Babesia microti - a complex species and its interrelationships

***CCT7* sequence data provide for an efficient method to address a large spectrum of questions, from the simple to the complex, relating to the phylogenetic relationships within the species complex *Babesia microti* including its interrelationships.**

Chiharu Morita, Masayoshi Tsuji, Takashi Kurosawa, Hiroshi Ueno, Shinichiro Fukumoto, Yutaka Tamura and Chiaki Ishihara, Gakujutsu Frontier Cooperative Research, Rakuno-Gakuen University**; Satoru Arai**, National Institute of Health, Japan and **Kim T.-S.** National Institute of Health, Republic of Korea.

In memory of the late **Professors Masayoshi Tsuji and Kiyoshi Takahashi** of the School of Veterinary Medicine, Rakuno-Gakuen University.

Corresponding author: Chiaki Ishihara, DVM, Ph.D.
Professor, School of Veterinary Medicine, Rakuno-gakuen University, Midorimachi 582, Bunkyo-dai, Ebetsu 069-8501, Japan

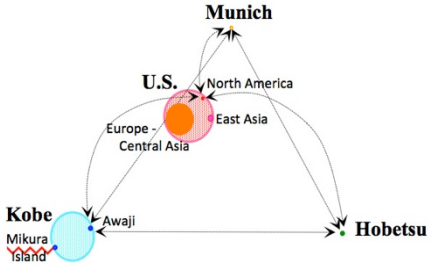
Babesiosis (Piroplasmosis)

Piroplasmosis is a tick-borne disease of man and animals, resembling malaria, that can exhibit severe sometimes fatal consequences. It is caused by a variety of protozoan haemoparasites (piroplasms) known as *Babesia, Theileria* and *Cytauxzoon*. Most human cases of piroplasmosis have been reported in the coastal areas of the northeastern United States though the etiologic agent, *B. microti* and its close relatives, infect a variety of small mammals - wood mice, voles and occasionally shrews are examples –that are distributed throughout the Holarctic temperate regions (both Nearctic and Palearctic).

We (Zaomoto et al., 2004A, B) and Goethert, H.K. and Telford, S.R. III (2003) previously reported the analysis of two DNA markers, small subunit ribosomal RNA (*18S rDNA*) and *β-tubulin* genes, to support the claim that *B. microti* is a complex species. Here, we present as further evidence the results from our investigation of the chaperonin containing TCP1, subunit 7 (eta) (*CCT7*) gene of these parasites.

General Information

Taxonomic position and general remarks of piroplasms

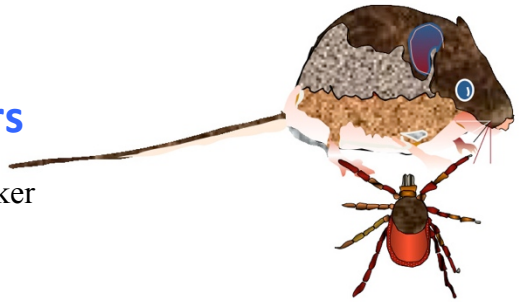


Genetic Structure of Parasite Populations

Lineage-specific patterns of population genetic variation

Phylogenetics and DNA Markers

CCT7 sequences as a multiple phylogenetic marker



Host-Parasite-Vector Interactions

Population genetics of parasite/host/vector interactions

General information

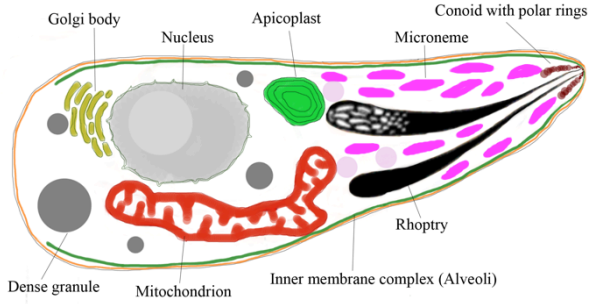


Fig. 1. Schematic drawing of a typical Apicomplexa

(1) ***Taxonomic position and general remarks***

Piroplasms (order Piroplasmida) are members of the phylum Apicomplexa, arguably, the largest taxon of unicellular obligate parasitic protists including over 6000 named species in existence though it is estimated that from 100- to 1000-times more as yet unnamed members are estimated to exist. These include piroplasma, hemosporidia (*Plasmodium* and *Leucocytozoon*), coccidia (*Eimeria*, *Isospora, Cyclospora, Toxoplasma, Sarcocystis*, *Frenkellia, Hepatozoon* and *Adelina*), cryptosporidia (*Cryptosporidium*) and gregarines (http://tolweb.org/Apicomplexa). This group derives its name from their common apical complex, a collection of anterior structures that allow the parasite to invade host cells (Fig.1). Most species in this phylum retain a single mitochondrion, a relic of a non-photosynthetic plastid-like organelle known as apicoplast, the secondary endosymbiotic origin of red-algal plastid. (Fig.1) (For further details see http://apiloc.biochem.unimelb.edu.au/apiloc/apiloc). The reduced function of these organelles is reflected in their highly compact genomes where genes have been lost or transferred to the nucleus, making the nuclear genome a mosaic of former organellar genes embedded in chromosomal DNA from both endosymbionts.

Molecular and cell-morphological data suggest that piroplasms evolved from Apicomplexa, perhaps as early as 550-600 million years ago (Fig. 2; Nguyen et al., 2007; Kuo & Kissinger 2008; Roy & Penny, 2006; Perkins & Schall, 2002). These results are, however, nearly 10 times greater than the estimated distances based on the associations to their mammalian hosts (Criado-Fornelio et al., 2003). In the absence of fossil data it is hard to know which estimate is the more precise making it, therefore, difficult to answer whether or not these obligate eukaryotic intercellular parasites evolved very quickly or in a similar fashion with multicellular eukaryotes.*1)

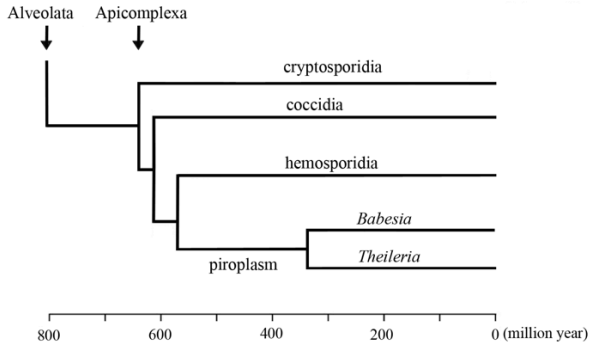


Fig. 2. Molecular evolution of apicomplexan lineages
After Nguyen et al., 2007.

(2) ***Members of this group, their biological characteristics and life cycles***

Piroplasmida are composed of more than 200 described species of genus *Babesia* (sensu stricto), *Theileria* and those of the group A (*Cytauxzoon* and its relatives), group B (*Babesia duncanni* and its relatives) and group C (*Babesia microti* and its relatives) as designated by Allsop & Allsop (2007). To date, seven distinct

*1): Organisms with shorter generation times and higher metabolic rates, such as eukaryotic microbes, are often assumed to have more DNA replication errors and, consequently, may have evolved more quickly. However, this estimation is questionable. A growing population generates mutations at a regular frequency and keeps them as the stocks of random genetic drifts (The vast majority of DNA mutations are evolutionary neutral and are mostly wasteful - "neutral theory"). Once an environmental insult emerges, a few mutation(s) hidden in a population, hypothetically, can be chosen by natural selection as beneficial to evade endangered situations. It can be delivered as a new highly dominant gene into the entire population as a consequence of the deaths of the allelo-types (sexual recombination may function as does the rapid delivery of "humanitarian aid" in times of crisis). If the environmental insults occur with similar frequencies in every eukaryotic taxon, evolutionary rates (the rates of selection and fixation of mutations) for all eukaryotes are presumed to be very close to each other. Many of singly cell eukaryotes, whose existing diversities remain to be discovered, will become discernable with the future acquisition of genomic and metagenomic sequences obtained from taxonomically more widely- and densely-sampled strains including as yet unknown species.

phylogenetic taxa[*2)] have been described that cause human disease. *B. microti* and its related organisms (members of Group C), are by far the most likely, while *B. divergens*, may do so on occasion and *B. bovis, B. canis, B. venatorum* (also called *Babesia* EU1), *Babesia* sp. similar to ovine babesias provisionally named KO1 (members of *Babesia* sensu stricto) and *B. duncani* (members of Group B) all only infrequently. Yet unrecognized species can cause infections in a variety of animal hosts and, perhaps, in some cases in humans, as recent molecular analysis of the implicated pathogens suggests that the host-range of many parasites in this group is less restricted than previously believed. (reviewed by Hunfeld et al., 2008; for further details see http://en.wikipedia.org/wiki/Babesia).

Organisms of this group divide by binary fission and have sexual and asexual phases (sexual reproduction occurs in the tick gut). The life cycle of this group of parasites can be separated into three stages. The initial gamogony is a sexual stage in which gametes produce and fuse inside the gut of the vector tick. This step is followed by sporogony, an asexual stage in the tick salivary gland. They further undergo merogony (also called as shizogony), an asexual dividing stage inside the vertebrate host (Fig. 3). During the tick bite, sporozoites are injected into the vertebrate host and infect either directly (*Babesia* sensu stricto) into red blood cells (RBC) or initially penetrate into lymphocytes (*Theileria*) / macrophages (*Cytauxzoon*), followed by an erythrocyte cycle. Based on DNA concentration results, the sporozoites are recognized as haploid, while the zygote and kinetes are diploid (Mackernstedt et al., 1990). Thus, the reduction division occurs during sporogony, as do the *Plasmodium* stages, and the parasite of this group will continue through merogony (also called shizogony) as a haploid organism.

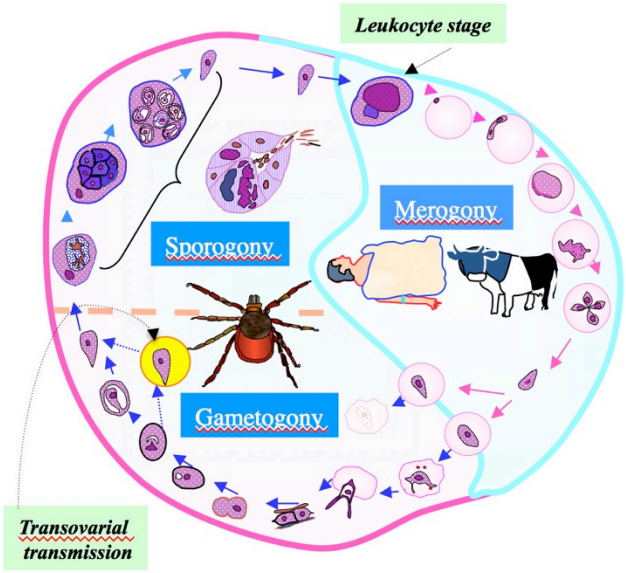


Fig. 3. Schematic Life cycle of Piroplasmida

Sexual recombination (typical outcrossing/xenogamy) requires two genetically different haploid gametes[*3)] either concurrently within a single larval/nymphal tick or gathered both into a nymphal tick, one carried from larval tick by transstadial transmission and the other introduced newly from an infected-vertebrate host. While in adult tick, mating is only fruitful for the parasites with the capacity to transmit transovarially (*Babesia* spp. sensu stricto group). Additionally, selfing (bi-parental inbreeding), may occur between the two genetically identical gametes which expand themselves by clonal expansion of haploid cell at the merogony stage (Fig. 3).

Yet another possibility is that tychoparthenogenesis may occur. Many other organisms exist that have a sexual life cycle permitting them to reproduce vegetatively: different types of organisms can reproduce by the modes (not only 2n-cells but possibly n- and 4n-cells), and at different rates and some of them actually alternate parthenogenesis and

[*2)]: Sexual reproduction allows shuffling of genes in a reproductive community and, consequently, the genetic traits such as pathogenicity and host specificity can be shared by a sexual species. The two decade prolonged "clonality/sexuality debate," has been settled and the classical view, that parasitic protists reproduce clonally, has been upset by the population genetic era. Genetic exchange is very frequent in many microbial species (reviewed by Tibayrenc 2003), although selfing (bi-parental inbreeding) and as well as arthenogenesis yet can be occur in many protists (see below).

[*3)]: Many morphological studies have reported that the parasites of this group produce gametes of different sizes (heterogamy/anisogamy). However, the evolutionary origin of heterogamy is poorly understood. There is no consensus among evolutionary biologists regarding the evolutionary origin or maintenance of sex and conflicting hypotheses abound. Therefore, care should be taken in use of the terms of hetero- or iso-gamy in this field of research until evolutionary process of sexual dimorphism has been elucidated.

sexual reproduction seasonally or in response to various environmental factors (reviewed by Heitman, 2006 and 2009).

(3) *Mixed and persistent infection with multiple genotypes*

It is known that many micro-parasitic diseases such as malaria and babesiosis, frequently have genetic diversity in pathogen populations even in a single host, especially in areas where disease is endemic (Anderson et al., 2000; Jinnai et al., 2010). This can arise because of co-infection, super-infection, or occasionally somatic mutation*4) during the course of the infection. Infection by multiple genotypes with tendency for long-term persistence (Fig 4, 5) may facilitate genetic admixture (also called xenogamy, outcrossing).*4)

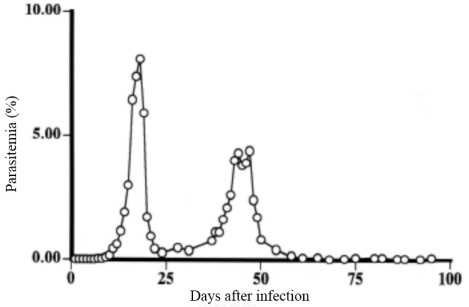


Fig. 4. Cyclical growth of a *T. orientalis* clone in a calf

Parasite was cloned by picking a single parasitized-RBC up under a microscopy from the blood of a grazing cow, which harbored C- and I- but not B-types of *T. orientalis* (also called *T. sergenti*) as determined by RFLP test on their p35 MPSP gene, and multiplied in a RBC-substituted SCID mouse. The resulting I-type clone was injected intravenously into a parasite-free splenectomized calf at a dose of 10^{10} parasitized RBCs. Peripheral blood smears were examined periodically under a microscope for the growth of parasite. Blood samples collected at around the first and second peaks were pooled separately and genotyped using the major piroplasm surface protein (MPSP) gene. The RFLP patterns yielded from the two sample pools were identical, indicating that *T. orientalis* clone grew cyclically without any sign of MPSP gene change in an immunologically intact, but splenectomized, calf.

Sample source and date (yr-mo-day)	Detection of parasites			Reciprocal IFA titers	
	Microscopy[a]	PCR[b]	Inoculation[c]	IgM	IgG
Patient					
1998-12-28[d]	ND[e]	−	ND	<100	<100
1999-05-24	+	+	+	12,800	1,600
1999-06-25	−	+	ND	12,800	800
1999-07-27	−	+	+	3,200	1,600
1999-08-31	+	+	+	12,800	102,400
1999-10-02	−	+	ND	12,800	51,200
1999-11-01	ND	−	ND	1,600	25,600
1999-11-29	ND	−	ND	800	25,600
1999-12-27	ND	−	ND	400	6,400
2000-01-29	ND	ND	ND	200	3,200
2000-03-08	ND	−	ND	200	3,200
2000-05-22	−	−	−	200	1,600
2000-07-19	−	−	−	100	800
Donor					
1998-12-22	ND	+	ND	200	25,600
1999-06-26	ND	−	ND	100	25,600
1999-07-27	−	−	+	50	25,600
1999-08-31	−	+	+	25	12,800
1999-10-02	−	+	+	50	25,600
1999-11-11	−	−	+	200	51,200
1999-12-20	−	−	−	100	51,200
2000-01-31	−	−	+	50	25,600
2000-03-06	−	−	+	100	25,600
2000-04-24	ND	+	ND	ND	ND

Fig. 5. Persistent infection of *B. microti* (Kobe-lineage) in a patient and a blood donor. After Wei et al., 2001.

[a]Thin-smeared blood films were microscopically observed; +, parasitemia detectable; −, not detectable. [b]Amplification by nested PCR; +, amplification positive; −, negative. [c]Inoculation into splenectomized hamster; +, parasitemia detected; −, not detected. [d]Because a blood sample from the patient was not available on this day, the results were estimated with extracts from the cross-match test papers made prior to blood transfusion. [e]ND, not done.

*4): It was once believed that once a cell in the process of organogenesis proceeded towards specialization it could no longer return to original state. It is now recognized that a molecular mechanism, epigenetic regulation, functions to stabilize the cellular phenotype permitting cells to return to a less specialized cell. The frequency of occurrence of this cell plasticity is, however, extremely low and often imperfect (reviewed by Kadereit & Hines, 2005). Consequently, in many eukaryotes, stem cells are quietly conserved awaiting the signal for cellular self-renew and the generation of gamates. Programed DNA rearrangements, on the other hand occurs commonly throughout cellular differentiation. It is unknown whether programed DNA rearrangement retainining functionally-differentiated cells and conserving resting cells is the basic process seen in unicellular eukaryotes. Some ciliates such as *Tetrahymen*a and *Paramecium* organize their two distinct genomes into separate nuclei and the smaller of which is known to be transcriptionally silent with an unrearranged genome preserved for sexual transmission. The larger of the nuclei that comes from small nucleus show DNA re-arrangement and is used as a "disposable genome." Apicomplexans such as Coccidia, Piroplasma, Haemosporida and Gregarinia have not been noted to generate small nuclei or to maintain stem-like silent cells. But malaria hypnozoites, a dormant stage that persists in hepatocytes, are recognized in some *Plasmodium* species (Cogswell, 2000). Hypnozoites behave like stem silent cells dividing by chance, perhaps asymmetrically giving rise to both one daughter stem-like silent cell and one activatet cell, which undergoes repeated merogonic cycles of multiplication. Piroplasms can cause persistent and relapsing infections. This recrudescence, however, may not be explained either by decreasing host immunity or by antigenic change of parasites to escape the host immune responses. *Theileria orientalis* sergenti were demonstrated to grow cyclically in a calf experimentally infected with a single biological parasite clone (Fig. 4). No sign of antigenic change could be demonstrated throughout the course of this infection. It is interesting to predict that for many apicomplexans, piroplasms included, stem cell-like resting cells reside for long periods in host animals and periodically generate both stem-like cells and functionally differentiated "disposable" cells. The findings described here are obviously insufficient to permit further speculation as to what may be the next paradigm shift.

Phylogenetics and DNA Markers

Today, the reconstruction of the evolutionary tree of piroplasms is based largely on the data derived from 18S ribosomal RNA gene (*18S rDNA*) analysis. These data alone, however, are at sometimes insufficient to reliably resolve evolutionary divergences at both the deep evolutionary tree branch level as well as at the more surface level. It is, consequently, advantageous to employ novel reliable phylogenetic DNA markers that can access both of these evolutionary tree relationships – the deep evolutionary branch as well as the surface branch - rather than to rely upon *18S rDNA* alone.

Here, we tested the accessibility of the matrix of intron locations to access these ancient events as we considered this method as suitable to identify the gain-and-loss events of introns when they occur. Additionally, we recommend the coring sequences (CDS) of *CCT7* gene and of the sequences of *CCT7* introns as the markers valuable for accessing closer evolutionary relationships within the group of parasites as of *B. microti* and its close relatives.

(1) *Presence/absence pattern of intron*

To determine the intron-exon structures in the genome of various parasites nested in the Allsop & Allsop's group C (*B. microti* and its relatives) we aligned each spliced cDNA sequences to the genome sequences from a parasite strain. Of the five piroplasma house keeping genes, *18S rDNA, β-tubulin* gene, 70 killo-dulton (kd) heat shock protein-encoding (*hsp70*) gene, 78 kd glucose-regulated protein (*grp78*) gene and *CCT7* gene, the latter two, *CCT7* and *grp78* genes, contained introns with the highest frequency (5 to 6 and 3 to 4 introns per gene, respectively), while *18S rDNA, β-tubulin* and *hsp70* genes had 0, 2 and 0 introns, respectively. Throughout the 22 strains (Fig. 6), all the introns in the *CCT7* gene had standard 5'-GT...AG-3' boundaries, and the coding sequences aligned confidently with no size heterogeneity at the exon-intron junctions. *CCT7* gene, that has been identified as a single-copy gene in *B. microti* genome (Nishisaka et al., 2001), unveiled patterns not described in the literature before and maximized the resolution of deep branches within piroplasma phylogeny. The intron presence/absence pattern for the parasite strains in the Allsop & Allsop's group C (from Gray to *B. rodhaini* in Fig. 6 and all of the 27 strains in Fig. 7) was surprisingly well confirmed for

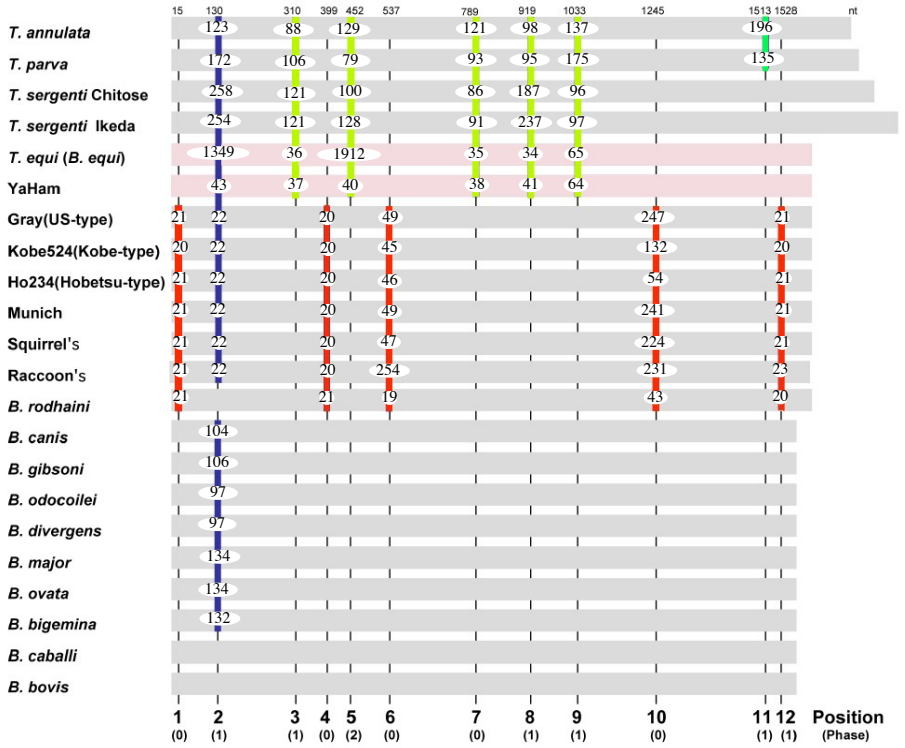


Fig. 6. Presence-absence matrix of introns within the *CCT7* gene of various piroplasma strains. After Nakajima et al., 2009; Fujisawa et al., 2011.

Tenuous transversal bar (gray or pink) shows *CCT7* gene and its nucleotide length (nt). Numerals on the mediastinum bars correspond to the size (nt) of intron: red, *B. microti*-group specific: yellow-green, *Theileria* specific: navy-blue, exist commonly in piroplasms. *T. equi* and YaHam strain, involving very deep branches in the phylogenetic trees based both on the genes, *18S rRNA, β-tubulin and CCT7*, are assembled clearly into genus *Theileria.* Very short, 19-23nt length, introns are exceptionally seen among *B. microti* and its relatives (Gray, Kobe524, Ho234, Munich, Squirrel, Raccoon and *B. rodhaini*).

all strains except for one case (*B. rodhaini* lost an intron at second position) and differed remarkably from those of other piroplasma genera *Babesia* sensu stricto and *Theileria*; of the 12 different positions, the 5 and 6 positions except one universal for all piroplasms (2nd location) were conserved exclusively in the group C and genus *Theileria*, respectively (Fig. 6). On the *grp78* gene, the intron presence/absence matrices were also found to be genus-specific (Dr. Nakajima, R., personal communication), suggesting that the presence/absence patterns of piroplasma introns should not be constrained by just one or two genes but is perhaps common in many genes.*5)

Notably, *T. equi* (*B. equi*) and the YaHam strains, both of which have very long *18S rDNA* branches in phylogeny and their taxonomic positions had long been debated, gave a typical pattern of genus *Theileria* (Fig. 6).

(2) *The smallest known introns*

To date, no nuclear introns as short as 25 nt have been recorded in any eukaryotes except in three other protists; i.e., *Paramecium tetraurelia* (20 - 33 nt; in macronuclear genome, Russel et al., 1994), *Bigelowiella natans* (18–21 nt; in nuclear morph genome, Gilson et al., 2006) and *Nyctotherus ovalis* (21–29 nt; macronulear genome, Richard et al., 2008). But, unexpectedly, the smallest known introns (from 19 to 23 nt in length) were frequently observed in the *CCT7* gene from *B. microti*-group and its relatives (Fig. 6, 7). From these parasites, we previously have detected the tiny introns as well as in the *β-tubulin* gene all at the two intron locations (Zamoto et al., 2004A) and *grp78* gene (all at the 1st, 2nd and 3rd but none at the 4th intron positions, Dr. Nakamima, R., personal communication). Thus, it is very likely that the rich abundance and instructive array of tiny introns (from 19 to 23 nt) can occur broadly through entire genome of this group of parasites.*5)

When the two intron traits are taken together, a characteristic presence/absence pattern and a carriage of tiny intron emerges that can provide a powerful phylogenetic tool to clarify the deep level of evolutionary relationships of piroplasms.*6)

Parasite Strains	Gene size (ORF)	Intron position											
		1	2	3	4	5	6	7	8	9	10	11	12
B. microti-group and their relatives													
U.S.													
North America													
Gray (U.S.A)	2009(1629)	21	22	-	20	-	49	-	-	-	247	-	21
GI (U.S.A.)	2009(1629)	21	22	-	20	-	49	-	-	-	247	-	21
East Asia													
NM69 (Hokkaido)	2010(1629)	21	22	-	20	-	49	-	-	-	248	-	21
AK2273 (Hokkaido)	2010(1629)	21	22	-	20	-	49	-	-	-	248	-	21
Vladivostock38 (USSR)	2010(1629)	21	22	-	20	-	49	-	-	-	248	-	21
Irkutsk16(USSR)	2010(1629)	21	22	-	20	-	49	-	-	-	248	-	21
Korea8 (Korea)	2010(1629)	21	22	-	20	-	49	-	-	-	248	-	21
Europe to Central Asia													
Xingiang1637 (China)	2009(1629)	21	22	-	20	-	49	-	-	-	247	-	21
HK (Germany)	2008(1629)	21	22	-	20	-	48	-	-	-	247	-	21
Munich													
Munich (Germany)	2003(1629)	21	22	-	20	-	49	-	-	-	241	-	21
Poland5 (Poland)	2003(1629)	21	22	-	20	-	49	-	-	-	241	-	21
Poland6 (Poland)	2003(1629)	21	22	-	20	-	49	-	-	-	241	-	21
UK5 (England)	2003(1629)	21	22	-	20	-	49	-	-	-	241	-	21
UK6 (England)	2003(1629)	21	22	-	20	-	49	-	-	-	241	-	21
Kobe													
Ko524 (Kobe)	1888(1629)	20	22	-	20	-	45	-	-	-	132	-	21
Hatahiyodori (Shimane)	1888(1629)	21	22	-	20	-	45	-	-	-	132	-	21
Mikura (Tokyo)	1916(1629)	20	22	-	20	-	72*	-	-	-	133	-	21
Hobetsu													
Ho234 (Hokkaido)	1813(1629)	21	22		20	-	46	-	-	-	54	-	21
Akkeshi (Hokkaido)	1813(1629)	21	22	-	20	-	46	-	-	-	54	-	21
Takanosu (Akita)	1813(1629)	21	22	-	20	-	46	-	-	-	54	-	21
Ot1 (Chiba)	1813(1629)	21	22		20	-	46	-	-	-	54	-	21
Awaji (Hyogo)	1813(1629)	21	22	-	20	-	46	-	-	-	54	-	21
Tsukiyo (Tokushima)	1813(1629)	21	22	-	20	-	46	-	-	-	54	-	21
Da116 (Shimane)	1813(1629)	21	22	-	20	-	46	-	-	-	54	-	21
Squirrel's		21	22	-	20	-	47	-	-	-	224	-	21
Raccoon's	2199(1629)	20	22	-	20	-	253	-	-	-	231	-	23
B. rodhaini	1752(1629)	20	-	-	21	-	19	-	-	-	43	-	20

Fig. 7. Size distribution of *CCT7* introns.
After Nakajima et al., 2009; Fujisawa et al., 2011.

CCT7 intron size (nt) by location is shown. Twenty-four strains of *B. microti*-group (9, 5, 3 and 7 strains of U.S.- , Munich, -Kobe- and Hobetsu lineage, respectively) and 3 other relatives (squirrel's, raccoon's and *B. rodhaini*) are included. More details are noted in Fig. 5 legend.

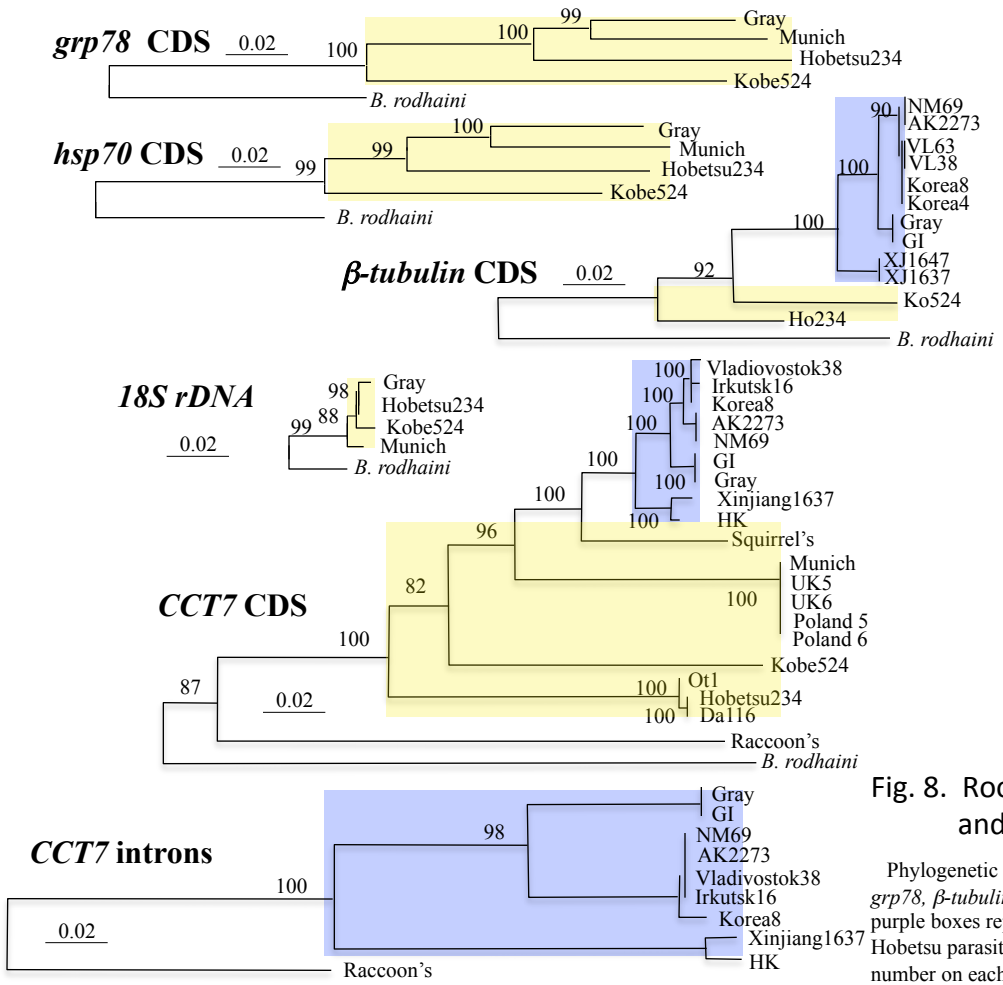
*5): *B. microti* whole genome has been sequenced very recently by Colliot, E., et al. (2012). Their results proved that *B. microti* genome structure is clearly different from that of *Babesia* sensu strict and *Theileria*.

*6): In the U.S. lineage, the frequency of substitutions throughout the 1st, 2nd, 4th, 6th, 10th and 12th introns was 1, 3, 1, 3, 22 and 1 time, respectively, and the Kobe lineage 1, 1, 1, 3, 8 and 1 time, respectively (4.8 to 13.6 mutations per 100 nt). Munich and Hobetsu lineage had 0 and 1 substitution, respectively. Contrary to the point mutations, the indel mutations did not occur in any of the tiny introns but was found exclusively in the 6th and 10th normal-size introns at rates of 2.0 to 2.4 times per 100 nt. The absence of indel mutation in the tiny introns is a curious infrequency when considered in the light of the long evolutionary history of the U.S. and Kobe lineages, a duration which would be nearly equivalent to one-third of the branching length of the entire *Babesia* sensu stricto and *Theileria* history (Fig. 8). This phenomenon may reflect a robust restraint on, or conservation of, the intron size and, thus, poses the question as to whether the processes of insertion/deletion of nucleotide sequences have a functional role in maintaining the intron size in a steady state.

The two properties, a characteristic intron-location pattern and the presence of very short introns, represent the basic differences by which higher taxonomic ranks of piroplasms, such as the order, class or even phylum can be delineated. This schema inevitably raises the question about the classification of piroplasms and others members within the phylum Apicomplexa at both the very deep evolutionary tree branches and the more surface level.

(3) *Intra-taxon size conservation at given intron locations*

It was once widely believed that individual introns could vary considerably in size within and between genes and among parasite strains (Fig. 6). However, all the *CCT7* introns, even at the 6th and 10th locations that show significant variation in size, have proven to be highly conserved by the lineage or sub-lineage of these parasites (Fig. 7). *CCT7* introns at their respective positions exhibited near uniformity in size within each given lineage, thereby suggesting that confident alignment of the intron sequences would be permitted. These two traits, a lineage-specific length variation of introns and a high-degree of conservation of CCT7 amino acid sequences, should both provide the reliable guides as to better taxon-sampling of intronic sequences. Due to their very nature, the molecular data sets can include only extant taxa. Otherwise, sparse (parasite strains of extant taxa are thought largely to be as yet undiscovered) and biased taxon sampling could occur and possibly lead to alignment errors and consequent incorrect phylogenetic



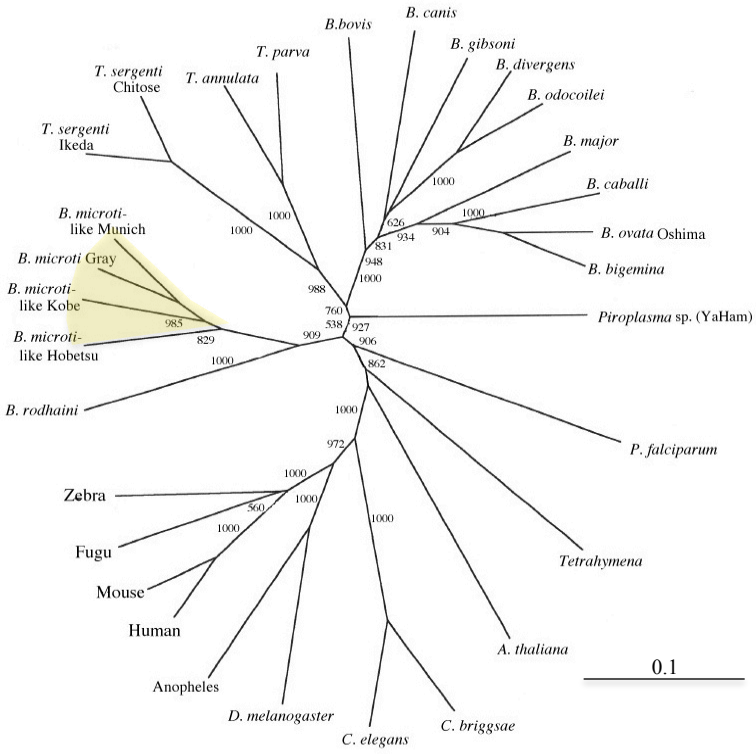


Fig. 8. Rooted and unrooted phylogenetic trees based on *CCT7* gene and 5 other gene regions of *B. microti* and its relatives

Phylogenetic trees constructed by the neighbor-joining method with the cording sequences of *18S rDNA* , *hsp70, grp78, β-tubulin* and *CCT7* genes and as well as with the combined sequences of the 6 *CCT7* introns. Yellow and purple boxes represent, respectively, the evolutionary distance among the 4 lineages of the U.S., Munich, Kobe and Hobetsu parasites and among the 9 strains in the U.S.lineage. Scale shows evolutionary distance in percent. The number on each node shows the percent occurrence in 1000 bootstrap replicates.

inferences (problem of long branch attraction).*7) Careful attention should be given to these "guides," and to curating the collected non-cording sequences of the internally transcribed spacer (ITS) regions of ribosomal DNA.*8)

(4) ***Nuclear DNA markers for assessing shallow phylogenetic relationships***

DNA sequences of the five piroplasma house keeping genes, *18S rDNA, hsp70, grp78, β-tubulin* and *CCT7* CDS, and as well as of the six *CCT7* introns were examined to evaluate shallow level of phylogenetic performance (Fig. 8). All these sequences except *CCT7* introns from *B. microti* and its closely and distantly related parasites, including *B. rodhaini*, formed a monophyletic clade (Zamoto et al., 2004A, Nakajima et al., 2009, Jinnai et al., 2009) distinct from all other *Theileria* and *Babesia* sensu stricto strains. Intron sequences were only readily aligned within each lineage but difficult globally across lineages because their big size diversity (see Fig. 7). Sequence divergence at intron regions of *CCT7* gene (combined with the 6 intron sequences) was compared with divergence at other DNA regions between the two samples, Gray and Xinjiang strains in the U.S. lineage.

A total of 7.6% diversity (29 nucleotide changes, 23 substitutions and 6 indels, over 383nt) in the introns was 63 and 2.6 times higher than those found in the *18S rDNA* (0.12%; 2 mutations over 1665 nt) and *CCT7* CDS (2.9%; 47 alterations over 1629 nt) sequences, respectively (Fujisawa et al., 2011). Thus, the intron sequences provide the most powerful tool, to our current knowledge, in micro-evolutionary studies of this group of parasites. The mitochondrial genome of apicomplexan species is a linear ~7kb in size, the smallest ever reported, encoding only three protein genes of *cox1, cox3*, and *cob* (Brayton et al 2007, Kairo et al 1994, Gray et al 1998). Sequence variations of these mitochondrial genes (*mitDNA*) among piroplasma species appear far less (Criado et al., 2006).

Population genetic structure

The complex species of *B. microti* and its close relatives, which distribute widely over the Northern Hemisphere in association with small mammals (mice, voles and shrews) and Ixodid vectors, are the most common causes of human piroplasmosis. Although these parasites have been studied for decades by applying molecular recognition principles, few studies have been conducted to measure the genetic variability and to describe the phylogeography of the parasite populations. A better understanding genetic diversity of these parasites may have far-reaching implications for public health strategies in preventing and reducing the disease prevalence and rational development of diagnostics, therapeutics and vaccines. Thus, genetic structure of this parasite group was investigated by analyzing the variability of nucleotide sequences of both *CCT7* CDS and *CCT7* introns from the 24 samples of different geographical origins (Nakajima et al., 2009; Fujisawa et al., 2011).

*7) The problem of "long branch attraction" is the tendency of highly divergent sequences (i.e. those with long terminal branches) to group together in a tree regardless of their true relationships. This is due in part to the rapidly evolving sequences, or to sequences without any close relatives, that have numerous unique mutations with respect to the rest of the tree (Reviewed by Baldauf, 2003). One explanation why this occurs is that there are only a limited number of possible states into which rapidly evolving sites may evolve; sequences either with the single change (A→C) or redundant changes (A→G→C/A→T→C) at a nucleotide position are indistinguishable and both will be treated as an identical change. If their branches are very long (i.e. if there are many such changes), these spurious similarities can override the true phylogenetic signal so that the subsequent sequences will be "attracted" to each other.

*8): *rDNA* CDS are highly conserved among species but ITS regions are variable due to insertions, deletions, and point mutations. Therefore, a comparison of sequences at ITS tracts between such remote species as human and frog is considered inappropriate though the sequences are occasionally used in analyzing piroplasma phyogenies at the scales (see Fig. 8).

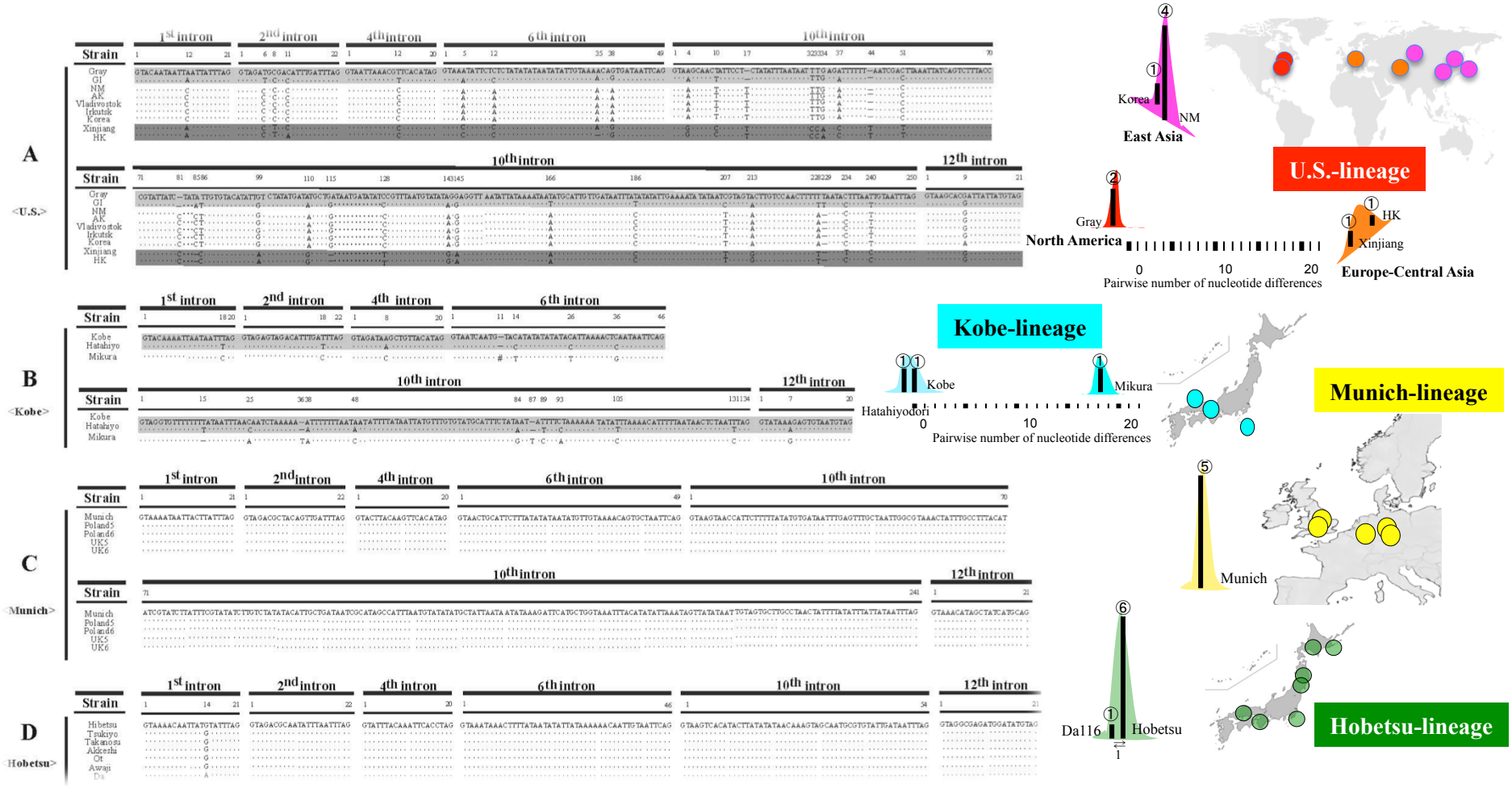


Fig. 9. Multiple sequence alignment of the *CCT7* introns

The individual intron at each position (1st, 2nd, 4th, 6th, 10th, 12th) was aligned by sequences with its same-position counterparts within lineage (A – D). Dots indicate identity with the consensus sequence. Line of letters with gray, dark-gray or white background highlights subgroups that appeared to be further divided within a lineage. #); A 27-nt insertion (CGCCT ATATA TATAT ATATA TGTTA TA) occurs after 10th position of the 6th intron in Mikura strain in the Kobe taxon.

Fig. 10. Frequency of mismatch distribution

Frequency distribution of the number of observed pairwise nucleotide differences among parasite samples for the combined sequences of *CCT7* introns. Hight of the black bar and numerals surrounded circle on top op the bar indicate sample incidence. The distance between two bars depicts the "pairwise number of nucleotide differences". Colored circle on map shows the geographical origins of parasite strains isolated.

(1) ***Frequency distribution of pairwise sequence mismatches of CCT7 introns***

Analysis of the combined intron sequences provided data for further subdivisions of the two known taxa, the U.S. and Kobe, into three and two separate clusters, respectively, based on the sequence similarities (Figs. 8, 9A, 9B, 10, 11). Nucleotide changes occurred most frequently

(90%≤) at the intra-taxon subgroup level and infrequently among individuals within a subgroup (Fig. 9A, 9B, 10, 11), indicating that the extent of *CCT7* intron variations is largely proportional to the micro-evolutionary distance and not to allele or genotype frequencies in a population. The Munich and Hobetsu taxa displayed no variation or, at most, only a very rare sequence variation, among geographically diverse samples (Fig. 9C, 9D, 10, 11), distributing independently either in Europe (England, Germany, Poland and Russia) or in Japan. This very low genetic diversity could be the result of natural selection favoring fragmentation and clonal proliferation, i.e., either the founder effect or a bottleneck event. The regional distributions of the Munich (found in Europe) and Hobetsu taxa (found in Japan) and their long evolutionary durations as represented through the branch lengths of the CDS phylogenies of *CCT7, β-tubulin*, *grp78* and *hsp70* genes (Fig. 8), seems to indicate a bottleneck event as the most plausible scenario for the clonal population structure of the two extant taxa. This bottleneck catastrophe would have occurred directly on the parasites themselves or indirectly on the host and vector.

In contrast these regional taxa, the Kobe taxon found in wild mouse and rat reservoirs have been shown to be distributed focally, as, in only a few narrowly defined areas in Japan - the Mikura Island in the Izu-Ogasawara (Izu-Bonin) Trench, Awaji Island close to Osaka and Daito Town on the western tip of Honshu, the main island of Japan (Fig. 9B, 10). Given that the Kobe lineage is especially likely to have risen from a population bottleneck, a plausible explanation for the evolutionary history may be that the lineage was once distributed widely only to become virtually extinct except for a few bare remnants in remote-like focal areas of Japan where they survive exhibiting pronounced genetic diversity. However, a founder effect, in which a few parasites would have dispersed to an isolated Pacific island in niches without any competition and evolved quickly is an alternative possibility. In fact, the sequence changes seem to have occurred almost solely in the Mikura strain but not in others, i.e., all but one of the 19 sequence differences including a 27-nt insertion were identified in a sample from Mikura island (Fig. 9B). Analyses of more intron data, difficult to obtain due to the scarce distribution of this taxon, will allow us to draw more accurate inferences.

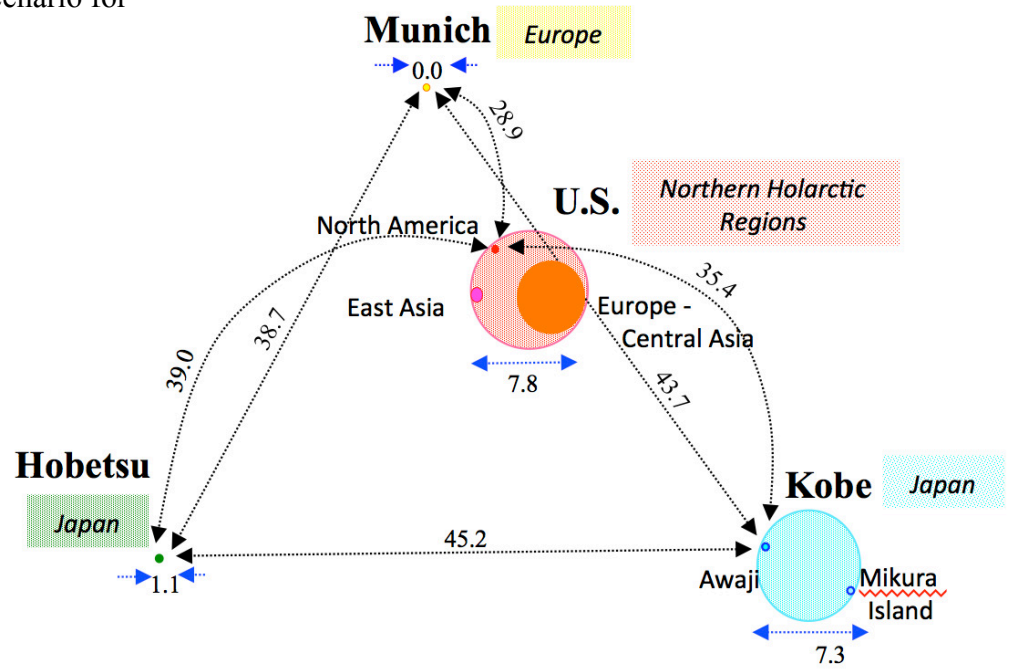


Fig. 11. Schematic patterns of population genetic variation

Percent of evolutionary distance (number of observed pairwise nucleotide differences) within and between the 4 lineages (U.S., Munich, Kobe and Hobetsu) of the *B. microti*-group parasites were generated in two ways. First, the within lineage diversity was measured by comparing with paired intronic sequences of all samples by each of the lineages or sub-lineages (see footnote in Fig. 9.) and the maximum extent of sequence diversity in percent is illustrated as to the diameter of circle. Second, percent of evolutionary distance was determined by comparing two pairs of *CCT7* ORF sequences from representative samples of each lineage and multiplied by the proportional vale {x2.6; described in the section of " (3) Nuclear DNA markers for assessing shallow phylogenetic relationships" }. A three dimensional representation of evolutionary distance is illustrated.

(2) *Levels of genetic diversity within and between populations of the four lineages in* B. microti*-group*

Levels of genetic diversity within and between populations of the four taxa in *B. microti*-group were compared by using two DNA markers, *CCT7* CDS and *CCT7* introns (Fig. 11). Two widely and narrowly distributed taxa (U.S., world-wide distribution, and Kobe, found only in focal areas in Japan) showed a significant level of intra-taxon genetic variability, and the maximum levels of intron sequence differences of 7.8 and 7.3 % for the U.S. and Kobe lineage, respectively, are far less (4-5 times lower) than those demonstrated between each pair of the populations of U.S., Munich, Kobe and Hobetsu taxa (Fig. 11). In addition, these taxa differ from one another in the presentation of several genetic and biological traits: (1) There are substantial levels of sequence divergences that are closely comparable to those found between pairs of the *β-tubulin* and *CCT7* CDS found in such well-recognized species as *B. odocoilei* and *B. divergence.* (2) *CCT7* introns, regardless of large size diversity among taxa (19-254 nt), exhibit uniformity in size within each given taxon (less than one base difference) at their respective positions, thus distinguishing them one from the other (Nakajima et al., 2009; Fujisawa et al., 2011). (3) a slight cross-reactivity of antigens are only demonstrated between the samples of the two different lineages (Tsuji et al., 2001).

Host-Parasite-Vector Interactions

The genetic population structure of most piroplasms remains largely unknown. This genetic population structure, however, is essential for understanding co-evolutionary interactions between parasites and their hosts or vectors. It is also of interest as it aids in predicting how fast phenotypes of interest, such as antigenic variants and drug resistance, originate and spread among populations i.e., whether or not they arise from mutations or are introduced from the gene flow from other populations.

Our results indicate that the patterns of genetic population structures based on the sequences of *CCT7* introns are different for each taxon of *B. microti*-group (Fujisawa et al., 2011; Nakajima et al., 2009). Hobetsu and Munich taxa, common to Japan and Europe, respectively, exhibit less or no genetic variability, whereas U.S. and Kobe taxa, widely distributed throughout the Northern hemisphere and within several narrowly spotted areas in Japan, show extreme genetic divergence among geographical samples (Fujisawa et al., 2011). The parasite population structures may mirror the migration of, and the adaptation process to, their host animals and/or vector ticks. To assess factors impacting population structure of these parasite lineages or sub-lineages in *B. microti*-group, taxonomic and geographical distance matrices of animal hosts and the vector ticks were compared.

(1) *Actual host range of parasites populations in nature*

The U.S. taxon is widely distributed throughout the Holarctic (both the Nearctic and Palearctic) temperate regions comprising the U. S. A., Germany, Russia, China, Korea and Japan, where the parasites are harbored by various small mammals such as *Peromyscus* and *Microtus* spp. (rodents), and *Blarina* (shrew) in North America, *Apodemus, Myodes* (formerly *Clethrionomys*), *Microtus* spp. (rodents) as well as *Sorex* spp. (shrew) in Eurasia, including Japan (Table 1, 2, Fig. 12; Zamoto et al., 2004A; reviewed by Hunfeld et al., 2008). The two regional taxa of the Hobetsu and Munich, biologically inseparable from U.S. parasites but distinctive phylogenetically by the *β-tubulin* and *CCT7* gene analyses, are common in Japan and Europe, respectively, where either of the two overlap in geographic distribution with the U.S. taxon by adaptation to the same or similar reservoir hosts at similar prevalence (Table 1, 2, Fig. 12; Tsuji et al., 2001; Zamoto et al., 2004B).

Table 1. Summary of field surveys for *B. microti* like parasites in small wild mammals in Hokkaido and Tohoku, Japan.

	Animal species	No. of animals captured	No. of positives per examined							
			Micro-scopically[a]	Nested PCR for beta-tubulin gene					IFAT[d]	Isolation[e]
				Univ.[b]	U.S.[c]	Hobetsu	Kobe	Munich		
Nemuro	*Clethrionomys rufocanus*	4	2/4	2/4	1/2	1/2	0/2	0/2	2/4	2/2
	Clethrionomys rutilus	10	1/10	1/10	1/1	1/1	0/1	0/1	1/10	1/1
	Sorex cauecutiens	3	1/3	1/3	0/1	1/1	0/1	0/1	1/3	0/1
Akkeshi	*Apodemus speciosus*	4	0/4	1/4	1/1	0/1	0/1	0/1	1/4	1/1
	Clethrionomys rufocanus	7	0/7	1/7	0/1	1/1	0/1	0/1	1/7	1/1
	Clethrionomys rutilus	2	0/2	0/2	·	·	·	·	0/2	·
	Tamias sibiricus	1	0/1	0/1	·	·	·	·	0/1	·
Horonobe	*Apodemus speciosus*	2	0/2	0/2	·	·	·	·	0/2	·
	Apodemus argenteus	3	0/3	0/3	·	·	·	·	0/3	·
	Clethrionomys rufocanus	1	0/1	0/1	·	·	·	·	0/1	·
	Sorex unguiculatus	2	0/2	0/2	·	·	·	·	0/2	·
Kiyosato	*Apodemus speciosus*	1	1/1	1/1	0/1	1/1	0/1	0/1	·	1/1
	Apodemus argenteus	2	0/2	0/2	·	·	·	·	·	·
	Clethrionomys rufocanus	2	0/2	0/2	·	·	·	·	·	·
	Sorex unguiculatus	1	1/1	1/1	0/1	1/1	0/1	0/1	·	1/1
Hobetsu[f]	*Apodemus speciosus*	10	7/10	7/10	0/7	7/7	0/7	0/7	7/10	7/7
	Apodemus argenteus	2	0/2	0/2	·	·	·	·	0/2	·
	Clethrionomys rufocanus	3	0/3	1/3	0/1	1/1	0/1	0/1	1/3	1/1
Ebetsu[f]	*Apodemus speciosus*	22	0/22	0/22	·	·	·	·	0/22	·
	Apodemus argenteus	15	0/15	0/15	·	·	·	·	0/15	·
	Clethrionomys rufocanus	7	0/7	0/7	·	·	·	·	0/7	·
	Sorex unguiculatus	1	0/1	0/1	·	·	·	·	0/1	·
Setana	*Apodemus speciosus*	11	0/11	0/11	·	·	·	·	0/11	·
	Apodemus argenteus	8	0/8	0/8	·	·	·	·	0/8	·
	Clethrionomys rufocanus	1	0/1	0/1	·	·	·	·	0/1	·
	Sorex cauecutiens	2	0/2	0/2	·	·	·	·	0/2	·
Okusihri	*Apodemus speciosus*	14	0/14	0/14	·	·	·	·	0/14	·
Shizukuishi	*Apodemus speciosus*	23	0/23	0/23	·	·	·	·	0/23	·
	Apodemus argenteus	10	0/10	0/10	·	·	·	·	0/10	·
	Eothenomys andersoni	1	0/1	0/1	·	·	·	·	0/1	·
	Microtus montebelli	1	1/1	1/1	0/1	1/1	0/1	0/1	1/1	1/1
	Urotrichus talpoides	2	0/2	0/2	·	·	·	·	0/2	·
Takanosu	*Apodemus speciosus*	17	7/17	7/17	0/7	7/7	0/7	0/7	8/17	7/7
	Apodemus argenteus	1	0/1	0/1	·	·	·	·	0/1	·
	Urotrichus talpoides	1	0/1	0/1	·	·	·	·	0/1	·
		197	21/197	24/197	3/24	22/24	0/24	0/24		23/24

a): A thin layer of blood smear was stained Giemsa's solution and examined under microscopically.

b): Nested PCR by using a set of primers broadly specific for all *B. microti* group parasits.

c): Nested PCR with lineage-specific primers

d): Indirect Fluorescent Antibody Test (IFAT) was performed by using the antigens as parasite strains either in the U.S., Kobe, Hobetsu or Munich lineage.

e): Isolation by inoculating blood samples into hamsters or erythrocyte-substituted SCID mice.

f): Samples used were those described previously (Tsuji et al., 2001).

Table 2. Summary of field survey of *B. microti* like parasites among small wild mammals in Japan from 1999 to 2000

Prefecture	Site	Species	No. of animals trapped	No. positive/no. tested by:				No. of animals with the following rDNA genotype[e]	
				Microscopy[a]	PCR[b]	IFAT[c]	Isolation[d]	Kobe	Hobetsu
Hokkaido	Hobetsu	*Apodemus speciosus*	10	7/10	7/10	7/10	7/9	0	7
		Apodemus argenteus	2	0/2	0/2	0/2	0/2		
		Clethrionomys rufocanus	3	0/3	1/3	2/3	1/3	0	1
	Ebetsu	*Apodemus speciosus*	22	0/22	0/22	0/22	0/11		
		Apodemus argenteus	15	0/15	0/15	0/15	0/8		
		Clethrionomys rufocanus	7	0/7	0/7	0/7	0/4		
		Sorex unguiculatus	1	0/1	0/1	0/1	ND		
Chiba	Ohtaki	*Apodemus speciosus*	2	2/2	2/2	2/2	2/2	0	2
Shiga	Yamanaka	*Apodemus speciosus*	1	1/1	1/1	1/1	1/1	0	1
Hyogo	Kobe	*Apodemus speciosus*	1	0/1	0/1	1/1	0/1		
	Miki	*Apodemus speciosus*	1	0/1	0/1	0/1	ND		
	Kanzaki	*Apodemus speciosus*	8	0/8	0/8	0/8	ND		
		Apodemus argenteus	1	0/1	0/1	0/1	ND		
	Awaji	*Apodemus speciosus*	7[f]	4/7	4/7	4/7	4/7	2	2
		Crocidura dsinezumi	1	0/1	0/1	0/1	0/1		
Shimane	Daito	*Apodemus speciosus*	14	4/14	7/14	4/8	3/4	0	3
		Apodemus argenteus	2	0/2	0/2	0/2	ND		
		Eothenomys smithii	3	0/3	0/3	0/3	ND		
Tokushima	Anan	*Apodemus speciosus*	11	1/11	2/11	3/11	2/3	0	2
Total			112	19/112	24/112	24/106	20/56	2	18

[a] Detection of parasitized RBCs by microscopy of thin-smear blood films.
[b] Detection of babesial rDNA by nested PCR.
[c] IFAT titers that were higher than 1:200 against either strain Kobe or strain Ho234 were taken as positive.
[d] Isolation of *B. microti*-like parasites by inoculation of blood specimens into splenectomized hamsters. ND, not done.
[e] Determined by sequencing of the rDNA amplified from the isolated parasites.
[f] Including two *A. speciosus* mice included in a previous study (35).

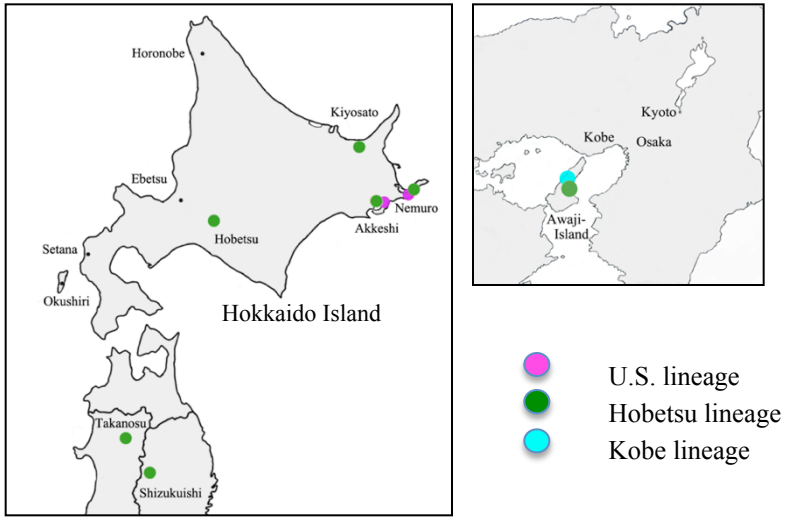


Fig. 12. Field surveys for *B. microti*-group parasites in small wild mammals. After Tsuji et al., 2001; Zamoto et al., 2004B.

Epizootiologic field surveys were carried out in two islands, Hokkaido and Awaji, in Japan. The blood specimens of wild small mammals were collected and tested by PCR for the presence of the *B. microti*-parasites. To classify the *B. microti*-group parasites detected, all of the *rDNA*-positive samples were further examined by lineage-specific PCR base on *β-tubulin* gene.

The Kobe lineage, also found in *Apodemus* and *Ruttus* spp., occurs only locally in a few narrowly defined areas such as the Mikura island in the Izu-Ogasawara (Izu-Bonin) Trench, Awaji island near Osaka, and Daito-town on the western tip of the main island of Japan (Wei et al, 2001; Tabara, et al., 2006), where the Hobetsu parasites have been shown to co-exist in similar reservoir hosts. (in Mikura island, the Kobe parasite was detected by chance from a wild rat despite the fact that local surveillance data has, to date, not reported its presence). Additionally, all the *B. microti*-group parasites with the exception of the Munich parasites have been associated with human infections. Hence, it is assumed that the parasites in *B. microti*-group have rather wide, overlapping host ranges with little differences in their prevalence with animals in each geographical region. (reviewed by Hunfeld et al., 2008).

(2) *Vector ticks*

In the U. S. A., *Ixodes scapularis* (also known as *I. dammini*) has been identified as the primary vector for *B. microti* parasites based on intensive laboratory and field ecologic studies, particularly on Nantucket Island, MA, where the mammalian and tick fauna are both scant (reviewed by Hunfeld et al., 2008). With regard to tick vectors elsewhere in the world very little was known. The question therefore, arose as to whether or not the regional Ixodid species was a competent vector for the regional lineages of the *B. microti*-group, particularly in the case when the two lineages co-occur. In selected areas in Japan, the U.S. and Kobe taxa are minimally sympatric with the widely distributed Hobetsu taxon (Tsuji et al., 2001; Zamoto et

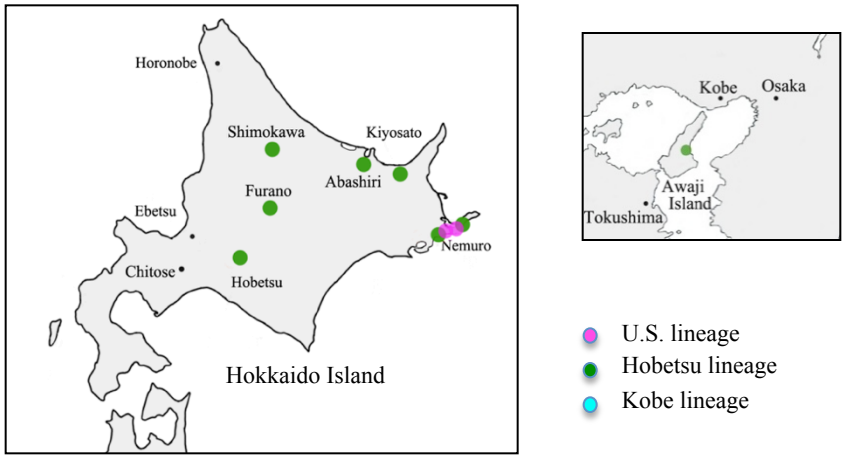


Fig. 13. Field surveys for *B. microt*-group parasites in *Ixodid* ticks in Japanese nature.

Epizootiologic field surveys were carried out in two islands, Hokkaido and Awaji. Ticks were tested by PCR for the presence of *B. microti*-parasites. To classify the parasites detected, all of the *rDNA*-positive samples were further examined by lineage-specific PCR base on *β-tubulin* gene. (After Zamoto-Niikura et al., 2012)

Table 3. Number of ticks collected in Hokkaido and Awaji islands, Japan.

Areas	Tick spacies						
	I. ovatus	*I. persulcatus*	*I. turdus*	*I. tanuki*	*H. flava*	*H. douglasi*	Total
Hokkaido	2744	1274	2	1	2	45	3708
Awaji	190	0	23	0	89	0	302

Table 4. Detction of *B. microti* like rDNA by PCR in the field collected ticks.

Tick species	Survey area	No. of ticks examined[a]	No. of ticks pooled	No. of ticks tested	No. of PCR-positive samples	Minimum infection rate(%)[b]
I.ovatus						
Hokkaido	Nemuro	48	2 to 3	19	4	8.3[c]
	Kiyosato	65	5	13	8	12.3
	Shimokawa	100	5	20	2	2.0
	Aibetsu	85	5	17	0	0
	Furano	115	5	23	3	2.6
	Hobetsu	140	5	28	10	7.1
	Ebestu	36	3	12	0	0
	Chitose	68	4 to 5	15	0	0
Awaji	Sumoto	180	5	36	11	6.1
	Subtotal	837		183	38	5.5
I. persulcatus						
Hokkaido	Nemuro	139	3 to 5	33	2	1.4
	(Nymph)	196	1	196	0	0
	Horonobe	42	3	14	0	0
	Kiyosato	105	5	21	0	0
	Shimokawa	15	5	3	0	0
	Aibetsu	85	5	17	0	0
	Furano	44	5	15	0	0
	Hobetsu	36	3	13	0	0
	Ebetsu	15	5	3	0	0
	Subtotal	1656		542	2	0.3
Other tick species						
	Six areas[d]	162	1	162	0	0

[a] Ticks examined were all adult except one case of *I. persulcatus* samples collected at Nemuro.
[b] Values(%) were calculatated by comparing the number of pools that were PCR positive for the *B. microti*-group to the total number of ticks examined. The calculation was based on the assumption that each PCR-positive pool contains at least one tick with detectable *B. microti*-group parasite(s).
[c] Considering the impact of the comparison of MIRs, the possible MIR would be between 2 and 8.3% when 5 ticks were pooled.
[d] Nemuro, Horonobe, Kiyosato, Hobetsu and Sumoto were included.

al., 2004B). We conducted field surveys in the two areas of sympatry at Nemuro on Hokkaido island and at Sumoto on Awaji island to assess the lineage- or sublineage-specific prevalence in ticks in order to ascertain what species of ticks are the natural vectors for the individual lineages of *B. microti*-group (Zamoto-Niikura et al., 2012).

By flagging vegetation at these spots and surrounding areas, 4010 ticks comprising 6 species were collected (Table 3). A nested PCR that detects *18S rDNA* of all *Babesia* species revealed that *I. ovatus* and *I. persulcatus* alone were positive. Lineage specific PCR for *rDNA*-positive samples demonstrated that *I. ovatus* and *I. persulcatus* carried, respectively, the Hobetsu and U.S. parasites. No Kobe-specific DNA was detected (Table 4); infected *I. ovatus* ticks were found at multiple sites in the two islands examined including Nemuro and Sumoto with minimum infection rates (MIR) of ~12.3%. However, totally 535 of *I. persulcatus* collected at the same areas (Nemuro in Hokkaido) were all negative for the Hobetsu-lineage. On the other hand, 2 out of 139 adult *I. persulcatus* harbored U.S. parasites (1.4%) at Nemuro and 48 of *I. ovatus* were all negative for the lineage (Table 4). Likewise, none of the *I. persulcatus* ticks, collected even in the areas where the Hobetsu parasites were endemic, was positive for the Hobetsu DNA (Table 4). Laboratory experiments confirmed the transmission, respectively, of the Hobetsu and U.S. parasites to hamsters via *I. ovatus* and *I. persulcatus*. These results indicate that, for the first time, the Hobetsu and U.S. parasites are vectored by different Ixodid species (Fig. 14), although the number of positive samples of U.S. lineage in *I. persulcatus* was small.

In most of study areas (urban and pre-urban forestry were for the most part free from infection), *I. ovatus* ticks and the Hobetsu parasites overlapped in geographic distribution (Figs. 12, 13). The U.S. parasites were detected only at Nemuro, regardless of the uniform distribution of the vector species, *I. persulcatus*, throughout Hokkaido where *I. ovatus* inhabited sympatrically (Figs. 12, 13). This discrepancy of the geographic occurrence between *I. persulcatus* ticks and U.S. parasites may in part be the result of the poor vectorial capacity of *I. persulcatus* of the difference of the MIRs for *I. persulcatus* (1.4%) and *I. ovatus* (8.3%) at Nemuro, where both tick species may have had an equal chance to be infected (Fig. 12). This lower vector activity could also contribute to minimizing regional risk of human infection with the parasites of U.S. lineage in Japan.[*9)] Indeed, a 1.4% MIR of *I. persulcatus* at Nemuro is significantly low (almost 1/10) compared to that of *I. scapularis* in Nantucket Island, MA, U. S. A., where a high prevalence of human infection has been reported (Piesman & Spielman, 1980; Liu, Y. 2012). We cannot predict accurately the specificity of tick vector(s) nor estimate definitively the vector capacity for the two sympatric taxa, the U.S. and Munich lineages in Europe in circumstances where most ecological and epidemiological studies have been carried out using *18S rDNA* for which the sequences do not precisely identify the two sympatric lineages of *B. microti*-group.

[*9)]: A trend for phylogenetic clustering of *CCT7* sequences (Fig. 14) of all U.S. lineage parasites was largely associated with the molecular classification of the sister tick species in the *I. ricinus* complex each of which transmitted the parasites with varying vector capacity with the subgroup-specification. Thus, the phylogenetic grouping at this shallow level was proven to elucidate the local risk of human infection. Similarly, extensive phylogenetic grouping of piroplasms, other than the *B. microti*-group, might also delineate the reproductively isolated populations, though the classification for piroplasms, has been made on the basis of pairwise evolutionary distances between the pairs of *18S rDNA* sequences. A genetic difference of 0.12% (2 mutations over 1665 nt) for the two sister sub-lineages, between the North American (Gray strain) and Europe-to-Central Asian (Xinjiang strain) subgroups, was 15 times lower than the distance found between well established closely-related piroplasma species, such as between *B. odocoilei* and *B. divergens* (1.8%) (Nakajima et al., 2009; Jinnai et al., 2009).

(3) ***Perspective on the recent evolution of the U.S. lineage parasites***

All parasites in the *B. microti*-group in Japanese nature respectively cluster in three groups, the U.S., Kobe and Hobetsu lineages, despite the sympatry of their hosts and their overlapping geographic distributions in a few particular areas (Figs. 12, 13). This supports an ancient and genetically independent evolution of these clusters. Likewise, despite the probable sympatry of the hosts, all parasites in the U.S. lineage distributing throughout temperate zone of North hemisphere, including Japan, cluster respectively in three sub-groups of the North American, Europe-to-Central-Asian and East Asian clusters (Fig. 14, 15). In view of their branching orders and shorter branch lengths on several gene trees as well as their geographical wide distributions (Figs. 8-10, 15), these subgroups may have arisen through rapid and most recent evolutionary radiations. Given the prior findings and our latest result (Fig. 14, Zamoto-Niikura et al., 2012), that indicate that these subgroups are independently vectored by the three sister tick species of *I. scapularis, I. ricinus* and *I. persulcatus*, it could be hypothesized that all these sister-taxa have likely diverged relatively recently from a common ancestor by spreading over the Holarctic (both the Nearctic

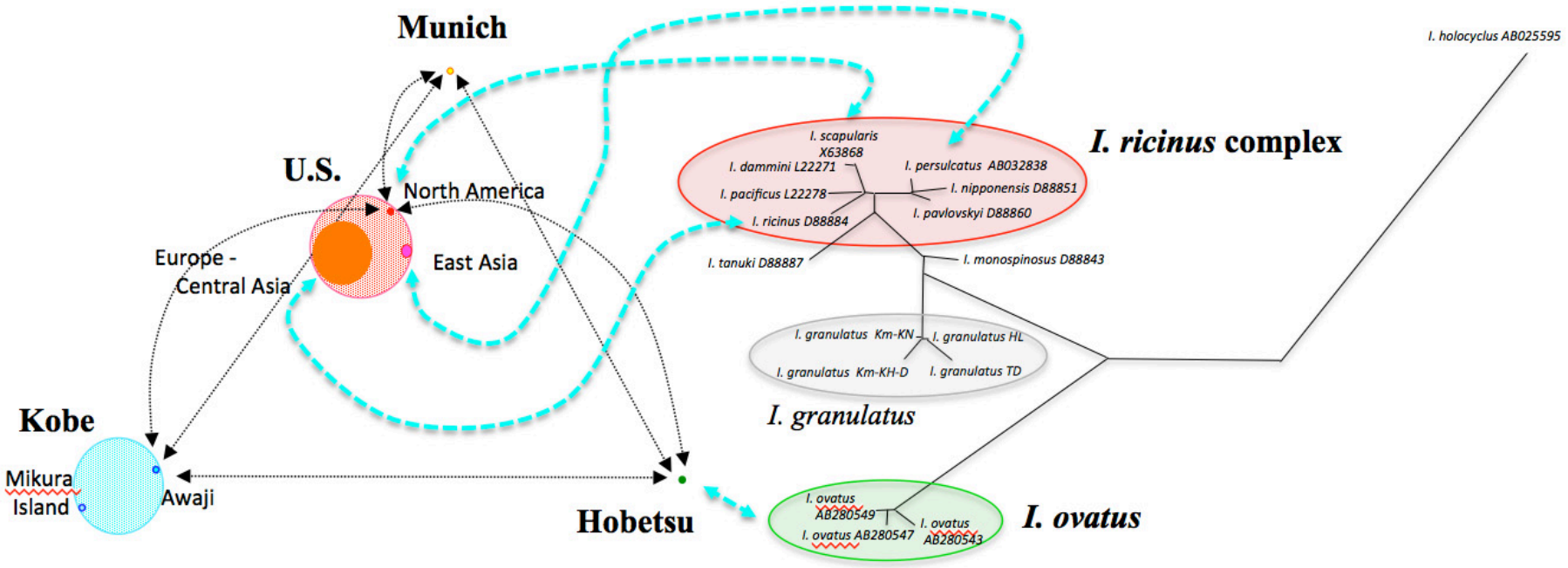


Fig. 14. Cross-species matches in vector specificity for *B. microti*-group parasites.

Schematic patterns of population genetic variation of *B. microti*-group parasites (left). Phylogenetic related ness of ITS2 sequences from various Ixodid ticks (right). Figure is modified from Zamoto-Niikura et al., 2012 (left, see legends in Fig. 11) and Chao et al., 2011 (right). Pale-blue dotted line represents the parasite lineage -primary vector relationship.

and Palearctic) temperate regions via the evolutionary journey of vector ticks to seek vertebrate hosts.*10) It has commonly been believed that parasites and their hosts speciate in synchrony, and our present results support this hypothesis – the co-evolution between the common ancestors of U.S. parasite and of *I. ricinus* complex (Nakajima et al., 2009; Fujisawa et al., 2011; Zamoto-Niikura et al., 2012), although strict co-evolution with congruent phylogenies has rarely been demonstrated.*11)

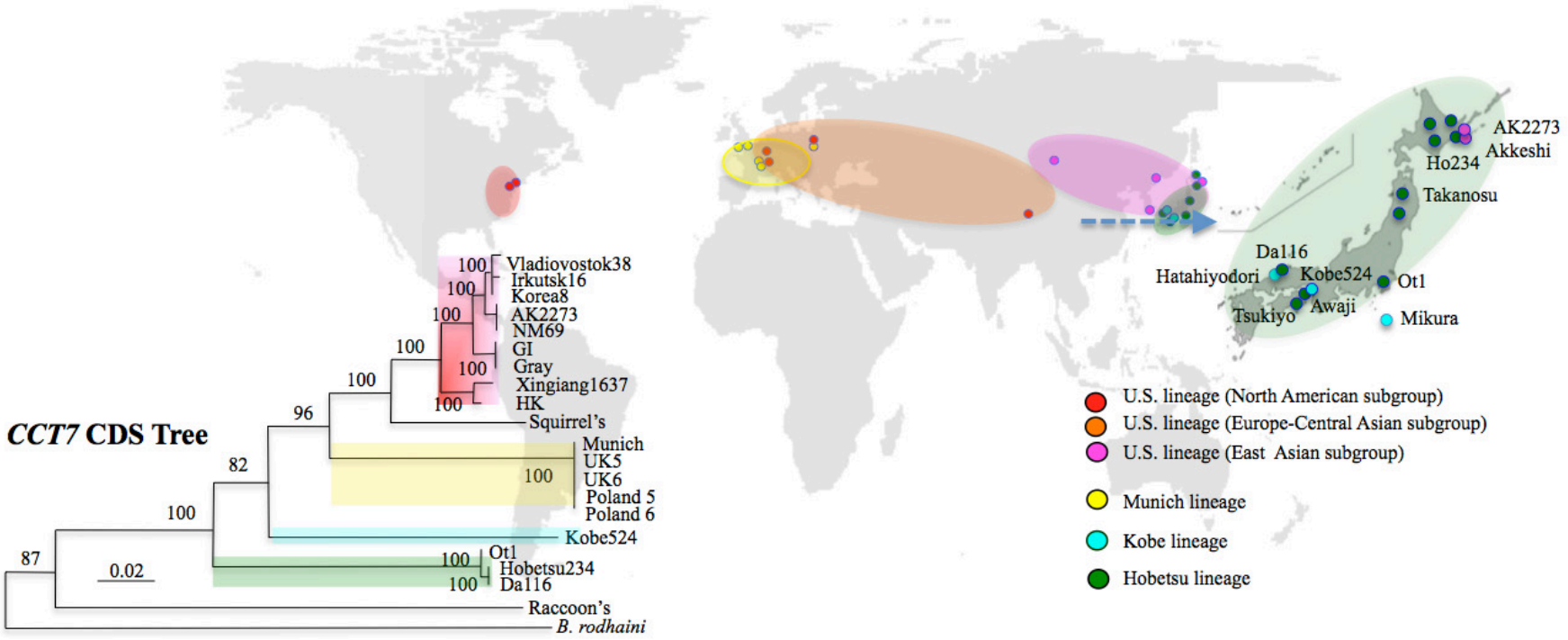


Fig. 15. Predicted distributions of the 4 lineages of *B. microti*-group and their phylogenetic relationships

Geographic origin of the parasite strains (see Figs. 6, 12, 13) and the spot where given lineage of parasites detected are all plotted on the Figure. Considerable endemic areas for each of the 4 parasite lineages are covered by transluent color. *CCT7* CDS tree shown as shown Fig. 6 is also placed on this figure for referring evolutionary relatedness of the parasite strains in the *B. microti*-group. After Nakajima et al., 2009; Fujisawa et al., 2011; Zamoto et al., 2004A, B; Zamoto-Niikura et al., 2012.

*10): In poor active dispersers such as ticks, genetic structuring must be caused by the evolutionary innovations following the intra-/inter-continental movements of their mammal hosts.

*11): Theoretical studies of speciation have been dominated by numerical simulations seeking to demonstrate that speciation in a given scenario can occur. Debates on diversification focus on speciation modes in the temporal dimension (gradual vs. instantaneous speciation, e.g. through hybrid speciation), the spatial dimension (allopatric vs. sympatric speciation), and on the general mechanisms driving divergence (ecological adaptation, sexual selection, or non-adaptive factors such as genetic drift), remain still unresolved.

(4) *Another plausible scenario for the most recent* B. microti *diversification*

Another most plausible scenario for the most recent piroplasma diversification can be a rapid diversification following host shift (adaptive speciation). Non-native exotic species invasions create almost ideal conditions for promoting evolutionary diversification by host shift: (1) establishment of allopatric populations of species (usually host mammals) in a new geographical regions; (2) generation of powerful selective forces on the invasive parasites, which promote vector shifts to the indigenous tick species; (3) and providing new opportunities for the parasites which has been endemic in native animals to switch to newly invaded mammal hosts. Hence, we started recently a prospective research in serial surveillance of piroplasma infection in raccoons (*Procyon lotor*), whose importation into Japan from North America began in the 1970s as pets and continued until 2000. Some escaped to live in the wild and the feral raccoons have now expanded their ranges throughout Japan (Japanese government has classified them as an alien "invasive species" soon to be exterminated). During the colonization process of feral raccoons, two different types of host-shifting events may be involved in piroplasma adaptation: i.e., (1) non-native exotic parasites will seek new vector species native to Japan to establish a novel life-cycle;[*12)] (2) native parasites will seek feral raccoons as a novel mammal host by escaping the established life cycle indigenous to Japanese nature.

piroplasma						
origin of parasite	prerequisite		vector type (transfered by)	resulting population bottleneck	prevalence of host infection at the initial stage of invasion	predicted population genetic structure of a parasite group that established in feral
	host shift to raccoons	vector shift				
North America	not necessary	no	one-host tick	no	high	genetically diverse
			three-host tick			
		yes	three-host tick	extreamly high*	very low	clonal
Japan	yes	no	three-host tick	only weakly	very low	genetically diverse
			one-host tick			

vector tick				
Origin of tick species	prerequisite	vector type (feed on)	provability of invasion into Japan	preference for feeding on raccoons
	host shift to raccoons			
North America	not necessary	one-host	easy	yes
	yes	three-host	very little	yes
Japan	yes	three-host	(residential)	yes
	not necessary	one-host		rare

Fig. 16. Possible patterns for the establishment of a new life-cycle for exotic/residential piroplasms in feral raccoons and, possibly, how exotic/residential tick species might infest feral raccoons in Japan.

Raccoon piroplasms endemic in North America are primarily considered to be vectored by three-host-feeding ticks. Raccoon piroplasms vectored by one-host-feeding ticks have, to date, not been described. These piroplasms, must, therefore, undergo a vector shift to the residential tick species in Japan since the establishment of an exotic three-host-feeding tick in Japanese nature is remote given the strict niche competitions against residential ticks in Japan. Two possibilities for consideration are (1) that exotic piroplasmas vectored by three-host-feeding ticks in North America may become established, despite the predicted extreme population bottlenecks, by vector-shifting to a three-host-feeding ticks native in Japan; and (2) that some piroplasms endemic in Japanese nature undergo host-shift during the process of population establishment of raccoons in Japan.

*: Bottleneck may occur at three separate stages; (1) host invasion usually by a small population, (2) adaptive mutation to resident tick-vector species in which an extremely rare mutant among a parasite population is acquired, and (3) completion of the vector-shift followed by a cessation of the old life-cycle

*12): In the rare instance of an exotic tick importation, despite an importation control by Japanese government, a tick population establishment, especially for three host ticks, must be assumed to be scarce due to a strong niche competitions at each of the three different host species in the whole life cycle, against native tick species. Indeed, there is no evidence for an established population of non-native tick vectors for raccoon parasites. The term "one-host-tick" is attempted to use broadly, in this report, even for a tick that normally prefer to have different host for each feeding stage but it, in certain circumstances, can complete its entire life cycle on a single host.

Theoretically, these piroplasms must have experienced three different categories of population bottlenecks during the course of their evolutionary adaptation: (a) a genetic bottleneck during the process of selecting a mutant clone enabling them to infect to a new vector tick/ mammal host; (b) a population bottleneck through a period of life-cycle interruption; (c) a population bottleneck corresponding to a severe reduction of the host's population size during an host invasion. Non-native exotic parasites undergo all these bottleneck processes. While in the case of native parasites, only (a) might prevail, and native parasites would have more chances to host-shift by shifting from established indigenous Japanese life-cycles. Consequently, non-native exotic parasites may face extinction during invasion but native parasites may suffer less severe consequences (Fig. 16).

Raccoons in eastern North Carolina, U.S.A. are reported to carry both *Babesia* sensu stricto and *B. microti*-like parasites, respectively, at the prevalence rates of 90% (37/41) and 83% (34/41) (Birkenheuer et al., 2007) and perhaps those parasites maintain substantial genetic variation within a population. However, the rates in feral raccoons in Japan were very low (Kawabuchi et al., 2005A; Jinnai et al., 2009; Table 5, Fig. 17). Only two *B. microti*-like parasite *rDNAs* could be detected in 387 raccoons examined (0.5%) and were both identical each other by sequences and also to that reported from wild raccoons in the U. S. A. (Birkenheuer et al., 2007). The four parasites of the *Babesia* sensu stricto group were clustered into a single clade (clade 1), a new and as yet unidentified taxon (Fig. 17). With regard to our hypothetical assortment, the *B. microti*-like parasite found in a clonal population with a very low prevalence rate is very likely to be non-native*13) but the parasites placed in the clade 1 of the *Babesia* sensu stricto group detected at low prevalence with a substantial genetic variability are more likely to be a species (or species complex) native to Japan, which may be endemic in wild mammals as yet

Table 5. Prevalence of piroplasma infections in feral raccoons captured in Sapporo city and its surrounding areas in Hokkaido, Japan.

Number of animals tested	Number of animals positive for infection		
	Clade 1	Clade 2	*B. microti*-like
387	3 (0.8%)	3 (0.8%)	2 (0.5%)

Two separate results were summarized. After Kawabuchi et al., 2005; Jinnai et al., 2009.

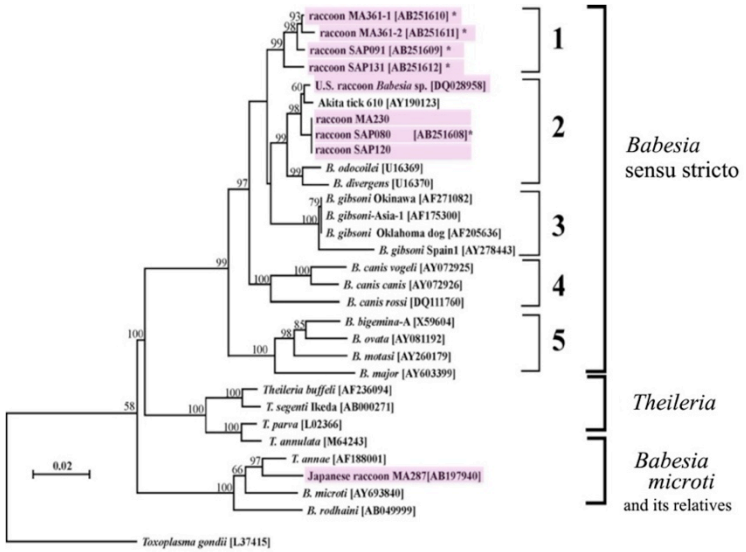


Fig. 17. *rDNA* tree of raccoon parasites and named piroplasms.
After Jinnai et al., 2009

Babesia parasites in raccoons and their close relatives are included. The GenBank accession number for each DNA sequence is given in parentheses. The number on each branch indicates the percent occurrence in 1000 bootstrap replicates. The asterisk indicates the *18S-rDNA* sequence originated by Jinnai et al., 2005. Parasites detected from raccoons are indicated as letterings on a colored background.

*13): That it may have kept its infection-cycle for years in the feral raccoons, as evidenced by the fact that the raccoons examined here were all aged less than four years (Kawabuchi et al., 2005A; Jinnai et al., 2009), possible, though we could not determined whether the parasite was transmitted through non-vector routes or by a tick species as yet unidentified.

unidentified. One additional clonal population (clade 2 in *Babesia* sensu stricto group) whose origins must at present remain obscure, since it is phylogenetically related closely to both parasites found in raccoons in U.S.A. and in a tick infested dog in Japan (Fig. 17).

Speciation generally occurs at a scale far too slow to be witnessed by humans. We can nonetheless look for clues whether the process of host/vector shifting is underway. A significant increase in infection prevalence is considered to be an initial sign for establishing new life cycle following host/vector shift, the first step toward parasite speciation, and this host/vector-fitting will occur relatively quickly as evidenced from a laboratory experiment showing RBC-shift by natural selection on randomly occurring parasite variants in a RBC-re-substituted SCID mouse (Kawabuchi et al., 2005B). Although it is challenging to identify specific genes linked to host/vector-shift even after the establishment of a clonal mutant strain (Kawabuchi et al., 2005B), study on the host/vector shifting process will provide most important insights into understanding *B. microti* evolution as well as genetic mechanisms behind host shifts.

(5) ***Perspective on population composition and structure for the piroplasms of domestic animals***

Through the geographic distribution and relatively recent evolutionary diversification of host/vector species, parasite co-evolution with host/vector species and/or new parasite establishment by host/vector-shifting may occur. Accordingly, the phylogenetic data are often intimately connected with host/vector-preference or with the landscape across which the evolutionary patterns evolved (Fig. 14, Criado-Fornelio et al., 2003). In contrast to the cases in wild animals, parasite population/lineages in domestic animals tend to have happened substantial genetic admixture in a blink of the eye, in terms of evolutionary time; i.e., various parasite populations that have once spread out and co-diverged with host/vector diversification and/or have newly acquired through host-shifting may have mixed during the host domestication/breeding process, whereas wild mammals, indigenous in different regions, admix to acquire desirable genetic traits for human beings or reduce inbreeding depression (Professor. Ueda, J., Rakuno-gakuen Univ. personal communication). A newly established hybrid piroplasma population in a domesticated/bred animal population may spread once again into new geographical ranges when trading of host animals occurs, either concurrently with the specific vector species or by newly acquiring resident tick species in the new geographic area.[*14)] Some of them may further admix with resident parasites in the emigrated area. [*15)]

Future studies on population structure of parasites and ticks with more density samplings and by using more sensitive molecular markers will provides new insights into understanding the relationship between the parasite population structure and their host/vector domestication/repopulation process as presented above (Perspective on the recent evolution of the U.S. lineage parasites), although there has yet been no data showing the evidence of admixture of parasite populations or of repopulation in relation to the tick feeding preference. Improving understanding will help to promote the development of a wide range of strategies to prevent and control babesiosis in domestic animals.

[*14)]: Parasites which are vectored by three-host-feeding ticks are presumed to have a tendency to establish new life-cycle by acquiring resident tick vectors since tropical tick species must inevitably prevail over existing tick species in extreme resource competition. While one-host-feeding tick species can easily establish themselves with less competition than that from native tick species, the parasites vectored by such one-host-feeding tick species might have invaded more easily invaded into new regions via moving domestic animals than the original tick vector species. The scenario may also mean that the invaded parasites vectored by three-host-feeding ticks have the advantage are of admixing with native parasites whereas the tropical parasites vectored by the one-host-feeding tick species invaded with host live-stock have a lesser chance of admixing with native parasite population.

[*15)]: Host/vector specificity had long been considered to be a sensitive marker that can divide parasites into separate sexual groups. However, it is now recognized that the host-range of many piroplasma parasites is less restricted than previously believed (reviewed by Hunfeld et al., 2008) and host/vector–shift might be more likely to happen than previously expected. Indeed, many parasites named as a single species occasionally can divide into separate clades, regardless of their common host-vector life-cycles.

Aknowledgement

I deeply acknowledge the full reach of his lifetime contributions of the late professor Masayoshi TSUJI to great advancement in this field of research and also grateful to the late professor Kiyoshi TAKAHASHI, a pioneer in the fields of piroplasma study, who lead us in new and promising directions. Parts of this report draw on the results from a “Gakujutsu-frontier” research by Drs. Morita, C., Tsuji, M., Kurosawa, T., Ueno, H., Fukumoto, S., Tamura, Y., and Ishihara, C. (Rakuno-Gakuen University), Dr. Arai, S. (NIH, Japan) and Kim, T.-S. (NIH, Republic of Korea).

I am deeply grateful to Drs. Hirata, H., Hagiwara, K., Takahashi, T., Asakawa, M., Nakajima, R., Jinnai, M., Kim, S.-J., and Fujisawa, K. (Rakuno-Gakuen University) for their magnificent contributions and insightful discussions. I also am most grateful to all of the graduate students of the Laboratory of Exp. Anim., School of Veterinary Medicine, Rakuno-Gakuen University for their generous time and energy providing special technical assistance, critical comments and comments. I would like to thank very much and acknowledge the invaluable support, contributions and constructive suggestions and insights given by: Drs. Zamoto-Niikura, A., Kamiyama, T. (NIH, Japan), Kawabuchi-Kurata, T. (Osaka Prefectural Institute of Public Health, Osaka, Japan), Y. Terada, Y. (National Institute of Animal Health, Japan), Takashima, I., Kariwa, H., Azuma, I., Tsubota, T., Arikawa, J., Yoshimatsu, K. (Hokkaido University, Sapppro, Japan), Nakao, M. (Asahikawa Medical Colledge, Asahikawa, Japan), Sanekata, T. (Tottori University, Tottori, Japan), Ikuta K., (Osaka University, Osaka, Japan), Isogai, H. (Sapporo Medical Colledge, Sapporo, Japan), Hioki, K. (CLEA, Tokyo, Japan), Kawai, S. (Dokkyo University School of Medicine, Tochigi Pref., Japan), Matsui, T. (Kobe University, Kobe, Japan), Saito-Ito, A. (Hyogo University of Health Science、Nishinomiya, Japan), Cho, S.-H. (NIH, Republic of Korea), and Wei, Q. (Inst. Lab. Anim. Sci., Chinese Acad. Med. Sci., China), and also Mr. Tabara DVM (Shimane Prefecture, Japan) and Ms. Y. Takita DVM (Morioka Zoological Park, Morioka, Japan). I express our debt of gratitude to all who donated parasites used in the study and to all who painstakingly helped capture the host animals and isolate the parasites.

With heartfelt appreciation, we also thank Dr. Danny H.- K. Jokl , (Columbia University Medical Center, NY, NY, U. S. A.) and Dr. Nell L. Kennedy (Rakuno Gakuen University) for critical discussion and help with the manuscript.

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

References

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., Ishihara, C. (2004A) Epizootiologic survey for *Babesia microti* among samall wild mammanls in Northeastern Eurasia and a geographic deversity in the *β-tubulin* gene sequences. J. Vet. Med. Sci., 66: 785-792.

Zamoto, A., Tsuji, M., Kawabuchi, T., Wei, Q., Asakawa, M., Ishihara, C. (2004B) U.S.-type *B. microti* isolated small wild mammals in Eastern Hokkaido, Japan. J. Vet. Med. Sci. 66: 919-926.

Goethert, H. K., Telford, S. R., III (2003) What is *Babesia microti* ? Parasitology. 127: 301-309.

Nguyen, H. D., Yoshihama, M. Kenmochi, N. (2007) The evolution of spliceosomal introns in Alveolates, Mol. Biol. Evol. 24: 1093-1096.

Kuo, C.-H., Kissinger, J. C. (2008) Consistent and contrasting properties of lineage-specific genes in the apicomplexan parasites *Plasmodium* and *Theileria,* BMC Evol. Biol. 8: 108.

Roy, S. W., Penny, D. (2006) Large-scale intron conservation and order-of-magnitude variation in intron loss/gain rates in apicomplexan evolution. Genome Res. 16: 1270-1275.

Perkins, S. L., Schall, J. J. (2002) A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences. J. Parasitol. 88: 972-978.

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A., Barba-Carretero, J. C. (2003) Molecular studies on *Babesia, Theileria* and *Hepatozoon* in southern Europe. Part II. Phylogenetic analysis and evolutionary history. Vet. Parasitol. 114: 173-194.

Allsop, M. E. P., Allsopp, B. A. (2006) Molecular sequence evidence for the reclassification of some *Babesia* species. Ann. N. Y. Acad. Sci. 1081: 509-517.

Tibayrenc, M. (2003) Genetic subdivisions with *Trypanosoma cruzi* (discrete typing units) and their relevance for molecular epidemiology and experimental evolution. Kinetoplastid Biol. Dis. 2: 12.

Hunfeld, K.-P., Hildebrandt, A., Gray, J. S. (2008) Babesiosis: Recent insights into an ancient disease. Int. J. Parasitol. 38: 1219-1237.

Mackenstedt, U., Gauer, M., Mehlhorn, H., Schein, E., Hauschild, S. (1990) Sexual cycle of *Babesia divergens* confirmed by DNA measurements. Parasitol. Res. 76: 199-206.

Heitman, J. (2006) Sexual reproduction and the evolution of microbial pathogens. Current Biol. 16: R711-R725.

Heitman, J. (2009) Love the one you're with. Nature 460: 807-808.

Anderson, T. J. C., Haubold, B., Williams, J. T., Estrada-Franco, J. G., Richardson, L., Mollinedo, R., Bockarie, M., Mokili, J., Mharakurwa, S., French, N., Whitworth, J., Velez, I. D., Brockman, A. H., Nosten, F., Ferreira, M. U., Day K. P. (2000) Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum.* Mol. Biol. Evol. 17: 1467-1482.

Jinnai, M., Kawabuchi-Kurata, T., Tsuji, M., Nakajima, R., Hirata, H., Fujisawa, K., Shiraki, H., Asakawa, M., Nasuno, T., Ishihara, C. (2010) Molecular evidence of a multiple genotype infection of wild Hokkaido brown bear（*Ursus arctos yesoensis*) by a new *Babesi*a sp. UR1. Vet. Parasitol. 173: 128-33.

Wei, Q., Tsuji, M., Zamoto, A., Kohsaki, M., Matsui, T., Shiota, T., Telford, III S. R., 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.

Kadereit, S., Hines, P.J. (2005). An overview of stem cell research. New England Law Review, 39, 607-622.

Cogswell, F. B. (1992) The hypnozoite and relapse in primate malaria. Clin. Microbiol. Rev. 5: 26-35.

Nakajima,R., Tsuji, M., Oda, K., Zamoto-Niikura, A., Wei, Q., Kawabuchi-Kurata, T., Nishida, A., Ishihara, C. (2009) *Babesia microti*-group parasites compared phylogenetically by complete sequencing of the *CCTh* gene in 36 isolates. J Vet Med Sci., J. Vet. Med. Sci. 71: 55-68.

Fujisawa, K., Nakajima, R., Jinnai, M., Hirata, H., Zamoto-Niikura, A., Kawabuchi-Kurata, T., Arai, S., Ishihara, C. (2011) Intron sequences from the *CCT7* gene exhibit diverse evolutionary histories among the four lineages within the *Babesia microti*-group, a genetically related species complex that includes human pathogens. Jpn. J. Infec. Dis. 64: 403-410.

Nishisaka, M., Yokoyama, N., Xuan, X., Inoue, N., Nagasawa, H., Fujisaki, K., Mikami, T. Igarashi, I. (2001) Characterisation of the gene encoding a protective antigen from *Babesia microti* identified it as eta subunit of chaperonin containing T-complex protein 1. Int. J. Parasitol. 31: 1673–1679.

Russell, C. B., Fraga, D., Hinrichsen, R. D. (1994) Extremely short 20-33 nucleotide introns are the standard length in *Paramecium tetraurelia*. Nucleic Acids Res. 22: 1221–1225.

Gilson, P. R., Su, V., Slamovits, C. H., Reith, M. E., Keeling, P. J., Mcfadden, G. I. (2006) Complete nucleotide sequence of the chlorarachniophyte nucleomorph: nature's smallest nucleus. Proc. Natl. Acad. Sci. U. S. A. 103: 9566–9571.

Reichard, M. V., Van Den Bussche, R. A., Meinkoth, J. H., Hoover, J. P., Kocan, A. A. (2005) A new species of *Cytauxzoon* from Pallas' cats caught in Mongolia and comments on the systematics and taxonomy of piroplasmids. J. Parasitol. 91: 420–426.

Baldauf, S.L. (2003) Phylogeny for the faint of heart: a tutorial. TRENDS in Genteics. 19: 345-351.

Jinnai, M., Kawabuchi-Kurata, T., Tsuji, M., Nakajima, R., Fujisawa K., Nagata, S., Koide, H., Matoba, Y., Asakawa, M., Takahashi, K., Ishihara, C. (2009) Molecular evidence for the new *Babesia* species in feral raccoons (*Procyon lotor*)in Hokkaido, Japan. Vet. Parasitol. 162: 241-247.

Brayton, K. A., Lau, A.O. T., Herndon, D.R., Hannick, L., Kappmeyer, L.S., Berens, S., J., Bidwell, S. L., Brown, W. C., Crabtree, J., Fadrosh, D., Feldblum, T., Forberger, H. A., Haas, B. J., Howell, J. M., Khouri, H., Koo, H., Mann, D. J., Norimine, J., Paulsen, I. T., Radune, D., Ren, Q., Smith Jr., R. K., Suarez, C. E., White, O., Wortman, J. R., Knowles Jr., D. P., McElwain, T. F., Nene, V. M. (2007) Genome sequence of *Babesia bovis* and comparative analysis of Apicomplexan Hemoprotozoa. PLoS 3: 1401-1413.

Kairo, A., Fairlamb, A.H., Gobright, E., Nene, V. (1994) A 7.1 kb linear DNA molecule of *Theileria parva* has scrambled rDNA sequences and open reading frames for mitochondrially encoded proteins. EMBO J. 13: 898-905.

Gray, M.W., Lang, B. F., Cedergren, R., Golding, G. B., Lemieux, C., Sankoff, D., Turmel, M., Brossard, N., Delage, E., Littlejohn, T. G., Plante, I., Rioux, P., Saint-Louis, D., Zhu, Y., Burger, G. (1998) Genome structure and gene content in protist mitochondrial DNAs. Nucleic Acid Res. 26: 865–878.

Criado, A., Martinez, J., Buling, A., Barba, J. C., Merino, S., Jefferies, R., Irwin, P. J. (2006) New data on epizootiology and genetics of piroplasms based on sequences of small ribosomal subunit and cytochrome b genes. Vet. Parasitol. 142: 238-247.

Tsuji, M., Wei, Q., Zamoto, A., Morita, C., Arai, S., Shiota, T., Fujimagari, M., Itagaki, A., Fujita, H., Ishihara, C. (2001) Human babesiosis in Japan: epizootiologic survey of rodent reservoir and isolation of new type of *Babesia microti*-like parasite. J. Clin. Microbiol. 39: 4316-4322.

Zamoto-Niikura, A., Tsuji, M., Wei, Q., Nakao, M., Hirata, H., Ishihara, C. (2012) Detection of two zoonotic *Babesia microti,* Hobetsu and U.S. lineages, respectively, in sympatric tick species, *Ixodes ovatus* and *Ixodes persulcatus,* in Japan. Appl. Environ. Microbiol. 78: 3424-3430.

Tabara, K., Arai, S., Kawabuchi, T., Itagaki, A., Ishihara, C., Satoh, H., Okabe, N., Tsuji, M. (2007) Molecular survey of *Babesia microti, Ehrlichia* species and *Candidatus Neoehrlichia mikurensis* in wild rodeents from Shimane prefecture, Japan. Microbiol. Immuol., 51: 359-367.

Piesman, J., Spielman, A. (1980) Human babesiosis on Nantucket Island: prevalence of *Babesia microti* in ticks. Amer. J. Trop. Med. Hyg. 29: 742-746.

Liu, Y. (2012) Invasion of *Babesia microti* in Northeastern U.S.A. http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-172530

Chao, L.-L., Wu, W.-J., Shih, C.-M. (2011) Species identification of *Ixodes granulatus* (Acari: Ixodidae) based on internal transcribed spacer 2 (ITS2) sequences. Exp. Appl. Acarol. 54: 51-63.

Kawabuchi, T., Tsuji, M., Sado, A., Matoba, Y., Asakawa, M., Ishihara, C. (2005A) *Babesia microti*-like parasites detected in feral raccoons (*Procyon lotor*) captured in Hokkaido, Japan. J. Vet. Med. Sci. 67: 825–827.

Birkenheuer, A. J., Marr, H.S., Hladio, N., Acton, A.E. (2007) Molecular evidence of prevalent dual piroplasma infections in North American raccoons (*Procyon lotor*). Parasitology, 135: 33-37.

Kawabuchi, T., Tsuji, M., Kuwahara, S., Nishida, A., Shimofurutachi, T., Oka, H., Ishihara, C. (2005B) Isolation of a human erythrocyte-adapted substrain of *Babesia rodhaini* and analysis of the merozoite surface protein gene sequences. J. Vet. Med. Sci. 69: 901-907.

Cornillot E, Hadj-Kaddour K, Dassouli A, Noel B, Ranwez V, Vacherie B, Augagneur Y, Brès V, Duclos A, Randazzo S, Carcy B, Debierre-Grockiego F, Delbecq S, Moubri-Ménage K, Shams-Eldin H, Usmani-Brown S, Bringaud F, Wincker P, Vivarès CP, Schwarz RT, Schetters TP, Krause PJ, Gorenflot A, Berry V, Barbe V, Ben Mamoun C. (2012) Sequencing of the smallest Apicomplexan genome from the human pathogen *Babesia microti. Nucleic Acids Res. 2012.* 1–13, doi:10.1093/nar/gks700