium* taxa vary in their degree of host adaptation or specificity as some are host generalists and others host specialists, but we used presence/absence data because were unable to look at individual *Cryptosporidium* taxa. The prevalence of *Cryptosporidium* adapted to a particular host species may be more likely to correlate with a specific host species density; whereas a generalist host species may correlate better with the entire host community. Therefore, conclusions based on the entire

community of *Cryptosporidium* parasites, which likely includes host generalists and host specialists, could mask differences in the population dynamics of individual taxa.

Determining host use patterns of *Cryptosporidium* taxa will aid in understanding *Cryptosporidium* transmission and identifying potential pathogens. Natural and experimental infections have shown that while some *Cryptosporidium* have a very narrow host range, many *Cryptosporidium* spp. can infect multiple host species. However, distinguishing major (principal) and minor (alternative) hosts can be difficult. Lootvoet et al. (Lootvoet et al., 2013) suggested that *Tracheliastes polycolpus* parasites preferred alternative hosts that were ecologically similar to the major host species, beaked dace (*Leuciscus burdigalensis*), regardless of host phylogenetics. *Cryptosporidium* deer mouse genotype III may illustrate a similar situation because the genotype is found in eastern grey squirrels (*Sciurus carolinensis*), American red squirrels (*Tamiasciurus hudsonicus*), and deer mice (*Peromyscus* spp.), but not in other cricetid rodents (Chapters 3 & 4; Feng et al., 2007). Deer mice are found in forested habitats, which they can share with squirrels, and prairie habitats, which they can share with meadow voles. However, despite deer mice being more closely related to meadow voles than squirrels, meadow voles do not host *Cryptosporidium* deer mouse genotype III. We identified *Cryptosporidium* deer mouse genotype III in 16.7% (6/36) and 22.7% (5/22), respectively, of genotyped tree squirrels and deer mice. Similarly, Feng et al. (Feng et al., 2007) found a similar prevalence of *Cryptosporidium* deer mouse genotype III in grey squirrels (42%; 5/12) and deer mice (37%; 21/37). Based on these data, it is not clear whether deer mice or squirrels are the preferred hosts for *Cryptosporidium* deer mouse genotype III. It is possible that squirrels and deer mice may host different subtypes of *Cryptosporidium* deer mouse genotype III. To test this hypothesis, it would be necessary to target more polymorphic loci, such as the gp60 gene. Designing PCR primers to target gp60 and other polymorphic loci is complicated by the lack of genomic sequence information for *Cryptosporidium* deer mouse genotype III.

We found that the extent of host adaptation and coevolution varied between different *Cryptosporidium* taxa, which may influence the risk of *Cryptosporidium* taxa to emerge as potential human pathogens. By using a multi loci approach, two *Cryptosporidium* species and at least 20 genotypes were identified in 13 rodent host species. Although the diversity (number of taxa) and prevalence of *Cryptosporidium* was quite high, very few of the *Cryptosporidium* species and genotypes

found are known human pathogens. The main exceptions were *Cryptosporidium* skunk genotype and *C. ubiquitum*, which were only found in tree squirrels. Subtyping of the gp60 gene revealed that the subtypes carried by tree squirrels have been responsible for human disease and water contamination (Li et al., 2014). Based on phylogenetic analyses, *Cryptosporidium* deer mouse genotype III & IV infecting tree squirrels and *Peromyscus* mice and just *Peromyscus* mice, respectively, are related to human-pathogenic *Cryptosporidium* and may eventually pose a threat human health. Particularly deer mouse genotype III as it does appear to have a broadening host range.

Recommendations for Future Work

Data from more loci and a greater number of isolates are needed to increase the accuracy of the *Cryptosporidium* species tree, which, in turn, will increase our ability to identify paralogous relationships (i.e. those that do not match the species trees). The current approaches to detecting paralogy in *Cryptosporidium*, including our approach and the single genome approach applied by Ikarashi et al. (Ikarashi et al., 2013), have limitations in detecting certain types of paralogs, such as pseudoorthologs, which are paralogs that appear to be orthologous because of differential, lineage-specific gene loss (Koonin, 2005).

The genomic organization of *Cryptosporidium* 18S rDNA may be important in explaining why copies are not divergent in some taxa (e.g. *C. tyzzeri*), and highly divergent in other taxa (Chapter 2) (Kváč et al., 2013). Within the microsporidian genus *Nosema*, the variant 18SrDNA copies in *N. bombycis* are dispersed across multiple chromosomes; in contrast, the non-variant 18S rDNA copies in *N. apis* are tandemly arrayed (Gatehouse and Malone, 1998; Iiyama et al., 2004; Liu et al., 2008). Ironside (Ironside, 2013) proposed that *Nosema* species undergo both birth-and-death and concerted evolution. Apart from one study on *C. parvum* (Le Blancq et al., 1997), little is known about the genomic organization of 18S rDNA in *Cryptosporidium,* and this gap in knowledge should be addressed in future studies.

It will be important for future research to determine the expression of heterogeneous 18S rDNA paralogs in *Cryptosporidium,* and the conditions under which the paralogs are expressed. For example, our data suggest *Cryptosporidium* chipmunk genotype II Types A and B are both functional, but we did not quantify expression (Chapter 2). One hypothesis is that these Types have adapted to different conditions allowing the *Cryptosporidium* to survive in hibernating chipmunks. This could be tested in

laboratory studies with hibernating and non-hibernating hosts. Determining the expression and functional significance of paralogous gene copies is important for understanding *Cryptosporidium* biology and evolution, and this information could be useful in the development of novel control strategies for cryptosporidiosis.

Our data suggest that wild rodents can be used as models for studying how evolution and ecology influence the expansion of the *Cryptosporidium* host range. A combination of natural infections, controlled experimental infections, and wider sampling of sympatric host species, and allopatric but related hosts are needed to determine how host habitat use, host species distribution, and the evolutionary relationships of the hosts have shaped specific *Cryptosporidium*-host relationships. This information will not only lead to a better understanding of basic *Cryptosporidium* biology and evolution, but also identify factors leading to the emergence of pathogenic *Cryptosporidium* in humans and other hosts.

Summary

Our data show that the extent of host adaptation varies among *Cryptosporidium* lineages in wild rodents and that caution should be used when inferring evolutionary relationships from 18S rRNA gene sequences. An understanding of both host and parasite ecology is essential to clarify *Cryptosporidium* taxonomy and understand the basic biology and evolution of *Cryptosporidium* parasites. Future research needs to take an interdisciplinary approach to study all levels of interactions and movement of *Cryptosporidium*, from molecules to the ecosystem level.

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APPENDIX. PUBLICATIONS

Peer Reviewed Publications

Stenger, B.L.S., Clark, M.E., Kváč, M., Khan, E., Giddings, C.W., Dyer, N.W., Schultz, J.L., McEvoy, J.M. 2015. Highly divergent 18S rRNA gene paralogs in a *Cryptosporidium* genotype from eastern chipmunks (*Tamias striatus*). Infection, Genetics, and Evolution 32: 113-123.

Kváč M, McEvoy J, Loudová M, Stenger B, Sak B, Květoňová D, Ditrich O, Rašková V, Moriarty E, Rost M, Macholán M, Piálek J. 2013. Coevolution of *Cryptosporidium tyzzeri* and the house mouse (*Mus musculus*). Int J Parasitol 43:805-817.

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Rašková V, Květoňová D, Sak B, McEvoy J, Edwinson A, Stenger B, Kváč M. 2013. Human cryptosporidiosis caused by *Cryptosporidium tyzzeri* and *C. parvum* isolates presumably transmitted from wild mice. J Clin Microbiol 51:360-362.

Book Chapter

Kváč M, McEvoy J, Stenger B, Clark M. 2014. Cryptosporidiosis in Other Vertebrates, p. 237-323. In Cacciò SM, Widmer G (ed.), Cryptosporidium: parasite and disease. Springer Vienna, Vienna, Austria.