# 北海道のエゾリス(Sciurus vulgaris orientis)から検出されたBabesia microti様原虫


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# *Babesia microti*-Like Parasites Detected in Eurasian Red Squirrels (*Sciurus vulgaris orientis*) in Hokkaido, Japan

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ABSTRACT. Six Eurasian red squirrels (*Sciurus vulgaris orientis*), victims of road traffic found during 2002 and 2004 near the Noppro Forest Park in Ebetsu, Hokkaido, Japan, were examined for the presence of *Babesia* parasites. Three of the six squirrels exhibited positive signals by nested PCRs targeting both the 18S rRNA and β-tubulin genes. Three squirrels proved to be infected with a *B. microti*-like parasite as evidenced by sequencing the amplified DNAs and by the morphology of the intraerythrocytic parasites. Genotypically, however, the parasite appeared to be of a new type, as it was clearly distinguishable from any of the known types that have previously been reported in various wild animals. This is the first report showing molecular evidence for the presence of *B. microti*-like parasites in Sciuridae.

KEY WORDS: *Babesia microti*, Eurasian red squirrel, Hokkaido, *Sciurus vulgaris*, Zoonosis.

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Since the recent discovery of the first human babesiosis case in Japan [3, 4], we have been conducting epizootiologic surveys on small wild mammals in the country that may serve as reservoirs for the human babesiosis agent. So far, three types of *Babesia microti*-like parasites, referred to as Kobe, Hobetsu, and U.S. types, have been found in Japan from various small wild rodent species, as well as from shrews [9, 10, 13]. The Kobe type has been isolated from Japanese large field mice (*Apodemus speciosus*) captured only in Awaji Island; this type of parasite was proven to be the causative agent in the Japanese index case patient of human babesiosis [4, 10]. The Hobetsu type is the most predominant type and is distributed throughout the major Japanese islands; this type is also mainly isolated from *A. speciosus* [9, 13] and is suggested to be infectious to human [1]. The U.S. type has very recently been found in Japan [13], and its distribution appears to be confined within a narrow area in the eastern part of Hokkaido. While both the Kobe and Hobetsu types have so far been reported only in Japan, the U.S. type has been found ubiquitously distributed in the temperate zones not only of North American but also of Eurasian Continents [11].

In 1973, Takahashi and Yamashita [6] reported on *Babesia* sp. in Eurasian red squirrels (*Sciurus vulgaris orientis*) that were captured or found dead near the Nopporo Forest Park, which is located adjacent to Rakuno Gakuen University in Ebetsu, Hokkaido, Japan. Although they described the morphology of the intraerythrocytic parasites in detail, precise species identification could not be made because the parasite did not morphologically match any of the other *Babesia* spp. previously described in squirrels [6, 7].

In the present study, we were given the opportunity to investigate Eurasian red squirrels found dead due to traffic accidents near the Nopporo Forest Park. As some of the squirrels had *Babesia* parasites very similar to those described by Takahashi and Yamashita [6], attempts were made to isolate the parasite and to obtain molecular evidence for species identification.

## MATERIALS AND METHODS

*Field collections*: In the years from 2002 to 2004, six Eurasian red squirrels that died from road traffic were found near the Nopporo Forest Park and brought into Rakuno Gakuen University. Blood specimens were collected from the ventricles of the hearts of two subjects (nos. 3222 and 3360) which were relatively in a fresh state (found in winter, probably within 24 or 48 hr of death); while the other four (nos. 0203, 0204, 3738, and 3739) were somewhat decomposed.

*DNA analyses*: DNA extraction and nested PCR were carried out according to the method described previously [11, 13]. For detection of *Babesia* parasites in the blood samples, nested PCR was carried out targeting both the 18S rRNA and β-tubulin genes. The PCR primers for the 18S rRNA gene, which are broadly specific for most of the hemoprotozoa in Piroprasmida, consisted of Piro0F (5'-GCC AGT AGT CAT ATG CTT GTG TTA-3') and Piro6R (5'-CTC CTT CCT YTA AGT GAT AAG GTT CAC-3') for the first round; and Piro1F (5'-CCA TGC ATG TCT WAG TAY AAR CTT TTA-3') and Piro5.5R (5'-CCT YTA AGT GAT AAG GTT CAC AAA ACT T-3') for the second round. The primers for the β-tubulin gene, which are highly specific for *B. microti*-like parasites, consisted of BmTubu93F (5'-GAY AGY CCC TTR CAA CTA GAA AGA GC-3') and BmTubu897R (5'-CGR TCG AAC ATT TGT TGH GTC ART TC-3') for the first round; and BmTubu192F (5'-ACH ATG GAT TCT GTT AGA TCY

GGC-3') and BmTubu782R (5'-GGG AAD GGD ATR AGA TTC ACA GC-3') for the second round. Amplified DNAs were sequenced according to the method described elsewhere [4, 11].

*Phylogenetic analysis*: Phylogenetic relationships were analyzed with the sequences of the 18S rRNA and β-tubulin genes using MacVector software version 8.0 (Accelrys Inc., San Diego, CA, U.S.A.). The parasites included for the analyses of the 18S rRNA and β-tubulin genes, and their sequence accession numbers in GenBank were as follows: Gray strain of U.S.-type *B. microti*, AY693840 and AB083377; Ko524 strain of Kobe-type *B. microti*-like parasite, AB032434 and AB083440; Ho234 strain of Hobetsu-type *B. microti*-like parasite, AB050732 and AB083441; Munich strains of *B. microti*, AB071177 and AB124587; *Babesia* sp. from Alaskan voles, AY144687 and AY144710; *Babesia* sp. from a skunk in the United States, AY144698 and AF546902; *Babesia* sp. from a raccoon in the United States, AY144701 and AY144708; *Babesia* sp. from Spanish dogs (=*Theileria annae* [12]), AY144700 and AY144709; and *B. rodhaini*, AB049999 and AB083442. Sequences corresponding to regions 464 to 1718 of AY693840 and 304 to 1205 of AB083377 were used for the analyses of the 18S rRNA and β-tubulin genes, respectively. They were aligned with the program CLUSTAL W Alignment [8], and a phylogenetic tree was constructed by the neighbor-joining method [5] from the aligned sequences using the program Phylogenetic Analysis in the MacVector software. Support for tree nodes was calculated with 1,000 bootstrap replicates using the bootstrap tree algorithm.

*Laboratory animals*: Isolation of *Babesia* parasites was attempted using three laboratory animals that had been splenectomized prior to use: a golden Syrian hamster (8-week-old male Std:Syrian, Japan SLC Inc., Hamamatsu, Japan), a Mongolian gerbil (10-week-old female MON/Jms/Gbs, Japan SLC Inc.), and a SCID mouse (9-week-old female NOD/shi-*scid*, maintained in the Laboratory Animal Facility in Rakuno Gakuen University [4, 10]). Blood specimen from squirrel no. 3360 was intraperitoneally inoculated into these animals (approximately 200 μl for each animal). Blood samples of the animals were examined once a week for 2 months to determine the presence or absence of *Babesia* parasites by both microscopy and nested PCR.

*Nucleotide sequence accession numbers*: The nucleotide sequences determined in this paper have been deposited in DDBJ under accession numbers AB219802 and AB219803.

## RESULTS

Nested PCR targeting the 18S rRNA gene gave rise to positive signals in three (nos. 0203, 3222 and 3360) of the six squirrels. The three amplified DNAs had identical sequences showing a high degree of similarity to those of *B. microti*-like parasites (Fig. 1A). The three squirrels also exhibited positive signals by nested PCR targeting the β-tubulin gene; their amplified DNA sequences were identi-

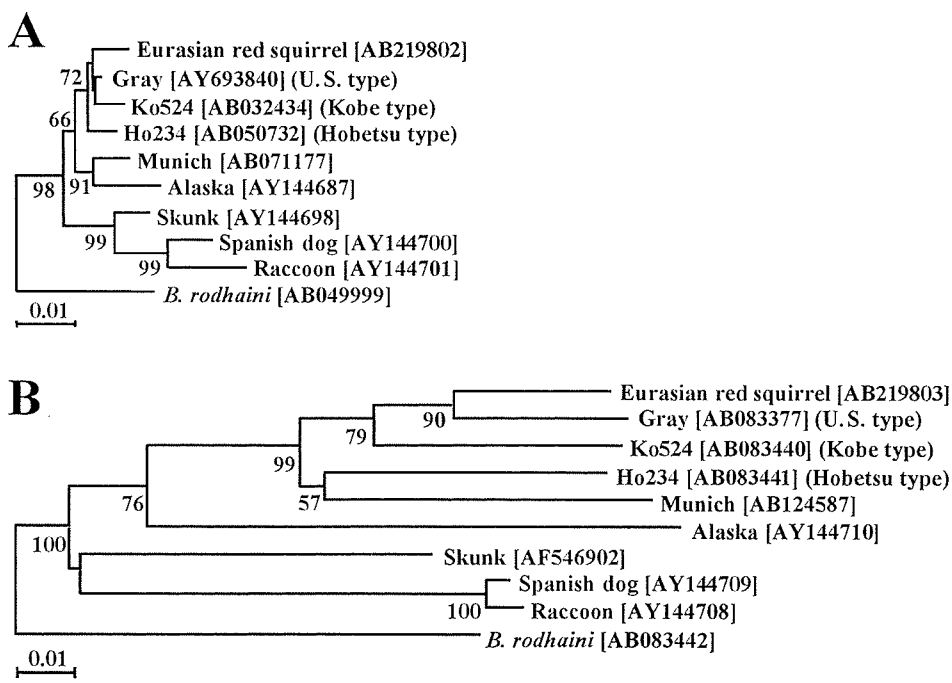


Fig. 1. Phylogenetic trees inferred from the sequences of 18S rRNA (A) and β-tubulin (B) genes. *B. microti*-like parasite in the Eurasian red squirrels and its close relatives were included in the analyses. The GenBank accession number for each DNA sequence is given in parenthesis. The number on each branch indicates the percent occurrence in 1,000 bootstrap replicates (numbers less than 50 are not shown). The scale bars in both A and B represent 0.01 substitutions per site.

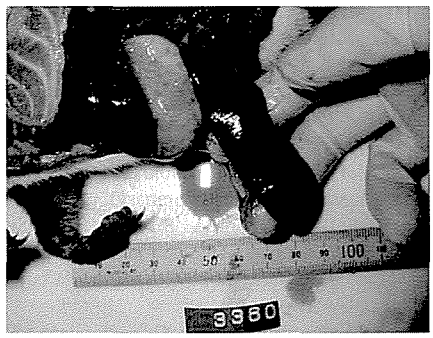


Fig. 2. A splenomegaly observed in the Eurasian red squirrel no. 3360.

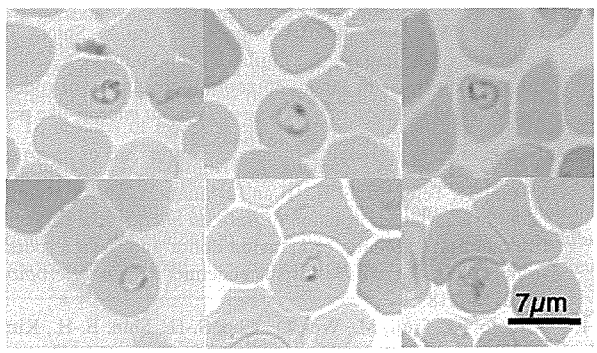


Fig. 3. Morphology of the *B. microti*-like parasites in blood smears from squirrels nos. 3222 (upper panels) and 3360 (lower panels).

cal, indicating that all three were infected by the same *B. microti*-like parasite. The sequences of the 18S rRNA and β-tubulin genes, together with those from closely related *B. microti*-group parasites, were used to construct phylogenetic trees, respectively (Fig. 1A and B). Although branching patterns in the two trees were similar, the interrelationships among the parasites inferred from the 18S rRNA tree were unclear. In contrast, the tree based on the β-tubulin gene sequences gave rise to better resolution, because sequence diversity in the β-tubulin gene is much greater than that in the 18S rRNA gene.

The two squirrels (nos. 3222 and 3360) whose bodies were in a relatively fresh condition enabled us to conduct some biological examinations. Squirrel no. 3360 had obvious splenomegaly (Fig. 2), a major clinical manifestation of babesiosis. Unclotted blood remaining in the hearts of both allowed us to prepare Giemsa-stainied thin-smear blood films, that clearly showed the presence of intraerythrocytic parasites (Fig. 3) morphologically very similar to the *Babesia* sp. described by Takahashi and Yamashita [6]. Since a relatively large amount of unclotted blood could be obtained from squirrel no. 3360, attempts were made for parasite isolation by intraperitoneal inoculations of the blood into three species of splenectomized laboratory animals. Development of parasitemia, however, was not detected in any of them by either microscopy or PCR.

## DISCUSSION

In this study, we presented the first molecular evidence for the presence of a *B. microti*-like parasite in Sciuridae. Phylogenetically, the parasite in the Eurasian red squirrels (*Sciurus vulgaris orientis*) is most closely related to the U.S.-type *B. microti* (=*Babesia microti sensu stricto*, which is regarded as the major causative agent of human babesiosis [2, 7]). Our finding, therefore, implies that the squirrels may serve as an additional reservoir for the human babesiosis agent, although the zoonotic potential of this newly identified parasite has yet to be proven.

In our earlier studies [9–11, 13], we conducted field surveys at various places in Japan, and also in some regions in the northeastern Eurasian Continent. Investigations of a large number of small wild mammals revealed that various species of the family Muridae (*Apodemus agrarius, A. peninsulae, A. speciosus, Clethrionomys rufocanus, C. rutilus, Eothenomys smithii, Lagurus luteus*, and *Microtus montebelli*) and some shrews (*Sorex unguiculatus* and *S. caecutiens*) carried *B. microti*-like parasites. Based on their 18S rRNA sequences, they were classified into three genotypes, designated as the Kobe, Hobetsu, and U.S. types [9, 11, 13]. Furthermore, regardless of the host species and place of collection, there were virtually no intragenotypic sequence variations. The *Babesia* sp. from squirrels differed from Kobe-, Hobetsu-, and U.S-type parasites in their 18S rRNA sequences, 1.47%, 1.52%, and 1.24%, respectively. The distinctive sequence detected in the present study from squirrels seems to, therefore, indicate a new variant different from either one of the three genotypes reported earlier [9, 11, 13].

Three decades ago, Takahashi and Yamashita [6] had reported infection of *S. vulgaris orientis* with a *Babesia* parasite. It is not sure whether the *Babesia* sp. described by them is identical to that shown in this study. However, we believe that this is probably the case, based on the parasite's morphology and place of sample collection, which is the Noppro Forest Park comprising 2,051 hectares, and surrounded by the urban areas of Sapporo, Ebetsu, and Kitahiroshima. The natural environment within the park has been relatively well preserved with most of the animal populations having only slight chances for interaction with populations in other areas. Thus, it is highly likely that the *Babesia* sp. infection in squirrels in the park has also been maintained for many years. *Sciurus vulgaris orientis* in Hokkaido Island is regarded as a subspecies of *S. vulgaris*, which is widely distributed in the northeastern Eurasian Continent. Although the prevalence of *Babesia* infection in squirrels was consistently high in both this and previous studies [6], whether or not this is also the case in other regions in Hokkaido Island and in Eurasian Continent need to be investigated.

Unfortunately, our attempt to isolate the *Babesia* parasite from a dead squirrel using three species of splenectomized

laboratory animals that were presumed to be susceptible to *B. microti* was unsuccessful. A low level of parasitemia (approximately 0.02%) may have accounted for this. However, recording of negative results may be important inasmuch as encountering such an opportunity is extremely rare. Our earlier request for capturing live squirrels in the park to obtain a sufficient amount of blood for parasite isolation was disapproved owing to a possible high risk of fatal injury to the animals, whose number in the park was estimated to be decreasing (probably less than 50; T. Kataoka *et al.*, unpublished data). Nevertheless, further efforts should be made towards achieving parasite isolation and determination of tick vector(s).

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