A comparison of the REAP patterns obtained from the outbreak isolates and other Japanese isolates is shown in Fig. 3. All outbreak isolates showed the same REAP pattern, designated as pattern S, while the patterns of the other Japanese isolates have been identified as P1 to P3 (14). REAP pattern S corresponds to pattern P3.

Japanese O:8 isolates have been classified into seven genotypes (I–VII) based on the combination of the results of PFGE by *Not*I and ribotyping by *Eco*RI (14). Our 30 outbreak isolates were classified into two genotypes, with 29 belonging to a genotype similar to the genotype VI of the isolates from wild rodents captured in Niigata Prefecture, and 1 outbreak isolate from a black rat belonging to a genotype similar to the genotype VII of the isolate from wild rodent captured in Yamagata Prefecture (Table 3).

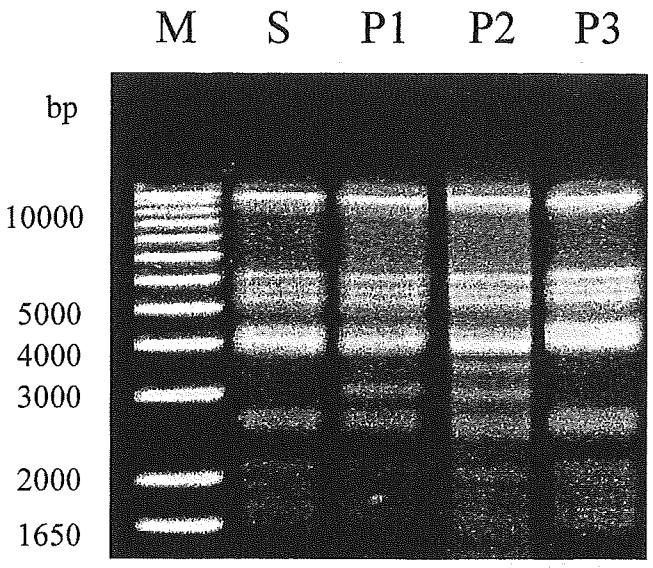

Fig. 3. Comparison of the REAP patterns of representative outbreak isolate (lane S) and Japanese isolates (lanes P1 to P3). Lanes: M, molecular weight marker; S, NY0212001 (squirrel monkey); P1, NY9306089 (wild rodent); P2, NY936005 (wild rodent); P3, YE9809001 (wild rodent).

Discussion

To the best of our knowledge, this is the first report of fatal cases of *Y. enterocolitica* serovar O:8 infection in monkeys anywhere in the world. There have been some reports of *Yersinia* spp. outbreaks in monkeys all over the world showing that monkeys are sensitive to bacteria of this genus (6, 15, 21, 31, 39). However, the majority of these outbreaks, which usually affect a large number of monkeys and show high mortality, are caused by *Y. pseudotuberculosis*. *Yersinia* outbreaks in breeding monkeys have also been reported in Japan, but all reported outbreaks were of *Y. pseudotuberculosis* (15, 21, 27, 29, 34, 39). Therefore, the present study indicates the need for more attention to the possibility of the occurrence of *Y. enterocolitica* serovar O:8 outbreaks, especially in countries such as the United States and Japan, where reservoirs of this serovar are found in nature (1, 8, 13, 18, 19, 22, 33, 35).

Moreover, although a human case of *Y. enterocolitica* serovar O:8 infection was reported in 1997, in Kanagawa Prefecture (16), located at the south of the Kanto region that lies in the central part of Honshu island, isolation of this pathogen from rodents has been reported only in wild rodents of the north area of Honshu island (13, 19). This study showed the presence of this pathogen in black rats living in the Kanto region, indicating that serovar O:8 strains are also present in this area.

Molecular genetic analysis of the 30 isolates from the analyzed samples showed that all but one had the same molecular genotype, suggesting that these isolates

Table 3. Typing results for *Yersinia enterocolitica* serovar O:8 isolated in Japan using PFGE and ribotyping

Genotype[a)]	PFGE pattern	Ribo-pattern	REAP pattern	Strain	Region	Source
I	A	R1	P1	YE93009	Aomori	Patient
II	A	R4	P1	YE89023	Aomori	Patient
III	B	R1	P2	APCC Y9314	Aomori	Patient
IV	B	R3	P2	YE87069	Aomori	Patient
V	C	R1	P1	NY9504002	Aomori	Wild rodent
VI	D	R1	P3	NY891001	Niigata	Wild rodent
VII	D	R2	P3	YE9809001	Yamagata	Wild rodent
VI′	D′	R1	P3	NY0212001	Saitama	Squirrel monkey
VII′	D′	R2	P3	NY0304008	Saitama	Black rat

[a)] Genotype was produced by combining the results obtaining using PFGE with *Not*I and ribotyping.
The prime (′) denotes a closely related type or pattern.

originated from a common source. Nevertheless, it is unlikely that the bacterium spread by direct transmission from the squirrel monkeys to the dark-handed gibbon because the enclosure for these two monkey species are separated and the people responsible for the squirrel monkeys do not work with the dark-handed gibbons or vice-versa. Moreover, the historical order in which the infection occurred in the different monkey species in the Zoological Garden, with the initial outbreak among the squirrel monkeys (December 2002 to January 2003) followed by the case of the dark-handed gibbon (April 2003), together with the results of molecular genetical analysis, suggests that O:8 infection occurred first in the colony of squirrel monkeys, and was then transmitted to the dark-handed gibbon. Since the isolates from black rats had the same molecular genotypes of the two monkey species, these rats might be the vector between the two species. Moreover, given that the prevalence of *Y. enterocolitica* serovar O:8 in the black rats captured in this area was relatively high, and considering the time lag between the infection of the two colonies of monkeys, the black rats might be considered a reservoir of strains of this serovar.

A comparison of the molecular genotypes of the isolates of the present study with other Japanese isolates analyzed by Hayashidani et al. (14) shows that the molecular genotype of 29 of the present isolates was highly similar to that of the strains isolated from wild rodents captured in Niigata Prefecture, which borders the Kanto region in the northwest, and that the genotype of the 1 isolate that differed from the other 29 showed a molecular genotype similar to that of an isolate from a wild rodent in Yamagata Prefecture, located in the northeast of Japan. It is tempting to speculate that the strains isolated in the present study might have originated in wild rodents. Future detailed epidemiological studies are necessary to elucidate the origin or the route of transmission of these strains.

Pathogenic *Yersinia* including *Y. enterocolitica* serovar O:8 are a cause of zoonotic disease, and we cannot deny the possibility of human infection from monkeys, especially when an outbreak occurs throughout a colony. Therefore, from the point of view of public health, it is important to develop preventive methods, such as effective vaccines, to prevent pathogenic *Yersinia* spp. infection in monkeys.

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FULL PAPER *Public Health*

Isolation of a Human Erythrocyte-Adapted Substrain of *Babesia rodhaini* and Analysis of the Merozoite Surface Protein Gene Sequences

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ABSTRACT. *Babesia rodhaini* is a rodent hemoparasite closely related to *B. microti*, the major causative agent of human babesiosis. We tested the infectivity of *B. rodhaini* for human erythrocytes by using the SCID mouse model in which the circulating erythrocytes were replaced with those of humans. Initially, parasites grew very poorly in the mouse model, but a variant capable of propagating in human erythrocytes emerged after an adaptation period of three weeks. In an attempt to identify parasite proteins involved in the alteration of host cell preference, an expression cDNA library of *B. rodhaini* was constructed and screened with immune mouse sera. Although we were able to obtain three merozoite surface protein (MSP) genes, sequences of these genes from both the parental strain and human erythrocyte-adapted substrain were identical. Our results suggest that *B. rodhaini* has potential ability to infect human erythrocytes, but development of this ability may not be brought about by an amino acid change in MSPs.

KEY WORDS: adaptation, *Babesia rodhaini*, human erythrocyte, merozoite surface protein.

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Babesia rodhaini is an intraerythrocytic protozoan parasite that has been used in many studies as a laboratory model for babesiosis [1, 4, 6, 10, 12, 20, 22, 26, 28, 29, 31]. According to the original description by van den Berghe *et al.* [36], the parasite was isolated from a wild rodent, *Thamnomys surdaster*, captured in Congo, and maintained by syringe-passages in laboratory mice. There is no other report describing isolation of this species of parasite from wild animals. Nonetheless, several laboratory strains of *B. rodhaini* are currently maintained in various laboratories in the world. Whether they are identical parasites originated from a single source is unclear. While abundant knowledge about the host-parasite inter-relationship has been gained through experimental infection studies with *B. rodhaini* [1, 6, 10, 12, 13, 20, 22, 26, 28, 31], little information is available about the epidemiology and zoonotic potential of this parasite.

Babesia microti is another rodent *Babesia* that has also been used in many studies [6, 9, 13, 20, 23, 25–27, 35]. Unlike *B. rodhaini*, however, the epidemiology of *B. microti* has been well studied [11, 14, 30], largely because the parasite is known as the causative agent of human babesiosis. The taxonomic relationship between *B. rodhaini* and *B. microti* has long been a matter of argument because of their morphological resemblance, immunological cross-reaction, and similar host range [6, 16, 20, 27]. In the literature, *B. rodhaini* was once regarded as a synonym of *B. microti* [16, 17, 30]. Molecular evidence from recent studies [9, 14, 21, 38] also suggested their close relationship, but phylogenetic distance between the two parasites clearly places them into distinct species [9].

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It is interesting that although both *B. rodhaini* and *B. microti* are capable of infecting the laboratory mice the outcomes of the respective infections differ dramatically. While *B. rodhaini* causes an acute fulminating disease and kills nearly 100 % of infected mice, *B. microti* causes a relatively mild, chronic disease that is usually non-lethal [6, 10, 13, 26]. Hence, these two species have served as useful experimental tools to elucidate mechanisms involved in virulence and pathogenesis of intraerythrocytic parasites [6, 13, 26]. Experimentally, both *B. rodhaini* and *B. microti* have wide host ranges, and susceptible laboratory animals include mice, rats, hamsters, gerbils, and monkeys [10, 23, 25, 27]. Natural infections by parasites closely related to *B. microti* or *B. rodhaini* have increasingly been reported in dogs [38], cats [8], lions [21], foxes [9], skunks [9], raccoons [2], and baboons [5]. Nonetheless, *B. microti sensu stricto* ("clade 1" in the reference [9]) is currently regarded as the sole etiologic agent of human babesiosis within the group of parasites.

Whether *B. rodhaini* is infective for humans is a simple question that has not been investigated experimentally. The major difficulty in addressing this question is the lack of a suitable experimental system. *In vitro* cultivation techniques available for other erythrocyte-dwelling protozoa, such as *Plasmodium falciparum* [32] and *Babesia bovis* [18], have not been established for this parasite. We developed the erythrocyte-replaced SCID mouse model for bovine hemoparasites [33] more than a decade ago, but it was only very recently that usefulness of this model for human babesiosis has successfully been demonstrated [24]. In the present study, we tested infectivity of *B. rodhaini* for human erythrocytes by using SCID mice in which the circulating red blood cells (RBCs) were replaced with those of humans (designated as the hu-RBC-SCID mouse model).

Because this study provided us with a variant substrain capable of multiplying in human erythrocytes, we also investigated the merozoite surface protein genes (*msp*) in an attempt to identify parasite proteins that may be involved in the alteration of host cell preference.

MATERIALS AND METHODS

Strain of parasite: The Australian strain of *Babesia rodhaini* used in this study has previously been described [35]. This strain has been shown to be highly virulent; inoculation of as few as 10 parasites into BALB/c mice resulted in 100% lethality. Convalescent sera containing antibodies against the parasites were prepared in BALB/c mice by infection and treatment with diazoaminodibenzamidine diaceturate (Ganaseg; Ciba-Geigy Japan, Takarazuka, Japan) according to the method described previously [1].

Laboratory animals: NOD/shi-*scid* mice [15], which were originally obtained from the Central Institute of Experimental Animals, Kawasaki, Japan, were maintained in the Laboratory Animal Facility in Rakuno-Gakuen University. BALB/c mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). All animals were housed in isolators at a temperature between 22 and 25°C and were provided with a γ ray-irradiated pellet diet and autoclaved tap water. Animal experimentation was carried out according to the Laboratory Animal Control Guidelines of Rakuno-Gakuen University.

Hu-RBC-SCID mouse model: The procedures for preparation of the hu-RBC-SCID mouse model were basically in accordance with those described previously [24, 34]. Three splenectomized NOD/shi-*scid* mice were used for the experiment, in which parasite infection was initiated by an intravenous injection of 2×10^8 parasitized RBCs. Human type-O RBCs were obtained from a healthy volunteer. Transfusions of 0.5 m*l* of a packed cell volume of human RBCs (approximately 5×10^9) were intravenously given to the mice on the days indicated. When the human RBC-adapted variant began to emerge in the mouse, 100 μ*l* of rat anti-mouse RBC monoclonal antibody, clone 2E11 [19], was intraperitoneally administered to remove mouse RBCs in the mouse. For monitoring infection, a small amount of blood was collected from the tail vein of the mice. The level of parasitemia, expressed as the percent of parasitized erythrocytes, was microscopically determined with Giemsa-stained thin-smear blood films. The percentage of human RBCs in the total RBCs were determined by flow cytometry (Cyto ACE-150; JASCO Co., Tokyo, Japan) with RBC samples stained with a biotin-labeled anti-human RBC mouse immunoglobulin G Fab fragment [34] and phycoerythrin-labeled streptavidin (Life Technologies, Rockville, MD, U.S.A.). For two colors analysis, RBC samples were additionally stained with SYTO16 as described previously [37].

Construction and screening of B. rodhaini cDNA library: Total RNA was extracted from *B. rodhaini*-infected RBCs (approximately 50% parasitemia) with Trizol reagent (Life Technologies) according to the manufacturer's instructions. Poly $(A)^+$ mRNAs were purified from total RNA with an oligotex-dT30 mRNA purification kit (TaKaRa, Otsu, Japan), and cDNAs were prepared with a Zap-cDNA synthesis kit (Stratagene, La Jolla, CA, U.S.A.). Synthesized cDNA fragments were ligated into *Eco*RI/*Xho*I site of the λ Uni-Zap XR vector, followed by mixing with Giga Pack III Gold Packaging Extract (Stratagene) to form phage particles. The cDNA library had a titer of 1.84×10^6 plaque-forming units. This expression cDNA library was then screened with serum antibodies from convalescent BALB/c mice. To reduce non-specific reactions, the serum antibodies were passed three times through a Sepharose 4B column on which Y1090/λgt11 lysate was immobilized (BioDynamics Laboratory, Inc., Tokyo, Japan). *Escherichia coli* strain XL-1 Blue MRF' infected with 3×10^4 phages were grown on agar plates at 42°C for 4 hr. Plates were then overlaid with nitrocellulose membranes pre-soaked with 100 mM isopropyl-β-D-thiogalactopyranoside, and incubated at 37°C for 3 hr. Plaques on the membranes were incubated with anti-*B. rodhaini* antibodies, followed by alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (heavy and light chains). Positive plaques were visualized by development with an alkaline phosphatase substrate kit IV containing 5 bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (Vector Laboratories, Inc. Burlingame, CA, U.S.A.). Plaque purification was repeated until all plaques exhibited positive signals. Phage clones were converted into plasmids by *in vitro* excision as described in the manufacturer's instructions (Stratagene). Plasmid DNAs were prepared with the Wizard plus SV minipreps DNA system (Promega, Madison, WI, U.S.A.), and used for sequencing.

Sequencing analysis: Nucleotide sequences were determined with plasmid DNAs for both strands. Sequencing reactions were carried out with an AutoRead sequencing kit (Amersham Phermacia Biotech, Uppsala, Sweden) with Cy5-labeled primers. Samples were analyzed with a DNA sequencer (ALFexpress; Amersham Pharmacia Biotech), and sequence data were processed with the associated software (ALFwin Sequence Analyser, version 2.00). With the cDNA clones obtained by immunoscreening, approximately 300 bases from both ends of the inserts were determined. Homology search in the DNA database of Japan (DDBJ) was carried out for each sequence using FASTA search.

Reverse transcription-PCR for MSPs: Total RNA was prepared from a human RBC-adapted variant substrain of *B. rodhaini* in the same manner as described above. After the RNA sample was treated with RNase free-DNase I (Amersham Pharmacia Biotech), reverse transcription (RT) reaction was performed with the Thermoscript RT-PCR system (Invitrogen, Corp., Carlsbad, CA, U.S.A.). PCR mixture consisted of 400 μM of each deoxynucleoside triphosphate, 0.25 μM of each primer (MSP1, forward 5'-TTTGTATAT-AGATGGCTGCTGT-3' and reverse 5'-GTTAGTTACA-CATGATAATAATCGT-3'; MSP2, forward 5'-TTGT ATATAAATGTCAGCTGTTAAA-3' and reverse 5'-ATT

GATTAAAGTCTAATATACGGCT-3'; MSP3, forward 5'-CCATATTATATATAAATGTCAGCTC-3' and reverse 5'-ACCAATAAGCTAGTTAGCATGAC-3'), 100 *ng* of template, and 2.5 U of LaTaq DNA polymerase (TaKaRa) in 20 μl of PCR buffer supplied with the enzyme. Thermal cycling was carried out in Program Temp Control System PC-320 (ASTEC, Fukuoka, Japan) with a program including 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 40 sec, and extension at 72°C for 90 sec. Amplified DNAs were purified with a Min Elute reaction clean up kit (Qiagen, Hilden, Germany), followed by cloning into pCR2.1-TOPO (Invitrogen).

Nucleotide sequence accession numbers: The nucleotide sequences described in this paper have been deposited in DDBJ under accession numbers listed in Table 1.

RESULTS

Parasite infection in hu-RBC-SCID mice: Prior to testing the infectivity of *B. rodhaini* for human RBCs, parasites were inoculated into three splenectomized NOD/shi-*scid* mice and propagated in the mouse RBCs. Only the result from a mouse in which a human RBC-adapted variant arose is depicted in Fig. 1. On day 2 post-infection (PI), when parasitemia became 38.3%, the mouse began to receive a transfusion of 5×10^9 human RBCs. Following daily transfusions given from day 3 to day 6 PI resulted in rapid decrease of parasitemia, which enabled the mouse to survive an otherwise lethal infection. Contrary to the decrease in parasitemia, the percentage of the human RBCs in the total blood cells increased, because only a very few human RBCs were infected by parasites, while leaving the mouse RBCs to be multiply infected and destroyed (Figs. 2 and 3). Transfusions of human RBCs were given less frequently from day 8 to day 24 PI, during which parasitemia became very low but

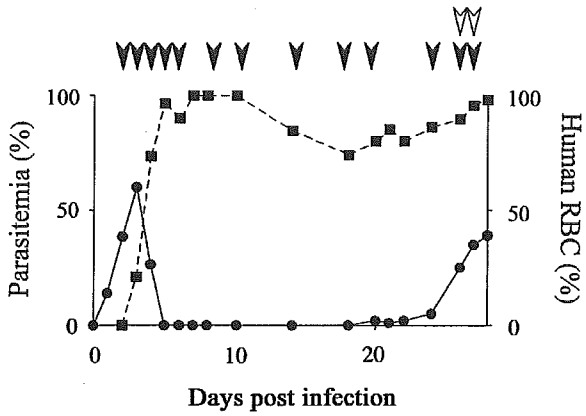

Fig. 1. *B. rodhaini* infection in the hu-RBC-SCID mouse model. A splenectomized NOD/shi-*scid* mouse was infected with *B. rodhaini* on day 0, and the peripheral blood samples were examined for the level of parasitemia (circles on a solid line) and for the rate of replacement with human RBCs (squares on a dotted line). Transfusions of human RBCs were given on days indicated by solid arrowheads. Anti-mouse RBC monoclonal antibody, 2E11, was administered on days indicated by open arrowheads.

Table 1. *B. rodhaini* cDNA clones obtained by immunoscreening

Gene	No. of clones obtained	cDNA clones	Accession number
msp1 (*p26*)	11	Br3–1, Br12–2[a)], Br16–1, Br17–1, Br22–2, Br25–1, Br28–2, Br30–1, Br34–1, Br39–3, Br42–1	AB183391
msp2 (*p26*)	5	Br4–1, Br11–2, Br23–4[a)], Br31–3, Br37–1	AB183392
msp3 (*p17*)	1	Br2–3[a)]	AB183393
hsp70[b)]	4	Br29–1[a)], Br38–1, Br41–1, Br42–3	AB188238
grp78[c)]	1	Br24–4[a)]	AB188239
Mouse *alpha-globin*	3	Br6–1, Br10–2, Br26–3	
Mouse *beta-globin*	2	Br5–1, Br14–2	
Unidentified[d)]	15	Br7–4, Br8–2, Br13–1, Br14–4, Br15–1, Br18–2, Br19–1, Br20–2, Br23–1, Br28–1, Br32–5, Br34–2, Br36–1, Br39–3, Br42–2	
Total	42		

a) Clones used for determination of full-size cDNA sequences that were reported to DDBJ.
b) Heat shock protein 70.
c) 78-kDa glucose-regulated stress protein.
d) Genes with significantly high sequence similarities were not found.

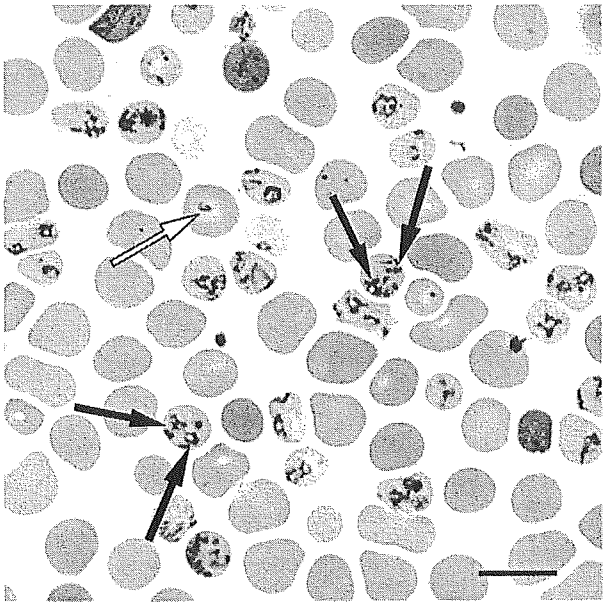

Fig. 2. Light micrograph of Giemsa-stained thin-smear blood film. The blood sample was obtained on day 4 PI from the mouse shown in Fig. 1. The human and the mouse RBCs are distinguishable owing to distinct tone of coloration and difference in size (mouse RBCs are smaller than human RBCs). Of the total RBCs, 73.7% and 26.3% were of human and mouse, respectively; most of the mouse RBCs contained multiple parasites (closed arrows), whereas only a few of the human RBCs contained a single parasite (open arrow). Bar, 10 μm.

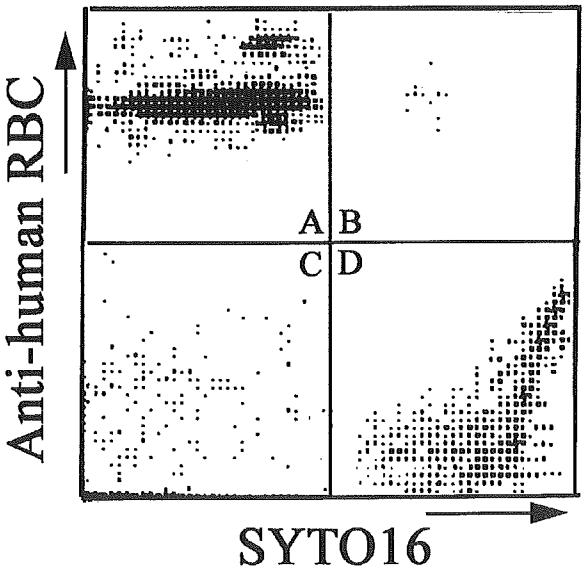

Fig. 3. Flow cytometry of the blood sample obtained from the hu-RBC-SCID mouse on day 4 PI shown in Fig. 2. Two-colour analysis showed that 71.8%, 1.84%, 5.6% and 20.7% of the red blood cells are in regions A (non-parasitized human RBCs), B (parasitized human RBCs), C (non-parasitized murine RBCs), and D (parasitized murine RBCs), respectively.

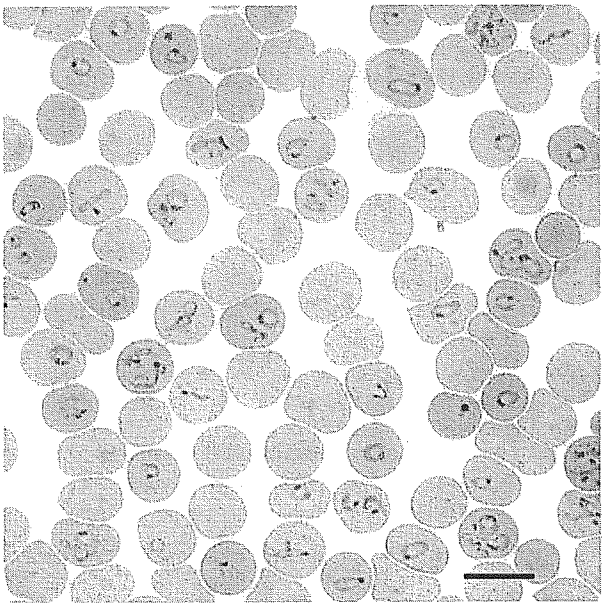

Fig. 4. Light micrograph of Giemsa-stained thin-smear blood film showing *B. rodhaini* propagating in the human RBCs in the hu-RBC-SCID mouse. Blood sample was obtained from the mouse shown in Fig. 1 on day 28 PI, when parasitemia was 39% and the human RBCs consisted of 98% of the blood cells in the mouse. Bar, 10 μm.

did not disappear completely. Apparent increase of parasitemia began on day 24 PI, at which time transfusions were again given daily. Anti-mouse RBC monoclonal antibody 2E11 [19] was also administered when parasitemia increased over 25%. On day 28 PI, when whole blood was harvested from the mouse and cryopreserved, the blood sample contained 98% human RBCs and 39% parasitemia (Fig. 4), indicating that parasites became capable of propagating in human RBCs. The infectivity for human erythrocytes was stable after passages into new hu-RBC-SCID mice. The two other mice used in the experiment also exhibited parasitemia profiles similar to that in Fig. 1, but because of unfortunate episodes, they were lost before yielding human RBC-adapted variants.

cDNA cloning for MSPs: Because the change of host cell preference is presumed to be associated with an alteration in parasite proteins involved in host cell attachment and invasion, we attempted to clone genes encoding merozoite surface proteins (MSPs) of *B. rodhaini*. Taking advantage of MSPs being immunodominant proteins, we constructed an expression cDNA library with mRNA from the parental strain of *B. rodhaini* and screened it with serum antibodies from convalescent mice. Immunoscreening yielded 42 cDNA clones, for each of which approximately 300 bases from both the 5' and 3' ends of the insert were sequenced. The results of FASTA search in DDBJ, shown in Table 1, revealed that 17 of the 42 clones encoded genes for three kinds of MSPs, designated as *msp1*, *msp2*, and *msp3*, which were similar or identical to those previously reported by

Snary and Smith [29] with the Antwerp strain of *B. rodhaini*. Eleven of the 17 clones encoded *msp1*, five *msp2*, and one *msp3*; the numbers are probably in proportion to the expression level of each gene. As shown in Fig. 5, *msp1* and *msp2* in this study were similar to the gene *p26* reported by Snary and Smith [29], whereas *msp3* in this study was

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p26   ATGGCTGCTGTTAAATTAACTTTATTTACTTTCTTGGCTGTTGCCTTTACCTATAACCCAATTTTCTGTGCTGGT   75
msp1  ...............................................................................   75
msp2  ...T.A.....................................G.........G.............--          73

p26   GCTGGTGCTGAAGGCTCTGCTGGTAATGCCGAGGGCCAACAAGAAAGCTCCGTTGATAGTTCTCCTACTGGTGGT  150
msp1  ...............................................................................  150
msp2  ----------.........G..A.....-------------------------------------------------   91

p26   GCCGGTAGTAATAATAATGCTGGAGGTGCTCCTGCCCCTCCTCCTGCAAATCCTGAGGGCCAACAAGAAGGCGCC  225
msp1  ...............................................................................  225
msp2  ------------------------------------------------.C.....................        114

p26   GTTGATAATACCAAACTTATAGCCGCTTTGAAGGTTACTGATGATGGTCTCAAGACTGATATCTCCACCACTTCT  300
msp1  ...............................................................................  300
msp2  ..........................G.....................AA....A........T..TGTTT.C.G.   189

p26   GAACA-AGTTCAAAGCAAATTGAAGACTCAATATACCACTTATCTTGGCAAGATTAATAAGGAAGGTAATGATGA  374
msp1  .....-.................................................................... .  374
msp2  -..T.C.T.AGCTG.......AC.TGG...T.TC.AT.AC...A..ATT.GA...G....T.....C.....A..  263

p26   ATACTTCAAAGATACCAAGGAAAA---A--TACGACCAAT-----TG--AATAATAATATTAAGGCCGCTATTGA  437
msp1  ........................---.--.........-----..--.....................  437
msp2  T..TA.T..G..GG....A.....GGA.CC..A.CT....AAAGC..TTG.CG..GC.........TCT.TC...  338

p26   TGCCTTTAAGGCTGCTGATGACTGGCATACTCAGGTTGATGCTGTTCTTAACTCCCTTTCCGAATTAGCTGAATT  512
msp1  ...............................................................................  512
msp2  ...--------.--A...........G..T....A.............................AT........  404

p26   CGTACAATCTGTTTACGATAAGGCCAACGGTAACTTGAAGGATGATAATGTCGCCAAGCTTGCTAGTGAAATGTA  587
msp1  ........................C.......T.....................................  587
msp2  ...............................................................................  479

p26   TAAGAACAAAGCTGATACCGTCAGATCTTTGGTAGGTTTCTATGAAGCTATTATGACTAGATGTTCAACTGAAGT  662
msp1  ..............................................G...C..T.AC.A...C..T.........  662
msp2  ...............................................................................  554

p26   TACTTCTGCTGCTGATGTTAGCGAGGAAATTAAGACCGCTATTACTGAAGCTAAGACTAAATTTGATGAATTGAA  737
msp1  ..................................A.A.........................................  737
msp2  ...............................................................................  629

p26   GAATGAAATTGAGGATCTCGTCACTAAGCTTAATTCTGAGAAGGATAAGGCTGCTGAGCACTTTGATGAACTTAT  812
msp1  .CCC.GT..C..A..AG.T.CTGG...AG...G...C..T..A..G.....................................  812
msp2  ...............................................................................  704

p26   GTATAAGATGTCAGAAATCTCTGAATACGCTAAGACTGCCTACTCAAAGATTCTCGAAAACAGAGATGAGAAACA  887
msp1  ....................................C.........T...G..A.T.....................  887
msp2  ...............................................................................  779

p26   AGTTGAATACAAGAAGGAAATCCAACACAAGCTTTTTAATTTCATCTCTGAAGCTATCAACATTTCAAGATCCGT  962
msp1  ....................................G.C......A.C.......C..G...C..G......A.  962
msp2  ...............................................................................  854

p26   TATCCCTGGCTTCTTCGCCATCGCTCTTTCCTTCCTCATGGTTGCTCTATAA                        1014
msp1  ......C............G..........TC..GC....C.CT..T.....                         1014
msp2  ..................................................                          906
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Fig. 5. Multiple-sequence alignment of *msp1* and *msp2* from the Australian strain of *B. rodhaini* (accession numbers AB183391 and AB183392, respectively) and the gene *p26* from the Antwerp strain (accession number M19145). Only sequences that differ from *p26* are shown. Gaps were inserted to maximize alignment. Start and stop codons are indicated by double underline. The underlined regions at the 5' and 3' ends encode hydrophobic amino acids for the putative signal peptide and phosphatidylinositol anchoring, respectively [29].

exactly the same as the gene *p17* [29]. The cDNA clones obtained by immunoscreening also contained genes encoding stress proteins (*hsp70* and *grp78*) and mouse globins, indicating that significant amounts of antibodies were produced against those proteins during *B. rodhaini* infection.

Msp genes in the variant substrain: Open reading frames (ORF) of the three *msp* genes were amplified by RT-PCR with mRNA from the human RBC-adapted variant of *B. rodhaini*. Sequencing analysis for each of the three genes, however, showed that they were identical to those of the parental strain.

DISCUSSION

This study showed that *B. rodhaini* is potentially infective for human RBCs, and represents the first report of such demonstration on a *Babesia* parasite with uncertain pathogenicity to humans. This result does not necessarily indicate that *B. rodhaini* is actually infectious and pathogenic to humans. However, precautions should be taken against this and other *B. microti*-like parasites that have increasingly been reported from a wide variety of wild animals [2, 5, 8, 9, 21, 38] as a group of potentially zoonotic pathogens.

Our study also demonstrated the usefulness of the hu-RBC-SCID mouse model not only for testing infectivity of hemoparasitic protozoa for human RBCs, but also for selection of variants that are infective for human RBCs. This mouse model clearly showed that the parental strain of *B. rodhaini* is poorly capable of infecting human RBCs (Figs. 1, 2, and 3). Daily transfusions of human RBCs given from day 3 to day 6 PI almost cleared out parasitemia in the mouse. In order to rescue the parasites, transfusions were given less frequently during the following days. This change induced anemia, which, in term, promoted erythropoiesis in the mouse, as evidenced by appearance of reticulocytes in the blood smear specimens. Human-mouse RBC chimera created in this manner enabled us to maintain a low level of parasitemia for a long period, contributing to emergence of variant parasites capable of propagating in human RBCs.

Failure of the parental strain of *B. rodhaini* to infect human RBCs can be the result of a disability at any of the three steps in parasite infection for host cells; namely, attachment, invasion, and multiplication. The present study showed that only a very few parasites were present in or on the human RBCs after transfusion into the SCID mouse with high parasitemia (Fig. 2), implying that the parental parasites suffer some disability in the early steps of infection. Therefore, we explored the merozoite surface proteins (MSPs), as they are presumed to be the most probable candidates that play roles in attachment or invasion [3, 7].

Snary and Smith [28], using monoclonal antibodies raised against the Antwerp strain of *B. rodhaini*, identified four MSPs having apparent molecular masses of 37, 35, 33, and 30 kDa. Complete ORF nucleotide sequences (accession number M19145) have been reported for two *msp* genes, designated as *p17* and *p26*, which were determined by cDNA cloning followed by genomic DNA analysis [29]. In the present study, we were able to obtain three *msp* genes from the Australian strain of *B. rodhaini*. One of them, *msp3*, was exactly identical to *p17*, whereas the other two, *msp1* and *msp2*, were very similar, but not identical, to *p26*. Difference in *msp* gene sequences between the Antwerp and Australian strains has also been reported by Igarashi *et al.* [12]. Curiously, the differences between *msp1* and *p26* were found only within the 3' half of *p26* (bases 538 to 1009 in Fig. 5), whereas those between *msp2* and *p26* were seen within the 5' half of *p26* (bases 4 to 504 in Fig. 5), implicating that some recombination events may have taken place among those *msp* genes. Such events would be anticipated because *B. rodhaini* has been shown to undergo antigenic variation in the presence of immune pressure [22, 31]. Most laboratory strains of *B. rodhaini* are presumed to be maintained for many years by syringe passages in susceptible laboratory animals even though the history of each strain is not known precisely.

We could not find any differences in the sequences of the three *msp* genes between the parental strain of *B. rodhaini* and its human RBC-adapted substrain. Gaining of infectivity for human RBCs, therefore, may not be attributable to a change in the amino acid sequence of MSPs. However, given that *B. rodhaini* is reported to have at least four MSPs [28], the final conclusion must await the results of cloning and sequencing analyses for all *msp* genes. In any case, the human RBC-adapted variant obtained in this study, in comparison with the parental strain, will provide us with significant insights into the mechanisms and molecules involved in determining host cell specificity.

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NOTE *Parasitology*

Babesia microti-Like Parasites Detected in Feral Raccoons (*Procyon lotor*) Captured in Hokkaido, Japan

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ABSTRACT. Raccoons (*Procyon lotor*), which have recently become feral in Japan, were examined for the presence of *Babesia microti*-like parasites. Out of 372 raccoons captured in the west-central part of Hokkaido, 24 animals with splenomegaly were selected and tested by nested PCR targeting the babesial 18S rRNA gene. *B. microti*-like parasites were detected in two of the 24 individuals, and their DNA sequences were identical to that of the *B. microti*-like parasite reported from raccoons in the United States, suggesting that the parasites were probably imported into Japan and that the life cycle of the parasite has already been established in the country. The potential risk of this *B. microti*-like parasite spreading among dogs and foxes in Japan will need to be carefully monitored, as parasitization by phylogenetically very close parasites has been reported from such animals.

KEY WORDS: *Babesia microti*, Hokkaido, Raccoon.

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The raccoon is a Carnivora species native to North America [9]. A large number of raccoons, however, have been imported as pets into Japan since the 1970s. In Hokkaido, the northernmost island of Japan, the accidental release and subsequent escape of these pet raccoons has resulted in a feral population, distributed mainly in the suburban areas of Sapporo, the biggest City on the island [1]. In 1997, the Hokkaido government approved of the control killing of feral raccoons only locally in response to agricultural and aquiculture damage. From 1999, the Hokkaido government started a feral raccoon management program, which enabled scientists in various fields to conduct collaborative investigations on captured raccoons [1, 7].

Parasites phylogenetically closely related to *Babesia microti*, referred to as *Babesia microti sensu lato*, have recently been reported from several Carnivoras including raccoons, foxes and skunks in the United States ("clade 2" in the reference [6]). *Babesia microti sensu stricto* ("clade 1" in the reference [6]) is most frequently found in small wild rodents and occasionally appears as a causative agent of human babesiosis [11]. Recently, a fulminating disease in dogs has emerged in Spain [2, 4, 5, 14], and the causative agent was named *Theileria annae* [14]. Phylogenetic studies, however, have subsequently made it clear that this parasite belong to the *B. microti* group [13], and that its closest relative is the one in raccoons [6], which have recently become feral in Europe also [9]. In the present study, we had an opportunity to obtain blood samples from raccoons captured in Hokkaido, and investigated them to determine whether they carried babesial infections.

Raccoons were captured from June to November in 2004 at 9 places in the west-central part of Hokkaido (Fig. 1) under the feral raccoon management program directed by the Hokkaido government. The number of captured raccoons in each region is shown in Table 1. The animals were euthanized according to the procedure approved by the program, and examined for sex, body weight, pregnancy, spleen size, and tick infestation, followed by tissue and blood collections. DNA samples were prepared from the blood samples with a DNA Extractor WB kit (Wako Pure Chemical Industries, Osaka, Japan), and partial sequences within the nuclear small subunit ribosomal RNA gene (18S rRNA gene) were amplified using 2 sets of nested PCR primers (Table 2), according to the procedure described in our previous studies [8, 12, 15]. One set of primers consisted of Bab1A and Bab4A for the 1st round and Bab2A and Bab3A for the 2nd-round PCR; a set that is highly specific for *B. microti*-like parasites and useful for their detec-

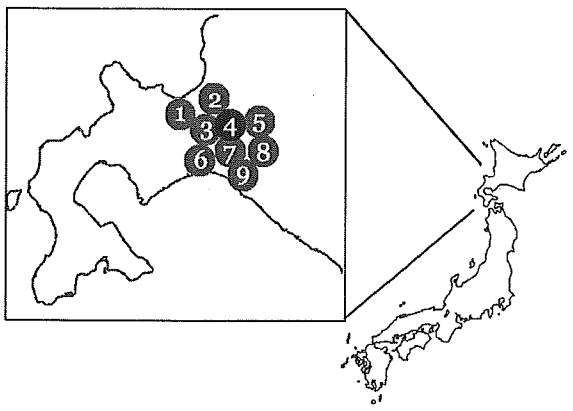

Fig. 1. Location of the study area for feral raccoons in the west-central parts of Hokkaido, Japan. Trapping reagion; 1, Sapporo; 2, Ebetsu; 3, Kitahiroshima; 4, Maoi; 5, Yubari; 6, Tomakomai; 7, Oiwake; 8, Hobetsu; 9, Mukawa.

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Table 1. Summary of the survey on raccoons captured in the west-central parts of Hokkaido, Japan

Region	No.of raccoon captured	Splenomegaly[a] /no. of raccoon	PCR positive[b] /splenomegaly
Sapporo	90	4/90	0/4
Ebetsu	42	4/42	0/4
Kitahiroshima	77	5/77	0/5
Maoi	34	6/34	2/6
Yubari	8	0/8	–
Tomakomai	45	4/45	0/4
Oiwake	12	1/12	0/1
Hobetsu	38	0/38	–
Mukawa	26	0/26	–
Total	372	24/372	2/24

a) Splenomegaly was judged by spleen size (in length) being more than 10 cm.
b) Nested PCR specific for *B. microti*-like parasites was carried out using Bab1A and Bab4A for the first round, and Bab2A and Bab3A for the second round.

Table 2. PCR primers used to amplify babesial 18S rRNA gene sequence

Primer	Sequence (5' to 3')	Orientation
Bab1A	5'-GTCTTAGTATAAGCTTTTATACAGCG-3'	Forward
Bab2A	5'-CAGTTATAGTTTATTTGATGTTCGTTTTAC-3'	Forward
Bab3A	5'-CGGCAAAGCCATGCGATTCGCTAAT-3'	Reverse
Bab4A	5'-GATAGGTCAGAAACTTGAATGATACATCG-3'	Reverse
Piro0F	5'-GCCAGTAGTCATATGCTTGTGTTA-3'	Forward
Piro1F	5'-CCATGCATGTCTWAGTAYAARCTTTTA-3'	Forward
Piro5.5R	5'-CCTYTAAGTGATAAGGTTCACAAAACTT-3'	Reverse
Piro6R	5'-CTCCTTCCTYTAAGTGATAAGGTTCAC-3'	Reverse

tion [8, 12, 15]. The other set of primers consisted of Piro0F and Piro6R for the 1st round, and Piro1F and Piro5.5R for the 2nd-round PCR; a set that is not highly specific for *B. microti*-like parasites but useful to obtain a near full-length 18S rRNA gene sequence. The DNA fragments amplified with the latter set of primers were cloned in a plasmid vector for sequencing as described previously [10]. At least 3 plasmid clones were sequenced for both strands to obtain a consensus sequence.

Out of 372 raccoons captured, 24 animals were found to have splenomegaly (Table 1). DNA samples were extracted from those 24 animals, and tested by the nested PCR highly specific for *B. microti*-like parasites (see above). A positive signal was obtained from 2 of the 24 samples (Table 1). Near full-length 18S rRNA gene sequences, 1684 bp in size, were amplified from those two samples, and their sequences determined (accession number AB197940). The sequences from the 2 samples were the same, and bases 428 to 1684 were identical to the sequence reported for a *B. microti*-like parasite from a raccoon in the United States (accession number AY144701). The 2 PCR-positive animals were both trapped within the Maoi region (Fig. 1). Ticks were not found on the 2 animals, but *Ixodes ovatus, I. persulcatus*, and *I. tanuki* were found on the other raccoons captured in this region. Although the level of parasitemia was very low (less than 0.01%), *Babesia* parasites were microscopically observed in a blood smear prepared from one of the two PCR-positive raccoons (Fig. 2).

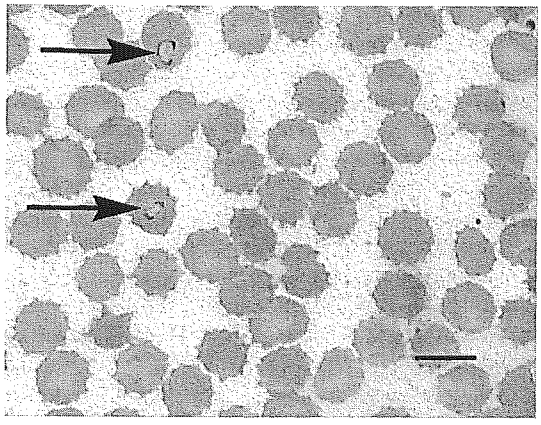

Fig. 2. Light micrograph of Giemsa-stained thin-smear blood film showing *Babesia microti*-like parasites. Bar, 10 μm.

Our study represents the first detection of *B. microti*-like parasites in Japanese raccoons. As evidenced by the sequence identity of the 18S rRNA gene, the *B. microti*-like parasite found in Japan is probably the same as that in the United States. The fact that raccoons became feral in Japan only very recently suggests two possibilities: namely, that

the parasite was also recently introduced into Japan, and that its life cycle has already been established within the detection area. In the present study, we could not identify the vector tick involved in the life cycle, but one of the three *Ixodes* ticks obtained from captured raccoons, namely, *I. ovatus, I. persulcatus*, and *I. tanuki*, may well be the candidate. Phylogenetically, the parasite found in this study is very closely related to the *B. microti*-like parasite causing newly emerging babesiosis in Spanish dogs [2, 4, 5, 14], for which *I. hexagonus* has been indicated to serve as the vector tick [3]. Speculating from the phylogenetic relationship, it would be interesting to investigate the hypothesis that the *B. microti*-like parasite in Spanish dogs might be a dog-adapted variant that was derived from the parasite in raccoons or foxes in the United States (personal communication with S. R. Telford).

The results presented in this study are still preliminary because only 24 out of the 372 samples were examined. These 24 raccoons, however, had splenomegaly, a major sign of babesiosis. Further studies are now underway to obtain more precise information about parasite prevalence and geographical distribution. Careful monitoring of not only raccoons but also dogs and foxes will be needed for assessment of the potential risk of this *B. microti*-like parasite spreading among these animals; such a field survey may also provide us with an unique opportunity for testing the hypothesis described above.

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Novel Mitochondrial Gene Content and Gene Arrangement Indicate Illegitimate Inter-mtDNA Recombination in the Chigger Mite, *Leptotrombidium pallidum*

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Abstract. To better understand the evolution of mitochondrial (mt) genomes in the Acari (mites and ticks), we sequenced the mt genome of the chigger mite, *Leptotrombidium pallidum* (Arthropoda: Acari: Acariformes). This genome is highly rearranged relative to that of the hypothetical ancestor of the arthropods and the other species of Acari studied. The mt genome of *L. pallidum* has two genes for large subunit rRNA, a pseudogene for small subunit rRNA, and four nearly identical large noncoding regions. Nineteen of the 22 tRNAs encoded by this genome apparently lack either a T-arm or a D-arm. Further, the mt genome of *L. pallidum* has two distantly separated sections with identical sequences but opposite orientations of transcription. This arrangement cannot be accounted for by homologous recombination or by previously known mechanisms of mt gene rearrangement. The most plausible explanation for the origin of this arrangement is illegitimate inter-mtDNA recombination, which has not been reported previously in animals. In light of the evidence from previous experiments on recombination in nuclear and mt genomes of animals, we propose a model of illegitimate inter-mtDNA recombination to account for the novel gene content and gene arrangement in the mt genome of *L. pallidum*.

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Key words: Acari — Double-strand break — End-joining repair — Gene order — Gene rearrangement — Illegitimate recombination — Mitochondrial genome — tRNA secondary structure

Introduction

Mitochondrial (mt) genomes of animals are usually circular, 15–20 kb long, and have 37 genes plus a large noncoding region (LNR; Boore 1999). Nucleotide (nt) sequences of mt genomes are often used in evolutionary studies. In recent years, arrangements of mt genes have also been used in evolutionary studies. Early work showed that recombination of mtDNA was extremely rare in mammals (Clayton et al. 1974; Zuckerman et al. 1984; Hayashi et al. 1985); this was generally thought to be the case for other animals too. Indeed, the view that animal mt genomes recombine rarely was a key part of the rationale for using mtDNA sequences in evolutionary biology. Further, whether or not mt genomes of animals recombine, and if they recombine, how they recombine, is critical to our understanding of the mechanisms of gene rearrangements in mt genomes (Boore 2000; Dowton and Campbell 2001).

Recent studies have challenged the view that mt genomes of animals do not recombine or recombine rarely. Lunt and Hyman (1997) reported illegitimate

(i.e., nonhomologous) intra-mtDNA recombination in a nematode: two double-strand breaks cleaved out a section of the large noncoding region; the two ends of this section then rejoined to form a minicircle. Tang et al. (2000) showed that in long-term cultured human cell lines the mt genomes with partial duplications could generate wild-type mt genomes and mt genomes with sections deleted, apparently by illegitimate intra-mtDNA recombination. Ladoukakis and Zouros (2001) showed that in heteroplasmic male mussels the male-type and female-type mt genomes could recombine by homologous recombination. The mt genomes of humans may also recombine, but this is controversial. Awadalla et al. (1999) reported that the linkage disequilibrium declined as the physical distance between two polymorphic sites in a mt genome increased. This was interpreted as evidence for homologous recombination in human mt genomes. However, four subsequent papers reported the reanalyses of the data of Awadalla et al. (1999) with alternative methods of measuring linkage disequilibrium (Jorde and Bamshad 2000; Kivisild and Villems 2000; Kumar et al. 2000; Parsons and Irwin 2000). These authors argued that the linkage disequilibrium did not decline as the physical distance between two polymorphic sites increased. More recently, Kraytsberg et al. (2004) showed that in the muscle tissue of a patient with paternal inheritance of mtDNA about 0.7% of the mt genomes contain both maternal and paternal segments. This indicates homologous recombination between the paternal-type and the maternal-type mt genomes in this patient. In summary, these studies indicate that recombination of animal mt genomes may be more common than previously thought but this remains controversial.

The subphylum Chelicerata is one of the four major lineages of the phylum Arthropoda. The Chelicerate comprises the horseshoe crabs, mites, ticks, spiders, scorpions, sea scorpions, sea spiders, and their kin. The nt sequences of entire mt genomes have been reported for 13 chelicerates: 1 horseshoe crab (Lavrov et al. 2000), 1 jumping spider (Masta and Boore 2004), 1 honeybee mite (Evans and Lopez 2002), 3 soft ticks (Shao et al. 2004, 2005), 4 prostriate ticks, and 3 metastriate ticks (Black and Roehrdanz 1998; Shao et al. 2004, 2005). The horsehoe crab, the soft ticks, and two prostriate ticks, *Ixodes hexagonus* and *I. persulcatus*, have exactly the same gene content and gene arrangement as the hypothetical ancestor of the arthropods (Lavrov et al. 2000), whereas the jumping spider, the honeybee mite, and the metastriate ticks have novel arrangements of mt genes relative to the hypothetical ancestor of the arthropods. The other two prostriate ticks, *I. holocyclus* and *I. uriae*, have the same gene arrangement as the hypothetical ancestor of the arthropods except that these two Australasian *Ixodes* ticks have duplicate LNRs, i.e., two separated LNRs with identical or highly similar nt sequences. The metastriate ticks also have duplicate LNRs. The presence of duplicate LNRs is thought to be a synapomorphy for the Australasian *Ixodes* ticks (Shao et al. 2005). The rearrangements of mt genes and the presence of duplicate LNRs are thought to be synapomorphies for the metastriate ticks (Black and Roehrdanz 1998; Campbell and Barker 1998; Murrell et al. 2003).

All of the Chelicerata studied so far, except the horseshoe crab and the jumping spider, are from the Parasitiformes. The Parasitiformes is one of the three major divisions of the Acari (the mites and ticks [Lindquist 1984]). Nucleotide sequences of entire mt genomes or arrangements of mt genes are not available for any mites from the other two divisions of the Acari, the Acariformes and the Opilioacariformes. Yet about two thirds of the 45,000 described species of Acari are in the Acariformes. In order to get a better understanding of the evolution of mt genomes in the Acari, we sequenced the entire mt genome of the chigger mite, *L. pallidum*, which is from the Acariformes. *L. pallidum* has a novel mt gene content and differs substantially in gene arrangement from the hypothetical ancestor of the arthropods and the other species of Acari studied. Here, we present the novel features of the mt genome of *L. pallidum* and discuss their implications for the transcription of mt genes, the replication of mt genomes, and the phylogeny of the Acari. Further, we propose a model of illegitimate inter-mtDNA recombination to account for the novel gene content and gene arrangement of *L. pallidum*.

Materials and Methods

Source of Mites, DNA Extraction, PCR Amplification, and nt Sequencing

The chigger mite, *L. pallidum*, was from a laboratory colony in Saitama, Japan (see Takahashi et al. 1994). Three mites were used in DNA extraction and the method of DNA extraction was described by Shao et al. (2004). Fragments of two mt genes, *cob* and *cox1*, were amplified with primers that were conserved among arthropods. Nucleotide sequences from these fragments were then used to design four PCR primers that were specific to *L. pallidum*. The mt genome of *L. pallidum* was then amplified in two fragments (~8 kb each) by long-PCR with the following primers: (1) Lp-cob-f (5′-TATGCAATTTTGCGATCAATTCCT-3′) with Lp-cox1-r (5′-ATCCGGGCAAAATAAGAATATACA-3′); and (2) Lp-coxl-f (5′-CTATGTTGTTGCTCATTTCCACTA-3′) with Lp-cob-r (5′-TAGTAATTACTGTTGCACCTCAAA-3′). Each 50-μl PCR contained 1 μl (~2.5 ng) of total DNA, 400 μM of each dNTP, 1 μ*M* of each prime, and 2.5 units of LA *Taq* polymerase (Takara Bio). GeneAmp PCR System 9700 (PE Biosystems) was used in PCR and the reaction conditions were 95°C for 1 min, followed by 37 cycles of 95°C for 40 sec, 57°C for 45 sec, 68°C for 10 min, and then 68°C for 10 min. Long-PCR fragments were sheared by son-

Table 1. Comparison of the 13 putative mitochondrial proteins of the chigger mite, *Leptotrombidium pallidum* (*L.p*), and the fruit fly, *Drosophilla yakuba* (*D.y*)

Protein	Length (aa)		Variation (%)[a]	Domains absent in *L.p*[b]
	L.p	*D.y*		
ATP6	207	224	−7.6	44–55
ATP8	49	53	−7.5	50–53
COX1	510	512	−0.4	None
COX2	216	228	−0.9	152–160
COX3	259	262	−1.1	None
COB	352	378	−6.8	353–378
NAD1	293	324	−13.8	294–324
NAD2	294	341	−13.8	2–9, 19–28, 250–270
NAD3	101	117	−13.6	10–19, 112–117
NAD4	408	446	−8.5	20–29, 70–85, 432–446
NAD4L	85	96	−11.5	41–48
NAD5	512	573	−10.6	2–9, 159–177, 406–434, 569–573
NAD6	142	174	−18.3	110–135

[a]Variation in length of protein = $(L.p - D.y)/D.y \times 100\%$.
[b]Numbers indicate the positions of domians in *D. yakuba*.

ication and then separated by gel electrophoresis. Fragments of the size 1.5–2.5 kb were excised from gels, purified, blunt-ended, ligated into pUC-18 vectors, and then transformed into competent cells. One hundred clones were picked at random for each long-PCR fragment and sequenced with BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

Sequence Analysis

The nt sequence of the entire mt genome of *L. pallidum* was assembled by aligning sequences from the 200 clones (100 clones from each fragment) with AutoAssembler (Applied Biosystem). Both strands of the mt genome were sequenced. On average, each nt was sequenced seven times. Protein-coding genes were identified by BLAST searches of GenBank (Altschul et al. 1990) and confirmed by comparisons of hydrophilicity profiles of putative proteins (Hopp and Woods 1981) with those of the fruit fly, *Drosophila yakuba* (Clary and Wolstenholme 1985). BLAST searches of GenBank did not identify *atp8*, *nad4L*, and *nad6* of *L. pallidum*; these three genes were identified by comparing the hydrophilicity profiles of three "orphan" open reading frames of *L. pallidum* with the hydrophilicity profiles of ATP8, NAD4L, and NAD6 of *D. yakuba*. rRNA genes were identified by BLAST searches of GenBank and confirmed by comparison with rRNA genes of *D. yakuba*. tRNA genes were identified with tRNAscan-SE (Lowe and Eddy 1997) or by eye and confirmed by comparisons with tRNA genes of the horseshoe crab, *Limulus polyphemus* (Lavrov et al. 2000). The mtDNA sequence of *L. pallidum* has been deposited in DDBJ (accession number AB180098).

Results and Discussion

General Features of the mt Genome of L. pallidum

The mt genome of *L. pallidum* is circular and has 16,779 bp. In addition to the 37 genes that are typical of the mt genomes of animals, the mt genome of *L. pallidum* has an extra gene for large subunit rRNA (*rrnL*), a pseudogene for small subunit rRNA (*PrrnS*), and four large noncoding regions (LNR). This genome is the third largest of the 49 mt genomes of arthropods that are in GenBank. Only the mt genomes of *Drosophila melanogaster* (19,517 bp; accession number NC_001709) and the kissing bug, *Triatoma dimidiata* (17,019 bp; NC_002609), are larger than the mt genome of *L. pallidum*. For *D. melanogaster* and *T. dimidiata*, the large sizes of the noncoding regions (4,600 and 2,472 bp, respectively) account for the large sizes of these mt genomes. For *L. pallidum*, however, the extra genes and the noncoding regions together (3,414 bp in total) account for the large size of the mt genome, In contrast to the large size of the mt genome, most mt genes of *L. pallidum* are shorter than those of other arthropods. The proteins inferred from the 13 protein-coding genes of this genome are shorter or substantially shorter (by 0.4–18.3%) than those of *D. yakuba*. Comparison of hydrophilicity profiles indicated that some domains of the proteins of *D. yakuba* are not present in *L. pallidum* (Table 1). All the 22 tRNA genes and *rrnS* of *L. pallidum* are substantially shorter than their counterparts in *D. yakuba* (see below).

Novel mt Gene Arrangement of L. pallidum *and Its Implications for mt Gene Transcription, mt Genome Replication, and Acari Phylogeny*

The arrangement of genes in the mt genome of *L. pallidum* differs drastically from that of the hypothetical ancestor of the arthropods, which has been retained in the horseshoe crab, the soft ticks, and at least two prostriate ticks, *I. hexagonus* and *I. persulcatus* (Fig. 1). Thirty-four of the 43 gene boundaries in *L. pallidum* are novel for an arthropod. Four features of the mt genome of *L. pallidum* are partic-

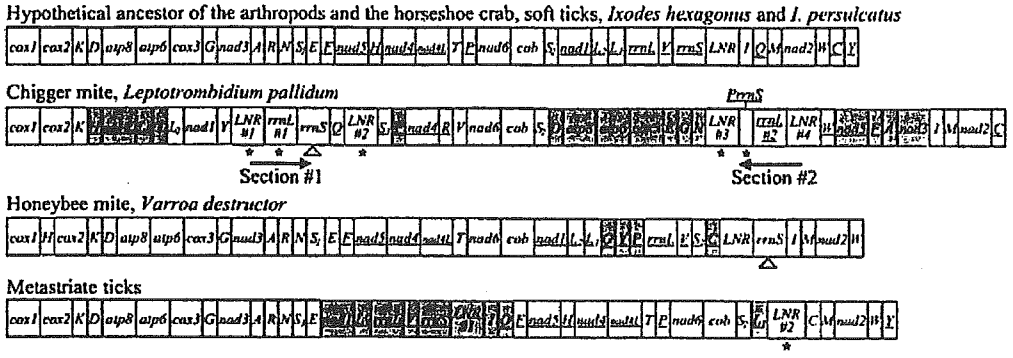

Fig. 1. Comparison of the gene content and gene arrangement of the mt genomes of the chigger mite, *Leptotrombidium pallidum*, the hypothetical ancestor of the arthropods and other species of the Acari studied to date. Abbreviations of protein-coding and rRNA genes: *atp6* and *atp8*, ATP synthase subunits 6 and 8; *cox1–3*, cytochrome oxidase subunits 1–3; *cob*, cytochrome *b*; *nad1-6* and *nad4L*, NADH dehydrogenase subunits 1-6 and 4L; *rrnL* and *rrnS*, large and small rRNA subunits. tRNA genes are shown with the single-letter abbreviations of their corresponding amino acids. The two tRNA genes each for leucine and serine are L_1 (anti-codon sequence nag), L_2 (yaa), S_1 (nct), and S_2 (nga). The circular genomes were linearized at the 5′ end of *cox1* to aid comparison. Genes are transcribed from left to right except those underlined, which are transcribed from right to left. Asterisks indicate genes or noncoding regions that are present in *L. pallidum* and metastriate ticks but not in the hypothetical ancestor of the arthropods. Dark gray–shaded boxes indicate genes which changed locations relative to the arrangement of mt genes of the hypothetical ancestor of the arthropods (Lavrov et al. 2000). Light gray–shaded boxes indicate genes that changed both location and orientation relative to those of the hypothetical ancestor of the arthropods. Triangles indicate the inversion of *rrnS* in the chigger mite, *L. pallidum*, and the honeybee mite, *Varroa destructor*. Thick arrows show the two 1,852-bp sections which have identical nt sequences but opposite orientations of transcription in *L. pallidum*.

ularly noteworthy. First, the two genes for NADH dehydrogenase subunits 4 and 4L (*nad4* and *nad4L*) are not adjacent but are 4,085 bp apart. Thus, these two genes may have their own mature mRNAs rather than share a single mature mRNA as is the case in fruit flies (Berthier et al. 1986) and possibly in most other arthropods in which these two genes overlap. Second, there are two copies of *rrnL* genes but one copy of *rrnS* gene. Thus, the large subunit rRNA may be expressed more than the small subunit rRNA. Third, there are four nearly identical LNRs. The transcription of mt genes and the replication of the mt genome of *L. pallidum* may, therefore, start at multiple sites if each of the four LNRs contains the initiation sites for transcription and/or replication, as is the case for the LNRs in the mt genomes of mammals (Taanman 1999) and fruit flies (Goddard and Wolstenholme 1980). Stem–loop structures in noncoding regions are associated with the initiation of replication or transcription (Wong and Clayton 1985; Clayton 1991; L'Abbe et al. 1991). Such structures are also present on both strands of the four LNRs of *L. pallidum* (Fig. 2A), Fourth, *rrnS* is immediately downstream of *rrnL* whereas in most animals *rrnS* is upstream of *rrnL*. In humans the two mt rRNA genes are transcribed together at a rate much higher than other mt genes; the transcription initiates at a site upstream of *rrnS* in the LNR and terminates at a site downstream of *rrnL* in a tRNA gene (Taanman 1999). A conserved heptamer, TGGCAGA, was thought to be the termination site of the transcription of the two rRNA genes in all eukaryotes examined (Valverde et al. 1994; Richard et al. 1998). This heptamer is always present at the 5′-end of the gene that is immediately downstream of *rrnL*; if the gene immediately downstream of *rrnL* is a tRNA gene, this heptamer is present at positions 8–14 of the 5′-end of that tRNA gene. In *L. pallidum*, this heptamer was not present at the 5′-end of *rrnS* or *PrrnS*, which is adjacent to the 3′-end of *rrnL1* or *rrnL2*. However, a heptamer, TGGTGTA, was present at positions 8–14 of the 5′-end of *trnQ*, which is adjacent to *rrnS*. The first three and the last nts of this heptamer are identical to those of the conserved heptamer that has been found in other eukaryotes examined. We propose that *rrnL1* and *rrnS* are transcribed together in *L. pallidum*: the transcription initiates at a site upstream of *rrnL1* in one of the four LNRs (LNR1) and terminates at the heptamer, which is downstream of *rrnS* in *trnQ*. This indicates that there may be functional constraints on the rearrangement of the two mt rRNA genes that keep the two genes to be close to each other and on the same strand, regardless of their relative positions.

Comparisons of the mt gene arrangements of the Acari studied so far show that the chigger mite, *L. pallidum* (Acariformes), and the honeybee mite, *Varroa destructor* (Parasitiformes), share the inversion of *rrnS*. In all other Acari studied so far *rrnS* is in the orientation that is ancestral for arthropods. Further, *V. destructor* (Parasitiformes) does not share any gene boundaries nor gene rearrangements with the metastriate ticks which are also from the Parasitiformes (Fig. 1). Inversions of rRNA genes in mt genomes are

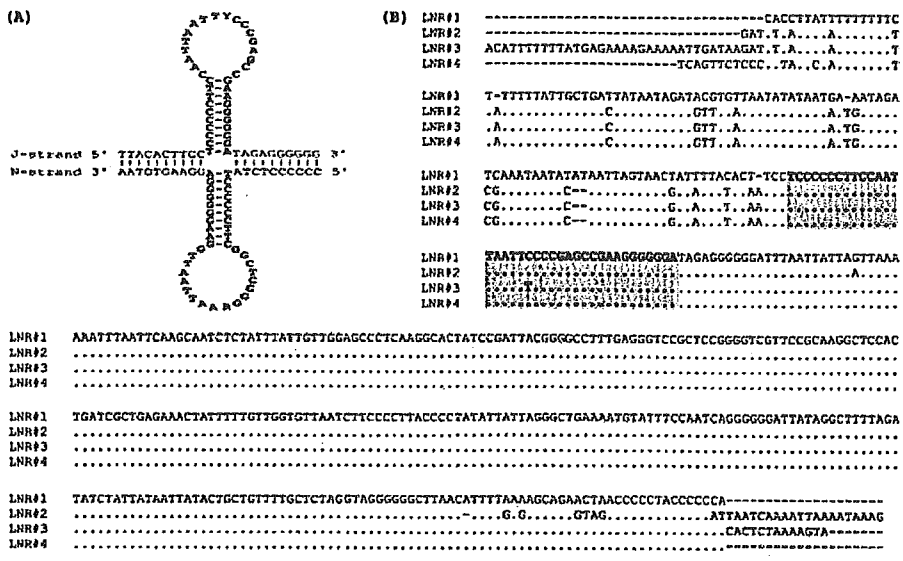

Fig. 2. (A) Stem–loops in the large noncoding regions (LNR) of the mt genome of the chigger mite, *Leptotrombidium pallidum*. The nts of the stem–loops are in boldface, whereas the nts that flank the stem–loops are in regular font. (B) Alignment of the nt sequences of the four large noncoding regions. Dashes indicate indels. Periods indicate nts that are identical to LNR1. Sequences that can form stem–loops are highlighted in gray.

rare and, thus are less likely to be homoplastic (Boore 1999). If the inversion of *rrnS* that is shared by the chigger mite (Acariformes) and the honeybee mite (Parasitiformes) is a synapomorphy, it would indicate that the honeybee mite (Parasitiformes) is more closely related to the chigger mite (Acariformes) than it is to the ticks (Parasitiformes), i.e., the Parasitiformes would be paraphyletic. This phylogenetic arrangement differs markedly from the current hypothesis of the phylogeny of the Acari that the Parasitiformes is monophyletic (Lindquist 1984). Broad sampling is needed to elucidate whether the inversion of *rrnS* in the chigger mite and the honeybee mite is a synapomorphy or evolved independently.

Unusual Secondary Structures of the mt tRNAs of L. pallidum

Like those of most animals, the mt genome of *L. pallidum* apparently encodes 22 tRNA genes. However, the tRNAs inferred from these genes have unusual secondary structures (Fig. 3). Nine tRNAs of *L. pallidum* (C, Q, E, H, L_2, F, P, T, and W) lack a T-arm and instead have a TV replacement loop. Ten tRNAs (A, R, N, G, I, L_1, S_1, S_2, Y, and V) lack D-arms and have a D-arm replacement loop. Only three tRNAs (D, K, and M) can form cloverleaf-shaped structures but none of these appear to have a T-loop. These changes caused substantial size reduction (by 9–31%) of the tRNAs of *L. pallidum* compared to those of the horseshoe crab and the fruit fly. Most of the mt tRNAs of *L. pallidum* are even shorter than the nematode, *Ascaris suum*, of which all putative mt tRNAs lack either a T-arm or a D-arm (Table 2).

For most animals, all 22 mt tRNAs can form cloverleaf-shaped secondary structures except tRNA-Ser(ncu), which lacks a D-arm and has a D-arm replacement instead (Wolstenholme 1992). In several exceptions, some of the 22 tRNAs, in addition to tRNA-Ser(ncu), lack either a T-arm or a D-arm, whereas the rest can form a cloverleaf-shaped structure (Yamazaki et al, 1997; Lavrov and Brown 2001; Masta and Boore 2004), Changes to the secondary structures of all the 22 mt tRNAs of *L. pallidum* have not been observed in arthropods before. Changes of this scale have only been observed previously in nematodes of the class Secernentea (Okimoto et al. 1992; Wolstenholme et al. 1987).

The unusual putative structures and small sizes of the mt tRNAs of *L. pallidum* beg the question: Are these tRNA genes functional? The analyses below support our view that the mt tRNA genes of *L. pallidum* encode a complete set of the functional tRNAs. First, there is no evidence to date that tRNAs can be imported from cytoplasm into mitochondrion in arthropods, although we cannot exclude such a possibility in *L. pallidum*. Second, 21 of the inferred 22 putative tRNAs of *L. pallidum* have anticodon sequences identical to those of *Li. polyphemus* and *D. yakuba* (Table 2). The only difference is the anticodon sequence of tRNA-Lys, which is TTT in *L. pallidum* but CTT in *Li. polyphemus* and *D. yakuba*. However, the use of anticodon TTT in *L. pallidum* is consistent with the colon composition of the mt proteins of *L. pallidum*: there are 128 AAA but only 19 AAG. Third, only tRNA-Phe and tRNA-Ser(gct) have three mismatches, whereas the other 20 tRNAs have either no mismatches or one or two mismatches (Fig. 3), Fourth, most tRNAs of *L. pallidum* share about 50% of their nt sequences with their counterparts in *Li. polyphemus*, in which all tRNAs can form a conventional cloverleaf-shaped structure except tRNA-Ser(gcu) (see Lavrov et al, 2000). Fifth, it has been

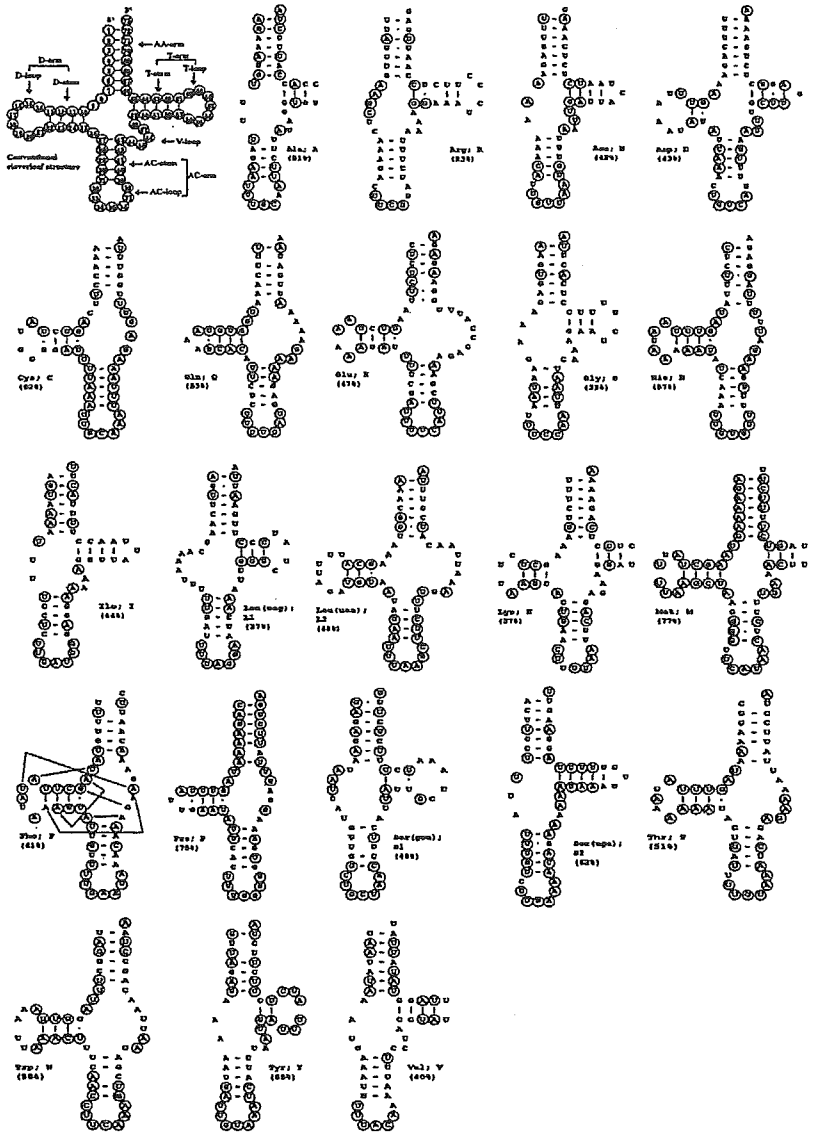

Fig. 3. Inferred secondary structures of the 22 tRNAs encoded by the mt genome of the chigger mite, *Leptotrombidium pallidum*. tRNAs are labeled with the abbreviations of their corresponding amino acids. The conventional cloverleaf secondary structures and the numbering of nts are after Sprinzl et al. (1989). Dashes indicate Watson–Crick bonds; dots indicate bonds between U and G. Circled nts are also present in the tRNAs of the horseshoe crab, *Limulus polyphemus*. The six pairs of nts that form a tertiary structure in tRNA-Phe of the nematode, *Ascaris suum*, are linked with lines in tRNA-Phe.

shown that in the nematode, *A. suum*, the mt tRNAs which lack either a T-arm or a D-arm are functional (Watanabe et al. 1994). Further, the six pairs of nts U^{8}–A^{14}, A^{9}–A^{23}, G^{10}–G^{L2}, A^{22}–A^{L3}, U^{15}–A^{L4}, and A^{26}–G^{L1}, which form the functional L-shaped tertiary structure of the tRNA-Phe of *A. suum* are also present in tRNA-Phe of *L. pallidum*: the first five pairs of nts in *L. pallidum* are identical to those in *A. suum* and the last pair in *L. pallidum*, A^{26}–A^{L1}, differs from to that in *A. suum* by one nt (Fig. 3). In both *L. pallidum* and *A. suum* the tRNA-Phe has a TV replacement-loop.

rRNA Genes, Large Noncoding Regions, and Illegitimate Inter-mtDNA recombination

There are two identical copies of *rrnL* (1,282 bp each), one copy of *rrnS* (601 bp), and a pseudogene of *rrnS*, *PrrnS* (212 bp), in the mt genome of *L. pallidum*. The size of the *rrnL* genes of *L. pallidum* is similar to that of *Li. polyphemus* (1,296 bp) and *D. yakuba* (1,326 bp), whereas *rrnS* of *L. pallidum* is only about 75% of the size of *rrnS* of *Li. polyphemus* (799 bp) and *D. yakuba* (789 bp). The two copies of *rrnL* in *L. pallidum* are 6,586 bp apart and identical in size and nt sequence but have opposite orientations of transcription. *PrrnS* is 5,772 bp away from *rrnS* and has identical sequence to the 5′-end of *rrnS* but has the opposite orientation of transcription. The presence of two copies of *rrnL* genes with identical sequences but opposite orientations in *L. pallidum* was confirmed by two specifically designed PCR tests. In each PCR test, we used only one *rrnL* specific primer (a forward primer in one test and a reverse primer in another test). Both PCR tests generated products of expected sizes (6.8 and 8.1 kb, respectively; data not shown). The four LNRs of *L. pallidum* are 460, 483,

Table 2. Comparison of the 22 putative tRNA genes of the chigger mite, *Leptotrombidium pallidum* (*L.p*), the horseshoe crab, *Limulus polyphemus* (*Li.p*), the fruit fly, *Drosophila yakuba* (*D.y*), and the nematode, *Ascaris suum* (*A.s*)

	Length (bp)				Anticodon		
Gene	*L.p* (%)[a]	*Li.p*	*D.y*	*A.s*	*L.p*	*Li.p*	*D.y*
trnA	47 (−28)	67	65	56	TGC	TGC	TGC
trnC	52 (−17)	64	63	58	GCA	GCA	GCA
trnD	58 (−15)	65	68	60	GTC	GTC	GTC
trnE	57 (−16)	66	68	59	TTC	TTC	TTC
trnF	54 (−18)	66	66	59	GAA	GAA	GAA
trnG	50 (−23)	64	65	56	TCC	TCC	TCC
trnH	54 (−18)	69	66	55	GTG	GTG	GTG
trnI	50 (−23)	67	65	61	GAT	GAT	GAT
trnK	57 (−20)	70	71	62	TTT	CTT	CTT
$trnL_1$	54 (−17)	69	65	56	TAG	TAG	TAG
$trnL_2$	60 (−9)	66	66	55	TAA	TAA	TAA
trnM	61 (−12)	70	69	61	CAT	CAT	CAT
trnN	50 (−23)	67	65	57	GTT	GTT	GTT
trnP	52 (−19)	67	64	56	TGG	TGG	TGG
trnQ	53 (−23)	66	69	55	TTG	TTG	TTG
trnR	56 (−13)	63	64	54	TCG	TCG	TCG
$trnS_1$	57 (−16)	64	68	51	GCT	GCT	GCT
$trnS_2$	52 (−21)	73	66	54	TGA	TGA	TGA
trnT	55 (−15)	66	65	55	TGT	TGT	TGT
trnV	50 (−31)	69	72	57	TAC	TAC	TAC
trnW	55 (−18)	68	67	57	TCA	TCA	TCA
trnY	49 (−28)	62	68	58	GTA	GTA	GTA
Average size	54 (−19)	67	67	57			

[a]Variation in length of genes relative to the genes of *D. yakuba*.

509, and 472 bp, respectively, The four LNRs share 94–98% of the 454 bp in the middle parts (Fig. 2B). LNR1 and 2 have opposite transcription orientations to those of LNR3 and 4.

Two sections of the mt genome of *L. pallidum* have identical nt sequences but have opposite orientations of transcription. These two sections are 1,852 bp long and are 5,772 by apart (Fig. 1). Section #1 has part of LNR1, the entire *rrnL*1, and part of *rrnS*, whereas Section #2 has part of LNR4, the entire *rrnL2*, and the entire *PrrnS*. This is the first report of a mt genome of an animal that has two distantly separated sections of identical nt sequences but opposite orientations of transcription. This arrangement cannot be accounted for by homologous recombination (Tsukarnoto and Ikeda 1998; Rokas et al. 2003) or by the other two well-known mechanisms of mt gene rearrangement: (1) tandem duplication followed by deletion (Moritz and Brown 1986; Macey et al. 1998; Boore 2000) and (2) illegitimate intra-mtDNA recombination (Lunt and Hyman 1997; Dowton and Campbell 2001). Homologous recombination may change the nt sequences of mt genes but would not change the gene content nor gene arrangement in the mt genomes. Tandem duplications may generate two identical sections in a mt genome but these two sections would always be adjacent to each other and in the same orientation. Illegitimate intra-mtDNA may change the position and/or orientation of a section of a mt genome but would not duplicate that section. In theory, tandem duplication and illegitimate intra-mtDNA recombination could act together to give a mt genome with two separated sections of identical or highly similar nt sequences but opposite orientations, like Sections #1 and #2 of *L. pallidum*. However, the most plausible explanation, in our view, is illegitimate inter-mtDNA recombination. Illegitimate inter-mtDNA recombination has been considered as a possible mechanism for gene rearrangement in animal mt genomes (Boore 2000) but there was little evidence for this mechanism until now. Indeed, *L. pallidum* is the first species of animal for which we need to invoke this mechanism to account for the novel mt gene content and gene arrangement. Evidence from experiments on recombination in nuclear and mt genomes further leads us to propose that the illegitimate inter-mtDNA recombination in *L. pallidum* may occur in two steps.

(1) *Double-strand breaks*: Consider two hypothetical, identical mt genomes, A and B (Fig. 4). Double-strand breaks cause a single break in A and two breaks in B. Thus, a section of genome B, S_B, is cleaved out. Double-strand breaks are a type of DNA damage that can be caupdsed by ionizing radiation, chemicals, oxidative stress or routine cellular processes (Tsukamoto and Ikeda 1998; Lakshmipathy and Campbell 1999). Double-strand breaks are harmful to cells and may lead to cell death or