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FAY AND RAUSCH 1969 REVISITED: *BABESIA MICROTI* IN ALASKAN SMALL MAMMALS

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ABSTRACT: The Holarctic distribution of *Babesia microti* within small rodents implies an ancient association. A seminal report of piroplasms in Alaskan voles suggested to us the possibility that *B. microti* entered North America within Eurasian microtine rodents dispersing through Beringian corridors. To test this hypothesis, we analyzed samples from Alaskan rodents by polymerase chain reaction for evidence of infection with *B. microti*; one-third of the rodents were found to be infected. Sequence analysis of the 18S rDNA gene demonstrates that Alaskan *B. microti* comprises a clade that infects microtines in several sites across North America and is distinct from a clade that is zoonotic.

Human babesiosis caused by *Babesia microti* was first described from sites along the northeastern United States terminal moraine (Spielman et al., 1985) and, later, from Minnesota and Wisconsin (Steketee et al., 1985). Until recently, cases of *B. microti* infection have remained limited to these areas, although this piroplasm was first described from microtine rodents in Portugal (Franca, 1910) and seems widely enzootic in Europe (Young, 1970; Krampitz and Baumler, 1978). Literature reports suggest, however, that *B. microti* has a Holarctic distribution, with infected rodents found in Russia (Telford et al., 2002), Japan (Shiota et al., 1984; Saito-Ito et al., 2000), Taiwan (van Peenen et al., 1977), California (Wood, 1962; van Peenan and Duncan, 1968), Wyoming (Watkins et al., 1991), Colorado (Burkot et al., 2000), Wisconsin/Minnesota (Steketee et al., 1985), upstate New York (Kirner et al., 1958), and much of western Europe. As with certain helminths, such a distribution suggests the hypothesis of an origin of the agent within Eurasia and introduction into North America via the Bering Land Bridge with microtine rodents (Rausch, 1994). An alternative hypothesis, introduction into North America from western Europe, seems unlikely because no Tertiary biogeographic corridor has been described between the eastern United States and western Europe; alternatively, errant migrant birds may transport ticks great distances. But, as with a hypothesis of introduction by human-associated activity (e.g., Norway rats within household goods), successful colonization of multiple sites by such low-probability events (as introduction by European birds) seems unlikely.

Fay and Rausch (1969) described the presence and distribution of a piroplasm in Alaskan voles. This seminal article provided details of the life cycle, particularly its transmission by *Ixodes angustus*, which ranges from Siberia into northern Maine (Keirans and Clifford, 1978). At the time, the identity of the Alaskan agent was tentatively assigned to *B. microti* because of its morphology and association with microtine rodents. The development of molecular phylogenetic tools for objectively probing the genetic relationships between organisms has provided new insight into the history of a wide range of taxa and now allows a reexamination of tentative identifications based on classical methods. The recent description of *Theileria youngi* from rodents in California (Kjemtrup et al., 2001), which previously might have been assigned to *B. microti* based on morphology, serves as an example of the utility of DNA-based methods as a complement to microscopy and life cycle information. It may be that the paucity of reports of human babesiosis in sites where ‘‘*B. microti*’’ has been reported from rodents may reflect, in part, misidentification of the parasite. Because the presence of *B. microti* in Alaska would provide pivotal evidence for its introduction via Beringia, it seems logical to revisit Alaska and confirm the identity of the piroplasm. Accordingly, we sampled rodents and insectivores from 5 major faunal zones of Alaska and analyzed them for evidence of *B. microti* infection by polymerase chain reaction (PCR), targeting the 18S rDNA. In addition, representative amplification products were sequenced and analyzed as a first attempt to describe the phylogeography of *B. microti*.

MATERIALS AND METHODS

Sample collection

Small mammals were snap-trapped or live-trapped during a biotic inventory through the Beringian Coevolution Project. In addition to other tissues, spleens were removed and immediately frozen in liquid nitrogen. There were 4 main collection areas: southeast Alaska-Klukwan, White Pass, Prince of Wales Island and Chichagof Island; Gulf of Alaska-Hinchinbrook Island, Montague Island, Cordova, Knight Island, Evans Island, and Kodiak; interior Alaska-Cantwell; Seward Peninsula-Bendeleben, Kotzebue, Noatak, Nome, and Solomon; and Yukon Delta-Yukon Delta. All mammal specimens (skin, skull, and frozen tissues) have been deposited in the University of Alaska Museum.

Polymerase chain reaction

DNA was extracted from spleens by using the Isoquick blood extraction kit (Orca Research, Bothell, Washington). Approximately 10 mg of spleen was homogenized in 100 μl of 4 M guanidium lysis solution, and the lysate was extracted according to the manufacturer's recommendations. DNA was resuspended in 50 μl of distilled water. Babesia 18S rDNA was amplified by PCR by using primers Bab1/Bab4 (Persing et al., 1992) as described previously. The 238-bp amplification products were detected by agarose gel electrophoresis. Random samples were amplified using PiroA/PiroB as described previously (Armstrong et al., 1998), excised from the gel, purified by spin column (QIAquick; QIAGEN, Valencia, California) and sequenced at the University of Maine Sequencing Facility (Orono, Maine) for sequence confirmation. PCR contamination control measures included physical separation of extraction, PCR setup, and electrophoresis; dedicated pipettors; inclusion of dUTP in PCR reactions; and appropriate negative extraction and amplification controls. Restriction polymorphism within the Bab1/4 target was identified that differentiated Alaska-derived *Babesia* from the standard Harvard *B. microti* strain (GI). MluI cuts the Alaska-derived *Babesia* once, creating fragments of approximately 130 and 120 bp, but it does not cut the Harvard *B. microti*. Conversely, XhoI cuts the Har-

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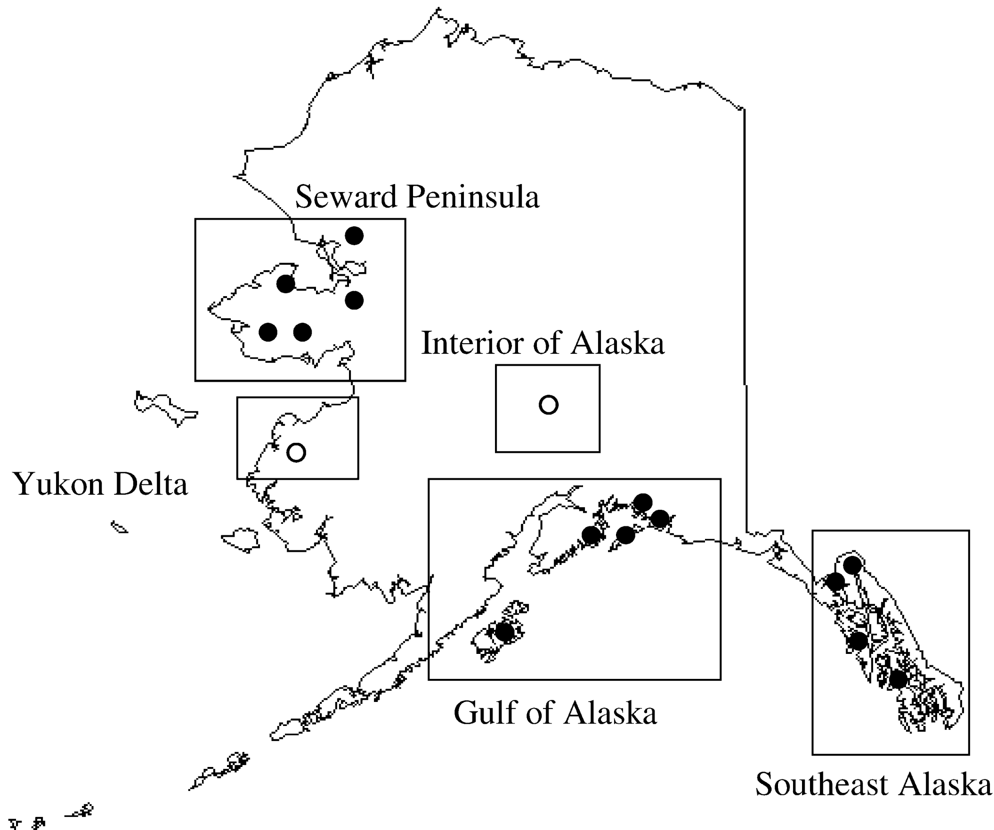

FIGURE 1. Areas of sample collection. Closed circles indicate the presence of *B. microti* in rodents. Open circles indicate no *Babesia* sp. was detected.

vard *B. microti* and not the Alaska-derived *Babesia*. All amplicons were subjected to restriction analysis to exclude the possibility of laboratory contamination.

Morphology

Blood smears were made from fewer than 20% of all animals. Thin smears were made from whole anticoagulated blood or from heart blood (dead animals), fixed with methanol in the field, and stained with Giemsa (pH 7.0). Slides from PCR-positive animals were examined by bright field microscopy at ×1,000. Randomly selected parasites (30 from each blood smear, for at least 3 *Microtus* sp. or *Clethrionomys* sp.) were measured under oil immersion by using a calibrated ocular micrometer; size indices (length × width of the parasite/diameter of the infected red blood cell) were compared by boxplot (MINITAB version 11.21, Minitab, Inc., State College, Pennsylvania).

Phylogenetic analysis

To obtain a larger portion of the 18S rDNA for phylogenetic analysis, primers A and B were used as described previously (Kjemtrup et al., 2000), or primer B was combined with the general piroplasm primer, PiroA (Armstrong et al., 1998). A 1,266-bp piece was amplified, sequenced, and aligned with other *Babesia* sequences from GenBank by using Clustal X (Smith et al., 1996) and adjusted by eye with GeneDoc (Nicholas and Nicholas, 1997). The maximum likelihood algorithm was used for phylogenetic analysis in PAUP (Swofford, 1998). The statistically best model was selected using Modeltest (Posada and Crandall, 1998). This model, TVM+I+G (transversional model with an estimated proportion of invariant sites and gamma distribution shape parameter), was then used in the maximum likelihood analysis. *B. odocoilei* and *B. divergens* were used as outgroups.

GenBank accession numbers

AB083375 *B. microti* China, AB05191 *B. microti* HK strain, AY144692 *B. microti* Switzerland, AB050732 *B. microti* Japan-Hobestu, AY144693 *B. microti* Russia, AY144694 *B. microti* Wisconsin, AF231348 *B. microti* GI strain, AB032434 *B. microti* Japan-Kobe, AB071177 *B. microti* Munich, AY144687 *B. microti* AK-Cleth., AY918951 *B. microti* AK-Microtus, AY918952 *B. microti* AK Sorex, AY144690 *B. microti* Maine, AY144699 *B. microti* Montana, AY144698 *Babesia* sp. skunk, AY144700 and AF188001 *B. annae*, AY144702 *Babesia* sp. fox, AY144701 *Babesia* sp. raccoon, AB070506 *Babesia* sp. Japan- *I. ovatus*, AB049999 and M87565 *B. rodhaini*, AF244913 *B. leo*, AF244912 and AY45707 *B. felis*, AF245279 *T. youngi*, U16370 *B. divergens*, and U16369 *B. odocoilei*.

RESULTS

In total, 297 rodents or insectivores were analyzed for evidence of infection, representing collections from every major Alaskan faunal zone (Fig. 1). *Babesia microti* was detected by amplification of the Bab1/4 target in 97 of 297 samples (Table I). All amplicons were readily distinguished from the GI strain of *B. microti* by restriction enzyme analysis. Animals that were sampled from coastal regions were frequently infected, with the exception of the Yukon Delta, whereas those from the central portion of the state were not infected. *Ixodes angustus* were noted from animals from Alexander Archipelago and south coast regions, whereas no ticks were noted from Cantwell. The presence of ectoparasites was not recorded from the Seward Peninsula and Yukon Delta.

TABLE I. Prevalence of *Babesia* in Alaskan rodents.

Site	Species	n	% Prevalence	95% CI*
Gulf of Alaska				
	Clethrionomys rutilus	35	54	(37–71)
	Microtus oeconomus	80	60	(48–71)
	Sorex spp.	21	14	(3–36)
	Combined	136	51	(43–60)
Southeastern Alaska				
	Microtus spp.	15	53	(27–79)
	Peromyscus keeni	5	60	(15–95)
	Combined	20	45	(23–68)
Interior of Alaska				
	C. rutilus	79	0	(0–5)
Yukon Delta				
	C. rutilus	26	0	(0–13)
Seward Peninsula				
	C. rutilus	20	35	(15–59)
	M. oeconomus	11	64	(31–89)
	Microtus miurus	5	40	(5–85)
	Combined	36	44	(27–62)

* CI, confidence interval.

Piroplasms within blood smears of infected *C. rutilus* were morphologically indistinguishable from *B. microti* GI strain within hamsters or in naturally infected New England *Peromyscus leucopus*. Size indices of parasites infecting *C. rutilus* and *M. oeconomus* suggest a host-related effect (Fig. 2).

We sequenced a 407-bp portion of the 18S rDNA from 6 selected samples, representing species of *Clethrionomys*, *Microtus*, and *Sorex*, and obtained identical sequences (data not shown). Because of the difficulty in obtaining large sequences from frozen splenic material, only 3 full-length sequences were obtained. These full-length sequences are identical within the sites of overlap to the other sequences. The Alaskan *B. microti* significantly differs from the GI strain of *B. microti*, with 97.4% sequence similarity (33 bp different) within the 1,266-bp portion that was sequenced.

Our phylogenetic analysis yielded the tree shown in Figure 3. The Alaskan *Babesia* sp. was identical to that from Maine and Montana (group B in Fig. 3). These sequences cluster together with *B. microti* sensu stricto (group A in Fig. 3) to the exclusion of *B. annae* and *B. rodhaini* but form their own distinct clade. Interestingly, a sequence from Munich, Germany (unpublished sequence from GenBank AB07177) also falls within group B. We conclude that although the Alaskan *Babesia* is closely related to *B. microti* sensu stricto, it is genotypically distinct.

DISCUSSION

As reported by Fay and Rausch (1969), *B. microti* commonly infects Alaskan rodents. Transmission is most intense in the coastal areas of Alaska, with almost one-half of the animals that were tested containing evidence of infection. None of the animals tested from Yukon Delta or from inland sites was positive. Although it is possible that our sample size was insufficient to detect a low prevalence of infection in the Yukon Delta or inland sites, the absence of *B. microti* in rodents from inland sites was noted by Fay and Rausch (1969) and attributed to the distribution of the vector *I. angustus*, which seems to be present only in the coastal areas of Alaska. The prevalence of gross splenomegaly, which has been used as a marker for *B. microti* infection, seems to coincide with the presence of *I. angustus* (Fay and Rausch, 1969). Our survey of small mammals also suggested that splenomegaly was more common in coastal sites, but quantitative comparisons have not been performed.

Phylogenetic analysis, from partial 18S rDNA sequences, demonstrates that all the parasites detected within Alaskan rodents and insectivores group within clade B of a putative *B. microti* species complex (Goethert and Telford, 2003), distinct from zoonotic clade A of the northeastern United States, or Wisconsin, or Japan. The Alaskan parasites are clearly not *T. youngi*, described from *Neotoma* spp. trapped in northern California (Kjemtrup et al., 2001). Whether the 2.6% divergence in the 18S rDNA represents geographic variation within an allopatric population of a widely distributed species or serves as a marker for a unique entity remains speculation (Goethert and Telford, 2003). Alaskan *B. microti* seem to be morphologically similar to parasites from sites across the Holarctic and has a similar host range (*Microtus* spp., *Clethrionomys* spp., and *Sorex* spp.). We have failed in a small number of attempts to pass infection by subinoculation of fresh infected blood or splenic homogenates to laboratory-reared *P. leucopus*, hamsters, or SCID mice (data not shown), which contrasts with the report of Fay and Rausch (1969) of infecting golden hamsters. It is possible that the infections we used during these experiments represented chronic infections, with predominantly sexual forms (gametocytes of Rudzinska et al., 1983), which would be poorly transmissible by syringe. The Alaskan parasites seem to be maintained by *I. angustus* (Fay and Rausch, 1969), which are classified within a subgenus (*Pholeoixodes*) different from that of the main *B. microti* vectors for much of the northeastern United States (*I. dammini* subgenus *Ixodes*), suggesting the possibility for vector-related divergence. Additional information (including establishing an experimental life cycle in laboratory-

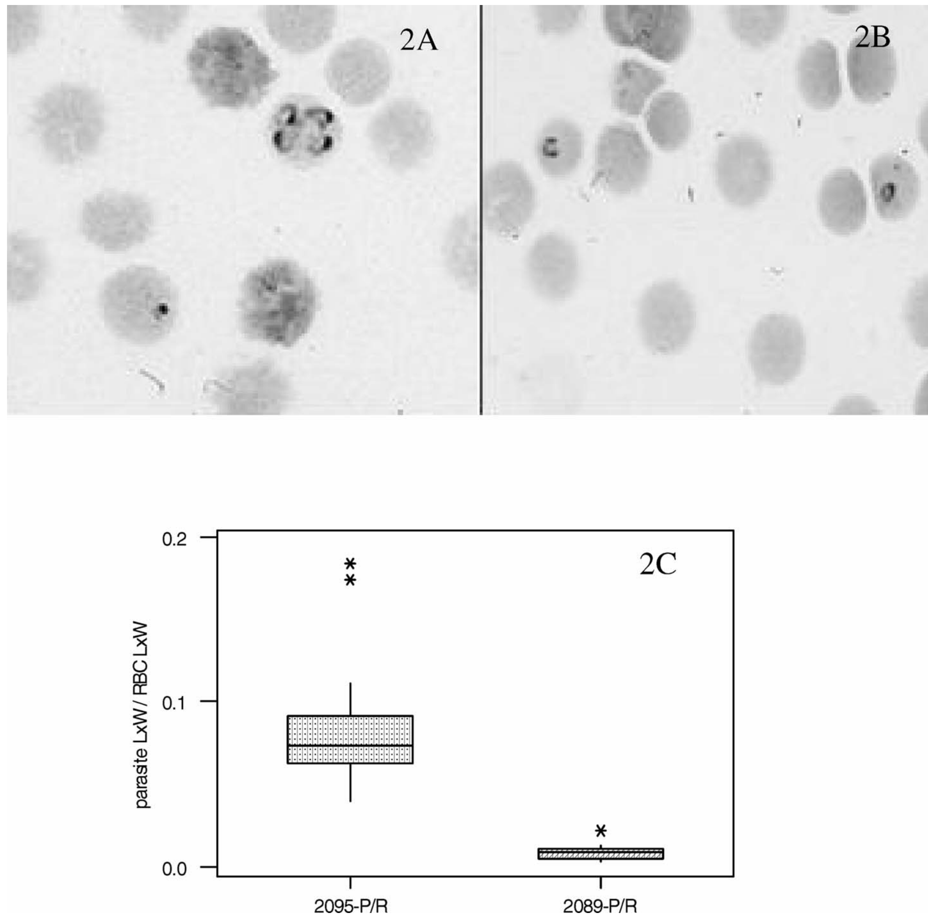

FIGURE 2. Morphology of *B. microti* infecting *Clethrionomys rutilus* (**A**) and *Microtus oeconomus* (**B**) Boxplot comparison of size indices calculated from representative parasites from each host (**C**) 2095 is *M. oeconomus* and 2089 is *C. rutilus*.

reared microtines; vector competence studies as well as sequencing of other genes) will be required to determine whether Alaskan *B. microti* deserve description as a new species.

Parasites seemed to morphologically differ with respect to infections in *M. oeconomus* and *C. rutilus*, as observed by Fay and Rausch (1969). Indeed, these authors suspected the presence of 2 different *Babesia* spp. Sequence data from the 18S rDNA gene do not support the hypothesis of 2 species, because we only obtained invariant sequences from species of *Clethrionomys*, *Microtus*, and *Sorex*. This gene, however, is known to be highly conserved and may not show divergence between species that have recently diverged; thus, we cannot exclude the possibility that there may be diverse parasites within Alaskan small mammals. In addition, we directly sequenced from PCR amplicons instead of cloning and sequencing them, which might have revealed rare copy variants. The differences in parasite size could be simply due to host factors. *Babesia* spp. are well known to be morphologically pleomorphic, depending on host or stage of infection.

The apicomplexan 18S rDNA gene has been demonstrated to have paralogous sequences that are differentially expressed during different stages of their life cycle (Long and Dawid, 1980). In *Plasmodium* spp., for example, the small subunit rDNA has many copies with sequence polymorphism (McCutchan et al., 1995). We do not believe that paralogous rDNA genes can account for the sequence divergence we find between Alaskan *B. microti* and others from elsewhere in the world, because detailed analyses of other *Babesia* spp. have detected only limited sequence polymorphism in their small subunit rDNA (Dalrymple, 1990; Reddy et al., 1991). Most of the sequence polymorphism noted was in the noncoding regions. For example, among 3 identified ssu rDNA genes in *B. bigemina*, 10 nucleotide differences were noted, 8 of which occurred in the 5′ and 3′ flanking regions. Only 2 bases differed in the coding regions corresponding to areas of the gene we sequenced. We found 33 bases different between the sequence from the Alaskan vole and *B. microti* GI strain, greatly exceeding that which would be expected from polymorphism due to paralogous sequences.

We had expected that *B. microti* from Alaskan rodents would comprise genotypes that might be considered basal to that which seems to be associated with human infection in the northeastern United States (clade A, GI-like strains, *B. microti* s.s.). Because GI-like strains have been reported from the western Urals (Telford et al., 2002), Japan (Saito-Ito et al., 2000), and Taiwan (Shih et al., 1997), such strains also should be found in Alaska if they had been introduced from Eurasia into North America through the dispersal of the reservoir or vector via Beringia. We did not detect this genotype among the 97 18S rDNA amplicons that were examined by restriction analysis.

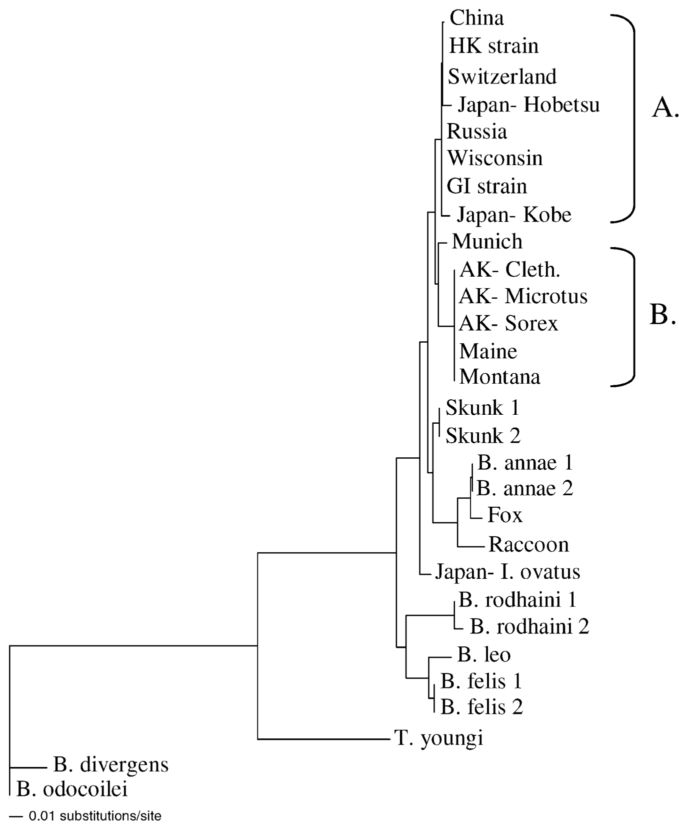

FIGURE 3. Phylogenetic analysis of *Babesia microti*-like parasites. Maximum likelihood analysis was done on a 1,266-bp piece of the 18S rDNA gene. *B. odocoilei* and *B. divergens* were used as outgroups. Group A consists of *B. microti* sensu stricto, and group B consists of *B. microti* parasites that are genetically distinct.

Although this finding might argue against the GI genotype's introduction into North America via Beringia, the complexity of rodent, parasite, and tick biogeography would suggest that additional information is needed. Microtine rodents have been present in North America for at least 5 million yr (Repenning, 1980), diversifying from forms that dispersed from Eurasia through Beringia in multiple events (Conroy and Cook, 2000). The Nearctic *M. pennsylvanicus*, a well-known host for *B. microti* (Tyzzer, 1938; Kirner et al., 1958; Spielman, 1976), apparently expanded its range within the last 50,000 yr to include much of North America, perhaps recolonizing southern and central Alaska. Parasite populations may have become isolated, mixed with others as habitat and animal distributions shifted with glacial advances or retreats, or became locally extinct (Rausch, 1994). Also, very little is known about the biogeography of the likely vector ticks, other than noting the reported wide range of *I. angustus* from Siberia to Maine. Molecular studies of a number of mammalian hosts have uncovered substantial phylogeographic structure (Cook et al., 2001; Brunhoff et al., 2003), suggesting that further investigation of geographic variation is warranted. Accordingly, future studies should concurrently analyze parasite, vertebrate host, and invertebrate vector population structure to explore the origins of *B. microti*.

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