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Mitochondrial genome of *Babesia orientalis*, apicomplexan parasite of water buffalo (*Bubalus bubalis*, Linnaeus, 1758) endemic in China

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Abstract

Background: Apicomplexan parasites of the genus *Babesia*, *Theileria* and *Plasmodium* are very closely related organisms. Interestingly, their mitochondrial (mt) genomes are highly divergent. Among *Babesia*, *Babesia orientalis* is a new species recently identified and specifically epidemic to the southern part of China, causing severe disease to water buffalo. However, no information on the mt genome of *B. orientalis* was available.

Methods: Four pairs of primers were designed based on the full genome sequence of *B. orientalis* (unpublished data) and by aligning reported mt genomes of *B. bovis*, *B. bigemina*, and *T. parva*. The entire mt genome was amplified by four sets of PCR. The obtained mt genome was annotated by aligning with published apicomplexan mt genomes and Artemis software v11. Phylogenetic analysis was performed by using *cox1* and *cob* amino acid sequences.

Results: The complete mt genome of *B. orientalis* (Wuhan strain) was sequenced and characterized. The entire mt genome is 5996 bp in length with a linear form, containing three protein-coding genes including cytochrome c oxidase I (*cox1*), cytochrome b (*cob*) and cytochrome c oxidase III (*cox3*) and six rRNA large subunit gene fragments. The gene arrangement in *B. orientalis* mt genome is similar to those of *B. bovis*, *B. gibsoni* and *Theileria parva*, but different from those of *T. orientalis*, *T. equi* and *Plasmodium falciparum*. Comparative analysis indicated that *cox1* and *cob* genes were more conserved than *cox3*. Phylogenetic analysis based on amino acid sequences of *cox1*, *cob* and *cox1 + cob*, respectively, revealed that *B. orientalis* fell into *Babesia* clade with the closest relationship to *B. bovis*.

Conclusions: The availability of the entire mt genome sequences of *B. orientalis* provides valuable information for future phylogenetic, population genetics and molecular epidemiological studies of apicomplexan parasites.

Keywords: *Babesia orientalis*, Mitochondrion, Mitochondrial genome, Phylogeny

Background

Mitochondria are essential organelles within cells and are responsible for energy transduction, metabolism, cell growth and survival [1-3]. Inside mitochondria, there is a genome called mitochondrial (mt) genome. Mt genomes are present in almost all eukaryotic cells and have remarkable variations in size, structure, and organization [4-6].

The largest mt genome has been found in muskmelons with an estimated size of 2400 kb [7-9]. The smallest mt genome of only 6 kb in length has been reported in an apicomplexan parasite (*Plasmodium*) [10,11].

The structure of mt genome contains two major types, the linear form and the circular form. The circular forms are usually present in animal mt genomes with the size ranging from 15 kb to 20 kb, containing 12-13 protein-coding genes, 22 transfer RNA (tRNA) genes and two ribosomal RNA (rRNA) genes, and gene arrangements in the genomes are extremely stable [12]. The linear forms have been documented in many apicomplexan parasites,

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including *Plasmodium*, *Babesia*, *Theileria* and *Eimeria* [5,10,11,13]. Compared with animal mt genomes, the mt genomes of apicomplexan parasites encode only three protein-coding genes (cytochrome c oxidase subunits I [*cox1*] and III [*cox3*], and cytochrome b [*cob*]) and six fragments of large subunit rRNA genes [14]. The gene arrangements are also different among animal mt genomes and apicomplexan parasites mt genome.

Meanwhile, in apicomplexan parasites, mitochondrial protein-coding genes have been extensively used as genetic markers for phylogenetic analysis at different taxonomic levels, serving as an ideal model for gene rearrangement, and evolutionary studies [13,15]. Most of the phylogenies of apicomplexan parasites were constructed using *cox1* or *cob* alone, however, in some cases, a combination of *cox1* and *cob* was employed to evaluate the phylogenetic relationships [13,16]. In addition, mt genome sequences are also very valuable for population genetic studies [17-19] as reported in *Plasmodium vivax*, *Plasmodium knowlesi*, *Trypanosoma cruzi* [20-22].

B. orientalis is a tick-borne, intra-erythrocytic protozoan parasite causing buffalo babesiosis characterized by fever, anemia, icterus, haemoglobinuria and high mortality [23,24]. This species was first reported in 1987 and then identified as a new species named *Babesia orientalis* in 1997 [25]. The new species was discovered initially based on the differences in morphology, transmission, pathogenicity and endemic areas, compared to *Babesia bigemina* and *Babesia bovis* [25], and later confirmed by the phylogenetic analysis based on 18S rRNA and heat shock protein 70 (HSP70) genes [26,27]. The disease caused by *B. orientalis* is one of the most important parasitic diseases of buffalo in central and south China, resulting in enormous economic losses [27,28]. In spite of its importance, very limited information was available about this parasite, especially at the molecular level, including mt genome sequences and structures.

In the present study, *B. orientalis* (Wuhan strain) mt genome was determined and annotated. The structure was characterized and compared with those of related species. In addition, the evolution of structural divergence in the apicomplexan mt genomes was discussed.

Methods

Parasite cultivation

Babesia orientalis (Wuhan strain) was cultivated according to the protocol of He et al. [29]. In brief, two, 1-year-old water buffalo, free of *B. orientalis* infection as confirmed by microscopy and real-time PCR [29], were splenectomized 14 days prior to *B. orientalis* infection. Each buffalo was subcutaneously injected with 4 ml of *B. orientalis*-infected blood (Wuhan strain, percentage parasitized erythrocytes, PPE 1%). Blood samples were

collected everyday to monitor the parasitemia until PPE reached 3%.

All the experimental animals were housed, fed and given clean drinking water in accordance with the stipulated rules for the regulation of the administration of affairs concerning experimental animals of P.R. China.

B. orientalis mitochondria DNA sequencing

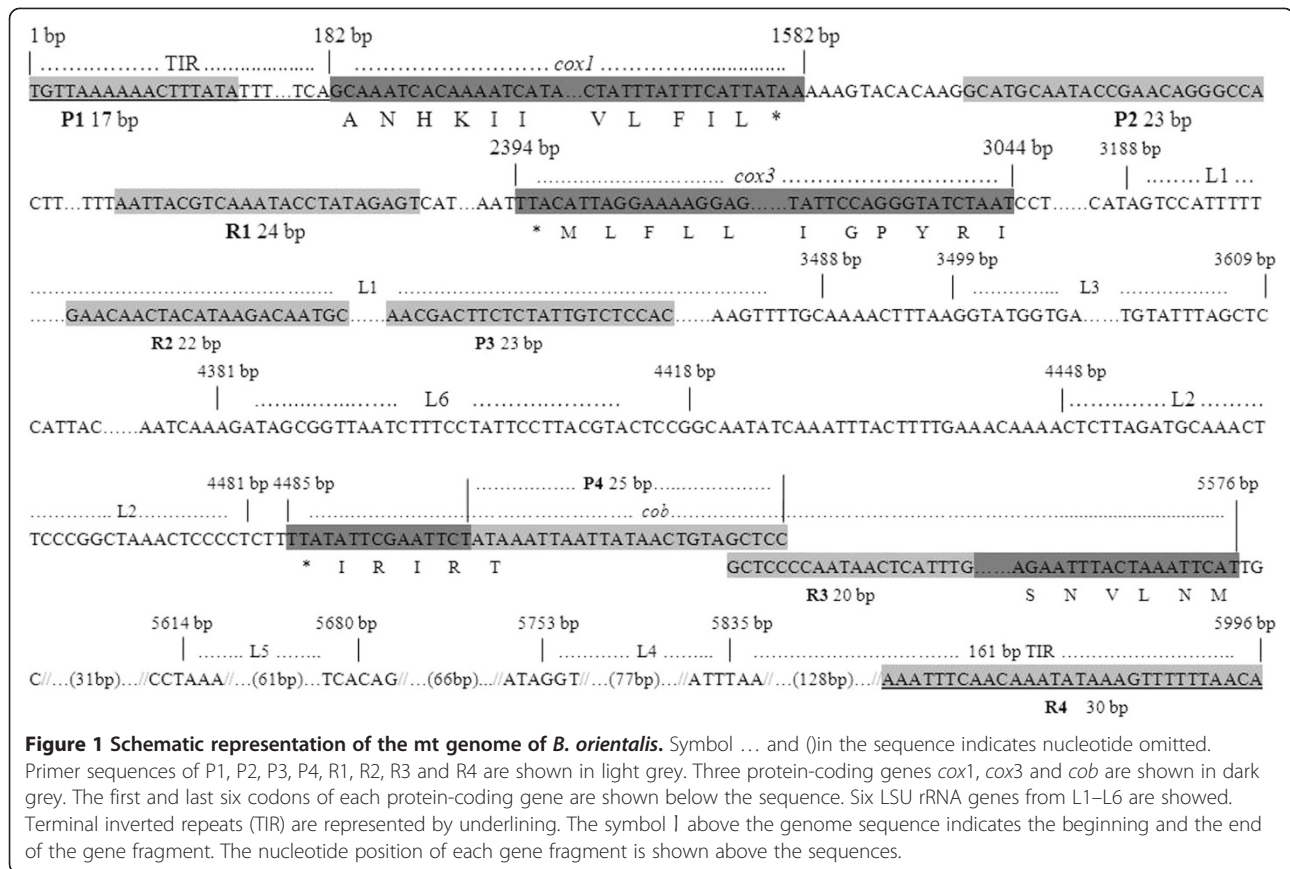
The blood from experimentally infected buffalo was collected in EDTA. Parasite genomic DNA was extracted from *B. orientalis*-infected blood using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The terminal inverted repeat (TIR) regions in the beginning and end of *B. orientalis* mt genome, 1-181 bp and 5835-5996 bp, were obtained by using *B. bovis* mt genome sequence to blast the full genome sequence of *B. orientalis* (unpublished data). Four pairs of primers were designed based on these two mtDNA sequences (1-181 bp and 5835-5996 bp) of *B. orientalis* and by aligning reported mt genomes of *B. bovis* (EU075182 and AB499088), *B. bigemina* (AB499085), and *T. parva* (Z23263 and AB499089) (Table 1 and Figure 1). The entire mt genome was amplified by P1 and R4. In order to confirm the mt genome, four sets of PCR were processed using primers P1 and R1, P2 and R2, P3 and R3, P4 and R4, respectively (Figure 1). Amplified products were purified and then ligated into the pMD19-T vector (TaKaRa Biotechnology), and the recombinant clones were sequenced using the ABI PRISM 377 DNA sequencer by following the manufacturer's instructions. The vector primers M13 (-47) and M13 (-48), as well as PCR primers, were used for the sequencing of mt genome.

Gene annotation and sequence analysis

Nucleotide sequences of *B. orientalis* mt genome (GenBank accession no. KF218819) were aligned with published mt genome sequences of *Plasmodium falciparum* (M76611), *B. bovis* (AB499088), *T. parva* (AB499089), and *T. annulata* (NT167255) by MAFFT (version 7) [30,31] with manual corrections. Protein-coding genes

Table 1 Primers used for cloning of *Babesiaorientalis* mt genome sequence

Primers	Sequences	Position
P1	5'-TGTTAAAAAAGCTTTATA-3'	1-17
R1	5'-ACTCTATAGGTATTTGACGTAATT-3'	1749-1726
P2	5'-GCATGCAATACCGAACAGGGCCA-3'	1592-1617
R2	5'-GCATTGTCTTATGTAGTTGTC-3'	3246-3225
P3	5'-AACGACTTCTCTATTGTCTCCAC-3'	3368-3390
R3	5'-CAAATGAGTTATTGGGGAGC-3'	5198-5179
P4	5'-ATAAATTAATTATACTGTAGCTCC-3'	5159-5183
R4	5'-TGTTAAAAAAGCTTTATATTTGTTGAAATTT-3'	5967-5996



were predicted by comparing the previously annotated sequences from these four related species. To identify putative rRNA genes, mt DNA sequences or annotated rRNA gene fragments from the four related species were used as queries by pair-wise comparison (Blastn) in NCBI. The entire *B. orientalis* mt genome was subjected to tRNAscan SE 1.21 (<http://lowelab.ucsc.edu/tRNAscan-SE/>) using Mito/Chloroplast model option and Nematode Mito model for analyzing the existence of tRNA gene. Results from both models were compared, final annotation was determined according to *B. bovis* mt genome annotation. *B. orientalis* mt genome was also annotated using appropriate mitochondrial codes in Artemis software v11 [32,33].

Nucleotide sequence identities were calculated by pair-wise comparison between 13 apicomplexan parasites including *B. orientalis*, another six *Babesia* species, five *Theileria* species and *P. falciparum* for each of the three protein-encoding genes. The data sets of *cox1* (472 aa), *cox3* (229 aa) or *cob* (358 aa) were aligned using MAFFT (version 7) employing the FFT-NS-i algorithm [31,34]. The alignment was manually edited using BioEdit 7.1.11 [35]. The nucleotide identities were determined through BioEdit 7.1.11.

Phylogenetic analysis

The concatenated amino acid sequences of *cox1*, *cox3* and *cob* from 15 apicomplexan parasites (Table 2) were used for similarity analysis. For phylogenetic analysis, *cox3* was not included, because *cox3* has been presented

Table 2 Mitochondrial genome sequences of apicomplexan parasites used in the present study

Family	Species	Size of mt DNA (bp)	GenBank accession no.
Babesiidae	<i>B. orientalis</i>	5996	KF218819
	<i>B. bovis</i>	6005	EU075182
	<i>B. bovis</i>	5970	AB499088
	<i>B. bigemina</i>	5924	AB499085
	<i>B. caballi</i>	5847	AB499086
	<i>B. gibsoni</i>	5865	AB499087
Theileriidae	<i>B. rodhaini</i>	6929	AB624357
	<i>T. parva</i>	5895	Z23263
	<i>T. parve</i>	5924	AB499089
	<i>T. equi</i>	8246	AB499091
	<i>T. orientalis</i>	5957	AB499090
Plasmodiidae	<i>T. annulata</i>	5905	NT167255
	<i>P. falciparum</i>	5967	M76611

in the nuclear genome rather than in mt DNA in some species, such as *T. thermophila*. A free-living ciliate, *Tetrahymena thermophila* [36], was used as the outgroup. The best-fit model of *cox1*, *cob*, and *cox1-cob* combined nucleotide substitution was determined by JmodelTest 0.1.1 [37] selected by AIC calculations. A general time reversible model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites (TIM1 + I + G) substitution mode was used in PAUP* v4b10 [38] to explore neighbour-joining, parsimony and maximum likelihood methods. MrBayes v3.1.2 [39,40] was used to explore Bayesian phylogeny. Consensus trees were edited in MEGA v4.0.2 [41].

All water buffalo studies were carried out in compliance with the regulations (No. 5 proclaim of the Standing Committee of Hubei People's Congress) approved by the Standing Committee of Hubei People's Congress, P. R. China. The animal protocols were approved by Laboratory Animals Research Centre of Hubei province and the ethics committee of Huazhong Agricultural University (Permit number: 4200696657).

Results and discussion

Characterization of *B. orientalis* mt genome

The full length mt genome of *B. orientalis* was amplified by P1 and R4, and a 5996 bp fragment was obtained. In

order to confirm the mt genome sequence, four overlapping fragments were amplified by using primer sets P1-R1, P2-R2, P3-R3 and P4-R4 with expected sizes of 1749 bp, 1655 bp, 1831 bp and 838 bp, respectively. These four overlapping fragments covered the entire genome of *B. orientalis* (Figure 1). Each of the PCR products were then cloned into pMD19-T vector, and sequenced. A 5996 bp mt genome sequence of *B. orientalis* (Wuhan strain) was obtained by assembling all the sequenced fragments.

Sequence analysis indicated that *B. orientalis* mt genome was arranged in a linear form. It containing three protein-coding genes, *cox1* (cytochrome *c* oxidase I), *cob* (cytochrome *b*), *cox3* (cytochrome *c* oxidase III) and six large subunit (LSU) rRNA gene fragments, but not any tRNA genes, which is consistent with those of other apicomplexan parasites studied to date (Figures 1 and 2) [11]. The mt genomes of apicomplexan parasites usually contain terminal inverted repeat (TIR) sequences with the size of around 440-450 bp [5]. However, in the mt genome of *B. orientalis*, 181 bp and 161 bp TIRs were identified from the beginning and the end, respectively (Figure 1). *cox1*, the first and fourth rRNA large subunit fragments (L1 and L4) are encoded by one strand of the mt genome, whereas *cox3*, *cob*, the second, the third, fifth and sixth fragments of large subunit rRNA genes (L2, L3, L5 and L6) are encoded by another strand

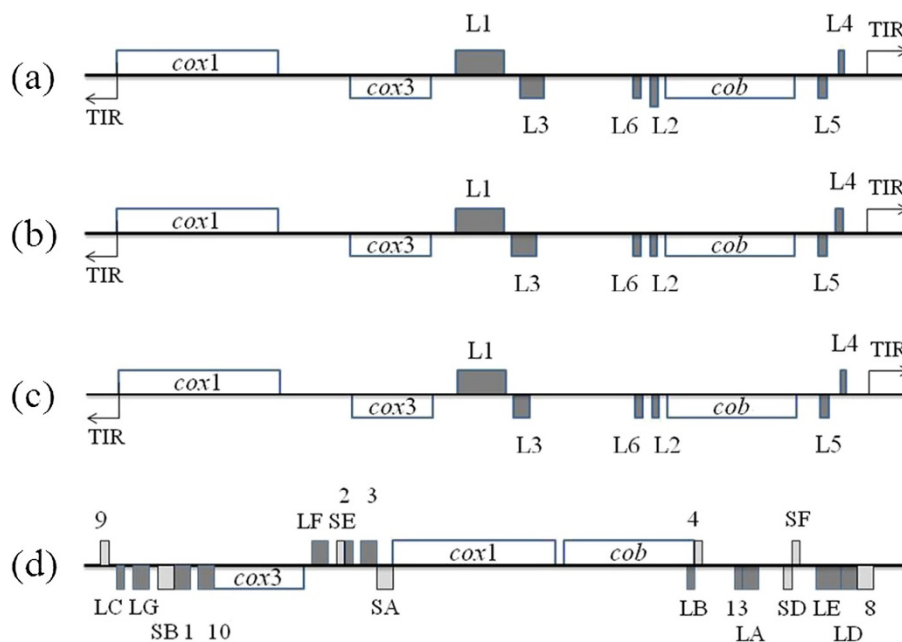


Figure 2 Schematic structure of mitochondrial genomes of *Babesia orientalis* (KF218819) (a) and three apicomplexan parasites including (b) *B. bovis* (AB499088), (c) *Theileria parva* (Z23263) and (d) *Plasmodium falciparum* (M76611). Gene names above the bold line are transcribed from left to right, and those below are transcribed from right to left. White boxes indicate protein-coding genes, *cox1* (cytochrome *c* oxidase I), *cox3* (cytochrome *c* oxidase III), and *cob* (cytochrome *b*). Fragments of LSU (L1 – L6, LA – LG, 1, 2, 3, 10 and 13) and SSU (SA, SB, SD – SF, 4, 8 and 9) rRNA genes are shown by dark and light gray boxes. TIR represents terminal inverted repeats.

Table 3 Nucleotide sequence identity (percentage) between *Babesiaorientalis* and 12 apicomplexan parasites

(a) Identity analyses based on the sequences of *cox1*

Species	1	2	3	4	5	6	7	8	9	10	11	12
1. <i>B. orientalis</i> (KF218819)												
2. <i>B. bovis</i> (EU075182)	80.6											
3. <i>B. bovis</i> (AB499088)	80.6	99.8										
4. <i>B. bigemina</i> (AB499085)	82.4	81.1	81.2									
5. <i>B. caballi</i> (AB499086)	82.2	80.9	81.1	85.0								
6. <i>B. gibsoni</i> (AB499087)	81.6	79.0	79.2	81.6	82.9							
7. <i>B. rodhaini</i> (AB624357)	68.4	67.0	67.0	66.3	67.5	69.5						
8. <i>T. parva</i> (Z23263)	73.9	71.2	71.1	72.7	72.8	74.3	66.5					
9. <i>T. parva</i> (AB499089)	74.8	72.1	72.0	73.6	73.7	75.2	67.4	98.6				
10. <i>T. equi</i> (AB499091)	38.9	39.3	39.3	38.9	39.0	41.0	38.7	39.1	39.3			
11. <i>T. orientalis</i> (AB499090)	74.0	72.1	72.1	73.0	73.5	74.6	66.9	78.1	79.1	38.0		
12. <i>T. annulata</i> (NT167255)	71.7	68.9	68.9	70.6	70.7	71.3	63.3	84.0	83.8	37.2	74.3	
13. <i>P. falciparum</i> (M76611)	61.1	60.9	60.9	60.2	60.2	62.1	61.5	59.6	60.1	36.0	60.0	58.0

(b) Identity analyses based on the sequences of *cox3*

1. <i>B. orientalis</i> (KF218819)												
2. <i>B. bovis</i> (EU075182)	80.9											
3. <i>B. bovis</i> (AB499088)	74.2	92.5										
4. <i>B. bigemina</i> (AB499085)	68.7	68.1	73.7									
5. <i>B. caballi</i> (AB499086)	65.6	67.2	72.4	77.6								
6. <i>B. gibsoni</i> (AB499087)	64.7	63.3	67.6	71.4	72.7							
7. <i>B. rodhaini</i> (AB624357)	34.4	34.6	34.2	33.0	32.2	32.2						
8. <i>T. parva</i> (Z23263)	53.1	52.3	47.5	47.5	49.3	49.4	28.3					
9. <i>T. parva</i> (AB499089)	54.1	53.7	56.8	56.8	59.0	59.1	31.6	83.5				
10. <i>T. equi</i> (AB499091)	29.6	29.8	29.7	28.8	29.3	31.8	32.8	29.5	33.3			
11. <i>T. orientalis</i> (AB499090)	33.0	34.3	34.4	35.8	32.8	34.3	51.6	28.4	31.8	33.0		
12. <i>T. annulata</i> (NT167255)	53.1	52.9	48.3	48.1	48.8	50.5	29.4	82.6	67.0	29.4	29.3	
13. <i>P. falciparum</i> (M76611)	45.8	44.4	42.3	43.5	43.2	46.6	31.7	40.6	43.2	32.5	32.5	41.3

(c) Identity analyses based on the sequences of *cob*

1. <i>B. orientalis</i> (KF218819)												
2. <i>B. bovis</i> (EU075182)	81.7											
3. <i>B. bovis</i> (AB499088)	81.7	99.9										
4. <i>B. bigemina</i> (AB499085)	81.0	80.3	80.4									
5. <i>B. caballi</i> (AB499086)	82.0	80.6	80.7	84.6								
6. <i>B. gibsoni</i> (AB499087)	78.1	77.1	77.0	78.2	78.5							
7. <i>B. rodhaini</i> (AB624357)	33.7	34.8	34.8	34.1	32.8	34.2						
8. <i>T. parva</i> (Z23263)	58.5	59.0	58.9	58.9	58.7	60.1	30.9					
9. <i>T. parva</i> (AB499089)	62.2	62.7	62.6	62.6	62.4	63.9	32.7	94.0				
10. <i>T. equi</i> (AB499091)	69.3	68.2	68.1	68.0	68.8	67.7	33.6	58.2	61.8			
11. <i>T. orientalis</i> (AB499090)	61.2	60.6	60.5	61.9	62.0	63.2	33.3	56.5	60.1	61.3		
12. <i>T. annulata</i> (NT167255)	62.7	62.2	62.1	62.6	62.5	64.1	32.9	68.8	73.1	61.7	60.6	
13. <i>P. falciparum</i> (M76611)	33.9	33.9	33.9	33.9	34.0	34.7	56.0	32.9	34.9	32.6	33.5	33.5

(Figure 2a). The arrangement and predicted transcriptional direction of three protein-coding genes are the same as that of *B. bovis* (Figure 2b), *B. gibsoni* (not shown), and *Theileria parva* (Figure 2c), however, it greatly differed from that of *T. orientalis*, *T. equi* (not shown) and *P. falciparum* (Figure 2d) [5].

In this study, BLAST analysis of the entire mt genome sequence of *B. orientalis* to NCBI databases revealed that the mt genome sequence of *B. orientalis* was most similar to those of *B. bovis* (EU075182 and AB499088), with an identity of 87%. The other related species were *B. caballi*, *B. bigemina* and *B. gibsoni*, showing their mt genome sequence identities of 86%, 85% and 84% with that of *B. orientalis*, respectively. However, nucleotide identities were greatly different for the sequences of three protein-coding genes *cox1*, *cox3* and *cob*, when compared among different species. Pairwise comparison has been carried out among 13 different apicomplexan species including *B. orientalis*, another six *Babesia* species, five *Theileria* species and *P. falciparum* (see Table 3). The nucleotide sequence differences among 13 species ranged from 38.7% to 99.8% for *cox1* (Table 3a), from 28.8% to 92.5% for *cox3* (Table 3b) and 32.7% to 99.9% for *cob* (Table 3c), respectively. These results indicated that *cox1* and *cob* genes were more conserved than *cox3* gene. The different extent of conservation in the protein-coding genes suggested that mt genome sequences could be used as a gene marker for population evolutionary studies.

Phylogenetic analysis

The majority of phylogenetic studies in the phylum apicomplexa have utilized 18S rRNA genes [27,42], which allow the analysis of the ancient relationships or strains and species differentiation by focusing on the highly conserved regions defining the critical secondary structure, or the more variable internal transcribed spacer regions, respectively. Because of different extent of conservation of three protein-coding genes and important function of mt genome, the extent of sequence difference might reflect the phylogenetic relationship across species of apicomplexan parasites. So the mitochondrion sequences may provide an alternative approach to conduct these studies [5,10,13].

To analyze the phylogenetic relationship of *B. orientalis* with other apicomplexan parasites, phylogenetic trees were constructed with the amino acid sequences of *cox1*, *cob* and *cox1 + cob* using neighbor-joining (NJ), maximum likelihood (ML), maximum parsimony (MP) and Bayesian phylogenetic methods. The trees obtained from three data sets by different methods were consistent with no significant changes in the topology or in the bootstrap values (Figures 3a,b, and c). The NJ trees constructed with *cox1*, *cob* and *cox1 + cob* sequences have

been presented as a representative (Figure 3). All three NJ trees displayed the same topology with high bootstrap values. *B. orientalis* appeared in the *Babesia* clade, and it's most close to *B. bovis*. However, the bootstrap value was higher in the tree constructed by *cox1 + cob* as compared to those of *cox1* and *cob*. These results suggested that the combined amino acid sequences of *cox1* and *cob* may be more reliable in studying evolutionary relationships than the sequences of single gene. The relationship of *B. orientalis* with other apicomplexan parasites revealed by all trees from mt genome sequences were consistent with that from the previous phylogenetic trees based on 18S rRNA and heat shock protein 70 (HSP70) gene [26,27]. These results demonstrated that the mt genome sequences are useful for the phylogenetic studies of apicomplexan parasites.

Conclusion

In this study, we first reported the 5996 bp linear mitochondrion genome of *B. orientalis*. This mt genome contains 6 LSU rRNA gene fragments and three protein-coding genes, but no tRNA gene. Gene arrangement in the mt genome of *B. orientalis* is similar to those of *B. bovis*, *B. gibsoni*, and *T. parva*, but different from those of *T. orientalis*, *T. equi* and *P. falciparum*. Phylogenies based on the amino acid sequences of *cox1* or *cob* alone and *cox1 + cob* combined all indicated that *B. orientalis* is closest to *B. bovis*, which is in agreement with the previous phylogenetic studies of *B. orientalis*. The availability of the entire mt genome sequences of *B. orientalis* provides valuable information for future phylogenetics, population genetics and molecular epidemiological studies of apicomplexan parasites.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All the authors contributed significantly to this study. LH and JLZ designed the experiments, carried out field and laboratory experiments, interpreted the results, and wrote the manuscript. YZ, QLZ, WJZ and HHF participated in cloning experiments and sequence analysis. MKK, MH and YQZ carried out editing and also helped in writing the manuscript. All the authors of the manuscript read and approved the final version of the manuscript.

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