Comparative genomic analysis of *Babesia duncani* responsible for human babesiosis

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**Abstract**

**Background:** Human babesiosis, caused by parasites of the genus *Babesia*, is an emerging and re-emerging tick-borne disease that is mainly transmitted by tick bites and infected blood transfusion. *Babesia duncani* has caused majority of human babesiosis in Canada; however, limited data are available to correlate its genomic information and biological features.

**Results:** We generated a *B. duncani* reference genome using Oxford Nanopore Technology (ONT) and Illumina sequencing technology and uncovered its biological features and phylogenetic relationship with other Apicomplexa parasites. Phylogenetic analyses revealed that *B. duncani* form a clade distinct from *B. microti*, *Babesia* spp. infective to bovine and ovine species, and *Theileria* spp. infective to bovines. We identified the largest species-specific gene family that could be applied as diagnostic markers for this pathogen. In addition, two gene families show signals of significant expansion and several genes that present signatures of positive selection in *B. duncani*, suggesting their possible roles in the capability of this parasite to infect humans or tick vectors.

**Conclusions:** Using ONT sequencing and Illumina sequencing technologies, we provide the first *B. duncani* reference genome and confirm that *B. duncani* forms a phylogenetically distinct clade from other Piroplasm parasites. Comparative genomic analyses show that two gene families are significantly expanded in *B. duncani* and may play important roles in host cell invasion and virulence of *B. duncani*. Our study provides basic information for further exploring *B. duncani* features, such as host-parasite and tick-parasite interactions.

**Keywords:** De novo assembly, *Babesia duncani*, Babesiosis, Phylogenetic analysis, Adaptive evolution, Invasion

**Background**

Human babesiosis, caused by the genus *Babesia*, is an emerging and re-emerging tick-borne infectious disease that is mainly transmitted by tick bites, blood product transfusion, and congenitally. There is a broad agreement that the main causative agents of human babesiosis are *B. microti*, *B. divergens*, *B. duncani*, *B. crassa*, and *B. motasi* [1–6]. Symptoms of babesiosis include fever, headache, multi-system organ failure, and even death [7, 8]. In recent years, an increasing number of cases of human babesiosis have drawn people's attention. During the past decades, the majority of knowledge has been obtained about *B. microti*, which is responsible for the majority of *Babesia* species infections in humans.
throughout the world. Traditionally, other *Babesia* spp. have been neglected because they cause relatively fewer cases in comparison with *B. microti* [9]. Compared with *B. microti*, *B. duncani* is characterized by a rapid increase in parasitemia and severe pathology, with mortality rates of around 95% in infected C3H, A/J, AKR/N, and DBA/1J mice [10, 11]. The recent emergence of significant human babesiosis cases caused by *B. duncani* in the Pacific coast region, as well as the observation of severe and even fatal human cases, has stimulated interest in this enigmatic species, including its biological features, phylogenetic relationships, and adaptive evolution.

As the highest virulent *Babesia* species as confirmed in animal models (such as in mice and hamsters), *B. duncani* was first reported in a 41-year-old man who contracted human babesiosis in Washington state in 1993 [12]. Since then, additional cases caused by this pathogen were documented in California and Canada [13, 14]. Earlier studies confirmed *B. duncani* in a clade with parasite *B. conradae* isolated from canines in California, based on its phylogenetic analysis targeting the 18S rRNA gene, whereas by targeting the ITS (Internal transcribed spacer) gene, *B. duncani* was placed in a distinct clade from other known *Babesia* spp. [15]. However, those controversial conclusions were recently challenged with the completeness of mitochondrial genome sequencing that placed this parasite in a clade with *T. orientalis* and *T. parva*, infecting buffalo and cattle, respectively, but distinct from other *Babesia* spp. and *Plasmodium* spp. [16]. The updated phylogeny-based classification of the Piroplasmida is challenging the previous taxonomic evolutionary analysis. *Babesia* spp. form a polyphyletic group, in terms of their phenotype and life history, which provides valuable information for understanding the taxonomy of the Piroplasmida [17]. The *B. duncani* lineage is classified as *Babesia sensu lato*, suggesting that *B. duncani* belongs to clade III of Piroplasmida [18].

In this context, we use genomic and transcriptomic data derived from *B. duncani* merozoites to assemble a reference genome and to perform a comparative analysis of genomes from apicomplexan parasites. Our analyses provide a better understanding of its evolution and key features correlated with its biology, such as gene family expansion and host cell invasion.

**Results and discussion**

**Genome assembly and annotation**

*Babesia duncani* genome was sequenced using ONT and Illumina platforms. Long reads derived from ONT (182,649 reads, median length 25,597 bp, total bases 4.7 Gb) were assembled into the draft assembly, which was further corrected using ONT long reads and Illumina reads (length 150 bp, total paired sequences 9,442,873, total bases 2.2 Gb). The overall coverage of sequencing data was evaluated using Jellyfish v2.3.0 (Fig. 1a) [19]. Eventually, a total length of 7.9 Mb with seven scaffolds was generated, which size is comparable to *B. microti*, *B. bovis*, and *Babesia* sp. Xinjiang, ranging from 6.4 to 8.4 Mb. However, the *B. duncani* genome is the second smallest *Babesia* spp. genome. GC content is similar between *B. microti* and *B. duncani* (Table 1), but lower than other Piroplasm parasites, such as *B. bovis*, *B. bigemina*, and *B. ovata*. *Babesia duncani* genome contains 9.1% repeated sequences, including 8.8% of unclassified repeats and 0.2% simple repeats, and classic transfer RNA genes. The completeness of the genome, evaluated by BUSCO (v5.1.3) using the core apicomplexan dataset (apicompexa_odb10), was 95.3% [20]. Comparisons of *B. duncani* reference genome with these of *B. bovis* and *B. microti* reveal some common features, including a similar GC

![Fig. 1](image-url) Sequencing depth estimation and density of gene length with variable exon number. a Sequencing depth was evaluated using Jellyfish. b Gene length increased with exon numbers
content, some degree of collinearity, rearrangements of large fragment, and conservation of gene content (Table 1, Figs. 2, and 3a).

We predicted 3759 protein-coding genes in the *B. duncani* genome, which is similar in the number of genes to those of other *Babesia* spp., 61.4% of which were proved by RNA-Seq data. Almost half of these genes (1636 genes) were annotated to Gene Ontology (GO) terms (Additional file 1: Table S1). A total of 369 (9.8%) of predicted proteins contained signal peptide sequences. A

|                      | *B. bigemina* | *B. bovis* | *B. duncani* | *B. microti* | *B. ovata* | *Babesia sp. Xinjiang* |
|----------------------|---------------|------------|--------------|--------------|------------|------------------------|
| Genome size (Mbp)    | 13.8          | 8.2        | 7.9          | 6.4          | 14.5       | 8.4                    |
| N50                  | 2,541,256     | 1,795,777  | 1,067,452    | 1,766,409    | 2,090,503  | 533,301                |
| GC (%)               | 50.63         | 41.69      | 37.68        | 36.17        | 49.27      | 43.87                  |
| Coding gene numbers  | 5079          | 3974       | 3759         | 3573         | 5044       | 3066                   |
| Ns per 100 kbp       | 0             | 0.01       | 0.01         | 1.59         | 0          | 591.11                 |
| Gene density (gene/Mb)| 368           | 484.6      | 475.8        | 558.3        | 347.9      | 365                    |
| Number of exons per gene | 2.6          | 2.8        | 2.7          | 7.7          | 2.5        | 3.3                    |

**Fig. 2** Genome collinearity analysis of *B. duncani* with *B. bovis* and *B. microti*. The collinear gene blocks were determined by MCScanX between genome scaffolds for three *Babesia* spp.
A total of 1625 genes are lacking introns, and the remaining 2134 (56.8%) genes are present with one or several introns. The percentages of genes containing one or even more introns are almost similar in lineage-specific gene families (52/89, 58.4%) and expanded gene families (12/21, 57.1%), whereas a significantly high percentage of multiple introns genes is observed in conserved housekeeping genes (1279/1900, 67%) ($p < 0.01$). Gene length is a positive correlation with the number of exons (Fig. 1b).

**Phylogenetic relationship with other Apicomplexa parasites**

There is an agreement that mitochondrial protein-coding sequences are commonly used to investigate the evolutionary and phylogenetic relationships of apicomplexan parasites. Recently, analysis of cytochrome c oxidase subunit I (*CoxI*), cytochrome b (*Cob*) protein sequences, and 18S rRNA revealed that *B. duncani* was defined in a clade with *Theileria* spp. (including *T. orientalis* and *T. parva*), whereas it has a relatively remote phylogenetic relationship with *Babesia* spp. (*B. bigemina, B. ovata, Babesia sp. Xinjiang, and B. bovis*), but itself forms a separate clade [16]. Obviously, each species responsible for human babesiosis forms a monophyletic clade. One reason for our results differing from previous reports is limited phylogenetic information on the single or a few genes used to analyze this relationship. Our results provide robust evidence to resolve the position for this human pathogen (Fig. 3b).

Estimating the dates of speciation across Apicomplexa is a challenging task, as there are no available fossil documents, whereas the increasing amount of apicomplexan parasite genome data enables estimation of divergence time, which was performed using a Generalized Phylogenetic Coalescent Sampler (G-PhoCS) [22]. *Babesia duncani* and other *Babesia* spp. infective to bovine and ovine species appear to have split around 351.5 million years ago (Mya). Interestingly, one of the *Babesia* species, *B. microti*, derived from a common ancestor with other Piroplasm parasites around 614.2 Mya (Fig. 4). This result is consistent with previous reports that revealed piroplasm parasite speciation events to have been earlier than those of their hosts and vectors [23, 24].

**Babesia duncani species-specific genes**

Multigene families are well known to play critical roles and evolve extremely rapidly during species evolution and adaptation to hosts. Completeness of *B. duncani*...
genome sequencing facilitates a better understanding of its adaption evolution, vector-pathogen and pathogen-host interactions, and the discovery of virulence factors. For Apicomplexa parasites, most large size of family is species-specific genes, such as fam gene families in *Plasmodium gallinaceum* and *Plasmodium relictum*, var genes in *P. falciparum*, pir genes in *P. vivax*, and *Plasmodium knowlesi*, msa in *Babesia* spp. [25–28]. Likewise, we identified the largest gene family in *B. duncani*, containing 89 members that encode a motif conserved across family members, which is a novel family, and is named *B. duncani* largest family 1 (blf1, Additional file 2: Table S2) [29–31]. Additionally, conserved motifs were identified in this gene family by MEME (Fig. 5) [32, 33]. Sixty-five out of 89 genes each encodes a protein with at least one predicted transmembrane helix, and the remainder of these are predicted to be exported. It is impossible to determine significant sequence similarity to other apicomplexan parasite genomes. Almost all of these genes are located in the subtelomeric region, and subtelomeric multigene families in *Plasmodium* spp. have been proved to be important for transporting proteins into/through the host cell (Fig. 6) [34]. RNA-Seq data proved that 53 out of 89 members of blf1 gene family are expressed in blood-stage.

We identify 223 species-specific genes without orthologs in other *Babesia* spp. and *Theileria* spp. included in this study, which were distributed across its whole genome (Fig. 6). Thirty-one of these genes present signal peptide, identified by signalp-5.0b (Additional file 3: Table S3), and almost all proteins encoded by these genes are secreted into the host cell environment using TMHMM, suggesting that these genes might involve in parasite and host/vector receptor interactions [35, 36]. Relative synonymous codon usage was estimated by CodonW v1.4.2 program (https://github.com/smsaladi/codonw-slim). No codon usage bias was observed between 223 species-specific genes and all orthologues in *B. duncani* (Fig. 7). The values of relative synonymous codon usage were tested by paired t test, and no significant difference was observed between species-specific genes and all orthologs (p = 0.997). Aligning with RNA-Seq data of *B. duncani*, 215 out of 223 *B. duncani* species-specific genes show evidence of expression in the blood-stage, meaning that the remaining eight genes might play a role in other stages of the life cycle. Ninety-eight of these genes are unlikely to ascribe their potential functions by BLASTP and Interproscan (v5.48-83.0), highlighting that limited efforts have been made to explore the content of genome that may play critical roles relating to host specificity and immune evasion [37, 38]. Species-specific genes may be an alternative source of evolutionary innovations and host adaptations, whereas their precise biological functions remain to be investigated.
Multigene families
Although we observed 46 expanded and 705 contracted gene families involving 61 genes gained and 710 genes lost, except for glycosylphosphatidylinositol-anchored protein family (GPI-AP) and serine esterase (SEA) (Fig. 8; Additional file 5: Table S5), almost all of gene families members slightly increased by 1 or 2 copies and decreased by 1 to 2 copies. Compared with B. microti, these expanded and contracted gene families showed no significant difference. These small expansions and contractions may be caused by an artifact of genome sequencing. GPI-APs have been identified in the membranes of apicomplexan, such as P. falciparum, T. gondii, B. bovis, Trypanosoma brucei, and Leishmania donovani [39–41]. Some of the GPI-APs that bind to receptors of erythrocytes in B. divergens and B. canis are expressed on the surface of parasite merozoites [42, 43]. Babesia divergens antigen Bd37 is a GPI-AP expressed on the surface of merozoites, which has been used to immunize animals against B. divergens infection [44]. Subsequently, a homologous GPI-AP of B. canis was proved to protect dogs against this parasite infection [45]. These results highlight the feasibility of a more general strategy involving GPI-AP to develop protective vaccines against Babesia spp., including B. duncani [46]. GPI-APs are also an attractive pool of antigens for vaccine and diagnostic test development.

Some of the GPI-APs, including BmGPI12 and BmGPI13 in B. microti, and GPI-anchored merozoite surface antigen-1 are highly expressed in red blood cell stages, suggesting their importance for membrane structure or function [47, 48]. BmGPI12 is also a sensitive diagnostic antigen for determining the prevalence of B. microti in affected countries [49, 50]. The performance of GPI-APs against B. duncani infection and in diagnostic assays needs to be investigated. Concerning the six-cysteine gene family in P. falciparum, some members (Pf48/45, Pf230, and Pf47) contribute to parasite fertilization, while PfP52 and PfP36 perform a vital role in sporozoite invasion of hepatocytes [51–54]. Mosquito stage-specific proteins of the six-cysteine family, such as P25, P28, P230, P48/45, and Pfs47, show significant efficiency in transmission blocking against Plasmodium; meanwhile, six-cysteine A and B emphasize candidates from this family blocking B. bovis transmission in vector ticks [55–58]. Twenty-three members of this gene family are identified in B. duncani. However, whether these members perform similar roles in B. duncani development and immune response remains largely unknown.

To complete the life cycle of Babesia spp., host cell invasion is initiated with interactions between parasite proteins and host receptors. Thrombospondin-related anonymous proteins (TRAP) contribute to sporozoites

### Table: Motif Identification of blf1

| Name             | p-value | Motif Locations |
|------------------|---------|-----------------|
| GWHPBECJ000001   | 1.03e-55|                 |
| GWHPBECJ000040   | 3.88e-119|                |
| GWHPBECJ000042   | 2.32e-97|                 |
| GWHPBECJ000043   | 1.51e-129|                |
| GWHPBECJ000044   | 3.70e-123|                |
| GWHPBECJ000095   | 5.11e-129|                |
| GWHPBECJ001717   | 1.66e-91 |                 |
| GWHPBECJ001718   | 2.02e-134|                |
| GWHPBECJ001719   | 5.34e-153|                |
| GWHPBECJ001720   | 3.79e-155|                |
| GWHPBECJ001722   | 8.21e-92 |                 |

**Motif Identification of blf1.** Each colored rectangle represents a different motif. Almost all members in this gene family present at least one of these motifs.
of *Plasmodium* infecting salivary glands/live cells and merozoites of *Babesia* infecting red blood cells [59, 60]. In some *Plasmodium* spp., such as *P. berghei* ANKA and *P. vivax* P01, a single copy of TRAP was identified in genomes [60]. However, we identified seven copies in *B. duncani*, revealing that they may contribute to a
more sophisticated process in terms of parasite-host interactions. Furthermore, transcriptional evidence of all these genes is found in blood stages, consistent with their role in red blood cell adhesion/invasion.

**Babesia duncani adaptive evolution**

Using pairwise Ka/Ks comparisons of the *B. duncani* genome with its closest sister species, we are able to discover species-specific adaptation to vectors or hosts. A branch-site model analysis was performed to determine the positive selection across 1437 orthologous genes that occurred in *B. duncani* and other piroplasm parasites. Within the *B. duncani* genome, we identified 38 genes that presented positive selection (Table 2). Furthermore, we determined these positive selection genes to fully examine whether they could perform specific functions in *B. duncani* life cycle. We noticed that some essential genes received selection pressure and took important roles in cellular process, including in transcription (high mobility group protein B1, AP2 domain transcription factor ap2ix-6, helicase), translation (tyrosyl-tRNA synthetase, RNA methyltransferase, ribosomal protein L24, N6-adenine-specific methylase), post-transcription modification (phosphatase methyltransferase, GPI ethanolamine phosphate transferase 3), and protein degradation (peptidase, 26S proteasome, ERAD-associated E3 ubiquitin-ligase, ubiquitin carboxyl-terminal hydrolase, ubiquitin carboxyl-terminal hydrolase).

We also observed positive selection events in some genes correlated with the survival environment of *B. duncani*. Selection was detected in a gene involved in taking nutrients from red blood cell plasma (heme oxygenase), implying adaptation to the internal environment of red blood cells. Positive selection was also found in genes contributing to maintaining the morphology of *B. duncani*, including cytoskeleton-associated protein (subpellicular microtubule protein 1) and erythrocyte-binding protein (CD2 antigen cytoplasmic tail-binding 2).

**Conclusions**

In conclusion, using ONT sequencing and Illumina sequencing technologies, we assembled and generated the first *B. duncani* reference genome, which is essential to better understand this species’ biological features. We confirmed that *B. duncani* forms a phylogenetically distinct clade from other Piroplasm parasites and estimated the speciation date of *B. duncani* that occurred later than that of *B. microti*, providing new insights into the evolutionary history of *B. duncani*. Two gene families present significant expansion in *B. duncani* and may play important roles in host cell invasion and virulence of *B. duncani*, using comparative genomic analyses. Whether these gene families perform predicted roles needs to be unraveled through genetic manipulation technology and functional studies. Genes identified in *B. duncani* presenting signal of positive selection perform diverse roles in transcription, translation, and post-translated modification processes. Our study provides basic information for further exploring *B. duncani* features, such as host-parasite and tick-parasite interactions.
Methods

Sequencing and preparing data

The first case of babesiosis, caused by *B. duncani* WA1, was reported in a 41-year-old man in Washington state. This parasite was obtained from ATCC (PRA-302™) and injected into hamsters. Sub-cloning of this parasite was not performed, as a continuous culture system in vitro has not been developed in our laboratory. When the parasitemia reached 10%, infected red blood cells were collected and merozoites of *B. duncani* were purified as previously reported with minor modification [61]. Briefly, host nucleated blood cells were removed using a syringe filter for white blood cells (PALL, USA). Following this, blood cells were washed three times with cold phosphate-buffered saline (PBS, pH7.4) and lysed by saponin (0.05% in PBS). Merozoites were collected by centrifugation at 10,000g for 30 min.

| No. of positive selection gene | Protein ID          | Protein length (aa) | Predicted protein function                                                                 |
|-------------------------------|---------------------|---------------------|---------------------------------------------------------------------------------------------|
| 1                             | GWHPBECJ001716      | 1815                | Predicted protein                                                                          |
| 2                             | GWHPBECJ000742      | 132                 | Protein DJ-1-like protein B                                                                 |
| 3                             | GWHPBECJ001735      | 105                 | Uncharacterized protein                                                                     |
| 4                             | GWHPBECJ001852      | 211                 | Tryptophanyl-tRNA synthetase                                                                |
| 5                             | GWHPBECJ001853      | 406                 | Conserved hypothetical protein                                                              |
| 6                             | GWHPBECJ001854      | 956                 | DEAD box ATP-dependent RNA helicase family member protein                                   |
| 7                             | GWHPBECJ001855      | 386                 | Coronin                                                                                     |
| 8                             | GWHPBECJ001856      | 1032                | ATP-dependent helicase rhp16                                                                |
| 9                             | GWHPBECJ001857      | 108                 | Ribosomal protein L24 family protein                                                        |
| 10                            | GWHPBECJ001858      | 87                  | EF-hand domain-containing protein                                                            |
| 11                            | GWHPBECJ001859      | 270                 | Methionine aminopeptidase 1                                                                 |
| 12                            | GWHPBECJ001860      | 284                 | CD2 antigen cytoplasmic tail-binding 2                                                       |
| 13                            | GWHPBECJ001861      | 420                 | Ubiquitin carboxyl-terminal hydrolase                                                        |
| 14                            | GWHPBECJ003724      | 552                 | ERAD-associated E3 ubiquitin- ligase                                                         |
| 15                            | GWHPBECJ001999      | 891                 | Hypothetical protein, conserved                                                              |
| 16                            | GWHPBECJ003331      | 969                 | GPI ethanolamine phosphate transferase 3                                                     |
| 17                            | GWHPBECJ000975      | 505                 | Uncharacterized protein                                                                     |
| 18                            | GWHPBECJ002933      | 986                 | Hypothetical protein                                                                         |
| 19                            | GWHPBECJ001102      | 233                 | Hypothetical protein                                                                         |
| 20                            | GWHPBECJ002272      | 276                 | Heme oxygenase (HO)                                                                         |
| 21                            | GWHPBECJ002296      | 250                 | ABC transporter ATPase                                                                      |
| 22                            | GWHPBECJ001213      | 170                 | Phosphatase methyltransferase                                                                |
| 23                            | GWHPBECJ000505      | 195                 | Putative rRNA methyltransferase                                                              |
| 24                            | GWHPBECJ000083      | 649                 | Exosome component 10                                                                        |
| 25                            | GWHPBECJ001479      | 361                 | Hypothetical protein                                                                         |
| 26                            | GWHPBECJ000708      | 460                 | AP-2 complex subunit alpha-2                                                                 |
| 27                            | GWHPBECJ001500      | 570                 | Hypothetical protein                                                                         |
| 28                            | GWHPBECJ001501      | 211                 | High mobility group b1                                                                       |
| 29                            | GWHPBECJ001502      | 1361                | Condensin complex subunit 1                                                                  |
| 30                            | GWHPBECJ001503      | 113                 | Subpellicular microtubule protein 1                                                           |
| 31                            | GWHPBECJ001504      | 833                 | 5′->3′ exoribonuclease                                                                       |
| 32                            | GWHPBECJ001505      | 318                 | Hypothetical protein                                                                         |
| 33                            | GWHPBECJ001506      | 376                 | Tyrosyl-tRNA synthetase                                                                      |
| 34                            | GWHPBECJ001507      | 264                 | Proteasome subunit alpha                                                                     |
| 35                            | GWHPBECJ001508      | 760                 | Peptidase, 59A/B/C family, catalytic domain-containing protein                               |
| 36                            | GWHPBECJ001509      | 1084                | Helicase SKI2W                                                                               |
| 37                            | GWHPBECJ003167      | 695                 | Elongation factor G                                                                         |
| 38                            | GWHPBECJ001820      | 76                  | Secreted ookinete protein                                                                    |
Genomic DNA was extracted using a commercial DNA extraction kit according to the manufacturer’s instructions (QIAamp DNA Blood Mini Kit; Qiagen, Hilden, Germany). The library for PromethION was constructed using a ligation kit (SQK-LSK109, Oxford Nanopore Technology, Oxford, UK) and then analyzed using two FLOMIN106 flow cells (v9.4.1). The raw FAST5 data were base called using Guppy (v3.2.2) [62]. A library of 400-bp paired-end reads of genomic DNA was prepared for genome correction and sequenced using the Illumina sequencing platform. Total RNA was extracted, and library construction was performed according to Illumina TruSeq mRNA library protocol.

De novo assembly
To remove hamster genomic DNA contamination, the NanoLyse software package was used to compare ONT sequencing data with *Cricetulus griseus* genome (https://www.ncbi.nlm.nih.gov/; accession number GCA_000223135.1) [63]. Eventually, 11,118 reads (account for 5.7% of ONT data) from host genomic DNA were removed. Low-quality reads, contained in ONT sequencing data, were filtered by NanoFilt [63]. Meanwhile, for Illumina sequencing data, low-quality base/reads and adaptor sequences were removed by trim_galore (https://github.com/FelixKrueger/TrimGalore) and contamination of host genome DNA (423,726 paired reads account for 4.49% of Illumina sequencing data) was depleted by aligning Illumina reads with *Cricetulus griseus* genome using bowtie2 [64].

In our previous study, genome assembly pipelines were developed for Piroplasm parasites [65]. Briefly, genome assembly of ONT reads was performed using NECAT (v0.0.1) and Canu (v2.2.2) with default parameters. Correction of raw draft genomes is a critical step in ONT reads assembly (https://ngdc.cnbc.ac.cn, CRA004588; https://www.ncbi.nlm.nih.gov/bioproject/PRJNA844476) [66, 67]. Draft genomes were improved by ONT reads self-correction using Medaka (v1.3.4). Further error correction was an essential step using Illumina data, which are available from the National Genomics Data Center (https://ngdc.cnbc.ac.cn) under accession number CRA004607 and the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov) under accession number PRJNA844476, using Pilon to generate the final assembly output [66–68]. To generate more contiguity assembly, we also merged assembly outputs from assemblies derived from distinct de novo tools (NEACT and Canu) (Fig. 9). Samtools was employed to determine the overall coverage of the genome assembly by mapping Illumina sequencing reads to it. We obtained 98.1% coverage of the assembly. Furthermore, the quality of assembly was evaluated using Benchmarking Universal Single-copy Orthologs (BUSCO v5.1.3) to determine the completeness using the core apicomplexan dataset (apicomplexa_odb10) [20, 69, 70].

Genome annotation
Before genome annotation, repeat sequences were masked to reduce the requirements of computed resources and to produce reliable annotation outcomes. For this purpose, a standard pipeline was performed including (1) simple tandem repeat sequences predicted using TRF (v4.09) program, (2) ab initio repeat identification using RepeatScout (v1.0.5), and (3) homologous alignment using RepeatMasker program [71–73]. The genome of masked sequences was performed gene structure annotation.

Gene structures were predicted by a combination of ab initio, homology alignment, and transcriptome data. In terms of ab initio, PASA (v2.3.1) was applied to produce candidate gene structures based on the longest open reading frame and a GFF3 file, which could be applied to obtain a set of gene structured for
training gene models of Augustus (v3.3.3) and GlimmerHMM (3.0.4) programs [64, 74–77]. Following this, we used Augustus and GlimmerHMM to predict the gene structure based on the trained gene models. Furthermore, ATT, exonerator (2.4.0), and GeneID (v1.4.5) programs were used to align with the UniProt apicomplexan database to identify candidate gene structures [78–80]. Illumina RNA-Seq reads (https://ngdc.cnbc.ac.cn, CRA004607) were mapped against the B. duncani genome with TopHat2 (v2.1.1) [81]. Mapped reads were processed using Cufflinks (v2.2.1) to generate annotation blocks were defined as at least five homologous genes in homologous scaffolds and gene synteny. The pairwise analysis. MCScanX (https://github.com/tanghaibao/jcvi/wiki/ MCscan-(Python-version)) was used to identify homologous scaffolds and gene synteny. The evidence of gene annotation from ab initio, homologous alignments, and transcriptional data was integrated into a non-redundant gene set by EvidenceModel (v1.1.1) [85].

**Collinearity analysis**

Three species with completed genomes, including B. duncani (https://www.cnbc.ac.cn/, GWHBECJ00000000), B. microti (GCF_000691945.2), and B. bovis (AAXT02000000), were selected for collinearity analysis. MCScanX (https://github.com/tanghaibao/jcvi/wiki/ MCscan-(Python-version)) was used to identify homologous scaffolds and gene synteny. The pairwise blocks were defined as at least five homologous genes in the 25-gene size window.

**Ortholog group identification and gene family expansion and contraction analysis**

Apicomplexan protein sequences were downloaded from NCBI and Plasmo DB databases. The orthologous group across 18 species, including B. duncani (https://www.cnbc.ac.cn/, GWHBECJ00000000), B. bigemina (GCA_000981445.1), B. bovis (AAXT02000000), B. microti (GCF_000691945.2), B. ovata (GCA_002897235.1), Babesia sp. Xinjiang (GCA_002095265.1), T. annulata (GCA_000003225.1), T. parva (GCA_000165365.1), T. equi (GCA_000342415.1), T. orientalis (GCA_000740895.1), Toxoplasma gondii (GCA_00006565.2), Neospora caninum (GCA_016097395.1), Plasmodium falciparum (GCA_00002765.3), P. vivax (GCA_00002415.2), P. yoelii (GCA_900002385.2), Cryptosporidium parvum (GCA_000165345.2), Eimeria tenella (GCA_000499545.1), and Tetrahymena thermophila (GCA_000189635.1), were identified using OrthoFinder (v2.5.4), which is a practical, fast, accurate, and comprehensive tool for comparative genomes [86, 87]. The program, based on amino acid sequence alignment, uses diamond, and the important parameter inflation index was set at 1.5 to balance sensitivity and selectivity. Identified ortholog groups were used for further analysis of gene family expansion and contraction with caf (v2.0) [88, 89].

**Phylogenetic analysis and divergence time estimation**

Three hundred ten single-copy orthologous that were present in 18 species of Apicomplexa parasites were aligned with MUSCLE, and ambiguous alignments were processed using Gblocks with default parameters (parameters: -t = p –b = h –p =n –b4 = 2) [90]. Then aligned sequences were concatenated by custom scripts to generate FASTA files for further phylogenetic analysis. The maximum likelihood phylogenetic trees were generated by RAxML with the best fit model LG+F+R5 [91]. ITOL was used to visualize and edit the labels of phylogenetic trees (http://itol2.embl.de/upload.cgi) [92].

The divergence time for Apicomplexa parasites was estimated using the mcmtree program with three correlated time points, 817 million years ago (Mya, divergence time between T. gondii and P. falciparum, ranging from 580 to 817 Mya), 470 Mya (divergence time between E. tenella and N. caninum), and 1290 Mya (divergence time between C. parvum and T. thermophila, ranging from 767 to 1344 Mya) [93–97].

**Ka/Ks analysis**

The nonsynonymous (Ka) and synonymous (Ks) substitution rates and positive selection strength (Ka/Ks) were calculated by KaKs_Calculator (v2.0) [98]. First, reciprocal BLAST was used to run the pairwise alignments between Babesia spp., the e-value was set to 10−5, and the number of hits for each pair of species was set to 5. Second, each pairwise protein sequence was aligned by MUSCLE, and pairwise nucleotide sequence alignments were generated by transforming protein alignments into codon alignments with ParaAT [99]. Third, Ka/Ks ratios were calculated based on pairwise codon alignments using KaKs_Calculator, and the models of KaKs_Calculator were invoked from PAML. M0 model (Branch site model) was used in this study [100].

**Abbreviations**

ONT: Oxford Nanopore Technology; ITS: Internal transcribed spacer; GO: Gene Ontology; CoxI: Cytochrome b; b/f: 1: B. duncani largest family 1; TRAP: Thrombospondin-related anonymous protein; GPI-AP: Glycosylphosphatidylinositol-anchored protein family; SEA: Serine esterase; PBS: Phosphate-buffered saline; Ka: Nonsynonymous; Ks: Synonymous.
Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12915-022-01361-9.

Additional file 1: Table S1. Protein annotations of B. duncani using BLASTP and Interproscan programs.

Additional file 2: Table S2. Babesia duncani largest gene family 1.

Additional file 3: Table S3. 223 species specific genes in B. duncani.

Additional file 4: Table S4. Multi-gene families in B. duncani.

Additional file 5: Table S5. Significantly expanded gene families in B. duncani.

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Authors' contributions

Manuscript: JW and KC. Analysis: JW, JC, and GW. Reagents/materials: JY, SZ, JX, YL, and GL. Supervision: JL, YH, and GG. All authors have read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in the article, its supplementary information files are publicly available in repositories. The genomic sequencing data of ONT reads and Illumina reads presented in this study have been deposited in the China National Center for Bioinformation (https://www.cncbc.ac.cn/, CRA0A4S86 and CRA0A46607) and the NCBI Sequence Read Archive (SRA) database under project accession number PRJNA844476 [66, 67].

Declarations

Ethics approval and consent to participate

The collection and manipulation of sheep blood samples were approved by the Animal Ethics Committee of the Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. All sampling procedures were handled in accordance with the Animal Ethics Procedures and Guidelines of the People's Republic of China (Permit No. LVRIAEC-2018-001). All the procedures conducted were according to the Ethical Procedures and Guidelines of the People's Republic of China (Permit No. LVRIAEC-2018-001). All the procedures conducted were in accordance with the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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