NOTE *Parasitology*

Babesia gibsoni internal transcribed spacer 1 region is highly conserved amongst isolates from dogs across Japan

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ABSTRACT. *Babesia gibsoni* is a tick-borne apicomplexan parasite of dogs that often causes fever and hemolytic anemia with highly variable clinical outcome. In this study, we sequenced the 254bp Internal Transcribed Spacer 1 region (ITS1) of 54 *B. gibsoni* isolates from 14 different geographical regions of Japan. The 54 isolates shared high sequence identity with each other and with *B. gibsoni* isolates reported in GenBank database (97.2–100%). Consistent with previous reports, phylogenetic analysis showed that *B. gibsoni* isolates from Japan formed the same clade with those from U.S.A., Australia, India and Taiwan. Our finding indicates that *B. gibsoni* ITS1 region is highly conserved among isolates from dogs in Japan, making it a useful genetic marker for molecular epidemiology of the parasite.
KEY WORDS: *Babesia gibsoni*, conserved gene, internal transcribed spacer 1 (ITS1), Japan

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Babesia gibsoni is a tick-borne intraerythrocytic protozoan parasite that causes piroplasmosis of dogs [2]. During the asexual stage in its natural hosts, *B. gibsoni* causes serious clinical signs, including remittent fever, progressive anemia, hemoglobinuria, marked splenomegaly and hepatomegaly leading to death [4]. *B. gibsoni* is mainly distributed in Asia, including Japan, Korea, Taiwan and Malaysia [7, 10, 11, 13]. Recently, *B. gibsoni* has also been reported in Bangladesh and India [1, 16].

The internal transcribed spacer 1 (ITS1) region located between the repeating array of the nuclear 18S, 5.8S and 28S rRNA genes demonstrates marked divergence among species and even among strains of the same species [5]. This has made the 18S rRNA, which is one of the genes having ITS1 region, to be widely used for the establishment of phylogenetic relationships and for the differentiation of the genotypes or subspecies of canine *Babesia* [9]. The rRNA genes, which have the ITS1 regions, are versatile genetic markers and have been used for phylogenetic analysis, evaluation of the evolutionary process and for the determination of taxonomic identities [6, 14]. Previous studies revealed that the ITS1 gene is highly conserved amongst *B. gibsoni* isolate from Australia, India, Japan, Taiwan and U.S.A. [3, 12]. However, Bostrom *et al*. [3] hypothesized that the single *B. gibsoni* isolate from Japan used in the study was not conclusive to rule out possible genetic variability of the ITS1 gene. Based on the above research gap, we investigated the genetic diversity of ITS1 gene in 54 *B. gibsoni* isolates from 14 different geographical regions of Japan.

Fifty-four blood samples (1 from Hokkaido, 1 from Oita, 1 from Shiga, 2 from Fukuoka, 2 from Kyoto, 2 from Mie, 3 from Kochi, 3 from Okayama, 3 from Yamaguchi, 4 from Hiroshima, 5 from Kagawa, 6 from Hyogo, 10 from Osaka and 11 from Wakayama) were collected from domestic dogs with clinical signs consistent with *B. gibsoni* infection from 14 different geographical regions in Japan between 2011 and 2014. All samples were provided by a commercial laboratory (Marupi Lifetech Co., Ltd., Osaka, Japan). The blood samples were drawn from the jugular veins of the dogs into vacutainer tubes containing an anticoagulant. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, GmbH, Hilden, Germany), following manufacturer's protocol and stored at −30°C until future use.

The *B. gibsoni* published primer set [12], BgITS1-F and BgITS-R, were used in this study to amplify the partial sequence of ITS1 region (254 bp) [12]. All the amplifications were performed in a 20 μl reaction mixture, which contained 2 μl of 30–100 $ng/\mu l$ template DNA, 10 pmol of each primer and 250 μM of each deoxynucleotide triphosphate, 2 μl of 10 ×Ex Taq buffer and 1 unit of Ex Taq DNA polymerase (Takara Bio, Kyoto, Japan). The thermal cycling condition was as described previously [12] using MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, U.S.A.). The amplified PCR product was checked by electrophoresis on 1.5% agarose gel. The resultant PCR products were purified with QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instruction. The purified PCR products were sequenced with BigDye v3.1 Terminator Cycle Sequencing Kit and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The BgITS1-F and BgITS-R primers were used for sequencing reactions. Each fragment was sequenced at least twice to reduce possibility of sequencing artifacts. The resultant nucleotide sequence was analyzed

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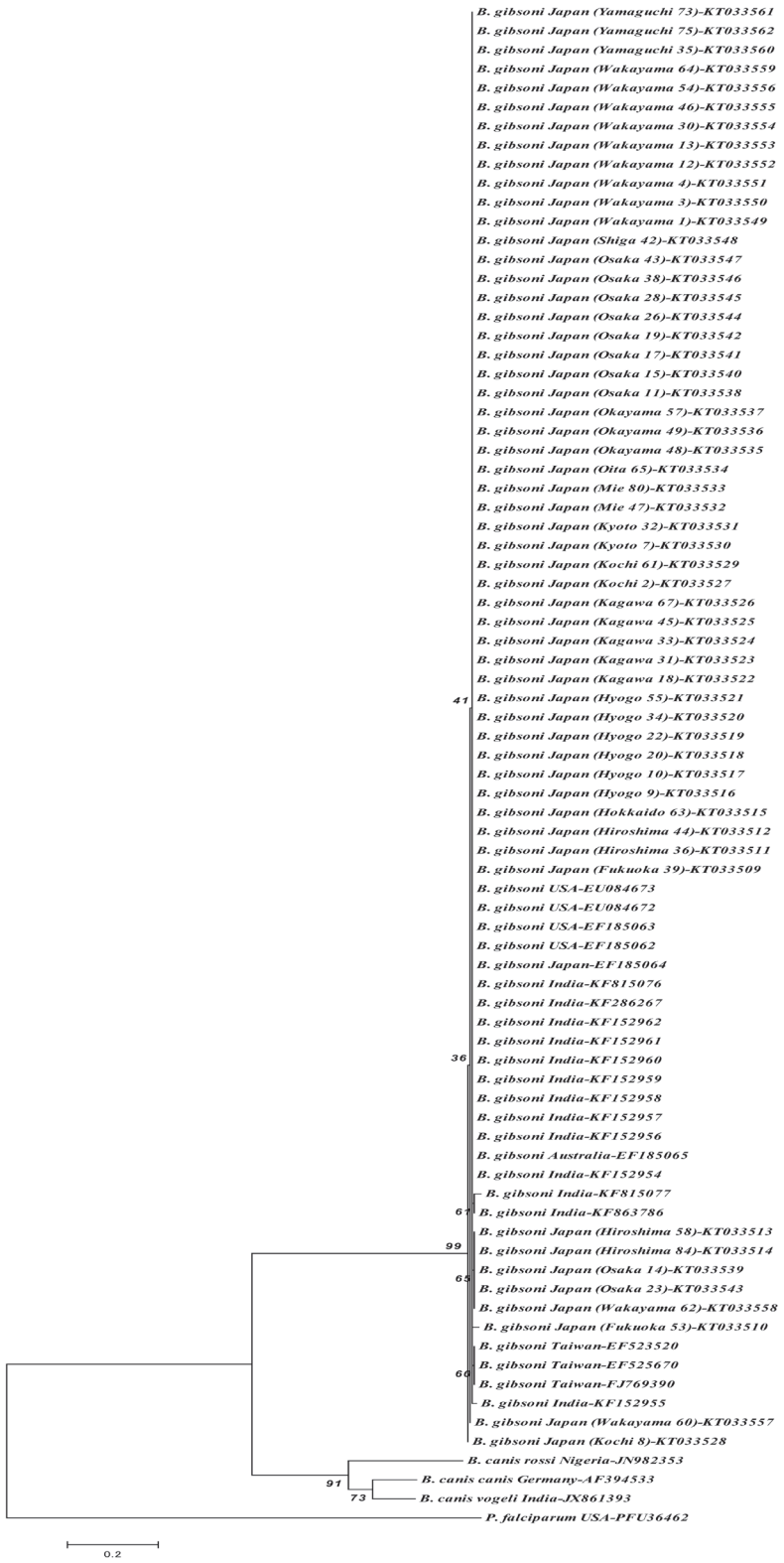

Fig. 1. Phylogenetic tree based on ITS1 region of *Babesia* isolates using Maximum Likelihood method (Kimura-two-parameter model). The percentage of replicate trees in which the associated species clustered together in the bootstrap method (1,000 replicates) is shown next to the branches. The *Plasmodium falciparum* ITS1 gene (PFU36462) was used as outgroup.

by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and percentage identity was determined. A phylogenetic analysis was done using MEGA5.0 software employing the Maximum Likelihood method (Kimura-two-parameter model) and bootstrap values [8, 15]. This analysis was determined for 1,000 replicates of the data sets. The *P. falciparum* ITS1 gene (PFU36462) was used as out group species to root the tree.

All the procedures were carried out according to ethical guidelines for the use of animal samples permitted by Obihiro University of Agriculture and Veterinary Medicine (Permit for animal experiment: 201109–7; DNA experiment: 1211–3).

A total of 54 nucleotide sequences were generated in the current study and deposited in the GenBank under accession numbers: KT033509-KT033562. BLAST analysis revealed that the nucleotide sequences of the 54 isolates were 97.2–100% identical with published sequences of *B. gibsoni* reported in GenBank database [3, 12]. A total of one to three nucleotide substitutions were observed in one or two isolates from each of the following areas; Fukuoka (n=1), Hiroshima (n=2), Kochi (n=1), Osaka (n=2) and Wakayama (n=2). However, phylogenetic analysis revealed that all the *B. gibsoni* genotypes formed a separate clade with high bootstrap support (99%) (Fig. 1). Within this clade, the Japan isolates were found interspersed with isolates from Australia, India, Taiwan and U.S.A. This finding is consistent with previous studies [3, 12]. This further supports the proposition of the clonal expansion of a single strain of *B. gibsoni* across territories [3, 12]. However, the large forms of *Babeisa* of dogs (*B. canis canis*, *B. canis rossi* and *B. canis vogeli*) formed separate clades away from *B. gibsoni* clade. This further supports the importance of the ITS1 gene as a molecular marker for differentiation of *B. gibsoni* from other babesia species [12]. In the present study, the sequences of 54 isolates of *B. gibsoni* from different geographical regions of Japan revealed that the ITS1 region was highly conserved with miniature intraspecific variations. Overall, our finding is a useful addition to the body of knowledge on conservation of the ITS1 gene in *B. gibsoni* and its importance in molecular phylogenetic studies [14].

In conclusion, this study revealed that the ITS1 region of *B. gibsoni* isolates from dogs in the different geographical location of Japan was highly conserved further clarifying previous hypothesis that it may be genetically diverse in Japan. This also consolidates its usefulness as a genetic marker for molecular epidemiology of *B. gibsoni* infection in dogs.

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