Epidemiological survey of tick-borne diseases in Japanese wildcats

日本産ヤマネコにおける マダニ媒介性疾患に関する疫学調査

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CONTENTS

General Introduction

The Iriomote cat (IC), *Prionailurus bengalensis iriomotensis*, and the Tsushima leopard cat (TLC), *Prionailurus bengalensis euptilura*, are the only two subspecies of wildcats that are indigenous to Iriomote-jima and Tsushima islands in Japan, respectively [46]. Although these cats are designated as a protected species, their current populations are estimated to be approximately only 100 [34]. Both species were listed in the Critically Endangered category in the Japanese government's official endangered species list [34]. The conservation of wildcats is important not only in terms of national property, but also for the conservation of biodiversity.

A major threat for Japanese wildcats is the expansion of human habitats resulting in the development of land and roads. These factors not only cause an increased risk of the cats being run over and killed by cars, but also may decrease the number of small animals serving as prey, and reduce and segregate the habitat in the future. In fact, sixty-four ICs were dead by traffic accidents between 1978 and 2015 (http://iwcc.a.la9.jp/ym.htm). Seventy-eight TLCs were also killed by car from 1992 to 2012 (http://kyusyu.env.go.jp/twcc/accident/index.html).

In addition to the road kill, exposures to infectious agents may also be a possible threat for Japanese wildcats. These wildcats are living in the field which many other

animal species are also inhabiting. Furthermore, the introduction of animals to islands, especially companion dogs and domestic cats, with the growth of the human population may increase the risk for the infection of diseases in those animals. Therefore, it is essential for the conservation of these wildcats to monitor infectious diseases.

Previous studies have shown that some wildcats were found to be seropositive against feline panleukopenia virus, feline calicivirus, feline coronavirus and/or feline syncytium forming virus [25, 50]. Furthermore, interspecies transmission of feline immunodeficiency virus (FIV) has been reported in TLCs [54]. In addition to viruses, hemotropic mycoplasmas (hemoplasmas) which are closely related to hemoplasmas in domestic cats have also been detected in some ICs [28]. Although great efforts have been paid for the detection of infectious agents in Japanese wildcats and actually found the evidence of their infections, we still have a room for discussion whether those infectious agents show pathogenicities against wildcats or not.

Infectious diseases can also be spread by ectoparasites, such as ticks, fleas and lice. Many tick species play important roles in transmitting numerous pathogens,

especially vector-borne ones such as *Bartonella*, *Ehrlichia*, *Anaplasma*, *Hepatozoon*, and *Babesia* in felids [8, 11, 17, 41]. Previous study revealed that these pathogens have been isolated from wildcats in the world and from ticks infested in these wildcats and domestic animals [5, 15, 50]. Tick infestations have often been observed in TLCs and ICs. Therefore, they are seemed to be at high risk for the exposure to tick-borne diseases. To date, *Hepatozoon felis* infection has been the only arthropod-borne disease reported in the two species of Japanese wildcats [40, 67]. However, no epidemiological surveys have been conducted to investigate the presence of these pathogens in Japanese wildcats and ticks.

In Chapter 1, I investigate the prevalence of tick-borne diseases in endangered Japanese wildcats using their blood samples. In addition, an epidemiological survey of tick-borne diseases in ticks collected from wildcats was also conducted in Chapter 2. A series of these studies were carried out to elucidate the role of infectious diseases on decreasing the population of Japanese wildcats and the role of ticks on the transmission of pathogens as a part of ongoing conservation activities.

Chapter 1

A molecular epidemiological study of tick-borne diseases in Japanese wildcats

ABSTRACT

The Iriomote cat (IC) and Tsushima leopard cat (TLC) are endangered wildcats in Japan. In the present study, an epidemiological survey of the tick-borne diseases caused by *Bartonella* spp., *Ehrlichia* spp., *Anaplasma* spp., *Hepatozoon* spp., and *Babesia* spp. infections was conducted as a part of ongoing conservation activities for these two species of wildcats. Blood samples from ICs and TLCs were collected between 2002 and 2012, and the prevalence of each pathogen was analyzed by PCR and DNA sequencing. The prevalence of *Bartonella henselae*, *Ehrlichia canis* and *Hepatozoon felis* was 4.6%, 9.3% and 72.0% in ICs, respectively. In TLCs, the prevalence of *Bartonella clarridgeiae*, *E. canis*, *Anaplasma bovis* and *H. felis*, was 7.1%, 7.1%, 21.4% and 100%, respectively. No *Babesia*-infected wildcats were detected in this study. Obvious pathogenicity of these pathogens in these two species of wildcats was not observed in the present study; however, continuous surveillance of infectious diseases will be necessary for the conservation of these endangered species.

INTRODUCTION

The Iriomote cat (IC), *Prionailurus bengalensis iriomotensis*, and the Tsushima leopard cat (TLC), *Prionailurus bengalensis euptilura*, are the only two subspecies of wild felids that are indigenous to Iriomote-jima and Tsushima islands in Japan, respectively [46]. Although these cats are designated as a protected species, their current populations are estimated to be approximately only 100 and there is a great fear that they will go extinct [34].

Infectious diseases could threaten their populations. Previous studies have shown that some wildcats were found to be seropositive against feline panleukopenia virus, feline calicivirus, coronavirus and/or feline syncytium forming virus [25, 50]. Furthermore, interspecies transmission of feline immunodeficiency virus (FIV) has been reported in TLCs [54]. In addition to viruses, hemotropic mycoplasma (hemoplasma) infections have also been detected in some ICs [28].

Infectious diseases can also be spread by ectoparasites, such as ticks, fleas and lice. Many tick species play important roles in transmitting numerous pathogens, especially vector-borne ones such as *Bartonella*, *Ehrlichia*, *Anaplasma*, *Hepatozoon*,

and *Babesia* in felids [8, 11, 17, 41]. Tick infestations have often been observed in TLCs and ICs. Therefore, they are seemed to be at high risk for the exposure to arthropod-borne diseases. To date, *Hepatozoon felis* infection has been the only arthropod-borne disease reported in the two species of Japanese wildcats [40, 67].

Bartonellosis, ehrlichiosis and anaplasmosis are representative arthropod-borne diseases in felids. It is known that domestic cats harbor *Bartonella henselae*, *Bartonella clarridgeiae*, and *Bartonella koehlerae*, although only a limited number of cats have been found to exhibit the disease [35, 42]. *Bartonella* infection was also shown in wild felids in North, Central and South America and Africa; however, its pathogenic potential has not been evaluated [15, 16, 51]. *Ehrlichia* and *Anaplasma* are Gram-negative, obligate intracellular bacterium, belonging to the family *Anaplasmataceae*. Clinical signs in affected animals are dependent on the species of the pathogen [22]. Domestic cats are susceptible to the infection of *Ehrlichia canis* and *Anaplasma phagocytophilum*, and display fever, anorexia and thrombocytopenia. These two pathogens have also been detected in wild felids; however, the virulence to these bacteria as well as to *Bartonella* is unknown [24, 49, 64].

Hepatozoon is one of tick-borne protozoa and belongs to genus apicomplexa.

There are many *Hepatozoon* spp. which infect a wide variety of mammals, birds, reptiles, and amphibians [9]. Most of *Hepatozoon* spp. are not so highly pathogenic with the exception of *Hepatozoon americanum* in dogs. Although *Hepatozoon* sp. in domestic cats is assumed to possess low virulence, some clinical abnormalities including anorexia, lethargy, weight loss, ocular discharge, icterus, and elevates levels of skeletal muscle-derived enzyme (creatine kinase and lactate dehydrogenase) may occur [9, 59]. *Hepatozoon* sp. infection of felids has been reported from several countries (e.g. India, Thailand, Tanzania, the United States, Brazil and Spain) [3, 5, 7, 37, 56, 75]. In Japan, hepatozoonosis has also been shown in domestic dogs, wild fox, wild Japanese marten, and wild Japanese black bear [43, 52, 81, 85]. In addition, there are a case report and a literature of histopathological evaluation for hepatozoonosis in ICs and TLCs [40, 67]. Therefore, a large scale molecular epidemiological survey in Japanese wildcats is necessary to understand the actual prevalence and the origin of *Hepatozoon*.

Babesia is the other tick-borne protozoan. *Babesia* infects to hematopoietic cells and often induces severe diseases such as hemolytic anemia and thrombocytopenia in host animals [71]. Babesiosis and similar cytauxzoonosis in domestic cats have

been reported in several countries including the United States, India, Thailand, and South Africa [58, 84]. Severe clinical symptoms are rarely observed in domestic cats infected with *Babesia*, however, we have to pay attention to their potential virulence to felids because it is well known that canine babesiosis induce severe clinical symptoms and they sometimes become life-threatening [71]. Babesiosis in cats has not been detected in Japan yet, however, it would be necessary to obtain basic information on epidemiology of *Babesia* in ICs and TLCs.

The Japanese wildcats are also likely to be susceptible to pathogens mentioned above, with those pathogenicity are unclear in endangered Japanese wildcats. In the present study, a molecular epidemiological survey of *Bartonella* spp., *Ehrlichia* spp., *Anaplasma* spp., *Hepatozoon* spp., and *Babesia* spp. infections in two Japanese wildcats, ICs and TLCs, was conducted to understand the prevalence and characteristics of these pathogens as a part of ongoing conservation activities.

MATERIALS AND METHODS

Sample collection and DNA extraction

Capturing and sample collection of two subspecies of wildcats were done under permissions by Ministry of Environment and Agency for Cultural Affairs in Japan. Capturing procedures were conducted under cooperation with Iriomote Wildlife Conservation Center and Tsushima Wildlife Conservation Center of Ministry of Environment. A series of ecological survey were performed between August 2002 and January 2012. During this period, 43 ICs and 14 TLCs were captured or found dead (Tables 1 and 2). Because some wildcats were retrapped, several blood samples were collected in a same cat. Accordingly, 62 blood samples from ICs and 29 from TLCs were collected in total (Tables 1 and 2). Blood samples were anti-coagulated with EDTA and subjected to complete blood count, serum biochemistry analyses and preparation of blood smear specimens. Blood smear specimens were stained with Wright-Giemsa. Based on examination of 1,000 neutrophils and/or erythrocytes under microscopic observations, parasitemia of hepatozoon and/or babesia were calculated.

After complete blood count and biochemistry analyses were performed, total DNA was extracted from blood samples. Total DNA was extracted from 200 µl of each blood sample using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) with a final elution volume of 200 µl. Extracted DNA was then used as a template to amplify the DNA from infectious pathogens, and as an internal control for PCR analyses. As an internal control, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified to determine the quality of all the examined samples as described previously [80]. All PCR samples were electrophoresed in a 2% agarose gel followed by ethidium bromide staining and were subsequently checked for the appearance and size of positive bands.

PCR analyses for the detection of pathogens

To PCR amplify pathogen-derived DNA, previously reported primers and procedures were used. Oligonucleotide sequences, product length and corresponding references are listed in Table 3.

Nested PCR reported by Ramersad *et al*. was applied to amplify the region between 16S and 23S ribosomal RNA (rRNA) genes from *Bartonella* species [60].

This PCR enabled the detection of distinct *Bartonella* species based on the size of amplified DNA fragments. In the first round of the PCR, forward primer P-bhenfa and reverse primer P-benr1 were used. In the second round PCR, forward primer N-bhenf1a and reverse primer N-bhenr were used. Reaction mixtures (50 µl) for both reactions contained primers (0.5 µM each), 0.2 mM of dNTP, 2 µl of template DNA, *Taq* DNA polymerase (1 unit), and the reagents recommended by the manufacturer (Takara, Kyoto, Japan). The PCR reaction for first round involved 30 cycles of denaturation (94°C, 1 min), annealing (48.2°C, 1 min) and polymerization (72°C, 1 min). The second round of PCR was also performed for the same number of cycles, denaturation and polymerizations, but the annealing temperature was set at 56°C.

Screening for the presence of *Ehrlichia* and *Anaplasma* was conducted based on the nested PCR strategy reported by Tabar *et al.* [75]. The first primer set, P-ehrf and P-ehrr, and the second set, N-ehrf and N-ehrr, were used. The PCR reaction for first round reaction involved 40 cycles of denaturation (94°C, 30 sec), annealing (62°C, 30 sec) and polymerization (72°C, 30 sec). The second round PCR consisted of 30 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min) and

polymerization (72°C, 1 min). To determine sequence of the 16S rRNA genes, 5' and 3' regions were separately amplified by using two sets of PCR [32]. The primers fD1 and EHR16SR were used to amplify the 5' region of the 16S rRNA gene. The PCR reaction for the 5' region involved 45 cycles of denaturation (94°C, 1 min), annealing (56°C, 30 sec) and polymerization (72°C, 1 min). For the 3' region, the primers EHR16SD and Rp2 were used. The PCR reaction for the 3' region consisted of 40 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min) and polymerization (72°C, 1 min).

Nested PCR was performed to amplify 18S rRNA derived from *Hepatozoon* and/or *Babesia* species as a screening PCR. For the first round PCR, two oligonucleotide, PIRO-F and PIRO-R, were constructed [6]. For the second PCR, BabgenF and BabgenR were used [10]. These primer sets are universal and recognize 18S rRNA genes from all of *Hepatozoon* and *Babesia*. Two kinds of pathogens could be differentiated each other based on the size of amplified DNA fragments and the expected size of DNA fragment from *Hepatozoon* spp and *Babesia* spp. was 267 bp, 230 bp, respectively. The amplification steps for the first round PCR consisted of initial heating at 94°C for 2 min, 30 cycles of denaturation

(94°C for 45 sec), annealing (45°C for 45 sec), polymerization (72°C for 45 sec), followed by a final extension at 72°C for 5 min. The second round PCR reaction involved initial heating at 94°C for 2 min, 30 cycles of denaturation (94°C for 40 sec), annealing (51.5°C for 40 sec), polymerization (72°C for 30 sec), followed by a final extension at 72°C for 1 min. If the samples showed positive result in the PCR screening, those were then subjected to detailed analysis which was able to differentiate the species of infected *Hepatozoon* and/or *Babesia* by PCR amplification of nearly the entire 18S rRNA gene and following DNA sequencing analysis. Four oligonucleotide primers were constructed to amplify the 18S rRNA gene from *Hepatozoon*: BT1 and HPF2P for the first round PCR, and HepF and RLB-R for the second round PCR [4, 18, 19, 31]. The first round PCR reaction involved initial heating at 94°C for 5 min, 25 cycles of denaturation (94°C for 30s), annealing (55.5°C for 45s), polymerization (72°C for 2 min), and a final extension at 72°C for 10min. For the second PCR, 1 μl of first PCR product was used as a template instead of extracted sample DNA and other components were same except primers. Cycling conditions were also same as the first round PCR with the exception of polymerization (72°C for 1min). For the *Babesia*-positive samples,

nested primers Bab F3 and HPF2Pfor the first reaction, and Bab F3 and Bab R3 for the second reaction were prepared [18, 21]. The first round PCR involved initial heating at 94°C for 5 min, 25 cycles of denaturation (94°C for 30 sec), annealing (65°C for 45 sec) and polymerization (72°C for 2 min), and a final extension at 72°C for 10 min. The second PCR was performed, under the same conditions as the first round except template (1 μl of first round product), annealing temperature (66°C) and duration of polymerization (1 min).

Nucleotide sequencing, BLAST and phylogenic analysis

DNA fragments obtained in PCRs mentioned above were inserted into a pCR2.1 plasmid vector (Invitrogen, Carlsbad, CA), and the nucleotide sequence of the inserted DNA fragments was determined by the dideoxy chain termination method (ABI Prism BigDye Primer Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA) using primers constructed from the M13 region (Forward, 5'-GTA AAA CGA CGG CCA G-3'; Reverse, 5'-CAG GAA ACA GCT ATG AC-3'). Nucleotide sequence data from each sample were subjected to BLAST analysis in the DNA data bank of Japan (DDBJ,

http://www.ddbj.nig.ac.jp/Welcome-j.html) to identify the nearest phylogenic neighbor and calculated the homology using GENETYX Ver. 10.0.3 software (GENETYX Corporation, Tokyo, Japan). Phylogenic analyses were performed for examine pathogens to determine the genetic relationship among those pathogens by a neighbor-joining method in the DNADIST program from the PHYLIP software package, as previously reported [23].

RESULTS

Internal control

G3PDH gene of all samples from wildcats was successfully amplified, allowing for all samples to be subjected to the molecular epidemiological survey for the infectious pathogens (data not shown).

Detection of pathogen-derived DNA, DNA sequencing and phylogenic analysis

Two samples from ICs and two samples from a single TLC were positive for *Bartonella* DNA. Nucleotide sequences of amplified DNA fragments from both ICs showed 100% homology with *B. henselae*, while two clones from TLC was found to have 100% similarity with *B. clarridgeiae* in a BLAST search. Finally, the DNA of *B. henselae* and *B. clarridgeiae* was detected from 4.6% (2/43 cats) in ICs and 7.1% (1/14 cats) in TLCs, respectively.

Four samples from four ICs and eight samples from four TLCs showed positive in the PCR screening for *Ehrlichia* and *Anaplasma*. These 12 samples were then analyzed by sequencing to determine the sequence of the 16S rRNA genes. PCR and

sequence analysis of the 16S rRNA suggested that six samples from four ICs and one TLC was derived from *Ehrlichia* and the other six samples from three TLCs were derived from *Anaplasma*. Representative clones were subjected to phylogenic analysis (Figure 1). *Ehrlichia* detected from ICs and TLC formed one cluster suggesting the similarity to *E. canis*. The detected *Anaplasma* from TLC formed one group predominantly with *Anaplasma bovis* (Figure 1). The prevalence of *E. canis* was 9.3% (4/43 cats) in ICs and 7.1% (1/14 cats) in TLCs, respectively. The prevalence of *A. bovis* was 21.4% (3/14 cats) in TLCs.

In the screening PCR for *Hepatozoon* and *Babesia*, DNA bands of the expected size were observed in 49 samples from 31 ICs and 28 samples from 14 TLCs, respectively. As all of amplified DNA fragments showed 267 bp in size, those seemed to be derived from *Hepatozoon* sp. Most of adult cats were PCR-positive. Negative results were mainly observed in immature cats including ICs D-043J, E-89J and E-92J, and TLC CMT-33. CMT-33 had been negative in 2010, however, the same cat converted to PCR positive at the time of recapturing in 2011. BLAST analysis revealed that all of DNA fragments showed high similarity (99.9%) to 18S rRNA gene of *H. felis* isolated from wild felid, Indian leopard (*Panthera pardus*

fusca), in Asia (GenBank/EMBL/DDBJ Accession number HQ 829443). The prevalence of *Hepatozoon* was 72.0% (31/43 cats) in ICs and 100% (14/14 cats) in TLCs in this study. No *Babesia*-derived genes were detected in ICs and TLCs in this survey.

Observation of blood smear specimens

Thirty-five blood smear specimens from 26 ICs and 9 from 8 TLCs were available (Tables 1 and 2). Although all of 34 wildcats showed positive results in PCR analyses, cytoplasmic gamonts of hepatozoon in neutrophils were microscopically detected in 24 out of 35 samples (68.6%). All of cats with microscopic parasitemia were ICs. Degrees of parasitemia in 24 samples ranged from 0.1% to 4.7%.

DISCUSSION

In this study, *Bartonella*, *Ehrlichia*, *Anaplasma* and *Hepatozoon* were detected in two Japanese wildcats by molecular techniques for the first time.

Bartonella is widely distributed in the world with a high seroprevalence of more than 20% in wild felids inhabiting areas with mild and moist climates [15, 16, 24, 51]. While Iriomote and Tsushima islands are located in the Subtropical and the Temperate Zones, respectively, the positive rates in our PCR based survey were low in both species of Japanese wildcats, IC and TLC. This discrepancy may be due to differences in the analytical strategies which arise when using serological and PCR-based surveys. A PCR-based survey detects DNA from a bacterium if it is currently present in the animal, whereas a serological survey is able to detect antibodies against bacteria from prior infection. Molia and colleagues showed that higher prevalences were detected in a serological survey in comparison to a PCR-based analysis even though they used same blood samples obtained from African lions and cheetahs for both analyses [51]. Similar findings were also reported in an epidemiological survey of *Bartonella* infection in domestic cats [72].

In the present study, we performed only PCR to detect *Bartonella*-derived DNA; however, more positive samples might have been obtained if a serological survey was conducted instead [44]. Another possible reason why low prevalence of *Bartonella* was observed might be due to low frequency of flea infestation in both of wildcats. Flea is a major vector of *Bartonella* species between cats. Since the infestation of flea was rarely detected in both ICs and TLCs, this fact might be also related to the low prevalence of *Bartonella* species in Japanese wildcats.

In the present study, *B. henselae* was detected in two ICs and *B. clarridgeiae* was observed in one TLC. Many reports have shown that antibody responses against *B. henselae* and *B. clarridgeiae* were observed in wild felids [15, 16, 35, 42, 51]. In addition, a previous epidemiological survey in domesticated cats and wild felids showed an almost equal prevalence of *B. henselae* and *B. clarridgeiae* infections or a slightly higher rate in *B. henselae* infection [75]. Furthermore, co-infection of *B. henselae* and *B. clarridgeiae* is found in some domestic cats and wild felids [75]. ICs and TLCs would be presumably susceptible to both *B. henselae* and *B. clarridgeiae*, however, the limited *Bartonella* infection in each wildcat species was observed in this study. It might have been due to a low prevalence of *Bartonella*

species, critical populations of both wildcat species, and limited habitats in isolated islands. As a previous epidemiological survey showed that the co-infection of *B. henselae* and *B. clarridgeiae* was uncommon in Japanese domestic cats, the incidence of co-infection may also be actually low in ICs and TLCs [45].

It has been reported that 0 to 20% of wild felids possess antibodies against *E. canis* [24, 49, 61]. Seroprevalence of *E. canis* in domestic cats varies ranging from 0 to 10%, while PCR analysis rarely detects *E. canis* derived DNA in blood [12, 33, 38, 72]. Detection rates of *E. canis* in this study were relatively higher than that of other previous studies. It is uncertain why high detection rates were observed in this study; however, the year of sample collection may be important factor. In ICs, the positive results were obtained from blood samples collected only from 2010. Three of four PCR positive ICs were repeatedly captured several times; however, those cats showed positive results transiently throughout this year. Although we do not know whether there was an increased chance of tick infestations and disease introduction into the island; various factors might have influenced a change in the detection rates. Only two samples were positive for *E. canis* in TLCs; however, those two were obtained from the same cat with a 4-year interval between detection.

The detailed mechanism for the development of bacteremia has to be evaluated in the large number of samples and serological test.

In the present study, *A. bovis* DNA has been detected in felids for the first time. A recent study revealed that 1.1% of domestic dogs were PCR positive for *A. bovis* infection [65]. *A. phagocytophilum* was also reported to infect ruminants and carnivores [2, 63]. These findings suggest that *Anaplasma* species have a wide host range including carnivores. Recent studies have shown that cattle and wild deer in Japan were infected with *A. bovis* [38, 39, 55]. Cattle are raised as farm animals and many wild deer inhabit, and heavy tick infestations are observed in wild deer in Tsushima Island. Therefore, it is thought that wild deer may serve as one of reservoirs for *A. bovis* in Tsushima Island. An epidemiological survey for farm animals and wild animals in Tsushima Island will be also necessary to understand the origin of *Anaplasma*.

Previous studies showed prevalence of *Hepatozoon* ranging from 4 to 100% in Brazilian wild felids (ocelot, little spotted cat, margay, and jaguarondi), Indian wild felids (Asiatic lion, Indian tiger and Indian leopard), African lions and cheetahs [5, 7, 48, 56]. The prevalence of *H. felis* in ICs and TLCs was 72.0% and 100%,

respectively, and these rates were higher than those of previous studies [5, 56]. This difference might be simply due to the frequency of infestation and numbers of ticks. The infestation of a number of ticks is observed in most of ICs and TLCs at the time of capture, however, previous studies revealed a low frequency of ectoparasite infestation in Brazilian wild felids and Indian wild felids mentioned above [5, 56]. Many infestations might have increased the opportunity of tick ingestion through a grooming behavior, and accordingly increase the infection rate of *H. felis*. Evaluation of possession rate of *H. felis* in ticks inhabiting in both islands will be helpful for the further understanding the reason why Japanese wildcats showed a high prevalence of this pathogen.

One interesting finding is that *H. felis* would have infected to the wildcats posteriori. Because negative PCR results were mainly observed in young and juvenile cats, and one juvenile TLC converted the infection status. These immature cats were considered to have had less frequent opportunities for infestation and ingestion of ticks than adult cats. This fact would have resulted in lower prevalence of *H. felis*. This finding also suggests that the major transmission route of *H. felis* is ticks but not a vertical transmission.

In this study, a microscopic parasitemia of *H. felis* was found in only ICs but not TLCs. Although exact reasons why only ICs showed parasitemia could not be determined, the difference of *H. felis* strain in ICs and TLCs might have been one possible reason. Previous histopathological study showed, in ICs, *Hepatozoon* sp. was detected in several tissues including heart, tongue, masseter muscle, and diaphragm. On the other hand, in TLCs, *Hepatozoon* sp. was only detected in heart [40]. Both wildcats inhabit in islands but those islands were geographically and climatically separated. Therefore, it is plausible that the virulence or host tropism of *H. fe*lis in each island is subtly different. The possible second reason is that immune responses against *H. felis* were different between ICs and TLCs. Low parasitemia level and mild clinical symptoms were observed in human malaria patients who have high antibodies titer against malaria serine repeat antigens [29]. TLCs might have more effective immune responses against *H. felis* than ICs and these immune responses could suppress the propagation of *H. felis* in their blood and tissues. As a third reason, the influence of infectious diseases other than *H. felis* could be included. Acute onset of hepatozoonosis in domestic dogs are often observed in the case with co-infections other than hepatozoon such as babesiosis, internal parasitic

diseases, dirofilariasis and others [27, 66, 68]. Although the analysis of infectious diseases has just started and the information is currently limited, wide ranges of pathogens should be evaluated to see their contribution to the development of acute onset of hepatozoonosis in wildcats.

In the present study, *Babesia* infection was not detected in wildcats. It is unlikely that Japanese wildcats do not have a susceptibility to these pathogens because other felids in other countries obviously reveal susceptibilities to those pathogens and a part of infected felids shows an onset of disease [71]. Therefore, it is likely that *Babesia* which has a tropism to felids have not been introduced to the islands. The results from nation-wide epidemiological survey conducted in our laboratory would also support this hypothesis because this survey failed to detect any evidence of *Babesia* spp. infection in domestic cats (data not shown).

Any wildcats showing clinical signs were not observed in this study even though those cats were PCR positive for examined infectious diseases. In addition, the cats killed by traffic accidents were also assumed not to have shown clinical signs at the time of death because the stomach was full of food at the time of necropsy. Furthermore, no dead bodies obviously being caused by infectious diseases have

been found until now. Therefore, the virulence of detected pathogens to IC and TLC would be not so high and I speculated that the infectious diseases might not be a main cause of decreasing the population of Japanese wildcats.

In this study, a molecular epidemiological survey for infectious diseases was conducted in two subspecies of Japanese wildcats. *B. henselae*, *B. clarridgeiae*, *E. canis*, *A. bovis* and *H. felis* were detected from Japanese wildcats. *H. felis* infection was observed in most of Japanese wildcats and *A. bovis* has been detected in felids for the first time. The infection source and infection route were not completely understood. The virulence of detected pathogens was assumed to be not so high and, therefore, these infectious diseases were not likely to be main cause of decreasing Japanese wildcats population.

Chapter 2

A molecular survey of arthropod-borne diseases in ticks obtained from Japanese wildcats

ABSTRACT

The Iriomote cat (IC), *Prionailurus bengalensis iriomotensis*, and the Tsushima leopard cat (TLC), *Prionailurus bengalensis euptilurus*, are endangered subspecies of leopard cats in Japan. In addition to the habitat destruction and road kills, infectious diseases may threaten their populations, and both subspecies are reported to be infected with arthropod-borne pathogens. Infestations of ectoparasites, especially ticks, have frequently been observed in ICs and TLCs. In the present study, ticks collected from these two subspecies of Japanese wildcats were morphologically identified and the prevalence of the pathogens *Bartonella* sp., *Babesia* sp., *Ehrlichia* sp., *Anaplasma* sp., *Hepatozoon* sp. and hemoplasmas in ticks were molecularly evaluated. Ticks were collected from captured ICs and TLCs between November, 2011, and January, 2012. The ticks *Haemaphysalis longicornis*, *H. hystricis*, and *Amblyomma testudinarium* were obtained from ICs, and *H. megaspinosa*, *Ixodes tanuki*, *H. campanulata*, *A. testudinarium* were collected from TLCs. The pathogens *Hepatozoon felis*, *Babesia* sp., and *Anaplasma bovis* were detected in ticks obtained from ICs, while *H. felis. Babesia* sp., *Ehrlichia* sp., *E.*

muris, '*Candidatus* Mycoplasma haemominutum', and *Bartonella henselae* were found in ticks from TLCs. Continuous monitoring and additional surveys will be necessary to understand the roles of ticks in vectoring Japanese wildcat diseases to protect and conserve these endangered animals.

INTRODUCTION

Many tick species play important roles in transmitting numerous pathogens, especially vector-borne ones such as *Bartonella*, *Ehrlichia*, *Anaplasma*, *Hepatozoon*, and *Babesia* in felids [8, 11, 17, 41]. Wild felids, except the animals living in captivity, appear to be at high risk of exposure to those pathogens because of their habitats and the difficulties in applying periodic prophylactics against ectoparasite infestations. Arthropod-borne diseases have been confirmed in many wild felid species worldwide [5, 15, 49]. Although most felids infected with such pathogens generally show only mild symptoms, their lives can be threatened in some situations, such as immune compromise caused by other diseases or by trauma [8, 11, 26, 41].

The Iriomote cat (IC), *Prionailurus bengalensis iriomotensis*, and the Tsushima leopard cat (TLC), *Prionailurus bengalensis euptilurus*, are the only two subspecies of leopard cats in Japan [46]. They inhabit only Iriomote Island and the Tsushima islands which are located in the Subtropical and the Temperate Zones, respectively. Their current populations are estimated to be approximately one hundred each, and there is a great fear that they will go extinct [34]. Infectious diseases could threaten

these populations. Arthropod-borne pathogens including *Bartonella*, *Ehrlichia*, *Anaplasma*, *Hepatozoon*, and/or hemotropic mycoplasma (hemoplasma) were detected in ICs and/or TLCs [28, 77, 78]. Infestations of ectoparasites, such as ticks and lice, have frequently been observed in ICs and TLCs. However, no surveys have been conducted to evaluate the tick species infesting these wildcats or the presence of arthropod-borne pathogens in those ticks.

In the present study, we morphologically identified ticks collected from the two endangered subspecies of Japanese wildcats, and then performed a molecular survey for *Bartonella* sp., *Babesia* sp., *Ehrlichia* sp., *Anaplasma* sp., *Hepatozoon* sp. And hemoplasmas to understand their prevalence in ticks and role of ticks on transmission of pathogens in Japanese wildcats cohorts as a part of ongoing conservation activities.

MATERIALS AND METHODS

Tick collection

As a part of ongoing ecological survey, 13 ICs and eight TLCs were captured between November 2011 and January 2012. Ticks were collected from the captured animals. Tick species, stage, and sex were identified morphologically under a microscope and the data were pooled for each cat and collection date. If multiple larvae and nymphs of same species obtained from the wildcat at the time of capture, they were gathered and pooled in one tube. Each adult tick was individually stocked in a single tube. Ticks were preserved in ethanol at −20°C until use. Wildcats were captured with permission from Ministry of the Environment and the Agency for Cultural Affairs in Japan. Capturing was conducted in cooperation with the Iriomote Wildlife Conservation Center and the Tsushima Wildlife Conservation Center of Ministry of the Environment. Capture of wildcats was carried out at night, and captured wildcats were released in the afternoon of the next day after physical examination and blood collection under anesthesia with medetomidine and ketamine.

DNA extraction and internal controls

The pooled ticks were frozen in liquid nitrogen and mechanically disrupted, then extraction buffer (100 mM Tris [pH 8.0], 100 mM NaCl, 10 mM EDTA, 0.5% sodium dodecyl sulfate) and protease K (20 μg/μL) were added, and the mixture was incubated at 55°C for more than 12 h. DNA was extracted using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. To validate the accuracy of the DNA extraction, the tick 16S ribosomal RNA (rRNA) gene was amplified in each sample as an internal control using the primers 16S-1 (5'-CCG GTC TGA ACT CAG ATC AAG T-3) and 16+1 (5'-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3') [14]. Each reaction mixture (50 μL) contained primers (0.5 μM each), 0.2 mM of dNTP, *Taq* DNA polymerase (1.25 U), 2 μL of template DNA, and reagents (Takara, Kyoto, Japan) recommended by the manufacturer. PCR amplification was performed with an initial heating at 94°C for 10 min; 40 cycles of denaturation (94°C for 1 min), annealing (56°C for 1 min), and polymerization (72°C for 1 min); and a final extension at 72°C for 10 min.

PCR amplification of pathogen-derived DNA

To PCR amplify pathogen-derived DNA, previously reported primers and procedures were used. Oligonucleotide sequences and corresponding references are listed in Table 4. Nested PCR was performed to amplify the 18S rRNA gene derived from *Hepatozoon* and *Babesia* species [13, 18, 20]. For the first round of PCR, two oligonucleotides, BT1 and HPF2P were constructed. For the second PCR, two primer sets, HEP-1 and HEP-4 and 455-479F and 793-772R, were used for *Hepatozoon* spp. and *Babesia* spp, respectively.

Because of the similarities between the 16S rRNA genes derived from *Ehrlichia* and *Anaplasma*, two steps of analyses, screening and detailed tests, were applied in this study. First, screening PCR was conducted based on the nested PCR strategy reported by Tabar *et al.* [75]. Primers P-ehrf and P-ehrr were used for the first PCR and primers N-ehrf and N-ehrr for the second. To determine the nearly complete sequences of the 16S rRNA genes, the 5′ and 3′ regions were separately amplified according to Inokuma *et al*. (2001); the primers fD1 and EHR16SR were used to amplify the 5′ region, and for the 3′ region, the primers EHR16SD and Rp2 were used [32].

To amplify the 16S rRNA gene from a broad range of hemoplasma species, a universal primer set consisting of one forward primer (MY-F) and two reverse primers (MY-R1 and MY-R2) were used according to Willi *et al*. (2009) [83]. Nested PCR was used to amplify the region between the 16S and 23S rRNA genes from *Bartonella* species [60]. In the first round of PCR, primers P-bhenfa and P-benr1 were used. In the second round, N-bhenf1a and N-bhenr were used.

DNA sequencing, BLAST analysis and phylogenic analysis

DNA fragments obtained from PCRs were inserted into a pCR2.1 plasmid vector (Invitrogen, Carlsbad, CA, USA), and the inserted nucleotide sequences were determined by the dideoxy chain termination method (ABI Prism BigDye Primer Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA, USA) using primers constructed for the M13 region (Forward, 5'-GTA AAA CGA CGG CCA G-3', nt 404–389; Reverse, 5'-CAG GAA ACA GCT ATG AC-3', nt 205–221). Nucleotide sequence data from each sample were subjected to BLAST analysis in the DNA data bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp/Welcome-j.html) to identify the nearest phylogenetic neighbor and calculated the homology using

GENETYX Ver. 10.0.3 software (GENETYX Corporation, Tokyo, Japan). Phylogenetic analyses were performed for *Hepatozoon* spp., *Babesia* spp., *Ehrlichia* spp. and *Anaplasma* spp. to determine the genetic relationship among those pathogens by a neighbor-joining method in the DNADIST program from the PHYLIP software package, as previously reported [23].

RESULTS

Identification of tick species

In total, 31 and 51 tick pools were collected from 13 ICs and eight TLCs, respectively. Three *Haemaphysalis longicornis*, 19 *Haemaphysalis hystricis*, and nine *Amblyomma testudinarium* pools were obtained from ICs and identified morphologically. These pools included 23 of larvae, eight of nymphs, and no adults (Table 5). Similarly, from TLCs, three *A. testudinarium*, eight *Haemaphysalis megaspinosa*, 25 *Ixodes tanuki*, and five *Haemaphysalis campanulata* pools were obtained. The remaining 10 tick pools were difficult to identify precisely, but nine of ten were suspected to be *I. tanuki*, and the other may have been *A. testudinarium*. There were one larva, 6 nymph, 12 adult male, and 32 adult female pools (Table 6).

Detection of pathogens in ticks by PCR and nucleotide sequencing analysis

Tick-derived 16S rRNA genes of all samples were successfully amplified (data not shown); thus, all tick-derived DNA samples were surveyed for pathogens by PCR.

Twenty-three (3 *A. testudinarium*, 3 *H. longicornis*, and 17 *H. hystricis*) and 30 (1 *A. testudinarium*, 12 *I. tanuki*, 6 *H. megaspinosa*, 2 *H. campanulata*, and 9 unidentified species) pools collected from ICs and TLCs, respectively, were positive for *Hepatozoon.* Sequencing and BLAST analysis revealed that all of these amplified DNA fragments were highly similar (98–99%) to the 18S rRNA gene of *Hepatozoon felis*. Phylogenic analysis revealed that representative clones obtained from wildcats also could be classified as *H. felis* and they were closely related to *H. felis* isolated in Japanese wildcats rather than strains isolated in other countries (Figure 2).

DNA derived from *Babesia* sp. was detected in two *A. testudinarium* pools from ICs and one *I. tanuki* pool from a TLC. Nucleotide sequencing and BLAST analysis revealed that DNA amplified from *A. testudinarium* had a similarity to *Babesia gibsoni* (91–92%). However, these two clones formed one cluster different from *B. gibsoni* in phylogenic analysis (Figure 3). The species of *Babesia* from *I. tanuki* could not be identified. Although this clone showed the highest identity with the *Babesia* sp. isolated in Japan (100%) but this clone formed one independent branch in a phylogenic analysis (Figure 3).

Ehrlichia was detected in four pools of *I. tanuki* and one unidentified tick pool obtained from TLCs. Partial nucleotide sequences of the 16S rRNA gene showed 91–99% similarity with *Ehrlichia muris* and *Ehrlichia chaffeensis,* and the phylogenic analysis revealed that two of the detected *Ehrlichia* sp. was closely related to *E. muris*. *Anaplasma* sp. was also detected in one *H. longicornis* pool, and it showed 99% homology with *Anaplasma bovis*. This clone belonged to a group of *A. bovis* in phylogenic analysis (Figure 4).

Although no tick pools from ICs were positive for hemoplasma, one *I. tanuki* pool from TLC was positive and had 99% similarity to the 16S rRNA gene of CMhm.

Finally, *Bartonella* sp. was detected from two *I. tanuki* pools and two unidentified tick pools collected from TLCs, but no positive results were obtained from the IC tick pools. Nucleotide sequences from all positive samples showed 98–100% homology to *Bartonella henselae*.

DISCUSSION

In the present study, an epidemiological survey was conducted for the presence of arthropod-borne pathogens in ticks collected from two subspecies of Japanese wildcats. As the results, *H. felis*, *B. gibsoni*, and *A. bovis* were detected in ticks obtained from ICs and *H. felis. Babesia* sp., *Ehrlichia* sp., CMhm, and *B. henselae* were found in ticks on TLCs.

Although a previous report suggested that wild felids were susceptible to *H. felis*, its precise vectors and its virulence toward felids remained unclear [11]. As shown in Chapter 1, the prevalence of *H. felis* in ICs and TLCs were reported to be 72.0% and 100%, respectively [77]. In this study, several tick species (*H. longicornis*, *H. hystricis A. testudinarium*, *H. megaspinosa*, *I. tanuki*, and *H. campanulata*) harbored *Hepatozoon*, all of which were confirmed to be *H. felis*. The present data suggested that those tick species are likely to be important vectors of *H. felis*. This finding might be also supported by the case of *Hepatozoon canis* infection, because several ticks of *Haemaphysalis* sp. were reported to be a vector for *H. canis* [1, 30, 53], and they were frequently observed on both subspecies of wildcats. However, we must

consider that the ticks examined in this study were not limited to unfed ones. In this study, the ticks might have just harbored (and not vectored) *Hepatozoon* acquired by feeding on wildcats infected with *H. felis* because consistent PCR results were obtained from most of wildcats and ticks (Tables 5 and 6). We must evaluate whether these ticks are authentic biological vectors of *H. felis* by examining the protozoan life cycle within the ticks.

Babesia was detected in *A. testudinarium* and *I. tanuki* in this study. The *Babesia* detected from two *A. testudinarium* was closely related to *B. gibsoni* in the phylogenetic analysis. However, I would not be able to make a definitive statement for these clones to be *B. gibsoni*, because these clones showed only 91-92% homologies against reported *B. gibsoni* strain. Therefore, I will have to state this clone as *B. gibsoni*-like strain. The definitive host of this *B. gibsoni*-like strain is suspected to be canids, however, additional analyses will be necessary. DNA of *Babesia* sp. in blood samples of both ICs and TLCs was not detected [77]. Furthermore, previous molecular epidemiological study in our laboratory failed to detect *Babesia* sp. in 1,770 Japanese domestic cats (unpublished data). Therefore, the definitive host of this *B. gibsoni*-like strain might be unlikely to be domestic cats

and wildcats. It was difficult to determine the species of *Babesia* in an *I. tanuki* from TLC even in a phylogenetic analysis. In Tsushima islands, many animal species, including carnivores, rodents and ruminants, inhabit Tsushima Island, so the origin of *Babesia* sp. in ticks from TLC will be difficult to identify. A broad range of animal species must be surveyed to identify the prevalence of *Babesia* species on this island.

Ehrlichia was found in ticks from the wildcats, although the species could not be determined in all of the detected *Ehrlichia* because the DNA fragments were too short for species-level identification. The prevalence of *Ehrlichia canis* in ICs and TLCs was 9.3% and 7.1%, respectively (Chapter 1). Unexpectedly, the phylogenic analysis revealed that two *Ehrlichia* sp. detected in ticks were closely related to *E. muris* and those were different from *E. canis* previously isolated from the Japanese wildcats. Antibody against *E. muris* was detected in various animals including mice, deer, monkeys, bears and dogs [39, 82]. As mentioned above, there are many animal species in Tsushima Island and identification of the original host of the detected *E. muris* was not achieved in the present study. We may need to analyze *Ehrlichia* sp. in ticks as well as broad range of host animals using a more sensitive and specific

PCR system. On the other hand, *E. muris* has been detected in several *Ixodes* tick species including *I. scapularis*, *I. persulcatus* and *I. ricinus* [62, 73, 79]. *E. muris* was detected in *I. tanuki* in the present study and, therefore, *Ixodes* ticks may be a vector of *E. muris*.

Inconsistent results were obtained regarding *Anaplasma* infection in wildcats and ticks. Our previous study revealed that only TLCs, and not ICs, were infected with *A. bovis* (Chapter 1); however, only ticks from ICs showed positive results for *A. bovis* in the present survey. The limited numbers of wildcats and ticks were positive for *A. bovis*, thus the exact reason for this discrepancy on the distribution of *A. bovis* is unclear. Additional study using many wildcats and ticks might be able to determine more accurate distribution of *A. bovis*.

Previous studies successfully detected CMhm from *I. ovatus* and Mhf from *Ixodes ricinus*, respectively; thus, tick is in *Ixodes* may be a vector of feline hemoplasmas [69, 76]. The results *I. tanuki* collected from TLCs was positive for CMhm also support those findings. Our previous analysis actually revealed that 7.1% of TLCs were infected with CMhm (unpublished data). However, conflicting results were observed in ticks from ICs. The prevalence of hemoplasmas in ICs was

found to be approximately 9 % [28], but no ticks possessing hemoplasma were identified on ICs. The transmission route of hemoplasmas is not yet completely understood, and ticks are not the only possible vector [74]. There may be other major transmission routes of hemoplasma in Japanese wildcat cohorts.

Similarly, a discordant result was also observed in our *Bartonella* analysis. In the previous survey, *B. clarridgeiae*, but not *B. henselae*, was detected in TLCs (Chapter 1). However, the present study showed that ticks collected from TLCs were positive for *B. henselae*. A larger-scale survey should be able to clarify the role of ticks in transmitting *Bartonella* and the prevalence of *Bartonella* spp. in wildcats, especially TLCs.

To my knowledge, this is the first report to have detected *H. felis*, *Babesia* sp., *Ehrlichia* sp., CMhm and *B. henselae* in *I. tanuki*. *I. tanuki* is distributed in East Asia, including Japan, China and Nepal. Most specimens are found on wild raccoon dogs and rodents, and occasionally on humans [47]. Although a nation-wide survey of tick infestations in Japanese domestic cats had been conducted, no cats hosting *I. tanuki* were reported [70]. Wildcat might be a preferred host for *I. tanuki* in Japan, because most of the ticks on TLCs were this species.

In this study, arthropod-borne pathogens were found in ticks collected from two subspecies of Japanese wildcats, although the exact roles of ticks in transmitting these pathogens could not be determined. Continuous monitoring and additional surveys would be necessary to understand the etiological role of tick infestations in Japanese wildcat disease and to conserve these endangered wildcats.

Conclusion

The Iriomote cat (IC) and Tsushima leopard cat (TLC) are endangered wildcats in Japan. Several factors are threatening their population. One of the most important factors is expansion of human activities resulting in the increases of risk for road kills. In fact, both species of Japanese wildcats were dead by road kills (http://iwcc.a.la9.jp/ym.htm; http://kyusyu.env.go.jp/twcc/accident/index.html). Infectious diseases could also be a potential threatening factor for their population. Therefore, in the present study, a series of epidemiological surveys of tick-borne infectious diseases caused by *Bartonella* spp., *Ehrlichia* spp., *Anaplasma* spp., *Hepatozoon* spp., and *Babesia* spp. were conducted as a part of ongoing conservation activities for these two species of wildcats.

In Chapter 1, epidemiological survey revealed that the prevalence of *Bartonella henselae*, *Ehrlichia canis* and *Hepatozoon felis* was 4.6%, 9.3% and 72.0% in ICs, respectively. In TLCs, the prevalence of *Bartonella clarridgeiae*, *E. canis*, *Anaplasma bovis* and *H. felis* was 7.1%, 7.1%, 21.4% and 100% respectively. Especially, *H. felis* infection was observed in most of wildcats and *A. bovis* has been detected in felids for the first time. This epidemiological information could be important for understanding the characteristics of infectious diseases not only in

Japanese wildcats but also in other wildcats.

Several viral and bacterial infections in Japanese wildcats have been reported in previous studies [25, 28, 50, 54]. In addition to those pathogens, I showed that Japanese wildcats were exposed to many other infectious agents and were susceptible to them. These results obtained in this study supported the hypothesis that infectious diseases possibly threat the population of Japanese wildcats. However, pathogenicities of detected pathogens were assumed to be not so high because obvious clinical signs or abnormalities in physical and blood examinations were not observed at the time of capture. No dead bodies obviously being caused by infectious diseases have also been found until now. In addition, *H. felis*, *E canis*, *B. henselae* and *B. clarridgeiae* were detected in foreign wildcats, but only mild symptoms were observed in most cases [5, 11, 49, 51]. It is speculated that detected pathogens in this study were likely to subclinically infect to Japanese wildcats and those might not cause critical symptoms them similar to infectious diseases in other wildcats and domestic cats [5, 11, 41, 57, 75]. Infectious diseases might have less important roles on decreasing Japanese wildcats population. Therefore, efforts to reduce a risk for the direct cause of death, such as a road kill, might be more

important than the control of infectious diseases at present. However, I believe that results obtained in this chapter would be valuable from a view of the first detection of analyzed pathogens in Japanese wildcats.

In Chapter 2, ticks collected from Japanese wildcats were morphologically identified and the prevalence of the pathogens including *Bartonella* sp., *Babesia* sp., *Ehrlichia* sp., *Anaplasma* sp., *Hepatozoon* sp. and hemoplasmas were molecularly evaluated in ticks. Furtheremore, the role of ticks on transmission of pathogens in Japanese wildcats cohorts were estimated. *Hepatozoon felis*, *Babesia* sp., and *Anaplasma bovis* were detected in ticks obtained from ICs, while *H. felis*. *Babesia* sp., *Ehrlichia* sp., *E. muris*, '*Candidatus* Mycoplasma haemominutum', and *Bartonella henselae* were found in ticks from TLCs. Consistent results were obtained between wildcats and ticks on *H. felis* infection in both of wildcat species, and CMhm infection in TLCs. The present data suggested that tick should be an important vector for these pathogens in Japanese wildcat cohort. On the other hand, discordant results were observed in *Bartonella*, *Ehrlichia*, *Anaplasma* and Mhf infection. These pathogens were detected in either wildcats or ticks. This finding suggests that there might be other transmission routes of these pathogens or might

be due to limited number of wildcats and ticks. A larger scale of survey will be necessary to clarify the authentic role of ticks for the transmission of pathogens.

In conclusion, I investigated the prevalence of arthropod-borne pathogens in Japanese wildcats and actually found several pathogens in two species of Japanese wildcats and ticks. Ticks were suggested to be a vector for some pathogens in Japanese wildcat cohort. Epidemiological information obtained in a series of study would be useful to understand characteristics of infectious diseases in wildcats. Pathogenicities of detected pathogens were assumed to be not so high, and I believe that the infectious diseases might not be a main cause of decreasing the population of Japanese wildcats at present. However, to date, only a little information about infectious disease in Japanese wildcats has been available and the present study is just a beginning of evaluation for infectious diseases in Japanese wildcats. We cannot exclude completely a potential of infectious diseases to threaten the habitation of Japanese wildcats. Therefore, further studies are necessary to understand the source, infection route and pathogenicity not only of pathogens evaluated in this study but also of many other infectious agents.

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Tables

Table 1. Profile of Iriomote cat evaluated in this study

Cat ID	Date	Alive/Dead	Detected pathogens [a]	Sample ID[b]	Cat ID	Date	Alive/Dead	Detected pathogens[a]	Sample ID[b]
D-043J	12/26/2009	Car-related death	-	I-27	W-119	11/11/2006	Car-related death	-	I-12
E-100	01/06/2012	Alive	Hf	I-60*	W-120	11/18/2006	Alive	Hf	I-15*
E-102	01/06/2012	Alive	-	I-59	W-121	11/18/2006	Alive	-	I-16
E-30	11/23/2003	Alive	Hf	I-42	W-126	01/09/2011	Alive	Hf	I-29*
E-33	11/26/2004	Alive	Hf	I-2	W-127	01/10/2010	Alive	Hf	I-32*
E-60	11/27/2004	Alive	Hf	I-3		01/08/2011	Alive	Hf	I-45*
	11/04/2005	Alive	Hf	I-4*		12/02/2011	Alive	Hf	I-56*
	02/04/2007	Alive	Bh, Hf	I-17*	W-129	01/09/2010	Alive	Hf	I-30*
	02/08/2010	Dying>Dead	Ec, Hf	I-33		12/18/2010	Alive	Hf	I-43*
E-67	11/04/2005	Alive	Hf	I-5		01/08/2011	Alive	Hf	I-46*
	11/18/2006	Alive	Hf	I-13		12/03/2011	Alive	Hf	I-58*
	01/27/2008	Alive	Bh, Hf	I-22*	W-130	11/25/2011	Alive	Hf	I-49*
	12/17/2006	Alive	Hf	I-39	W-131	07/22/2009	Car-related death	-	I-26
E-70	02/06/2006	Alive	Hf	I-8*	W-134	01/09/2010	Alive	Ec, Hf	I-31*
E-72	02/07/2006	Alive	Hf	I-9*		10/17/2010	Alive	Hf	I-44
E-82	01/08/2012	Alive	Hf	I-62*		06/22/2011	Alive	Hf	I-48
E-83	11/17/2008	Alive	Hf	I-24	W-135	04/16/2010	Car-related death	Hf	I-36
	12/01/2011	Alive	Hf	I-55*	W-137	09/09/2010	Car-related death	Ec	I-41
E-84	12/03/2008	Dead	-	I-25	W-140	01/09/2011	Alive	Hf	I-47*
E-89J	01/07/2010	Car-related death	Ec	I-28		11/26/2011	Alive	Hf	I-50*
E-91	02/14/2010	Car-related death	Hf	I-34	W-143	11/26/2011	Alive	Hf	I-51*
	02/17/2010	Dead	Hf	I-35	W-145	01/08/2012	Alive	Hf	I-61*
E-92J	05/10/2010	Alive	-	I-37	W-146	11/26/2011	Alive	Hf	I-52*
E-98	12/02/2011	Alive	Hf	I-57*	W-148	11/26/2011	Alive	-	I-53
Ed-61	12/09/2003	Dead	-	I-1	W-149	11/26/2011	Alive	Hf	I-54*
W-101	02/04/2007	Alive	Hf	I-18*	W-48	02/07/2006	Alive	-	I-10
W-106	12/11/2005	Alive	Hf	I-6*		10/21/2006	Alive	-	I-38
	02/04/2007	Alive	Hf	I-19*	W-71	01/28/2006	Alive	Hf	I-7*
W-108	02/04/2007	Alive	Hf	I-20*	W-87	02/07/2007	Alive	Hf	I-21*
W-113	11/18/2006	Alive	Hf	I-14*	W-99	01/27/2008	Car-related death	Hf	I-23
W-118	10/22/2006	Alive	Hf	I-11*					

[a] Ab, *Anaplasma bovis*; Bh, *Bartonella henselae*; Ec, *Ehrlichia canis*; Hf, *Hepatozoon felis*

[b] Astarisk indicates the blood samples whose boold smear specimens were available.

Table 2. Profile of Tsushima leopard cat evaluated in this study

Cat ID	Date	Alive/Dead	Detected pathogens[a]	Sample ID[b]
CMF-20	11/09/2006	Alive	Hf	T-5
CFS-18	08/23/2002	Alive	Hf	T-2*
CFS-26	11/10/2006	Alive	Hf	T-7*
CFT-17	08/23/2002	Alive	Hf	T-1
CFT-24	12/12/2008	Alive	Hf	T-9*
	12/24/2009	Alive	Ab, Hf	T-13
	12/25/2010	Alive	Ab, Hf	T-19
	12/24/2011	Alive	Hf	T-27
CFT-25	11/10/2006	Alive	Hf	T-8*
	12/26/2009	Alive	Hf	T-17
	12/24/2011	Alive	Hf	T-29
CFT-27	12/12/2008	Alive	Ab, Hf	T-10*
	12/24/2009	Alive	Hf	T-14
	12/26/2010	Alive	Ab, Hf	T-22
	12/23/2011	Alive	Ab, Hf	T-24
CFT-28	12/262009	Alive	Hf	T-18
	12/24/2011	Alive	Hf	T-26
CMM-19	08/23/2002	Alive	Ec, Hf	T-3
	11/03/2005	Alive	Hf	T-4*
	11/09/2006	Alive	Ec, Hf	T-6*
CMS-29	12/12/2008	Alive	Hf	T-11*
	12/24/2009	Alive	Bc, Hf	T-15
	12/25/2010	Alive	Bc, Hf	T-20
	12/24/2011	Alive	Hf	T-28
CMS-32	12/24/2009	Alive	Hf	T-16
CMS-34	12/24/2011	Alive	Ab, Hf	T-25
CMT-33	12/25/2010	Alive	-	T-21
	11/01/2011	Alive	Hf	T-23
MM-22	12/12/2008	Alive	Hf	T-12*

[a] Ab, *Anaplasma bovis*; Bc, *Bartonella clarridgeiae*; Ec, *Ehrlichia canis*; Hf, *Hepatozoon felis*

[b] Astarisk indicates the blood samples whose boold smear specimens were available.

Table 3. Primers used in pathogen-specific PCR in Chapter 1.

Pathogen	Gene	Name	Sequence (5' to 3')	References
Hepatozoon sp., *Babesia* sp.	18S rRNA	PIRO-F	AGT CAT ATG CTT GTC TTA	Ano *et al*., 2001
		PIRO-R	CCA TCA TTC CAA TTA CAA	
		BabgenF	GAA ACT GCG AAT GGC TCA TTA	Baneth et al., 2004
		BabgenR	CGG TAG GCC AAT ACC CTA CCG TC	
		BT1	GGT TGA TCC TGC CAG TAG T	Criado-Fornelio *et al*., 2007
		HPF2P	GAC TTC TCC TTC TTT AAG TGA TAA G	
Hepatozoon sp.		HepF	ATA CAT GAG CAA AAT CTC AAC	Inokuma et al., 2002
		RLB-R	TCT TCG ATC CCC TAA CTT TC	Allison et al., 2011
Babesia sp.		Bab F3	ATG TCT AAG TAC AAG CTT TTT ACG GT	Devos et al., 2004
		Bab R3	AAA GGC GAC GAC CTC CAA TCC CTA GT	
Ehrlichia sp., *Anaplasma* sp.	16S rRNA	P-ehrf	GGG GAT GAT GTC AAR TCA GCA C	Tabar *et al*., 2008
		P-ehrr	CAC CAG CTT CGA GTT AAG CCA AT	
		N-ehrf	ART CAG CAC GGC CCT TAT RG	
		N-ehrr	GAC CCG AGA ACG TAT TCA CC	
	5' region of 16S rRNA	fD1	AGA GTT TGA TCC TGG CTC AG	Inokuma *et al*., 2001
		EHR16SR	TAG CAC TCA TCG TTT ACA GC	
	3' region of 16S rRNA	EHR16SD	GGT ACC YAC AGA AGA AGT CC	
		Rp2	ACG GCT ACC TTG TTA CGA CTT	
Bartonella sp.	16S-23S rRNA intergenic spacer region	P-bhenfa	TCT TCG TTT CTC TTT CTT CA	Rampersad *et al*., 2005
		P-benr1	CAA GCG CGC GCT CTA ACC	
		N-bhenf1a	GAT GAT CCC AAG CCT TCT GGC	
		N-bhenr	AAC CAA CTG AGC TAC AAG CC	

Table 4. Primers used in pathogen-specific PCR in Chapter 2

Pathogen	Gene	Name	Sequence (5' to 3')	Product length	References
Hepatozoon sp., *Babesia* sp.	18S rRNA	BT1	GGT TGA TCC TGC CAG TAG T	1669bp	Criado-Fornelio *et al*., 2007
		HPF2P	GAC TTC TCC TTC TTT AAG TGA TAA G		
Hepatozoon sp.		HEP-1	CGC GAA ATT ACC CAA TT	665 bp	Criado-Fornelio *et al*., 2006
		HEP-4	TAA GGT GCT GAA GGA GTC GTT TAT		
Babesia sp.		455-479F	GTC TTG TAA TTG GAA TGA TGG TGA C	339bp	Birkenheuer *et al*., 2003
		793-772R	ATG CCC CCA ACC GTT CCT ATT A		
Ehrlichia sp., *Anaplasma* sp.	16S rRNA	P-ehrf	GGG GAT GAT GTC AAR TCA GCA C	256bp	Tabar *et al*., 2008
		P-ehrr	CAC CAG CTT CGA GTT AAG CCA AT		
		N-ehrf	ART CAG CAC GGC CCT TAT RG	192bp	
		N-ehrr	GAC CCG AGA ACG TAT TCA CC		
	5' region of 16S rRNA	fD1	AGA GTT TGA TCC TGG CTC AG	787bp	Inokuma *et al*., 2001
		EHR16SR	TAG CAC TCA TCG TTT ACA GC		
	3' region of 16S rRNA	EHR16SD	GGT ACC YAC AGA AGA AGT CC	1028bp	
		Rp2	ACG GCT ACC TTG TTA CGA CTT		
Hemoplasmas	16S rRNA	MY-F	AGC AAT RCC ATG TGA ACG ATG AA	104bp	Willi *et al*., 2009
		MY-R1	TGG CAC ATA GTT TGC TGT CAC TT		
		MY-R2	GCT GGC ACA TAG TTA GCT GTC ACT		
Bartonella sp.	16S-23S rRNA intergenic spacer region	P-bhenfa	TCT TCG TTT CTC TTT CTT CA	186bp	Rampersad *et al*., 2005
		P-benr1	CAA GCG CGC GCT CTA ACC		
		N-bhenf1a	GAT GAT CCC AAG CCT TCT GGC	149bp	
		N-bhenr	AAC CAA CTG AGC TAC AAG CC		

Table 5. Tick species and stages collected from Iriomote cats and the pathogens detected in those ticks and ICs.

Cat ID	Date	Species[a]	Stage[b]	Detected pathogens in tick[c]	Detected pathogens in IC[c,d,e]
E-100	01.06.12	Hh	L	Hf	Hf
E-102	01.06.12	Hh	N	-	-
E-83	12.01.11	Hh	L	Hf	Hf
E-98	12.02.11	Hh	L	Hf	Hf
		Hh	N	Hf	
		At	L	Hf	
W-127	12.02.11	Hh	L	Hf	Hf
		At	L	-	
W-129	12. 03. 11	Hh	L	Hf	Hf
		At	L	*B. gibsoni*-like strain	
	12.18.11	Hh	L	-	NA
	01.08.12	Hh	L	Hf	NA
		Hh	N	Hf	
		At	L	*B. gibsoni*-like strain, Hf	
W-130	11.25.11	Hh	L	Hf	Hf
		At	L	-	
W-134	12.18.11	Hh	L	Hf	NA
W-140	01.09.12	At	N	-	NA
		Hl	N	Ab, Hf	
		At	L	-	
		Hh	L	Hf	
	11.26.11	At	L	-	Hf
		Hh	L	Hf	
W-143	11.26.11	Hh	L	Hf	Hf
		At	L	Hf	
W-145	01.08.12	Hh	N	Hf	Hf
		Hh	L	Hf	
W-146	11.26.11	Hl	N	Hf	Hf
		Hh	L	Hf	
W-149	11.26.11	Hl	N	Hf	Hf
		Hh	L	Hf	

[a] At, *A. testudinarium*; Hl, *H. longicornis*; Hh, *H. hystricis*. [b] L, Larva; N, Nymph; A, Adult. [c] Ab, *Anaplasma bovis*; Hf, *Hepatozoon felis*

[d] NA, Not available

Table 6. Tick species and stages collected from Tsushima leopard cats and the pathogens detected in those ticks and TLCs.

Cat ID	Date	Species[a]	Stage/ Sex[b]	Detected pathogens in tick[c]	Detected pathogens in TLC[c, d, e]
CFT-24	12.25.11	It	A/F	-	Hf
		It	A/M	-	
	12.23.11	Hm	A/F	Hf	Hf
		At	N	-	
		It	A/F	-	
		It susp	A/F	Bh, Hf	
		It	A/F	Hf	
		It	A/F	Hf	
		It	A/F	*Ehrlichia* sp., Hf	
CFT-25	12.24.11	Hm	A/M	Hf	Hf
		At	N	-	
		Hm	A/F	Hf	
	12.25.11	It	A/F	Hf	Hf
		At	N	Hf	
		It susp	A/F	Bh, Hf	
		It	A/M	Bh	
CFT-27	12.23.11	Hc	A/F	Hf	Hf, Ab
		It	A/M	*Ehrlichia* sp.	
		Hm	A/F	Hf	
		It	A/F	-	
		It	A/F	Hf	
		It susp	A/F	-	
		It susp	A/F	Hf	
	12.26.11	Hm	A/M	Hf	Hf, Ab
		Hm	A/F	Hf	
		It	A/M	Hf	
		It	A/F	CMhm, Hf	
		It	A/F	-	
CFT-28	12.23.11	It	A/M	*Ehrlichia* sp., Hf	Hf
		Hm	L	-	
		It	A/F	Hf	
		Hc	N	-	
CMS-29	12.23.11	It	A/F	Hf	Hf
		It	A/M	-	
		It susp	A/F	Hf	
		Hm	A/F	Hf	
		Hc	A/F	Hf	
		It susp	A/F	Hf	
		It	A/F	Hf	
		It	A/M	-	
CMS-34	12.24.11	It	A/M	-	Hf, Ab
		Hc	A/F	-	
		It susp	A/F	*Ehrlichia* sp., Hf	
		It susp	A/F	Hf	
CMS-35	12.25.11	It	A/F	Hf	NA
		It	A/M	*Ehrlichia* sp.	
		At susp	N	Hf	
CMT-33	12.25.11	Hc	N	-	NA
		It	A/F	*Babesia* sp., Bh	
		It	A/M	-	
		It susp	A/F	-	

[a] At, *A. testudinarium*; It *I. tanuki*; Hm, *H. megaspinosa*; Hc, *H. campanulata*; Hl, *H. longicornis*; Hh, *H. hystricis;* susp, suspicion.

[b] L, Larva; N, Nymph; A, Adult; F, Female; M, Male.

[c] Ab, *Anaplasma bovis*; Bg, *Babesia gibsoni*; Bh, *Bartonella henselae*; CMhm, '*Candidutus* Mycoplasma haemominutum'; Hf, *Hepatozoon felis*

[d] NA, Not available

Figure Legends

Figure 1

Phylogenetic relationship of the partial 16S rRNA gene sequences of *Ehrlichia* and *Anaplasma* isolated from ICs, TLCs and other animals. Six clones of *E. canis* 16S rRNA from four ICs (E-60, E-89J, W-134 and W-137J) (AB723707–AB723710) and one TLC (CMM-19) (AB723711 and AB723712) and four clones of *A. bovis* 16S rRNA gene from two TLCs (CFT-24 and CFT-27) (AB723713–AB723716) were analyzed. Pathogen names, host species, country of isolation (if available), and the GenBank accession numbers or WGS database (in parentheses) of compared sequences are shown in the phylogenetic tree. These included: *E. canis* from a cat in Taiwan (EU178797), a dog in Taiwan (EU143637), a dog in Thailand (EU263991), a dog in China (AF162860), and a dog in Japan (AF536827); *E. chaffeensis* (AAIF01000035); *E. ewingii* (M73227); *A. bovis* from a dog in Japan (HM131218) and a raccoon in Japan (GU937021); *A. centrale* (AF283007); *A. marginale* (FJ226454); *A. ovis* (AJ633052); *A. platys* from dogs in Japan (AF536828) and Thailand (EF139459); and *A. phagocytophilum* (AB196721). Numbers under internal nodes indicate the percentages of 1,000 bootstrap replicates that supported the branch.

Figure 2

Phylogenetic relationship of the partial 18S rRNA gene sequences of *Hepatozoon* isolated from ticks, ICs and TLCs, and other animals. Twenty-three clones of *H. felis* 18S rRNA gene from ticks on ICs (AB983385- AB983407) and 30 clones from ticks on TLCs (AB983408-AB983437) were analyzed. Pathogen names, host species, country of isolation (if available), and the GenBank accession numbers (in parentheses) of compared sequences are shown in the phylogenetic tree. These included: *H. felis* from a Bengal tiger in India (HQ829446), a cat in Israel (KC138534), an Asiatic lion in India (HQ829439), an Iriomote cat in Japan (AB771538), and a Tsushima leopard cat in Japan (AB771576); *H. canis* from a dog in India (JX112783) and a Golden Jackal in Hungary (KJ634654), *H. americanum* (AF176836); *H. ursi* from an Asiatic black bear in Japan (EU041717); *H. catesbianae* (AF130361); *H. sipedon* (JN181157); *H. avorgbor* from a brown house snake in Ghana (EF157822); *H. domerguei* from a chameleon Madagascar (KM234649); *B. gibsoni* from a dog in Taiwan (FJ769388); and *Theileria parva* (L02366). Numbers under internal nodes indicate the percentages of 1,000 bootstrap

replicates that supported the branch.

Figure 3

Phylogenetic relationship of the partial 18S rRNA gene sequences of *Babesia* isolated from ticks, ICs and TLCs, and other animals. Two clones of *B. gibsoni* 18S rRNA gene from ticks on ICs (AB983383 and AB983384) and a clone of *Babesia* sp. from a tick on a TLC (AB983382) were analyzed. Pathogen names, host species, country of isolation (if available), and the GenBank accession numbers (in parentheses) of compared sequences are shown in the phylogenetic tree. These included: *B. canis vogeli* from a dog in Italy (AY072925); *B. canis canis* from a dog in Croatia (AY072926); *B. gibsoni* from a dog in India (KF928958), a dog in Taiwan (FJ769388), a dog in Japan (AB478329), and a dog (AF175300); *B divergens* (Z48751); *B. odocoilei* from a white-tailed deer in USA (U16369); *B. Bigemina* (X59607); *Babesia* sp. (AB649052); and *Theileria parva* (L02366). Numbers under internal nodes indicate the percentages of 1,000 bootstrap replicates that supported the branch.

Figure 4

ßPhylogenetic relationship of the partial 16S rRNA gene sequences of *Ehrlichia* and *Anaplasma* isolated from ticks, ICs and TLCs, and other animals. Five clones of *Ehrlichia* sp. 16S rRNA gene from ticks on TLCs (AB983377-AB983381) and a clone of *A. bovis* from a tick on an IC (AB983376) were analyzed. Pathogen names, host species, country of isolation (if available), and the GenBank accession numbers (in parentheses) of compared sequences are shown in the phylogenetic tree. These included: *E. canis* from a dog in Thailand (EF139458), a Tsushima leopard cat in Japan (AB723711), an Iriomote cat in Japan (AB723708), and a dog in Japan (AF536827); *E. muris* from a large Japanese field mouse in Japan (AB196302); *E. chaffeensis* (U23503); *E. ruminantium* (DQ647616); *E. ewingii* (U96436); *A. bovis* from a dog in Japan (HM131218), a Tsushima leopard cat in Japan (AB723716), and a deer in China (KJ659043); *A. centrale* (AF283007); *A. marginale* (FJ226454); *A. ovis* (AJ633052); *A. platys* from dogs in Japan (AF536828); *A. phagocytophilum* (AB196721); and *Rickettsia rickettsii* (U11021). Numbers under internal nodes indicate the percentages of 1,000 bootstrap replicates that supported the branch.

Figures

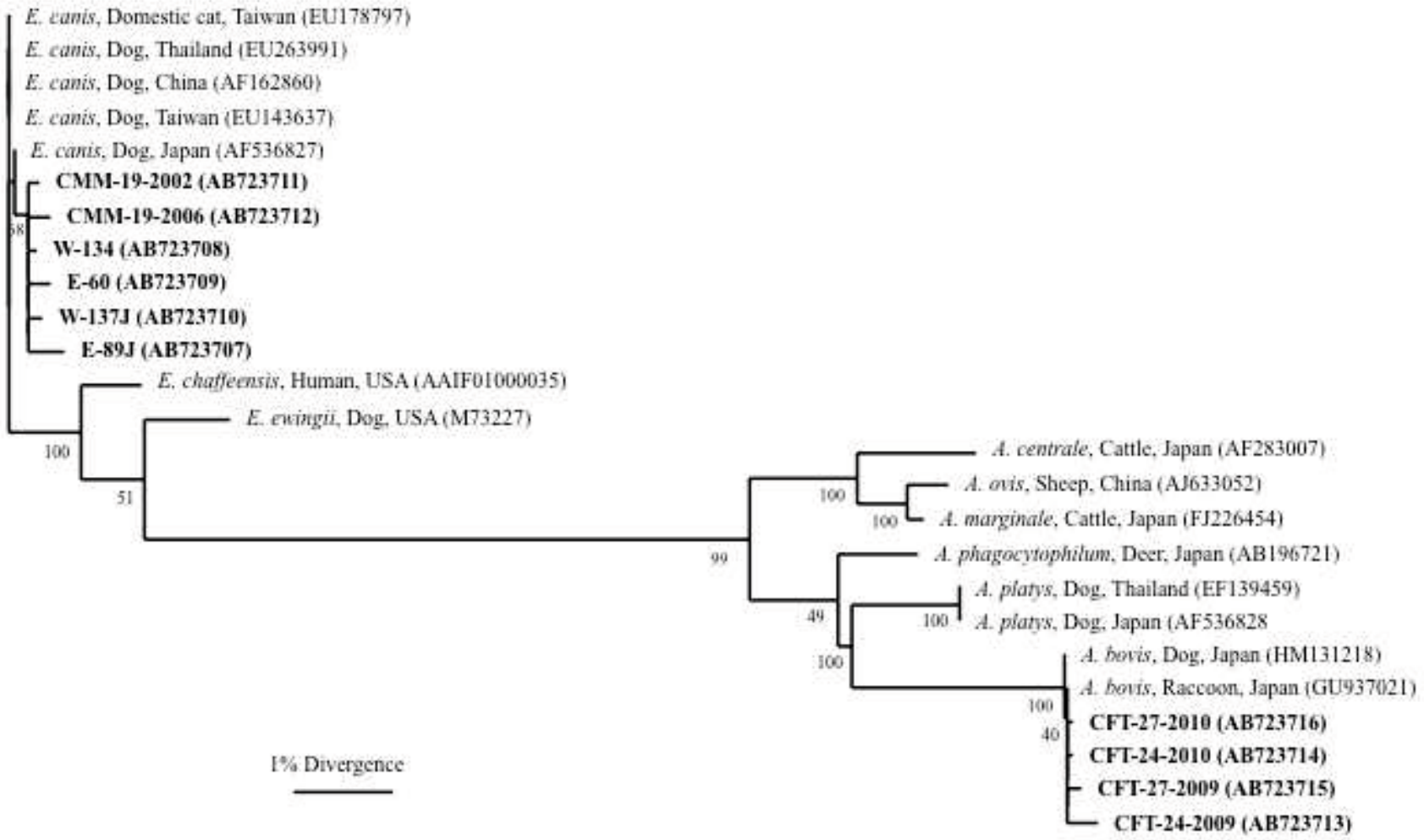
E. canis, Domestic cat, Taiwan (EU178797)
E. canis, Dog, Thailand (EU263991)
E. canis, Dog, China (AF162860)
E. canis, Dog, Taiwan (EU143637)
E. canis, Dog, Japan (AF536827)
CMM-19-2002 (AB723711)
CMM-19-2006 (AB723712)
W-134 (AB723708)
E-60 (AB723709)
W-137J (AB723710)
E-89J (AB723707)
E. chaffeensis, Human, USA (AAIF01000035)
E. ewingii, Dog, USA (M73227)
A. centrale, Cattle, Japan (AF283007)
A. ovis, Sheep, China (AJ633052)
A. marginale, Cattle, Japan (FJ226454)
A. phagocytophilum, Deer, Japan (AB196721)
A. platys, Dog, Thailand (EF139459)
A. platys, Dog, Japan (AF536828
A. bovis, Dog, Japan (HM131218)
A. bovis, Raccoon, Japan (GU937021)
CFT-27-2010 (AB723716)
CFT-24-2010 (AB723714)
CFT-27-2009 (AB723715)
CFT-24-2009 (AB723713)
100
51
99
100
100
49
100
100
100
40
1% Divergence

Figure 1

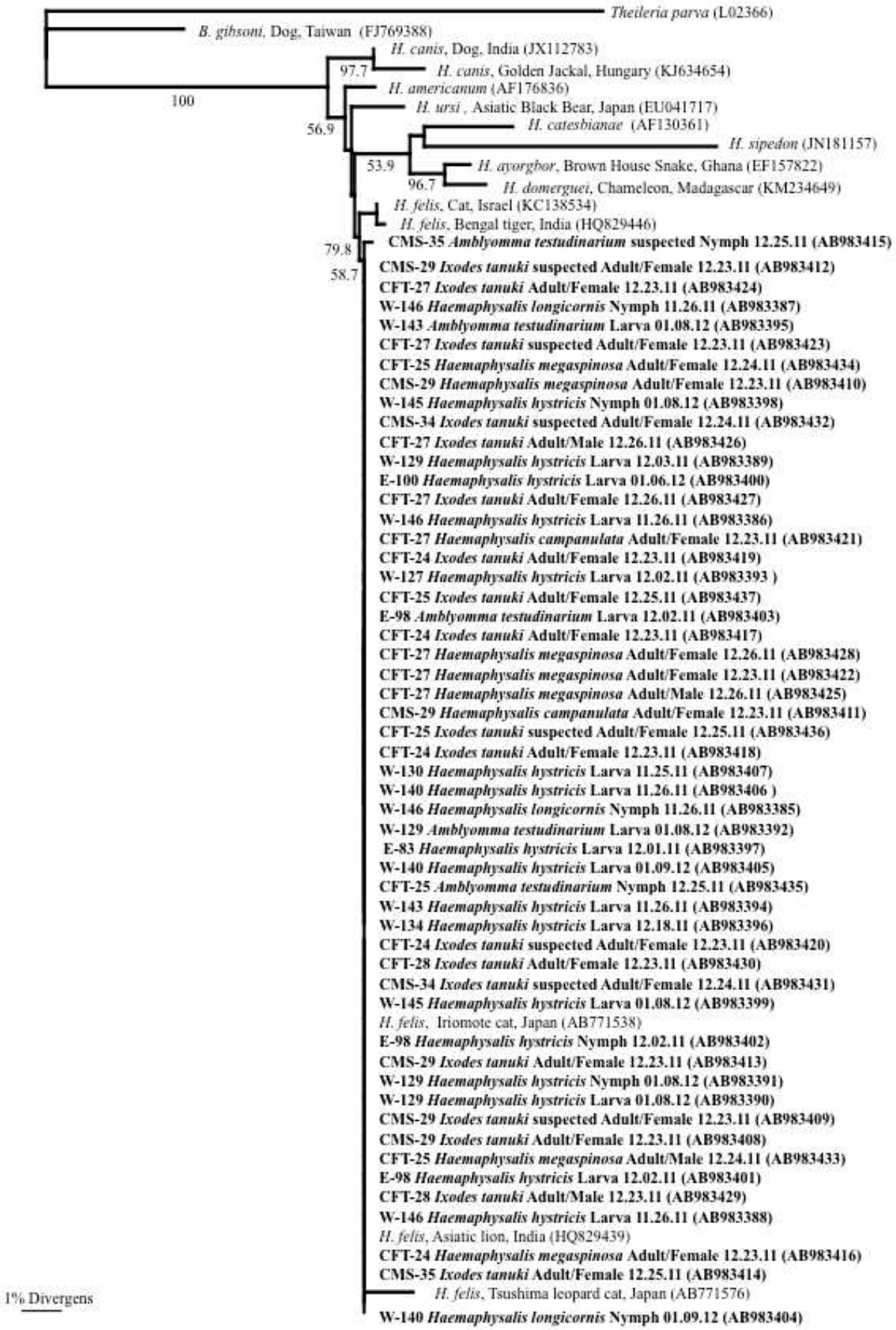
Theileria parva (L02366)
B. gibsoni, Dog, Taiwan (FJ769388)
H. canis, Dog, India (JX112783)
97.7
H. canis, Golden Jackal, Hungary (KJ634654)
H. americanum (AF176836)
100
H. ursi , Asiatic Black Bear, Japan (EU041717)
56.9
H. catesbianae (AF130361)
H. sipedon (JN181157)
53.9
H. ayorghor, Brown House Snake, Ghana (EF157822)
96.7
H. domerguei, Chameleon, Madagascar (KM234649)
H. felis, Cat, Israel (KC138534)
H. felis, Bengal tiger, India (HQ829446)
79.8
CMS-35 Amblyomma testudinarium suspected Nymph 12.25.11 (AB983415)
58.7
CMS-29 Ixodes tanuki suspected Adult/Female 12.23.11 (AB983412)
CFT-27 Ixodes tanuki Adult/Female 12.23.11 (AB983424)
W-146 Haemaphysalis longicornis Nymph 11.26.11 (AB983387)
W-143 Amblyomma testudinarium Larva 01.08.12 (AB983395)
CFT-27 Ixodes tanuki suspected Adult/Female 12.23.11 (AB983423)
CFT-25 Haemaphysalis megaspinosa Adult/Female 12.24.11 (AB983434)
CMS-29 Haemaphysalis megaspinosa Adult/Female 12.23.11 (AB983410)
W-145 Haemaphysalis hystricis Nymph 01.08.12 (AB983398)
CMS-34 Ixodes tanuki suspected Adult/Female 12.24.11 (AB983432)
CFT-27 Ixodes tanuki Adult/Male 12.26.11 (AB983426)
W-129 Haemaphysalis hystricis Larva 12.03.11 (AB983389)
E-100 Haemaphysalis hystricis Larva 01.06.12 (AB983400)
CFT-27 Ixodes tanuki Adult/Female 12.26.11 (AB983427)
W-146 Haemaphysalis hystricis Larva 11.26.11 (AB983386)
CFT-27 Haemaphysalis campanulata Adult/Female 12.23.11 (AB983421)
CFT-24 Ixodes tanuki Adult/Female 12.23.11 (AB983419)
W-127 Haemaphysalis hystricis Larva 12.02.11 (AB983393)
CFT-25 Ixodes tanuki Adult/Female 12.25.11 (AB983437)
E-98 Amblyomma testudinarium Larva 12.02.11 (AB983403)
CFT-24 Ixodes tanuki Adult/Female 12.23.11 (AB983417)
CFT-27 Haemaphysalis megaspinosa Adult/Female 12.26.11 (AB983428)
CFT-27 Haemaphysalis megaspinosa Adult/Female 12.23.11 (AB983422)
CFT-27 Haemaphysalis megaspinosa Adult/Male 12.26.11 (AB983425)
CMS-29 Haemaphysalis campanulata Adult/Female 12.23.11 (AB983411)
CFT-25 Ixodes tanuki suspected Adult/Female 12.25.11 (AB983436)
CFT-24 Ixodes tanuki Adult/Female 12.23.11 (AB983418)
W-130 Haemaphysalis hystricis Larva 11.25.11 (AB983407)
W-140 Haemaphysalis hystricis Larva 11.26.11 (AB983406)
W-146 Haemaphysalis longicornis Nymph 11.26.11 (AB983385)
W-129 Amblyomma testudinarium Larva 01.08.12 (AB983392)
E-83 Haemaphysalis hystricis Larva 12.01.11 (AB983397)
W-140 Haemaphysalis hystricis Larva 01.09.12 (AB983405)
CFT-25 Amblyomma testudinarium Nymph 12.25.11 (AB983435)
W-143 Haemaphysalis hystricis Larva 11.26.11 (AB983394)
W-134 Haemaphysalis hystricis Larva 12.18.11 (AB983396)
CFT-24 Ixodes tanuki suspected Adult/Female 12.23.11 (AB983420)
CFT-28 Ixodes tanuki Adult/Female 12.23.11 (AB983430)
CMS-34 Ixodes tanuki suspected Adult/Female 12.24.11 (AB983431)
W-145 Haemaphysalis hystricis Larva 01.08.12 (AB983399)
H. felis, Iriomote cat, Japan (AB771538)
E-98 Haemaphysalis hystricis Nymph 12.02.11 (AB983402)
CMS-29 Ixodes tanuki Adult/Female 12.23.11 (AB983413)
W-129 Haemaphysalis hystricis Nymph 01.08.12 (AB983391)
W-129 Haemaphysalis hystricis Larva 01.08.12 (AB983390)
CMS-29 Ixodes tanuki suspected Adult/Female 12.23.11 (AB983409)
CMS-29 Ixodes tanuki Adult/Female 12.23.11 (AB983408)
CFT-25 Haemaphysalis megaspinosa Adult/Male 12.24.11 (AB983433)
E-98 Haemaphysalis hystricis Larva 12.02.11 (AB983401)
CFT-28 Ixodes tanuki Adult/Male 12.23.11 (AB983429)
W-146 Haemaphysalis hystricis Larva 11.26.11 (AB983388)
H. felis, Asiatic lion, India (HQ829439)
CFT-24 Haemaphysalis megaspinosa Adult/Female 12.23.11 (AB983416)
CMS-35 Ixodes tanuki Adult/Female 12.25.11 (AB983414)
H. felis, Tsushima leopard cat, Japan (AB771576)
W-140 Haemaphysalis longicornis Nymph 01.09.12 (AB983404)
1% Divergens

Figure 2

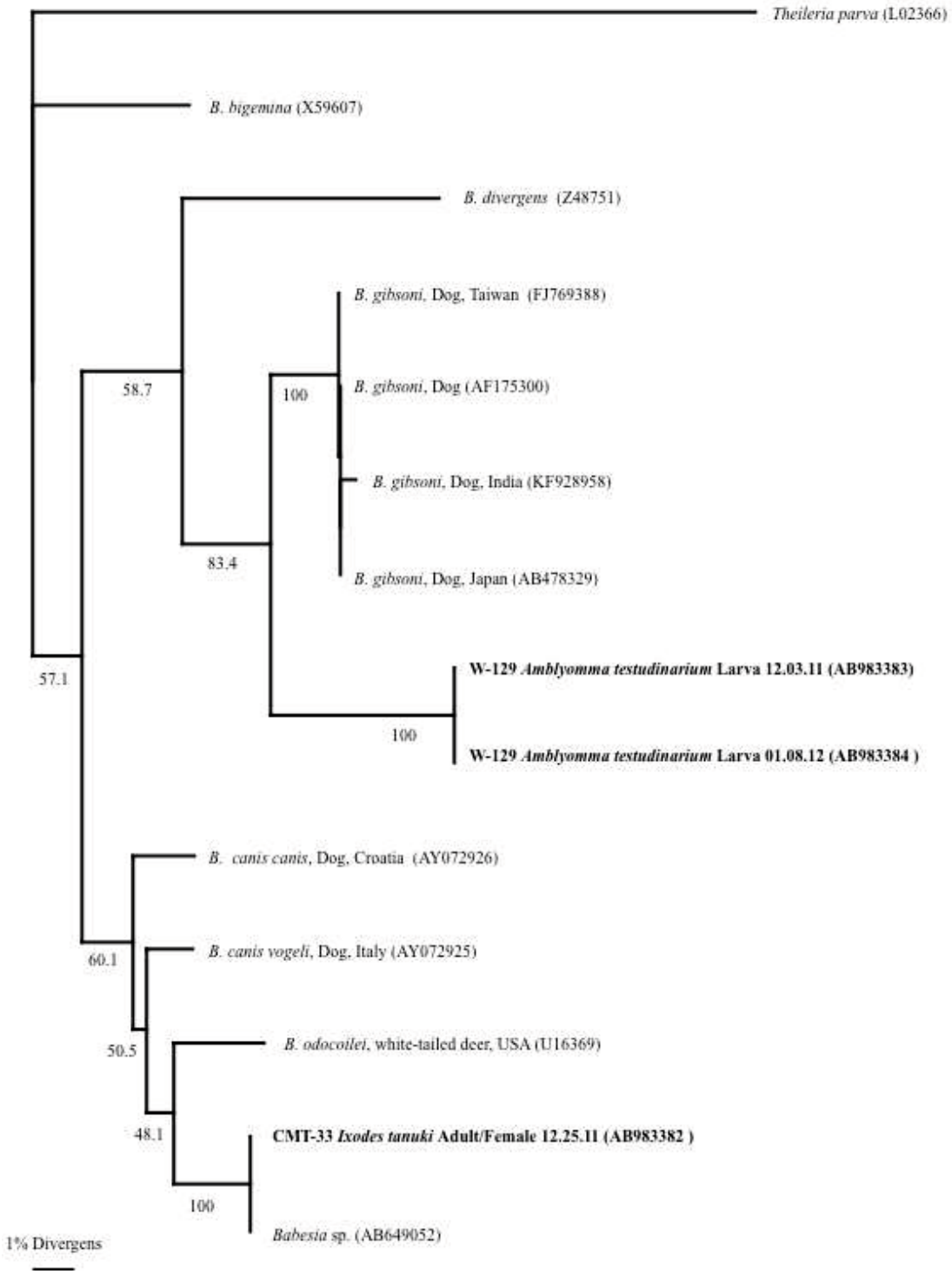
Theileria parva (L02366)
B. bigemina (X59607)
B. divergens (Z48751)
B. gibsoni, Dog, Taiwan (FJ769388)
B. gibsoni, Dog (AF175300)
B. gibsoni, Dog, India (KF928958)
B. gibsoni, Dog, Japan (AB478329)
W-129 *Amblyomma testudinarium* Larva 12.03.11 (AB983383)
W-129 *Amblyomma testudinarium* Larva 01.08.12 (AB983384)
B. canis canis, Dog, Croatia (AY072926)
B. canis vogeli, Dog, Italy (AY072925)
B. odocoilei, white-tailed deer, USA (U16369)
CMT-33 *Ixodes tanuki* Adult/Female 12.25.11 (AB983382)
Babesia sp. (AB649052)
58.7
100
83.4
57.1
100
60.1
50.5
48.1
100
1% Divergens

Figure 3

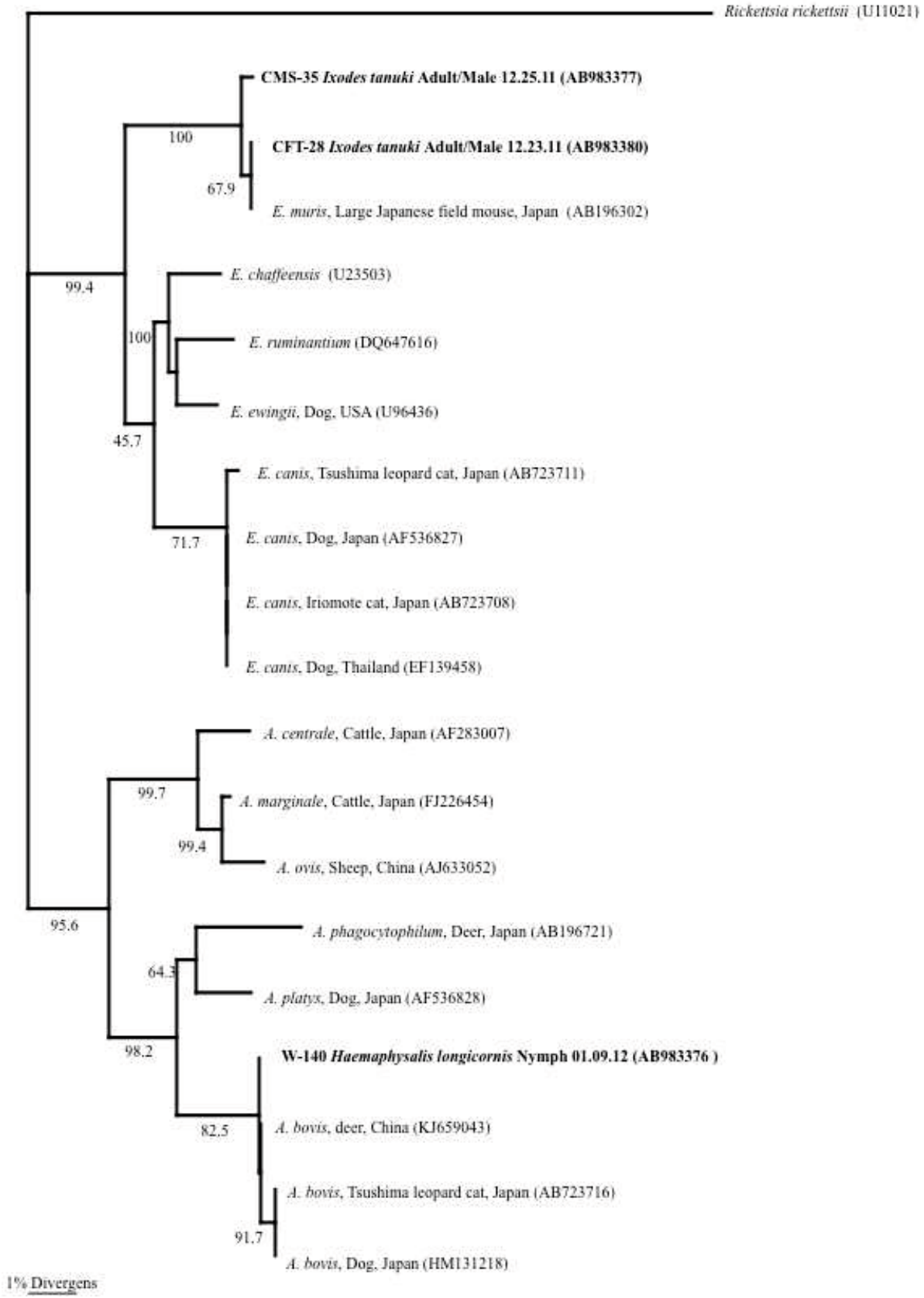
Rickettsia rickettsii (U11021)
CMS-35 Ixodes tanuki Adult/Male 12.25.11 (AB983377)
100
CFT-28 Ixodes tanuki Adult/Male 12.23.11 (AB983380)
67.9
E. muris, Large Japanese field mouse, Japan (AB196302)
E. chaffeensis (U23503)
99.4
100
E. ruminantium (DQ647616)
E. ewingii, Dog, USA (U96436)
45.7
E. canis, Tsushima leopard cat, Japan (AB723711)
71.7
E. canis, Dog, Japan (AF536827)
E. canis, Iriomote cat, Japan (AB723708)
E. canis, Dog, Thailand (EF139458)
A. centrale, Cattle, Japan (AF283007)
99.7
A. marginale, Cattle, Japan (FJ226454)
99.4
A. ovis, Sheep, China (AJ633052)
95.6
A. phagocytophilum, Deer, Japan (AB196721)
64.3
A. platys, Dog, Japan (AF536828)
98.2
W-140 Haemaphysalis longicornis Nymph 01.09.12 (AB983376)
82.5
A. bovis, deer, China (KJ659043)
A. bovis, Tsushima leopard cat, Japan (AB723716)
91.7
A. bovis, Dog, Japan (HM131218)
1% Divergens

Figure 4