Insights into the phylogenetic relationship and drug targets of *Babesia* infective to small ruminants from its mitochondrial genomes

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Research

Keywords: Babesia motasi, Babesia sp., cytochrome bc1 complex, drug target, mitochondrial genome, phylogenetic relationship

Posted Date: March 17th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-17485/v1

Version of Record: A version of this preprint was published at Parasites & Vectors on July 29th, 2020. See the published version at https://doi.org/10.1186/s13071-020-04250-8.

Abstract

Background: Babesiosis, a tick-borne disease caused by protozoans of the genus *Babesia*, is widespread in subtropical and tropical countries. Mitochondrion is an essential organelle that is responsible for energy transduction and metabolism, calcium homeostasis and cell signaling. Mitochondrial genomes could provide a new insight to understand and explore the population genetics, biological features of the pathogens, and evolutionary relationships. However, there is limied information on the mitochondrial genomes of ovine *Babesia* spp. in China.

Methods: Herein, we sequenced, assembled and annotated the mitochondrial genomes of six ovine *Babesia* isolates, and analyzed genome size, gene content, genome structure and cytochrome b (*cob*) amino acid sequences, and performed comparative mitochondrial genomics and phylogenomic analyses among apicomplexan parasites.

Results: The mitochondrial genomes range from 5767 to 5946 bp in length with a linear form and contain three protein-encoding genes, cytochrome c oxidase I (*cox1*), cytochrome c oxidase III (*cox3*) and *cob*, six large subunit rRNA gene (LSU), and two terminal inverted repeats (TIR) on both ends. The cob sequence analysis indicated that the binding site of anti-Babesia drugs targeted on the cytochrome bc1 complex. *Babesia microti* and *Babesia rodhaini* have a dual flip-flop inversion of 184-1082 bp, whereas other *Babesia* spp. and *Theileria* spp. have one pair of TIR, 25-1563 bp. Phylogenetic analysis indicated that six ovine *Babesia* isolates were divided into two clades, *Babesia* sp. and *Babesia motasi*. *B. motasi* isolates were further separated into two subclades (*B. motasi* Lintan/Tianzhu and *B. motasi* Ningxian/Hebei).

Conclusions: The data provided new insights into the population genetics, taxonomic relationships, molecular epidemiological studies and drug targets of apicomplexan parasites.

Background

Babesia are tick-transmitted haemoprotozoa causing babesiosis characterized by fever, anaemia, jaundice and haemoglobinuria. Babesiosis of small ruminants has great economic importance. In sheep and goats, the main causative agents are *Babesia ovis* and *Babesia motasi*, transmitted by *Rhipicephalus* spp. and *Haemaphysalis* spp. and reported in Asia, South America, Africa, the Far East and Europe [1-3]. *Babesia* sp. Xinjiang (BspXJ), *Babesia* sp. Dunhuang (BspDH), *B. motasi* Lintan (BmLT), *B. motasi* Tianzhu (BmTZ), *B. motasi* Hebei (BmHB) and *B. motasi* Ningxian (BmNX) have been isolated from sheep in China during the period of 2000-2010. The six ovine *Babesia* parasites have different characteristics in serology, pathogenicity, vector specificity and virulence. For instance, BspXJ and BspDH have low-virulence and are transmitted by the *Hyalomma* spp. ticks, whereas BmLT, BmHB, BmNX and BmTZ are transmitted by *Haemaphysalis* spp. and cause a range of mild to severe clinical manifestations. BspXJ/DH is serologically distinct from *B. motasi*, and there are also with serological difference between isolates of *B. motasi* [4-12]. The prevalence of *B. motasi* and *Babesia* sp. were 30.4-31.7% and 36.0-43.5%, respectively, which indicated that ovine *Babesia* spp. are widespread in China [11, 13-15].

Mitochondrion is an essential organelle that is responsible for energy transduction and metabolism, calcium homeostasis and cell signaling [16]. So far, sequencing of the mitochondrial genomes have been performed for *Babesia bovis*, *Babesia bigemina*, *Babesia orientalis*, *Babesia caballi*, *Babesia gibsoni*, *Babesia canis*, *Babesia vogeli*, *Babesia rossi*, *Babesia conradae*, *B. microti*, *B. rodhaini*, *Babesia duncani*, *Theileria equi*, *Theileria orientalis* [17-22]. Mitochondrial genomes could provide a new insight to understand and explore the population genetics, biological features of the pathogens, and evolutionary relationships. The cytochrome *bc*1 is the important complex III of the mitochondrial electron transport chain, which is essential to cellular respiration and energy conversion [23]. The Q cycle reaction mechanism of *bc*1 assumes a separated quinone reduction (Qi) and quinol oxidation (Qo) site. Inhibitors of cytochrome *bc*1 complex may be divided into two types that act on Qi and Qo sites according to the binding site. Some studies suggest that yeast *bc*1 complex structure could be used as a model for discovering new antimalarial drugs [24, 25]. The mutations in the cytochrome b (*cob*) gene have been found to be the molecular basis of drug resistance [25-28]. Several anti-*Babesia* drugs (for instance diminazene aceturate) have demonstrated to be ineffective owing to drug resistance [29, 30]. Therefore, there is an urgent need to develop new drugs.

However, there is limited information on the mitochondrial genomes of ovine *Babesia* spp. in China. Although the mitochondrial genomes of BspXJ and BmLT were sequenced by using Illumina technology, they have not been verified using PCR amplification and sequencing using Sanger dideoxy chain-termination method [31]. In this study, the mitochondrial genomes of six ovine *Babesia* isolates were sequenced, assembled, annotated and compared. And they were used to clarify phylogenetic relationships and classification of the *Babesia* infective to small ruminants in China, and to determine novel molecular markers for identification studies of *Babesia* species. The current study will provide valuable information for understanding of mitogenome evolution among apicomplexan parasites, identifying diagnostic markers and screening drug targets.

Methods

Parasites and isolation of genomic DNA

The purified merozoites of BspXJ, BspDH, BmLT, BmTZ, BmHB and BmNX were provided by the vectors and vector-borne diseases laboratory in Lanzhou Veterinary Research Institute, China [32]. Genomic DNA was extracted from the merozoites using a QIAamp DNA Blood Mini Kit (Qiagen,

Hilden, Germany). The DNA concentration and quality were measured using the 260/280 nm absorbance ratio on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Amplification and sequencing

The PCR primers were designed by aligning with the reported genomic sequences of *B. bovis* and *B. bigemina* (AB499088 and AB499085) (Table S1). Mitochondrial genome fragments were amplified from the genomic DNA of BspXJ, BspDH, BmLT, BmTZ, BmHB and BmNX, respectively. The PCR products were cloned into pGEM®-T Easy Vector (Promega, Beijing, China) for subsequent sequencing using a BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3730 DNA analyser (Applied Biosystems) by Sangon Biotech company.

Genome assembly and annotation

Mitochondrial genomic fragments were assembled with the CLC Genomics Workbench v.7.5.1 (Qiagen, Redwood City, CA, USA). The mitogenome annotation was performed using the DOGMA (http://dogma.ccbb.utexas.edu/) [33] and Artemis [34], followed by application of BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify homologous proteins in apicomplexan parasites in the GenBank database. The tRNA genes were identified using tRNAscan-SE v.2.0 (http://lowel ab.ucsc.edu/tRNAs can-SE/) with the default search mode and other mitochondrial sequence sources [35]. The rRNA genes were annotated by searching previously reported rRNA sequences of *B. bovis*, *B. microti*, *B. orientalis*, and *T. parva*. Mauve (http://gel.ahabs.wisc.edu/mauve) was used to generate the genome comparisons [36]. The mitochondrial genomes of six ovine *Babesia* were annotated and deposited in GenBank (accession numbers: MK962313, MK962314 and MN605889–MN605892).

Sequence alignment and amino acid conservation of *cob* gene

We used MegAlign software to compare the amino acid sequences of the *cob* genes of *Saccharomyces cerevisiae*, *Bos taurus*, *Toxoplasma gondii*, *Theileria parva*, *B. microti*, *B. duncani*, *Plasmodium falciparum*, BspXJ/DH, BmLT/TZ and BmNX/HB; and referred to the results of the previous researches [24-28, 37-41] (that use mitochondrial cytochrome *bc*1 complex from *S. cerevisiae* to explicate the binding residues of the inhibitor (acting on *bc*1 complex) by X-ray crystallography, spectroscopy and *cob* sequence analysis) to found main resistance mutations and drug binding residues of *cob* gene in the genus *Babesia*.

Phylogenetic analysis

The concatenated sequences of *cob* and cytochrome c oxidase I (*cox1*) amino acid residues from each species were put into multi-alignment using Clustal W with further manual verification. Subsequently, MEGA v.6.06 (http://www.megasoftwa re.net/) software was applied to conduct phylogenetic analysis. A bootstrap phylogenetic tree was created by the maximum likelihood (ML) method or neighbour-joining (NJ) method, using a distance matrix corrected for nucleotide substitutions based on the JTT with Freqs model. In addition, phylogeny of the whole mitochondrial nucleotide sequence was constructed by the ML method based on the Kimura 2-parameter model. A bootstrap analysis was used to assess the robustness of the clusters using 1000 replicates.

Results

Sequence analysis of the mitochondrial genomes of ovine *Babesia*

Sequence analysis revealed that six ovine *Babesia* mitochondrial genomes were formed of linear DNA, ranging from 5767 to 5946 bp in length, with a high A + T content of 70.05–70.87% (Table 1). Mitochondrial genomes of six ovine *Babesia* contained three protein-encoding genes, *cox1*, cytochrome c oxidase III (*cox3*), *cob*, six large subunit rRNA gene (LSU), and two terminal inverted repeats (TIR). The transcriptional direction of *cox3*, LSU3, LSU6, LSU2, *cob* and LSU5 was from 3' to 5', whereas the direction of *cox1*, LSU1 and LSU4 was from 5' to 3' (Figure 1; Figure S1). The start codons of *cox3* and *cob* genes of ovine *Babesia* were ATA and ATG, respectively. The initiation codon of *cox1* gene of BspXJ/DH was ATA, whereas that of BmLT/TZ and BmNX/HB was ATG. Most of the mitochondrial protein-encoding genes had TAA as a termination codon, followed by TGA (Table 2).

Comparison of genomic data sequenced by the Sanger and Illumina method

The mitochondrial genomic sequences of BspXJ and BmLT sequenced using Illumina method (designated BspXJ-Illumina and BmLT-Illumina) were compared with those obtained in this study using the Sanger method (designated BspXJ-Sanger and BmLT-Sanger). The results indicated that two sets of data from Sanger and Illumina have some differences in the size of the mitochondrial genome, the A + T contents, the number of LSU, and the start and stop codons used in the encoding genes (Table 2). Furthermore, there were differences in the bases at several nucleotide positions along the full-length genome between the Illumina and Sanger data (Table 3).

Cob gene sequence analysis

The results of amino acid sequence alignment indicated that all *cob* genes contain the highly conserved PEWY motif. The residue at position Leu275 of *S. cerevisiae* is a key determinant of efficacy of atovaquone and myxothiazol binding to the *bc*1 complex. This position is occupied by a Leu in the *T. parva*, *B. microti*, *B. duncani*, BspXJ/DH, BmLT/TZ and BmHB/NX sequence, whereas a Phe is present in *T. gondii*, bovine and *P. falciparum* sequence (Figure 2). The inhibitors of Qi and Qo sites include atovaquone, stigmatellin, myxothiazol, endochin-like quinolone (ELQ), antimycin A and NQNO. The amino acid changes conferring atovaquone resistance in the yeast numbering system included five mutations (I269M, F278I/A, Y279C/S, L275F and L282V). The drugs (target protein is Qo site of *bc*1 complex) binding residues of cob of ovine *Babesia* included Met128, Gly132, Glu259, Leu262, Phe265 and Tyr266. And drugs (target protein is Qi site of *bc*1 complex) binding residues of cob of ovine *Babesia* involved His187, Ser191 and Asp214 (Table 4).

Phylogenetic analysis

Phylogenetic trees were constructed with the concatenated amino acid sequences of *cob* and *cox1* gene using ML and NJ methods. The two approaches showed no significant changes in the topology. The piroplasms were divided into six groups: classical *Babesia* infective to ruminants, canine, equine, *Theileria*, *B. duncani* and *B. microti*/*B. rodhaini*. *Babesia* infective to ruminants were separated into four clades: *B. motasi*, *B. bigemina*, *B. orientalis*/*B. bovis*, *Babesia* sp. XJ/DH. Furthermore, *B. motasi* were further divided into two subclades: *B. motasi* LT/TZ and *B. motasi* NX/HB (Figure 3). The grouping results of the phylogenetic tree constructed using mitochondrial nucleotide sequences were consistent with *cox1* and *cob* amino acid trees (Figure S2).

Discussion

In the present study, we assembled and annotated the mitochondrial genomes of six ovine *Babesia* isolates and performed a mitochondrial genomic analysis with published mitochondrial genomes of apicomplexan parasites. The mitochondrial genomes of six ovine *Babesia* is highly similar to most of *Babesia* spp. in respect of genome size, high A + T content, genome form, and gene content. However, the mitochondrial genomes of ovine *Babesia* are smaller than *B. microti*, *B. rodhaini* and *Theileria equi*, and larger than *Toxoplasma gondii* [19, 20, 42]. The tRNA genes were not found in the mitochondrial genomes of six ovine *Babesia*, which is consistent with other apicomplexan parasites. We speculate that they may be directly encoded by the nuclear genome. The order and transcriptional direction of three protein-encoding genes are the same in *B. bovis*, *B. bigemina*, *B. gibsoni* [19], but different from *B. microti*, *T. equi*, *T. orientalis* and *Plasmodium falciparum* [19, 20].

In the reported mitochondrial genomes of piroplasma, *B. microti* and *B. rodhaini* have a dual flip-flop inversion system, range from 184–1082 bp in length [20]. However, one pair of TIR was found at the mitochondrial genomes of other *Babesia* spp. and *Theileria* spp., range from 25–1563 bp in length [17-19, 21, 31]. These findings indicated that the number and size of TIRs are one of the main causes of different mitochondrial genome sizes. We also found that different numbers of LSUs are responsible for the size of the mitochondrial genomes. The TIRs are considered to play a crucial role in the replication and stabilization of the linear mitochondrial genomes [43]. In the published mitochondrial genome of apicomplexan parasites, the sequences of the coding genes and LSU are basically the same. Therefore, we speculated that differences in lengths and sequences of TIRs may be responsible for divergence in host-specific and in vitro culture characteristics of protozoa.

The difference between the first-generation and second-generation sequencing data is mainly caused by the nucleotide substitutions, deletions and insertions, which is caused by the different sequencing techniques, assembly and annotation software used. And settings of a few parameters are different, which is one of the reasons for the difference between the two sets of data. The results of Illumina sequencing method are more prone to errors. The Sanger approach is more accurate and thus more appropriate for further studies.

Mitochondria are essential organelles and play an important role in the energy metabolism and the growth and development of apicomplexan parasites [44]. Cytochrome *bc*1 is an integral membrane protein complex, which is essential to cellular respiration. The highly conserved of the *cob* gene binding to inhibitors is the molecular basis for the effect of the drugs on yeast, fungi and parasites [41]. The drug binding residues of *cob* gene of six ovine *Babesia* are completely consistent. In the conserved PEWY region of the *cob* gene, Phe278 (yeast number of *cob*) is present in most organism, whereas *T. gondii* and *B. taurus* at this position are Tyr and Ala, respectively. Studies have reported that the L275F mutation in yeast has no effect on enzyme activity, but IC50 has increased ten-fold [28]. Compared with the wild type of yeast, the mutation Y279S indicated a B40-fold increased IC50 (B1.7 mM) for atovaquone [28], and Y268S in the *P. falciparum* numbering system results in a 3,000-fold loss of atovaquone sensitivity. In addition, the mutations M133I, L271V and Y268N of *cob* gene of *P. berghei* confer resistant to atovaquone [41]. Therefore, we conclude that the mutations of *cob* gene are largely responsible for the efficacy of drugs (target protein is *bc*1 complex) in apicomplexan parasites.

Currently, atovaquone and ELQs have been reported for the treatment of human babesiosis and malarial by modifying of the drug target by disruption of cytochrome *bc*1 complex [25, 27, 28, 39, 41]. In 2019, a *B. motasi*-like parasite has been detected in human blood in Korea [45], which suggests that *B. motasi* may be potentially zoonotic. Therefore, we should investigate the infection of *B. motasi* in human in China, evaluate the zoonotic potential of *B. motasi*, and the effect of inhibitors binding to cytochrome *bc*1 complex.

In this study, the taxonomical relationships of *Babesia* infective to sheep and goats are consistent with the reported phylogenetic analyses based on *cob*, *cox1*, *cox3*, nuclear small subunit (SSU) and internal transcribed spacer (ITS) [5, 6, 8, 31, 46]. The ovine *Babesia* are divided into two species: *Babesia* sp. and *B. motasi*. *B. motasi* further fell into two small clades, named BmLT/TZ and BmNX/HB. With the expect of *B. conradae*, piroplasm infective to the same host fell into one clade. These findings indicated that the mitochondrial genome data are valuable for the population genetic, phylogenetic and molecular epidemiological studies.

Conclusions

In conclusion, we reported the mitochondrial genome of six ovine *Babesia* infective to small ruminants in China. The phylogeny based on the concatenated amino acid sequences indicated that there are two species of *Babesia* (*Babesia* sp. and *B. motasi*) infective to small ruminants in China, and that the four *B. motasi* possibly belong to two subspecies (BmLT/TZ and BmNX/HB). The possible efficacy of inhibitor (target protein is *bc*1 complex) should be evaluated on six ovine *Babesia* in future work. Further studies are needed to analysis TIR and proteins functions could open new insight for characterize *Babesia* phylogenetic relationships, biology and therapy.

Abbreviations

DNA: deoxyribonucleic acid; PCR: polymerase chain reaction; *cob*: cytochrome b; *cox1*: cytochrome c oxidase I; *cox3*: cytochrome c oxidase III; LSU: large subunit ribosomal RNA; TIR: terminal inverted repeats; Qi: quinone reduction; Qo: quinol oxidation; ELQ: endochin-like quinolone; SSU: small subunit ribosomal RNA; ITS: internal transcribed spacer

Declarations

Acknowledgements

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated or analyzed during this study are included in this published article and its additional files. The newly generated sequences were submitted to the GenBank database under the accession numbers MK962313, MK962314 and MN605889–MN605892.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was financially supported by the National Key Research and Development Programme of China (2017YFD0501200), 973 Programme (2015CB150300), ASTIP (CAAS-ASTIP-2016-LVRI), NBCIS (CARS-37) and Jiangsu Co-innovation Center Programme for Prevention and Control of Important Animal Infectious Diseases and Zoonoses.

Authors' contributions

GQG and XXW designed the study, wrote the manuscript, and performed the experiments. JMW, JLL, AHL, XH and QJX extracted the genomic DNA and sequenced the PCR fragments. HY, JXL and YQL reviewed the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Mitochondrial genome sequences of apicomplexan parasites used in the present study

Taxon	Size/bp	A+T contents (%)	Form	Protein-encoding genes	Original host	Country of origin	Accession number
Babesia sp. Xinjiang (BspXJ-Sanger) [a1]	5767	70.87	Linear	*cox1, cox3, cob*	Sheep	China	MK962313
Babesia sp. Dunhuang (BspDH)	5767	70.85	Linear	*cox1, cox3, cob*	Sheep	China	MK962314
Babesia motasi Lintan (BmLT-Sanger) [b1]	5836	70.05	Linear	*cox1, cox3, cob*	Sheep	China	MN605889
Babesia motasi Tianzhu (BmTZ)	5836	70.13	Linear	*cox1, cox3, cob*	Sheep	China	MN605890
Babesia motasi Ningxian (BmNX)	5946	70.10	Linear	*cox1, cox3, cob*	Sheep	China	MN605891
Babesia motasi Hebei (BmHB)	5946	70.06	Linear	*cox1, cox3, cob*	Sheep	China	MN605892
Babesia sp. Xinjiang (BspXJ-Illumina) [a2]	6020	71.30	Linear	*cox1, cox3, cob*	Sheep	China	KX698108
Babesia motasi Lintan (BmLT-Illumina) [b2]	5790	69.97	Linear	*cox1, cox3, cob*	Sheep	China	KX698109
Babesia bovis T2Bo	6005	70.49	Linear	*cox1, cox3, cob*	Bovine	USA	EU075182
Babesia bovis	5970	70.35	Linear	*cox1, cox3, cob*	Bovine	Japan	AB499088
Babesia bigemina	5924	69.82	Linear	*cox1, cox3, cob*	Bovine	Japan	AB499085
Babesia orientalis	5996	71.10	Linear	*cox1, cox3, cob*	Water buffalo	China	KF218819
Babesia caballi	5847	70.43	Linear	*cox1, cox3, cob*	Equine	USA	AB499086
Babesia gibsoni	5865	72.24	Linear	*cox1, cox3, cob*	Canine	Japan	AB499087
Babesia gibsoni (WH58)	5865	72.21	Linear	*cox1, cox3, cob*	Canine	China	KP666169
Babesia canis canis	5769	71.90	Linear	*cox1, cox3, cob*	Canine	USA	KC207822
Babesia canis vogeli	5603	71.19	Linear	*cox1, cox3, cob*	Canine	USA	KC207825
Babesia canis rossi	5838	71.24	Linear	*cox1, cox3, cob*	Canine	USA	KC207823
Babesia conradae	5608	72.41	Linear	*cox1, cox3, cob*	Canine	USA	KC207826
Babesia microti	11109	64.36	Linear	*cox1, cox3, cob*	Murine, human	Germany	AB624353
Babesia microti	11109	64.36	Linear	*cox1, cox3, cob*	Murine, human	Germany	AB624354
Babesia microti	11109	64.36	Linear	*cox1, cox3, cob*	Murine, human	Germany	AB624355
Babesia microti	11109	64.36	Linear	*cox1, cox3, cob*	Murine, human	Germany	AB624356
Babesia rodhaini	6929	70.69	Linear	*cox1, cox3, cob*	Murine	Australian	AB624357
Babesia rodhaini	6929	70.69	Linear	*cox1, cox3, cob*	Murine	Australian	AB624358
Babesia rodhaini	6929	70.69	Linear	*cox1, cox3, cob*	Murine	Australian	AB624359
Babesia rodhaini	6929	70.69	Linear	*cox1, cox3, cob*	Murine	Australian	AB624360
Babesia duncani	5893	68.15	Linear	*cox1, cox3, cob*	Human	USA	MH107387
Babesia sp. Coco	5612	71.22	Linear	*cox1, cox3, cob*	Canine	USA	KC207824
Cytauxzoon feil	5945	70.83	Linear	*cox1, cox3, cob*	Feline	USA	KC207821
Theileria annulata	5905	70.57	Linear	*cox1, cox3, cob*	Bovine	Turkey	NT167255
Theileria parva	5924	70.07	Linear	*cox1, cox3, cob*	Bovine	Kenya	AB499089
Theileria parva	5895	70.01	Linear	*cox1, cox3, cob*	Bovine	Kenya	Z23263
Theileria orientalis	5957	70.72	Linear	*cox1, cox3, cob*	Bovine	Japan	AB499090
Theileria equi	8246	70.94	Linear	*cox1, cox3, cob*	Equine	USA	AB499091
Plasmodium berghei	5957	68.84	Linear	*cox1, cox3, cob*	Murine	Turkey	AB558173
Plasmodium malariae	5968	70.12	Linear	*cox1, cox3, cob*	Human	Japan	AB489194
Plasmodium knowlesi	5957	69.48	Circular	*cox1, cox3, cob*	Human, macaques	Malayan	AY722797
Plasmodium vivax	5947	69.50	Linear	*cox1, cox3, cob*	Human	Malaysia	DQ396549
Plasmodium falciparum	5967	68.38	Circular	*cox1, cox3, cob*	Human	India	KT119882
Toxoplasma gondii	2607	64.90	Linear	*cox1, cob*	Cat, human	RH	JX473253

[a] a1 and a2 is the same sample, which sequenced using Sanger method and Illumina method, respectively.

[b] b1 and b2 is the same sample, which sequenced using Sanger method and Illumina method, respectively.

Table 2 Gene contents and initiation and termination codons of encoding genes of the piroplasma mitochondrial genomes

Species	5' TIR (bp)	*cox1* (Start, stop codons)	*cox3* (Start, stop codons)	LSU1 (bp)	LSU3 (bp)	LSU6 (bp)	LSU2 (bp)	*cob* (Start, stop codons)	LSU5 (bp)	LSU4 (bp)	3' TIR (bp)
BspXJ/DH	25	1434 (ATA, TAA)	639 (ATA, TGA)	302	111	37	36	1092 (ATG, TAA)	69	82	25
BmLT/TZ	35	1434 (ATG, TAA)	639 (ATA, TGA)	297	111	37	36	1092 (ATG, TAA)	70	82	35
BmNX/HB	101	1434 (ATG, TAA)	639 (ATA, TGA)	297	111	37	36	1092 (ATG, TAA)	70	82	101
B. gibsoni (AB499087)	74	1434 (TTG, TAA)	642 (ATT, TAA)	306	111	43	35	1092 (TTA, TAA)	70	82	74
B. bovis (AB499088)	119	1434 (ATG, TAA)	639 (ATA, TAA)	302	111	38	35	1092 (ATG, TAA)	68	82	119
B. duncani (MH107387)	48	1302 (ATG, TAA)	588 (ATG, TAA)	298	111	44	34	1092 (ATG, TAA)	69	82	48
B. bigemina (AB499085)	65	1434 (ATG, TAA)	639 (ATA, TGA)	299	111	37	36	1092 (ATG, TAA)	70	82	65
B. caballi (AB499086)	62	1434 (ATA, TAA)	639 (ATA, TAA)	301	111	37	35	1092 (ATG, TAA)	68	82	62
T. parva (AB499089)	94	1440 (ATT, TAA)	642 (ATT, TAA)	301	111	38	38	1092 (ATG, TAG)	68	82	94
T. orientalis (AB499090)	47	1437 (ATA, TAA)	642 (ATT, TAA)	310	111	38	38	1092 (ATA, TAA)	69	82	47

Table 3 Comparison of *Babesia* sp. Xinjiang and *Babesia motasi* Lintan mitochondrial genome were sequenced using Illumina (BspXJ-Illumina and BmLT-Illumina) and the Sanger method (BspXJ-Sanger and BmLT-Sanger)

Position[a]	1	2-195	179-185	218	5992-5993	5994-6052	5999-6044
Babesia sp. Xinjiang (BspXJ-Sanger, MK962313)		-...-			TT	-...-	
Babesia sp. Xinjiang (BspXJ-Illumina, KX698108)		AA...TT			AA	CT...TG	
Babesia motasi Lintan (BmLT-Sanger, MN605889)	A		GT...TT	-			AA...TT
Babesia motasi Lintan (BmLT-Illumina, KX698109)	T		-...-	G			-...-

[a]Position numbers given BspXJ (GenBank: MK962313)

Key: -, base deletion

Table 4 Main resistance mutations and drug binding residues of cytochrome *b* in *Saccharomyces cerevisiae*, *Plasmodium falciparum*, *B. microti*, *B. duncani* and six ovine *Babesia*

Target protein	Drug	Amino acid changes conferring drugs resistance in the yeast numbering system	*S. cerevisiae* residues	*P. falciparum* residues	*B. microti* residues	*B. duncani* residues	BspXJ/DH residues	BmLT/TZ residues	BmNX/HB residues
Qo site of *bc*1 complex	atovaquone	Ile^{269}→Met, Phe^{278}→Ile, Phe^{278}→Ala, Tyr^{279}→Cys, Tyr^{279}→Ser, Leu^{282}→Val, Leu^{275}→Phe	Glu^{272}, Leu^{275}, Phe^{278}, Tyr^{279}	Glu^{261}, Phe^{267}, Tyr^{268}	Glu^{265}, Leu^{268}, Phe^{271}, Tyr^{272}	Glu^{257}, Leu^{260}, Phe^{263}, Tyr^{264}	Glu^{259}, Leu^{262}, Phe^{265}, Tyr^{266}	Glu^{259}, Leu^{262}, Phe^{265}, Tyr^{266}	Glu^{259}, Leu^{262}, Phe^{265}, Tyr^{266}
	stigmatellin	Leu^{275}→Phe	Glu^{272}	Glu^{261}	Glu^{265}	Glu^{257}	Glu^{259}	Glu^{259}	Glu^{259}
	myxothiazol	Leu^{275}→Phe	Leu^{275}	-	Leu^{268}	Leu^{260}	Leu^{262}	Leu^{262}	Leu^{262}
	ELQ-110	Met^{139}, Gly^{143}, Glu^{272}	Met^{139}, Gly^{143}, Glu^{272}	Met^{133}, Gly^{137}, Glu^{261}	Met^{134}, Gly^{138}, Glu^{265}	Met^{126}, Gly^{130}, Glu^{257}	Met^{128}, Gly^{132}, Glu^{259}	Met^{128}, Gly^{132}, Glu^{259}	Met^{128}, Gly^{132}, Glu^{259}
Qi site of *bc*1 complex	antimycin A	Asn^{31}, Ser^{34}, Gly^{37}, Met^{221}, Phe^{225}, Lys^{228}, Asp^{229}	Ser^{34}, His^{202}, Lys^{228}, Asp^{229}	His^{192}, Asp^{218}	His^{193}, Asp^{220}	His^{185}, Asp^{212}	His^{187}, Asp^{214}	His^{187}, Asp^{214}	His^{187}, Asp^{214}
	ELQ-300	Ile^{26}→Leu, Asp^{229}	Ile^{26}, Asp^{229}	Ile^{22}, Asp^{218}	Asp^{220}	Ile15, Asp^{212}	Asp^{214}	Asp^{214}	Asp^{214}
Qi and Qo sites of *bc*1 complex	NQNO	Trp^{30}, Asn^{31}, Gly^{33}, Gly^{37}, His^{204}, Ser^{206}, Met^{221}, Phe^{225}	His^{202}, Ser^{206}, Asp^{229}	His^{192}, Ser^{196}, Asp^{218}	His^{193}, Ser^{197}, Asp^{220}	His^{185}, Ser^{189}, Asp^{212}	His^{187}, Ser^{191}, Asp^{214}	His^{187}, Ser^{191}, Asp^{214}	His^{187}, Ser^{191}, Asp^{214}

Figures

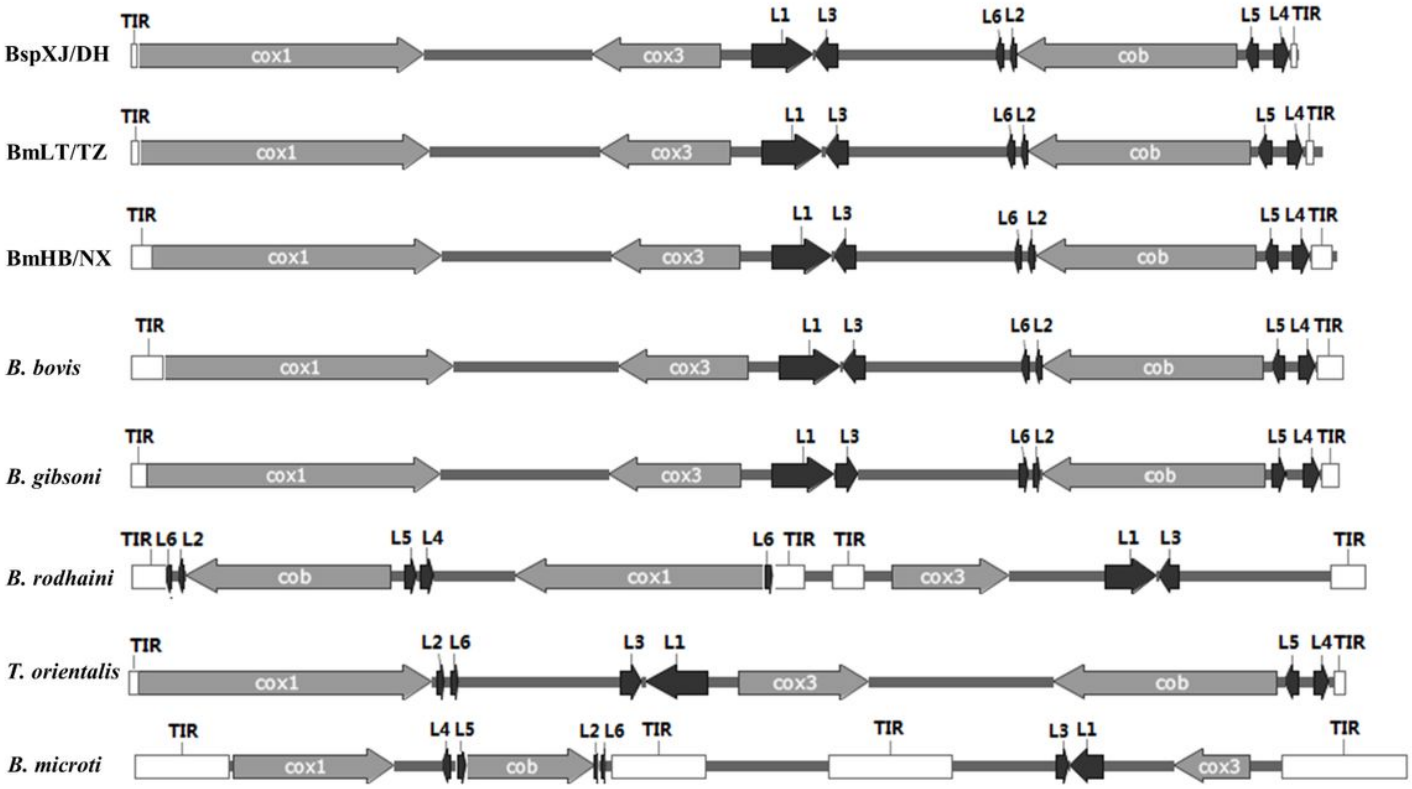

Figure 1

Comparison of the mitochondrial genomes of six ovine Babesia isolates and other apicomplexan parasites. It was performed using SnapGene software and Adobe Photoshop. The different shades of gray represent different gene types. In detail, white represents TIR, 40% grey represents protein-encoding genes, and 80% grey represents LSU

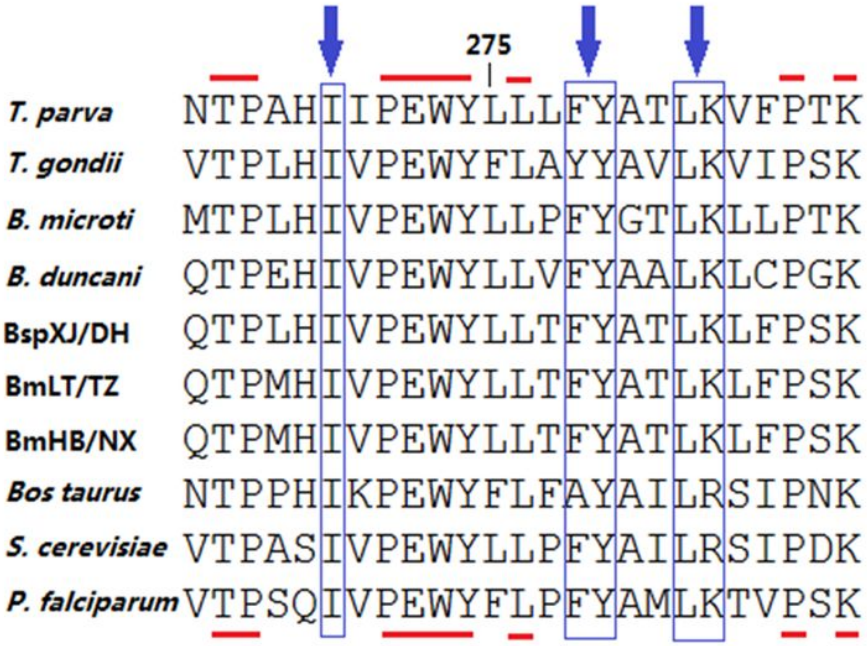

Figure 2

Amino acid sequence alignment of the conserved PEWY region of the cytochromes b of six ovine Babesia, Theileria parva, Toxoplasma gondii, B. microti, Plasmodium falciparum, Saccharomyces cerevisiae, Bos taurus and B. duncani. The alignment was constructed using MegAlign. The number 275 points to the residues at position Leu275 of S. cerevisiae. The residue at position 275 is a key determinant of efficacy of ligand (atovaquone) binding to the bc1 complex. Arrows indicate amino acid positions altered in atovaquone-resistant parasites. Red horizontal lines indicate positions that are completely conserved in all species.

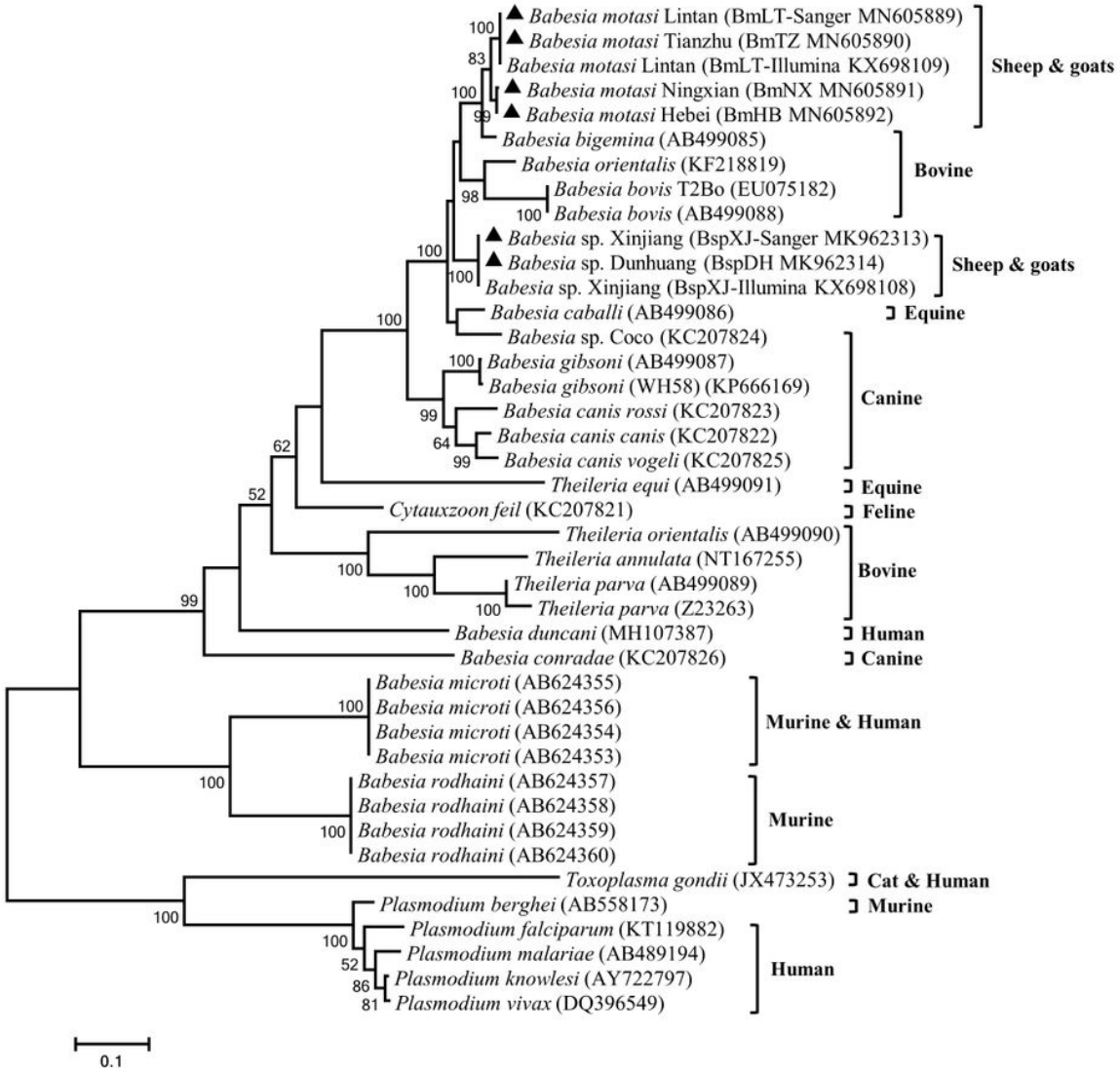

Figure 3

Phylogenetic relationships of Babesia infective to small ruminants in China and other apicomplexan parasites. Phylogeny was inferred with a maximum likelihood analysis of amino acid sequence of cox1 and cob genes based on distances calculated with the JTT with Freqs model. Bootstrap values > 50% from 1000 replicates are shown on the nodes. Babesia obtained in this study is shown as triangles

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