Investigation of the Piroplasm Diversity Circulating in Wildlife and Cattle of the Greater Kafue Ecosystem, Zambia

David Squarre
  Research Center for Zoonosis Control, Hokkaido University

Yukiko Nakamura
  Research Center for Zoonosis Control, Hokkaido University

Kyoko Hayashida
  Research Center for Zoonosis Control, Hokkaido University

Naoko Kawai
  Research Center for Zoonosis Control, Hokkaido University

Herman Chambaro
  Research Center for Zoonosis Control, Hokkaido University

Boniface Namangala
  School of veterinary Medicine, University of Zambia

Chihiro Sugimoto
  Research Center for Zoonosis Control, Hokkaido University

Junya Yamagish (✉ junya@czc.hokudai.ac.jp)
  Hokkaido Daigaku

Abstract

Background: Piroplasms are vector-borne intracellular haemoprotozoan parasites that infect wildlife and livestock. Wildlife species are reservoir hosts to a diversity of piroplasms and play an important role in the circulation, maintenance and evolution of these parasites. The potential for likely spillover of both pathogenic and non-pathogenic piroplasms from wildlife to livestock is underlined when common ecological niche is shared in the presence of a competent vector.

Method: To investigate piroplasm cryptic variation and diversity in parasite community in wildlife and cattle population of the greater Kafue ecosystem, we utilized PCR to amplify the 18S rRNA V4 hyper-variable region and meta-barcoding strategy using illumina MiSeq sequencing platform and amplicon sequence variant (ASV) based bioinformatics pipeline to generate high resolution data which discriminate sequences down to a single nucleotide difference.

Results: A parasite community of 45 ASVs and 23 piroplasm species consisting of 4 genera of *Babesia*, *Theileria*, *Hepatozoon* and *Colpodella* was identified in wildlife and cattle population from the study area.

Conclusion: The findings of this study demonstrates the usefulness of illumina MiSeq sequencing to describe the area specific parasite composition, which is critical in the application and establishment of disease control strategy options of ideal diagnostic tools to employ and area specific vaccine development.

Introduction

Piroplasmida is an order of intracellular heamoprotozoan parasites that belong to the phylum Apicomplexa. The species of genera *Theileria* and *Babesia* cause clinical disease in vertebrate host which include domestic and wild animals(1,2). The parasites are transmitted by vectors of ixodid ticks and cause considerable socio-economic impact on livestock production in sub-Saharan Africa, threatening livelihoods, food security, and cultural heritage(3). The *Theileria* and *Babesia* genera are characterized by a wide diversity of species and genotypes(4,5). This diversity is a consequence of many factors including genetic recombination, mutations and evolutionary process of evading host's and vector's immune response, leading to the selection pressure of isolated population occasioning genetic drift(6,7). Wildlife plays an important role in the circulation, maintenance and evolution of these parasites. African buffalos (*Syncerus caffer*) for example, are reservoir hosts of *Theileria parva* and hence play a vital role in theileriosis (8,9). The buffalo-derived *T. parva* causes severe acute infection in cattle resulting in mortalities(10). This form of theileriosis, also referred to as corridor disease is thought to be only transmitted from buffalo to cattle and not between cattle because cattle acutely die before piroplasms emerge or are sufficient to infect new ticks. Corridor disease is usually self-limiting and the occurrence is intermittent in areas where cattle and African buffalo associate in the presence of the vectors(10,11). Conversely, cattle derived *T. parva* causes infection in cattle but due to an immune response and sometimes coupled with chemotherapy, some cattle survive and become asymptomatic carriers leading to the continuous spread of the parasite among cattle. This type of theileriosis is referred to as East Coast fever (ECF)(12).

Although *Theileria* is by far the most important piroplasm causing measurable effect on livestock production, *Babesia* also cause a wide range of infectious diseases in domestic animals. Redwater in cattle, canine babesiosis, and equine piroplasms are caused by *Babesia bigemina/B. bovis*, *B. canis,* and *B. caballi/B. equi*, respectively. Several wildlife species are natural hosts of a wide diversity of piroplasms that are pathogenic and non-pathogenic to domestic animals.

The Kafue ecosystem, measuring 68,000 Km^2 in size, is a large conservation area in central Zambia. It is composed of the Kafue national park (22,400 km^2) and nine adjacent game management area (GMAs) that act as a buffer to the national park. The national park is host to numerous wildlife species and particularly is devoid of human settlements and livestock. The GMAs that immediately surround the park are notably characteristic of wildlife cohabiting with communities and their livestock, thus forming a wildlife-livestock interface area(13,14). The potential for likely spillover of arthropod-borne pathogens such as piroplasms from wildlife to livestock occurs when a common ecological niche is shared in the presence of a competent vector(15). In addition to the interface in conservation areas, the growing game ranching/farming industry in Zambia has integrated wildlife and livestock farming, creating widespread patches of ex-situ wildlife-livestock interface areas across the country. The primary source of wildlife for game farms are the conservation areas such as the Kafue ecosystem. Game ranching is hinged on a large volume of translocation, stocking and restocking of wildlife species including known wildlife reservoirs for important piroplasms such as buffalo that affect the livestock. This has the potential of spreading parasites and creating a vortex of piroplasmid parasites across the country.

In Zambia, livestock diseases caused by piroplasms are controlled by several approaches as outlined in the tick and tick-borne disease control strategy and the east coast fever (ECF) control strategy (2016-2021) (16,17). The control measures include; vector control (use of acaricide), calf immunization of live vaccine (for theileriosis), chemotherapy and stock movement controls. Clinical examination and use of microscopy to demonstrate piroplasms in blood smear and schizonts in lymph biopsy smears are common and primary diagnostic methods used. Serological methods such as indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA), based on monoclonal antibodies and polymorphic immunodominant molecule (PIM), are routinely used to detect seroconversion and identify possible asymptomatic carrier animals for stock movement control purposes(12,18). However, due to several closely related genotypes within parasite species, these methods have a general

limitation on sensitivity and specificity. Non-discrimination and cross-reaction of closely related species or genotypes is a common problem associated with serological assays in mixed infection(19,20). Indeed, cross-reactions among *T. parva, T. taurotragi* and *T. annulata* are common(21). Other cross reactions are also demonstrated between *T. parva* and *T. sp* (buffalo)(22,23). Molecular method of reverse line blot polymerase chain reaction (RLB-PCR) assay which is based on the simultaneous amplification of the V4 hyper-variable region of the 18S rRNA gene of theileria, babesia and other piroplasms followed by hybridization of specific probes of piroplasm parasites of interest, has been reliably used to detect diverse species/genotypes of piroplasms(24–26). However, the major hindrance to this method is the masking of new or novel species or genotypes in the presence of known species/genotype in mixed infections leading to narrow conclusion(25). The real-time hybridization test that amplify the 18S rRNA V4 hyper-variable region and uses two probe sets for *T. parva* and *Theileria* genus, have been designed to detect targeted species (27). This assay also has a limitation with cross-reactions of similar or closely related species, leading to diagnostic challenges. Suppression of the qPCR signal of *T. parva* in buffalo with mixed infection of *T. sp.* (buffalo) and *T. sp.* (bougasvlei) has been reported, which may lead to false-negative result (27,28). Thus, the complex species and genotypic diversity and natural occurrence of mixed infections of both pathogenic and non-pathogenic piroplasms in domestic animals and wildlife pose diagnostic and interpretation challenge of pathogenic parasites(22).

Highlighting the cryptic species/genotype diversity of circulating parasites in wildlife, livestock and vector population is essential to understand disease ecology and piroplasmid parasite community composition(29,30). It is important to identify species and genotypes known to cause diagnostic problems and account for interpretations given the diagnostic limitation related to cross reactions, suppression and non-discrimination inherently associated with piroplasm diversity and mixed infections. This also has implication on the choice of assays to adopt in control options such as calf immunization, and in epidemiology studies of piroplasm infections(21,22,29–31). Understanding the parasite community also provides basic information for the selection of live or recombinant vaccines to be used in a specifc area (32). To investigate parasite diversity, deep amplicon sequencing of the 18S rRNA V4 hyper-variable region and next generation sequencing (NGS) technology of illumina MiSeq are applied to generate in-depth high resolution data of the parasite community. This study uses genomics and bioinformatic tools to investigate and establish a diversity of piroplasm community in wildlife and cattle in a discrete geographical region of the greater Kafue ecosystem of Zambia.

Methods

Sample collection and DNA extraction

The Greater Kafue ecosystem (Figure 1.) is a conservation area located in central Zambia (14°03²S / 16°43²S and 25°13²E / 26°46²E) and measuring about 67,806Km^2 in size. The sample collection from wild animals was done between May and August in 2017 and 2018. Whole blood samples were collected in EDTA tubes from 210 individual wild animals comprising 12 wildlife species in the Kafue National Park (Table 1). Additional 230 blood samples were collected from cattle in the interface between the GMA and open area in Zambia's Itezhi Tezhi district between April and May, 2019 (Table 1). From each blood sample collected, genomic DNA was extracted using the DNA Isolation Kit for mammalian blood (Roche Applied Science, Indianapolis, USA) for wild animal samples and QuickGene DNA whole blood kit S (Kurabo, Osaka, Japan) for cattle samples as per manufacturers protocol. A 200 ml of DNA was eluted in Eppendorf tubes and stored at -80 °C until analysis.

RLB-PCR amplification and library preparation

Amplification of the V4 hypervariable region of the 18S rRNA gene was obtained by piroplasms specific RLB-PCR using primers RLBF and RLBR (Table 2). A reaction mix of 10 mL containing 5.0 mL Ampdirect plus buffer (Shimadzu, Kyoto, Japan), 3.95 mL PCR grade water, 0.05 mL Bio Taq HS (Bioline, London, UK), 0.5 mL DNA template and 0.2 mL each of the RLB primers. The thermocycler conditions were 94 °C for 10 min denaturation and 40 cycles of 94 °C for 1 minute, annealing at 50 °C for 1 minute, extension at 72 °C for 1.5 minutes and final extension at 72 °C for 10 minutes. The second PCR adding sequencing tag was conducted using 100 times diluted 1st PCR amplicons as template. The reaction volume of 10 mL comprised the same volumes of reagents as the first RLB-PCR but instead replaced the primer with 10 mM illumina-tagged RLB primers (Table 2). The thermocycler conditions were the same as the first PCR except amplification was set at 12 cycles. The illumina-tagged amplicons from the second PCR were then diluted 50 times and 1mL was added to a 20 mL reaction mixture for Index PCR. The other reagents included 4 mL of 5X buffer, 1.4 mL MgCl$_2$ (25mM), 0.5 mL 10mM dNTP mix, 1mL mix illumina-index primer (Table 2), 11.975mL nuclease-free water and 0.125 mL KAPATaq Extra. The indexing PCR was run with thermocycler condition of 95 °C initial denaturation for 5 minutes followed by 15 cycles of 92 °C for 30 seconds, 45 °C for 30 seconds and 72 °C for 30 seconds and final extension at 72 °C for 15 minutes. The obtained amplicons were quantified by agarose gel electrophoresis, pooled then purified using Wizard SV Gel and PCR Clean-Up system (Promega, Madison, WI, USA).

Amplicon sequencing and Bioinformatic analysis

The RLB-PCR amplicon library was sequenced with the MiSeq using a 300 bp paired-end sequencing protocol and the MiSeq sequencing reagent kits v3 (Illumina) with 25% PhiX DNA spike-in control according to the manufacturer's instructions. Quality control and filtering were conducted with Trimmomatic(33) using the following parameter; TRAILING:20, SLIDINGWINDOW:4:15 and MINLEN:36. Concatenation between forward and reverse reads and primer trimming were conducted with AMPtk(34), allowing minimum merged length of 400 bp. Primer sequences to be trimmed

were GAGGTAGTGACAAGAAATAACAATA and TCTTCGATCCCCTAACTTTC for forward and reverse reads, respectively. A set of amplicon sequence variants (ASVs) were generated by DADA2 and LULU in the AMPtk package using the default parameters. The obtained sequences were annotated based on sequence homology with the Basic Local Alignment Tool (BLAST), and non-redundant nucleotide database by NCBI using -max_target_seqs 1, -perc_identity 70, -qcov_hsp_perc 70 and -evalue 1e-20 as a set of parameters (35). Operational taxonomic units (OTUs) was further generated by clustering the ASVs using usearch(36) with 99% similarity as clustering threshold. Observed ASVs in each sample were filtered out if number of the assigned reads were less than 1% of the total number of assigned reads.

Phylogenetic analyses

Phylogenetic relationship among ASVs were analyzed using Neighbor-Joining method(37) implemented in MEGA X(38). The evolutionary distances were computed using the maximum composite likelihood method(39) and default parameters with 10,000 bootstraps. Visualization and annotation were conducted using iTOL v5.5(40). Each clade was annotated based on sequence similarity obtained by the blast analysis.

Results

Detection of piroplasm parasite by PCR, taxonomical annotation and host tropism

Out of 210 wild animals and 230 cattle, 131 and 147 tested positive for piroplasm parasites by RLB-PCR, respectively. Of the 12 wildlife species sampled and screened, 7 species were infected by piroplasms (Table 1, Supplementary Table S1). All the positive amplicons were subjected to sequence analysis to identify their taxonomic classification. In total, 2.80M raw reads had been obtained from 278 PCR positive samples, then merged into 2.46M contigs (Supplementary Table S1).

A total of 45 ASVs of the V4 hyper-variable region of the 18S rRNA gene corresponding to Apicomplexan parasites were obtained from both wildlife species and cattle sampled from the greater Kafue ecosystem (Table 3). The taxonomic assignment of ASV using blastn resulted into the identification of four genera, *Theileria*, *Babesia*, *Hepatozoon* and *Calpodella*, which consisted of 11, 3, 2 and one known species and 36, 6, 2 and one ASVs, respectively (Supplementary Table S1)

In the phylogenetic analysis, we observed both *Theileria* and *Babesia* clade (Figure 2). The *Theileria* clade consisted of subclade for *T. velifera*, *T. mutans*, *T. parva* and *T. taurotragi*.

The *T. velifera* subclade consisted of seven ASVs and sequence similarity to *T. velifera* was 98.7% to 100%, suggesting all of these ASV belong to *T. velifera.* The subclade was further divided into two groups based on sequence similarity. One was ASV6, 29 and 55 which were detected only in buffalo, while ASV7, 64 and 92 were cattle specific and ASV1 was detected in cattle and impala (Da049).

A similar correlation among sequence similarity and hosts was observed in the *T. mutans* clade. ASV26, 42 and 60 were buffalo-specific and ASV3, 5, 16 were detected from both buffalo and cattle. All of them had more the 99.5% similarity to reference sequences of *T. mutans.*

Interestingly, most of the observed ASVs in the *T. parva* clade were buffalo specific except ASV15 (*T. parva*) and ASV11 (*T*.sp buffalo) which were detected in both buffalo and cattle (Na032 and Na142, respectively) (Supplementary Table S1). ASV25 showed 100% similarity to *Theileria sp. bougasvlei* but was adjacent to the *T. parva* clade.

T. taurotragi was detected only in four cattle. ASV46 and 62 had more than 99.8% similarity to a *T. taurotragi* reference sequence but ASV35 had 97.8% similarity implying this can be categorized in a subspecies.

There were two additional clades in *Theileria*. One consisted of ASV8, 9 14, 23, 49 and 85. It was almost specific to hartebeest even though ASV8 and 23 were detected from a wild dog (Da082). They showed high similarity to *Theileria* spp. without species name. The other consisted of ASV2, 47 and 106 specific for impala. ASV4 was detected from both hartebeest and sable but also found in a buffalo (Da109). ASV12 and 13 were detected from Sitatunga.

The *Babesia* clade consisted of subclade for *B. bigemina* and *B. occultans*. ASVs in the *B. bigemina* subclade showed more than 99.8% similarity to *B. bigemina* reference sequences and detected only in cattle. *B. occultans* was also detected only in cattle.

A *Hepatozoon canis* sequence was detected from a lion and another *Hepatozoon* sp. was detected from a wild dog. Interestingly, a colpodellidae sequence, ASV57, was also detected from cattle.

Discussion

We have comprehensively described piroplasm parasite community in wildlife and cattle population of the greater Kafue ecosystem by a meta-barcoding strategy using illumina MiSeq sequencing platform and ASV based bioinformatics pipeline. This approach has an advantage of generating high resolution data which enables discriminating sequences down to a single nucleotide difference(41,42). This is ideal to reveal

cryptic variation and diversity in parasite community in a population which is ordinarily masked when looked at genus or species level(30). The robustness of RLB-NGS (illumina MiSeq) approach in piroplasm investigations is well demonstrated in previous studies done on laboratory isolates, trial livestock and captive wild/domestic buffalos(30,43). Here we present the use of this method in different species of wild populations in the national park and in the wildlife-livestock interface area. A revealed parasite community of 45 ASVs and 23 piroplasm species consisting 4 genera (*Babesia*, *Theileria*, *Hepatozoon* and *Colpodella*) were identified in wildlife and cattle population from the study area.

The study identified 16 sequences of *Theileria* species, subspecies and variants (Table 4). As an important natural reservoir host, buffalo had the highest diversity of ten theileria species or sub-species infection compared to other wildlife species. Importantly, three genotypes of *T. parva* (OTU23 comprising ASV15, 86 and 101) were obtained from buffalo, providing important epidemiological data for cattle in the area. This finding is consistent with a previous report from a serological study involving buffalos (15). Indeed, *T. parva* ASV15 was detected in cattle (Na032), suggesting possible spillover of *T. parva* from wildlife to domestic animals.

The presence of non-pathogenic *T. sp. (buffalo)* (26 of 53; 49.1%) and *T. sp. (bougasvlei)* (10 of 53; 18.9%) in buffalo (Table 4) is of diagnostic importance as it affects the accurate detection of *T. parva* in mixed infections when performing hybridization PCR assay diagnosis(44). In addition to buffalo, this study found *T. sp* (buffalo) circulating in cattle population (1 of 230; 0.4%). This finding deviates from previous studies in southern Africa which classified *T. sp* (buffalo) as a buffalo specific parasite(22,45). In fact, other studies conducted in Kenya also identified *T. sp* (buffalo) from cattle, suggesting that *T. sp* (buffalo) infection in cattle occurs in the field where buffalo and cattle share pasture(46,47). *T. sp* (buffalo) is considered to transiently infect cattle and no carrier state is proved, same as Corridor disease of buffalo-derived *T. parva* infection. Nevertheless, more knowledge on the infection epidemiology and pathogenicity to cattle will be required. Further, the presence of *T. taurotragi* circulating in cattle population is consistent with findings in other similar studies(25) .The characterization of parasite community and molecular ecology raises awareness on the consequences and limitations of specific diagnostic tests and further cautions the interpretation of the results used for diagnostics or surveillance in a specified area.

Babesia was predominantly observed in cattle but also detected in Wild dogs. *Babesia bigemina* (31 of 230; 13.5%) and *B. occultans* (4 of 230; 1.7%) were the only species detected in cattle (Table 4), of which *B. bigemina* is a pathogenic parasite to cattle causing a clinical disorder of babesiosis, also known as Redwater. These findings are similar to other comparable studies in southern Africa where the presence of *Babesia* in cattle and wild animals particularly buffalo was assessed(45). To the best of our knowledge, this is the first report of the non-pathogenic *B. occultans* in Zambia. However, its specific vectors, impact on cattle, diagnostic consequence in *Babesia* mixed infection or implication of infection to wildlife are not evaluated.

Despite not being classified in the order of piroplasm but Euccidiidae, Apicomplexan species of *Hepatozoon canis* and *Hepatozoon sp.* were detected in African lion and wild dog samples, respectively. Divergent to other arthropod-borne parasites transmitted through the vector`s salivary glands at the time of feeding, *Hepatozoon* are transmitted to the canid host exclusively by ingestion of infected vectors (ticks) during grooming(48,49). They cause subclinical infection in wild canids while domestic dogs yield to clinical infection(50). Previous studies on free ranging wild canids in Zambia have indicated the widespread presence of *Hepatozoon sp*. in lions(51). This finding highlights the considered epidemiologic role of wild canids as sylvatic reservoir of canine *Hepatozoon* and presents the risks of likely spillover of *Hepatozoon* infections to domestic canids in the interface area.

Genera of *Colpodella* are part of apicomplexan organisms that are originally known to be free-living. However, recent studies have revealed the parasitic nature of *Colpodella* sp. as a Human Erythrocyte Parasite (HEP) that has lately been reported to cause relapsing fever and neurological symptoms in human(52,53). Furthermore, the detection of *Colpodella* sp. in ticks suggests that this parasite may potentially be transmitted by a tick vector(52). We detected a *Colpodella* sequence from one of the cattle samples, with the sequence similarity of 79.6% with the reported human cases (GQ411073; *Colpodella* sp. HEP). The sequence detected from our cattle sample showed perfect match (100% identity) to GenBank MN103986 (Colpodellidae clone PL31), reported in raccoon dog in Poland(54). Thus, the detection of *Colpodella* sp. from cattle sample implies to support those findings that some of the *Colpodella* species are associated with vertebrates, and possibly cause disease. It is largely undetermined what vector is involved, how the parasite is maintained, and the risk that the cattle may pose for human infection. It would be important to determine the zoonotic scale of *Colpodella* infection to rule out incidental infections.

The study has revealed the piroplasms parasite community circulating in wildlife and cattle population but also highlighted the parasites that are present in both wildlife and cattle which may signify the possible overspill of wildlife parasites into cattle population in common instances of sharing common resource niche of pasture and water at the wildlife-livestock interface. This is particularly important since Zambia`s cattle population stronghold is in the Itezhi Tezhi district which is adjacent to the KNP. This is cardinal as accurate diagnosis and effective control (vaccinations) of piroplasms need to take the parasite community data into account.

The growth of the game ranching in Zambia has steered an increased large scale movement of seed stock of wildlife from national parks and GMAs to game ranches dispersed throughout the country. Conversely, restocking programs of depleted conservation areas with wildlife from national parks with profuse numbers to restore ecological functions of these areas in line with commitment of the global theme of decade of ecosystem restoration(55). Despite the obvious conservation benefit of these programs there is a potential risk of spread of endemic piroplasms

and its vectors into expanded new areas to the detriment of livestock production in the absence of deliberate and specific strategy of accurate diagnosis and wildlife movement controls(56)

The fine scale results of metagenomic analysis of parasite communities has applications in the assessment and development of area specific vaccines candidates. This implies the vaccination programs conforming to local strains and not vaccinations based on cross reacting strains. This prevents introduction of foreign strains to the area from vaccination with exotic cross reacting strains.

Molecular epidemiology based on the strength of knowledge of cryptic parasite community and diversity is essential in identifying and tracing source of infection or outbreaks. Mapping of these parasites in all major livestock landscapes beyond the Kafue ecosystem using metagenomic approach may benefit the piroplasm control in Zambia through high resolution data to precisely guide diagnosis, vaccination and movement controls

Declarations

Competing interests

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

The permission to collect blood samples from the free ranging wildlife of the Kafue ecosystem and authority to use the samples for this study was granted by the Department of National Parks and Wildlife (DNPW) in Zambia (TJNPW/8/27/1). The blood samples from cattle were collected under the permission from ethics approval Ref. No.2019-Feb-081 (ERES Converge IRB, Lusaka, Zambia).

Authors` contributions

DS, YN collected samples and wrote the Manuscript. DS, YN, NK, KH and JY conducted laboratory experiments and analyzed the data. HC collected samples. JY, KH, BN and CS designed the study, supervised the work and critically revised the manuscript. All authors read and approved the final Manuscript

Consent of publication

Not applicable.

Availability of data and materials

Data supporting the conclusions of this article are included within the article and its additional file. Representative ASV sequences were deposited in the GenBank database under the accession numbers MT814722- MT814766

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Tables

Table 1. Detection of piroplasma parasites in wildlife species and cattle from the Kafue ecosystem

	Species sampled	Number	RLB-PCR positive	Piroplasma prevalence (%)
1	Impala *(Aepyceros melampus)*	106	65	61.3
2	Hartebeest *(Alcelaphus buselaphus)*	47	19	40.0
3	Sable *(Hippotragus niger)*	8	8	100
4	Lion *(Panthera leo)*	4	1	25.0
5	Wild dog *(Lycaon pictus)*	2	2	100
6	Sitatunga *(Tragelaphus spekii)*	4	3	74.0
7	Buffalo *(Syncerus caffer)*	53	33	62.3
8	lechwe *(Kobus leche leche)*	9	-	-
9	cheetah *(Acinonyx jubatus)*	1	-	-
10	Vevert monkey *(Chlorocebus pygerythrus)*	1	-	-
11	Baboon *(Papio ursinus)*	1	-	-
12	warthog *(Phacochoerus africanus)*	17	-	-
13	cattle (Bos taurus)	230	147	63.9
	Total	**483**	**278**	**57.6**

Table 2. Primers used for piroplasm parasite detection

Primer`s target region	Primer name	Primer sequence (5`-3`)	Reference
18S rRNA V4 hyper-variable region	Reverse Line Blot - F (RLB-F)	GAGGTAGTGACAAGAAATAACAATA	(24)
	Reverse Line Blot - R (RLB-R)	TCTTCGATCCCCTAACTTTC	
illumina-tag for RLB	Forward primer adapter	ACACTCTTTCCCTACACGACGCTCTTCCGATCT[RLB-F]	
	Reverse primer adapter	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT[RLB-R]	
illumina-index primer	illumina-i5 primers	AATGATACGGCGACCACCGAGATCTACAC[index] ACACTCTTTCCCTACACGACGCTCTTCCGATCT	
	illumina-i7 primers	CAAGCAGAAGACGGCATACGAGAT[index] GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	

Table 3. Diversity of piroplasm detected in wildlife and cattle samples collected from the Kafue ecosystem. OTU[a] = Operational Taxonomic Unit, ASV[b] = Amplicon Sequence Variant

Genus	Species	Subspecies	Blast Top Hit	ID	OTU[a]	ASV[b]
Theileria	*T. verifera*	*T. verifera*	*T. velifera* KSA_D_Th6	LC431550	1	1,7,64,92
		T. verifera A	*Theileria cf. velifera A*	GU733375	2	6
		T. verifera B	*Theileria cf. velifera (Syncerus caffer)* clone H4a	JN572701	1	29,55
	T. mutans	*T. mutans*	*Theileria mutans* isolate MT15	KU206320	11	3,16
		T. mutans MSD	*Theileria sp.* B15a	JN572700	12	5
		T. mutans-like 1	*Theileria cf. mutans 1 (Syncerus caffer)* clone C21b	JN572699	13	26
		T. mutans-like 2	*Theileria cf. mutans 2 (Syncerus caffer)* clone Q15d	JN572696	14	42,60
	T. parva		*Theileria parva* isolate F45P16	MH929322	23	15
			Theileria parva	AF013418	23	86,101
	T. sp (buffalo)		*Theileria sp. ex Syncerus caffer* MCO-2011 clone V8b	HQ895982	23	10,11,18,22
	T. sp (bougasvlei)		*Theileria sp.* KS-2015 isolate CAT79	KP410267	22	25
	T. taurotragi		*Theileria taurotragi*	L19082	20,21	35,46,62
	T. sp (sable)		*Theileria sp.* BM-2010/sable	GU733378	16	8,14,85
	T. sp (waterbuck)		*Theileria sp.* NG-2013c isolate waterbuck 39 clone 6	KF597072	19	4
	T. sp. (tsessebe)		*Theileria sp. ex Damaliscus lunatus* clone TS22_11	HQ179766	16	9,23,49
	T. sp (giraffe)		*Theileria sp.* NG-2012b isolate 44 clone 2	JQ928925	15	2,6,47
	T. sp (bongo)		*Uncultured Theileria sp.* isolate BNG13	MH569462	17	12
			Uncultured Theileria sp. isolate BNG10	MH569463	18	13
Babesia	*B. bigemina*		*Babesia bigemina* clone PR28CL7	MH050387	7	32,71
			Babesia bigemina isolate B_bi11	EF458200	7	67
			Babesia bigemina clone PR38CL1BBIG	MH047819	7	19
	B. sp		*Babesia sp.* 9 1093 cl1	KX218437	10	58
	B. occultans		*Babesia occultans* isolate Trender1	KP745626	6	28
Hepatozoon	*H. canis*		*Hepatozoon canis* isolate 70	MK645969	8	40
	H. sp		*Hepatozoon sp.* 2 BCS-2013 isolate L4	KF270665	9	82
Colpodellidae			*Uncultured Colpodellidae* clone PL31	MN103986	3	57

Table 4. Prevalence of detected piroplasms in the sampled wildlife species and cattle from the Kafue ecosystem. ASVs = Amplicon Sequence Variants

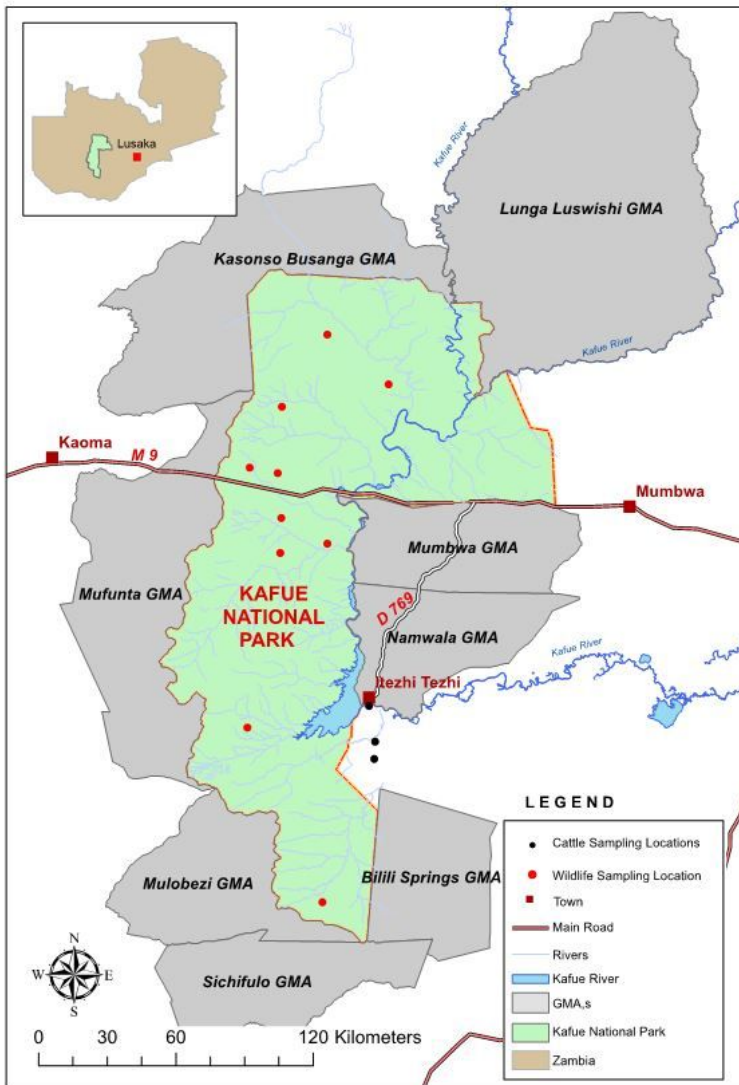| #	Piroplasm species	ID	Impala	Hartebeest	Sable	Sitatunga	Lion	Wild dogs	Buffalo	Cattle	Coresponding ASVs
1	*T. verifera*	LC431550	1 (0.9%)							136 (59.1%)	1, 7, 64, 92
2	*T. verifera* A	GU733375							24 (45.3%)		6
3	*T. verifera* B	JN572701							9 (17.0%)		29,55
4	*T. mutans*	KU206320							15 (28.3%)	139 (60.4%)	3,16
5	*T. mutans* MSD	JN572700							14 (26.4%)	113 (49.1%)	5
6	*T. mutans-like 1*	JN572699							8 (15.1%)		26
7	*T. mutans-like 2*	JN572696							6 (11.3%)		42,60
8	*T. parva*	MH929322							7 (13.2%)	1 (0.4%)	15
9	*T. parva*	AF013418							4 (7.5%)		86,101
10	*T. sp (buffalo)*	HQ895982							26 (49.1%)	1 (0.4%)	10,11,18,22
11	*T. sp (bougasvlei)*	KP410267							10 (18.9%)		25
12	*T. taurotragi*	L19082								4 (1.7%)	35,46, 62
13	*T. sp (sable)*	GU733378		15 (31.9%)				1 (50%)			8,14,85
14	*T. sp (waterbuck)*	KF597072		12 (25.5%)	8 (100%)				1 (1.9%)		4
15	*T. sp. (tsessebe)*	HQ179766		8 (17.0%)				1 (50%)			9,23,49
16	*T. sp (giraffe)*	JQ928925	65 (61.3%)								2,47,106
17	*T. bongo*	MH569462				3 (75.0%)					12
18	*T. bongo*	MH569463				2 (50.0%)					13
19	*B. bigemina*	MH050387								7 (3.0%)	32,71
20	*B. bigemina*	EF458200								2 (0.9%)	67
21	*B. bigemina*	MH047819								22 (9.6%)	19
22	*B. sp*	KX218437						1 (50%)			58
23	*B. occultans*	KP745626								4 (1.7%)	28
24	*H. canis*	MK645969					1 (25.0%)				40
25	*H. sp*	KF270665						1 (50%)			82
26	*Colpodellidae*	MN103986								1 (0.4%)	57
	Total head sampled		106	47	8	4	4	2	53	230	

Figures

Figure 1

Map of the Kafue ecosystem consisting of the Kafue national Park and the game management areas (GMAs) showing sampling sites of wildlife and cattle
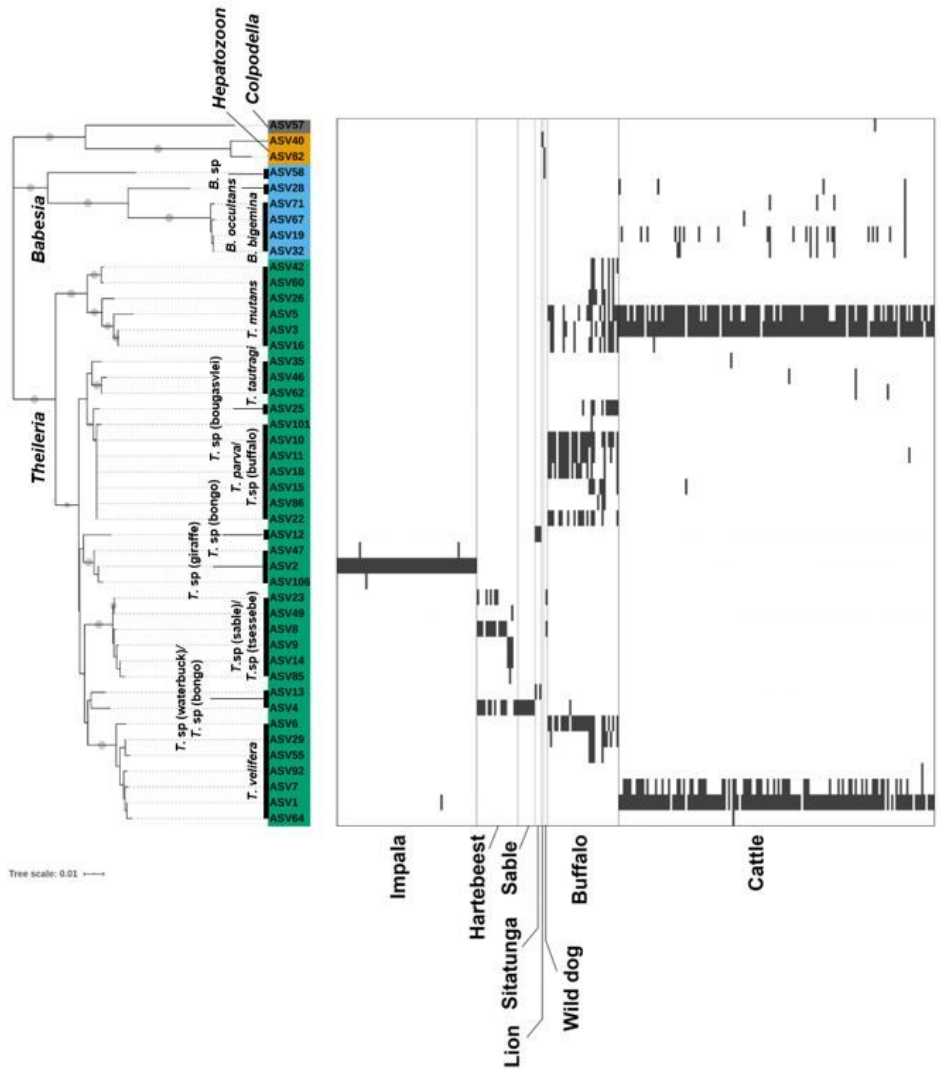
Figure 2

Phylogenetic tree of ASVs and the map of positive ASVs per animal species. On the top is the Neighbor-Joining tree of 45 ASV sequences, using a total of 411 positions in the final dataset. Bootstrap values larger than 70 are shown as proportionate size circles for each node. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Below is a table showing the positive wildlife and cattle samples for each ASVs.

Supplementary Files

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