Table 2. Isolation of *Babesia* parasites from field collections

Animal species	No. of field animals tested	Experimental animals used for isolation	No. of isolated/No. of innoculated	No. of isolates with the following rDNA genotype[a)]		
				U.S.	Hobetsu	Kobe
Apodemus speciosus	16	Hamster	16/16	1	15	0
Clethrionomys rufocanus	4	Hamster	4/4	1	3	0
Clethrionomys rutilus	1	Hamster	1/1	1[b)]	0	0
Microtus montebelli	1	Hamster	1/1	0	1	0
		Girbil	1/1	0	1	0
Sorex unguiculatus	1	Hamster	0/1	–	–	–
		Girbil	1/1	0	1	0
Sorex caecutiens	1	Hamster	0/1	–	–	–
		Home musk shrew	0/2	–	–	–
		NOD/shi-*scid*	0/2	–	–	–
Total	24		24/30	3	20	0

a) Genotype was determined by sequencing analysis of near full-size rDNA gene.
b) The primaly isolate predominantly consisted of U.S.-type parasite, but the presence of Hobetsu-type parasite was demonstrated by passage in mice.

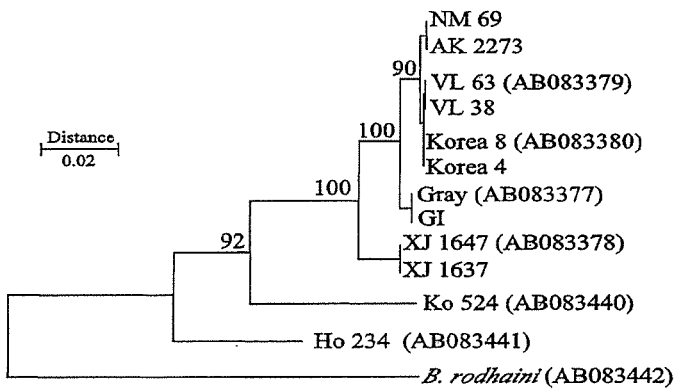

Fig. 2. Phylogenetic tree constructed with sequences of the β-tubulin genes of the U.S.-type *B. microti* and other closely related parasites. The U.S.-type parasites were collected from various places; NM69 and AK2273 from Hokkaido in Japan; VL63 and VL38 from Vladivostok in Russia; Gray and GI from the northeastern Unites States; XJ1647 and XJ1637 from Xinjiang in China. The strains Ko524 (Kobe-type) and Ho234 (Hobetsu-type) were also from Japan. GenBank accession numbers are given in parenthesis. The number on each branch shows the percent occurrence in 1,000 bootstrap replicates.

firming mixed infections by both U.S.- and Hobetsu-type parasites.

Sequencing analysis of the β-tubulin gene: Sequencing analysis was also carried out for the β-tubulin gene of the 23 parasite isolates. Of the 23 β-tubulin gene sequences, 20 were identical to that of strain Ho234 (the type strain of Hobetsu type; GenBank accession no. AB083441), and the other 3 were very close, but not exactly identical, to that of the Gray strain (GenBank accession no. AB083377), the isolate from the United States index case patient [5]. With the β-tubulin gene sequences from the U.S.-type *B. microti* strains (NM69 and AK2273) isolated in Japan, together with those previously reported for the other U.S.-type *B. microti* parasites [29], we constructed a phylogenetic tree to analyze their interrelationship (Fig. 2). The U.S.-type *B. microti* isolated in Japan (NM69 and AK2273) was found to be quite closely related to those found in South Korea (Korea4 and 8) and Far East Russia (VL38 and 63), but relatively distantly related to those found in the United States (Gray and GI) or the northwestern China (XJ1637 and 1647).

Antigenic analysis: To examine the antigenic relationship between the U.S.-type parasites isolated from Japan (NM69 and AK2273) and those from the United States (Gray and GI), IFATs were carried out for pair wise comparison. Both

Table 3. Results of IFATs with *B. microti* parasites isolated from U.S. and Japan

Sera	Reciprocal titer against the following parasite:			
	Gray	GI	NM69	AK2273
anti-Gray	25,600	12,800	1,600	800
anti-GI	12,800	12,800	1,600	800
anti-NM69	<100	100	12,800	6,400
anti-AK2273	400	200	25,600	25,600

the Japanese and the Unites States parasites showed higher titers against the homologous than against the heterologous antiserum (Table 3). Similar results were also obtained in Western blot analysis (Fig. 3), in which we found that many of immunodominant antigens recognized by the homologous antiserum were poorly cross-reactive against the heterologous antiserum.

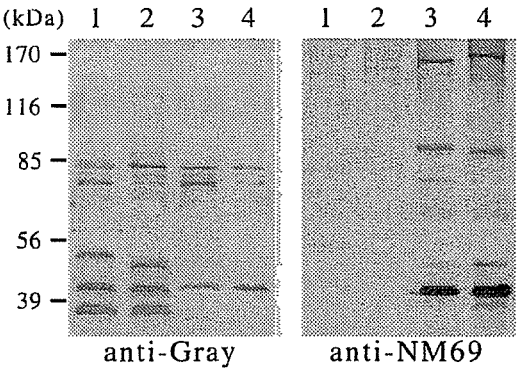

Fig. 3. Western blot analysis with the U.S.-type *B. microti* isolated in the United States (Gray and GI; lanes 1 and 2, respectively) and Japan (AK2273 and NM69; lanes 3 and 4, respectively). Parasite antigens were detected with anti-Gray mouse serum (left panel) and with anti-NM69 hamster serum (right panel).

DISCUSSION

This study represents the first isolation of U.S.-type *B. microti* in Japan. Previously, this type of *B. microti* was presumed not to exist in the country, because of its apparent absence in our field survey [23]. Recently, however, we found the presence of U.S.-type parasites in Northeastern Eurasia [29], which gave us motivation to seek this type of parasite in Japan. We focused on Hokkaido Island because this island had been connected with Eurasian Continent until approximately ten thousand years ago, and some species of small wild rodent seen in Northeastern Eurasia also exist on Hokkaido Island. The presence of U.S.-type *B. microti* in Japan appeared to be confined to a narrow area in the eastern part of Hokkaido Island. Although the exact reason for this is currently unclear, it may be related to the fact that natural environment in this area is so well preserved as to accommodate a large number of wild-life animals.

Finding of the U.S. type in this study, in addition to the Hobetsu and Kobe types in previous studies [23], demonstrated that there are three types of *B. microti*-like parasites in Japan. While the Hobetsu type is the major parasite that is widely distributed in Japan, the U.S. type appeared to be the predominant parasite that is distributed throughout the temperate zones of the North American and Eurasian Continents [4, 5, 28]. A major factor contributing to this difference is probably the geographic isolation, owing to which many mammals distributed in Japanese Islands are unique species, including *A. speciosus*, the major reservoir host for the Hobetsu type *B. microti*-like parasites.

We originally defined the three genotypes, namely, U.S., Hobetsu, and Kobe types, based on the sequences of their rDNA. All the U.S.-type *B. microti* isolated to date were reported to have virtually identical rDNA sequences [4, 5, 28, 29], regardless of the place of parasite isolation and also regardless of the host animal species. Sequencing analyses on the rDNA of the Hobetsu- and Kobe-type isolates also indicate that there are no sequence variations in their rDNA. Such high degree of sequence conservation, therefore, makes the rDNA sequence an ideal genotypic marker. However, since the number of nucleotides useful to distinguish between types is only a few, sequencing of the near full-size rDNA is needed for genotype determination. In this and previous studies [29], we included sequence analyses of the β-tubulin gene in addition to the rDNA. This enabled us to determine not only genotype but also intragenotypic variation, because sequence variation in the β-tubulin gene is much greater than that in the rDNA. Phylogenetic analysis of the β-tubulin gene sequences clearly demonstrated the presence of geographic diversity within the U.S.-type *B. microti* which could not previously be seen by analysis of rDNA.

Diversity of the U.S.-type *B. microti* was also observed in the parasite antigens recognized by the host animals. Western blot analysis clearly showed that the U.S.-type parasites isolated in Japan and those in the United States had several immunodominant antigens that were poorly cross-reactive against heterologous antiserum. This antigenic diversity indicates the necessity of a local isolate as an antigen for sensitive serodiagnosis of local infections. Indeed, in our previous study [29], we failed to detect specific antibodies in the serum samples from wild rodents collected in South Korea and Far East Russia, despite that their infection by U.S.-type *B. microti* was clearly detected by PCR. In that study, antibody detection was carried out by IFAT using the Gray strain of *B. microti* which was obtained from the United States. Recently, however, we were able to detect specific antibodies in the same serum samples by using the strain NM69, a U.S.-type parasite isolated in this study, as an antigen of IFAT (M. Tsuji, unpublished data). This also suggests that the antigenicities of the U.S.-type parasites distributed in Korea and Far East Russia are closely related with that in Japan, which is consistent with their close relationships in the phylogenetic tree (Fig. 2).

The results of the present survey reconfirmed our previ-

ous conclusion that *A. speciosus* serves as the major host for *B. microti*-like parasites in Japan. Besides *A. speciosus*, however, we also found *C. rufocanus, C. rutilus, M. montebelli, S. unguiculatus*, and *S. caecutiens* harboring *B. microti*-like parasites. These species of Rodentia and Insectivora may, therefore, serve as additional reservoir hosts for the agents of human babesiosis in the country. Interestingly, preference of experimantal hosts appeared to be somewhat different between the parasites infecting rodents and those infecting insectivores: all *B. microti*-like parasites derived from wild rodents were isolated by inoculation into hamsters which, however, appeared to be refractory to infection by the parasites derived from insectivores. Shrews have been suggested to play roles as reservoirs of not only babesiosis but also Lyme borreliosis in the Unites States [21] and Japan [15].

Previously, we speculated that the apparent absence of the U.S.-type *B. microti* in Japan might be relevant to the seemingly rare occurrence of symptomatic human babesiosis cases in the country [2, 23], which is in contrast to the relatively frequent clinical case reports from areas of endemicity in the Unites States [11, 20]. It was hypothesized for this speculation that the U.S.-type *B. microti* may be more pathogenic and virulent than the Hobetsu-type *B. microti*-like parasite, the major type widely distributing throughout Japan. Therefore, careful monitoring for the residents in the eastern area of Hokkaido Island, where the U.S.-type parasites have been found to be enzootic, may provide us with a good opportunity to test that hypothesis.

ACKNOWLEDGMENTS. We thank Murayama, H., Nishida, A. and Iwabu, Y. Rakuno-Gakuen University, for their excellent technical assistance. This work was supported in part by Grants-in-Aid (nos. 12450139 and 14560271) from the Ministry of Education, Science and Culture of Japan, by Health Science Grants for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare of Japan, by a Grant-in-Aid (no. 2002–1) to Cooperative Research from Rakuno-Gakuen University, by a Grant-in-Aid to Akkeshi-ko and Betsukanbeushi-shitsugen Research Project 2000 from Akkeshi-cho, Hokkaido, and by Gakujutsu Frontier Cooperative Research in Rakuno-Gakuen University.

REFERENCES

1. Abe, H., Ishii, N., Kaneko, Y., Maeda, K., Miura, S. and Yoneda, M. 1994. A Pictorial Guide to the Mammals of Japan. Tokai University Press, Tokyo (in Japanese).
2. Arai, S., Tsuji, M., Kaiho, I., Murayama, H., Zamoto, A., Wei, Q., Okabe, N., Kamiyama, T. and Ishihara, C. 2003. Retrospective seroepidemiological survey for human babesiosis in an area in Japan where a tick-borne disease is endemic. *J. Vet. Med. Sci.* **65**: 335–340.
3. Burkot, T. R., Schneider, B. S., Pieniazek, N. J., Happ, C. M., Rutherford, J. S., Slemenda, S. B., Hoffmeister, E., Maupin, G. O. and Zeidner, N. S. 2000. *Babesia microti* and *Borrelia bissettii* transmission by *Ixodes spinipalpis* ticks among prairie voles, *Microtus ochrogaster*, in Colorado. *Parasitology* **121**: 595–599.
4. Duh, D., Petrovec, M. and Avsic-Zupanc, T. 2001. Diversity of *Babesia* infecting European sheep ticks (*Ixodes ricinus*). *J. Clin. Microbiol.* **39**: 3395–3397.
5. Gleason, N. N., Healy, G. R., Western, K. A., Benson, G. D. and Schultz, M. G. 1970. The "Gray" strain of *Babesia microti* from a human case established in laboratory animals. *J. Parasitol.* **56**: 1256–1257.
6. Gray, J., von Stedingk, L.V., Gurtelschmid, M. and Granstrom, M. 2002. Transmission studies of *Babesia microti* in *Ixodes ricinus* ticks and gerbils. *J. Clin. Microbiol.* **40**: 1259–1263.
7. Healy, G. R., Spielman, A. and Gleason, N. 1976. Human babesiosis: reservoir of infection on Nantucket Island. *Science* **192**: 479–480.
8. Homer, M. J., Aguilar-Delfin, I., Telford, S. R., III, Krause, P. J. and Persing, D. H. 2000. Babesiosis. *Clin. Microbiol. Rev.* **13**: 451–469.
9. Hussein, H. S. 1980. *Ixodes trianguliceps*: seasonal abundance and role in the epidemiology of *Babesia microti* infection in north-western England. *Ann. Trop. Med. Parasitol.* **74**: 531–539.
10. Karbowiak, G., Stanko, M., Rychlik, L., Nowakowski, W. and Siuda, K. 1999. The new data about zoonotic reservoir of *Babesia microti* in small mammals in Poland. *Acta Prasitologica* **44**: 142–144.
11. Kjemtrup, A. M. and Conrad, P. A. 2000. Human babesiosis: an emerging tick-borne disease. *Int. J. Parasitol.* **30**: 1323–1337.
12. Koyanagi, Y., Tanaka, Y., Kira, J., Ito, M., Hioki, K., Misawa, N., Kawano, Y., Yamasaki, K., Tanaka, R., Suzuki, Y., Ueyama, Y., Terada, E., Tanaka, T., Miyasaka, M., Kobayashi, T., Kumazawa, Y. and Yamanoto, N. 1997. Primary human immunodeficiency virus type 1 viremia and central nervous system invasion in a novel hu-PBL-immunodeficient mouse strain. *J. Virol.* **71**: 2417–2424.
13. Matsui, T., Inoue, R., Kajimoto, K., Tamekane, A., Okamura, A., Katayama, Y., Shimoyama, M., Chihara, K., Saito-Ito, A. and Tsuji, M. 2000. First documentation of transfusion-associated babesiosis in Japan. *Rinsyo Ketsueki* **41**: 628–634 (in Japanese with an English summary).
14. Medlin, L., Elwood, H. J., Stickel, S. and Sogin, M. L. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* **71**: 491–499.
15. Nakao, M. and Miyamoto, K. 1993. Long-tailed shrew, *Sorex unguiculatus*, as a potential reservoir of the spirochetes transmitted by *Ixodes ovatus* in Hokkaido, Japan. *Jpn. J. Sanit. Zool.* **44**: 237–245.
16. Saito-Ito, A., Tsuji, M., Wei, Q., He, S., Matsui, T., Kohsaki, M., Arai, S., Kamiyama, T., Hioki, K. and Ishihara, C. 2000. Transfusion-acquired, autochthonous human babesiosis in Japan: isolation of *Babesia microti*-like parasites with hu-RBC-SCID mice. *J. Clin. Microbiol.* **38**: 4511–4516.
17. Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425.
18. Shiota, T., Kurimoto, H., Haguma, N. and Yoshida, Y. 1984. Studies on *Babesia* first found in murine in Japan: epidemiology, morphology and experimental infection. *Zentralbl. Bakteriol. Mikrobiol. Hyg. [A]* **256**: 347–355.
19. Spielman, A., Clifford, C. M., Piesman, J. and Corwin, M. D. 1979. Human babesiosis on Nantucket Island, USA: description of the vector, *Ixodes (Ixodes) dammini*, n. sp. (Acarina:

Ixodidae). *J. Med. Entomol.* **15**: 218–234.
20. Telford, S. R., III, Gorenflot, A., Brasseur, P. and Spielman, A. 1993. Babesial infections in humans and wildlife pp. 1–47. *In*: Parasitic Protozoa, 2nd ed. vol. 5 (Kreier, J. P. ed.), Academic Press. Inc., New York.
21. Telford, S. R., III, Mather, T. N., Adler, G. H. and Spielman, A. 1990. Short-tailed shrews as reservoirs of the agents of Lyme disease and human babesiosis. *J. Parasitol.* **76**: 681–683.
22. Thompson, J. D., Higgins, D. G. and Gison, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673–4680.
23. Tsuji, M., Wei, Q., Zamoto, A., Morita, C., Arai, S., Shiota, T., Fujimagari, M., Itagaki, A., Fujita, H. and Ishihara, C. 2001. Human babesiosis in Japan: epizootiologic survey of rodent reservoir and isolation of new type of *Babesia microti*-like parasites. *J. Clin. Microbiol.* **39**: 4316–4322.
24. van Peenen, P. F., Chang, S. J., Banknieder, A. R. and Santana, F. J. 1977. Piroplasms from Taiwanese rodents. *J. Protozool.* **24**: 310–312.
25. Walter, G. 1984. *Babesia microti* (strain "Hannover I"): transmission by *Ixodes ricinus* and course of parasitemia in the bank vole (*Clethrionomys glareolus*) and the field vole (*Microtus agrestis*). *Acta Trop.* **41**: 259–264.
26. Watkins, R. A., Moshier, S. E., O'Dell, W. D. and Pinter, A. J. 1991. Splenomegaly and reticulocytosis caused by *Babesia microti* infections in natural populations of the montane vole, *Microtus montanus*. *J. Protozool.* **38**: 573–576.
27. Wei, Q., Tsuji, M., Zamoto, A., Kohsaki, M., Matsui, T., Shiota, T., Teleford, S. R. III and Ishihara, C. 2001. Human babesiosis in Japan: isolation of *Babesia microti*-like parasites from an asymptomatic transfusion donor and from a rodent from an area where babesiosis is endemic. *J. Clin. Microbiol.* **39**: 2178–2183.
28. Zahler, M., Rinder, H. and Gothe, R. 2000. Genotypic status of *Babesia microti* within the piroplasms. *Parasitol. Res.* **86**: 642–646.
29. Zamoto, A., Tsuji, M., Wei, Q., Cho, S.-H., Shin, E.-H., Kim, T.-S., Leonova, G. N., Hagiwara, K., Asakawa, M., Kariwa, H., Takashima, I. and Ishihara, C. 2004. Epizootiologic survey for *Babesia microti* among small wild mammals in Northeastern Eurasia and a geographic diversity in the β-tubulin gene sequence. *J. Vet. Med. Sci.* **66**: 785–792.

FULL PAPER *Public Health*

Epizootiologic Survey for *Babesia microti* among Small Wild Mammals in Northeastern Eurasia and a Geographic Deversity in the β-Tubulin Gene Sequences

Aya ZAMOTO[1], Masayoshi TSUJI[1]*, Qiang WEI[1], Shin-Hyeong CHO[3], E-Hyun SHIN[3], Tong-Soo KIM[3], Galina N. LEONOVA[4], Katsuro HAGIWARA[1], Mitsuhiko ASAKAWA[1], Hiroaki KARIWA[2], Ikuo TAKASHIMA[2] and Chiaki ISHIHARA[1]

[1]*School of Veterinary Medicine, Rakuno-Gakuen University, Ebetsu 069–8501,* [2]*School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan,* [3]*Department of Medical Zoology, National Institute of Health, Seoul 122–701, Korea,* [4]*Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences Siberia Branch, Vladivostok 690028, Russia*

(Received 7 October 2003/Accepted 10 February 2004)

ABSTRACT. We previously reported that small wild rodents in Japan harbor two types of novel *Babesia microti*-like parasites (designated as Hobetsu and Kobe types), but not the type commonly found in the northeastern United States (U.S. type) where human babesiosis is endemic. To determine whether these new types of parasites are distributed in places surrounding Japan, an epizootiologic survey was undertaken in three geographically distant areas in northeastern Eurasia; South Korea, Vladivostok in Russia, and Xinjiang in China. Blood samples were collected from a total of 387 animals comprising 24 species. DNAs extracted from the samples were tested by nested PCR targeting babesial nuclear small-subunit rRNA gene (rDNA), which revealed that small rodents harboring *B. microti* exist in all three survey areas. Sequence analysis showed that all PCR-positive samples had rDNA sequences virtually identical to that of U.S.-type *B. microti*. However, when β-tubulin gene sequences were compared, evident geographic variations were seen. By use of primers specific for each of the β-tubulin genes of Kobe-, Hobetsu-, and U.S.-type parasites, a type-specific PCR was developed. Parasite with Hobetsu- or Kobe-type sequence was not detected from any of the three survey areas. These findings suggest that U.S.-type *B. microti* is widely distributed among small wild mammals in temperate zones of not only North America, but also Eurasia, whereas that Hobetsu- and Kobe-type parasites may be uniquely distributed in Japan.

KEY WORDS: *Babesia microti*, β-tubulin, Epidemiology, Eurasia, wild rodent.

J. Vet. Med. Sci. 66(7): 785–792, 2004

Babesia microti is a tick-transmitted, intraerythrocytic protozoan parasite frequently seen in small wild rodents. This parasite is known to be the causative agent of human babesiosis, an emerging tick-bone zoonosis that is endemic mainly in the northeastern and upper midwestern United States [19, 29]. Extensive epidemiological studies have been conducted in the regions, revealing that the white-footed mouse (*Peromyscus leucopus*) and the black deer tick (*Ixodes scapularis*) serve as the rodent reservoir and the tick vector, respectively, for the agent of human babesiosis [19, 29].

In the literature, there are many reports that document the parasitization of *Babesia* among wide varieties of rodent species in various places in the world, including North America [3, 14, 33], Europe [15, 16, 18], Africa [8] and East Asia [28, 32]. The parasites reported in those studies are referred to as *B. microti*, suggesting that a single parasite species may widely be distributed throughout the world. However, because identification of the species in most studies was made primarily on the basis of parasite morphology and host species, and also because the taxonomy of rodent *Babesia* spp. has historically been quite complicated [20, 21], the possibility that several similar, but significantly distinct, parasite species might have been regarded as the same species can not be excluded. The possible presence of distinct "*B. microti*" in different places might give an easier explanation for the geographically biased occurrence of symptomatic human babesiosis cases, which have been reported frequently from the United States but only very rarely from other regions in the world.

Our previous investigations on the first Japanese case of human babesiosis [25, 34] proved that the patient was infected by blood transfusion from an asymptomatic carrier. A following epidemiological survey [31] further revealed that two types of *B. microti*-like parasites, namely Kobe and Hobetsu, are enzootic among Japanese small wild rodents, and that *Apodemus speciosus*, a species of Muridae unique to Japan, serves as the major reservoir of the agent of human babesiosis. Both the Kobe- and Hobetsu-type parasites were closely related phylogenetically to the U.S.-type parasite (=*B. microti* sensu stricto), but these three types were clearly distinguishable from one another antigenically and genotypically [31]. Parasites with a nuclear small-subunit rRNA gene (rDNA) sequence that is virtually identical to that of the United States *B. microti* have also been reported from Europe [7, 35], but to date not from Japan [31]. The objective of the present study was to conduct epizootiologic surveys in various places in northeast Eurasia for detection of *Babesia* parasites in small wild mammals.

MATERIALS AND METHODS

Field collections: Epizootiologic field surveys were car-

* CORRESPONDENCE TO: TSUJI, M., School of Veterinary Medicine, Rakuno-Gakuen University, 582–1 Bunkyodai-midorimachi, Ebetsu 069–8501, Hokkaido, Japan.

Fig. 1. Map of eastern Asia, showing locations of survey sites (●).

ried out in three geographically distant areas in the northeastern Eurasia: South Korea, Vladivostok in Russia, and Xinjiang Uygur autonomous region in China (Fig. 1). Samples from South Korea were collected in 2001 for this study, and obtained from 154 animals comprising three species of Rodentia and one of Insectivora. Field collections were carried out at eight locations; Odaesan National Park, Sobaeksan National Park, Songnisan National Park, Chirisan National Park, Soraksan National Park, Inchon in Kyodong, Tokyusan National Park, and Samsan in Inchon City. The blood specimens, which were stored in a dried form on blood sampling papers (Toyo Roshi, Tokyo), were brought to Japan and processed for subsequent analyses. Small wild mammals collected in these places are listed in Table 1. At two survey points near Vladivostok, 68 animals comprising five species of Rodentia were trapped in 1999. The serum samples collected were used for a previous study [22], and the remaining blood clots that had been stored at −80°C were made available for the present study. In Xinjiang, 165 small wild mammals comprising 19 species of Rodentia were captured during 1998 and 1999 at eight locations, namely Wuchai wan, Wulong-gu, Fuhai, Bulzin, Hanashi Lake, Jadenggu, Fuwen, and Kamushite [2]. The survey in Xinjiang was originally conducted for other purposes [2, 13], and the remaining stocks of dried bloods absorbed on filter papers were made available for this study.

DNA preparation: The samples collected near Vladivostok were stored as frozen clotted blood. DNAs from these samples were prepared with a whole blood DNA extraction kit (DNA Extractor WB Kit; Wako Pure Chemical Industries, Osaka) according to the instruction manual supplied by the manufacturer, except that clotted blood was mashed with a pestle (Handy Pestle; Toyobo, Osaka). The blood samples collected in Xinjiang and South Korea had been stored in a filter paper-absorbed, dried form. These filter papers were cut into small pieces and soaked in 500 μl of phosphate buffered saline (PBS) containing 0.05% NaN_3 for 2 days at 4°C. After centrifugation at 15,000 × g for 10 min, the supernatants were transferred to new tubes and used for IFA tests. The pellets were resuspended in 500 μl of TNE buffer (150 mM NaCl, 10 mM Tris-HCl, and 100 mM EDTA, pH 8.0) containing 1% sodium dodecyl sulfate and 100 $\mu g/ml$ of proteinase K, and incubated at 55°C overnight. DNA was purified by phenol-extraction followed by ethanol precipitation. DNA pellets were dissolved in 30 μl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5).

Detection of B. microti-specific DNA in blood samples: Nested PCR for detection of *Babesia* parasites in the blood specimens of the field collections was carried out according to the previously published protocol [31, 34]. All oligonucleotide primers used for various PCR are listed in Table 2. Approximately a 1/30 volume of the final DNA preparations described above was used for the first-round PCR, which was followed by the second-round PCR with 1 μl of the first-round product. Two kinds of primer sets were used to detect babesial rDNA: one set, Bab1A-4A (Bab1A and Bab4A for the first-round PCR, followed by Bab2A and Bab3A for the second round), was highly specific for rDNA of all three types of *B. microti*-like parasites (GenBank accession numbers U09833, AB050732, and AB032434 for U.S., Hobetsu, and Kobe types, respectively), and the other set, Bab5–8 (Bab5 and Bab8 for the first-round PCR, followed by Bab6 and Bab7 for the second round), was broadly specific for various piroplasms belonging to *Babesia* and *Theileria*. In order to minimize erroneous amplification during nested PCR, both DNA preparation described above and PCR detection were carried out in an isolated laboratory room with specialized equipment. Unused filter tips were always used for pipetting to avoid cross-contaminations.

Sequencing analysis of rDNA: In order to determine gen-

Table 1. Summary of the field surveys for *B. microti* among small wild mammals in Northestern Eurasia

Place	Animal species	No. of animals	No. of positives by nested PCR					Positive samples[d]
			rDNA		β-tubulin[c]			
			Bab5–8[a]	Bab1A-4A[b]	U.S.	Hobetsu	Kobe	
South Korea								
	Apodemus agrarius	146	30	16	16	0	0	Korea8 and others
	Apodemus peninsulae	2	2	1	1	0	0	Korea138
	Clethrionomys regulus	2	0	0				
	Crocidura lasiura	4	0	0				
	subtotal	154	32	17	17	0	0	
Vladivostok								
	Apodemus peninsulae	32	2	1	1	0	0	VL63
	Apodemus agrarius	28	0	0				
	Clethrionomys rufocanus	4	2	1	1	0	0	VL38
	Microtus fortis	2	0	0				
	Tamias sibiricus	2	0	0				
	subtotal	68	4	2	2	0	0	
Xinjiang								
	Citellus erythrogenys	1	1	1	0	0	0	XJ1648
	Citellus undulatus	7	7	0				
	Cricetulus migratorius	6	2	0				
	Cricetulus longicaudatus	9	4	0				
	Cricetulus eversmanni	10	7	0				
	Cricetus cricetus	1	1	0				
	Microtus arvalis	5	5	0				
	Lagurus luteus	3	3	2	2	0	0	XJ1637 and XJ1647
	Clethrionomys rufocanus	1	1	0				
	Clethrionomys rutilus	15	12	0				
	Meriones meridianus	28	12	0				
	Meriones tamariscinus	4	2	0				
	Apodemus uralensis	2	1	0				
	Apodemus peninsulae	14	5	0				
	Mus musculus	1	0	0				
	Alactagulus pumilio	1	1	0				
	Allactaga sibirica	20	14	0				
	Dipus sagitta	32	15	0				
	Euchoreutes naso	5	2	0				
	subtotal	165	53	3	2	0	0	
	total	387	131	22	21	0	0	

a) Nested PCR with primers Bab5 and Bab8 for the first round, and primers Bab6 and Bab7 for the second round.
b) Nested PCR with primers Bab1A and Bab4A for the first round, and primers Bab2A and Bab3A for the second round.
c) Genotyping of β-tubulin genes by type-specific PCR.
d) Identification numbers of the positive samples.

otypes of *B. microti*, near full-size rDNA sequences (approximately 1.6 kb) were amplified from DNA samples of field collections by nested PCR with the primer sets of Piro1F and rRNA-3', followed by Bab1A and Piro6R (Table 2). The procedures of DNA sequencing have been described previously [25].

Sequencing analysis of β-tubulin gene: The 1651 bp sequence encoding the full-size β-tubulin gene of the Gray strain was elaborated by combining three overlapping sequences. The first sequence contained a 1214 bp partial sequence of the β-tubulin gene, which was amplified from the parasite genomic DNA with a set of primers Tubu63F and Tubu-3' (Table 2). The second sequence was obtained by reverse transcription-PCR [27] from mRNA of Gray strain, which amplified the 551 bp 3'-end region of the β-tubulin mRNA. The third sequence contained both 5'- and 3'-end regions of the genomic β-tubulin gene, which were amplified by inverse PCR [27] from fragmented genomic DNAs of the Gray strain made by *Hinc* II-digestion followed by self-ligation. Amplification of β-tubulin gene sequences from various geographic samples was carried out by nested PCR with UStubu127F and UStubu1637R primers for the first-round PCR, and with another set of primers, UStubu161F and UStubu1609R, for the second-round PCR. The specific PCR products, approximately 1.5 kb in size, were cloned and sequenced as described previously [25]. Sequences of β-tubulin genes from genomic DNAs of Hobetsu- and Kobe-type parasites and of *Babesia rodhaini*

Table 2. Oligonucleotide primers used for amplification of babesial rDNA and β-tubulin genes

Primers	Oligonucleotide sequences (5'–3')
Primers for rDNA	
Piro1F	CCATGCATGTCTWAGTAYAARCTTTTA
Bab1A	GTCTTAGTATAAGCTTTTATACAGCG
Bab2A	CAGTTATAGTTTATTTGATGTTCGTTTTAC
Bab3A	CGGCAAAGCCATGCGATTCGCTAAT
Bab4A	GATAGGTCAGAAACTTGAATGATACATCG
Bab5	AATTACCCAATCCTGACACAGG
Bab6	GACACAGGGAGGTAGTGACAAGA
Bab7	CCCAACTGCTCCTATTAACCATTAC
Bab8	TTTCGCAGTAGTTCGTCTTTAACA
Piro6R	CTCCTTCCTYTAAGTGATAAGGTTCAC
rRNA-3'	ATCCTTCYGCAGGTTCACCTAC
Primers for β-tubulin	
Tubu63F	CAAATWGGYGCMAARTTYTGGGA
Tubu-3'	TCGTCCATACCTTCWCCSGTRTACCAGTG
UStubu127F	ACATTTTCCCCATAGAACTTTATATCG
UStubu161F	CTAACCATTTAAATTGTTTATAATGAG
UStubu1609R	AAACAACTGTGTTAATATGTGAGT
UStubu1637R	AATTAACAGTACAGGAACTAGGTTA
BmTubu 93F	GAYAGYCCCTTRCAACTAGAAAGAGC
BmTubu192F	ACHATGGATTCTGTTAGATCYGGC
BmTubu782R	GGGAADGGDATRAGATTCACAGC
BmTubu897R	CGRTCGAACATTTGTTGHGTCARTTC
Tubu-Ko5'[a)]	CAAATGTTTTTTATAACCAGACGAGCG
Tubu-Ko3'[a)]	GAAAGGAATAAGATTCACAGTGAGCT
Tubu-Ho5'[a)]	AAGAGCTAACGTTTTTTACAATCTATCAAG
Tubu-Ho3'[a)]	CGCAAATCCAATCATAAAAAAGTTTAGTC
Tubu-US5'[a)]	GCAAAYGTTTTYTATAACCAGTTTAGTG
Tubu-US3'[a)]	GAAATGCAATCTCGGGAAGGTAATGA

a) A single mismutch indicated by underline was introduced to enhance type specificity.

were amplified by PCR with a set of primers Tubu63F and Tubu-3' (Table 2). Analyses for DNA sequences and phylogenetic relationships were done by using the MacVector software package, version 7.0 (Genetic Computer Group Inc., Madison, Wis, U.S.A.). The sequences were aligned with the program Clustal W Alignment [30], and a phylogenetic tree was constructed by the neighbor-joining method [26] from the aligned sequences with the program Phylogenetic Analysis in the MacVector software. Support for tree nodes was calculated with 1,000 bootstrap replicates by use of the bootstrap tree algorithm.

Type-specific PCR: Nested PCR for specific amplification of each of the β-tubulin genes from the U.S.-, Hobetsu-, and Kobe-type of *B. microti*-like parasites was developed. A set of primers used for the first-round PCR were BmTubu93F and BmTubu897R, which were designed to equally amplify all three types of β-tubulin gene sequences. The second-round PCR was carried out with four sets of primers (Table 2). One set, BmTubu192F and BmTubu782R, was designed to universally amplify most *B. microti* group parasites, and the other three sets were designed to specifically amplify each of the three types (Tubu-US5' and Tubu-US3', Tubu-Ho5' and Tubu-Ho3', and Tubu-Ko5' and Tubu-Ko3' for U.S., Hobetsu, and Kobe types, respectively). In each of these oligonucleotide primers (Table 2), an artificial single mismatch was introduced to a critical position near the 3' end in order to enhance the specificity of primers. The PCR mixtures contained 400 μM each deoxynucleoside triphosphate, 0.25 μM each primer, an appropriate amount of template DNA, and 1 U of La *Taq* DNA polymerase (Takara Biochemical, Otsu) in 20 μl of the PCR buffer supplied with the enzyme. Thermal cycling was carried out in a GeneAmp PCR system 9600 thermal cycler (Perkin Elmer, Norwalk, Conn, U.S.A.) with 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 60 s, and extension at 72°C for 90 s.

Serological analysis: The indirect immunofluorescent antibody test (IFAT) was carried out to detect specific antibodies against *B. microti* in PBS-eluates from blood specimens of field collections. The Gray strain was used as the antigen for IFAT. The method of IFAT has been described previously [25].

Reference strains of B. microti: The strains Gray and GI (U.S. type), Ho234 (Hobetsu type), and Ko524 (Kobe type) have been described in our previous study [25, 31], and were propagated in hamsters. The Australian strain of *B. rodhaini*, kindly provided by the National Institute of Animal Health, Tsukuba, Japan, was propagated in BALB/c mice. *Babesia canis vogeli*, Okinawa isolate, has been described in an earlier study [1].

Nucleotide sequence accession number: The sequence data generated in the present study were submitted to DNA Data Bank of Japan and were given accession numbers as listed in Table 3.

RESULTS

Epizootiologic survey: Small wild mammals examined in the present study are listed in Table 1. Blood samples were collected from 154 animals comprising four species in South Korea, from 68 animals comprising five species in Vladivostok, and from 165 animals comprising 19 species in Xinjiang. DNAs extracted from these samples were tested by nested PCR, for which two kinds of primer sets were used (Table 2): one set, Bab1A-4A, was highly specific for the rDNAs of three types of *B. microti*-like parasites (GenBank accession numbers U09833, AB050732, and AB032434 for U.S., Hobetsu, and Kobe types, respectively), the other set, Bab5–8, was broadly specific for various piroplasms belonging to *Babesia* and *Theileria*. The results of these PCR amplifications are summarized in Table 1, which revealed that small wild rodents harboring *B. microti* exist in all three places surveyed in the present study.

Sequencing analysis of rDNA: Twenty-two samples, which were PCR-positive with the primer set of Bab1A-4A, were subjected to another PCR for amplification of nearly full size rDNA sequences. Sequencing analysis revealed that both the 17 specimens from South Korea and the two specimens from Vladivostok had identical rDNA sequences which were exactly the same as that reported for a *B. microti*

Table 3. DNA sequence data generated in the present study

Accession no.	Gene	Size (bp)	Parasite (genotype)	Place[b)]
AB083374	rDNA[a)]	1708	*Babesia canis*	Okinawa, Japan
AB083375	rDNA	1665	*B. microti*, XJ1647 (U.S.)	Xinjiang, China
AB083376	rDNA	1598	*Babesia* sp., XJ1648	Xinjiang, China
AB083377	β-tubulin	1651	*B. microti*, Gray (U.S.)	U.S.A.
AB083378	β-tubulin	1476	*B. microti*, XJ1647 (U.S.)	Xnjiang, China
AB083379	β-tubulin	1448	*B. microti*, VL63 (U.S.)	Vladivostok, Russia
AB083380	β-tubulin	1482	*B. microti*, Korea8 (U.S.)	South Korea
AB083440	β-tubulin	1214	*B. microti*-like, Ko524 (Kobe)	Kobe, Japan
AB083441	β-tubulin	1214	*B. microti*-like, Ho234 (Hobetsu)	Hokkaido, Japan
AB083442	β-tubulin	1212	*B. rodhaini*	Uncertain

a) Nuclear small-subunit rRNA gene.
b) Place of parasite isolation or field sample collection.

isolate from United States (GenBank accession number U09833). The two specimens from *Lagurus luteus* in Xinjiang had rDNA sequences which were also identical to that of U.S.-type *B. microti*, except for two nucleotide substitutions at positions 605 and 1601 (GenBank accession number AB083375). Another sample from *Citellus erythrogenys* in Xinjiang, that was positive by *B. microti*-specific PCR, was found to have an rDNA sequence (GenBank accession number AB083376) with the highest similarity to that of *Babesia canis* (GenBank accession number AB083374). There were 131 specimens that were PCR-positive with the primer set of Bab5–8, but PCR-negative with the primer set of Bab1A-4A. Sequencing analysis of those rDNAs revealed that all of them were probably not derived from *B. microti* nor any closely related parasite, but may rather be derived from some other protozoan species belonging to Eimeriida, such as *Isospora*, *Sarcocystis*, and *Hepatozoon* spp.

Sequence analysis of β-tubulin gene: To further verify the presence of *B. microti* in the blood samples that were positive by rDNA-based PCR, babesial β-tubulin gene was also amplified from each of those samples and sequenced. Whereas the samples collected within a survey area had identical β-tubulin gene sequences, apparent sequence variations were seen among the samples obtained from distant geographic areas. We also amplified and sequenced partial β-tubulin gene sequences from two Japanese *B. microti*-like parasites (Kobe and Hobetsu types) and *B. rodhaini* (another rodent *Babesia*) for phylogenetic analysis. All of them contained two introns, the presence of which was experimentally confirmed by sequencing cDNA from β-tubulin mRNA. A neighbor joining tree constructed with those sequences was depicted in Fig. 2, showing that all the parasites detected in the present survey were closely related to *B. microti* from the United States.

Development of type-specific PCR: Designing primers specific for the β-tubulin genes of U.S.-, Hobetsu-, and Kobe-type parasites (Table 2), we developed type-specific nested PCR. The specificity of this PCR system is shown in Fig. 3a, demonstrating formation of a single positive signal which was specific for each type of the three *B. microti*-like parasites, namely Kobe- Hobetsu- and U.S.-type. The specificity of PCR products could further be verified without sequencing in aid of restriction enzyme digestion (Fig. 3b). The results obtained by this system were consistent with those obtained by rDNA-based PCR and sequencing (Table 1). A parasite with β-tubulin gene sequence of Hobetsu- or Kobe-type was not detected in any sample collected from the three survey areas.

Serological assay: The PBS-eluates from the blood samples, in which the presence of *B. microti* was detected by PCR, were tested by IFAT to detect specific antibodies against *B. microti*. Gray strain of *B. microti* [11] was used as the U.S.-type parasite antigen for IFAT. However, we were not able to find any sample which reacted with this parasite antigen (IFAT titers below 1:64).

DISCUSSION

In the present survey, we were able to demonstrate that U.S.-type *B. microti* is widely distributed among small wild rodents in northeastern Eurasia. Parasites with rDNA sequences that are virtually identical to that of *B. microti* from the United States (GenBank accession number U09833) have also been reported from Europe [7, 35]. Accordingly, it became increasingly clear that a single parasite species (*B. microti* sensu stricto, which is described as the U.S. type in this study) is distributed throughout the temperate zones of the Northern Hemisphere.

We previously reported that small wild rodents in Japan harbored two types of novel *B. microti*-like parasites, namely Hobetsu and Kobe types [31], whereas that U.S.-type *B. microti* has so far not been isolated in the country. Both the Hobetsu- and Kobe-type parasites have been isolated mainly from the large Japanese field mouse, *A. speciosus*, which is a major species of Muridae unique to Japanese Islands, indicating that these two types of *B. microti*-like parasites may also be unique to the country. Although some taxonomic criteria, such as morphology, pathogenicity, and ecological niche, place these Japanese parasites within the species of *B. microti*, our previous study clearly demonstrated that they differed from U.S.-type *B. microti* antigenically and genotypically [31]. If we are able to obtain further evidence for the geographic isolation of these two Japanese *B. microti*-like parasites, it may be reasonable to

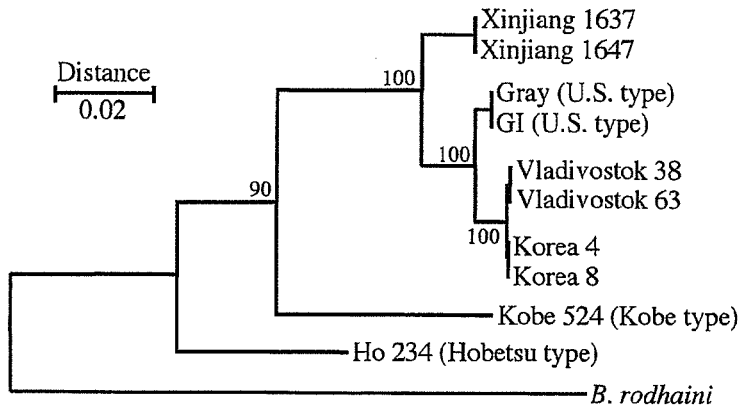

Fig. 2. Phylogenetic tree constructed by the neighbor-joining method with β-tubulin gene sequences of several *B. microti* group parasites collected at various places. The number on each branch shows the percent occurrence in 1,000 bootstrap replicates.

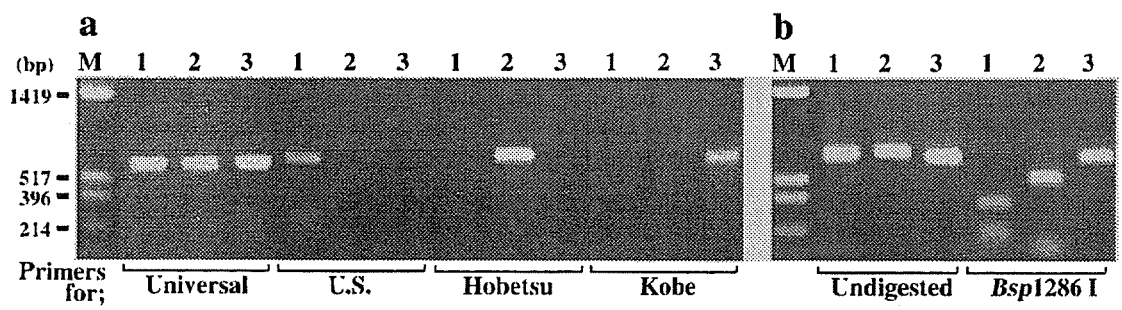

Fig. 3. Type-specific PCR targeting the β-tubulin genes of U.S.-, Hobetsu-, and Kobe-type parasites. Lanes; 1, Gray strain (U.S. type); 2, Ho234 (Hobetsu type); 3, Ko524 (Kobe type); M, DNA size makers. (a) Parasite DNA samples were amplified with either a set of the universal primers or each set of the three type-specific primers described in Materials and Methods. (b) Digestion of the specifically amplified PCR products by *Bsp*1286I, showing the presence of unique sites to each type.

propose separating them into new species or at least new subspecies.

The present survey showed that *Apodemus agrarius, A. penisulae, Clethrionomys rufocanus*, and *Lagurus luteus* were the rodent species harboring *B. microti* in northeastern Eurasia. *A. penisulae* and *C. rufocanus* are also known to exist on Hokkaido Island in Japan, because this island had been connected with Eurasian Continent via Sakhalin Island during the latest ice age until approximately ten thousand years ago. Hence, despite the apparent absence of U.S.-type *B. microti* in Japan, it may be possible to find this type in that region of the country. A sample from *Citellus erythrogenys* trapped in Xinjiang, China, contained a *Babesia* parasite whose rDNA was the most closely related to that of *B. canis vogeli* isolated from a dog in Okinawa Japan [1]. Whether this parasite is involved in any disease of dogs or humans is currently unknown, although a human case that was suggested to be due to *B. canis* has been reported [23].

The presence of *Babesia* parasites in the blood specimens was successfully determined by specific amplification of a short target sequence within the rDNA. However, sequencing near full length rDNA (approximately 1.6 kb) was needed for subsequent genotypic classification into Hobetsu, Kobe, and U.S. types, since the numbers of nucleotide sequences useful for type discrimination were only a very few; the three types of rDNAs showed more than 99.2% similarities [31]. Owing to such high degree of sequence conservation, the rDNA was not an appropriate target for further study on an intragenotypic, geographic variations. In fact, all the rDNA sequences amplified from the field collections in the present survey were virtually identical. Thus, we have analyzed β-tubulin gene sequences in addition to rDNA. All the β-tubulin gene sequences from the three survey areas exhibited high degrees of sequence similarity to that of *B. microti* isolated in the northeastern United States (GenBank accession number AB083377). Sequences obtained within a single survey area were identical, whereas significant numbers of base substitutions were seen among sequences obtained from distant areas, demonstrating intragenotypic, geographic variations. Most substitutions were found at the third nucleotide of the triplet genetic codon, therefore, resulted in little amino acid substi-

tution.

Sequencing analysis was carried out also for the β-tubulin genes of Hobetsu- and Kobe-type *B. microti*-like parasites and *B. rodhaini*. All of them contained two introns. While the position of the first intron is precisely conserved for most parasites in phylum Apicomplexa [4–6, 24], the presence and position of the second intron are probably unique to *B. microti* group (*B. microti* and closely related parasites, including *B. rodhaini*), because the other parasites belonging to genera *Babesia* and *Theileria* apparently lack it (M. Tsuji, unpublished data). The two introns were very small in size, 20 and 22 bp for the first and second ones, respectively, which are comparable to the smallest introns found in chloroplast nucleomorph of Chlorarachniophyte algae [10]. Since such introns may be too small to be recognized by the splicing mechanism known for general spliceisomal introns [12], *B. microti* may have a unique splicing mechanism. The introns in the β-tubulin genes of other *Babesia* and *Theileria* parasites are much longer than the first ones of *B. microti* group [4]. Gain and loss of introns have been regarded as a significant event in phylogenetic evolution [9]. Such features may be regarded as an additional line of evidence which supports the proposal that parasites in this group should belong to neither *Babesia* nor *Theileria*, and be re-classified as an independent genus [17, 35].

Phylogenetic analysis of the β-tubulin genes showed that sequence variations seen among the Kobe-, Hobetsu-, and U.S.-type parasites were much greater than those seen within various U.S. type *B. microti* from geographically distant areas. Whereas the former may be regarded as the interspecies variation, the latter may represent the intraspecies one. Evolutionary distances among these three types were comparable to those between some parasites in Piroplasmida that are well established to be distinct species, such as the distances between *Theileria parva* and *T. annulata* and between *Babesia bigemina* and *B. caballi* (M. Tsuji, unpublished data). This finding was in contrast to that reported in the previous studies based on rDNA sequences [25, 31], which showed that all the U.S.-, Hobetsu-, and Kobe-type parasites were quite closely related with each other. Because the degree of sequence conservation in the rDNA is exceptionally high, the phylogenetic analysis based on this gene may give rise to a significant underestimation. Further analyses with multiple genes will be needed to unequivocally address this issue.

We were able to develop type-specific nested PCR using primers that were specific for each β-tubulin gene of Kobe-, Hobetsu-, and U.S.-type parasites. This type-specific PCR easily and quickly generated results that were consistent with those obtained by rDNA-based PCR followed by sequencing analysis. Therefore, this system provided us with a much easier way of detection and typing *B. microti*-like parasites than that targeting their rDNA, and will become a powerful tool for facilitating epidemiological studies in much larger scale. With this type-specific PCR, we are currently conducting a field survey to identify vector ticks in Japan which transmit each of Hobetsu- and Kobe-type *B. microti*-like parasites.

Our serological assay failed to detect any specific antibody against *B. microti*. Because a local parasite isolate from any of the three survey areas has not been available in the present study, Gray strain, an isolate from the United State index case patient [11], was used as the U.S.-type parasite antigen for IFAT. However, variation in the β-tubulin gene sequences suggests that *B. microti* from different regions may significantly vary in their antigenicity. For more sensitive antibody detection, therefore, isolation of local parasites with homologous antigenicity may be needed.

ACKNOWLEDGMENTS. The authors thank Jiang, W., Yan,S.-S., Chai, J.-J. and Liao, L.-F. Xinjiang Institute for Endemic Diseases Control and Research, and Slonova, R. Institute of Epidemiology and Microbiology in Russian Academy of Medical Sciences Siberia Branch, for collection of wild mammals. We also appreciate their excellent technical assistance of Kim, S.-J. and Matsubara, Y. Rakuno-Gakuen University. This work was supported in part by a grant-in-aid from the Ministry of Education, Science and Culture of Japan (nos. 10041210, 12450139, and 13575029), by Health Science Grants for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare of Japan, and by Gakujutsu-Frontier Cooperative Research in Rakuno-Gakuen University.

REFERENCES

1. Arai, S., Tsuji, M., Kim, S. J., Nakade, T., Kanno, Y. and Ishihara, C. 1998. *Babesia canis* infection in canine-red blood cell-substituted SCID mice. *Int. J. Parasitol.* **28**: 1429–1435.
2. Asakawa, M., Hagiwara, K., Liao, L., Jiang, W., Yan, S., Chai, J., Oku, Y. and Ito, M. 2001. Collection record of small mammals in Xinjiang Uygur, 1998 and 1999 with brief review of its mammalian fauna. *Biogeography* **3**: 13–31.
3. Burkot, T. R., Schneider, B. S., Pieniazek, N. J., Happ, C. M., Rutherford, J. S., Slemenda, S. B., Hoffmeister, E., Maupin, G. O. and Zeidner, N. S. 2000. *Babesia microti* and *Borrelia bissettii* transmission by *Ixodes spinipalpis* ticks among prairie voles, *Microtus ochrogaster*, in Colorado. *Parasitology* **121**: 595–599.
4. Caccio, S., Camma, C., Onuma, M. and Severini, C. 2000. The β-tubulin gene of *Babesia* and *Theileria* parasites is an informative marker for species discrimination. *Int. J. Parasitol.* **30**: 1181–1185.
5. Caccio, S., Homan, W., van Dijk, K. and Pozio, E. 1999. Genetic polymorphism at the β-tubulin locus among human and animal isolates of *Cryptosporidium parvum*. *FEMS Microbiol. Lett.* **170**: 173–179.
6. Delves, C. J., Ridley, R. G., Goman, M., Holloway, S. P., Hyde, J. E. and Scaife, J. G. 1989. Cloning of a β-tubulin gene from *Plasmodium falciparum*. *Mol. Microbiol.* **3**: 1511–1519.
7. Duh, D., Petrovec, M. and Avsic-Zupanc, T. 2001. Diversity of *Babesia* infecting European sheep ticks (*Ixodes ricinus*). *J. Clin. Microbiol.* **39**: 3395–3397.
8. el-Kady, G. A., Makled, K. M., Morsy, T. A. and Morsy, Z. S. 1998. Rodents, their seasonal activity, ecto- and blood-para-

sites in Saint Catherine area, South Sinai Governorate, Egypt. *J. Egypt. Soc. Parasitol.* **28**: 815–826.
9. Hartung, F., Blattner, F. R. and Puchta, H. 2002. Intron gain and loss in the evolution of the conserved eukalyotic recombination machinery. *Nucleic Acids Res.* **30**: 5175–5181
10. Gilson, P. R. and McFadden, G. I. 1996. The miniaturized nuclear genome of eukaryotic endosymbiont contains genes that overlap, genes that are cotranscribed, and the smallest known spliceosomal introns. *Proc. Natl. Acad. Sci. U.S.A.* **93**: 7737–7742.
11. Gleason, N. N., Healy, G. R., Western, K. A., Benson, G. D. and Schultz, M. G. 1970. The "Gray" strain of *Babesia microti* from a human case established in laboratory animals. *J. Parasitol.* **56**: 1256–1257.
12. Guo, M. and Mount, S. M. 1995. Localization of sequences required for size-specific splicing of a small *Drosophila* intron *in vitro*. *J. Mol. Biol.* **253**: 426–437.
13. Hagiwara, K., Asakawa, M., Liao, L., Jiang, W., Yan, S., Chai, J., Oku, Y., Ikuta, K. and Ito, M. 2001. Seroprevalence of Borna disease virus in domestic animals in Xinjiang, China. *Vet. Microbiol.* **80**: 383–389.
14. Healy, G. R., Spielman, A. and Gleason, N. 1976. Human babesiosis: reservoir of infection on Nantucket Island. *Science* **192**: 479–480.
15. Hussein, H. S. 1980. *Ixodes trianguliceps*: seasonal abundance and role in the epidemiology of *Babesia microti* infection in north-western England. *Ann. Trop. Med. Parasitol.* **74**: 531–539.
16. Karbowiak, G., Stanko, M., Rychlik, L., Nowakowski, W. and Siuda, K. 1999. The new data about zoonotic reservoir of *Babesia microti* in small mammals in Poland. *Acta Parasitologica* **44**: 142–144.
17. Kjemtrup, A. M., Thomford, J., Robinson, T. and Conrad, P. A. 2000. Phylogenetic relationships of human and wildlife piroplasm isolates in the western United States inferred from the 18S nuclear small subunit RNA gene. *Parasitology* **120**: 487–493.
18 Krampitz, H. E. 1979. *Babesia microti*: morphology, distribution and host relationship in Germany. *Zentralbl. Bakteriol. Hyg. I. Orig. A* **244**: 411–415.
19. Krause, P. J. and Telford, S. R., III. 1999. Babesiosis. pp. 236–248. *In*: Protozoal Diseases. (Gilles, H. M. ed.), Arnold, London, England.
20. Levine, N. D. 1971. Taxonomy of the piroplasms. *Trans. Am. Microsc. Soc.* **90**: 2–33.
21. Levine, N. D. 1988. The protozoan phylum Apicomplexa, vol. 2. CRC Press, Boca Raton, Fla, U.S.A.
22. Lokugamage, K., Kariwa, H., Hayasaka, D., Cui, B. Z., Iwasaki, T., Lokugamage, N., Ivanov, L. I., Volkov, V. I., Demenev, V. A., Slonova, R., Kompanets, G., Kushnaryova, T., Kurata, T., Maeda, K., Araki, K., Mizutani, T., Yoshimatsu, K., Arikawa, J. and Takashima, I. 2002. Genetic characterization of hantaviruses transmitted by the Korean field mouse (*Apodemus peninsulae*), Far East Russia. *Emerg. Infect. Dis.* **8**: 768–776.
23. Marsaudon, E., Camenen, J., Testou, D., Bourree, P., Samson, P. and Luneau, F. 1995. Human babesiosis: *Babesia canis* causing a 40 day period of anuria. *Ann. Med. Intern.* **146**: 451–452.
24. Nagel, S. D. and Boothroyd, J. C. 1988. The α- and β-tubulins of *Toxoplasma gondii* are encoded by single copy genes containing multiple introns. *Mol. Biochem. Parasitol.* **29**: 261–273.
25. Saito-Ito, A., Tsuji, M., Wei, Q., He, S., Matsui, T., Kohsaki, M., Arai, S., Kamiyama, T., Hioki, K. and Ishihara, C. 2000. Transfusion-acquired, autochthonous human babesiosis in Japan: isolation of *Babesia microti*-like parasites with hu-RBC-SCID mice. *J. Clin. Microbiol.* **38**: 4511–4516.
26. Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425.
27. Sambrook, J. and Russell, D. W. 2001. Molecular Cloning: a Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.,U.S.A.
28. Shiota, T., Kurimoto, H., Haguma, N. and Yoshida, Y. 1984. Studies on *Babesia* first found in murine in Japan: epidemiology, morphology and experimental infection. *Zentralbl. Bakteriol. Mikrobiol. Hyg. [A]* **256**: 347–355.
29. Telford, S. R., III, Gorenflot, A., Brasseur, P. and Spielman, A. 1993. Babesial infections in humans and wildlife. pp. 1–47. *In*: Parasitic Protozoa, 2nd ed., vol. 5. (Kreier, J. P. ed.), Academic Press. Inc., New York, N. Y., U.S.A.
30. Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673–4680.
31. Tsuji, M., Wei, Q., Zamoto, A., Morita, C., Arai, S., Shiota, T., Fujimagari, M., Itagaki, A., Fujita, H. and Ishihara, C. 2001. Human babesiosis in Japan: epizootiologic survey on rodent reservoir and isolation of new type of *Babesia microti*-like parasite. *J. Clin. Microbiol.* **39**: 4316–4322.
32. van Peenen, P. F., Chang, S. J., Banknieder, A. R. and Santana, F. J. 1977. Piroplasms from Taiwanese rodents. *J. Protozool.* **24**: 310–312.
33. Watkins, R. A., Moshier, S. E., O'Dell, W. D. and Pinter, A. J. 1991. Splenomegaly and reticulocytosis caused by *Babesia microti* infections in natural populations of the montane vole, *Microtus montanus*. *J. Protozool.* **38**: 573–576.
34. Wei, Q., Tsuji, M., Zamoto, A., Kohsaki, M., Matsui, T., Shiota, T., Telford, S. R., III and Ishihara, C. 2001. Human babesiosis in Japan: isolation of *Babesia microti*-like parasites from an asymptomatic transfusion donor and from a rodent from an area where babesiosis is endemic. *J. Clin. Microbiol.* **39**: 2178–2183.
35. Zahler, M., Rinder, H. and Gothe, R. 2000. Genotypic status of *Babesia microti* within the piroplasms. *Parasitol. Res.* **86**: 642–646.

FULL PAPER *Public Health*

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

Masayoshi TSUJI[1], Aya ZAMOTO[1], Takako KAWABUCHI[1], Tomomi KATAOKA[2], Rui NAKAJIMA[1], Mitsuhiko ASAKAWA[1] and Chiaki ISHIHARA[1]

[1]*School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu 069 8501 and* [2]*Tama Forest Science Garden, Forestry and Forest Products Research Institute, 1322 Todori, Hachioji, Tokyo 193 0843, Japan*

(Received 27 September 2005/Accepted 1 March 2006)

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.

J. Vet. Med. Sci. 68(7): 643 646, 2006

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 Piroplasmida, 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-

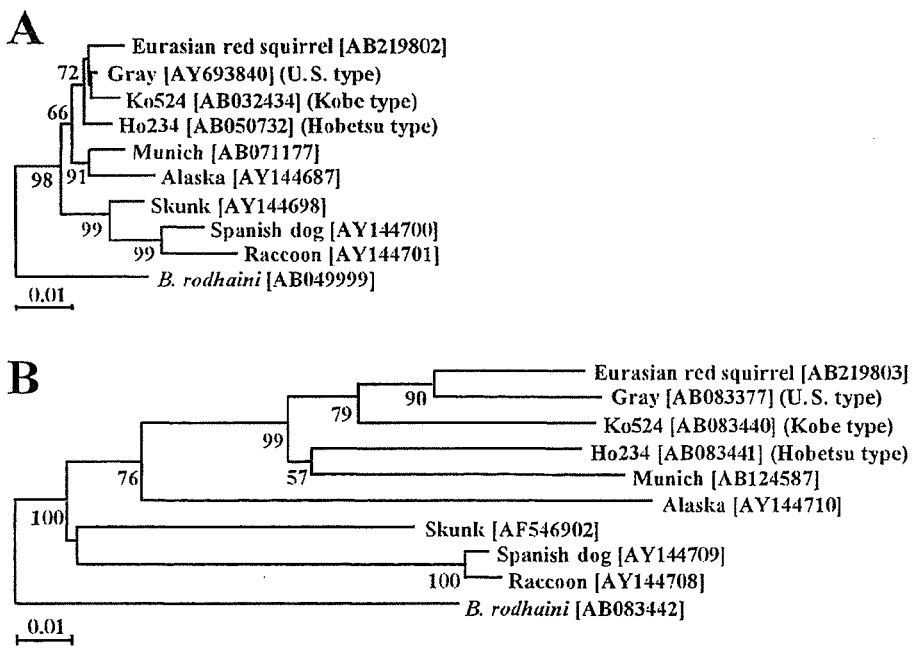

Fig. 1. Phylogenetic trees inferred from the sequences of 18S rRNA (A) and β-tubulin (B) genes. *B. microti*-like parasite in the Eurasian red squirrels and its close relatives were included in the analyses. The GenBank accession number for each DNA sequence is given in parenthesis. The number on each 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.

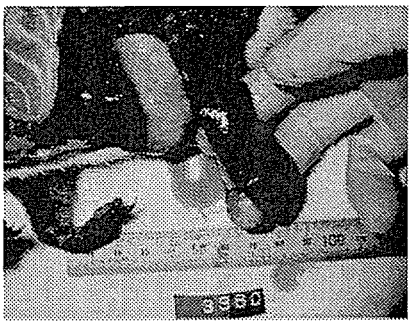

Fig. 2. A splenomegaly observed in the Eurasian red squirrel no. 3360.

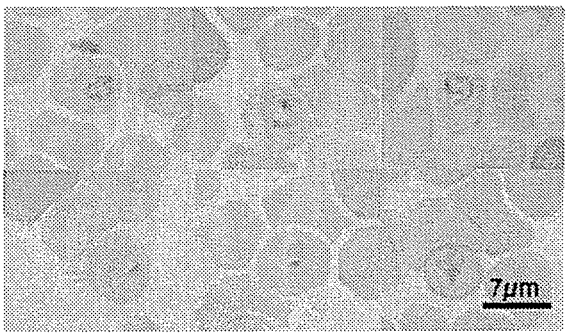

Fig. 3. Morphology of the *B. microti*-like parasites in blood smears from squirrels nos. 3222 (upper panels) and 3360 (lower panels).

tubulin gene; their amplified DNA sequences were identical, 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).

ACKNOWLEDGMENTS. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan, by a Health Science Grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare of Japan, and by Grants-in-Aid from Gakujutsu Frontier Cooperative Research and High Technological Research Centers in Rakuno-Gakuen University.

REFERENCES

1. Arai, S., Tsuji, M., Kaiho, I., Murayama, H., Zamoto, A., Wei, Q., Okabe, N., Kamiyama, T. and Ishihara, C. 2003. Retrospective seroepidemiological survey for human babesiosis in an area in Japan where a tick-borne disease is endemic. *J. Vet. Med. Sci.* **65**: 335 340.
2. Goethert, H. K. and Telford, S. R., III. 2003. What is *Babesia microti*? *Parasitology* **127**: 301 309.
3. Matsui, T., Inoue, R., Kajimoto, K., Tamekane, A., Okamura, A., Katayama, Y., Shimoyama, M., Chihara, K., Saito-Ito, A. and Tsuji, M. 2000. First documentation of transfusion-associated babesiosis in Japan. *Rinsyo Ketsueki* **41**: 628 634 (in Japanese with English summary).
4. Saito-Ito, A., Tsuji, M., Wei, Q., He, S., Matsui, T., Kohsaki, M., Arai, S., Kamiyama, T., Hioki, K. and Ishihara, C. 2000. Transfusion-acquired, autochthonous human babesiosis in Japan: isolation of *Babesia microti*-like parasites with hu-RBC-SCID mice. *J. Clin. Microbiol.* **38**: 4511 4516.
5. Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406 425.
6. Takahashi, K. and Yamashita, S. 1973. *Babesia* sp. from *Sciurus vulgaris orientis* Thomas in Hokkaido. *J. Coll. Dairying.* **5**: 1 6 (in Japanese with English summary).
7. Telford, S. R., III, Gorenflot, A., Brasseur, P. and Spielman, A. 1993. Babesial infections in humans and wildlife. pp. 1 47. *In*: Parasitic Protozoa, 2nd ed., vol. 5 (Kreier, J. P. ed.), Academic Press. Inc., New York.
8. Thompson, J. D., Higgins, D. G. and Gison, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673 4680.
9. Tsuji, M., Wei, Q., Zamoto, A., Morita, C., Arai, S., Shiota, T., Fujimagari, M., Itagaki, A., Fujita, H. and Ishihara, C. 2001. Human babesiosis in Japan: epizootiologic survey of rodent reservoir and isolation of new type of *Babesia microti*-like parasites. *J. Clin. Microbiol.* **39**: 4316 4322.
10. Wei, Q., Tsuji, M., Zamoto, A., Kohsaki, M., Matsui, T., Shiota, T., Teleford, S. R. III and Ishihara, C. 2001. Human babesiosis in Japan: isolation of *Babesia microti*-like parasites from an asymptomatic transfusion donor and from a rodent from an area where babesiosis is endemic. *J. Clin. Microbiol.* **39**: 2178 2183.
11. Zamoto, A., Tsuji, M., Wei, Q., Cho, S.-H., Shin, E.-H., Kim, T.-S., Leonova, G. N., Hagiwara, K., Asakawa, M., Kariwa, H., Takashima, I. and Ishihara, C. 2003. Epizootiologic survey for *Babesia microti* among small wild mammals in Eastern Eurasia with finding of divergence of β-tubulin genes. *J. Vet. Med. Sci.* **66**: 785 792.
12. Zahler, M., Rinder, H., Schein, E. and Gothe, R. 2000. Detection of a new *Babesia microti*-like species in dogs. *Vet. Parasitol.* **89**: 241 248.
13. Zamoto, A., Tsuji, M., Kawabuchi, T., Wei, Q., Asakawa, M. and Ishihara, C. 2004. U.S.-type *Babesia microti* isolated from small wild rodents in eastern Hokkaido, Japan. *J. Vet. Med. Sci.* **66**: 919 926.

Microbiol. Immunol., 48(7), 501–505, 2004

New World Relapsing Fever *Borrelia* Found in *Ornithodoros porcinus* Ticks in Central Tanzania

Harumi Mitani[1], Alison Talbert[2], and Masahito Fukunaga*[,1]

[1]*Laboratory of Molecular Microbiology, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, Hiroshima 729–0292, Japan, and* [2]*Mvumi Hospital, PO Box 32, Mvumi, Dodoma, Tanzania*

Received February 20, 2004; in revised form, March 22, 2004. Accepted April 9, 2004

***Abstract*: Ticks were collected from 8 houses in Mvumi Mission village, near Dodoma, Tanzania. All ticks were examined for *Borrelia* infestation by flagellin gene-based nested polymerase chain reaction. All houses were highly infested with ticks, and all ticks collected were of the *Ornithodoros porcinus* species. Fifty-one out of 120 ticks were infected with spirochetes, and a flagellin gene sequence comparison showed that most of the spirochetes belonged to *Borrelia duttonii*, which is the causative agent of tick-borne relapsing fever in East Africa. The rest of the spirochetes were quite different from *B. duttonii* and instead resembled the New World tick-borne relapsing fever borreliae. Phylogenetic analysis using 16S ribosomal RNA gene sequences also supported the interpretation that the spirochete was a *Borrelia* species distinct from previously described members of the genus.**

***Key words*: Phylogenetic analysis, *Ornithodoros porcinus*, Relapsing fever borreliae**

Tick-borne relapsing fever (TBRF) is found in Africa, Europe, western Asia, and North America (2). The disease is caused by several species of relapsing fever borreliae and is transmitted by various species of *Ornithodoros* and *Argas* ticks (15). Specific relationships often exist between *Borrelia* species and vector tick species, and certain *Borrelia* species are reported to be transmitted by a single tick species (18). *Ornithodoros moubata* complex ticks, such as *O. moubata* and *Ornithodoros porcinus*, are distributed in East Africa and are known to carry *Borrelia duttonii*, which is responsible for Old World TBRF (22). Ticks live in close proximity with humans, and the sole reservoir host for borreliae is thought to be humans. The disease is endemic in central Tanzania, where clinical and epidemiological studies have been conducted (20). TBRF causes a significant amount of morbidity and mortality in the Dodoma region, mainly in children under five and pregnant women (12). People in Dodoma Rural District live in traditional dwellings constructed of mud with earth-covered roofs, and house infestation with *O. moubata* complex ticks is very high (20). Residents sleep on the floor on animal skins, and it is probable that nocturnal tick bites are frequent and pass unnoticed because tick feeding takes only about 30 min.

Cutler et al. (3) have successfully cultivated *B. duttonii* in artificial BSKII medium from pediatric patients in Mvumi Hospital (which is 40 km southeast of the Tanzanian capital Dodoma), and five clinical isolates were characterized genetically. The sequences of 16S ribosomal RNA gene (*rrs*) and flagellin B gene (*flaB*) of *B. duttonii* were quite similar to those of *Borrelia recurrentis*, the causative agent of louse-borne relapsing fever, which is transmitted by the human louse, *Pediculus humanus* (4, 13). We have reported that *O. moubata* complex ticks collected from the houses in Dodoma Rural are highly infected with spirochetes, based on flagellin gene-based nested PCR (9). While most of the borrelia in ticks were *B. duttonii*, we have also found a different spirochete in *O. porcinus* ticks. In this study, we extended our work (8, 14) to elucidate the phylogenetic position of unidentified *Borrelia* found in *O. porcinus* ticks (9) and to screen spirochete infection rates in ticks collected from tick-infested houses in Mvumi village.

Materials and Methods

Ticks, collection sites, and tick species identification. Adult and nymphal ticks were collected from 8 houses

**Address correspondence to* Dr. Masahito Fukunaga, Laboratory of Molecular Microbiology, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, Hiroshima 729–0292, Japan. Fax: +81–84–936–2024. E-mail: maff@fupharm.fukuyama-u.ac.jp

Abbreviations: *flaB*, flagellin B gene; PCR, polymerase chain reaction; *rrs*, 16S ribosomal RNA gene; TBRF, tick-borne relapsing fever.

in Mvumi village (6° 34′60″ S, 37° 10′0″ E) in October and November 2002. Tick species were all *O. porcinus* with morphological characterization, and we also sequenced the mitochondrial *rrs* gene to confirm their species. Mitochondrial gene sequencing is as described previously (11). Ticks were all numbered based on the householder's initials (Table 1).

Culture. Ticks were washed in 3% hydrogen peroxide and dipped in 70% ethanol for 5 min. Ticks were then washed twice in distilled water and dissected individually in BSKII medium. Half the internal tissues were inoculated into 13 ml of BSKII medium within 15 ml culture tubes at 32 C and the rest were used for PCR examination. Cultures were examined for spirochetes with a dark-field microscope and passaged into fresh medium every week.

DNA preparation and PCR amplification. Individual ticks were frozen with liquid nitrogen, crushed manually, suspended in 50–200 μl of 0.02 N NaOH, and boiled 2 min as described previously (9). The aqueous phase was separated by centrifugation, and 1 μl of solution was used for PCR amplification. The primer sets were based on identical sequences in flagellin and 16S rRNA genes of *Borrelia* species (7). The positions listed refer to *B. duttonii flaB* and *rrs* genes: *Bfpad*, 5′-GAT CAA GCA CAA TAT AAC CAT ATG CA-3′ (376–401); *Bfpbu*, 5′-GCT GAA GAG CTT GGA ATG CAA CC-3′ (442–464); *Bfpcr*, 5′-TGA TCA GTT ATC ATT CTA ATA GCA-3′ (765–788); *Bfpdu*, 5′-AGA TTC AAG TCT GTT TTG GAA AGC-3′ (805–828); Bor16F, 5′-GCT GGC AGT GCG TCT TAA GCA TGC-3′ (26–49); Bor16SR, 5′-GTG ACG GGC GGT GTG TAC AAG-3′ (1373–1393). DNA was amplified using *Taq* polymerase (*Ex Taq*, Takara Bio, Japan) according to the manufacturer's protocol. The PCR was performed with the primer sets described above, each containing 50 μl of the reaction mixture, using 30 cycles (40 s at 94 C, 60 s at 50 C, 60 s at 72 C) with GeneAmp PCR system 9700 (Applied Biosystems Japan). If no PCR product was obtained at the first PCR, nested PCR was performed by taking 1 μl of the PCR product under the same conditions. A different set of conditions (40 s at 94 C, 30 s at 67 C, 90 s at 72 C, 35 cycles) was used for borrelial *rrs* amplification. Sequences of both *flaB* and *rrs* PCR products were determined directly in both directions by using custom-synthesized sequencing primers and a BigDye terminator cycle sequencing FS ready reaction kit (Applied Biosystems) as described previously (10, 19).

Sequence analysis. Nucleotide sequences were aligned using the CLUSTAL W software package (21), and neighbor-joining phylogenetic tree construction (17) and bootstrap analysis were done by the Kimura-2-parameter distance method (13) supplied on the web site (www.ddbj.nig.ac.jp). Pairwise alignments were performed with an open-gap penalty of 10, a gap extension penalty of 0.5, and a gap distance of 8. Multiple alignments were also done with the same values, and phylogenetic branches were supported by bootstrap analysis with 100 replications.

Accession numbers. The accession number of the mitochondrial genome sequence is AB105451 (*O. porcinus*), and the sequence determination procedures were the same as were described previously (1). The database accession numbers for the *flaB* reported in this paper are AB057546 (GC9), AB057548 (MK7), AB105128 (MK8), AB057547 (VS4), AB113313 (EM14), and AB113314 (MS3), and the *rrs* sequence accession number is AB113315 (VS4).

Results and Discussion

Borrelia *Infection Rates in Ticks*

Ticks used in this study were all nymphal and adult *O. porcinus* species. As shown in Table 1, each of the 8 houses was highly infested with *O. porcinus* ticks, and those ticks harbored spirochetes at levels from 19 to 86%, with an average of 43% (51 out of 120 ticks) as determined by PCR amplification of the targeted *flaB* gene of *Borrelia*. The sequences of the nested PCR products (341 nucleotides) were determined and aligned with the other flagellin gene sequences (6, 7, 16). Fifty-one *flaB* sequences obtained in this examination were classified into two major groups (Fig. 1). Forty-two of the sequences were *B. duttonii* and the remaining 9 were the new type *Borrelia*. Between the two groups there were 21 nucleotide substitutions, resulting in 7 amino acid substitutions, and 3 nucleotide deletions (data not shown).

Classification of Borrelia *Partial Gene Sequences*

The three sequences (VS4, EM14, and MS3) differed from each other by 1 nucleotide at each locus in the *flaB* gene. The deduced flagellin protein sequences were identical over 113 residues (Fig. 1). The sequences were branched deeply from the sequences of *B. duttonii*, *B. recurrentis*, *B. crocidurae*, and *B. hispanica*, all *Borrelia* species that cause Old World relapsing fever. The sequences of MK7, GC9, and MK8 were all included into the same cluster of Old World relapsing fever borreliae. The new type of borrelia found in *O. porcinus* ticks rather resembled *B. hermsii*, *B coriaceae*, *B. turicatae*, and *B. parkeri*, all of those borrelias distributed in North America. *B. miyamotoi* and *B. lonestari* are the species found in hard-bodied ixodid ticks exclusively, but they are closely related to relapsing

Table 1. Source of *Ornithodoros moubata* species complex ticks and detection of spirochetes in ticks by PCR and culturing of their internal tissues

Householder's initials	PCR		Culture	
	Borrelia positive	New type *Borrelia*	*Borrelia* positive	New type *Borrelia*
MK	12/18	1/18	1/27	0/27
DN	2/7	1/7	NT[a)]	
EM	6/17	1/17 (EM14)	NT	
JA	4/21	0/21	NT	
MS	11/22	4/22 (MS3)	2/40	0/40
RC	3/5	1/5	NT	
GC	7/23	0/23	NT	
VS	6/7	1/7 (VS4)	NT	
Total	51/120 (42.5%)	9/120 (7.5%)	3/67 (4.5%)	0/67 (0%)

[a)] NT, Not tested.

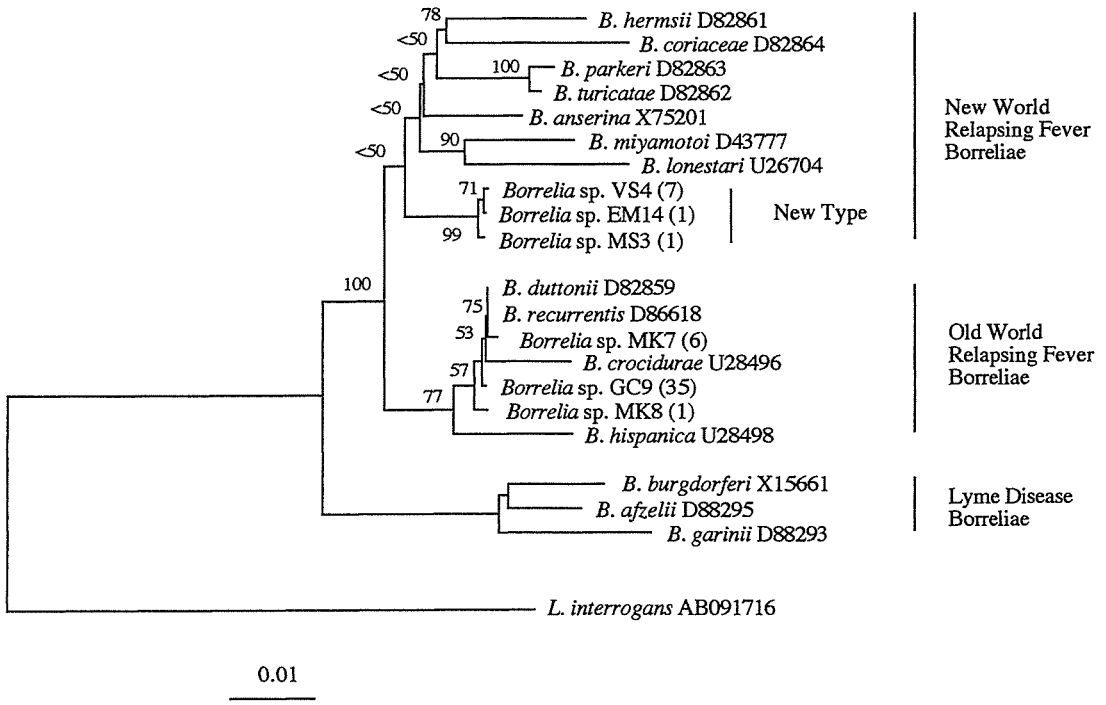

Fig. 1. Phylogenetic tree derived from the nucleotide sequences of the *flaB* gene of the new type of borrelia. The neighbor-joining tree was constructed using the sequences of partial flagellin genes. The bar represents the calculated distance value. Bootstrap values (100 duplications) are indicated to the left of each relevant branch. The numbers in parentheses indicate the number of tick-infected borrelia sequences determined. Accession numbers for the *flaB* shown in this tree are AB057546 (GC9), AB057548 (MK7), AB105128 (MK8), AB057547 (VS4), AB113313 (EM14), and AB113314 (MS3), and the other accession numbers of reference strains are all indicated in the figure. *Leptospira interrogans flaB* sequence (X17547) was used as the out group sequence.

fever borreliae (4, 6, 8). Most of the borrelias found in *O. porcinus* ticks are clustered into the *B. duttonii-B. recurrentis* clade.

The partial *rrs* sequence in VS4 was 1,368 nucleotides and there were 17 transitions between that of *B. duttonii* strain Ly. The *rrs* sequences in EM14 and MS3 were identical to that of the VS4. Further phylogenetic analysis was done by comparison of *rrs* sequences (Fig. 2). The *rrs* sequence found in tick (VS4) showed the following similarities to the other borrelial *rrs* sequences: *B. burgdorferi*, 95.7%; *B. duttonii*, *B. recurrentis*, *B. crocidurae*, *B. hispanica*, 98.5%; *B. anserina*, 98.2%; *B. hermsii*, 98.5%; *B. parkeri*, 98.7%; *B. turicatae*, 98.8%. The neighbor-

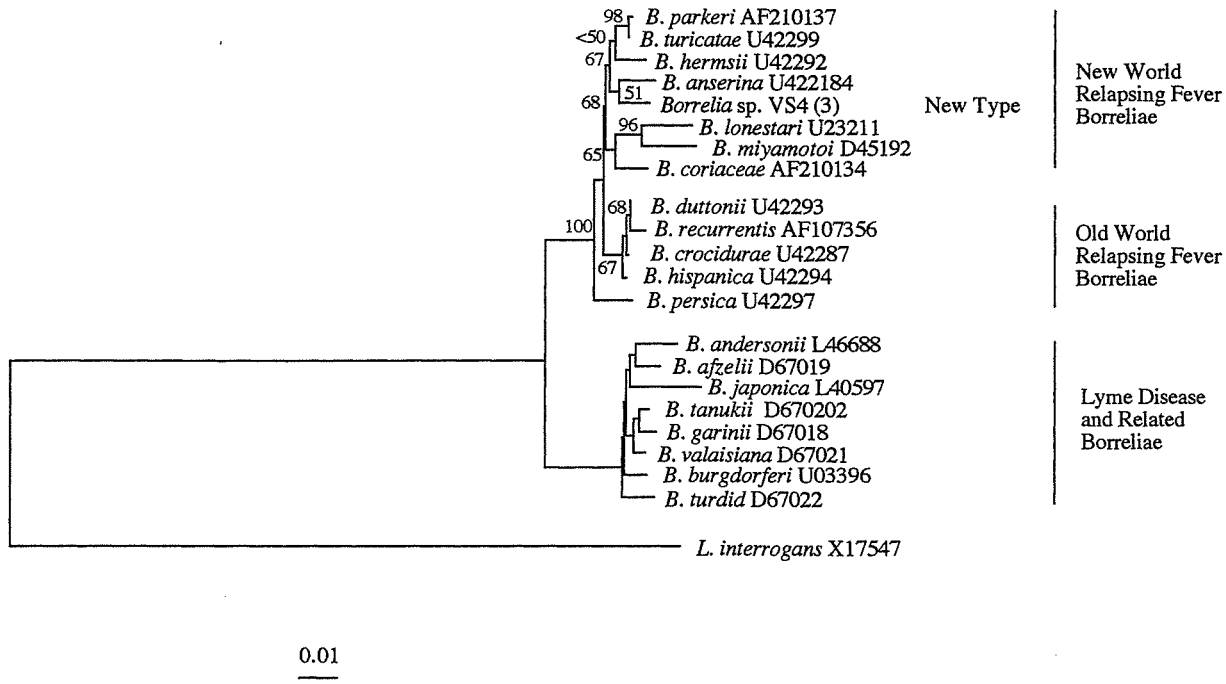

Fig. 2. Phylogenetic tree derived from the nucleotide sequences of the *rrs* gene of the new type of borrelia. The neighbor-joining tree was constructed using the sequences of partial 16S ribosomal RNA genes. The bar represents the calculated distance value. Bootstrap values (100 duplications) are indicated to the left of each relevant branch. The accession number for the tick *Borrelia rrs* sequence is AB113315 (VS4), and the other accession numbers are all shown in this figure. *Leptospira interrogans rrs* sequence (AB091716) was used as the out group sequence.

joining phylogenetic tree showed that the new type of borrelia found in *O. porcinus* ticks clustered with a group containing the New World relapsing fever borrelia species and a bird borreliosis agent. Bootstrap values in both trees supported the sequence being clustered with a non-Old World relapsing fever borreliae group and being most closely related to New World relapsing fever borreliae.

Borrelia *Culture*

Cutler et al. (3) successfully cultivated 5 TBRF borrelia strains from 12 blood samples of pediatric patients but these were all *B. duttonii*. To date, *B. duttonii* has only been cultivated from human blood specimens and not from ticks. Our recent investigation using the nested PCR for *flaB* gene for *Borrelia* detected the new type spirochetes in blood samples from both healthy children (5/11) and children with fever (1/6) in a village 12 km from Mvumi Mission (5, 14). This raises the possibility that the new *Borrelia* may be a causative agent for TBRF but successful culture will be necessary to confirm this.

To attempt *Borrelia* culture from *O. porcinus* ticks, two houses were chosen, and 67 ticks (27 ticks from the house MK and 40 ticks from the house MS) were used to cultivate the new type of *Borrelia* (Table 1). These ticks were estimated to be infected with new type of borrelia at 6% (1/18) and 18% (4/22), respectively, by PCR data (Table 1), and PCR examination of their internal tissues also showed 14/27 and 18/40 were *Borrelia* positive. However no new type spirochete grew in the BSKII media, and the three successful cultures from the ticks were all *B. duttonii*.

It seems likely that many of the spirochetes found in the ticks in this area are the new *Borrelia* species, not *B. duttonii*, and that *O. porcinus* acts as a competent vector for TBRF locally. Whether the new type *Borrelia* is an etiological agent for TBRF in central Tanzania remains to be determined. At least we must realize that *O. porcinus* ticks can transmit both *Borrelia* species in the Dodoma region and that one of these species resembles North American TBRF *Borrelia*. The evolution and adaptive radiation of borrelia is of interest.

We thank W. Kisinza and the people of Mvumi village for their help in collecting ticks, and to Y. Ushijima for her technical assistance. This study was funded partly by a grant to MF (no. 14570250) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.