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Insights into the mitochondrial genome structure and phylogenetic placement of *Theileria velifera* in comparison to other apicomplexan parasites

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In this study, we sequenced the complete mitochondrial genome of *Theileria velifera* and compared it with other Apicomplexan parasites. The mitochondrial genome of *T. velifera* is a linear monomer molecule spanning 6,125 bp, and it encodes 3 protein-coding genes (PCGs): *cox1*, *cob*, and *cox3*. Besides, it contains 5 large subunit (LSU) rRNA gene fragments and terminal inverted repeats (TIR) at both ends. Moreover, the mitochondrial genomes of most Apicomplexan parasites in this study are typically around 6,000 bp in length and are linear in structure, featuring three PCGs. The start codons observed in *Thaleria* spp. and *Babesia* spp. parasites predominantly include ATN, GTN, and TTN, while the end codons are mainly TAA, TAG, and TGA. Phylogenetic analysis showed that *T. velifera* was closely related to *T. annulata*, *T. parva*, *T. taurotragi* and *T. lestoquardi*. The complete mitochondrial genome sequence of *T. velifera* was examined and compared to other Apicomplexan parasites in this study, offering fresh perspectives on the evolution and phylogenetic connections among these parasites.

Keywords *Theileria velifera*, Apicomplexa, Mitochondrial genome, Phylogenetic analysis, Evolutionary relationship

Parasites of the genus *Theileria* are blood-borne parasite transmitted by ticks that primarily resides in the red blood cells, lymphocytes, and macrophages of ruminants like bovines, sheep, and equines, leading to theileriosis[1–4]. Clinical manifestations include loss of appetite, fever, jaundice, anemia, hemoglobinuria, and swollen lymph nodes[3,5]. Severe infections can result in death due to delayed intervention or inadequate treatment. Parasites of the genus *Theileria* can be categorized into two types based on their ability to transform host cells: those that can cause infected host cells to multiply indefinitely as the number of parasites increases is referred to as malignant *Theileria*, and those that do not, known as benign *Theileria*[6–8]. Theileriosis, caused by both types, has resulted in significant economic losses in the livestock industry due to high morbidity and mortality rates in animals. In sub-Saharan Africa, countries like Burundi, the Democratic Republic of the Congo, Rwanda, southern Sudan, Tanzania, Uganda, Zambia, and Zimbabwe, experience annual deaths of at least 100,000 cattle, and costs hundreds of millions of dollars in annual economic losses across much of southern Europe, Africa and South Asia[9,10]. Furthermore, given that theileriosis is primarily transmitted by ticks, the expansion of suitable

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habitats for these vector ticks, driven by global environmental and climatic changes, has led to ongoing exposure of animals to infected ticks. Consequently, this situation contributes to the persistent worsening of theileriosis[11]. In addition, although drug treatment can alleviate the clinical symptoms of sick animals, it does not completely eliminate the pathogens within their bodies. These animals will continue to carry the pathogens throughout their lives, creating conditions for the spread and persistence of the disease[12]. Consequently, the theileriosis, which is caused by the genus *Theileria*, significantly impairs both the quality and yield of livestock products, thereby hindering the sustainable development of the livestock industry.

Accurate identification of the genus *Theileria* species is a prerequisite for diagnosing theileriosis. By studying the types and distribution of the genus *Theileria*, we can gain insights into the epidemic characteristics, transmission routes, and patterns of theileriosis. Moreover, identifying specific the genus *Theileria* species that pose significant threats to animal health enables the implementation of targeted pharmacological interventions and control strategies. Consequently, precise identification of the genus *Theileria* species plays a crucial role in the effective prevention and control of theileriosis. However, in the identification of parasites of the genus *Theileria*, our approach has primarily relied on traditional scientific methodologies, specifically utilizing morphological characteristics, vector analysis, pathogenicity studies, host specificity data, and epidemiological information as the foundational criteria[13]. And traditional taxonomic methods are often influenced by subjective factors, leading to biased identification results. Moreover, these methods have limitations in distinguishing species with very similar morphologies or the same species at different developmental stages. Hence, the selection of a rapid and accurate identification method is crucial for the effective control of theileriosis.

Mitochondria are found in most eukaryotic organisms and are crucial for energy production and cellular function maintenance[14]. These organelles possess their own genome, known as the mitochondrial genome. Due to its greater reliability and faster evolutionary rate compared to nuclear DNA, mitochondrial DNA has become a popular molecular marker in phylogenetic studies[15]. This allows for a more in-depth examination of the taxonomic relationships among various organisms and serves as a foundation for population genetics research[16]. The current data on *Theileria* mitochondrial genomes are limited, which presents challenges for classification, identification, phylogenetic research, and the prevention and control of theileriosis. It is essential to enhance our understanding of *Theileria* mitochondria through genome research to acquire more genetic data. This will establish a basis for accurate identification and molecular biology studies of *Theileria*.

T. velifera belongs to the phylum Apicomplexa, class Piroplasmea, order Piroplasmida, family Theileriidae and genus *Theileria*, it is primarily transmitted by the ticks, specifically the *Amblyomma* genus, and is mainly parasitic on bovines[17]. It is commonly found in African countries, such as Ethiopia, Mozambique, South Africa, Guinea, and Kenya[18]. In China, *T. velifera* is mainly prevalent in the southwest region, particularly in Yunnan Province. Because *T. velifera* can infect wild and domesticated bovine worldwide, it can cause significant economic losses[19,20]. Despite the significance of *T. velifera*, there is a notable scarcity of biological information regarding this parasite, particularly at the molecular level, which includes details about its mitochondrial genome sequence and structure. Consequently, this study presents the first sequencing and analysis of the complete mitochondrial genome of *T. velifera*. This work enriches and fills a gap in the mitochondrial gene database of the genus *Theileria*, providing valuable information for the identification of the genus *Theileria* and its molecular epidemiology research. At the same time, the structure and organization of mitochondrial genomes in Apicomplexan parasites were analyzed in this study. Phylogenetic trees were constructed using 2 PCGs, *cox1* and *cob*, to explore evolutionary relationships. The findings presented in this article contribute to a better comprehension of the mitochondrial genome of *T. velifera* and lay the groundwork for future investigations into phylogenetic relationships among various Apicomplexan species at the mitochondrial genome level.

Materials and methods

Sample collection, DNA extraction and mitochondrial genome sequencing

To collect blood samples from bovine infected with *T. velifera*, with the consent of the animal owner, we utilized disposable venous blood collection needles and sterile EDTA anticoagulant tubes, obtaining a total of 331 venous blood samples (0.5 ml each) from cattle raised by farmers in Tengchong City, Yunnan Province (25°02′N, 98°50′E). Among these, 2 blood samples were confirmed to be *T. velifera*-positive by amplification and sequencing of the ribosomal 18 S rRNA in Yunnan Provincial Institute for Endemic Disease Prevention and Control (Yunnan, China), and these specimens were obtained for the purpose of this study. To extract a sufficient amount of DNA, we utilized the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions, 200 µl EDTA anticoagulant blood was extracted from a positive specimen tube to extract total genomic DNA. Then the isolated DNA samples were stored in a -20 ℃ freezer, while the remaining anticoagulant blood was transferred to a clean and sterile EP tube as a reference specimen and stored at -20 ℃ in the Parasitology Museum of Dali University, Yunnan, the voucher number is DLUP2022101804. This study was approved by the Animal Ethics Committee of Dali University (Application number: 2022-SL-280, Approval number: 2022-P2-280). The extracted DNA was utilized to create an Illumina paired-end library, followed by sequencing of the mitochondrial genome on the Illumina Novoseq 6000 platform at Harbin Botai Biotechnology Co., Ltd. in Heilongjiang, China.

Genome assembly, annotation and data analysis

The raw reads by sequencing had a certain proportion of low-quality reads. In order to ensure the accuracy and reliability of the subsequent information analysis results, the raw reads were filtered first to obtain clean reads, followed by genome assembly using IDBA software[21]. Subsequently, the mitochondrial genome was annotated using the MITOS web server[22], then the mitochondrial gene sequences of related parasites were queried by BLAST in the GenBank database and the homologous proteins were compared and confirmed. These rRNA genes were further annotated by probing formerly reported rRNA gene fragments of *T. parva*, *T. orientalis*, and

T. uilenbergi. Additionally, ClustalW was used to align the identified rRNA sequences with those previously reported. And tRNAscan-SE v.2.0 was employed to search for tRNA genes in default search mode, as well as other mitochondrial sequence sources[23]. The base component bias was determined using the formulas AT-skew = (A-T) / (A + T) and GC-skew = (G-C) / (G + C). Nucleotide composition analysis was conducted using DNAStar V7.1, while relative synonymous codon use (RSCU) calculation was performed using CodonW 1.4.2. The mitochondrial genome was visualized on the Gene Graphics drawing online website (https://www.genegraphics.net/).

Phylogenetic analysis

The nucleotide sequences of *cox1* and *cob* from Apicomplexan parasites were retrieved from GenBank (Table 1). The *cox3* gene was omitted due to significant variation in *Theileria* and *Babesia* spp[24]. Subsequently, Clustal W in MEGA 11.0 software was employed to align the *cox1* and *cob* nucleotide sequences of these Apicomplexan parasites, genetic distance analysis of these sequences was then conducted using MEGA 11.0 software. The concatenated amino acid sequence of *cox1* + *cob* was utilized to construct the phylogenetic tree using MEGA 11.0 software, the amino acid sequences of these Apicomplexan parasites were subjected to Clustal W multiple sequence alignment. The phylogenetic tree was then constructed using maximum likelihood (ML) analysis with 1,000 bootstrap repeats, based on JTT and Freqs models. Finally, the tree was visualized using iTOL[25] (iTOL: Interactive Tree Of Life (embl.de)).

Results and discussion

General characterization of *Theileria velifera* mitochondrial genome

The complete mitochondrial genome of *T. velifera* has been submitted to GenBank with entry number ON684327. Sequence analysis revealed a linear structure for the complete mitochondrial genome of *T. velifera*, spanning a total length of 6,125 bp. It includes 3 PCGs (*cox1*, *cox3*, and *cob*), 5 LSU rRNA gene fragments (LSU1, LSU3, LSU4, LSU5, and LSU6), and 2 TIRs at the both ends. And no tRNA was found in the mitochondrial genes of the parasite, aligning with previous findings that Apicomplexan parasites typically have three PCGs, fragmented rRNA genes and without tRNA presence[24,26,27]. In mitochondrial genes of *T. velifera*, the three PCGs are 1,428 bp (*cox1*), 636 bp (*cox3*), and 1,173 bp (*cob*) in length. The sizes of the 5 LSU rRNA fragments were 299 bp (LSU1), 111 bp (LSU3), 82 bp (LSU4), 69 bp (LSU5), and 38 bp (LSU6) (Table 2). The TIR values for both initiation and termination were 64 bp. This contrasts with previous studies on the mitochondrial genome of Apicomplexan parasites, where the terminal reverse repeat size is typically around 440–450 bp (TIR)[24]. The complete mitochondrial genome of *T. velifera* exhibited base composition percentages of A = 33.49%, T = 35.46%, C = 15.36%, and G = 15.69%, with AT and GC contents of 68.95% and 31.05% respectively. The AT Skew and GC Skew values were calculated as -0.029 and 0.011 (Table 2). Among the mitochondrial genes, *cox1*, LSU1, and LSU4 were found to be encoded by one strand, while *cox3*, LSU3, LSU6, *cob*, and LSU5 were encoded by the other strand (Fig. a).

Protein-coding genes and rRNA of *Theileria velifera*

The total length of the three PCGs of *T. velifera* is 3,237 bp, accounting for 52.85% of the complete genome. Meanwhile, we also calculated the amino acid usage of the three PCGs (Table 3) and the relative synonymous codon usage (RSCU) (Fig. 1). The analysis results indicate that the three PCGs collectively encode 1,076 codons (excluding stop codons). Among these, the most frequently used codon is UUU (75), while CCC, UGA, CGC, CGA, and CGG are not utilized. Additionally, leucine (Leu) has the highest utilization rate at 14.13%, followed by serine (Ser) at 9.94% and Valine (Val) at 9.01%. In contrast, Arginine (Arg) (0.46%) and cysteine (Cys) (1.30%) exhibit the lowest utilization rates among the amino acids. The study calculated the RSCU values of PCGs, with values above 1 indicating a strong codon use preference, values equal to 1 indicating no preference, and values less than 1 indicating weak preference[28]. The top five codons by RSCU are AGA (4.55), CCA (2.75), UUA (2.17), GCU (2.10), and UCU (2.07). Interestingly, the third position of these codons is either A or U, reflecting a bias towards the use of bases A and T. Furthermore, it was observed that most codons with RSCU values greater than 1 end with A or U, while those ending with G or C tend to have RSCU values less than 1, a pattern consistent with other metazoans[29,30].

The mitochondrial genome of *T. velifera* contains five LSU rRNA fragments, which is compared to the region of the rRNA LSU of other Apicomplexan parasites studied, designated LSU1-LSU6, the LSU2 fragment was not found in the mitochondrial genome of *T. velifera*. LSU1, LSU3, and LSU6 of *T. velifera* are primarily situated between the *cox3* and *cob* genes, while LSU4 and LSU5 are predominantly located near the *cob* gene and terminal TIR (Fig. 2a).

Comparison of the mitochondrial genome of *Theileria velifera* with other apicomplexan parasites

In this study, we compared *T. velifera* in this study with Apicomplexan parasites available on NCBI in terms of length, PCGs, mitochondrial genome structure, A + T content, and parasitic host (Table 1). This study revealed that while *Haemoproteus* spp., *Polychromophilus murinus* and *Legerella nova* possess circular mitochondrial DNA, *T. velifera* and other Apicomplexan parasites have linear mitochondrial DNA[24,31–38]. The mitochondrial genome length of *T. velifera* and most Apicomplexan parasites is approximately 6,000 bp. However, *T. equi* (8,246 bp), *Babesia rodhaini* (6,929 bp), and *B. microti* (10,547 bp and 11,109 bp) have significantly longer mitochondrial genome lengths compared to other Apicomplexan parasites. *B. microti*, for example, has a genome length almost twice that of most other Apicomplexan parasites. Among these Apicomplexan parasites examined in this study, *T. equi has two cox3*-like genes, while *T. velifera* and other Apicomplexan parasites possess one *cox3* gene. And none of them have mitochondrial tRNA genes, so some scholars believe that their deletion can be compensated by

Species	Size/bp	Protein-encoding genes	Form	Main host	A + T%	Country	GenBank accession number
Theileria velifera	6125 bp	cox1, cox3, cob	Linear	Bovine	68.95	China	ON684327
Theileria parva	5924 bp	cox1, cox3, cob	Linear	Bovine	70.07	Japan	AB499089
Theileria parva	5808 bp	cox1, cox3, cob	Linear	Bovine	69.89	Germany	MW172714
Theileria parva	5808 bp	cox1, cox3, cob	Linear	Bovine	69.89	Germany	MW172710
Theileria parva	5808 bp	cox1, cox3, cob	Linear	Bovine	69.89	Germany	MW172715
Theileria taurotragi	5800 bp	cox1, cox3, cob	Linear	Bovine	72.03	Germany	NC_053926
Theileria annulata	5905 bp	cox1, cox3, cob	Linear	Bovine	70.57	UK	NT_167255
Theileria orientalis	5882 bp	cox1, cox3, cob	Linear	Bovine	71.17	China	OM735582
Theileria orientalis	5957 bp	cox1, cox3, cob	Linear	Bovine	70.72	Japan	AB499090
Theileria luwenshuni	6042 bp	cox1, cox3, cob	Linear	Sheep	68.45	China	NC_070079
Theileria lestoquardi	5811 bp	cox1, cox3, cob	Linear	Sheep	70.83	Germany	NC_053925
Theileria uilenbergi	6000 bp	cox1, cox3, cob	Linear	Sheep	69.07	China	MZ231018
Theileria equi	8246 bp	cox1, cox3-like, cob, cox3-like	Linear	Equine	70.94	Japan	AB499091
Babesia bovis	6005 bp	cox1, cox3, cob	Linear	Bovine	70.49	USA	NC_009902
Babesia bovis	6005 bp	cox1, cox3, cob	Linear	Bovine	70.49	USA	EU075182
Babesia bovis	5970 bp	cox1, cox3, cob	Linear	Bovine	70.35	Japan	AB499088
Babesia bigemina	5924 bp	cox1, cox3, cob	Linear	Bovine	69.82	Japan	AB499085
Babesia motasi	5946 bp	cox1, cox3, cob	Linear	Sheep	70.06	China	MN605892
Babesia motasi	5946 bp	cox1, cox3, cob	Linear	Sheep	70.10	China	MN605891
Babesia motasi	5836 bp	cox1, cox3, cob	Linear	Sheep	70.13	China	MN605890
Babesia rodhaini	6929 bp	cox1, cox3, cob	Linear	Murine	70.69	Japan	AB624360
Babesia rodhaini	6929 bp	cox1, cox3, cob	Linear	Murine	70.69	Japan	AB624359
Babesia microti	10,547 bp	cox1, cox3, cob	Linear	Murine, Human	64.82	USA	NC_034637
Babesia microti	11,109 bp	cox1, cox3, cob	Linear	Murine, Human	64.36	Japan	AB624353
Babesia microti	11,109 bp	cox1, cox3, cob	Linear	Murine, Human	64.36	Japan	NC_031328
Babesia duncani	5893 bp	cox1, cox3, cob	Linear	Human	68.15	USA	NC_039721
Babesia canis rossi	5838 bp	cox1, cox3, cob	Linear	Canine	71.24	USA	KC207823
Babesia canis canis	5769 bp	cox1, cox3, cob	Linear	Canine	71.90	USA	KC207822
Babesia gibsoni	5865 bp	cox1, cox3, cob	Linear	Canine	72.21	China	KP666169
Babesia gibsoni	5865 bp	cox1, cox3, cob	Linear	Canine	72.24	Japan	AB499087
Babesia caballi	5847 bp	cox1, cox3, cob	Linear	Equine	70.43	Japan	AB499086
Plasmodium berghei	5957 bp	cox1, cox3, cob	Linear	Murine	68.84	Japan	AB558173
Plasmodium knowlesi	5957 bp	cox1, cox3, cob	Linear	Monkey, Human	69.48	Thailand	NC_007232
Plasmodium vivax	5989 bp	cox1, cox3, cob	Linear	Human	69.46	China	MZ440348
Plasmodium falciparum	5967 bp	cox1, cox3, cob	Linear	Human	68.41	USA	M76611
Plasmodium fragile	5977 bp	cox1, cox3, cob	Linear	Monkey	69.58	Thailand	NC_012369
Plasmodium fieldi	5983 bp	cox1, cox3, cob	Linear	Monkey	69.75	Japan	AB354574
Plasmodium chabaudi chabaudi	5949 bp	cox1, cox3, cob	Linear	Rodent	69.10	Japan	AB379663
Plasmodium mexicanum	5991 bp	cox1, cox3, cob	Linear	Bird	68.95	Japan	AB375765
Plasmodium floridense	6002 bp	cox1, cox3, cob	Linear	Lizard	68.18	USA	NC_009961
Toxoplasma gondii	2607 bp	cox1, cox3, cob	Linear	Human, Cat	64.90	Norway	JX473253
Eimeria anseris	6179 bp	cox1, cox3, cob	Linear	Geese	65.27	China	MH758793
Eimeria necatrix	6212 bp	cox1, cox3, cob	Linear	chicken	64.97	China	OP800505
Eimeria stiedai	6277 bp	cox1, cox3, cob	Linear	Rabbit	65.24	China	OQ427352
Eimeria mitis	6408 bp	cox1, cox3, cob	Linear	Chicken	67.34	Canada	KC409031
Leucocytozoon pterotenuis	5896 bp	cox1, cox3, cob	Linear	Bird	68.76	USA	KM610046
Leucocytozoon fringillinarum	5874 bp	cox1, cox3, cob	Linear	Bird	67.84	USA	KT162004
Leucocytozoon caulleryi	5959 bp	cox1, cox3, cob	Linear	Chicken	68.80	Japan	NC_015304
Leucocytozoon sabrazesi	5935 bp	cox1, cox3, cob	Linear	Chicken	67.03	USA	NC_009336
Leucocytozoon majoris	6684 bp	cox1, cox3, cob	Linear	Bird	67.56	USA	FJ168563
Haemoproteus nisi	5959 bp	cox1, cox3, cob	Circular	Bird	71.30	France	OR078931
Haemoproteus antigonis	5980 bp	cox1, cox3, cob	circular	Bird	67.98	China	OR662132
Haemoproteus tartakovskyi	5992 bp	cox1, cox3, cob	Circular	Bird	68.19	Sweden	CM004177
Haemoproteus belopolskyi	5988 bp	cox1, cox3, cob	Circular	Bird	67.92	Germany	NC_082945
Haemoproteus payevskyi	5989 bp	cox1, cox3, cob	Circular	Bird	68.31	Germany	NC_082944
Nycteria heischi	5989 bp	cox1, cox3, cob	Linear	Bat	68.26	France	KX090648
Continued							

Species	Size/bp	Protein-encoding genes	Form	Main host	A + T%	Country	GenBank accession number
Nycteria gabonensis	5999 bp	cox1, cox3, cob	Linear	Bat	68.71	France	KX090647
Nycteria medusiformis	6091 bp	cox1, cox3, cob	Linear	Bat	70.23	France	KX090645
Polychromophilus murinus	6001 bp	cox1, cox3, cob	Circular	Bat	68.81	Thailand	OP380909
Legerella nova	6750 bp	cox1, cox3, cob	Circular	Pill millipede	63.66	Canada	NC_088457
Isospora serinuse	6223 bp	cox1, cox3, cob	Linear	Bird	65.74	Australia	NC_034000
Isospora manorinae	6223 bp	cox1, cox3, cob	Linear	Bird	65.72	Australia	KX276861
Isospora picoflavae	6236 bp	cox1, cox3, cob	Linear	Bird	65.33	Canada	NC_065382
Isospora lugensae	6257 bp	cox1, cox3, cob	Linear	Bird	66.28	Australia	MW303519
Isospora amphiboluri	6264 bp	cox1, cox3, cob	Linear	Bird	66.35	Canada	KR108297
Caryospora bigenetica	6313 bp	cox1, cox3, cob	Linear	Serpent, Cat	66.01	Canada	KP658102
Cyclospora cayetanensis	6274 bp	cox1, cox3, cob	Linear	Human	66.72	USA	NC_038230
Klossiella equi	6569 bp	cox1, cox3, cob	Linear	Equidae	65.35	Canada	MH203050

Table 1. Comparative analysis of the Mt genome of apicomplexan parasites.

Region	Position	Size(bp)	A%	G%	T%	C%	A + T%	G + C%	AT Skew	GC Skew
Whole genome	1–6125	6125	33.49	15.69	35.46	15.36	68.95	31.05	-0.029	0.011
cox1	322–1749	1428	26.4	19.89	41.32	12.39	67.72	32.28	-0.220	0.232
cox3	3187–2552	636	31.6	17.61	39.78	11.01	71.38	28.62	-0.115	0.231
LSU1	3359–3657	299	33.44	18.06	30.43	18.06	63.88	36.12	0.047	0.000
LSU3	3778–3668	111	35.14	16.22	26.13	22.52	61.26	38.74	0.147	-0.163
LSU6	4600–4563	38	39.47	26.32	18.42	15.79	57.89	42.11	0.364	0.250
cob	5863–4691	1173	28.39	18.76	37.94	14.92	66.33	33.67	-0.144	0.114
LSU5	5886–5818	69	40.58	20.29	34.78	4.35	75.36	24.64	0.077	0.647
LSU4	5960–6041	82	25.61	26.83	32.93	14.63	58.54	41.46	-0.125	0.294

Table 2. Composition and skewness of *Theileria velifera* mitochondrial genome.

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	75	1.60	UCU(S)	30	2.07	UAU(Y)	39	1.63	UGU(C)	12	1.71
UUC(F)	19	0.40	UCC(S)	2	0.14	UAC(Y)	9	0.38	UGC(C)	2	0.29
UUA(L)	55	2.17	UCA(S)	29	2.00	UAA(*)	2	2.00	UGA(W)	0	0.00
UUG(L)	46	1.82	UCG(S)	4	0.28	UAG(*)	1	1.00	UGG(W)	23	1.00
CUU(L)	31	1.22	CCU(P)	6	0.75	CAU(H)	21	1.50	CGU(R)	5	1.03
CUC(L)	2	0.08	CCC(P)	0	0.00	CAC(H)	7	0.50	CGC(R)	0	0.00
CUA(L)	14	0.55	CCA(P)	22	2.75	CAA(Q)	17	1.89	CGA(R)	0	0.00
CUG(L)	4	0.16	CCG(P)	4	0.50	CAG(Q)	1	0.11	CGG(R)	0	0.00
AUU(I)	41	1.28	ACU(T)	27	1.64	AAU(N)	34	1.45	AGU(S)	20	1.38
AUC(I)	5	0.16	ACC(T)	1	0.06	AAC(N)	13	0.55	AGC(S)	2	0.14
AUA(M)	50	1.56	ACA(T)	30	1.82	AAA(K)	15	1.58	AGA(S)	22	4.55
AUG(M)	46	1.00	ACG(T)	8	0.48	AAG(K)	4	0.42	AGG(S)	2	0.41
GUU(V)	38	1.57	GCU(A)	21	2.10	GAU(D)	19	1.65	GGU(G)	36	1.67
GUC(V)	7	0.29	GCC(A)	6	0.60	GAC(D)	4	0.35	GGC(G)	6	0.28
GUA(V)	40	1.65	GCA(A)	11	1.10	GAA(E)	25	1.61	GGA(G)	42	1.95
GUG(V)	12	0.49	GCG(A)	2	0.20	GAG(E)	6	0.39	GGG(G)	2	0.09

Table 3. Codon usage in the mitochondrial genome of *Theileria velifera*.

functional replacement of cytoplasmic tRNA imports[39–41]. *T. velifera* and most Apicomplexan parasites analyzed here typically exhibit a high A + T content of around 70%[14]. However, it was observed that *B. microti*, *L. nova* and *Eimeria necatrix* have slightly lower A + T levels compared to other Apicomplexan parasites, approximately 64%. In terms of parasitic hosts, the different species of Apicomplexan parasites in this study showed differences, *Theileria* and *Babesia* spp. can be parasitic on different hosts, including bovine, sheep, equine, canine, murine, and humans. On the other hand, *Plasmodium* spp. primarily infect humans, murine, monkey, rodent, bird and lizard. *Toxoplasma gondii* mainly infect humans and cats. *Eimeria* spp. parasitizes chickens, geese, and rabbits.

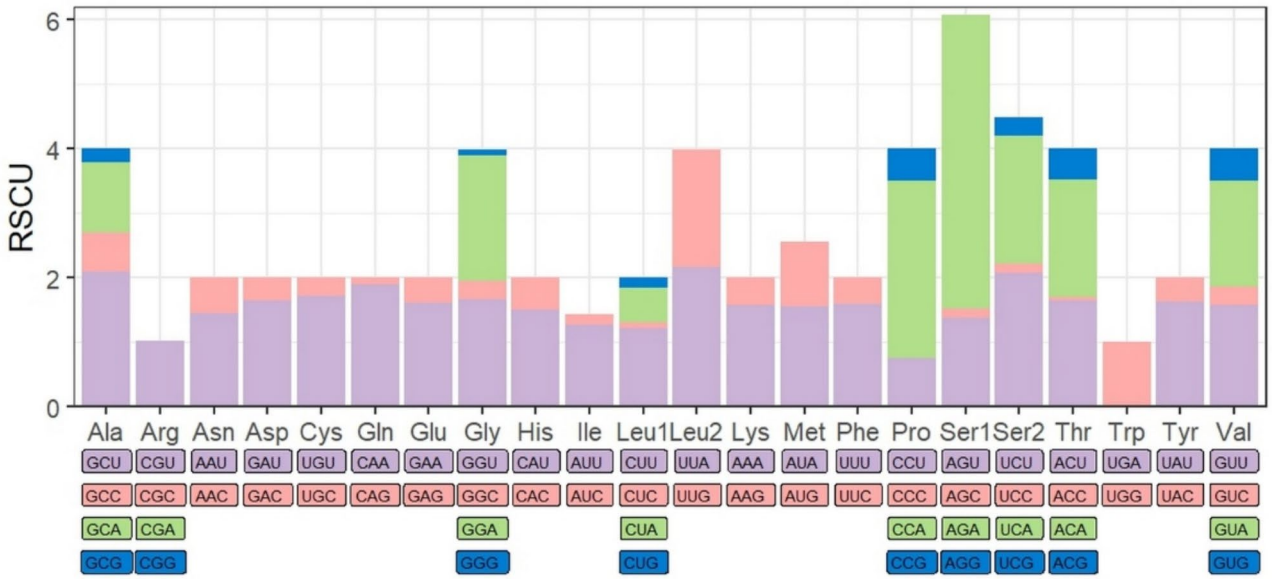

Fig. 1. *Theileria velifera* protein-coding gene relative synonymous codon usage (RSCU).

And the primary parasitic hosts of *Leucocytozoon* spp. are birds and chickens, the main hosts for *Haemoproteus* spp. and *Isospora* spp. are exclusively birds. *Nycteria* spp. primarily parasitize bats[14,27,42,43].

In a comparative analysis of start and stop codons in PCGs, *T. velifera* was compared with other species of the genus *Theileria* and *Babesia* in this study (Table 4). The start codons for *cox1*, *cox3*, and *cob* in *T. velifera* were ATA, ATG, and TTG, respectively, while the stop codons were TAA, TAA, and TAG, respectively. The analysis revealed that the start codons forms used in these parasites are ATN, GTN, and TTN, with ATN being the most commonly utilized. Additionally, it was observed that GTN and TTN are more frequently used in *cox1* compared to *cox3* and *cob*. In addition, the start codon of the *cox1* gene of *T. annulata is* CAT. The distribution of stop codons included TAA, TAG, and TGA, with TAA being the most prevalent, followed by TAG. In the start codons of the PCGs of these parasites examined in this study, in addition to the commonly used codon pattern ATN, several special start codons are also utilized, including GTG, GTT, CAT, TTA, TTG, and TTT. These special start codons are subject to correction during mRNA editing and do not affect the normal translation of the protein[44].

We conducted an analysis of the distribution and transcription orientations of PCGs, LSU, and TIR in *T. velifera* with other *Thaleria* spp. and *Babesia* spp. parasites in this study (Fig. 2). In terms of PCGs, *T. equi* contains two *cox3*-like fragments, *cox1* and *cob* genes, while *Thaleria* spp. and *Babesia* spp. parasites have three PCGs: *cox1*, *cox3*, and *cob*. The distribution order of these genes varies among species, in this study, with *T. velifera* and most *Theileria* and *Babesia* spp. having the order *cox1*, *cox3*, *cob*. In terms of gene length, there was no significant difference in the size of *cox1*, *cox3*, and *cob* between *T. velifera* and other *Thaleria* spp. and *Babesia* spp. parasites. Additionally, when comparing the expression directions of *cox1*, *cox3*, and *cob* genes in these species, it was observed that the majority of species shared the same expression direction as *T. velifera*. Specifically, the expression direction of the *cox1* gene was 5' to 3', while for *cox3* and *cob* genes, the transcription direction was 3' to 5'[16,45,46].

In terms of rRNA, *T. velifera* and other species of the genus *Theileria* and *Babesia* only contain LSU, typically five or six copies. Research indicates that rRNA fragmentation is a common occurrence in various natural organisms and not exclusive to the mitochondrial genome of Apicomplexan organisms, this phenomenon has been observed in Apicomplexans, Dinoflagellates, Oxytricha, Paramecium and Tetrahymena, with rRNA fragments dispersed across the mitochondrial genome and encoded on either strand[47,48]. However, the process by which these rRNA fragments assemble to form ribosomes remains unclear[49]. Interestingly, most parasites of the genus *Theileria* and *Babesia* in this study, LSU1 and LSU3 are found between *cox3* and *cob* genes, while LSU2 and LSU6 are also in this region. LSU4 and LSU5 are mostly located between *cob* gene and the terminal TIR. Additionally, LSU1 and LSU3, as well as LSU4 and LSU5, are transcribed in opposite directions in most genus *Babesia* and *Theileria* species, whereas LSU2 and LSU6 have the same transcription direction.

In the context of TIR, *T. velifera* and *Thaleria* spp. and *Babesia* spp. do possess TIR sequences. Notably, in *T. equi*, TIR sequences at both ends exhibit a significant overlap with *cox3*-like sequences. Furthermore, most the genus *Babesia* and *Theileria* species, including *T. velifera*, have a single pair of TIR sequences located at both ends of linear mitochondria, whereas *B. rodhaini* and *B. microti* have two long pairs of TIR sequences, named IRa and IRb, and a flip-flop inversion in between[50]. Reports have indicated individual TIR lengths ranging from 184 to 1,082 bp for *B. rodhaini* and *B. microti*, and 1,563 bp for *T. equi*, which are notably longer than the typical TIR length previously documented (440–450 bp). Furthermore, in terms of total mitochondrial length, *B. rodhaini*, *B. microti*, and *T. equi* exhibit considerable length compared to other *Babesia* and *Theileria* spp. species. These

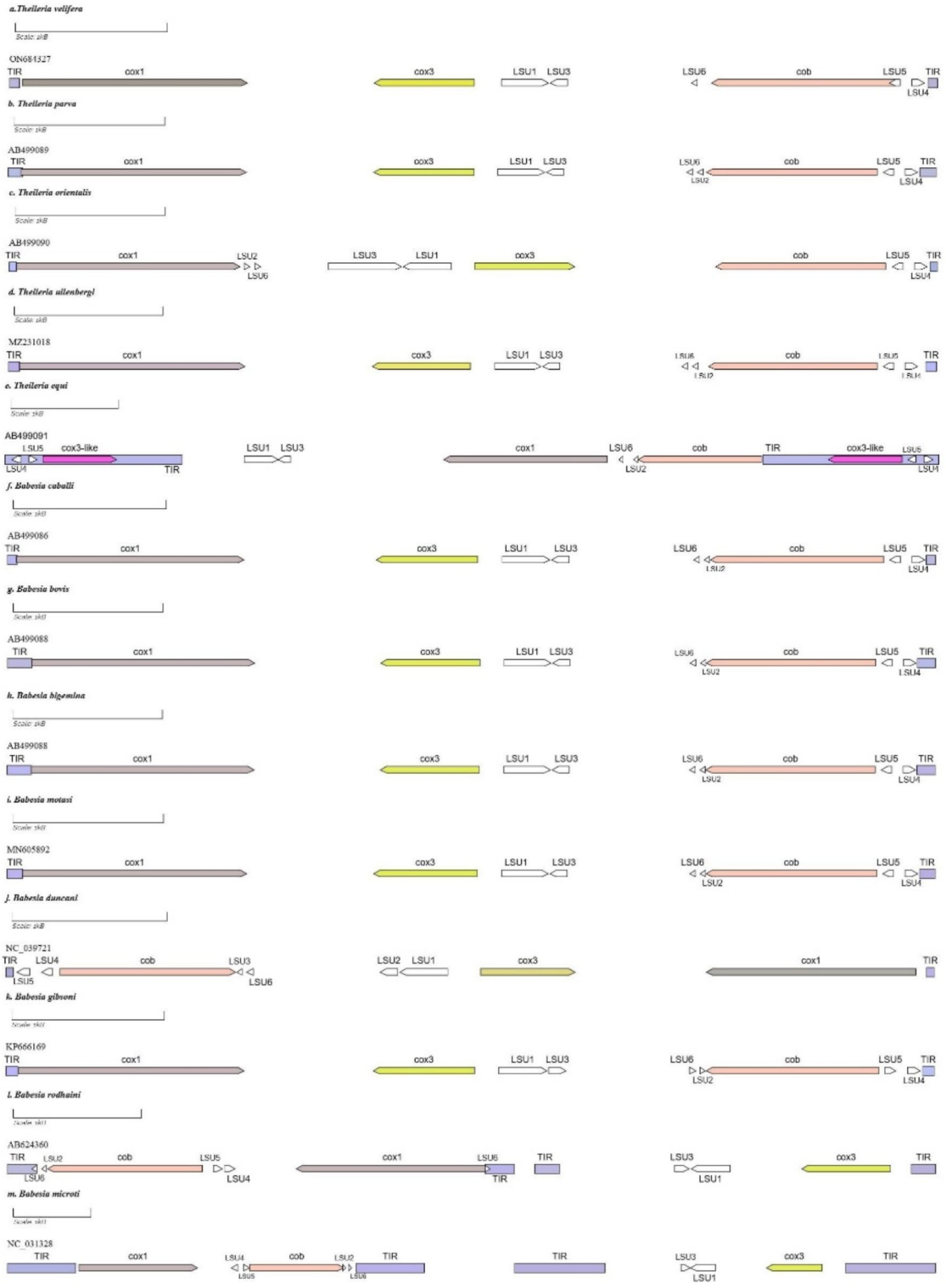

Fig. 2. Mitochondrial genome structure of *Theileria velifera* (ON684327) (**a**) and other *Thaleria* spp. and *Babesia* spp. parasites, including: (**b**) *Theileria parva* (AB499089), (**c**) *Theileria orientalis* (AB499090), (**d**) *Theileria uilenbergi* (MZ231018), (**e**) *Theileria equi* (AB499091), (**f**) *Babesia caballi* (AB499086), (**g**) *Babesia bovis* (AB499088), (**h**) *Babesia bigemina* (AB499085), (**i**) *Babesia motasi* (MN605892), (**j**) *Babesia duncani* (NC_039721), (**k**) *Babesia gibsoni* (KP666169), (**l**) *Babesia rodhaini* (AB624360), (**m**) *Babesia microti* (NC_031328), arrows indicate the direction of gene transcription. The protein-coding genes include *cox1*, *cox3*, and *cob*, *cox1* represented by a brown arrow, *cox3* by a yellow arrow, *cob* by a red arrow, and the *cox3*-like gene of *Theileria equi* is shown in purple arrow, LSU is represented by white arrows. TIR represents the end reverse repeat sequence, represented by a blue box.

Species	GenBank accession number	cox1 (start, stop codons)	cox3 (start, stop codons)	cob (start, stop codons)
Theileria velifera	ON684327	ATA/TAA	ATG/TAA	TTG/TAG
Theileria parva	AB499089	ATT/TAA	ATT/TAA	ATG/TAG
Theileria parva	MW172714	GTG/TAA	ATT/TAA	ATG/TAG
Theileria parva	MW172710	GTG/TAA	ATT/TAA	ATG/TAG
Theileria parva	MW172715	GTG/TAA	ATT/TAA	ATG/TAG
Theileria taurotragi	NC_053926	GTT/TAG	ATG/TAA	ATG/TAA
Theileria orientalis	AB499090	ATA/TAA	ATT/TAA	ATA/TAA
Theileria orientalis	OM735582	ATG/TAA	ATT/TAA	ATA/TAA
Theileria luwenshuni	NC_070079	GTG/TAG	ATG/TAG	GTG/TAA
Theileria uilenbergi	MZ231018	ATG/TAA	ATG/TAG	ATG/TAA
Theileria annulata	NT_167255	CAT/TAA	ATG/TAA	ATG/TAA
Theileria lestoquardi	NC_053925	GTT/TAA	ATT/TAA	ATG/TAA
Theileria equi	AB499091	ATT/TAA	TTA/TAG	ATG/TAA
Babesia bovis	AB499088	ATG/TAA	ATA/TAA	ATG/TAA
Babesia bovis	EU075182	ATG/TAA	ATT/TAA	ATG/TAA
Babesia bovis	NC_009902	ATG/TAA	ATT/TAA	ATG/TAA
Babesia bigemina	AB499085	ATG/TAA	ATA/TGA	ATG/TAA
Babesia motasi	MN605892	ATG/TAA	ATA/TAG	ATG/TAA
Babesia motasi	MN605891	ATG/TAA	ATA/TAG	ATG/TAA
Babesia motasi	MN605890	ATG/TAA	ATA/TAG	ATG/TAA
Babesia rodhaini	AB624360	TTA/TAA	ATA/TAA	ATG/TAA
Babesia rodhaini	AB624359	TTA/TAA	ATA/TAA	ATG/TAA
Babesia microti	NC_031328	ATG/TAG	ATT/TAG	ATA/TAG
Babesia microti	AB624353	ATG/TAG	ATT/TAG	ATT/TAG
Babesia microti	NC_034637	ATG/TAG	ATA/TAA	ATA/TAG
Babesia duncani	NC_039721	ATG/TAA	ATG/TAA	ATG/TAA
Babesia canis canis	KC207822	TTG/TAA	ATT/TAA	ATG/TAA
Babesia canis rossi	KC207823	ATG/TAA	TTT/TAA	ATG/TAA
Babesia gibsoni	KP666169	ATG/TAA	ATG/TAA	TTA/TAA
Babesia gibsoni	AB499087	TTG/TAA	ATT/TAA	TTA/TAA
Babesia caballi	AB499086	ATA/TAA	ATA/TAA	ATG/TAA

Table 4. Initiation and termination codons in protein-coding genes of the genus *Theileria* and *Babesia* parasites mitochondrial genomes.

findings suggest that the amount and size of TIR may play a significant role in the variations in mitochondrial genome sizes. Simultaneously, other researchers argue that TIR plays a pivotal role in the replication and stability of linear mitochondrial genomes[14,24,50].

Phylogenetic analysis

Due to the high divergence of the *cox3* gene in the genus *Theileria* and *Babesia* parasites, we obtained nucleotide sequences of *cox1* and *cob* from Apicomplexan parasites from GenBank. Combined with the *cox1* and *cob* sequences of *T. velifera* measured in this study, we conducted an analysis of genetic distances among these Apicomplexan parasites based on the *cox1* and *cob* genes (Supplementary Table S1). The results indicate that among the Apicomplexan parasite species examined in this study, *T. velifera* exhibited a genetic distance ranging from 0.276 to 0.356 from other species within the *Thaleria* spp., 0.318 to 0.465 from the *Babesia* spp., and 0.426 to 0.438 from the *Plasmodium* spp. The genetic distance from *Leucocytozoon* spp. is between 0.425 and 0.444, from *Haemoproteus* spp. it is between 0.424 and 0.448, and from *Isospora* spp. it is between 0.453 and 0.460. Furthermore, the genetic distance to the *Eimerium* spp. was found to be 0.452 to 0.467, while the distance to *T. gondii* was 0.486. Notably, *T. velifera* displayed the closest genetic distance to *T. taurotragi* (0.276) and the furthest distance to *T. gondii* (0.486). Additionally, within the genus *Babesia* examined in this study, *T. velifera* was genetically more closely related to most *Babesia* spp. than to *T. equi*.

Due to the high divergence of the *cox3* gene in the genus *Theileria* and *Babesia*, we utilized the *cox1* and *cob* amino acid sequences from the mitochondria of Apicomplexan parasites available in the NCBI database, along with the *cox1* and *cob* sequences from the mitochondrial genome of *T. velifera* obtained in our study (Table 1). These concatenated amino acid sequences were employed as a dataset for the construction of a phylogenetic tree (Fig. 3). In phylogenetic analysis, the evolutionary tree is primarily divided into two major branches. The first major branch comprises the species of the order Haemosporida, Eugregarinida, and Eucoccidiorida. Within the order Haemosporida, the parasites of the genus *Plasmodium* form a distinct subgroup, while the parasites of the genus of *Haemoproteus*, *Leucocytozoon*, and *Nycteria* are grouped together. This clustering indicates a relatively

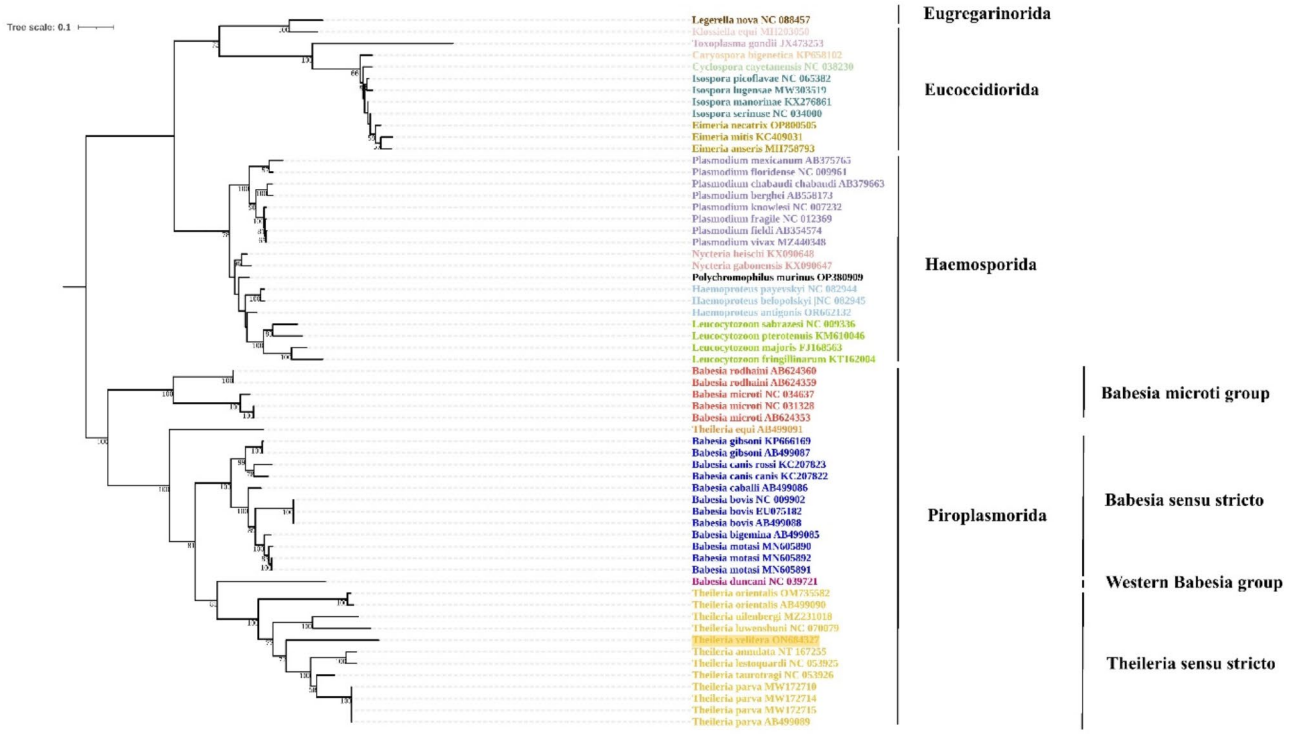

Fig. 3. The phylogenetic relationship between *T. velifera* and Apicomplexan parasites was analyzed using JTT and Freqs models. Maximum likelihood analysis was performed based on the amino acid sequences of *cox1* and *cob* genes to infer the phylogeny, with *T. velifera* highlighted in orange background. Bootstrap values > 50% from 1000 replicates are shown on the nodes.

close relationship among the parasites of the *Haemoproteus*, *Leucocytozoon*, and *Nycteria* genus. At the same time, in the order Eucoccidiorida, the genus of *Isospora* and *Eimeria* species are relatively close. Another large branch is mainly the parasite of the order Piroplasmorida, mainly including the genus *Theileria* and *Babesia*, which further divides into five main subgroups: (1) Babesia microti group including *B. rodhaini* and *B. microti*, (2) Western Babesia group containing *B. duncani*, (3) Babesia sensu stricto encompassing species such as *B. gibsoni*, *B. canis*, etc., (4) *T. equi*, and (5) Theileria sensu stricto comprising species like *T. orientalis*, etc. This classification aligns with previous research[51,52]. In this study, *T. velifera* was first clustered with *T. annulata*, *T. lestoquardi*, *T. taurotragi*, and *T. parva*. and then with *T. uilenbergi*, *T. luwenshuni* and *T. orientalis*, indicating a closer relationship between *T. velifera* and *T. annulata*, *T. lestoquardi*, *T. taurotragi*, and *T. parva* when compared to *T. uilenbergi*, *T. luwenshuni* and *T. orientalis*. This relationship is further supported by the 18s RNA phylogenetic tree[17,18].

Theileria spp. from the branch containing *T. velifera* initially grouped with *B. ducani*, then with Babesia sensu stricto, and finally with *T. equi*. This suggests that *T. velifera* is more closely related to *B. duncani* and Babesia sensu stricto when compared to *T. equi*. Furthermore, it indicates that Theileria sensu stricto, which includes *T. velifera*, shares a common ancestral branch with *B. duncani* and Babesia sensu stricto[53,54]. Studies have demonstrated that *B. duncani* is classified as a distinct member of a new clade when compared to other Apicomplexan parasites, as indicated by 18 S rRNA and neighbor-joining tree analysis. Additionally, 18 S rRNA analysis reveals that *B. duncani* and *B. conradae* are part of the same clade within the Western Babesia group. However, due to the lack of identification of the *cox1* and *cob* genes in *B. conradae*, this species was not included in the analysis conducted[51,53,54]. Furthermore, numerous studies have provided evidence that Babesia sensu stricto forms a monophyletic group, a conclusion supported by both molecular and biological data. Numerous studies have demonstrated that *T. equi* belongs to a distinct phylogenetic lineage separate from Theileria sensu stricto, *B. ducani*, and Babesia sensu stricto. This differentiation has been supported by the analysis of two molecular markers, 18 S rDNA and RPS8. Furthermore, the mitochondrial genome structure of *T. equi* differs significantly from that of Theileria sensu stricto including *T. velifera*, *B. duncani*, and Babesia sensu stricto. These findings increasingly suggest that *T. equi* occupies a unique branch within the system[27,51]. As a result, it can be concluded that mitochondrial genome sequences play a crucial role in phylogenetic investigations of Apicomplexan parasites.

Conclusion

In this study, the mitochondrial genome of *T. velifera* is reported for the first time, revealing three PCGs, five LSUs, and a pair of TIRs, but lacking tRNA. A comparative analysis with other Apicomplexan parasites was conducted to enhance our systematic understanding of their evolutionary relationships. The findings indicate structural and

phylogenetic similarities between the mitochondrial genome of *T. velifera* and Babesia sensu stricto, Theileria sensu stricto, while *T. annulata*, *T. lestoquardi*, *T. taurotragi*, *and T. parva* show greater similarity. This study presents robust molecular evidence for the accurate identification of *T. velifera* and establishes a theoretical foundation for subsequent genomic research. Furthermore, it provides a molecular basis for epidemiological investigations of *T. velifera*. And by comparing the mitochondrial genomes of different Apicomplexan parasites, this research enhances our comprehensive understanding of the phylogenetic relationships among these organisms. Overall, this work will contribute valuable insights into the phylogeny and population genetics of Apicomplexan parasites, while also providing critical molecular evidence for further investigations into their evolutionary mechanisms and genetic diversity.

Data availability

The nucleotide sequences of the T. velifera mitogenome were deposited in GenBank (National Center for Biotechnology Information (nih.gov)) under accession number ON684327.

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Author contributions

Conception and design of the study: XY, SBT and CBD. Acquisition of data: XY, SBT, CBD, SL and MND. Analysis and interpretation of data: XY, SBT, YQC, ZL and ML. Drafting the article: XY, SBT and DDJ. Revising the article critically for important intellectual content: CHD, YJS and QFZ. All authors have made substantial contributions to the study. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

The animal studies described in this research were approved by the Experimental Animal Management Committee of Dali University (Application Number: 2022-SL-280, Approval Number: 2022-P2-280). Furthermore, approval from the animal owner was obtained prior to the collection of bovine venous blood. The study was conducted in accordance with relevant local laws and institutional guidelines, and all experiments involving the use of animals adhered to the ARRIVE guidelines..

Additional information

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