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**Molecular evidence of the multiple genotype infection of a wild Hokkaido brown bear (*Ursus arctos* yesoensis) by *Babesia* sp. UR1**

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running head: BABESIA IN BROWN BEAR

**Abstract**

A frozen-stored blood clot of a wild brown bear cub□*Ursus arctos* yesoensis□ that had been captured in Hokkaido, Japan was examined for piroplasma infection using polymerase chain reaction (PCR). Two 18S ribosomal RNA gene (SSU rDNA) sequences were generated . One 1565-bp sequence showed the highest similarity with *B. gibsoni* (95.9% identity) but, phylogenetically, was found to belong to a distinct lineage. The other sequence (1709-bp) could not be definitively assigned to a described taxon, sharing only limited homology to the closest named species, (90.1% identity with *C. felis*). In order to enhance information obtained from the SSU rDNA sequence , further detection and sequence analysis of the *CCTη* gene sequence was done revealing the simultaneous presence of three closely-related genotypes (all in a monophyletic lineage) within a single bear host. This finding suggested the possibility that a new *Babesia* species (*Babesia* sp. UR1) may have been maintained in nature in wild brown bears. While the parasite's biology is yet unknown, to our knowledge, this is, excepting the single case documentation in 1910 of a hemoparasite in a bear at Russian zoo, the first reported case of piroplasms inhabiting a bear species .

*Key Words*: *Babesia* sp. UR1, *Cytauxzoon* sp., wild brown Bear, *Ursus arctos* yesoensis, multiple genotype infection.

## 1. Introduction

For case management and wildlife conservation programs to be effective, accurate identification of the causative agents for infectious diseases of wild animals, especially bears, in their rapidly dwindling wild habitats is essential., In 1910, Yakimof et al. reported an intra-erythrocytic protozoon on the blood smear of a brown bear living at a Russian zoo (Stiles and Baker, 1935).This case up to the present, is the of only report of piroplasma presence in bears regardless of species despite the lack of information regarding the taxonomic position, geographic distribution, tick vector specificities and description of clinical disease manifestations.. The morphological descriptions by light microscopy were, by current criterion, inadequate, so that at present, absent the original research materials,futher description of the organism is not possible..

In the present study, we had the opportunity to investigate piroplasma in a frozen-stored blood clot from a dead Hokkaido brown bear cub (*Ursus arctos* yesoensis). A nested PCR with subsequent nucleotide sequencing allowed us to detect the parasite sequences of 18S ribosomal gene (SSU rDNA) and also the *η subunit of the chaperonin-containing t-complex polypeptide 1* (*CCTη*) gene, that has been reported to have greater sensitivity for detecting small phylogenetic differences within a single species (Nakajima et al., 2009). We present the first molecular evidence for the multiple sequevar (sequence variants or *CCTη*-genotypes) infection in an Hokkaido brown bear with a possible new *Babesia* species (*Babesia* sp. UR1) as well.as the presence of a new bear *Cytauxzoon* sp.

## 2. Materials and Methods

### *2.1. Blood sample*

We obtained a bear blood specimen preserved for 6 months at -20 °C as a frozen scrapping of blood clot formed in the cardiac vasculature of a dead male cub of Hokkaido brown bear (*Ursus arctos* yesoensis). On May 15, 2005. a bear cub of 5.6 kg body weight (about 3 months old) and separated from his mother had been found exhausted in the wild in Honbetsu-town, Hokkaido, Japan and captured under the wildlife management plan by the Hokkaido prefecture The cub had received an intravenous infusion therapy and milk feedings but died at the 6th day of his protective care. According to the veterinarian's record, the cub had been heavily infested with ticks of the genus *Ixodes* and *Haemophysalis* and had received a spot-on type tick removal treatment (Fortreon®, Bayer, Tokyo, Japan). It had also been diagnosed with anemia defined as a 79 mg/ml hemoglobin concentration, a 3.9 x$10^6$/ ml total red blood cell counts and a 26.3% hematocrit. However, no blood smear examination was demonstrated in the 6 days of his care nor was there documentation of splenomegaly or icterus at necropsy.

### *2.2. Detection and sequencing of parasite SSU rDNA and CCTη gene*

Parasite DNA was extracted from the frozen thawed blood specimen with a whole blood DNA extraction kit (DNA Extractor WB Kit; Wako Pure Chemical Industries, Osaka, Japan) in accordance with the instruction manual. Nested PCR was carried out as described previously (Zamoto et al., 2004) with minor modifications as reported below. We always practiced ‘safe PCR’ technique

utilizing barrier pipette tips, a dedicated room for pre- and post-PCR, and negative control (no template) samples for each set of reactions. Since recombination artifacts have been noted between haplotypes in many of the specimens with mixed infections (Beser et al., 2007), we made a modification of accepted PCR practice in order to minimize the error. LA Taq™ DNA polymerase (Takara Bio Inc. Shiga, Japan) was used instead of Taq DNA polymerase, and reduced PCR cycles (25 cycles for each round of nest PCR). To detect the parasite's SSU rDNA, we used a set of primer pairs consisting of Piro0F and Piro6R for the 1st-round, and Piro1F and Piro5.5F for the 2nd-round PCR that target broadly to SSU rDNA of all the parasites in the order Piroplasma that includes *Babesia*, *Theileria* and *B. microti*-group (Kawabuchi et al., 2005). The use of this primer set makes it possible to obtain a nearly full-length SSU rDNA sequence. PCR amplicons were purified from 1% agarose gel with MinElute Gel Extraction Kit (QIAGEN K.K., Tokyo, Japan). The resulting products were cloned into a plasmid vector (pCR4-TOPO, Invitrogen, Carlsbad, CA, USA) and sequenced individually. Sequence determination was done by the fluorescent dideoxy termination using the DTCS DNA Sequencing kit with a CEQ8000 automated sequencer (Beckman Coulter, Inc., CA, USA) in accordance with the instruction manual. A minimum of triplicate experiments were performed to exclude possible jumping PCR artifacts. Plasmid clones isolated independently from each PCR amplification were sequenced individually, compared to confirm results and selected three or more sequences were used to construct a consensus SSU rDNA sequence. We also carried out another nested PCR to detect *CCTη* gene sequences that was present as a single-copy gene (Nishisaka et al., 2001) and allowed us more precise assignment of closely related *CCTη*-genotypes within a single species (Nakajima et al., 2009). A set of nested primers used was TBcct35F (5'-TGAAGGARGGMACNGAYACWTCYCARGG-3') and TBcctR0 (5'-GTYTCRTCDATDSWNAGNACHWGGCANGCNGCYTCDGTNGC-3') for the 1st-round

and CCT-262F (5'-CARGATGAYGARGTKGGDGATGGWACBAC-3') and Pircct-665R (5'-AABGTYTTYTTRAANGCHACDCCYTT-3') for the 2nd-round PCR that enabled us to detect partial *CCTη* sequences of *Babesia* (Nakajima et al., 2009). Resulting PCR amplicons of about 350 bp were purified and were cloned into a plasmid vector. In total, ten plasmed clones were picked randomly and sequenced subsequently. We included only sequences present in two or more plasmid clones with identical sequence. While sequences detectable as single plasmid clones were omitted as being potentially either polymerase errors, or indistinguishable from polymerase errors , they,nonetheless actually existed as rare genotypes within a mixed infection (Speksnijder et al., 2001).

*2.3. Phylogenetic analysis*

The sequences were aligned with the program Clustal W Alignment as described previously (Kawabuchi et al., 2005). Phylogenetic analyses of the SSU rDNA and *CCTη* gene sequences were performed using the neighbor-joining method with the program Phylogenetic Analysis in the Mac Vector software package, version 8.0 (Genetic Computer Group Inc., Madison, Wis, USA). Support for tree nodes was calculated with 1,000 bootstrap replicates by use of the bootstrap tree algorithm.

*2.4. Nucleotide sequence accession number*

The GenBank accession numbers of the reference strains used for SSU rDNA sequence analyses were, respectively, as follows; *Cryptosporidium muris*, AY642591; *Babesia rodhaini*, AB049999; *B. microti* from raccoons in Japan, AB197940; *B. microti*, U09833; *T. parva*, L02366; *T. sergenti* Ikeda, AB000271; *T. equi*, EU642511; *Cytauxzoon manul*, AY485690; *C. felis*, AY531524;

131 *B. caballi*, Z15104;*B. bovis*, ATTXT01000001; *B. major*, AY603399; *B. ovata*, AY603401; *B.*
132 *bigemina*, X59604; *B. canis rossi*, DQ111760; *B. canis canis*, AY072926; *B. canis vogeli*, AY07295;
133 *B. gibsoni* 'Oklahoma dog', AF205636; *B. gibsoni* Asia1, AF175300; *B.gibsoni* 'Okinawa dog',
134 AF271082; *Babesia* sp. AJB2006, DQ028958; *Babesia* sp. Akita 610, AY190123; *B. divergens*,
135 U07885; *B. odocoilei*, U16369. The *CCTη* sequences used to analysis and their GenBank accession
136 numbers were respectively as follows; *B. bovis*, AB367921; *B. canis vogeli*, AB367922; *B. caballi*,
137 AB367927; *B. major*, AB36726; *B. ovata*, AB367928; *B. divergens*, AB367925; *B. odocoilei*,
138 AB367924; *B. gibsoni*. AB367923. The *CCTη* gene of *T. parva* and *B. bigemina* were obtained from
139 the Institute for Genomic Reserch (TIGER) website (http://www.tiger.org), and from the Sanger
140 Institute website (http://www.sanger.ac.uk), respectively. The SSU rDNA and the CCT*η* sequence
141 data generated in the present study were submitted to DNA Data Bank of Japan and were given
142 accession numbers as follows; the SSU rDNA sequences of *Babesia* sp. UR1 and *Cytauxzoon* sp.
143 were AB480557 and AB480558, respectively, and the three *CCTη* sequences, representative of the
144 separate plasmid clones, of *Babesia* sp. UR1 were AB497048, AB497049 and AB497050.
145

## 3. Results

### *3.1. Molecular identification of parasites*

From a frozen-stored blood clot of a wild bear cub, two piroplasma specific PCR bands were successfully amplified (Fig. 1). PCR amplicons were purified and were cloned into plasmid vectors. Cloned DNA sequencing with subsequent alignment on each set of homologous SSU-rDNA sequences allowed us to construct two different consensus sequences corresponding to each of the two PCR bands. The two SSU rDNA sequences determined were 1565 bp (GenBank:AB480557) and 1709 bp (GenBank:AB480558), respectively. The shorter sequence exhibited a moderate degree of sequence similarities with *B. gibsoni* Asia1 (GenBank:AF175300, 95.9% identity) and with *B. canis canis* (GenBank:AY072926, 94.1% identity) . The latter sequence, however, shared only limited homologies to the closest known piroplasma species (90.1% and 90.2% of identities with *Cytauxzoon felis* and *Cytauxzoon manul*, respectively) . Phylogenetically, one babesial SSU rDNA can be regarded as "new" assemblage ( designates as *Babesia* sp. UR1) occupying alone branch separate from the dog parasites of *B. gibsoni* and of *B. canis* (Fig. 2). The other parasite branched nearly at the base of the genus *Cytauxzoon* (provisionally designates as *Cytauxzoon* sp.) (Fig. 2).

### *3.2. Infection of a single bear cub with three distinct* Babesia *genotypes*

Mixed infections of a single mammalian host by multiple strains of the same *Babesia* species are common in highly endemic area of the parasite infection (Arai et al., 1998). This is potentially of importance since vector ticks could pick up multiple genotypes that could mate and recombine.

168 Thus, we carried out another nested PCR to detect *CCTη* gene this allowing for more precise
169 assignment of genotypes of bear *Babesia* (Nakajima et al., 2009). Of the ten randomly selected
170 plasmid clones, three sequences were present in two or more plasmid clones with identical sequence .
171 Primary sequence comparisons between each pairs of these three sequences recovered only slight
172 differences from 0.3 to 3.4% (1 to 12 sequence alterations in 350 bp). All these three *CCTη* sequences
173 formed a well-supported single clade distinctly separated from the nearest relative of *B. gibsoni* (Fig.
174 3), indicating that phylogenetically a single group of babesial parasite () proven at a detectable level
175 by PCR, in triplicate (i.e. one each for the three sequevars), to have multiplied in the bear cub.

## 4. Discussion

Using molecular methodology, we searched for the presence of piroplasma in a frozen-stored blood clot of a wild Hokkaido brown bear cub. Two nearly full-length SSU rDNA sequences of 1565 bp and 1709 bp, respectively, were determined. Phylogenetically, the shorter DNA sequence was allocated to the genus *Babesia.* Sequence divergences between this parasite and each the closest published strains in separate clades (4.1% and 5.9% differences to *B. gibsoni* and to *B. canis,* respectively) were almost equivalent to those seen between pairs of well-recognized species (2.8% difference between *B. odocoilei* and *B. divergence*). While the other sequence, a deeply branched novel phylotype could be assigned in the genus *Cytauxzoon* (*Cytauxzoon* sp.), it was clearly different from the closest known *Cytauxzoon* species (90.1% and 90.2% identities with *C. felis* and *C. manul,* respectively) (Fig. 2). These results indicate that the two yet unnamed piroplasma parasites (*Babesia* sp. UR1 and *Cytauxzoon* sp.) were infected in our bear cub. To predict whether the bear *Babesia* (*Babesia* sp. UR1) was endemic in the area where the bear was captured or whether it was detected only accidentally, we analyzed this bear infection using *CCTη* gene phylogeny, which would enable us to arrive at a more precise characterization of *Babeisa* parasites. The results provided evidence for the presence of multiple *Babesia* strains within a single phylogenetic group in a single bear (Fig. 3), suggesting that *Babesia* sp. UR1 may have been the endemic species in Hokkaido, Japan.

It is becoming increasingly obvious that protozoan infections particularly in areas where the disease is highly endemic frequently contain multiple genotypes of the same parasite species in man and animals (Anderson et al., 2000, Arai et al., 1998, Balmer et al., 2008, Bruce et al., 2007, Susomboon et al., 2008, Villena et al., 2004). Although the relative importance of the two reproductive mechanisms, selfing and out-crossing, can vary within and among parasitic species in different

199 regions and epidemiological settings (Annan et al., 2007, Mzilahowa et al., 2007, Mzilahowa et al.,
200 2007), co-existence of multiple genotypes can, theoretically, be an advantage of parasites with
201 mating-type bias. Presumably, it would allow the opportunity for recombination among those strains
202 carrying beneficial genetic differences such as, virulence, transmission, immune induction and drug
203 resistance. Concurrent detection of three different sequevars that grouped with *CCTη* gene
204 (*CCTη*-genotypes) in the same monophyletic clade seems to be noteworthy, when taking into
205 account the parasites were recovered from a single bear cub. Evidence for the co-existence of three
206 different *CCTη*-genotypes of *Babesia* sp. UR1 within a single host, equivalent to a triplicate proof of
207 successful proliferation of a single species of parasite () in the brown bear (parasite must have
208 multiplied more than $2x10^6$ times in this 5.6 kg cub which have $2x10^{12}$ RBCs if the PCR-detection
209 limit is set at $10^{-6}$), indicates that brown bears are quite susceptible to infection with this *Babesia*
210 species. However, identification of piroplasma growth in bears does not distinguish whether bears
211 are accidental or reservoir hosts and, thus, further studies are needed before reaching a conclusion.
212 A heavy tick infestation (around 50 ticks) on the cub was noted by a veterinarian, which might have
213 occurred during the period from April to May, corresponding to the period from when the bear
214 emerged from hibernation to the day the cub was captured. We were given the opportunity to
215 examine 11 frozen-stored ticks (five *Ixodes* and six *Haemaphysalis* ticks) by nested PCR using the
216 above protocol and we successfully generated one consensus SSU rDNA sequence from a single
217 *Ixodes ovatus* tick (data not shown). The sequence of 1709 bp was identical to the *Cytauxzoon* sp. that
218 generated from the bear cub, suggesting that *I. ovatus* seems to be a possible candidate for the vector
219 of this new bear *Cytauxzoon* sp. However, presence of parasite DNA in ticks does not prove the
220 ability of arthropods to maintain and transmit parasites or confer epidemiologic importance, thus, the

vector competence of this and other tick species for this newly detected bear parasites, *Babesia* sp. UR1 and *Cytauxzoon* sp., requires further examination.

Piroplasms of wild animals appear to be incidental findings unrelated to disease excepting those fatal cases where conditions exist that reduce the host resistance following a change of environment and close confinement (Penzhorn, 2006). In the case of our bear cub found separated from his mother and exhausted, the anemia recorded by a veterinarian was conceivably caused not from the heavy tick infestation alone but from the piroplasma infection aggravated by stress factors. Additional epidemiological and experimental studies are needed to further clarify the clinical manifestations of the two newly identified two bear parasites, *Babesia* sp. UR1 and *Cytauxzoon* sp., though examination of such wild animals and, isolation and propagation of bear piroplasms of solid host specificity will be challenging.

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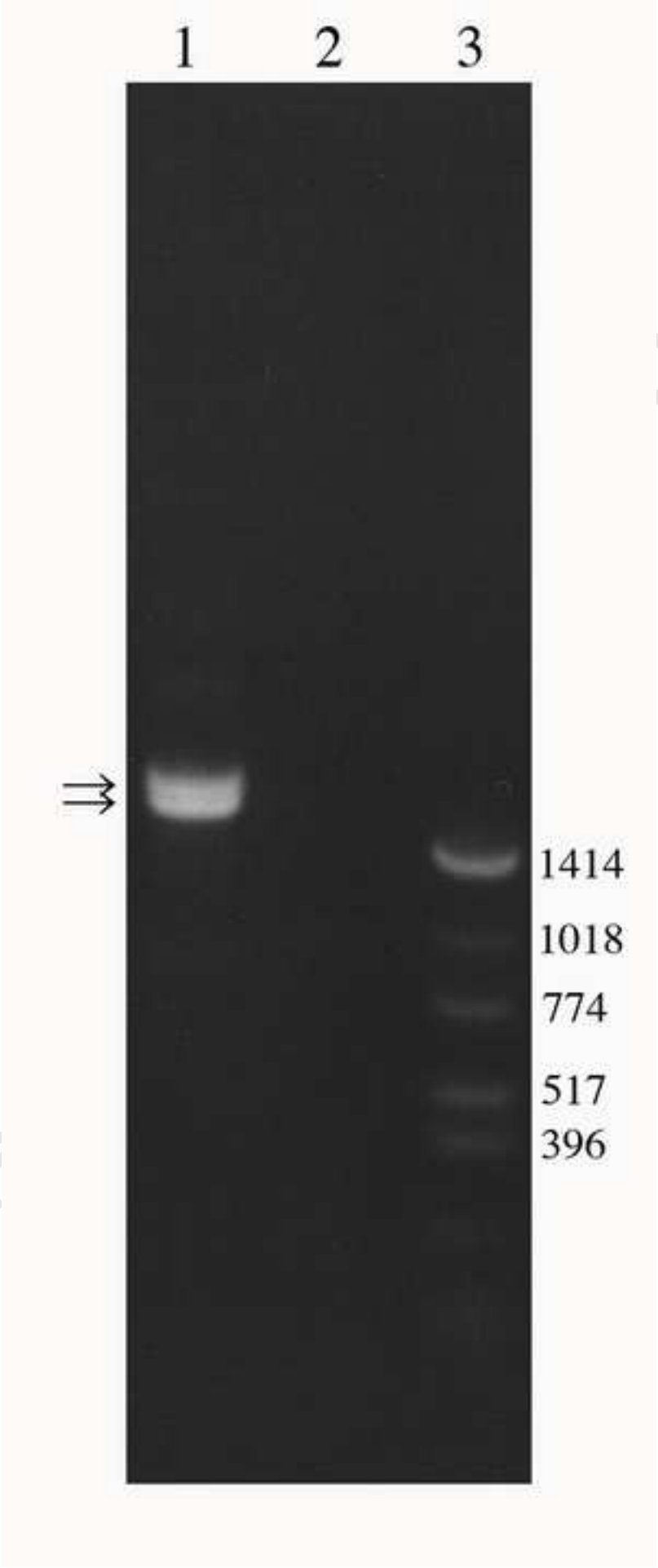

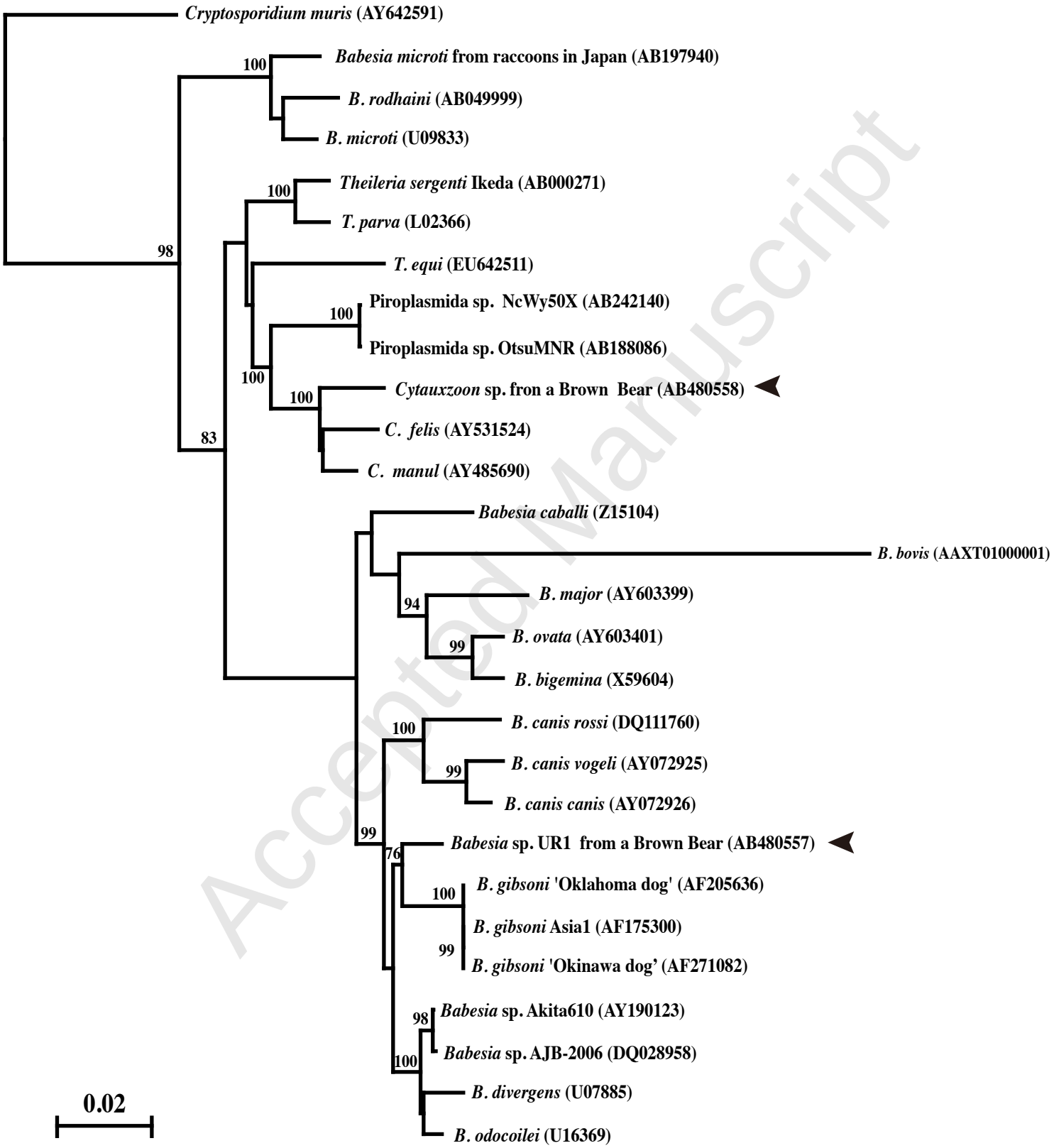

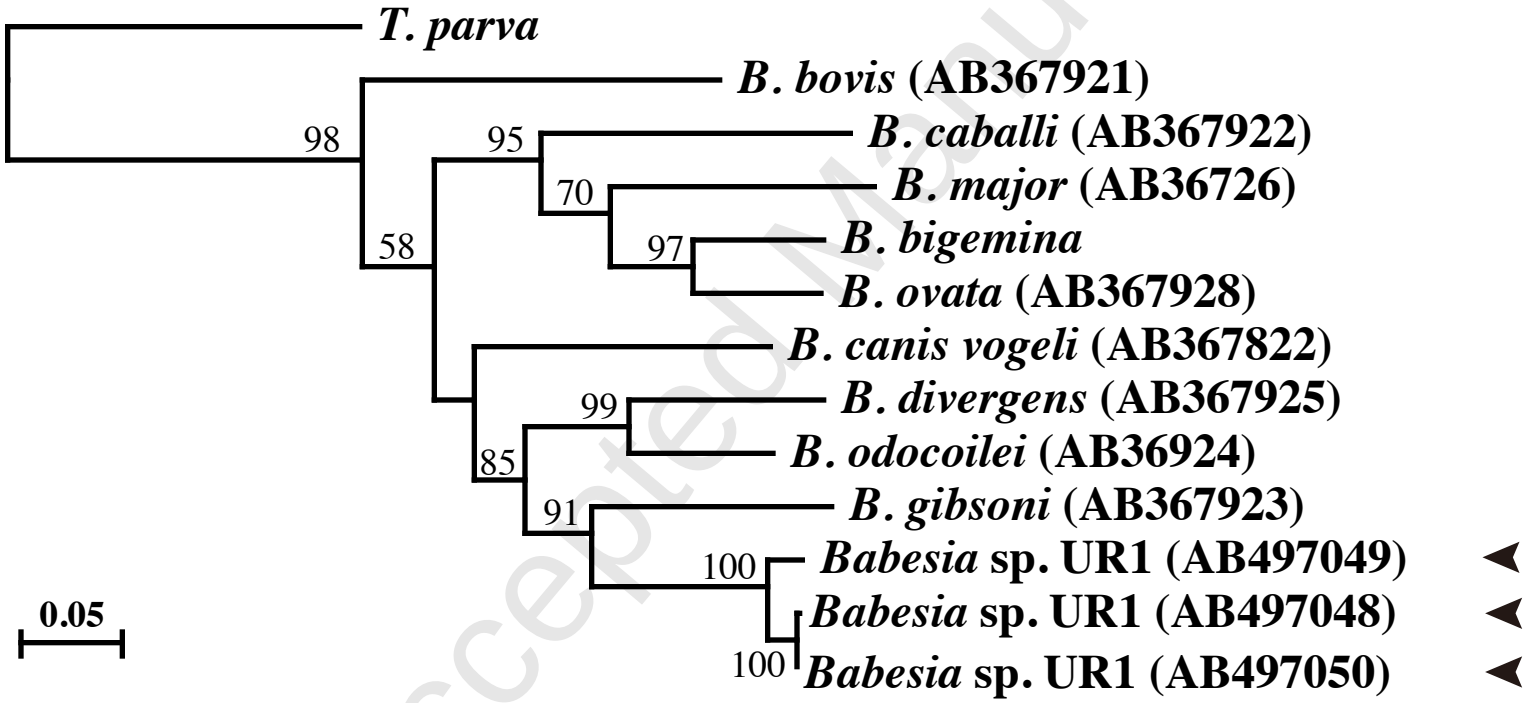

**Figure legends**

**Fig. 1**: Agarose gel showing two nested-PCR amplicons of piroplasma SSU-rDNA. PCR-products were visualized by ethidium-bromide stainining. Lane 1, two PCR amplicons (marked with arrows) from the blood clot of a dead bear cub; lane 2, PCR control without template; lanes 3, ladder molecular weight markers.

**Fig. 2**: Phylogenetic trees derived from SSU-rDNA. The tree was constructed by the neighbor-joining method. Numbers at the nodes indicate bootstrap support from 1000 replications. *Cryptosporidium muris* was used as outgroup. The scale bar represents 0.02 substitutions per site. The arrowhead indicates the SSU rDNA sequence originated from this study.

**Fig. 3**: Phylogenetic trees derived from CCT$\eta$ nucleotide sequences. The tree was constructed by the neighbor-joining method. Numbers at the nodes indicate bootstrap support from 1000 replications. *Theileria parva* was used as outgroup. The scale bar represents 0.05 substitutions per site. The arrowhead indicates the CCT$\eta$ sequence originated from this study.