Phylogenetic analysis based on 28S rRNA of *Babesia* spp. in ruminants in China

Huitian Gou · Guiquan Guan · Miling Ma · Aihong Liu · Zhijie Liu · Qiaoyun Ren · Youquan Li · Jifei Yang · Ze Chen · Hong Yin · Jianxun Luo

Abstract Molecular phylogenetic analyses are mainly based on the small ribosomal RNA subunit (18S rRNA), internal transcribed spacer regions, and other molecular markers. We compared the phylogenetic relationships of *Babesia* spp. using large subunit ribosomal RNA, i.e., 28S rRNA, and the united 28S + 18S rRNA sequence fragments from 11 isolates of *Babesia* spp. collected in China. Due to sequence length and variability, the 28S rRNA gene contained more information than the 18S rRNA gene and could be used to elucidate the phlyogenetic relationships of *B. motasi*, *B. major*, and *B. bovis*. Thus, 28S rRNA is another candidate marker that can be used for the phylogenetic analysis of *Babesia* spp. However, the united fragment (28S + 18S) analysis provided better supported phylogenetic relationships than single genes for *Babesia* spp. in China.

Keywords *Babesia* · Large subunit ribosomal RNA (28S rRNA) · Phylogenetic analysis · Small ribosomal RNA subunit (18S rRNA)

Introduction

Babesiosis is a tick-borne disease that is sometimes fatal to ruminants. The etiologic agents of this disease are hemoprotozoa from the phylum Apicomplexa (genus *Babesia*), which infect a variety of domestic and wild animals, and the disease hinders livestock production in farming areas (Edelhofer et al. 2004; Teleord et al. 1993). Ixodid ticks are vectors that

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transmit *Babesia* spp., which feed on many different hosts (particularly ruminants). The causative agents of bovine babesiasis in China are *Babesia bigemina*, *B. bovis*, *B. ovata*, *B. major*, and *Babesia* U sp. Kashi. These parasites are transmitted by *Rhipicephalus microplus*, *Haemaphysalis punctata*, *Hyalomma anatolicum anatolicum* and other hard ticks (Guan et al. 2006; Luo et al. 2005a, b; Yin et al. 1996). Ovine babesiasis is caused mainly by *Babesia ovis*, *B. motasi*, and *Babesia* sp. Xinjiang in China. *Haemaphysalis punctata*, *Hae. qinghaiensis*, *Hae. longicornis*, and *H. a. anatolicum* are known to be vectors of ovine *Babesia* spp. (Guan et al. 2009, 2010; Niu et al. 2009).

Generally, the classic identification of piroplasms is based on their morphology, host specificity, the vector tick, transmission mode, and epidemiological data. DNA markers also play an important role in piroplasm phylogenetic analysis. The small subunit ribosomal RNA (18S rRNA) gene has been used in evolutionary studies of *Babesia* spp. for many years (Ahmed et al. 2006; Armstrong et al. 1998; Gozar and Bagnara 1993; Luo et al. 2005a, b). However, species diversity means that one DNA marker cannot reflect the phylogenetic relationships of species clearly. Thus, more gene targets have been introduced for the phylogenetic analysis of piroplasms, including internal transcribed spacers (ITS) (Liu et al. 2008; Niu et al. 2009) and the major piroplasm surface protein (MPSP) (Liu et al. 2010; Sarataphan et al. 1999). 28S rDNA is part of the rDNA gene complex, which occurs in tandem repeats arranged in ribosomal clusters in the genome (Long and Dawid 1980). Recently, various molecular phylogeny studies have focused on 28S rDNA (Hedin and Bond 2006; Subbotin et al. 2006; Winchell et al. 2004). However, phylogenetic analysis of *Babesia* using 28S rDNA has not been reported.

The current study compared the nuclear rRNA genes, consisting of 28S rRNA, 18S rRNA, and united 28S + 18S rRNA, with other markers to provide a greater understanding of the phylogenetic relationships of *Babesia* spp. in China.

Materials and methods

Parasites and animals

Eleven Chinese isolates of *Babesia* included six bovine *Babesia* (*B. bigemina* Kunming, *B. bovis* Shanxian, *B. ovata* Zhangjiachuan, *B. ovata* Lushi, *B. major* Yili, and *Babesia* U sp. Kashi), and five ovine *Babesia* (*B. motasi* Lintan, *B. motasi* Ningxian, *B. motasi* Tianzhu, *B. motasi* Hebei, and *Babesia* sp. Xinjiang). All isolates were stored as EDTA–blood stabilates in liquid nitrogen. Detailed data for these isolates are provided in Fig. 1 and Table 1.

Cattle and sheep aged 6–12 months were purchased from an area where babesiosis had not been reported. Thirty days before the study started, all ruminants used in this experiment were splenectomized. Ten days prior to the experiment, blood films were taken from the ears of each animal and tested for hemoparasites. Only calves that were negative for hemoparasites were used in the study.

DNA extraction and sequencing

Six cattle and five sheep were each inoculated with 10 ml of cryopreserved infected blood containing different *Babesia* isolates. When parasitemia was >5 %, erythrocytes were isolated from venous blood that was collected using heparin as anticoagulant. Parasite DNA was isolated using a QIAamp DNA MiniKit (Qiagen, Hilden, Germany), according
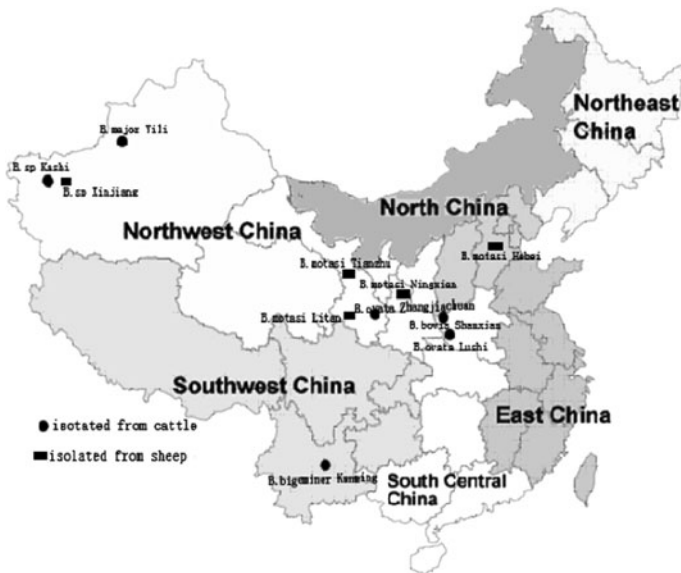
Fig. 1 Sites where *Babesia* spp. were isolated from cattle and sheep in China

to the manufacturer's instructions. The amount of DNA isolated was assessed photometrically. Control DNA was isolated from the venous blood of uninfected cattle and sheep.

A pair of *Piroplasma* universal primers for 28S rRNA sequence were designed based on the 28S rRNA sequences of two strains of *Theileria parva* (GenBank accession number: AF218825, AF013419). The primers were as follows: forward, 5′(1011)-CTAGTAACGG CGAGCGAAGA-3′ (1030); reverse, 5′(4056)-AGGCGTTCAGTCATTATCCAA-3′(4036). The numbers in parentheses refer to the nucleotide positions of the consensus sequences. PCR amplification was performed in a final volume of 50 μl containing 100 ng of genomic DNA, 0.72 pmol of each primer, 160 μM deoxynucleotide triphosphates, 0.9 U Taq DNA polymerase, PCR buffer (Mg^{2+} free), 1.75 mM $MgCl_2$ (all reagents purchased from TaKaRa Company, Japan). Amplification cycles were carried out in a DNA Thermal Cycler 2400 (Perkin-Elmer Life Sciences, USA). The reaction was incubated at 94 °C for 5 min to denature the genomic DNA and the thermal cycle reaction program was as follows: 1 min at 94 °C, 1 min at 63 °C, 3 min at 72 °C for 35 cycles, with a final extension step at 72 °C for 8 min. Samples were stored at 4 °C until analysis. PCR products were visualized by ethidium bromide staining on 1.0 % agarose gel and then cloned into pGEM-T Easy vectors (Promega, USA). To ensure the accuracy of the results, at least five recombinant clones were sequenced from each isolate using an ABI BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and analyzed on an ABI PRISM 3730 DNA sequencer.

Sequence alignment and phylogenetic analysis

Contigs were assembled with Lasergene SeqMan and the resulting sequences were checked against GenBank for contamination. All contigs were checked by eye for ambiguous nucleotides in the regions sequenced for both strands. We also downloaded the 18S rRNA sequence for each isolate from GenBank.

Table 1 Origins of the piroplasma and GenBank accession numbers for the 28S rRNA and18S rRNA gene used in this study

Species	Origin	Host	Type	Length (bp)	Accession number
Babesia major	Yili (China)	Cattle	28S	2,955	JN391438
			18S	1,693	AY603399
B. ovata	Lushi (China)	Cattle	28S	2,961	JN391436
			18S	1,685	AY603401
B. ovata	Zhangjiachuan (China)	Cattle	28S	2,967	JN391430
			18S	1,687	AY603400
B. bigemina	Kunming (China)	Cattle	28S	2,965	JN391440
			18S	1,693	AY603402
B. bovis	Shannxian (China)	Cattle	28S	2,878	JN391431
			18S	1,653	AY603398
Babesia U sp.	Kashi (China)	Cattle	28S	2,950	JN391439
			18S	1,689	AY726556
B. motasi	Lintan (China)	Sheep	28S	2,960	JN391432
			18S	1,683	AY260181
B. motasi	Ningxian (China)	Sheep	28S	2,957	JN391433
			18S	1,687	AY260182
B. motasi	Tianzhu (China)	Sheep	28S	2,957	JN391435
			18S	1,680	DQ159072
B. motasi	Hebei (China)	Sheep	28S	2,959	JN391434
			18S	1,683	DQ159074
Babesia sp.	Xinjiang (China)	Sheep	28S	3,017	JN391437
			18S	1,715	DQ159073
Theileria annulata	Inner Mongolia (China)	Cattle	28S	3,037	JN696678
			18S	1,758	EU083801
T. annulata	Xinjiang (China)	Cattle	28S	3,040	JN696671
			18S	1,741	EU073963
T. ovis	Xinjiang (China)	Sheep	28S	3,056	JN696672
			18S	1,764	FJ603460

Distance matrices for the aligned sequences with all gaps were calculated using the Kimura two-parameter method in MEGA 4 (Tamura et al. 2007). Phylogenetic trees were constructed for 18S rRNA, 28S rRNA, and 28S + 18S fragments using two different analytical methods, i.e., maximum parsimony (MP) and Bayesian inferences (BI). In maximum parsimony, the dataset was analyzed by heuristic search (Swofford 2002) with a 50 % majority consensus rule. Gaps were treated as missing data, with equal weighting for transitions and transversions, and a heuristic search was performed using the TBR branch-swapping algorithm. All trees were subjected to bootstrap analysis with 1,000 replicates to obtain bootstrap value support. Bayesian inference (BI) analysis of each dataset were conducted separately using MrBayes 3.1 (Ronquist and Huelsenbeck 2003). All Bayesian analyses were initiated using a GTR + I + Γ model and no initial values assigned to these parameters. The analyses were conducted with four chains of 1.0×10^5 generations for the

18S rRNA and 28S rRNA dataset and 2.0×10^5 generations for the 28S + 18S fragment dataset. After discarding the burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50 % majority rule consensus tree. Posterior probabilities (PP) were attributed to appropriate clades. Three *Theileria* isolates (*Theileria annulata* Xinjiang, *T. annulata* Inner Mongolia, and *T. ovis* Xinjiang) were used as outgroups. Finally, the phylogenetic trees generated using the two methods were viewed with the Tree View program (version 32).

Results

Amplification of 28S rRNA genes

Only 28S rRNA gene amplification products were generated from the genomic DNA of *Babesia* spp. The length of the PCR products ranged from 2,878 to 3,017 bp. The length of 18S rRNA gene downloaded from GenBank ranged from 1,653 bp to 1,693 bp. Thus, the 28S + 18S fragment length ranged from 4,531 to 4,732 bp.

Sequence alignment

Table 2 shows the percentage similarity values for 28S rRNA and 18S rRNA among the 11 *Babesia* isolates, indicating that the average divergence value for 28S rRNA (7.47 %) was greater than 18S rRNA (3.75 %). This showed that 28S rRNA sequence was more variable than the 18S rRNA sequence.

Of the 4,754 homologous sites (included gaps) in our aligned 28S + 18S fragment, there were 1,048 variable sites and 597 parsimony-informative sites according to the criterion of maximum parsimony. The nucleotide frequencies for all the taxa were 0.260, 0.228, 0.228, and 0.284 for T, C, A, and G, respectively.

Phylogenetic analysis

To determine the phylogenetic position of *Babesia* spp., we constructed 18S rRNA, 28S rRNA and 28S + 18S fragment trees using multiple algorithms. The topologies of the MP and BI trees were highly congruent. The MP trees are shown along with the bootstrap proportions (BP) and posterior probabilities (PP) from the Bayesian analyses. Figure 2a–c shows the three trees calculated for the 28S rRNA, 18S rRNA, and 28S + 18S fragments, respectively, demonstrating that all three trees shared similar topology. An examination of the bootstrap values and posterior probabilities of these trees showed that the 28S + 18S tree was slightly better than two other trees. Moreover, there were also some differences among these three trees, e.g., the relationship of four *B. motasi* isolates and the phylogenetic position of *B. major*. The *B. bigemina* and *B. ovata* branches were also different in the three rRNA gene trees.

Discussion

Previous phylogenetic studies have compared 28S rRNA, 18S rRNA, and united 28S + 18S fragment datasets and shown that 28S rRNA and 18S rRNA genes produced

Table 2 Sequence similarity of 28S rRNA and 18S rRNA of *Babesia* spp. from China isolates

Isolates		Similarity of 28S rRNA (%)										Similarity of 18S rRNA (%)									
		2	3	4	5	6	7	8	9	10	11	2	3	4	5	6	7	8	9	10	11
B. motasi Lintan	1	95.0	99.9	99.9	91.4	91.4	91.4	92.0	87.3	89.0	85.1	98.8	99.2	97.3	95.7	95.8	95.7	94.6	93.5	93.7	87.8
B. motasi Ningxian	2	100	94.9	95.0	90.8	90.6	91.1	91.7	85.8	88.7	84.8	100	98.1	97.9	96.1	95.9	95.7	95.0	93.7	94.0	88.3
B. motasi Tianzhu	3		100	99.9	91.4	91.3	91.6	91.9	86.8	89.1	85.1		100	98.0	96.4	96.4	96.4	95.2	94.2	94.4	88.2
B. motasi Hebei	4			100	91.4	91.3	91.6	91.9	86.8	89.1	85.1			100	96.5	96.4	96.3	95.1	94.1	94.5	88.3
B. ovata Lushi	5				100	95.8	95.8	90.7	86.1	88.8	85.4				100	98.4	97.8	95.1	94.8	94.3	88.5
B. ovata Zhangjiachuan	6					100	97.2	90.6	87.2	89.2	85.0					100	98.2	95.3	95.1	94.7	88.7
B. bigemina Kunming	7						100	90.6	87.6	89.5	85.1						100	95.0	94.5	94.5	88.5
B. major Yili	8							100	85.9	87.8	84.9							100	93.8	95.0	87.6
Babesia sp. Xinjiang	9								100	91.4	85.0								100	97.2	88.5
Babesia U sp. Kashi	10									100	86.1									100	88.9
B. bovis Shannxian	11										100										100

broadly similar trees, while 18S rRNA sequences were better than 28S rRNA at resolving higher order relationships. In general, the united fragment results were more robust than 28S rRNA and 18S rRNA (Mallatt and Sullivan 1998; Mallatt and Winchell 2002; Medina et al. 2001; Winchell et al. 2002). This was confirmed in the current study. To lower order relationships in the current study, the 28S rRNA genes were slightly better than the 18S rRNA genes based on the percentage similarity and bootstrap values. It was not entirely clear why the 18S rRNA genes provided fairly poor resolution. One important reason may be that the 28S rRNA sequences contained almost twice as many nucleotides as the 18S rRNA sequences and the sequences were more variable than 18S rRNA sequences (Winchell et al. 2004).

Our laboratory isolated several large *Babesia motasi* strains from the field-collected blood of sheep and goats from different regions in China (Guan et al. 2002; Liu et al. 2007). These strains were known as *B. motasi* Lintan, *B. motasi* Ningxian, *B. motasi* Tianzhu, and *B. motasi* Hebei (Niu et al. 2009). According to the 28S + 18S fragment and 28S rRNA phylogenetic trees, *B. motasi* Lintan and *B. motasi* Tianzhu fall into one clade, whereas *B. motasi* Hebei and *B. motasi* Ningxian fall into another clade. This result was different from the 18S rRNA tree. *B. motasi* Ningxian, which was isolated from the eastern part of Gansu Province in China, was highly virulent in both sheep and goats, and was similar to European *B. motasi* based on its morphology. The tick *Hae. longicornis* was known to be the vector of *B. motasi* Ningxian. However, the other three *B. motasi* isolates were transmitted by *Hae. qinghaiensis* with low virulence in sheep and goats (Guan et al. 2002). Furthermore, the result of an enzyme-linked immunosorbent assay (ELISA), developed using merozoite antigens of *B. motasi* Lintan purified from an in vitro culture suggested there were no cross-reactions between *B. motasi* Lintan and anti-*B. motasi* Ningxian, whereas anti-*B. motasi* Tianzhu antibodies cross-reacted with *B. motasi* Lintan (Guan et al. 2010). Thus, the information derived from the 28S + 18S and 28S trees may reflect the phylogenetic relationships of *B. motasi*, where *B. motasi* Ningxian was different from the other three isolates so they may be different subspecies of *B. motasi*.

Babesia major Yili is a large *Babesia* species that is infectious to cattle, which is transmitted by *Hae. punctata* (Yin et al. 1996). It was isolated from pasture in the Xinjiang Uygur Autonomous Region of China. Liu et al. reported that *B. major* Yili was different from several strains of *B. ovata* from China based on the ITS gene, whereas other researchers found it was very difficult to discriminate between these two species (Liu et al. 2008; Lu et al. 1988). From this study, although the phylogenetic position of *B. major* Yili was different in three trees, it was obvious that *B. major* Yili was distant from the two strains of *B. ovata*.

We also found that *Babesia* U sp. Kashi and *Babesia*. sp. Xinjiang always fell into one clade with a highly supported value in three phylogenetic trees and they shared common biological characteristics, e.g., both were isolated in Xinjiang Uygur Autonomous Region of China, they were transmitted by *H. a. anatolicum*, and that had low virulence in the host. However, *Babesia* U sp. Kashi infected cattle only whereas *Babesia* sp. Xinjiang infected only sheep (Guan et al. 2009; Luo et al. 2002, 2005a, b). The lack of sufficient isolates of these two species in this study may be an important cause of this confusion. However, the different mechanisms of the long-term coevolution between the parasite and host may also be an explanation (Kempf et al. 2009; Marques et al. 2011).

A

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                                              0.51/64.3 ┌── B. motasi Lintan
                                         1.00/100 ──────┤
                                                        └── B. motasi Tianzhu
                                    1.00/100 ───────────── B. motasi Hebei
                              1.00/94.6 ──────────────────── B. motasi Ningxian
                        1.00/100 ─────────────────────────── B. major Yili
                                              1.00/78.4 ┌── B. ovata Zhangjiachuan
                                         1.00/100 ──────┤
                                                        ├── B. bigemina Kunming
                                                        └── B. ovata Lushi
                1.00/65                 0.97/100 ┌──────── Babesia U sp. Kashi
                                                 └──────── Babesia sp. Xinjiang
                                    ──────────────────────── B. bovis Shanxian
                                              1.00/100 ┌── T. annulata Xinjiang
                                         1.00/100 ──────┤
                                                        ├── T. annulata Inner Mongolia
                                                        └── T. ovis Xinjiang
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B

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                                              1.00/52.3 ┌── B. motasi Lintan
                                         1.00/60.6 ──────┤
                                                         └── B. motasi Ningxian
                                    1.00/98.0 ──────────── B. motasi Tianzhu
                              0.88/86.6 ──────────────────── B. motasi Hebei
                                              1.00/56.8 ┌── B. ovata Lushi
                                         1.00/97.8 ──────┤
                                                         ├── B. ovata Zhangjiachuan
                1.00/97.0 ──────────────────────────────── B. bigemina Kunming
                                    ──────────────────────── B. major Yili
                                         0.96/100 ┌──────── Babesia U sp. Kashi
                                    0.86/58.5 ─────┤
                                                  ├──────── Babesia sp. Xinjiang
                                                  └──────── B. bovis Shanxian
                                              1.00/100 ┌── T. annulata Xinjiang
                                         1.00/100 ──────┤
                                                        ├── T. annulata Inner Mongolia
                                                        └── T. ovis Xinjiang
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C

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                                              1.00/100 ┌── B. motasi Lintan
                                         1.00/100 ──────┤
                                                        └── B. motasi Tianzhu
                                    1.00/100 ───────────── B. motasi Hebei
                              1.00/89.0 ──────────────────── B. motasi Ningxian
                        1.00/100 ─────────────────────────── B. major Yili
                                              0.98/96.0 ┌── B. ovata Zhangjiachuan
                                         1.00/100 ──────┤
                                                        ├── B. bigemina Kunming
                1.00/89.0                               └── B. ovata Lushi
                                              1.00/100 ┌──── Babesia sp. Xinjiang
                                                       └──── Babesia U sp. Kashi
                                    ──────────────────────── B. bovis Shanxian
                                              1.00/100 ┌── T. annulata Inner Mongolia
                                         1.00/100 ──────┤
                                                        ├── T. annulata Xinjiang
                                                        └── T. ovis Xinjiang
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◀ **Fig. 2** Phylogenetic trees of 11 *Babesia* isolates based on their rRNA genes. These trees were calculated using the BI and MP methods. *Trees* were constructed with: **a** 28S rRNA sequences, **b** 18S rRNA sequences, **c** a combined data set of 28S + 18S sequences. The posterior probability and bootstrap values supporting each node are shown. Three *Theileria* isolates were used as outgroup

In conclusion, our study was the first attempt to unravel the phylogenetic relationships in the molecular level distribution of Chinese *Babesia* spp. using 28S rRNA sequences. Our results suggest that 28S rRNA sequences could provide useful genetic markers for the specific identification and genetic characterization of *Babesia* spp. in China and elsewhere.

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