First molecular detection of tick-borne pathogens in dogs from Jiangxi, China

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ABSTRACT. In this study, blood samples obtained from 162 dogs in Jiangxi, China, were employed in molecular screening of canine tick-borne pathogens by PCR and sequencing. *Babesia* spp. gene fragment was detected in 12 (7.41%) dogs. All samples were negative for *Hepatozoon* spp., *Ehrlichia canis*, *Coxiella* spp., *Borrelia* spp., *Rickettsia* spp. and *Anaplasma platys*. Species-specific PCR analysis further confirmed that 8 (4.94%) and 4 (2.47%) dogs were infected by *Babesia canis vogeli* and *Babesia gibsoni*, respectively. Based on our analyses, *Babesia* spp. infection in Jiangxi appeared not related to age, gender, breed, usage, activity and health status or tick infestation history of the dogs. This is the first molecular report of *Babesia canis vogeli* and *Babesia gibsoni* in dogs from Jiangxi, China.

KEY WORDS: *Babesia* spp., dog, Jiangxi, tick-borne pathogen

Many tick-borne protozoan and bacterial agents are significant causes of morbidity and mortality in domestic dogs and potentially of great public health importance. To date, three protozoan (*Theileria*, *Babesia* and *Hepatozoon*) and five bacterial (*Anaplasma*, *Ehrlichia*, *Rickettsia*, *Coxiella* and *Bartonella*) genera have been reported in domestic dogs around the globe [2–5, 9, 17, 18, 21, 25, 37]. These pathogens infect blood cells and cause thrombocytopenia, anemia, leukopenia and organ damage. Clinical manifestation mainly includes fever, anorexia and weight loss [10, 11]. Furthermore, subclinically infected companion dogs are considered to be a reservoir or carrier for human tick-transmitted infectious agents, such as *Borrelia burgdorferi*, *Rickettsia conorii*, *R. rickettsii*, *Ehrlichia chaffeensis*, *E. ewingii*, *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Francisella tularensis* and *Babesia microti* [1, 7, 8, 10, 21, 26, 27, 30].

The current dog population in China is estimated to be between 150 and 200 million [22]. However, until 2015, very few data were available on tick-borne infections in dogs. In 2015, Xu *et al.* reported a prevalence of tick-borne pathogens in ten provinces of China, including Xinjiang, Gansu, Shaanxi, Inner Mongolia, Beijing, Henan, Jiangsu, Shanghai, Guangdong and Yunnan. This study, in combination with previous investigations, evidenced the existence of *Dirofilaria immitis*, *Babesia canis vogeli*, *B. gibsoni*, *Ehrlichia canis*, *Hepatozoon canis* and *Theileria orientalis* infections among the Chinese dog population [16, 29, 31, 36]. In addition, a large number of ticks, belonging to the genera *Rhipicephalus*, *Haemaphysalis* and *Ixodes,* were collected from dogs and detected positive for *B. canis vogeli*, *B. gibsoni*, *B. microti*, *Rickettsia* spp., *Anaplasma platys* and *A. phagocytophilum* infections [29, 34–36]. Meanwhile, the seroprevalence of *B. gibsoni* in different types of dogs in East China including Jiangxi, Fujian, Anhui, Jiangsu, Zhejiang, Shanghai and Shandong was assessed in the same year [6]. Jiangxi, located in the mid-eastern part of China, has a bio-environment that remarkably facilitates tick development and tick-borne pathogen transmission. However, data on tick-borne infections in this area are limited, and therefore, this study aimed to achieve a better understanding of these etiological agents. Molecular techniques were used to examine and characterize various tick-borne pathogens in dogs from Jiangxi province.

The current dog population in Jiangxi province is estimated to be between 5 and 6 millions. Most of them comprise pet, police and guard dogs. These dogs are mainly distributed in communities, villages, pet dog breeding centers and a police dog base. In our study, 18 samples collected from a community and 25 samples collected in a pet dog breeding center were obtained in
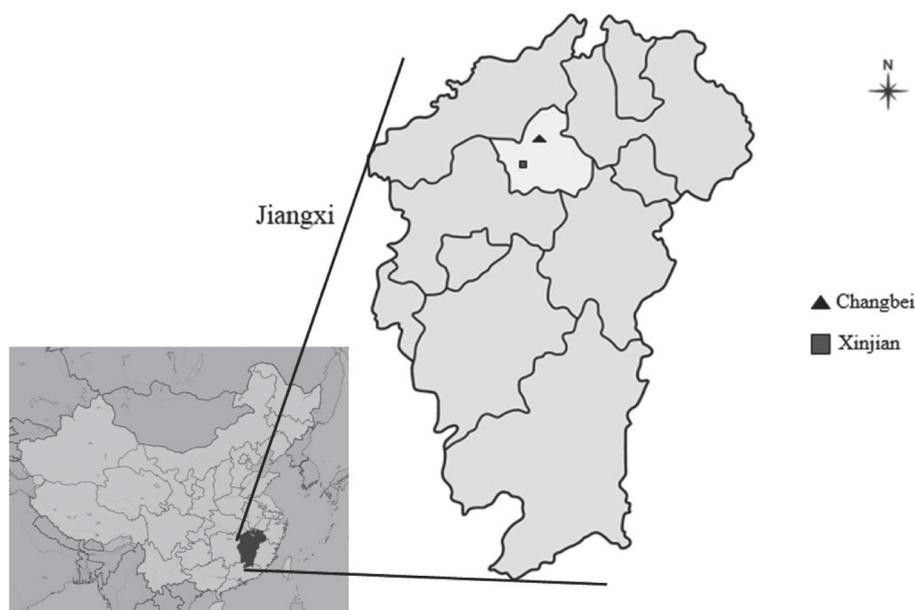
Fig. 1. Geographical location of Changbei and Xinjian where dog blood samples were collected. Legends indicate the location of sampling sites. ■, all samples collected from police dog base or veterinary hospital. ▲, samples obtained from pet dog owners or in pet dog breeding center.

Changbei district. Meanwhile, 40 samples were collected in a veterinary hospital and 79 samples in the police dog base in Xinjian district (Fig. 1). Blood sampling was conducted between March and April 2016 during the peak period of tick infestation in dogs. Approximately 5 m*l* aliquot of blood was collected from the cephalic vein by using sterile vacutainer tubes with EDTA. 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 (Animal experiment access num: 28–100; DNA experiment approval num:1219–3).

The majority of sampled dogs had high risk of exposure to tick infestation. During dog blood collection, we observed some ticks attaching themselves to dogs. Most (78.40%) of the dogs were adult, and 46.30% were female. The study samples included mainly German shepherd (20.37%), Rotterman (12.96%), Samoyed (8.64%), Golden Retriever (8.02%), Rottweiler (7.41%) and Springer (6.79%). The other breeds altogether represented 35.81% of the samples. The majority (63.58%) of sampled dogs were reported as having "absence of previous tick infestation", while 36.42% had been infested by ticks previously. A large number of dogs (73.46%) lived or worked outside. Compared to the 75.31% of healthy dogs, 24.69% of the dogs were diseased with leptospirosis, back leg twitches, fungi infection, ascites and /or cough.

Genomic DNA was extracted from a volume of $200\mu l$ of whole blood using the QIAamp® DNA blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendation. DNA concentrations and purities were determined by measuring the absorbance at 230 nm, 260 nm and 280 nm with a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, U.S.A.).

Obtained DNA samples were then used in PCR amplification in combination with DNA sequencing for detection of canine tick-borne pathogens. Previously developed PCR assays based on 18S rRNA, 16S rRNA, thrombospondin-related adhesive protein (*TRAP*) gene, *gltA*, *groESL* and *fla* [12–15, 19, 20, 24, 33] were carried out for the detection of *Babesia* spp., *Babesia canis, B. gibsoni*, *E. canis*, *Hepatozoon* spp., *A. platys*, *Rickettsia* spp., *Borrelia* spp. and *Coxiella* spp. All primers, expected size of the products and annealing temperatures are summarized in Table 1.

Babesia spp. were detected in 7.41% (12/162) dogs. Amplification of a 454 bp fragment of the 18S rRNA gene in the *Babesia* spp.-positive samples confirmed the presence of *B. canis vogeli* in 8 (4.94%) dogs. PCR reactions prepared using species-specific primers targeting *B. gibsoni* thrombospondin-related adhesive protein (*TRAP*) gene revealed 4 (2.47%) positive among the 12 samples containing *Babesia* spp. DNA. The percentage of *Babesia* spp. infections in our study is higher than reported earlier in Nanjing, but lower than that in Taixing, east China [36]. *B. gibsoni TRAP* sequences were amplified from 4 dog samples, corroborating previous report of sero-prevalence of this microorganism in pet dogs from Jiangxi [6]. The *B. gibsoni* infection rate in this study is almost similar to Luoyang (3.1%), Sanmenxia (3.81%) and Pingdingshan (3.54%), central China [29], but higher than Zhejiang (0.27%) and lower than Jiangsu (7.8%), east China [6]. However, the occurrence of *B. canis vogeli* infection in dogs is not well documented in China, with only two studies having reported this protozoan parasite in tick vectors collected from police dogs [35, 36].

Babesia canis and *B. gibsoni* cause some discomfort or even more severe clinical manifestations among dog populations. *B. canis vogeli,* like *B. canis canis* and *B. canis rossi*, belongs to the *B. canis* subspecies. It is a benign protozoan pathogen of dogs causing mild, often clinically inapparent manifestations, and sometimes thrombocytopenia [3]. *B. gibsoni* infection, however, is a real threat to canine health. The acute form of the disease in dogs is typically related to fever, anemia, thrombocytopenia,

Table 1. Sequences of oligonucleotides used for target gene PCR amplification

Pathogen	Target gene	Primer	Annealing temperature (°C)	PCR products size (bp)	Reference
Babesia spp.	18S rRNA	F: GCATTTAGCGATGGACCATTCAAG	60	209	[19]
		R: CCTGTATTGTTATTTCTTGTCACTACCTC			
Babesia canis	18S rRNA	F: GTTTATTAGTTTGAAACCCGC	59	456	[15]
		R: GAACTCGAAAAAGCCAAACGA			
Babesia gibsoni	*TRAP*	F: AAGCCAACATCAAGGAAAGC	58	679	This study
		R: TTCTGGTATGCGGCAGTGTA			
Ehrlichia canis	*gltA*	F: TTATCTGTTTGTGTTATATAAGC	53	1,372	[12]
		R: CAGTACCTATGCATATCAATCC			
Hepatozoon spp.	18S rRNA	F: ATACATGAGCAAAATCTCAAC	57	666	[14]
		R: CTTATTATTCCATGCTGCAG			
Anaplasma platys	*groESL*	F: AAGGCGAAAGAAGCAGTCTTA	58	724	[13]
		R: CATAGTCTGAAGTGGAGGAC			
Rickettsia spp.	*gltA*	F: GCAAGTATCGGTGAGGATGTAAT	50	401	[20]
		R: GCTTCCTTAAAAATTCAATAAATCAGGAT			
Borrelia spp.	*fla*	F: ACATATTCAGATGCAGACAGAGGT	60	665	[33]
		R: GCAATCATAGCCATTGCAGATTGT			
Coxiella spp.	16S rRNA	F: ATTGAAGAGTTTGATTCTGG	48[a)]	1,457	[24]
		R: CGGCTTCCCGAAGGTTAG			

a), To minimize nonspecific amplification, a so-called touchdown PCR program was used: 3 min at 95°C; this was followed by two cycles of 30 sec at 95°C, 30 sec at 58°C and 2 min at 72°C, and then, two cycles identical to the previous two cycles, but with an annealing temperature of 58°C; after every following two cycles, the annealing temperature was lowered by 2°C until it reached 50°C. Then, an additional 30 cycles of 30 sec at 95°C, 30 sec at 48°C and 2 min at 72°C were followed by the touchdown PCR program.

splenomagaly and hepatomegaly [23].

Rhipicephalus sanguineus, *R. haemaphysoides*. *Haemaphysalis longicornis*, *Dermacentor variabilis* and *Ixodes ricinus* are major vectors of *Babesia* species of dog around the globe [7, 8, 28–30]. In China, *R. sanguineus* is the dominant tick species among dog populations, although sometimes *R. haemaphysoides* and *H. longicornis* can be seen on some dogs [7, 29, 35]. In the sampling sites of this study, *R. sanguineus* ticks are frequent during the tick active season which ranges from March to June. Both *B. canis vogeli* and *B. gibsoni* were found to infect *R. sanguineus*, *H. longicornis* and *R. haemaphysaloides* collected on dogs around China [7, 29, 35]. However, a previous study performed by Wang *et al.* (2013) at the same place to our sampling sites did not detect *Babesia* spp. in *R. sanguineus* collected from police dogs. Other tick species, including *Haemaphysalis campanulata* and *H. verticalis*, which were collected from police dogs, were also tested negative for *Babesia* spp. infection [35]. Our detection of *Babesia* spp. DNA in dog blood contradicted the absence of *Babesia* spp. in ticks and suggests that ticks might not play a pivotal role in the transmission. Our hypothesis is partially supported by the evidence that *B. gibsoni* infection can be transmitted not only through tick bites, but also by dog bites and blood transfusion, as well as from mother to infant [23, 30].

Tick-borne *Babesia* spp. are related to detailed exposure variables, such as frequency of contact between animal and parasite, animal or human population, distribution of tick vectors, tick control measures and human behavior/animal management system. Exposure variable analyses revealed that *Babesia* spp. infection in the dogs was not significantly associated ($P > 0.05$) with factors, such as age, gender, breed, dog use, previous history of tick infestation, dog activity or health status. These data support a previous survey in Australia that indicated that breed is not related to tick infection, as opposed to the pivotal role of age and ecto-parasite infestation history in the transmission of these infections [11]. Previous history of tick infestation in this study was reported by police dog trainers, pet dog owners or keepers during sample collection, and therefore, the reality and accuracy of the information were actually unverifiable.

Despite the fact that *E. canis*, *A. platys*, *Rickettsia* spp., *Coxiella* spp., *Borrelia* spp. and *H. canis* have been detected in blood samples collected from dogs [3, 4, 18, 21, 37], their presence was not identified in any of the samples of our study. A study conducted by Xu *et al.* in 2015 showed the presence of *E. canis* and *H. canis* in dogs from China. In detail, dogs positive for *H. canis* were examined in the north of Yangtze River, for example in Shaanxi, Henan, Beijing, Xinjiang and Jiangsu, and *E. canis* was also detected in the north of this river, including Jiangsu and Beijing. However, there were no infections with *H. canis* and *E. canis* in dogs from the south of Yangtze River [36]. Jiangxi is a province located in the south of Yangtze River, our finding is therefore consistent with the study of Xu *et al.* (2015). This result supports the thought that the dogs in the study area are not important reservoirs/carriers of the above etiological agents.

Babesia spp. (n=12), *B. canis* (n=8) and *B. gibsoni* (n=4) positive DNA samples were randomly selected for cloning and sequencing of amplicons. Phylogenetic analysis was performed using the sequences of this study and those reported from other regions. Both SeqMan and MEGA 7 programs were used to build the alignment of all selected sequences, and then, we constructed a neighbor-joining phylogenetic tree using MEGA 7 program. The confidence of internal branches was estimated by bootstrapping with 1,000 replications.
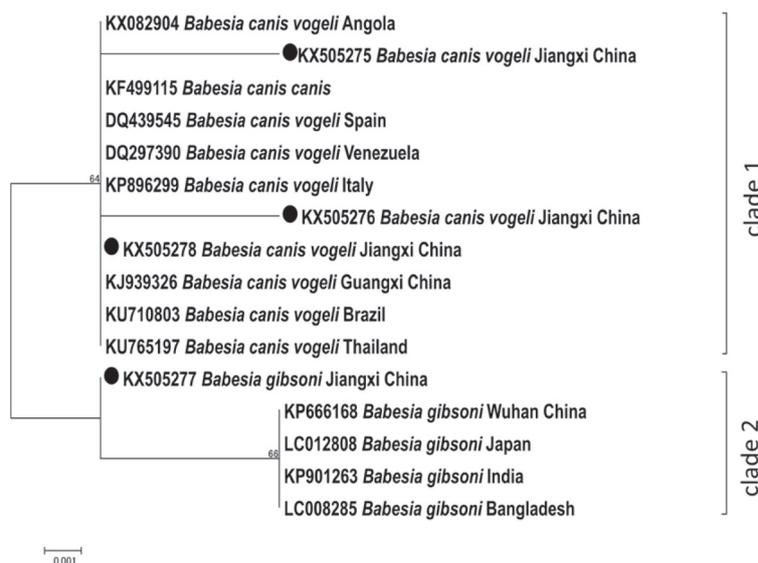
Fig. 2. Phylogenetic tree of *Babesia* spp. based on 209 bp 18S rRNA gene fragment.
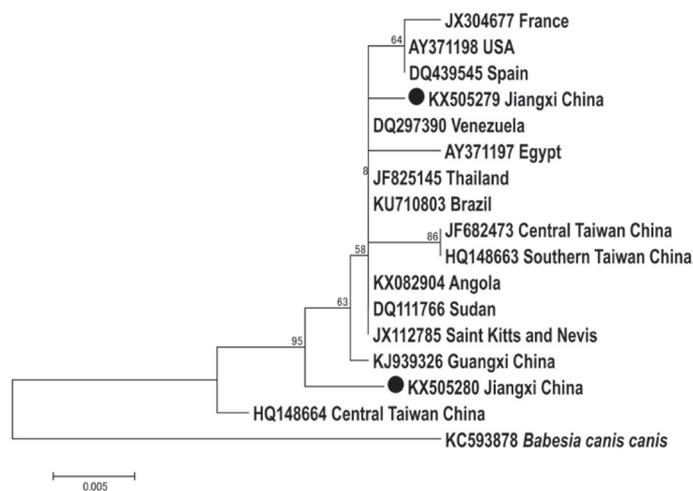● sequences obtained in this study.

Fig. 3. Phylogenetic tree of *Babesia canis vogeli* based on 456 bp fragment
of 18S rRNA gene. *B. canis canis* was employed as out group.

A 209 bp band was amplified with the primers targeting 18S rRNA fragment from *Babesia* spp. positive samples, while the species-specific primers yielded a 456 bp product for *B. canis* and a 679 bp product for *B. gibsoni*.

Babesia spp. 18S rRNA, *B. canis* 18S rRNA and *B. gibsoni TRAP* phylogenetic trees were constructed based on sequences generated in this study and selected sequences from GenBank database. The *Babesia* spp. tree (Fig. 2) found two major clades. Three sequences (KX505275, KX505276 and KX505278) in our study formed a clade (clade 1) with *B. canis vogeli* (DQ297390, DQ439545, KX082904 and KJ939326) and *B. canis canis* (KF499115), while the remaining sequence (KX505278) clustered with *B. gibsoni* sequences (KP666168, LC012808, KP901263 and LC008285). The sequences generated in the present study were highly (99.99%) conserved and showed 99.98–100% homology to those from other regions (countries).

Further phylogenetic analyses (Fig. 3) revealed that the *B. canis* gene sequences (KX505279 and KX50527980) obtained in this study were obviously different from *B. canis canis* (KC593878) and formed a clade with the *B. canis vogeli* sequences previously isolated in Europe (JX304677), Middle-east (AY371197), Africa (DQ111766), South America (KU710803), Guangxi in China (KJ939326) and Taiwan (HQ148664, JF682473 and HQ148663). Therefore, the isolates classified as *B. canis* in the previous dendogram are considered to be *B. canis vogeli*, not *B. canis canis*. The 679 bp fragments of *B. gibsoni TRAP* gene isolated in Jiangxi (KX528450 and KX528451) showed 99.99% similarity to each other and shared 99.98–99.99% homology to sequences deposited in GenBank. Jiangxi isolates formed a sub-cluster with the isolates from Japan (KR013043, AB478341, AB478342 and
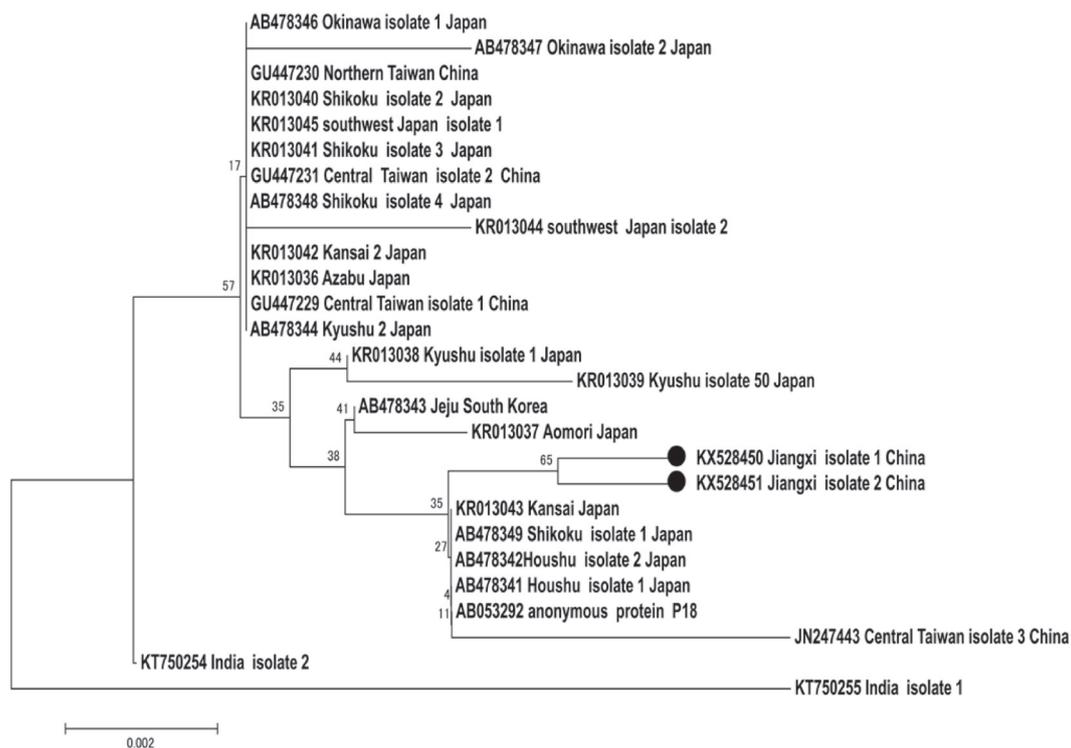
Fig. 4. Phylogenetic tree of *B. gibsoni* based on 679 bp fragment of TRAP gene.

AB478349) and China (JN247443) on the phylogenetic tree, far from the isolates from India (KT750254 and KT750255) (Fig. 4).

The nucleotide sequence of *B. gibsoni TRAP* in Jiangxi isolate 1 (KX528450) differed from the homologous sequences deposited in GenBank by 4 bps located at the positions 142, 360, 421 and 671. The *B. gibsoni* Jiangxi isolate 2 (KX528451) and isolate 1 (KX528450) were different at 2 nucleotide positions, namely, the nucleotides numbers 420 and 451 (Fig. 5).

Babesia spp. and *B. canis vogeli* in this study appear to be remarkably similar to previously described species with 99–100% homology to the reference sequences from the world. *B. gibsoni* TRAP (BgTRAP) gene encodes TRAP, a type-Itransmembrane protein and demonstrates abundant genetic diversity in Asian isolates [32]. However, fragments of the gene generated in dogs from Jiangxi shared 99.98–99.99% homology to the sequences deposited in GenBank and formed a sub-cluster with isolates from Japan and other regions of China.

This is the first molecular report documenting the existence of two canine tick-borne *Babesia* species in Jiangxi, mainland China. In the future, veterinary clinicians and dog administrators should take babesiosis into consideration in the prevention and treatment of canine diseases in this region.

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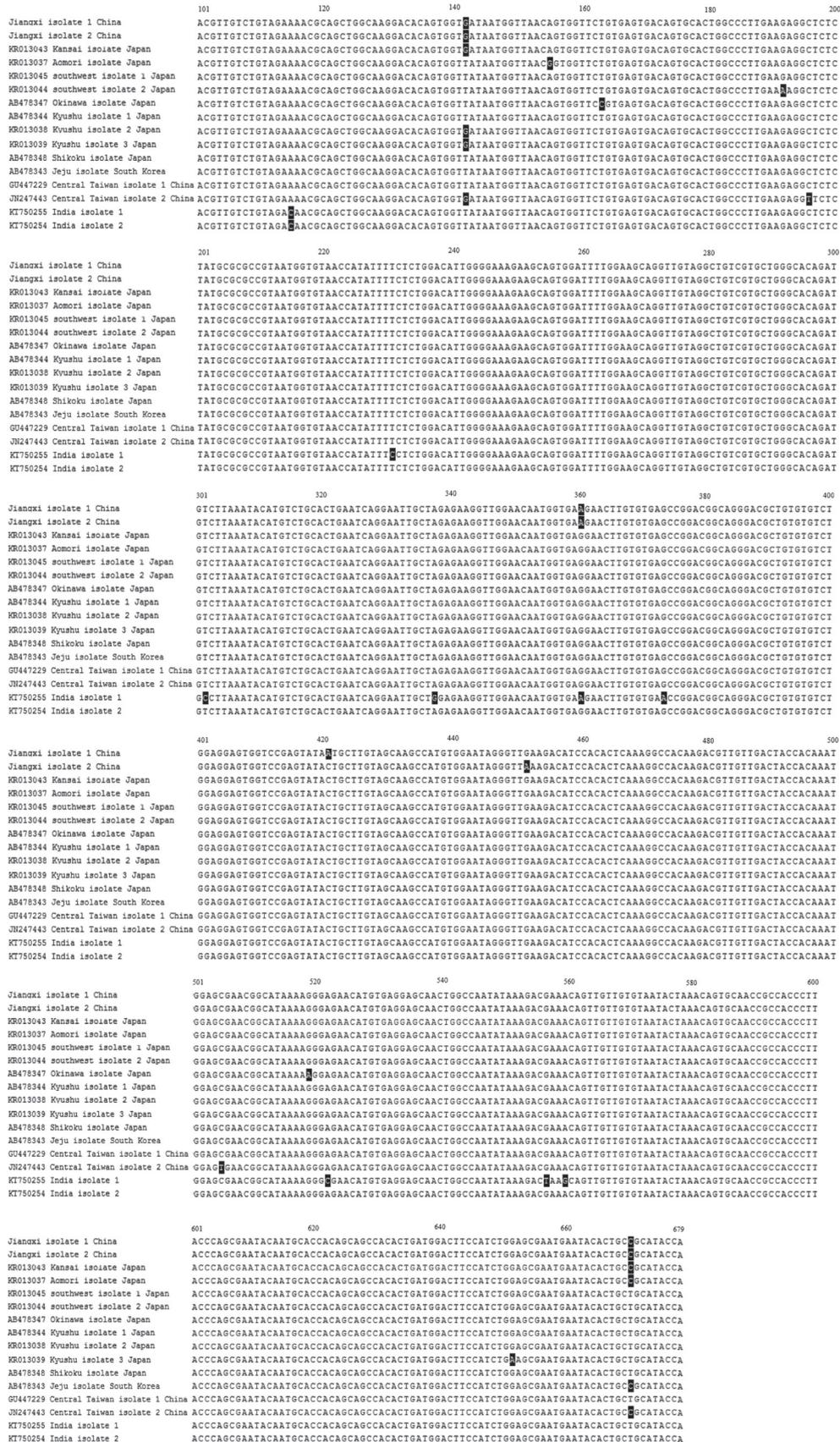
Fig. 5. Comparison of the partial 679 base-pair nucleotide sequences of *B. gibsoni* TRAP gene. Comparison between the nucleotide sequences of *Babesia gibsoni* TRAP gene from Jiangxi dog in this study with nucleotide sequences of *B. gibsoni* TRAP gene from Japan, South Korea and Taiwan, China. Dark rectangular box in each sequence demonstrates the variable bases at the corresponding positions compared with the bases in other sequences.

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