



Research paper

Mitochondrial genomes of two *Babesia* taxa from sheep in China as a foundation for population genetic and epidemiological investigations☆

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ABSTRACT

Here, we sequenced, assembled and annotated the mitochondrial (mt) genomes of two operational taxonomic units of *Babesia* from sheep from China using a deep sequencing-coupled approach. Then, we defined and compared the gene order of these mt genomes (~5.8 to 6.2 kb in size), assessed sequence differences in mt genes among *Babesia* taxa and evaluated genetic relationships among these taxa and related apicomplexans (*Theileria*) for which mt genomic data sets were available. We also identified mt genetic regions that might be useful as markers for future population genetic and molecular epidemiological studies of *Babesia* from small ruminants. We propose that the sequencing-bioinformatic approach used here should be applicable to a wide range of protists of veterinary importance.

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1. Introduction

Babesiosis is an economically important disease caused by tick-borne apicomplexan protists of the genus *Babesia* (Schnittger et al., 2012; Uilenberg, 2006). This disease is characterised principally by haemolytic anaemia, haemoglobinuria, fever and icterus (Yabsley and Shock, 2013). Most economic impact worldwide appears to relate to babesiosis of cattle (Gohil et al., 2013; Schnittger et al., 2012), caused by *Babesia bovis*, *B. bigemina* and *B. divergens*, but the socioeconomic importance of babesiosis in small ruminants is also acknowledged to be considerable (Uilenberg, 2006).

In sheep and goats, the main causative agents are *B. ovis*, *B. motasi* and *B. crassa* (transmitted by ticks of the genera *Rhipicephalus* and *Haemaphysalis*) (Uilenberg, 2006), each of which can cause relatively severe disease. However, in China other distinct taxa of ovine *Babesia* have been recorded. For instance, *Babesia* sp. Lintan (*Bl*) (Guan et al., 2002) and *Babesia* sp. Xinjiang (*Bx*) (Guan et al., 2001) have been isolated, and are reported to display marked differences in vector specificity

and virulence/pathogenicity (cf. Liu et al., 2007). Interestingly, while the former taxon is transmitted by *Haemaphysalis* spp. and causes mild to severe disease, the latter uses *Hyalomma anatolicum* and is associated with subclinical infection (Liu et al., 2007).

Although some basic research has been conducted to provide insights into the biology, epidemiology and immunology of *Bl* and *Bx* (Bai et al., 2002; Guan et al., 2001, 2002, 2010b, 2012b, 2012c; Niu et al., 2016), there have been few genetic studies (Guan et al., 2010a, 2015; Niu et al., 2013; Yin et al., 1997) and no genomic investigations of these two or any other *Babesia* taxa from small ruminants. Exploring their mitochondrial (mt) genomes would provide an avenue to better explore their systematic status and also defining a repertoire of genetic markers for population genetic studies (cf. Hikosaka et al., 2013). Previous studies have sequenced and characterised the mt genomes of *B. bigemina*, *B. bovis*, *B. orientalis* (bovids); *B. caballi* (equids); *B. gibsoni* (canids); *B. microti* and *B. rodhaini* (rodents) (Brayton et al., 2007; Cornillot et al., 2012; He et al., 2014; Hikosaka et al., 2010, 2012). While most of these studies used PCR and/or cloning-based approaches (He et al., 2014; Hikosaka et al., 2010, 2012), some have utilised direct, deep sequencing of total genomic DNA (Brayton et al., 2007; Cornillot et al., 2012).

In the present study, we employed Illumina technology to directly sequence the mt genomes of *Bl* and *Bx* from genomic DNA, and a custom bioinformatics platform to annotate them. Using the mt genomic data sets, we then undertook a phylogenetic analysis to assess the

☆ Note: Nucleotide sequence data reported in this article are publicly available in the GenBank database under accession nos. PRJNA338323 and PRJNA338210.

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relationships of *Bl* and *Bx* with all *Babesia* species and other related piroplasmids (i.e., *Theileria*) for which mt genomic data were publicly available. Finally, we discussed the implications of these data sets of *Bl* and *Bx* for future epidemiological and population genetic applications, and the applicability of the present sequencing-bioinformatic approach for protists of veterinary importance.

2. Materials and methods

2.1. Parasite materials and isolation of genomic DNA

Merozoites representing clonal lines of each *Babesia* sp. Lintan and *Babesia* sp. Xinjiang (designated *Bl* and *Bx*, respectively) were maintained in sheep erythrocytes in a continuous in vitro culture, and amplified in parasite-free, splenectomised sheep (Guan et al., 2012a); animal experiments were approved (permit SYXK2010-0001) by the Science and Technology Department of Gansu province, China. Merozoites were purified from blood as described previously (Guan et al., 2012a), and high molecular genomic DNA was isolated using the Gentra Puregene kit (Qiagen) according to the manufacturer's protocol. DNA amounts were measured using a fluorometer (Qubit, Invitrogen), and genomic DNA quality was verified by agarose gel electrophoresis and using a BioAnalyzer (2100, Agilent).

2.2. Sequencing of mt genomes, assembly and annotation

For each *Bl* and *Bx*, one paired-end (500 bp insert size) and two mate-pair (2 kb and 5 kb) libraries (Illumina) were built from high molecular weight genomic DNA, assessed for quality and size distribution using a BioAnalyzer and then sequenced using Illumina technology (HiSeq; 2×100 reads for paired-end libraries, and 2×49 reads for mate-pair libraries). For each taxon, the genomic reads were filtered for quality using the program Trimmomatic v.0.36 (Bolger et al., 2014), assembled using the program SPAdes v.3.5.0 (Bankevich et al., 2012) and scaffolded using the program SSPACE v.3.0 (Boetzer et al., 2011). The mt genomes were extracted from genomic assemblies and annotated using an established approach (Hikosaka et al., 2012). In brief, each protein-encoding mt gene was identified by local alignments (six reading frames) using amino acid sequences conceptually translated from corresponding genes from each mt genome of *B. bovis* (accession nos. EU075182 and AB499088; Brayton et al., 2007; Hikosaka et al., 2010) and *B. bigemina* (AB499085; Hikosaka et al., 2010). To predict ribosomal RNA genes, mt DNA sequences from *B. bovis* (EU075182 and AB499088) and *B. bigemina* (AB499085) were used as queries employing suggested algorithm parameters (Freyhult et al., 2007) in NCBI BLAST 2.2 (Altschul et al., 1990). Inverted repeat sequences were identified using a 'self-against-self' BLASTN search (Altschul et al.,

1997) employing a threshold of >20 nucleotides. Annotated sequence data were imported using the program SEQUIN (available via <http://www.ncbi.nlm.nih.gov/Sequin/>) for the final verification of the mt genome organization/annotation prior to submission to the GenBank database.

2.3. Sliding window analysis

Sliding window analysis was performed on aligned, concatenated nucleotide sequences of all genes of complete mt genomes representing nine recognised taxa of *Babesia* (cf. Table 1) using the program DnaSP v.5 (Rozas et al., 2003). The sequences were aligned using the program MUSCLE v.3.8 (Edgar, 2004); keeping the nucleotides in frame, there were no ambiguously aligned regions. A sliding window of 300 bp (10 bp-steps) was used to estimate nucleotide diversity (π) within and among members (pairwise) of each *Babesia* taxon using DnaSP v.5. Nucleotide diversity for the alignments was plotted against mid-point positions, and gene boundaries were defined. Separating the analyses in this manner allowed a pairwise comparison of general patterns among *Babesia* taxa as well as the identification of conserved regions and areas with potential for the definition of mt genetic markers with low, medium or high variability among taxa.

2.4. Phylogenetic analysis

Nucleotide or amino acid sequence conceptually translated from the protein-encoding genes from each of the mt genomes (cf. Table 1) were aligned using MUSCLE, ensuring accurate alignment of homologous characters. Aligned blocks of sequences were concatenated, and the alignment was manually adjusted. Subsequently, phylogenetic analysis of sequence data was conducted by Bayesian inference (BI) using Monte Carlo Markov Chain (MCMC) analysis in MrBayes v.3.2.3 (Huelsenbeck and Ronquist, 2001). Sequence data for the *cox3* gene were excluded from phylogenetic analysis, due to high divergence among *Babesia* species (see Section 3). The likelihood parameters set for BI analysis were based on the Akaike Information Criteria (AIC) test in jModeltest v.2.1.7 (Darriba et al., 2012). The number of substitutions (Nst) was set at 6, with a gamma-distribution and a proportion of invariable sites. Posterior probability (pp) values were calculated by running 2,000,000 generations with four simultaneous tree-building chains. Trees were saved every 100th generation. At the end of each run, the standard deviation of split frequencies was <0.01, and the potential scale reduction factor approached one. A 50% majority rule consensus tree for each analysis was constructed based on the final 75% of trees generated by BI. To ensure convergence and insensitivity to priors, analyses were run three times. *Plasmodium falciparum* was used as the outgroup. Unrooted trees were viewed in the program FigTree ([**Table 1**](http://</p>
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Taxa of piroplasmids, their host and geographical origins, and mt genomes used in the present study.

Taxon	Original host	Country of origin	Mt genome size (bp)	Accession	Reference:
<i>Babesia</i> sp. Lintan (<i>Bl</i>)	Bovidae (<i>Ovis</i>)	China	5790	PRJNA338323	This study
<i>Babesia</i> sp. Xinjiang (<i>Bx</i>)	Bovidae (<i>Ovis</i>)	China	6020	PRJNA338210	This study
<i>Babesia bovis</i>	Bovidae (<i>Bos</i>)	United States	6005	EU075182	Brayton et al. (2007)
<i>B. bovis</i>	Bovidae (<i>Bos</i>)	Japan	5970	AB499088	Hikosaka et al. (2010)
<i>B. bigemina</i>	Bovidae (<i>Bos</i>)	Japan	5924	AB499085	Hikosaka et al. (2010)
<i>B. orientalis</i>	Bovidae (<i>Bubalus</i>)	China	5996	KF218819	He et al. (2014)
<i>B. caballi</i>	Equidae (<i>Equus</i>)	United States	5847	AB499086	Hikosaka et al. (2010)
<i>B. gibsoni</i>	Canidae (<i>Canis</i>)	Japan	5865	AB499087	Hikosaka et al. (2010)
<i>B. microti</i>	Rodentia (<i>Mus</i>)	Germany	11,109	AB624353	Hikosaka et al. (2012)
<i>B. rodhaini</i>	Rodentia (<i>Mus</i>)	Australia	6929	AB624357	Hikosaka et al. (2012)
<i>Theileria annulata</i>	Bovidae (<i>Bos</i>)	Turkey	5905	NT167255	Pain et al. (2005)
<i>T. parva</i>	Bovidae (<i>Bos</i>)	Kenya	5895	AB499089	Hikosaka et al. (2010)
<i>T. orientalis</i>	Bovidae (<i>Bubalus</i>)	Japan	5957	AB499090	Hikosaka et al. (2010)
<i>T. equi</i>	Equids (<i>Equus</i>)	United States	8246	AB499091	Hikosaka et al. (2010)
<i>Plasmodium falciparum</i>	Hominidae (<i>Homo</i>)	India	5967	KT119882	Tyagi and Das (2015)

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