

Assessing *Babesia microti* Sensu Lato in Small Mammals in Northeast Florida

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Abstract

***Babesia microti* is the causative agent of human babesiosis. Black-legged ticks (*Ixodes scapularis*) are the only proven vector and white-footed mice (*Peromyscus leucopus*) serve as the main reservoir in northeastern United States. The aim of the present study was to determine if *B. microti* is present in small mammals in southeastern United States and to compare strains found in this study to reference strains from around the world. Blood samples were obtained from cotton mice, cotton rats, a flying squirrel, golden mice, rice rats, Virginia opossums, wood rats, and raccoons in northeastern Florida. DNA was extracted from the blood, and portions of the *B. microti* DNA (from 18S and β -tubulin genes) were amplified via nested PCR assays. Of the eight species tested, only cotton rats and raccoons tested positive for *B. microti*. Representative samples of the amplified DNA from these two species were sequenced and compared phylogenetically to reference strains of *Babesia* species. The *B. microti* found in the cotton rats most closely resembles *B. microti* sensu stricto, which is pathogenic to humans, and the *B. microti* found in the raccoons most closely resembles other raccoon strains of *B. microti*.**

Introduction

Victor Babes first described *Babesia* species in 1888. A few years later Smith and Kilbourne demonstrated ticks as the vector for *Babesia* (1893). *Babesia microti* is an intraerythrocytic protozoan that causes human babesiosis. Human Babesiosis is often asymptomatic or results in mild, malaria-like symptoms within weeks after infection (Homer et al. 2000). Symptoms typically include fatigue, headache, nausea and chills, but also include many others (Boustani & Gelfand 1996, Homer et al. 2000). Clinical signs include fever, anemia, jaundice, and dark urine (Homer et al. 2000, Krause 2003). Symptoms can be more severe, even leading to death in humans who are asplenic, immunodepressed or very old (Boustani & Gelfand 1996, Homer et al. 2000, Krause 2003). Due to nonspecific symptoms and coinfection with other tick-borne diseases, namely Lyme disease, human babesiosis is often misdiagnosed as the flu and, therefore underreported (Sherr 2005).

Blood smears, hamster inoculation, and indirect (immuno)fluorescent antibody tests (IFATs) are useful for diagnosing human babesiosis (Boustani & Gelfand 1996, Homer et al. 2000, Krause 2003). Polymerase Chain Reaction (PCR) assays also provide an easy and sensitive diagnostic tool, especially in cases of low parasitemia (Persing et al. 1992, Krause et al. 1996). According to Persing et al. (1992), PCR amplification and gel electrophoresis methods are sensitive enough to detect as few as three organisms when using an extra chemiluminescent probe. The primers used for PCR amplification of *B. microti* commonly target the 18S and β -tubulin genes (Goethert and Telford 2003, Zamoto et al. 2004), and phylogenetic trees can be created to characterize the amplified *B. microti* target sequences.

The only proven vector of *B. microti* in the United States is the black-legged tick (*Ixodes scapularis*). Black-legged ticks advance through three life stages in their two-year life span. Three-legged larvae emerge about a month after eggs are laid in the spring. The larvae feed once in the summer and can potentially acquire infection of *B.*

microti (Boustani and Gelfand 1996). The fed larvae then molt into nymphs in the following summer and feed usually between May and July of the second year (Spielman et al. 1985, Piesman et al. 1987, Boustani and Gelfand 1996). In fact, transmission of *B. microti* occurs most frequently in May and June, indicative of the importance of nymphal ticks as vectors of *B. microti* in northeastern United States (Spielman et al. 1985, Piesman et al. 1987, Mather et al. 1996). This is mainly because the small size of the nymphs makes it harder to notice on the body. Moreover, since both the larval and nymph stages feed on small mammals, and nymphs retain pathogens from the larval stage, the nymph can infect small mammals that serve as hosts for the next generation of larvae (Boustani and Gelfand 1996). The nymphs molt into adults and feed once more in the fall of the second year. The adults detach from the hosts, the females lay eggs, and the adults die (Spielman et al. 1985).

Small to large mammals serve as hosts for black-legged ticks in the eastern United States, including Florida, which allows the opportunity for transmission of *B. microti*. Although larval and nymphal tick hosts are often small rodents, typically mice, they can attach to larger mammals like raccoons as well (Spielman et al. 1985, Piesman et al. 1987, Boustani and Gelfand 1996). Lizards also serve as primary hosts for larval and nymphal stages (Spielman et al. 1985). The adult ticks typically feed on deer, thus deer density may be a limiting factor on tick abundance. Small mammal hosts for black-legged ticks in Florida include the cotton rat, flying squirrels, golden mice, cotton mice, wood rat, and rice rats. In Florida, deer, raccoons, opossums, and other medium-sized mammals serve as hosts for adult ticks. Once infected, mammals serve as reservoirs for *B. microti* but are not severely affected (Homer et al. 2000). Although mammals can pass *B. microti* vertically, this rarely occurs (Krause 2003). Mammals cannot transmit *B. microti* horizontally because the ticks are a necessary part of the *B. microti* life cycle (Homer et al. 2000). In addition to tick transmission, humans can acquire infection via blood transfusions (Krause 2003). To date, *B. microti* has not been reported from ticks or wild vertebrates in Florida.

The purpose of this study was to determine the presence of *B. microti* in mammals trapped in northeast Florida and to compare the relationship of the *B. microti* strains found in this study to reference strains around the world. This study presents the first published evidence of the presence of *B. microti* in wild vertebrates in northeast Florida as well as evidence for the possibility of a vector other than the established black-legged tick. This information can help determine the risk of human exposure to *B. microti* and enhance practice of preventive precautions against tick bites.

Materials and Methods

Study sites. Mammals were trapped at three locations. One site, Talbot Islands Geological Park (TIGP), is located in northeast Duval County, Florida. Most of the island is typical salt marsh, flooding often with spring tides. Another site, the Guana River State Park and Wildlife Management Area (GRSPWMA), is located 30 miles south of Jacksonville and is dominated by live oak and other species typical of maritime hammock habitat, pine flatwoods, and abundant saw palmetto understory. The third site, the University of North Florida Wildlife Sanctuary (UNFWS), is located on the UNF campus in southeast Jacksonville and also has mixed hardwood wetlands, pine flatwoods, and saw palmetto.

Sample collection and storage. Blood was collected from nine raccoons trapped during a study by Dr. Joe Butler, UNF Department of Biology. His research was studying the effect of raccoon removal on the nesting and hatching success of diamondback terrapins, and was located on an island within TIGP (unpublished). Raccoons were trapped from March to May of 2005 and on an as-needed basis thereafter. Ten Tomahawk #108 live traps were baited with sardines and sometimes peanut butter. Once the raccoons were anesthetized using ketamine, a 2-3 mL sample of whole blood was collected. Most of the nine samples were collected from a heart stick using an 18G needle. Each sample was inserted into two vacuum sealed tubes, with one of the tubes containing EDTA anticoagulant. The samples were stored on ice until they were brought back to the lab, where a few drops from each whole blood sample were absorbed into Nobuto filter paper strips (Advantec MFS Inc., Pleasanton, Calif.) and allowed to air dry. The whole blood samples and Nobuto strips were stored in the refrigerator (0-4°C).

The blood samples from the rest of the mammals were collected previously by Dr. Kerry L. Clark, UNF Department of Public Health. Eight raccoons were trapped by Dr. Clark at TIGP again using Tomahawk traps. The blood samples were collected and stored in the same fashion as just described. The other seven rodent species were trapped at GRSPWMA and UNFWS. The rodents were trapped from April to September of 1999 and from July to September of 2000 using live Sherman traps baited with wild bird seed. Once anesthetized using ketamine, each rodent had their tail clipped, and a sample of about 100µl of blood was absorbed onto a Nobuto strip. As with the samples from the raccoons, the Nobuto strips were allowed to air dry and stored refrigerated (5°C).

Microscopic examination for Babesia microti. A few drops of the whole blood from the raccoons trapped at TIGP in 2005 was also used to create thin blood smear slides, which were allowed to air dry, fixed with methanol, and then stained with geimsa. The slides were examined for ring and cross forms, characteristic of *Babesia* piroplasms, under oil immersion and pictures were taken using a digital camera.

DNA extraction. DNA was extracted from the Nobuto strips within a class II biological safety cabinet (NuAire, Plymouth, Minn.) utilizing standard precautions to prevent contamination, including using aerosol barrier filter pipette tips. The Masterpure Complete DNA Purification Kit (Epicentre Technologies Inc., Madison, WI) was used with slight modifications of the manufacturer's protocol for whole blood samples. A 5mm x 5mm slice of blood-soaked Nobuto strip from each sample was incubated overnight at 55°C in a microtube with 600µl of Red Cell Lysis Solution. After centrifuging the microtubes, the supernatant was discarded from each microtube, and 300µl of 1× Tissue and Cell Lysis Solution and 10.0µl (200µg) of Proteinase K were added to the pelleted Nobuto piece and cells. Then the samples were vortexed and incubated again overnight at 55°C. The supernatant was transferred to a clean microtube to discard the Nobuto piece. Then 200µl of MPC protein precipitate reagent was added to the samples, which were then vortexed, chilled, and centrifuged to pellet the cell proteins. The supernatant was transferred to new microtubes and the DNA was precipitated with 600µl of ice cold 100% 2-propanol and 20µg of glycogen. The DNA was rinsed once with 500µl of 75% ethanol and then once with 500µl of 100% ethanol. Residual ethanol was removed and the samples were allowed to air dry. Finally, the samples were resuspended with 50µl of TE buffer and stored in the freezer (-20°C).

DNA amplification and visualization. The pipettes, pipette tips, and PCR tubes were exposed to UV light within the PCR clean cabinets (CleanSpot workstation [Coy Laboratory Products, Grass Lake, Mich.] or PCR workstation [CBS Scientific, Del Mar, Calif.]) prior to setup. Nested polymerase chain reactions (PCR's) were used to amplify *B. microti* specific gene fragments. Samples were tested using several sets of primers. One set of primers specific to the 18S rRNA gene included BAB1, BAB2, BAB3, and BAB4 (Zamoto et al. 2004) and was used to first screen all samples for *B. microti* sensu lato. Another three primers targeting the 18S rRNA gene were used to amplify larger fragments of the gene for sequence analysis. These were RLB-R, 247Fa, and 1038 (Nagore et al. 2004). Another primer set specific to the β -tubulin gene included UStubu127F, UStubu161F, UStubu1609R, and UStubu1637R (Zamoto et al. 2004). Sterile water was used as a negative control and a previously positive sample from a cotton rat was used as a positive control for each PCR.

Samples were primarily amplified using 20 μ l of master mix (Hot Master Mix, Eppendorf), 20 μ l of deionized H₂O, 2.5 μ l of each primer, and 5 μ l of the extracted DNA for a total volume of 50 μ l. Reactions were carried out in an automated DNA thermal cycler (Geneamp PCR System 2400 [Perkin-Elmer, Norwalk, Conn.] or PTC 200 [MJ Research, Watertown, Mass.]). The initial denaturation occurred at 95°C for 1 minute, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 65°C for 45 seconds. Samples were secondarily amplified using the same reaction mixture as the primary reaction, except that 1 μ l of the primary reaction product was used for the DNA template, but the reaction mixture still totaled 50 μ l. The secondary reactions included 30 cycles with the same parameters as the primary reaction.

A small sample (5 μ l) of the amplified gene fragments was loaded with dye into wells of a 2% agarose gel. After electrophoresis and staining with ethidium bromide, amplified fragments were visualized using ultraviolet illumination.

DNA sequence analysis. To remove primer dimers and other nonspecific amplification by-products, the samples were purified using the Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and sequenced at Seqwright (McCombie et al. 1992). These sequences were compared to sequences from reference strains obtained by using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) to search the GenBank database (National Center for Biotechnology Information) and compared to each other via the BLAST 2 Sequences (Tatusova and Madden 1999). The GenBank accession numbers for the reference strains used for the 18S rRNA gene target in this study include: AY693840 *Human (Gray Strain)*, BMU09833 *House Mouse*, AB190435 *Field Mouse (Japan)*, AB085191 *Bank Vole (Germany)*, AF231349 *Castor-bean Tick (Germany)*, AB241632 *White-bellied Rat (China)*, AB112050 *Field Rat (Taiwan)*, AY789075 *Field Vole (Poland)*, AY534602 "*Spanish Dog*", AB197940 *Raccoon (Japan)*, AB049999 *Babesia rodhaini*, AY452708 *Babesia leo*, and AY452698 *Babesia felis*. The GenBank accession numbers for the reference strains used for the β -tubulin gene target in this study include: AB083377 *Gray Strain*, AY144722 *Black-legged Tick (Massachusetts)*, AY144725 *Human (Connecticut)*, AY144723 *Shrew (Maine)*, AY144718 *Bank Vole (Russia)*, AY144726 *Castor-bean Tick (Switzerland)*, AY144716 *Bank Vole (Maine)*, AY144714 *Vole (Montana)*, AY144710 *Vole (Alaska)*, AY144709 *Dog (Spain)*, AY144708 *Raccoon (Massachusetts)*, AF546902 *Skunk (Massachusetts)*,

AY144707 *Fox (Massachusetts)*, and AB083442 *Babesia rodhaini*. The sequences obtained in this study and the sequences from the reference strains obtained via GenBank were aligned using Clustal X (Thompson et al. 1997). After editing, ~820 nucleotides of the 18S rRNA and ~715 nucleotides of the β -tubulin gene were compared respectively. The tree-building program MEGA version 2.1 (Kumar et al. 2001) was used to create neighbor-joining (Saitou & Nei 1987) and maximum parsimony (Swofford et al. 1996) phylogenetic trees for both the 18S rRNA and the β -tubulin gene sequences. Bootstrap analysis (Felsenstein 1985) values based on 1,000 replicates were included to estimate node reliability of the trees obtained with each method.

Results

Eight species of mammals were trapped and sampled, for a total of 83 mammals. The results of the PCR amplifications from the samples are shown in Table 1. Only the blood samples from cotton rats (*Sigmodon hispidus*) and raccoons (*Procyon lotor*) amplified the *B. microti* 18S rRNA or β -tubulin specific gene targets per visualization in agarose gels. To confirm that the gene targets were from *B. microti*, the amplified samples were sequenced, and the sequences were identified as *B. microti* strains via BLAST searches. Additionally, intraerythrocytic structures typical of *Babesia* were seen in the raccoon blood smears. Although the resolution made determining the shape of these structures difficult, there was at least one discernable structure (Figure 1) that morphologically resembled the ring form of *B. microti*.

Table 1 Summary of PCR results for *Babesia. microti* in small mammals collected in northeast Florida (1999-2000, 2005). Number of PCR positive animals/Number tested of each species^a

County	Site ^b	Cotton mouse	Cotton rat	Flying squirrel	Golden mouse	Raccoon	Rice rat	Wood rat	Virginia opossum	Total
Duval	TIGP	--	--	--	--	15/17	--	--	--	15/17
	UNFWS	0/30	2/2	0/1	0/2	--	--	--	0/1	2/36
St. Johns	GRSPWMA	0/10	6/13	--	0/1	--	0/3	0/2	0/1	6/30
Total		0/40	8/15	0/1	0/3	15/17	0/3	0/2	0/2	23/83

^aCotton mouse (*Peromyscus gossypinus*), Cotton rat (*Sigmodon hispidus*), Flying squirrel (*Glaucomys volans*), Golden mouse (*Ochrotomys nuttalli*), Raccoon (*Procyon lotor*), Rice rat (*Oryzomys palustris*), Wood rat (*Neotoma floridana*), Virginia opossum (*Didelphis virginianus*)

^bTIGP = Talbot Islands Geological Park; UNFWS = University of North Florida Wildlife Sanctuary; GRSPWMA = Guana River State Park and Wildlife Management Area

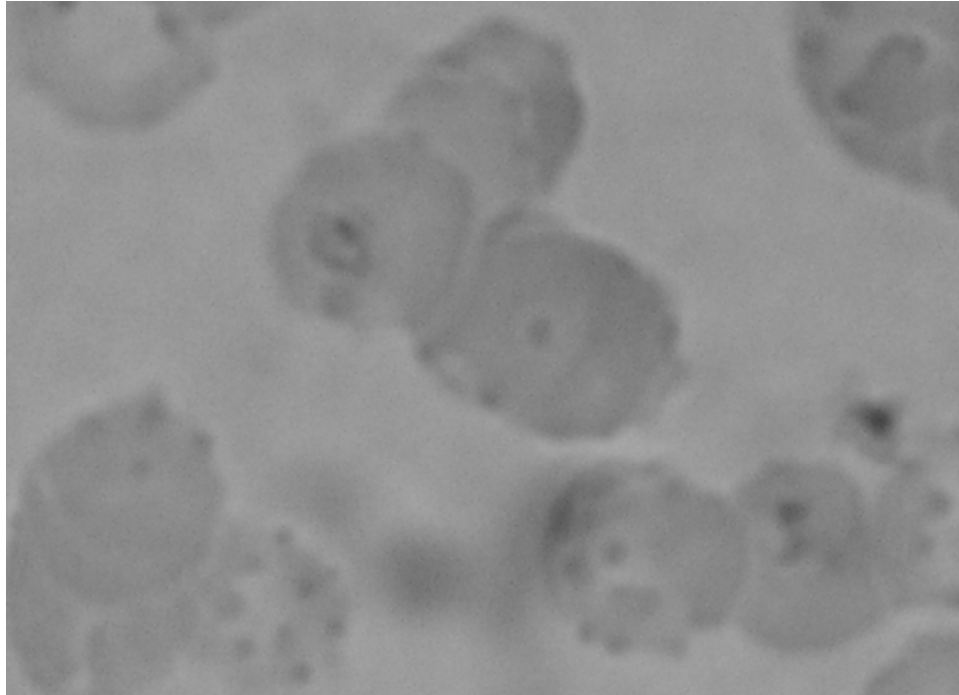
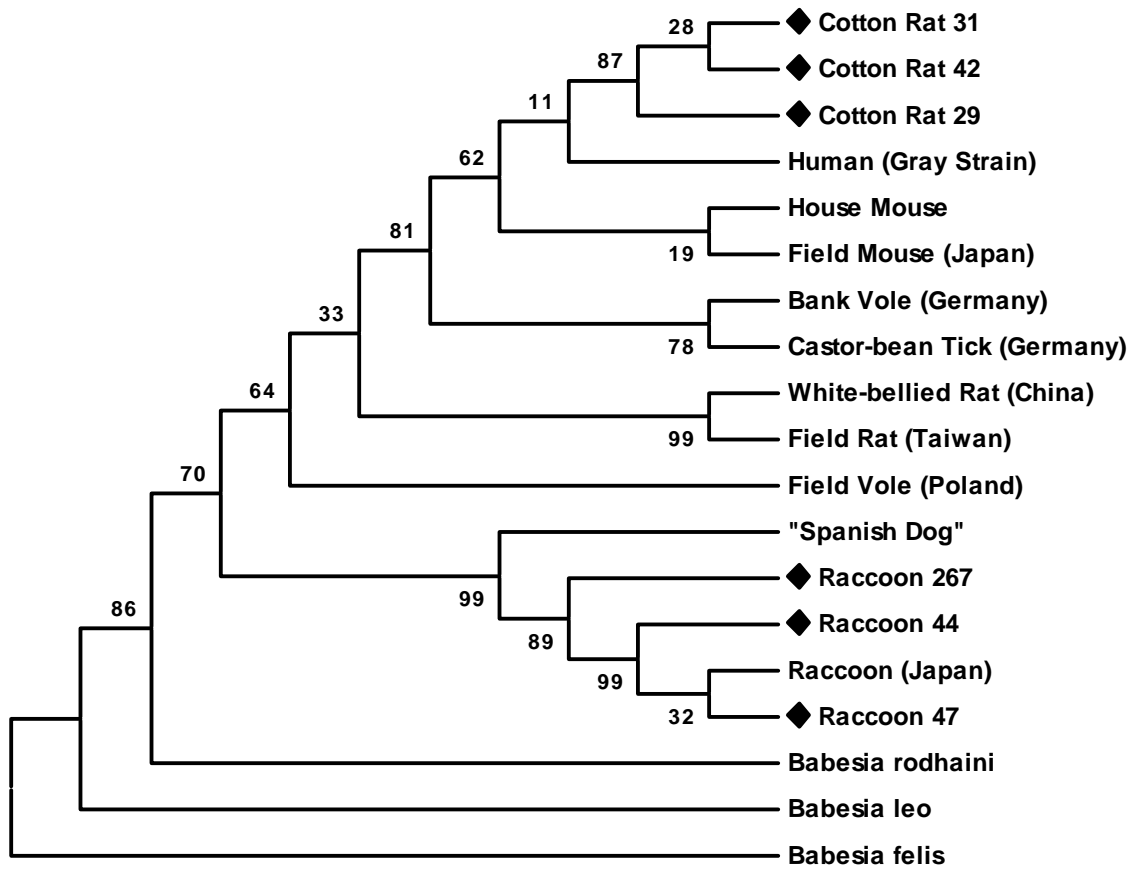


Figure 1 Ring-like structure, characteristic of *B. microti*, inside red blood cells. The blood smear, from a raccoon from Talbot Islands in 2005, was stained using geimsa and viewed under oil immersion at 1,000x.

Phylogenetic analysis of the 18S rRNA gene by maximum parsimony shows a branching of the *B. microti* sequences found in the cotton rats and the sequences found in the raccoons into separate clades (Figure 2A). This separation is more clearly seen in the neighbor-joining method when looking at the relatively long branch distance between cotton rat strains and raccoon strains as opposed to between cotton rat strains themselves (Figure 2B). According to the BLAST 2 Sequence comparison, this difference was 6% between the cotton rat 29 and Florida raccoon 47 18S rRNA sequences for example. Both phylogenetic trees also show a clustering of *B. microti* sequences from the cotton rats with sequences from other rodents, a castor-bean tick, and a human, as well as a clustering of *B. microti* sequences from the Florida raccoons with a sequence from the Japan raccoon and the “Spanish dog” (Figure 2A & B). More specifically, the *B. microti* sequence from the cotton rat 29, for example, was 99% similar to the human pathogenic gray strain sequence (accession number AY693840), 99% similar to the sequence from the house mouse (accession number BMU09833), and 99% similar to the sequence from the castor-bean tick from Germany (accession number AF231349) according to BLAST pairwise distances. The sequence from Florida raccoon 47 was 99% similar to the sequence from the Japan raccoon (accession number AB197940) and 97% similar to the sequence from the “Spanish dog” (accession number AY534602).

(A)



(B)

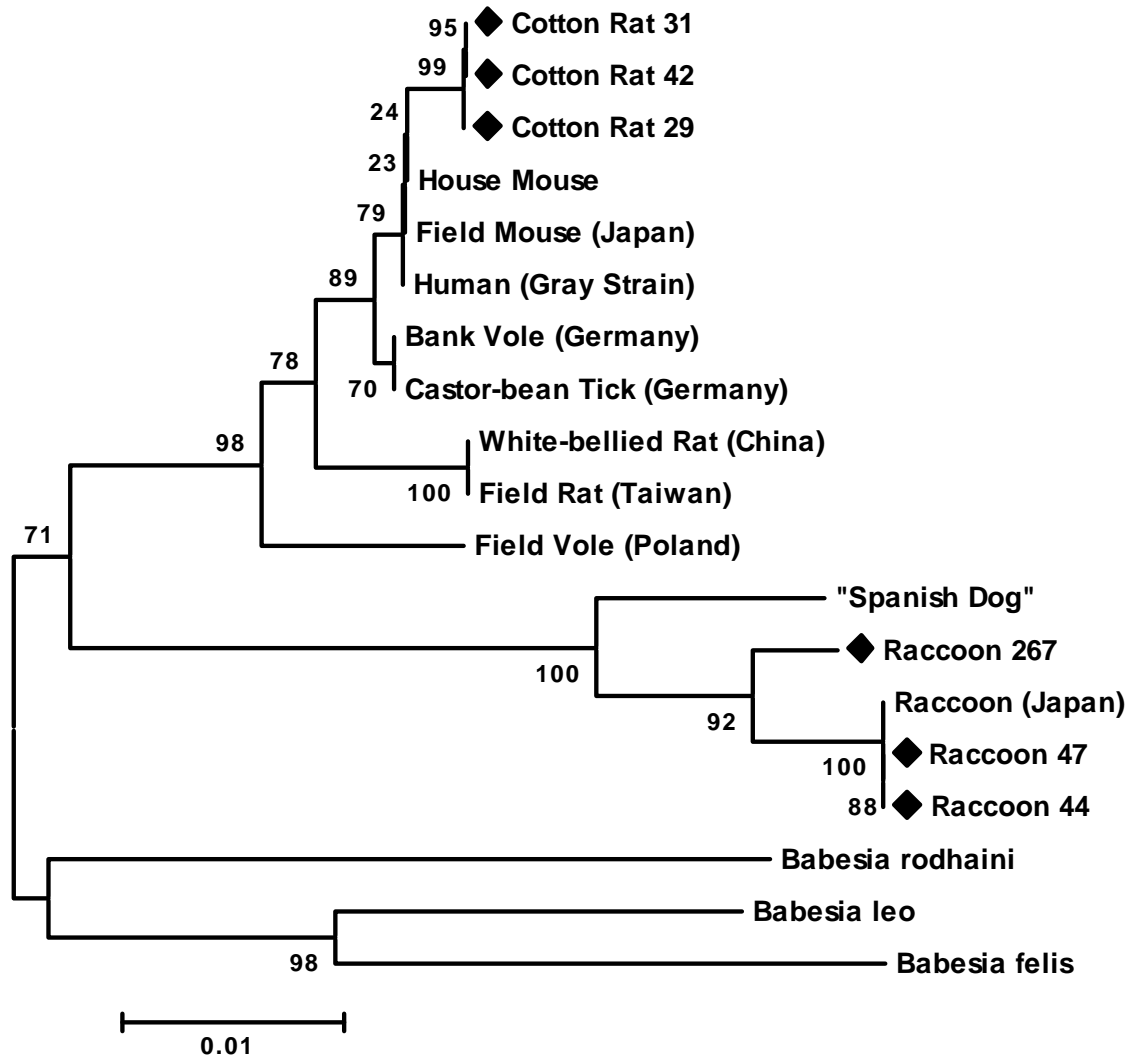
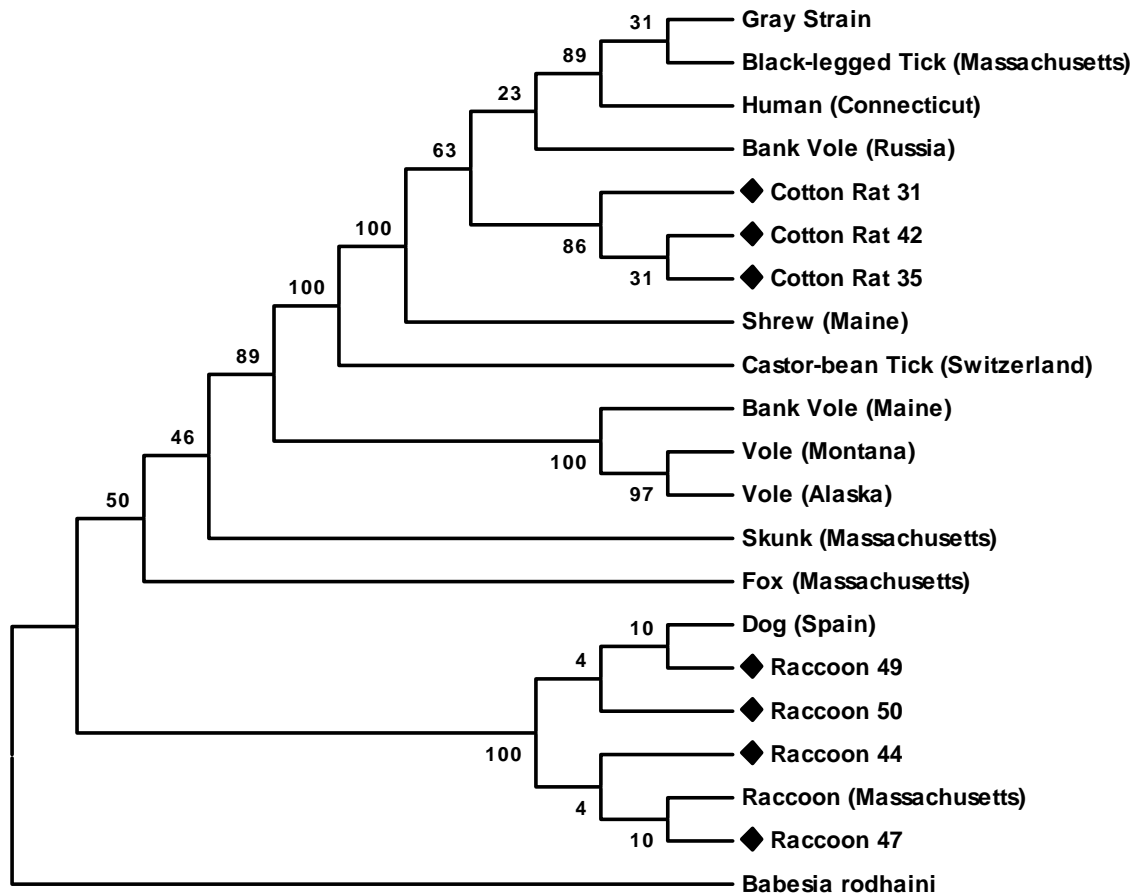


Figure 2 Unrooted phylogenetic trees based on a comparison of *Babesia microti* 18S rRNA sequences obtained from Florida small mammals (denoted by the diamonds) with other *Babesia microti* sensu lato species. *Babesia rodhaini*, *Babesia leo*, and *Babesia felis* were included as outgroups. Bootstrap values, as percentages of 1,000 replicates, are shown at the branch nodes. Shown are phylogenetic trees created by (A) maximum parsimony and (B) neighbor-joining methods.

Although somewhat different, the phylogenetic trees based upon the analysis of the *B. microti* β -tubulin gene (Figure 3) show roughly the same clustering as seen in Figure 2. The *B. microti* sequences from the cotton rats again branch into a separate clade from the sequences from the raccoons (Figure 3A & B). According to the BLAST 2 Sequence comparison, there was a difference of 18% between the cotton rat 31 and Florida raccoon 50 β -tubulin sequences for example. Also, the sequences from the cotton rats are still clustered with the sequences from the pathogenic gray strain, other rodents, as well as the black-legged tick, while the sequences from the raccoons are still clustered with the sequence from the "Spanish dog" as well as with a sequence from another

raccoon (Figure 3A & B). The neighbor-joining tree in Figure 3B shows the similarities within these clades more clearly as does the BLAST pairwise distances. The sequence from cotton rat 31 is 98% similar to the sequence from the human from Connecticut (accession number AY144725), 98% similar to the sequence from the shrew from Maine (accession number AY144723), and 98% similar to the sequence from the black-legged tick from Massachusetts (accession number AY144722). The sequence from Florida raccoon 50 is 98% similar to the sequence found in the Massachusetts raccoon (accession number AY144708) and 98% similar to the sequence found in the dog from Spain (accession number AY144709).

(A)



(B)

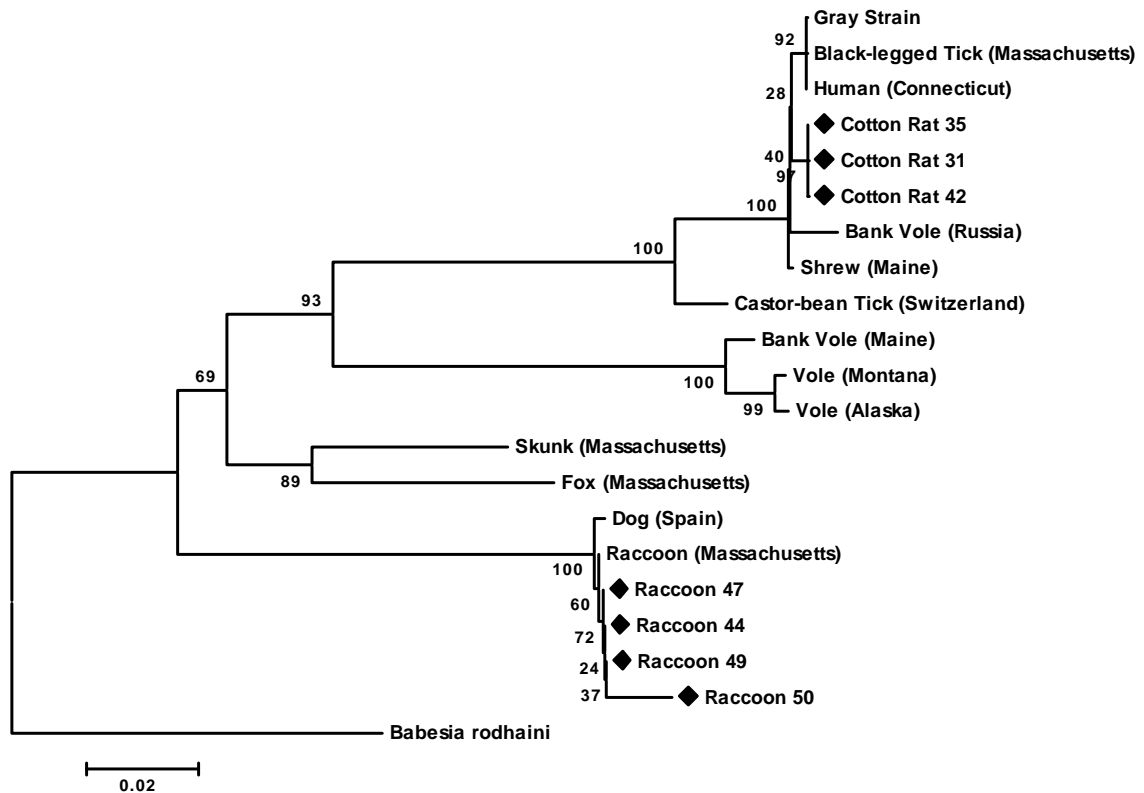


Figure 3 Unrooted phylogenetic trees based on a comparison of *Babesia microti* β -tubulin sequences obtained from Florida small mammals (denoted by the diamonds) with other *Babesia microti* sensu lato species. *Babesia rodhaini* was included as an outgroup. Bootstrap values, as percentages of 1,000 replicates, are shown at the branch nodes. Shown are phylogenetic trees created by (A) maximum parsimony and (B) neighbor-joining methods.

Discussion

It is clear that *B. microti* is present in small mammals, specifically raccoons and cotton rats, in northeast Florida. Sequence analysis of the amplified 18S rRNA and β -tubulin gene fragments confirmed the presence of *B. microti*. Additionally, the ring form of *B. microti* microscopically visualized from blood smears morphologically resemble the ring form of *B. microti* described by Homer et al. (2000). It is difficult to determine from morphology whether or not the intraerythrocytic organism is indeed *B. microti* as opposed to the morphologically similar *Plasmodium*. However, the sequence analysis of the amplified samples established the presence of *B. microti* in cotton rats and raccoons. Therefore, it is very likely that the ring forms were in fact *B. microti*. The presence of *B. microti* in small mammals in northeast Florida is not surprising because the only proven vector, the black-legged tick, is common and abundant in northeast Florida. Plus, *Babesia* species have been found in raccoons in Florida (Schaffer et al. 1978) and even in Duval County (Telford and Forrester 1991).

The phylogenetic results show a significant variation between the *B. microti* strains from the cotton rats and strains from the raccoons. The strains found in the cotton rats in this study cluster with strains found in other small rodents, such as mice, rats, and

voles (Figures 2 and 3). At the same time, strains found in the raccoons in this study cluster with other raccoons and a dog (Figures 2 and 3), which coincides with the 18S and β -tubulin phylogenetic trees by Goethert and Telford (2003). This difference between the strains from the cotton rats and raccoons raises the question of why these strains seem to be host specific. It is possible that horizontal or vertical transmission (Krause 2003) play a role in transmission of *B. microti*, but these modes of transmission are rare and not well studied. Another explanation could be the existence of a different tick vector for each mammal species or group (e.g. rodents versus carnivores). This is possible because ticks can be host specific themselves, thereby leading to a host specific *B. microti*. For example, Fish and Dowler (1989) found adult black-legged ticks on medium-sized mammals in general (although most abundant on opossums), while American dog ticks (*Dermacentor variabilis*) and raccoon ticks (*Ixodes texanus*) were most abundant on raccoons. This could explain why the strains from the cotton rats cluster with the black-legged tick and the strains from the raccoons do not. Perhaps the black-legged tick is host specific for the cotton rats and some other tick, such as *I. texanus*, is host specific for the raccoons.

Another question raised by the separate grouping of the strains from the cotton rats and from the raccoons is about the pathogenicity of the strains. Note that the strains from the cotton rats cluster with the grey strain from humans, while the strains from the raccoons do not (Figures 2 and 3). This suggests that the strains from the cotton rat may possibly be pathogenic to humans. If the strains from the cotton rats were pathogenic to humans, it is possible that these strains pose a risk to humans in the study area, since black-legged ticks do bite humans there. However, conclusions about pathogenicity cannot be made without further tests.

One weakness of this study is the small sample size. Although there were 83 animals trapped, there were few of each individual species. For example, only one flying squirrel and two Virginia opossums were tested. It is possible that more species of mammals are infected with *B. microti*. In fact, the two species that were collected the most in this study were the ones that tested positive for *B. microti*. So in the future, a larger sample size is highly recommended. Furthermore, it is also recommended to test more than just the eight species of mammals trapped in this study. This would give a better understanding of the prevalence of *B. microti* in northeast Florida.

Another recommendation for future studies would be to test various tick species as possible vectors for *B. microti*. As suggested in this study, it is possible that another vector besides the black-legged tick plays a role in *B. microti* transmission in the study area. It is recommended to first test different tick species for the presence of *B. microti*. Then test these species for their ability to acquire, maintain, and transmit the organism. This is particularly important to determine because several human biting species are common in the area, including Lone Star ticks (*Amblyomma Americanum*), American dog ticks, and black-legged ticks. These ticks feed on either rodents, raccoons, or both, and could potentially become infected with and transmit *Babesia* to humans.

Although the vector is not known in this region, this study confirmed the presence of *B. microti* in small mammals in northeast Florida, which demonstrates the presence of a possible human pathogen in natural hosts in the area. Secondly, this study demonstrated the possibility of different tick-host life cycles for the different phylogenetic groups of *B. microti*. In conclusion, this pilot study has shown the need for

further research on the ecology of *B. microti* in order to more accurately assess human risk. These future studies should address the natural reservoir hosts, tick vectors, vector competence, prevalence of infection, and genetic variation of *B. microti* in the Southeast.

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