Symbiosis of parasites in the blood, gut and skin of Cameroonian *Bos taurus* and *Bos indicus* cattle

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Abbreviations

AEZ	Agro ecological zone
BCS	Body condition score
BoLA	Bovine Leucocyte Antigens (equivalent to MHC)
Bs-SNPs	Breeds-specific Single Nucleotide Polymorphism
gGAPDH	glycosomal Glyceraldehyde 3-Phosphate Dehydrogenase Genes
GWAS	Genome Wide Association study
HSP70	Heat Shock Protein 70
Indels	an Insertions and deletions of bases in the genome of an organism
ITS1	Internal Transcript Spacer 1
ITS2	Internal Transcript Spacer 2
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
QTLS	Quantitative Trait Loci
rRNA	ribosomal RNA
S.	Setaria
s.l.	Sensu lato
SNP	Single Nucleotide Polymorphism
spp.	Species
SSU	Small Subunit
T.	Trypanosoma
TBDs	Tick-borne diseases
TNF	Tumour Necrosis Factor
WGS	Whole genome sequencing

Summary in English

Parasitism is a wide-spread lifestyle adopted by more than 50 % of living organisms on Earth. Under natural conditions, almost every host species is simultaneously infected with multiple parasite species (viruses, bacteria, protozoan, fungi and helminths) over the course of their lifetime. However, our knowledge on interspecific interactions of all the different parasites species that live together have been poorly understood. This symbiotic associations can be either synergistically with mutualistic benefit from parasites and host or antagonistically with elimination of one parasite species or harm to the host.

In this thesis, I investigated factors that structure parasite communities with emphasis on symbiosis of parasites in a free ranging population of African indigenous cattle breeds (ca. 1300 animals). The Sudan Savannah and the Sahel habitats of Cameroon are endemic for trypanosomes, tick and tick-borne pathogens, gastro-intestinal helminths and filarial nematodes.

In order to get a better understanding of the whole parasite communities, blood, skin and faecal samples of Zebu and taurine cattle were examined using microscopy, PCR and Sanger sequencing. The cattle body condition, live-weight and the haematocrit was measured. As expected, almost all animals were infected with at least one parasite.

Using molecular tools, I found seven species of trypanosomes and fifteen tick-borne pathogens (TBD) were found in the blood, co-occurring with the microfilariae of *Setaria labiatopapillosa*. I found an antagonistic polarizing effect with view to the presence of either pathogenic or non-pathogenic trypanosomes, while mutualistic associations with TBPs lead to protection of cattle against pathogenic TBD and exotic breeds invasion.

Using microscopy, 15 genera/species of helminths and protozoa were identified in the gut and five species of *Onchocerca* filariae were found in the skin, respectively. There was mutualistic association between flukes, helminths, sporozoan, *Eimeria* spp. and *Onchercerca* filarial species. Antagonistic associations were found between different parasite communities. The facilitating factors were tropical climatic conditions, vectors abundance and host susceptibility. The newly introduced Zebu cattle were more susceptible to parasite-caused pathology than the indigenous

taurine cattle. A subset of 700 cattle was genotyped for 53,714 single nucleotide polymorphisms (SNPs) and the whole-genome of 5 cattle was sequenced (WGS). Our search for the genomic regions under selection at the genome-wide level revealed novel genomic variants and pathways associated with tropical adaptation, diseases susceptibility and immunological regulation.

Zusammenfassung auf Deutsch

Parasitismus ist ein weit verbreiteter Lebensstil von mehr als 50 % aller derzeit lebenden Organismen. Unter natürlichen Bedingungen ist fast jede größere Art im Laufe ihres Lebens gleichzeitig mit mehreren Klassen und Arten von Parasiten (Viren, Bakterien, Protozoen, Pilzen und Helminthen) befallen. Unser Wissen über die interspezifischen Interaktionen aller in einem Wirt co-existierenden Parasiten ist jedoch unzureichend und zumeist durch eine medizinische Sicht geprägt. Eine wertneutral als symbiotisch bezeichnete Assoziation kann entweder synergistisch mit mutualistischem Nutzen von Parasit(en) und Wirt sein oder antagonistisch mit der Eliminierung einer Parasitenart oder des Wirts enden.

In dieser Arbeit untersuche ich ökologische, biologische und genetische Faktoren, die strukturierte Parasitengemeinschaften beeinflussen, mit Schwerpunkt auf der Symbiose von Parasiten in einer frei lebenden Population afrikanischer Rinderrassen (ca. 1300 Tiere wurden untersucht). Die Sudan Savanne und die Sahel-Zone Kameruns sind endemisch für Trypanosomen, Zecken und durch Zecken übertragene Krankheitserreger, gastrointestinale Helminthen und filariforme Nematoden, die von blutsaugenden Insekten übertragen werden.

Blut-, Haut- und Kotproben von Zebu- und taurinen Rindern wurden mittels Mikroskopie, PCR und Sanger-Sequenzierung untersucht. Der Körperzustand des Rindes, das Lebendgewicht und der Hämatokrit wurden gemessen. Wie erwartet waren fast alle Tiere mit mindestens einer Art von Parasiten befallen. Mit molekularen Methoden (PCR, Sequenzierung) fand ich 7 Arten von Trypanosomen und 15 zeckenübertragene Krankheitserreger (TBP) im Blut, zusammen mit den Mikrofilarien von *Setaria labiatopapillosa*. Pathogene und nicht-pathogene Trypanosomen stehen in einem antagonistischen Verhältnis, in welchem die viel häufigeren apathogenen Arten vor der Nagana schützen, ebenso wie mutualistische Assoziationen bei Zecken-übertragenen Bakterien und Piroplasmen dazu führen, dass Rinder vor pathogenen Arten und exotischen Rinder geschützt werden.

Im Verdauungssystem der Rinder liessen sich 15 Arten von Helminthen und Eimerien mikroskopisch nachweisen. In der Haut fand ich 5 Arten von Mikrofilarien.

Mutualistisch sind die Assoziationen zwischen Egeln, Helminthen, dem Sporozoon *Eimeria* spp. sowie den Mikrofilarien von *Onchocerca* Nematoden. Es wurden

antagonistische Assoziationen zwischen verschiedenen Parasitengemeinschaften gefunden.

Fördernde Faktoren für Parasitenbefall waren tropische Klimabedingungen, Vorkommen der biologischen Vektoren und genetisch prädisponierte Anfälligkeit der Wirte. Neu eingeführte Zebu-Rinder waren anfälliger für parasitenbedingte Pathologie als die einheimischen taurinen Namchi und Kapsiki Rinder.

Eine Teilmenge von 700 Rindern wurde hinsichtlich 53.714 einzelner Nukleotidpolymorphismen (SNPs) und 5 Rinder durch Ganzgenomsequenzierung (WGS) genotypisiert. Genomische Regionen, die mit Parasiten-Resistenz korrelierten, verweisen auf Varianten und Gene, die mit tropischer Anpassung, Krankheitsanfälligkeit und immunologischer Regulierung verbunden sind.

List of publications in the cumulative thesis

a) accepted publications:

1. **Paguem, A**., Abanda, B., Ndjonka, D., Weber, J.S., Ngomtcho, S.C.H., Manchang, T.K., Mamoudou, A., Eisenbarth, A., Renz, A., Kelm, S., Achukwi, M.D. 2019. Widespread co-endemic occurrence of *Trypanosoma* species infecting cattle in the Sahel and Guinea Savannah zones of Cameroon. *BMC Veterinary Research*. 25:344.
2. Abanda, B., **Paguem, A**., Achukwi, M.D., Renz, A., Eisenbarth, A. 2019. Development of a low-density DNA microarray for detecting tick-borne bacterial and piroplasmid pathogens in African cattle. *Tropical Medecine and Infectious. Diseases*. 4, 64. doi: 10.3390/tropicalmed4020064.
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2. **Paguem, A.,** Abanda, B., Ngwasiri N.N., Eisenbarth, A., Streit, A, Renz, A., Achukwi, M.D. Host specificity and phylogeny of trichostrongylidae of domestic ruminants in the Guinea savannah of the Adamawa highland in Cameroon. *Veterinary Parasitology: Regional Studies and Reports*

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Abanda B., M. Schmid M., **Paguem A**., H., Achukwi M.D., S. Preuß S., A. Renz A., Eisenbarth A. Genetic analyses and genome-wide association studies on parasitic and microbial pathogen resistance of *Bos taurus* and *Bos indicus* cattle breeds in Cameroon. *Animal Genetics*

Author contributions

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Author	Author position	Scientific ideas%	Data generation %	Analysis& Interpretation%	Paper writing%
Paguem, A.	1	30	50	50	30
Abanda, B.	2	10	25	0	10
Ndjonka, D.	3	0	0	0	5
Weber, J.S.	4	0	0	10	5
Ngomtcho, S.	5	0	0	5	5
Manchang, T.	6	5	5	0	5
Mamoudou, A.	7	5	5	0	5
Eisenbarth, A.	8	10	0	5	5
Renz, A.	9	10	0	10	10
Kelm, S.	10	20	5	10	10
Achukwi, M.D.	11	10	10	10	10
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Author	Author position	Scientific ideas%	Data generation %	Analysis& Interpretation%	Paper writing%
Abanda, B.	1	30	50	50	40
Paguem, A.	2	10	10	10	10
Achukwi, M.D.	3	10	0	5	10
Renz, A.	4	20	0	5	10
Eisenbarth, A.	5	30	20	30	30
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Author	Author position	Scientific ideas%	Data generation %	Analysis& Interpretation%	Paper writing%
Abanda, B.	1	40	50	50	50
Paguem, A.	2	10	20	10	10
Mamoudou, A.	3	0	10	0	5
Manchang, T.	4	0	10	0	5
Renz, A.	5	25	0	10	10
Eisenbarth, A.	6	25	10	30	20
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Author	Author position	Scientific ideas%	Data generation %	Analysis& Interpretation%	Paper writing%
Paguem, A.	1	20	20	30	40
Abanda, B.	2	10	10	0	10
Achukwi, M.D.	3	10	0	0	10
Baskaran, P.	4	10	35	30	10
Czemmel, S.	5	10	35	30	10
Renz, A.	6	20	0	5	10
Eisenbarth, A.	7	20	0	5	10
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Author	Author position	Scientific ideas%	Data generation %	Analysis& Interpretation%	Paper writing%
Paguem, A.	1	30	30	30	30
Abanda, B.	2	10	20	20	10
Ngwasiri, N.N.	4	0	15	0	5
Eisenbarth A.	5	5	0	10	10
Streit, A.	6	20	20	20	20
Renz, A.	7	15	0	10	10
Achukwi, M.D.	8	20	15	10	15
Titel of Paper:		Host specificity and phylogeny of trichostrongylidae of domestic ruminants in the Guinea savannah of the Adamawa highland in Cameroon			
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1- General Introduction

1.1. Multi-parasite infections

Parasitism is one of the most successful strategy displayed by living organisms (Poulin and Morand, 2000) and represents the predominant biological symbiotic interaction (Poulin, 1995; Windsor, 1998). It is well recognized that parasites account for more than 50% of all species on Earth (May, 1988; 1992). Therefore, they have a great importance not only for the ecology, behaviour, and evolution of free-living organisms but also for biodiversity and ecosystem function (Lafferty *et al*., 2006; Kuris *et al*., 1980; Dunne *et al*., 2013).

Parasites can be classified into macro-parasites and micro-parasites. Macro-parasites include eucaryotic protozoa, helminths and arthropods (Anderson and May, 1979). Within their hosts, they tend to have sexual reproduction, long generation duration and a continuously occurring re-infestation (aggregation). Generally, they cause morbidity rather than mortality in their definitive host (Tompkins *et al*., 2011). For example, the lifespan of *Onchocerca volvulus* in the West African savannah is up to 10 years in the human host (Schulz-Key and Karam, 1986).

Micro-parasites comprise viruses, rickettsiae, bacteria and some eukaryotic protozoa, and are characterized by microscopic size, high multiplication rate in the end-host and short generation times. They may also cause high mortality and morbidity in the host. Taken together micro-parasites follow an alternative strategy (death or immunity of infected hosts) and macro-parasites a simultaneous strategy (premunition, super- and re-infestation, Wenk and Renz, 2003, 2013; Anderson and May, 1979).

Most free-living organisms including human and cattle are concurrently infected with a number of micro-parasites and macro-parasites (multi-parasitism) over most of their lives (Cox, 2001, Petney and Andrews, 1998). Parasite-community ecologists have adopted two approaches and tools (e.g. food web network) to examine the complex dynamics of multi-parasitism (Pedersen and Fenton, 2007). The first approach for classification of parasite communities is based on patterns of species occurrence (presence and absence) and tests for community structuring by comparing observed species distributions against the null models (Janovy *et al*.,

1995). The second approach quantifies pair-wise associations between species, inferring interspecific interactions from correlations in species abundance (Nilssen *et al*.,1998) or more complex models that control for biotic and abiotic factors (Lello *et al*., 2004).

Parasite communities have been classified by Holmes and Price (1986) in three hierarchical levels:

> **First and basically**, a parasite infra-community, which comprises all members of a given species of one parasite within a single host. For example, all *Onchocerca ochengi* adult worms living in one cattle
>
> **The second hierarchical level** includes the component community defined as all parasite infra-communities within a given host population. For example, all helminths, protozoa and bacteria living in one cattle.
>
> **The third hierarchical level** is the compound community, which consists of all parasite communities within an ecosystem of different domestic and wild hosts and vectors.

Tropical and subtropical climatic zones are well known to harbour the richest biodiversity of parasites and hosts (Bordes and Morand, 2011; Vaumourin *et al.,* 2014). In Sub Saharan Africa, for example in West and Central Africa, savannahs and forest areas are populated with blood-feeding arthropods like mosquitoes, blackflies, gnats, tse-tse flies, tabanids and ticks which include vector species for a number of important parasites (e.g. trypanosomes, filariae, tick-borne pathogens and viruses). The humid climate of the rainy season favours the transmission of soil-transmitted helminths (Phiri *et al*., 2010; Rushton and Heffernan, 2002). However, parasite diversity and the interspecific interactions of parasite communities within one ruminant host have been poorly investigated in livestock and wildlife.

In the following, three types of infra-communities of parasites shall be presented:

1.1.1 *Trypanosoma* diversity, potential hosts and life cycles

Table 1 shows the ability of trypanosomes to colonize and potentially co-infect different hosts and blood-feeding arthropods. The life-cycle is depicted in Figure 1.

Stercorarian trypanosomes develop in the posterior gut of the insect and infective metatrypanosomes are excreted in the faeces of the insect onto the skin of the host. In contrast, the salivarian trypanosomes develop in the anterior gut of insect and infective stage are inoculated in the host during blood-feeding.

Table 1. Diversity of *Trypanosoma* species infecting various hosts and their known vectors

Sections	**Subgenus**	**Species**	**Hosts (Human and domestic animals)**	**Size (µM)**	**Vector**
Salivaria	Trypanozoon	*T. evansi*	Bovine, equine	15-36	*Glossina* spp.
					Tabanidae spp.
	Trypanozoon	*T. equiperdum*	Equine, donkey	15-36	None
	Trypanozoon	*T. brucei brucei*	Bovine, sheep, goat, dogs	11-42	*Glossina* spp.
	Trypanozoon	*T. brucei gambiense*	Human, Bovine, sheep, goat, dogs	23-30	*Glossina* spp.
	Trypanozoon	*T. brucei rhodesiense*	Human, Bovine, sheep, goat	23-30	*Glossina* spp.
	Nannomonas	*T. simiae*	Pigs	9-24	*Glossina* spp.
	Nannomonas	*T. congolense*	Bovine, sheep, goat	9-24	*Glossina* spp.
	Duttonella	*T. vivax*	Bovine, sheep, goat	21-25	*Glossina* spp.
					Tabanidae spp.
					Stomoxys spp.
Stercoraria	Pycnomonas	*T. suis*	Pigs	14-19	*Glossina* spp.
	Schizotrypanum	*T. cruzi*	Human, dogs, cats	16-21	*Triatominae*
	Megatrypanum	*T. theileri*	Cattle and Bovinae	24-61	*Tabanidae, Ixodes*
	Trypanosoma	*T. grayi*	Crocodiles	78-80	*Glossina*

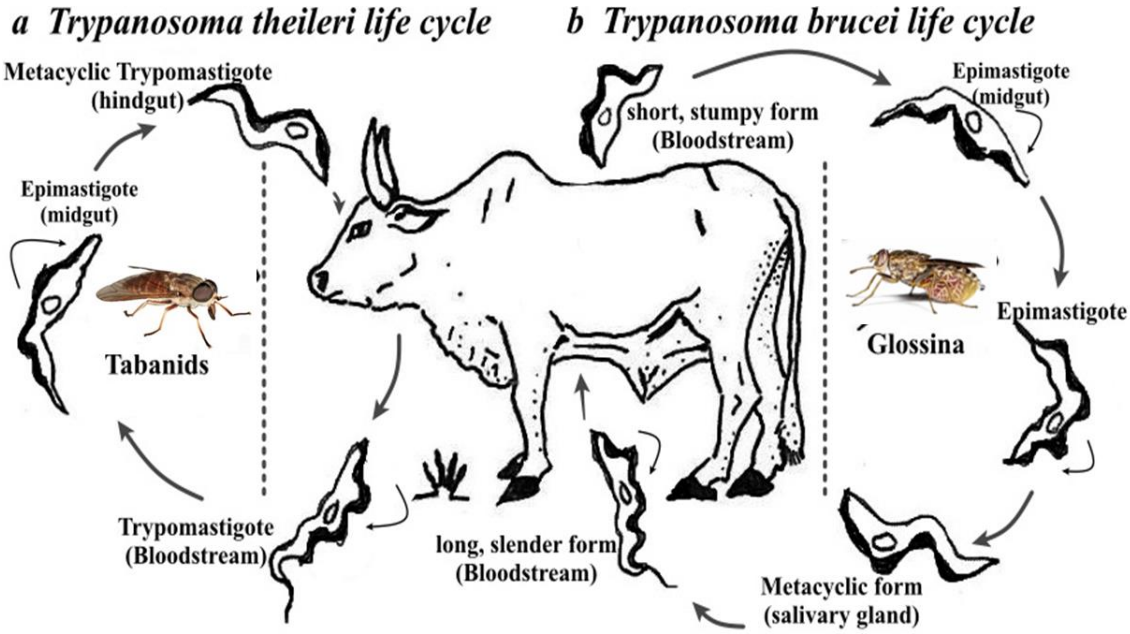


Figure1. Life-cycle of Stercoraria and Salivaria: Schematic representation of the life-cycle of *Trypanosoma theileri* (Stercoraria, a) and *Trypanosoma brucei* (Salivaria, b). Extracellular trypanomastigotes within the blood of a vertebrate host are taken up by the blood-feeding arthropods during their blood meal.
(a) In the Stercorarian section, ingested trypomastigotes differentiate into epimastigotes within the midgut of tabanids (or ticks). After 1 week, they differentiate to metacyclic trypomastigotes in the hindgut. When the arthropod host feeds, it often defecates at the same time, releasing infective metacyclic trypanosomes which may contaminate the wound or mucosa.
(b) The salivarian *T. brucei* completes its life cycle in the tsetse fly. The ingested trypanomastigotes start to develop in the crop and continue dividing by fission over a period of 1-2 weeks in the midgut. Then they migrate through the hemocoel, eventually entering the salivary gland, and accumulate there. Within the salivary gland, the parasites transform into epimastigote forms and continue to divide by binary fission. Some epimastigotes differentiate into infectious metacyclic trypomastigotes. After inoculation into the bloodstream of the new vertebrate host the parasite continues to divide in this form and the cycle starts again. Figure created by A. Paguem.

1.1.2 *Onchocerca* co-infecting cattle

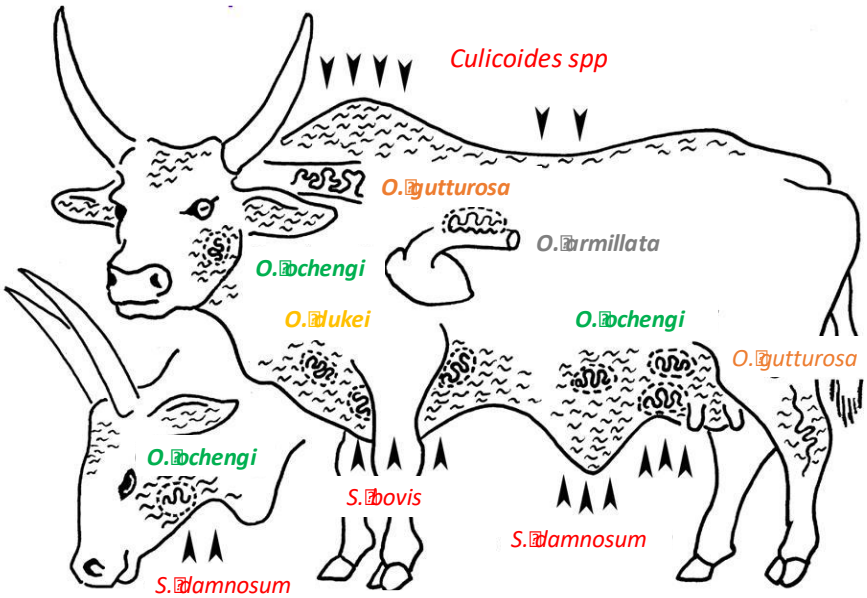
The second example of co-infections are the bovine filarial nematodes (Figure 2). Five species, namely *O. ochengi*, *O. gutturosa*, *O. armillata*, *O. dukei* and *Setaria* spp. share different habitats in the natural host, Zebu cattle (Renz, 2001; Wahl *et al*, 1994). Adults of *O. armillata* and *O. gutturosa* live attached in the aorta and the ligaments of neck and knee, respectively. While the adults of *O. ochengi* and *O. dukei* live in intradermal or subcutaneous connective tissue of the host (nodules). *Setaria digitata* and *S. labiatopapillosa* adults live freely in the peritoneal cavity. First-

stage larvae of filarial parasites ('microfilariae') either dwell in the skin (all *Onchocerca* species) or in the blood (*Setaria*). *Setaria* microfilariae are taken up by mosquitoes (Sundar *et al*., 2015). *Setaria labiatopapillosa* is common across the world, while *S. digitata* is restricted to Asian cattle (Sundar *et al*., 2015). All those filarial parasites do not cause any pathologies to the infected animal.

Most of the microfilariae produced by *Onchocerca* species migrate into the skin and are taken up by either *Culicoides* or *Simulium* flies. *Onchocerca ochengi* is of particular interest because it is a sister species of *O. volvulus*, the causative agent of human onchocerciasis. Both species share the same blackfly vector, *Simulium damnosum* s.l. Therefore, it has been established as the best filarial model for drug screening, immunological tests and epidemiological studies (Renz *et al*., 1995; Achukwi *et al*., 2000; Makepeace and Tanya, 2016).

The vectors and life cycle of *Onchocerca ochengi* as an example is shown in Figure 2.

(a)



(b)

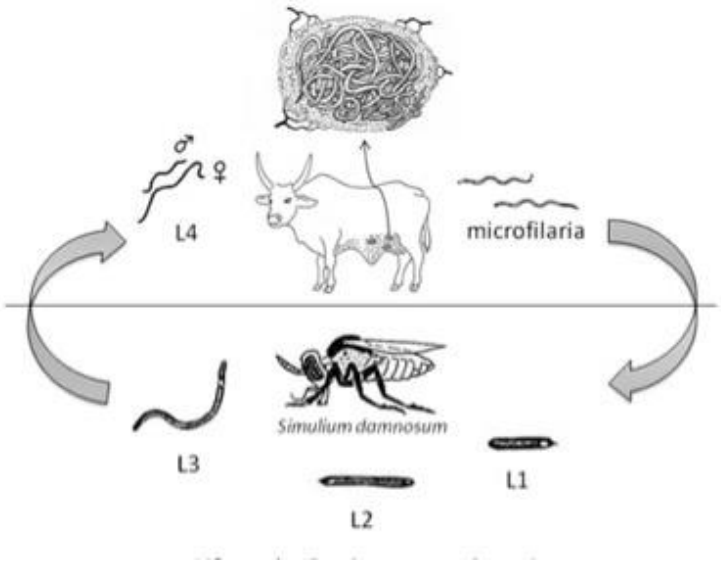


Figure 2. Diversity of *Onchocerca* filarial parasites of cattle in Cameroon (a) and life cycle of *Onchocerca ochengi* (b).
(a) *Onchocerca* adult filariae in Zebu cattle. The black arrow indicates the biting sites of natural vectors (red colour). **(b)** Female and male worms (sex-ratio on average 1:1) live surrounded by connective tissue (nodule) in the skin of cattle, normally around the belly, udder/ scrotum and umbilical region (penis). One productive female gives birth to approx.1, 000 offspring per day. These microfilariae migrate to the skin predominantly around the inguinal region and belly where they are ingested by the vector *S. damnosum* s.I. in the search of a blood meal. In the blackfly, they penetrate through the peritrophic membrane of the gut and settle in the fibres of the flight muscle, to develop to the so-called sausage stage (L1). After 2 to 3 days they moult to the encysted L2 stage, and 4 to 5 days later to the infective larva (L3). During the next (usually third or subsequent) blood meal they enter a new host through the labellum of the proboscis, and develop via another moulting stage (L4) to male or female adults after 9 to 12 months. Their life expectancy exceeds 10 years. Graphs are taken with copyright permission of A. Renz.

1.1.3 Gastrointestinal helminths and protozoans co-infecting cattle

The third example is the diversity of rumen and gut helminths, i.e. nematodes and trematodes, and a protozoon, e.g. *Eimeria* spp., co-infecting cattle. Figure 3 summarizes different tissues colonized by helminths and *Eimeria* spp. infecting the gastro intestinal tract of cattle.

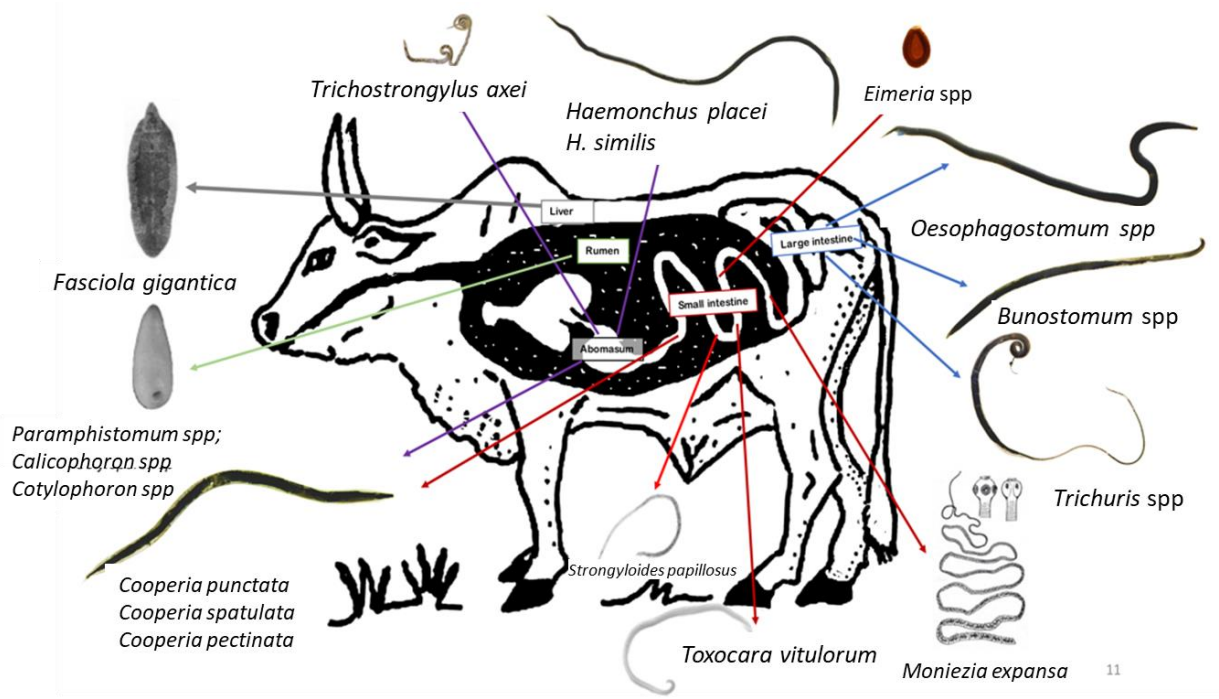


Figure 3. Diversity of parasitic helminths and sporozoan *Eimeria* infra-community in the African cattle gut. The colour of the arrow indicates that the parasites are found within the same site of the gastro-intestinal tract. *Fasciola gigantica* are located within the bile duct in the liver ('liver flukes') and *Paramphistomum* species live in the rumen ('rumen-flukes'). Figure credited by A. Paguem.

Figure 4 displays the life cycle of nematodes and trematodes.

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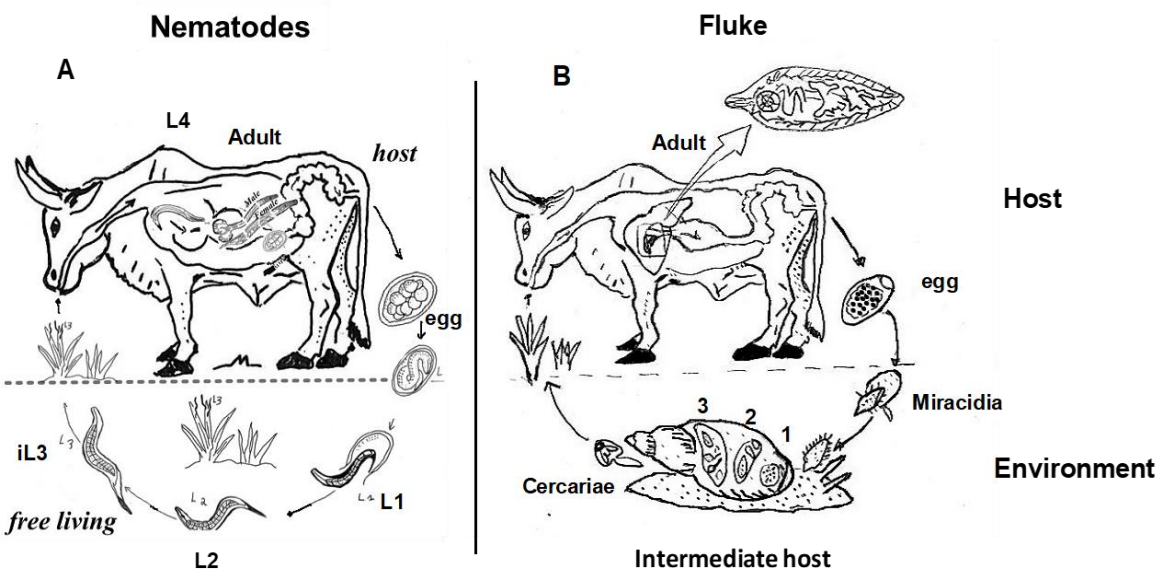


Figure 4. Schematic presentation of the life cycle of nematodes (Trichostrongylidae) (A) and trematodes (rumen- and liver flukes) (B). Adult female nematodes and trematodes produce eggs that are passed out of the host with the faeces.
(A) Under optimum condition in the external environment, first-stage larvae (L1) of gastro-intestinal nematodes can develop and hatch within 24 hours. L1 grow and develop to the second stage larvae (L2) that in turn grows and develop into third-stage larvae (L3), which is the infective stage. After ingestion, L3 develop into fourth-stage larvae (L4), which then develop into immature adults. Sexually mature adult nematodes develop within 2 to 4 weeks after ingestion of the L3 unless arrested larvae development occurs.
(B) The eggs of digenean trematodes usually are passed in the faces from the final host. Under suitable conditions (water and warmth), the miracidium hatches from the egg in the water and searches a suitable intermediate host snail (e.g. *Bulinus* spp., *Eustralorbis* spp., *Oncomelania* spp. and *Biomphalaria* spp.), they actively penetrate the tissue and develop into sporocysts (1), rediae (2) and cercariae (3) (Soulsby, 1982; Roberts and Suhardono, 1996). Cercariae leave the snail and encyst on the vegetation. Metacercariae reach the definitive host by oral ingestion of contaminated herbage or, in the case of Schistosomatidae, the cercariae actively penetrate the skin of the definitive host. After swallowing of metacercariae by the final host, excystation occurs in the intestinal tract (ileum), and the immature stages migrate to their predilection sites (liver for *Fasciola hepatica and F. gigantica* or reticulum or rumen for paramphistomidaes). Figure created by A. Paguem.

1.2. Factors facilitating multi-parasitism

1.2.1 Seasonality and the climatic conditions

Higher temperatures and precipitation favor the growth of many disease vectors and facilitate the persistence of infective larvae of helminths in nature. Such climatic conditions are given during the rainy season in the tropical regions. However, based on the climate change and global warming of the earth, the global distribution and diversity of parasites is predicted to increase as more favorable condition are provided for the development of parasites and their transmission (Benning *et al*., 2002).

Another major environmental factor that favours the multi-parasitism is the seasonality. For example, gastrointestinal helminths seem to have a seasonal pattern, with faecal egg outputs, reported to be following rainfall patterns (Fall *et al*., 1999; Waruiru *et al*., 2002). In contrast, a high prevalence of flukes (*Fasciola* spp. and *Paramphistomum* spp.) was observed during the dry season where the population of the intermediate hosts (freshwater snails) is restricted around the last remaining drinking ponds.

1.2.2 The spatial and temporal distribution

Host species living on large geographical zones harbour a higher diversity of parasitic species than host species living in the more restricted geographical areas (Morand, 2000; Rosenzweig, 1995; Morand, 2015). The overlapping spatial and temporal distribution of parasites increases the likelihood for a host to be parasitized by several parasite species (Morand, 2000; Batchelor *et al*., 2009; Davies and Pedersen, 2008; Wardrop *et al*., 2013). For example, *Plasmodium falciparum* and soil-transmitted helminths (*Ascaris lumbricoides*, *Trichuris trichiura* and the hookworms *Necator americanus*, *Strongyloides stercoralis* and *Ancylostoma duodenale*) have an overlapping geographical distribution and co-infect more than one-third of the world's population (review by Naing *et al*., 2013)

1.2.3 Host susceptibility

Host susceptibility to multi-parasites infections can be markedly affected by its physical condition, immunity falls, gender, age, pregnancy and the host's history of exposure (Ezenwa and Jolles, 2011; Lello *et al*., 2008). Individuals at the youngest or at the oldest age, pregnant and lactating females are highly predisposed to co-infection with multiple parasites (loc. cit.). In addition, animals with poor nutritional status and body condition (i.e. anorexic, anaemia) may be subject to multiple parasite coinfections (Stephenson *et al*., 2000).

Host genetics is also predicted to have an effect on multi-parasite occurrences (Ruiz-Lopez *et al*., 2012). It is well known that certain breeds of livestock tolerate infection better than others (Murry *et al.*, 1979; Dwinger *et al*., 1994). This is especially true for the West African Short-horned taurine cattle (Muturu, Baoule, Laguna, Samba, and Dahomey) and the N'Dama, which is also of West Africa. These cattle have existed in the region for over 5,000 years. Susceptibility studies have shown the N'Dama to be the most resistant breed followed by the smaller West African short-horned cattle, but the large Zebu (*Bos indicus*) is the most susceptible (Murray *et al*., 1979). In Cameroon, the Zebu cattle like Gudali and Fulani are more susceptible to parasite infections than the indigenous taurine breeds like Doayo (Namchi) and Kapsiki (Murry *et al*., 1990; Achukwi *et al.,* 1997). To be precise, one should distinguish "resistant" (defined as

an individual's ability to block the reproduction of a pathogen) from "tolerant" (defined as an individual's ability to control the population of a parasite, and limit the impact of the infection on the host' health, Murry *et al*., 1990; Achukwi *et al.,* 1997). However, such studies have rarely considered more than one single parasitic organism.

Genetic methods for host genotyping

Two quantitative genetic methods have been used to identify the genetic background of susceptibility to parasites, namely the heritability index (Kruuk, 2004; Lynch and Walsh, 1998) and genome wide association studies (GWAS).

The heritability (h^2) broadly measures the proportion of variation in the given phenotypic trait within the host population that can be attributed to genetic variation Heritability estimates range from 0 to 1, with higher values indicating that all of the variability of the targeted trait is due to genetics factors rather than environmental factors. For example, in sheep and cattle the heritability to gastrointestinal nematodes (Strongyle faecal egg count) ranged between 0.2 and 0.4 (Leighton *et al*., 1989; Bisset *et al*., 1992; Bishop *et al*., 2004).

Genome Wide Association Studies (GWAS) have profited from major advances in nucleotide sequencing, high-throughput genotyping and bioinformatics pipelines tools, which have contributed significantly to the identification of genes associated with complex traits (Visscher *et al*., 2012). For example, two single nucleotide polymorphisms (SNPs) loci surrounding the protein tyrosine phosphatase receptor T and myosin genes explained 21% of the *Mycobacterium bovis* resistance phenotype in European cattle (Bermingham *et al*., 2014). In addition, the major histocompatibility complex, which comprises a multigene family (class I, II and III) that acts at the interface between the immune system and infectious pathogens, has attracted much attention in studies of association between genetic variants and disease resistance (Cox, 2001; Jankovic *et al*., 2001).

In cattle, this complex is known as bovine leucocyte antigen (BoLA) and is located on chromosome 23; BTA23 (Goszczynski *et al*., 2017). Genetic variation in BoLA has been associated with resistance or susceptibility to several pathogens and diseases such as mastitis, leucosis and parasitic infections (Takeshima and Aida, 2006; Wegner *et al*., 2003; Baxter *et al*., 2009; Dietz *et al*., 1997). Association

between MHC (BoLA-DR/DQ) alleles and resistance against *Dermatophilus congolensis* have been reported by *Maillard et al*. (2003).

1.3. Symbiosis and multi-parasites interactions

Symbiosis has been defined by Anton Debary (1879) as different species living together. In this light, multi-parasites interactions can either be considered under ecological aspects, for example via competition for space (blood, gut, skin), or via concurrence for resources (i.e. blood, host digested food) and/or on the level of immunological reactions through modulation of the host's defence system that can facilitate or inhibit multi-parasites infections (Pedersen and Fenton, 2007).

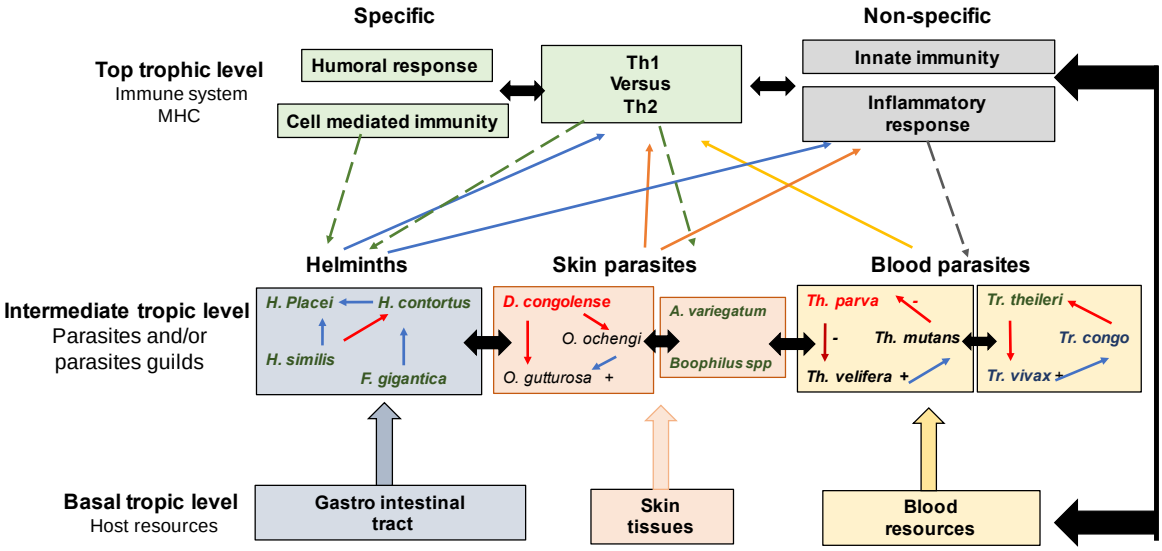


Figure 5. A hypothetical within-host parasite community interaction network. (Adapted from Pedersen and Fenton, 2007). Parasite interactions were compiled by A. Paguem using data from Woolhouse *et al*., 2015; Lay, 2003; Urquhart *et al.,* 1972; Kaufmann *et al*., 1992). Th = *Theileria*, Tr = *Trypanosoma*. Blue arrows indicate positive interactions while red arrows indicate negative interactions.

1.3.1 Competition for space and resources

Closely related parasites or strains that share the same space are more likely to cooperate in order to exploit their hosts economically and maximize their chance of transmission (Cox, 2001). This synergistic action (which can be regarded as mutualism or commensalism) leads to density-dependent regulation of the

population sizes and subsequent reduction of the virulence of one strain. It also decreases the cost imposed on the host fitness (Cox, 2001). For example, indigenous African cattle concurrently co-infected with *Theileria parva* (a tick-borne protozoan causing East Coast fever) and with the less pathogenic species *Th. mutans* or *Th. velifera* show a reduction of 89% in mortality if compared to a single *Th. parva* infection (Woolhouse *et al*., 2015). In contrast, distantly related parasites sharing the same habitat in the host, food or resources may compete with each other and this antagonistic effect lead to either exclusion of one parasite or increased virulence of one species and decreased transmission due to the host death. Competition for red blood cells (RBCs) between malaria parasites and bloodsucking helminths can regulate malaria population dynamics (Budischak *et al*., 2018). In her diploma thesis, K. Lay investigated the interactions between *Dermatophilus congolensis*, Bovine Popular Stomatitis Virus (BPSV) and bovine filarial *O. ochengi* in Northern Cameroon (Lay, 2003). Cattle co-infected with dermatophilosis and BPSV showed lower microfilarial density as compared to mono infected ones. She also found a strong fertility reduction (gravidity, fecundity and mff productivity) in female filariae from those animals co-infected (Lay, 2003). This antagonistic interaction of *D. congolenesis* and BPSV on filarial nematodes may be caused through the modulation of the host's immune system.

1.3.2 Host immunomodulation

Infected hosts mount a protective immune response (Cox, 2001). The Th (T helper) lymphocytes (Th1 and Th2) are the key cellular defence against any infectious agent and the Th1 and Th2 cells are mutually exclusive (Jankovic *et al*., 2001; Ben-Smith *et al*., 2003). The Th1 cells produce cytokines (IL-2 and IFN-γ) that drive the activation of macrophages and elimination of micro-parasites. Conversely, Th2 cells produce cytokines (IL-4, IL-5, IL-10 and IL- 13) that lead to the production of specific antibodies, eosinophils proliferation and subsequently elimination of macro-parasites like helminths and even ectoparasites like ticks (Cox, 2001; Jankovic *et al*., 2001).

All parasites secrete or excrete products which can polarize the Th1/Th2 response and modulate the immune system for their own survival. The best studied examples of immune-suppression are infections with African trypanosomes, malaria

parasites and nematode worms (Greenwood, 1974; Hudson and Terry, 1979; Behnke, 1987). In the concurrent competition for blood resources, the immuno-suppression by *Trypanosoma* spp. enhances the pathogenicity of gastrointestinal worms such as *Haemonchus contortus* (Urquhart *et al*., 1972; Kaufmann *et al*., 1992). In addition, cattle concurrently co-infected with *F. hepatica* and *Mycobacterium bovis*, *the fluke worms* down-regulate the Th1 responses (by IFN-γ), with subsequent predominance of Th2 responses, in order for the parasite to survive and reproduce (Kelly *et al*., 2018).

1.4. Consequence of multi-parasitism

Multiple parasite infestations may interact with each other and influence the outcome of the host health and fitness (Thumbi *et al*., 2014). However, the significance of interactions between species and the processes that shape within host parasite communities remain unclear (Pedersen and Fenton, 2007). Concurrent parasite infections can either increase or even decrease the severity of disease in comparison to those infected with one parasite species alone (Krause *et al*., 1996; Graham *et al*., 2005). For example, co-infection of laboratory mice by *Toxoplasma gondii* and *Nippostrongylus brasiliensis* can result in prolonged and increased egg output of the gastrointestinal worm (Liesenfeld *et al.*, 2004). And the second example is the co-infected HIV and tuberculosis that induced early mortality (Marshall *et al*., 1999; Fenton and Perkins, 2010).

From the above-cited studies, it is evident that pathogen-pathogen interactions occur, and that the effect observed on the hosts differs in strength and direction dependent on the specific coinfection combinations and the mechanisms by which pathogen-pathogen interactions occur. Knowledge of parasite-parasite interactions and the occurrence of different parasites in cattle is still limited. We do not know which coinfections are prevalent among domestic animals in Cameroon, how the different parasites interact, synergistic or antagonistic, and what is the implication of these interactions on the host survival and reproduction.

2- Objectives and expected output

Multiple parasite species co-infections occur in natural populations. These host species were laboratory model animals or/ and well control farm animals (Thumbi *et al*., 2014). In the ecological context, natural habitats of multiple parasite species and multiple host species overlap. In order to fill this gap of the biodiversity of multiple parasite coinfections and parasites symbiosis in the natural population, we selected indigenous African cattle as a study model because they are free ranging populations, which occupy large savannah areas endemic to many infectious parasites. Indigenous taurine breeds are more resistant or tolerant to most endemic diseases than Zebu cattle. We predicted that:

1) Indigenous African cattle are exposed and infected with a high biodiversity of pathogenic and non-pathogenic parasites.
2) Indigenous African cattle are susceptible to multi-parasitism.
3) Taurine cattle are more tolerant to multiple parasitism than Zebu cattle because of their longer co-evolution.
4) Taurine cattle do possess genetic variants and markers in their genome under natural selection that confer disease resistance.

The objective of the present dissertation is to better understand the ecology and evolution of multi-parasite communities in indigenous African cattle breeds, their associated fitness costs and subsequently the consequences of multi-parasite communities on epizootiology, biology diagnostic and control of cattle parasites in Central Africa.

Molecular techniques were employed, in order to identify the rich biodiversity of salivarian and stercorarian trypanosomes, tick-borne bacteria and piroplasmids species found in blood samples, filarial parasites found in the skin and gastrointestinal helminths. New strains and species were discovered and their phylogenetic evolution analysed. The possible parasite-parasite interspecific interactions were evaluated and the associated fitness cost highlighted. Individual worms of the genera *Haemonchus*, *Trichostrongylus* and *Cooperia* were examined using nuclear ribosomal markers 18 SSU and ITS 2 in order to identify the various species infecting domestic animal and to infer their evolutionary relationships.

Furthermore, the analysis of the whole genome of African indigenous cattle breeds coupled with SNPs genotyping evaluation lead to the discovery of novels SNPs and indels and revealed several genes and pathways associated with heat

stress, haemostasis response, blood parasitic control, trypanotolerance, immunological regulation and productivity. These findings shall improve our understanding of the epidemiology and transmissibility of multi-parasite infections and help to understand their implication in livestock-management and disease-control. Finally, yet importantly they will aid the genetic conservation management of endangered Doayo (Namchi) and Kapsiki cattle breeds.

3- Materials and Methods

3.1. Study areas

A cross-sectional study was carried out in five geographical areas localized in two large agro-ecological zones (AEZs) in Northern Cameroon: The Sahel (Far North region and a larger part of the North region) and the Guinea savannah (Adamawa region with a little part of the North region) (see Figure 6). The two AEZs are lying between latitudes 7 to 10°N and 11 to 15°E and the cover an area of 164,000 km^2. A strong climatic gradient runs through the wet high Guinea savannah in the Adamawa up to the dry Sudano-Sahelian zone in the Far North region. The rainy season in the Guinea savannah zone is from April to October, whereas in the Sudano-Sahelian zone it is from June to September. Annual rainfall ranges from 1400 to 1700 mm in the Guinean savannah and 800–1400 mm in the Sudano-Sahelian zone. The Guinea savannah of the Adamawa plateau has a suitable climate and pasturelands for extensive cattle rearing. Overall, this plateau contributes to about 38% of beef production in the country.

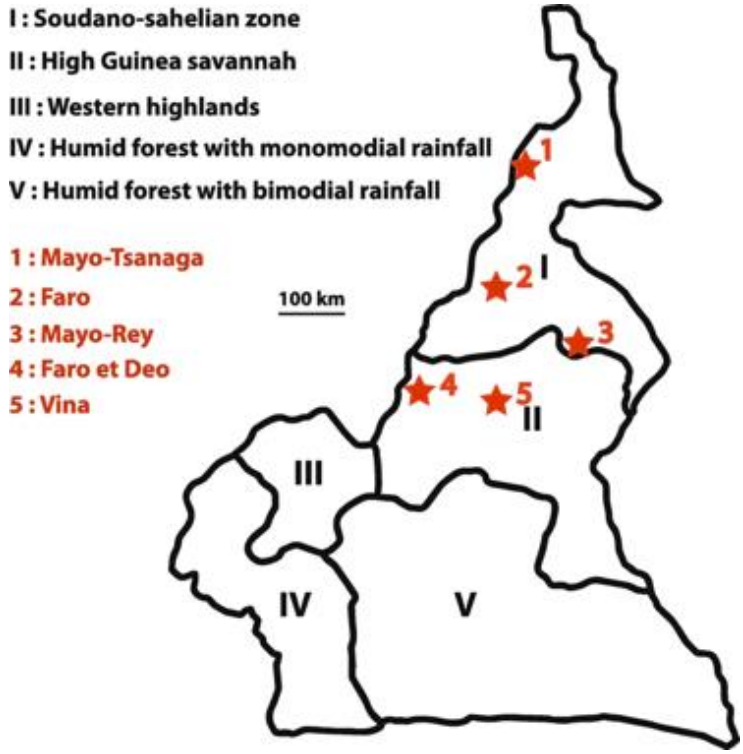


Figure 6. Map of the study area. Geographic map showing five Agro-Ecological Zones of Cameroon (based on information from the Institute of Agricultural Research for Development, IRAD, 2009). The cattle sampling areas (red stars) were located in the climate zones Guinea wet savannah and Sudano-Sahelian dry savannah. (Map depicted in Fig.6 is from Paguem *et al*., 2019).

3.2. Animal selection

53 cattle herds (approx. 1300 animals) located in the high Guinea and Sudano-Sahelian savannah were surveyed in a cross-sectional study over a period of 12 months. Sampled animals comprised humped Zebu cattle breeds (*Bos indicus*): Gudali (n= 650), White Fulani (n= 60), Red Fulani (n= 57) and Bokolodji (n= 6). Non-humped taurine cattle breeds were the autochthonous Doayo (Namchi) (n= 205) and Kapsiki (n= 205) and the introduced European taurine composites: Charolais X Gudali (n= 29) and Hybrids (n= 37).

In addition, data derived from a longitudinal observation of the experimental DFG-COBE cattle herd (27 Zebus Gudali) exposed to the natural transmission of endemic parasites since their birth over a period up to 96 months, with the special interest on the filarial nematode *O. ochengi*, were also included.

3.3. Field work and sampling procedure

For each herd visited, approximately 10% of the cattle were sampled using a systematic random approach (see Paguem *et al.,* 2019 for more details). In the Faro and Mayo Tsanaga divisions only the indigenous taurine cattle breeds Doayo (Namchi) and Kapsiki, respectively, were examined and sampled. From each animal, physical examinations were made and the following variables recorded: breed, sex, body condition score (BCS) using the method described by Pullan for White Fulani (Pullan, 1978) on a scale from 0 to 5 (0–2: poor condition, 3–4: good condition and 5 very good condition or fat), and age by dentition categorized as young (< 2.5 years), mature (> 2.5–5 years) and older (> 5 years).

Moreover, animal live weight was determined by thoracic girth measurement using the animal weight measure tape for cattle (SIFAB, Cameroon) and the weight standardized using the formula $LW = 0.000141\ HG^{2.873}$ (where LW is live weight in kg, HG is thoracic girth measurement in cm) as recommended by Dineur and Thys,1985, for taurine Kapsiki, and $LW = 1{,}513 - 37.97\ HG + 0.3093 HG^2 + 0.000749\ HG^3$ and $LW = -438 + 4.88 HG - 0.001823\ HG^2$ for Fulani and Gudali respectively as recommended by Buvanendran *et al*., 1980. In many farms only very few males were present in the herds causing the random selection to be applied on the animals

found in the herd without balancing for sex proportions.

3.3.1. Assessment of packed cell volume (PCV) and trypanosome detection by microscopy

Approximately 5 ml of blood per animal was collected from the jugular vein in 9 ml ethylene diamine tetra acetic acid (EDTA) treated vacutainer tubes (Greiner Bio-One, Frickenhausen, Germany) and analyzed for packed cell volume (PCV) (Paris *et al*., 1982). Briefly, approximatively 70 µl of collected whole blood was transferred into heparinized micro-hematocrit capillaries and centrifuged for 5 min at 12,000× rpm in a hematocrit centrifuge (Hawksley & Sons Limited, Lancing, UK). The solid cellular phase in relation to the liquid serum phase was measured using the Hawksley micro hematocrit reader (MRS Scientific, Wickford, UK). A PCV below the threshold level of 26% was considered anaemic. Thereafter, the capillary tube was cut and the buffy coat and the upper most layer of RBC's was extruded on to a clean microscope slide and covered with a cover-slip (22 × 22 mm). Approximately 200 fields of the preparation were examined for the presence of motile trypanosomes with a conventional microscope with a 40x objective lens (Paris *et al*., 1982). Trypanosome species were identified by reference to the following criteria described by Murray *et al*. (1983): *Trypanosoma brucei*: Various sizes, rapid movement in confined areas; undulating membrane traps the light into 'pockets' moving along the body. *Trypanosoma congolense*: Small, sluggish, adheres to RBCs by anterior end. *Trypanosoma theileri*: More than twice the size of pathogenic trypanosomes, tends to rotate; the posterior end is clearly visible, very long, sharp and rigid.

The remaining whole blood was centrifuged at 3000× rpm for 15 min. Plasma was collected for immunological studies (not applicable here) and the remaining fraction (red blood cells and buffy coat) was used for DNA isolation.

3.3.2. Whole body palpation for *Onchocerca* nodule count and skin snips for microfilarial density

A nodule count was done according to method described by Renz *et al*., 1995. In brief, the animal was maintained at lateral recumbency and whole-body

palpation was done to determine nodule loads. Then three skins snips were collected from each animal and transferred into 1.5 ml tube containing RPMI 1640 medium supplemented with L glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin (Gibco, Ltd, Paisley, Scotland), one near umbilicus, second between umbilicus and udder/scrotum and the last one close to udder/scrotum (Renz *et al*., 1995; Achukwi *et al*., 2000). Skin snips were weighed; mff species identified using the methods described by Wahl *et al*., 1994. Microfilarial density per mg of skin were calculated as follow (mff density per mg skin = (total mff count1+mff count2 + mff count3) / (weight of skin snip 1 + skin snip2 + skin3)).

3.3.3. Gastro intestinal worms and liver fluke diagnostic fecal egg count

Fresh faeces were taken directly from the rectum of each animal, placed in plastic bags and labelled with the animals' code and stored in cool box. Faecal samples were processed for nematode, trematode, cestode eggs and protozoa cystes identification using the McMaster method with little modification. Brief, 3g of feaces were weighed per animal and transferred into a 50 ml falcon tube, then 45ml of salt solution saturated at room temperature were added (density,1.2, prepared by adding NaCl to 5 l of distilled water until no more salt went into solution and the excess settled on the bottom of the container). The faecal suspension was poured three times through a wire mesh (aperture of 250 mm) to remove large debris. Then, 0.5 ml aliquots were added to each of the two chambers of a McMaster slide (http://www.mcmaster.co.za). Both chambers were examined under a light microscope using a 10x objective magnification and the FEC, expressed as EPG for each helminth species, were obtained by multiplying the total number of eggs by 50 (Levecke *et al*.,2011). The Benedeck sedimentation technique was used to identify the eggs of trematodes (*Paramphistomum* and *Fasciola* spp). Three g of faecal sample were weighed and transferred into a 50 ml tube, then 45 ml of tape water was added and mixed for 3 mins. Then the faecal homogenate was successively filtered through a tea filter and tissue filter. The resulting faecal suspension of these filtrations was incubated for 5 min at room temperature and the supernatant discarded; two successive steps of addition of 45 ml tape water follow by incubation at room temperature for 3 min was performed. The remaining sediment was

transferred into a Petri-dish and egg of *Fasciola* spp and *Paramphistomum* spp were counted under a dissecting microscope at 50× objective magnification.

3.3.3.1. Morphological and molecular identification of trichostrongylid worms

Thirty-three gastro-intestinal tracts (GI) were collected, of which thirteen originated from adult female Gudali short-horn Zebu cattle (*Bos indicus*). Ten GI came from West African dwarf goats and ten from Djallonke sheep slaughtered in Ngaoundéré. Immediately after slaughtering of the animals, the two ends of the abomasum, small intestines and large intestines were sealed separately by ligation with a thin rope. Each abomasum, small intestine and large intestine was processed separately. The samples were brought to the Programme Onchocercoses laboratory in Ngaoundéré, sliced open and the content washed off with tap water. The mucosa was carefully examined and washed to remove any adhering worms. The collected contents were passed through sieves of 200 and 100 µm diameter, respectively. Collected nematodes were separated under a dissecting microscope into groups according to their length and shape and transferred into clean petri dishes containing phosphate buffered saline (PBS). They were later identified to their genus and/or species as described by Hansen and Perry (1990). A proportion of 20% of the female trichostrongylid worms belonging to the genera *Haemonchus*, *Trichostrongylus* and *Cooperia* were randomly selected for molecular analysis and preserved in 95% ethanol and stored at -20^{o}C until DNA extraction was performed. Details of molecular approaches, primers used for PCR and sequencing can be taken form Paguem *et al*., submitted (a).

3.3.3.2. Morphological and molecular identification of *Pamphistomum*

3.3.3.2.1. *Paramphistomum* sampling

Live specimens of *Paramphistomum* (ca. 2000 flukes) were collected from the rumen of 30 Zebu cattle and 10 West African Dwarf goats and ten from Djallonke sheep slaughtered in Ngaoundéré. The samples were brought to the Programme Onchocercoses laboratory in Ngaoundéré. After washing thoroughly in the

Phosphate buffer saline for clear tissue debris, the specimens were preserved in the 70% of ethanol until being processed for morphological and molecular studies.

3.3.3.2.2. Light microscopy examination

Out of all recovered adult flukes, 10 flukes were arbitrarily picked from each infected animal.

The collected flukes were placed on Petri-dishes and observed through a stereo microscope to determine the morphology following the standard guidelines given by Urquhart *et al*.,1996 e. g. body shape, anterior sucker, posterior sucker (acetabulum), terminal genitalium and tegumental papillae Then, flukes were flattened between two glass slides and fixed in Bouin's fluid at room temperature for 24 h. After that, these specimens were washed in water and then stained for 24 h in 0.5% Borax carmine and subsequently distained in 1% hydrochloric acid until a pink color was observed. The acid was thoroughly washed out from all specimens with water. Thereafter, the specimens were dehydrated through 50-100% alcohol dilutions each for 1 h, and cleared with xylene for 30 mins. The cleared specimens were mounted using DPX and covered with a cover slip. The mounted slides were allowed to dry at the air and were observed under the light microscope. Parasites were processed for whole mounting and stained by Borax carmine according to the procedure given by Singh and Srivastava (1977). The species were identified according to the criteria outlined by Eduardo (1973).

3.3.3.2.3. Scanning electron microscopy examination

For scanning electron microscopy evaluation, adult flukes were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (Sigma-Aldrich, USA), pH 7.2, at 4℃ for 2 h. They were washed three times with the same buffer, and re-fixed in 1% osmium tetroxide (Sigma-Aldrich) with 0.1 M sodium cacodylate buffer (pH 7.2) at 4℃ for 1 h. After washing with distilled water, the flukes were dehydrated with increasing concentrations of ethanol (from 50% to 100%), and then dried in a HCP-2 critical point drying apparatus (Polaron 3100) using liquid carbon dioxide as a transitional medium for 15 min. The specimens were then mounted on aluminium stubs and coated with gold in an ion-sputtering apparatus (SPI-Model sputter coater;

Structure Probe, USA) for 4 min. Finally, the specimens were examined with a Zeiss Evo LS10 electron microscope (Zeiss, Germany). All the SEM analysis were done at the Departement of Evolutionary Biology of Invertebrates (Prof. Betz), Institute of Evolution and Ecology, University Tübingen.

3.3.3.2.4. Molecular identification

Genomic DNA was extracted from 70 individual samples using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer's instruction. The internal transcribed spacer region 2 (ITS2) was amplified and sequenced. 2 µl of gDNA were used as template for PCR amplification with Go Taq G2 DNA polymerase (M7845, Promega, USA) and primers (GA1 5'-AGAACATCGACATCTTGAAC-3' and BD2 5'-TATGCTTAAATTCAGCGGGT-3') according to the manufacturer's instructions. **Cycling protocol:** An initial denaturation step at 95°C for 60 sec was followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 2 min, extension at 74°C for 90 sec and a final extension step of 5 minutes at 74° C (Lotfy *et al*., 2010). Amplified products were subject to electrophoresis on 2% agarose gels. The selected positive PCR products were sent for sequencing (Macrogen, Netherlands). The chromatograms were visually inspected for ambiguous signals and the sequences were analyzed using Geneious bioinformatics software.

3.4. DNA extraction from buffy coat

Samples of 300 µl of the erythrocyte and cellular fraction were purified using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer's instruction. For sample preservation, 50 µl of trehalose enriched 0.1× Tris EDTA (TE) solution (c = 0.2 M, Sigma-Aldrich, Taufkirchen, Munich, Germany) was added as DNA stabilizing preservative in the tube containing the extracted DNA, vortexed and spun down. All samples were stored at room temperature in a dry and light-protected environment after being left to dry at 37 °C. Rehydration was done in the laboratory in Tübingen using 75 µl 0.1× TE buffer at 35 °C for at least 10 min until the pellet was completely resolved, and immediately stored at − 20 °C.

3.5. Molecular identification of trypanosomes

A nested PCR was performed that targets a variable region of the ITS-1 rDNA locus using generic primers that have the potential to recognize different species of trypanosomatids as described previously (Adams *et al.,* 2006). Positive samples with ITS-1 rDNA PCR were screened with a second PCR of the gGAPDH locus using modified nested reactions (Hannaert *et al.*, 1998; Hamilton *et al*., 2004). Details of molecular approaches, primers used for PCR and sequencing can be taken form Paguem *et al*., 2019. This work was done at the Faculty of Biology and Chemistry, University of Bremen (Prof. Kelm).

3.6. Molecular identification of tick-borne bacteria and piroplasmids

Targeted amplicon sequencing of the ribosomal small subunits, 16S and 18s RNA genes are common markers to investigate the diversity of microbial communities. Since the ribosomal RNA genes sequence is similar but not identical in different organisms, degenerate primers can be used for sequencing. The 18s and 16s mitochondrial regions, respectively, for the genera of *Babesia/Theileria, Borrelia* and *Anaplasma/Ehrlichia* were PCR amplified using group-specific primers. Details about the used primers, PCR, microarray and sequencing conditions are described in Abanda *et al*. (2019 a, 2019b).

3.7. Phylogenetic analysis

All alignments and phylogenetic analyses were done using the MEGA 7.0 software package with default settings. All figures shown are based on alignments using muscle and tree reconstruction using the neighbour joining or maximum likelihood methods. The trees were evaluated by 1000 bootstrap repetitions. For a more detailed, see the corresponding manuscripts and publications. As a control, alignments were also done with Clustal W and trees were also reconstructed using the maximum parsimony and the minimal evolution methods.

3.8. Statistical analysis

Descriptive statistics were performed to summarize parasites frequency, percentage, and proportion in study sites and co-infection levels according to region and breed. Multivariate logistic regression (MLG) analysis and descriptive statistics were performed using R v.3.4.2 (www.R-project.org) with the ISLR package for the MLG. The symbiotic association between parasite-parasite and independent variables were examined by computing the Generalised Linear Mixed Model (GLM) at 95% confidence intervals (CI). Each parasites species was used independently as outcome in separate equations. The other variables (PCV, BCS, age, sex, region, and breed) were used as cofactors. All cattle breeds with less than 10 sampled individuals and all parasite species with less than 10 infected animals were excluded from the GLM. A p-value below 0.05 was considered statistically significant.

3.9. Whole genome sequencing of taurine and Zebu

One representative individual of each of the five different cattle breeds (Namchi, Kapsiki, White Fulani, Red Fulani and Gudali) was selected. Genomic DNA was extracted from the buffy coat as described in 3.4.

The quality and concentration of the gDNA isolate was verified by fluorescent methods using Picogreen (Life Technologies). Libraries were generated from 2 µg of genomic DNA per specimen using the Illumina TruSeq DNA PCR-Free Library Prep Kit (Illumina, San Diego, CA, USA) following the manufacturer's protocol. 2x 150bp paired-end libraries sequencing was conducted on the Illumina HiSeq4000 platform with the manufacturer's proprietary TruSeq SBS Kit V3-HS at the Genetic Diagnostics and Sequencing Services (CeGat) in Tuebingen (http://www.cegat.de/en/). Raw Illumina reads were mapped to the reference *Bos taurus* Hereford breed genome UMD3.1 using BWA-MEM. For comparison with other cattle breeds, whole genome raw sequencing data from NCBI Sequence Read Archive (SRA) was extracted for the breeds Holstein (SRR934414), N'Dama (SRR3693376) and Brahman (SRR6649996) in order to study genetic variability among the breed in relation to the parasites resistance and susceptibility. Variants calling, annotations and downstream analysis were done by Stefan Czemmel and Praveen Baskaran

from the Quantitative Biology Center (QBIC) of the University Tübingen. Details of methodological approaches can be taken form Paguem *et al*., submitted (b).

3.10. **Bovine genotyping**

A subset of 721 animals from different cattle breeds were genotyped at the Institute of Animal Genetics at the University of Hohenheim (https://www.uni-hohenheim.de/en/organization/institution/animalgenetics-and-breeding) for 53,714 single nucleotide polymorphisms (SNPs). 74 ng/µL of gDNA was used as template for genotyping at the Illumina HiScanSQ platform with the Illumina BovineSNP50 v3, which features 53 714 SNP probes distributed across the whole cattle genome, and provides an average inter-SNP spacing of ~37.4 kb (https://www.illumina.com/products/by-type/microarray-kits/bovine-snp50.html). Genotyping was conducted over three days, and included overnight whole-genome amplification, followed by fragmentation, precipitation and re-suspension of the samples in a hybridization buffer. Hybridization of the DNA to the bead chips occurred overnight for 20 hours in a hybridization oven at 48 ºC. After hybridization, the bead chips were washed, stained and dried. Processed bead chips were imaged with the Illumina iScan Reader, after which data were transferred to Illumina GenomeStudio 1.9.0 software for analysis. SNPs quality control and GWAS was done by Abanda Babette, M. Schmid and S. Preuß at the Institute of Animal Genetics at the Animal Genetics at the University of Hohenheim. The manuscript is in preparation.

4- Results and Discussion

Figure 7 summarizes the overall diversity of parasite communities in 1300 cattle examined. We found that 90% of examined animals were infected with at least one parasite species. More than 50% of animals were infected with multiple component communities of parasites. The *Babesia*/*Theileria* and *Anaplasma*/*Ehrlichia* were the most abundant communities (90% of the animal infected) and the least prevalent community was blood filariae.

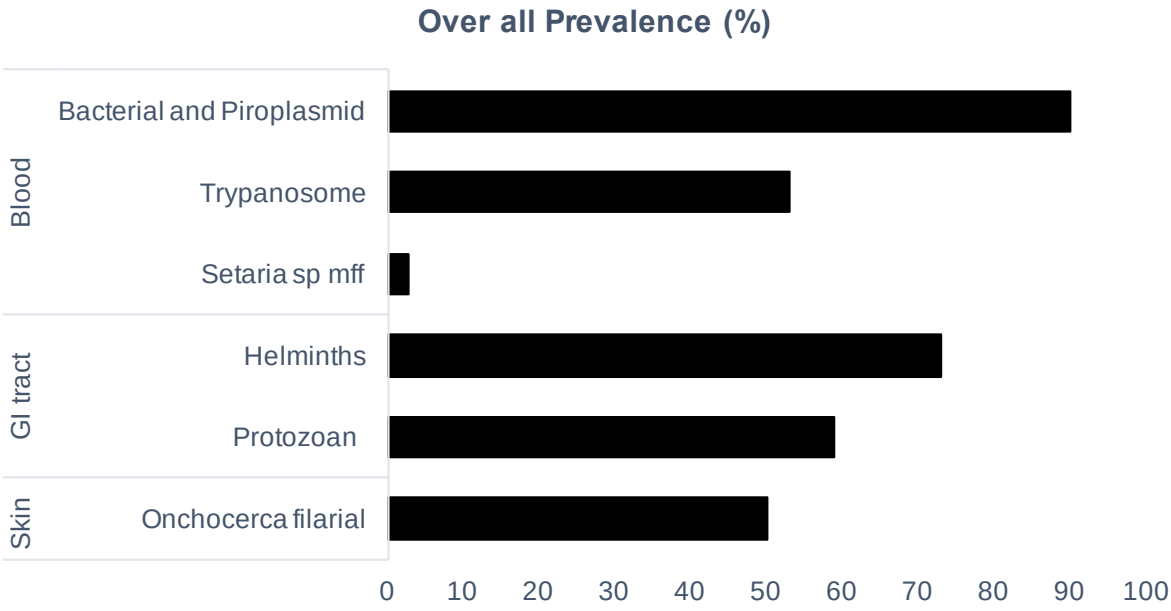


Figure 7. Overall prevalence of component community of parasites living in cattle blood, gut and skin**. G**I=gastro intestinal tract**.** Figure credited by A. Paguem.

4.1. Component community of blood parasites in various bioclimatic zones and cattle breeds

4.1.1. Susceptibility and diversity of trypanosomes (Paguem *et al*., 2019).

The overall prevalence of *Trypanosoma* spp. detected by microscopy was 5.9 % (56/953). Three species, namely *T. vivax* (2.3%) *T. brucei* (3.7%) and *T. congolense* (3.0%) were identified. Within the *T. brucei* group several specimens could not be identified beyond doubt and were named *T. brucei*-like. More surprising *T. brucei* like

organism look very similar to bacterial *Borrelia theileri* in the shape, size and movement in buffy coat slide preparation. These bacteria had never been reported in our study areas, but were found in 17.7% of the animals examined (Abanda *et al*., 2019b). Therefore, we believe that this bacterium might occasionally be misidentified as trypanosome when the buffy coat is examined by light microscopy.

By using the much higher sensitive DNA-based nested PCR (targeting ITS1), 53.2% of the animals were found infected with at least one trypanosome species (Paguem *et al*., 2019). Out of 56 trypanosome-positive cases identified by microscopy, only 41 were detected by nested PCR giving the concordance rate of 73.2% between both techniques. Among the eight species of trypanosomes identified by size estimation of the ITS1 region, five of them were confirmed by sequencing of ITS1 and gGAPDH, namely *T. brucei* spp., *T. congolense* spp., *T. vivax*, *T. theileri* and *T. grayi*. The remaining last three species *T. godfreyi*, *T. evansi* and the Bodonidae could not be identified beyond doubt. The hypothesis of some Bodonidae that co-evolved from free-living to parasitic forms is rising up from our study and previous work done by Ngomtcho *et al*. (2017). In our sample selection, there are 10 confirmed cases of Bodonidae infecting cattle.

The second parasite that may have undergone host change was *T. grayi*, which is naturally a parasite of crocodiles and reptiles (Hoare, 1972; Kelly *et al*., 2014). We found 50 of 358 animals infected either with *T. theileri* or with *T. grayi*. However, the mode of transmission and the effect of the parasite on the cattle host remains unclear. Although a previous study points out that Bodonidae and *T. grayi* might be potentially pathogenic (Ngomtcho et al., 2017), this is maybe attributed to the poor health status and poor feeding conditions of the study animals.

Nuclear gGAPDH markers revealed the presence of two strains of *T. vivax* (clade A and clade C) and at least five clades of *T. theileri* (clades IIB, IIA, IA+IB, Uganda and SitaBip1 isolate). This diversity of *Trypanosoma* species circulating in the Guinee savannah and Sahel agro-ecological zones is more complex than previously reported. It may reflect the abundance and biodiversity of domestic animals, wild animals and humans as potential hosts (Fig.9).

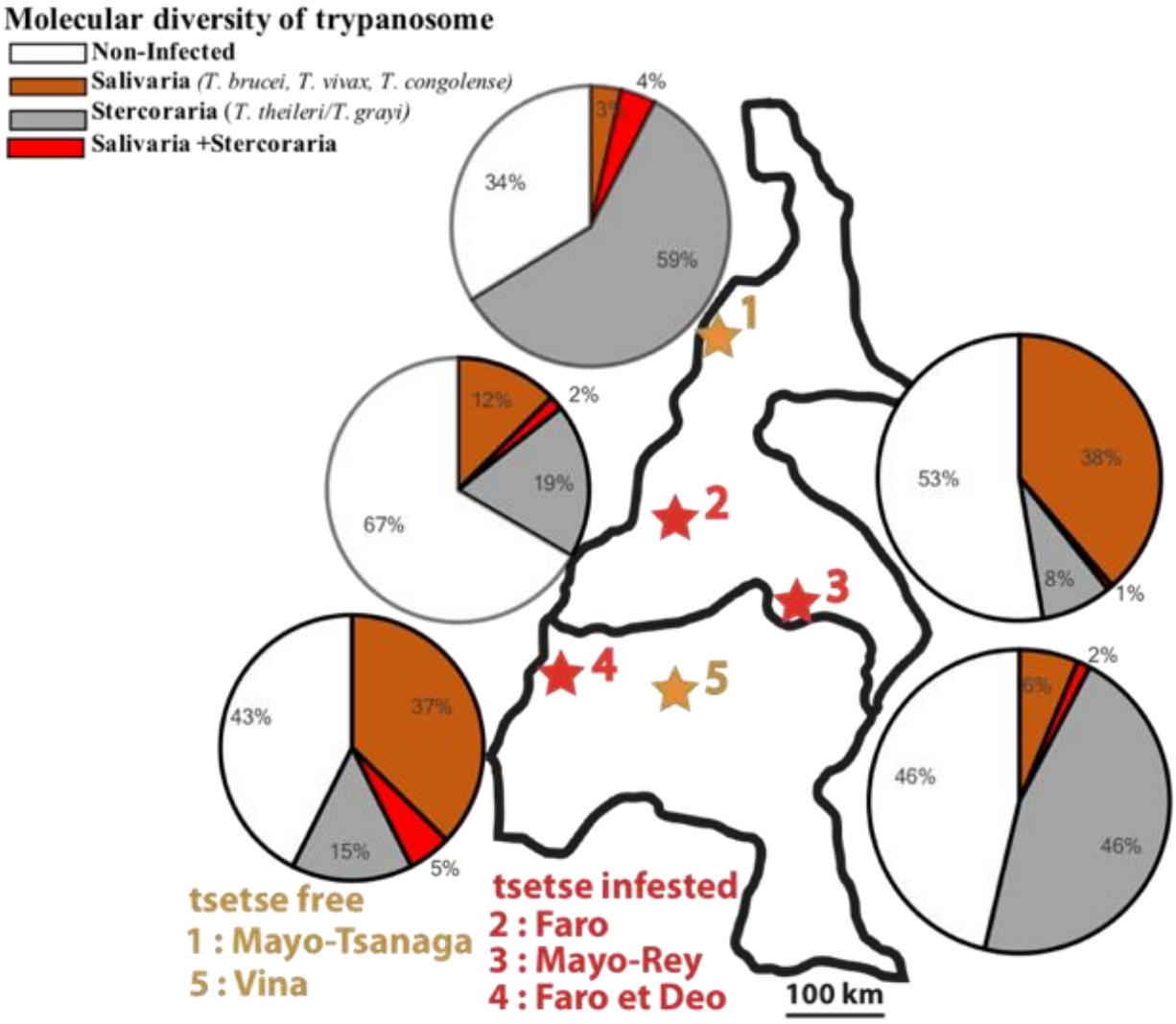
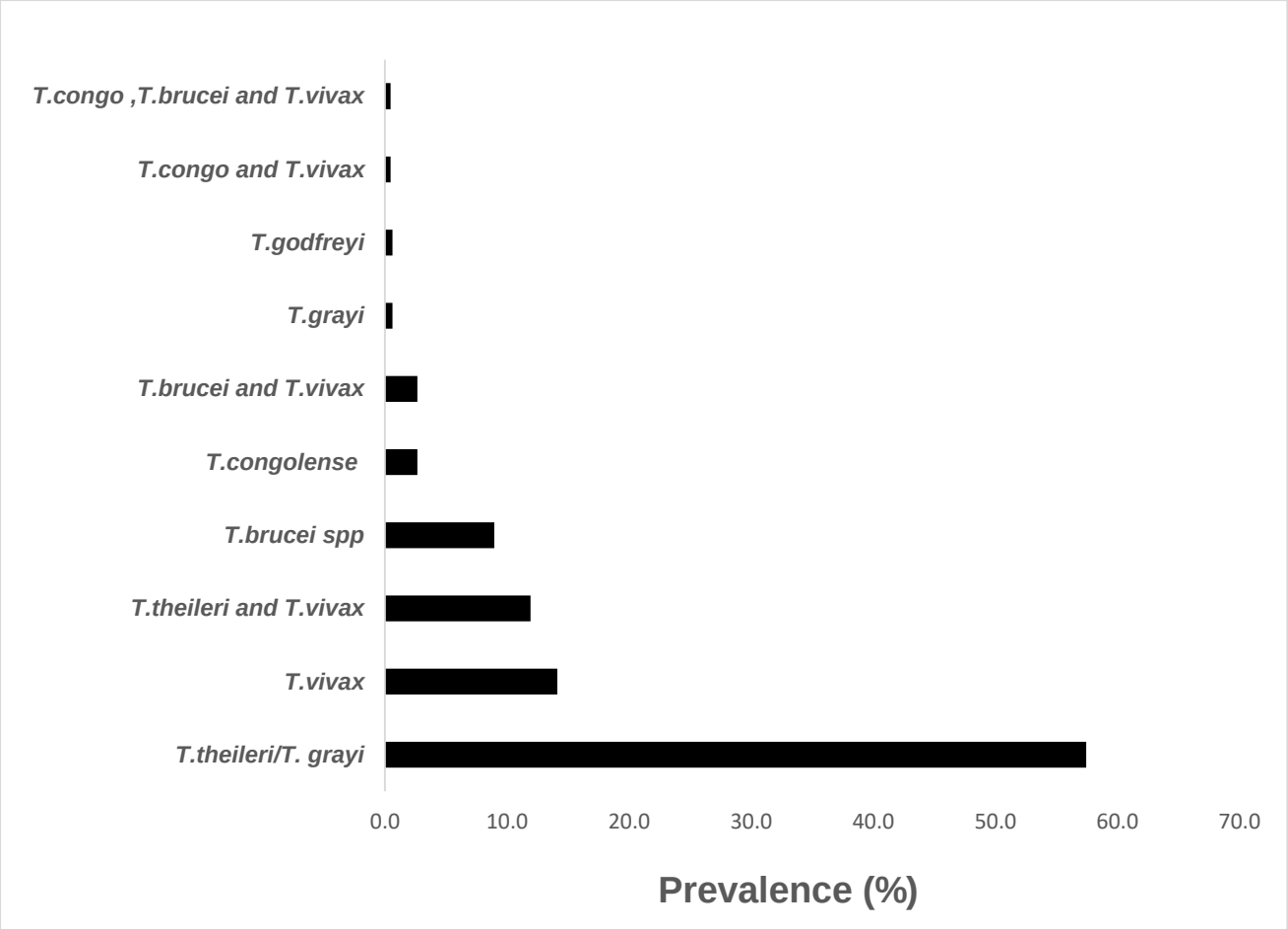


Figure 8: Distribution of Salivaria (*T. brucei*, *T. vivax* and *T. congolense*) and Stercoraria (*T. theileri* and *T. grayi*) in tsetse-free and tsetse-infested areas. From Paguem *et al*., 2019

A


T.congo ,T.brucei and T.vivax
T.congo and T.vivax
T.godfreyi
T.grayi
T.brucei and T.vivax
T.congolense
T.brucei spp
T.theileri and T.vivax
T.vivax
T.theileri/T. grayi
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
Prevalence (%)

B

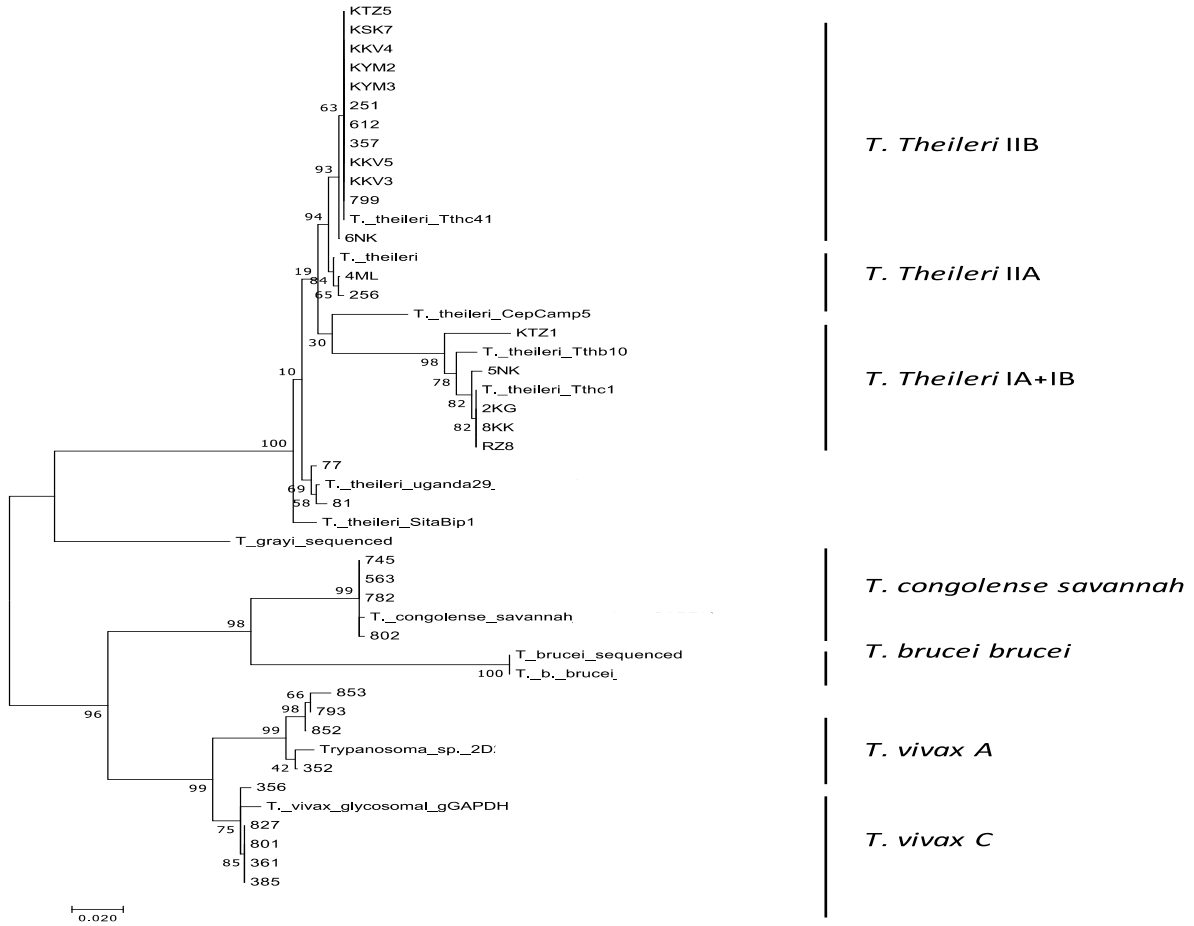


Figure 9. Diversity of trypanosomes in Northern Cameroon. A) Molecular identification using ITS1. **b)** Phylogenetic analysis by Maximum Likelihood methods based on the gGAPDH-encoding gene. Adapted from Paguem *et al.*, (2019)

The predominance of trypanosome species/strains was correlated with the presence or absence of tsetse vector flies (Fig. 8). In the tsetse free areas, the predominant group belonged to the Stercoraria (*T. theileri* and*T. grayi,* prev = *57.5%*), while Salivaria (*T. brucei*, *T. vivax* and *T. congolense*) predominated in the tsetse-infested areas. Indeed, our data suggest that competition between Stercoraria and Salivaria for the blood space may result in over-dominance of one type with few limited cases of concomitant infections (Fig. 8).

Risk factors of trypanosome co-infections were, in the order of importance, age of the animal, PCV, body condition, sex and sampling areas (+/- tsetse flies) (Paguem *et al*., 2019). For comparison of susceptibility of the different cattle breeds, trypanosomosis (ie. the prevalence of - even very few – trypanosomes in a cattle host) is distinct to trypanosomiasis, which is the acute form of the disease caused by

many parasites in the blood. Note that most Stercoraria infections generally do not cause apparent disease, i.e. they are non- or low pathogenic.

All cattle breeds were susceptible to trypanosome infection. However, Doayo (Namchi) cattle were less infected than the other taurine breed Kapsiki and Zebu cattle. Salivaria infections in Kapsiki and Zebu cattle were correlated with low PCVs and poor BCS, whereas no correlation was observed in Namchi cattle (Paguem *et al.,* 2019).

In a previous study N'Dama, Namchi, Kapsiki and Zebu (Gudali) cattle were experimentally challenged to pathogenic *T. congolense* (Achukwi *et al*., 2007). N'Dama and Doayo (Namchi) developed less severe anaemia, lost less weight and were able to limit the level of parasitaemia better than the Zebu Gudali and Kapsiki cattle. Kapsiki and Zebu were found to be trypano-susceptible whereas N'Dama and Doayo (Namchi) are trypanotolerant (Achukwi *et al*., 1997). Namchi, like other West African taurine cattle breeds are also resistant to many other infectious diseases such as foot and mouth diseases virus, dermatophilosis and tick infestations (Achukwi *et al*., 2007).

4.1.2. Susceptibility and diversity of bacteria and piroplasmid (Abanda *et al*., 2019a, 2019b)

PCR screening for tick-borne bacteria and piroplasmids had shown that 90% of cattle were co-infected with *Babesia*/*Theileria* (79%, n=993), *Anaplasma*/*Ehrlichia* (76%, n= 959), *Borrelia* (18%, n=225) and *Rickettsia* (14%, n= 180) (Abanda *et al*., 2019a; 2019b). In total, 12 different species or genotypes were identified namely *Th. mutans, Th. velifera*, *Theileria. sp B15a*, *A. platys*, *A. marginale*, *A. centrale*, *Anaplasma. sp Hadesa*, *Borrelia theileri*, *Rickettsia africae*, *R. felis, Ehrlichia ruminantium* and *E. canis*, (loc. cit. and Abanda *et al*., 2019b). The most frequent co-infection was that of *Th. mutans* and *Th. velifera* (60%; Figure 10 and Figure 11). These two-species differ from *Th. parva,* the etiological agent of East Coast fever (ECF). *Th. mutans* and *Th. velifera* are transmitted by *Amblyomma* spp. In contrast, *Th. parva* is transmitted by *Rhipicephalus appendiculatus [syn. Boophilus microplus]* and *Th. mutans* and *Th. velifera* multiply in erythrocytes rather than in lymphocytes (Coetzer and Tustin, 2004).

Indigenous East African calves that are concurrently co-infected with those two less pathogenic species of *Theileria* were protected against East Coast Fever (Woolhouse *et al*., 2015). This phenomenon is termed “heterologous protection” (Coetzer and Tustin, 2004). The same phenomenon is observed in African buffalos that rarely suffer from clinical ECF by carrying a higher prevalence of *Th. mutans* and *Th. velifera* co-infections (loc. cit.). Therefore, “heterologous protection” may exist in cattle in our study areas, despite the absence of the vector *Boophilus microplus* (Awa *et al.,* 2016). Nevertheless, it is believed that with the climate change and human or livestock migrations, *B. microplus* and *Th. parva* will eventually arrive in Central Africa (Awa *et al*., 2015). *Boophilus microplus* has already colonized a number of West African countries during the last two decades including Nigeria, which is close to the study area. Animal co-infected with *Th. mutans* and *Th. velifera* are naturally protected again ECF.

We found a positive association between *Theileria* spp. and *Anaplasma* spp. Figure 10 summarizes the diversity and occurrence of TBDs species. *Babesia/Theileria* and *Anaplasma/Ehrlichia* co-infections were highly prevalent in the study sites. This, combined with the observed abundance of the various tick species may indicate a status of endemic (or enzootic) stability of those pathogens. 90% of all examined calves (less than 2.5 years old) were positive for TBDs, however, no association was observed between PCV, body condition, age and infection status.

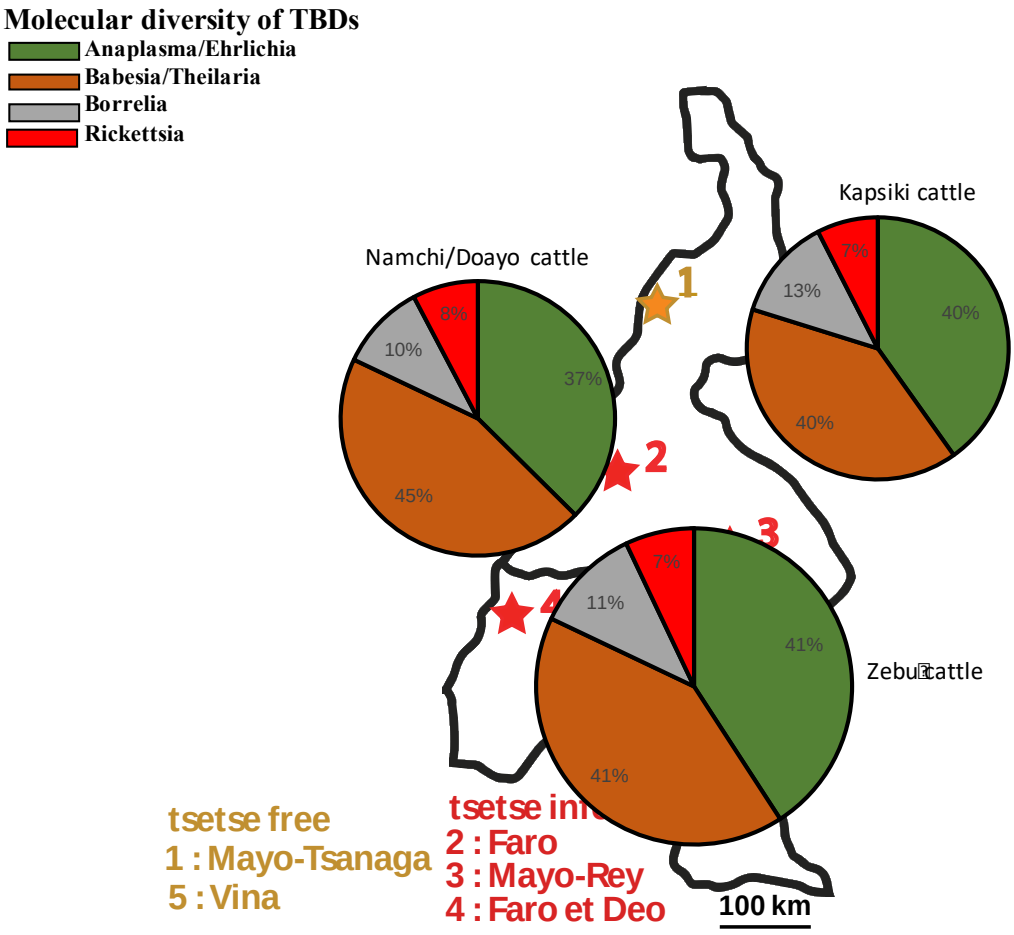


Figure 10. Molecular diversity of *Anaplasma/Ehrlichia*, *Babesia/Theileria*, *Borrelia* and *Rickettsia* in the Northern Cameroon. Adapted from Abanda *et al*., 2019b

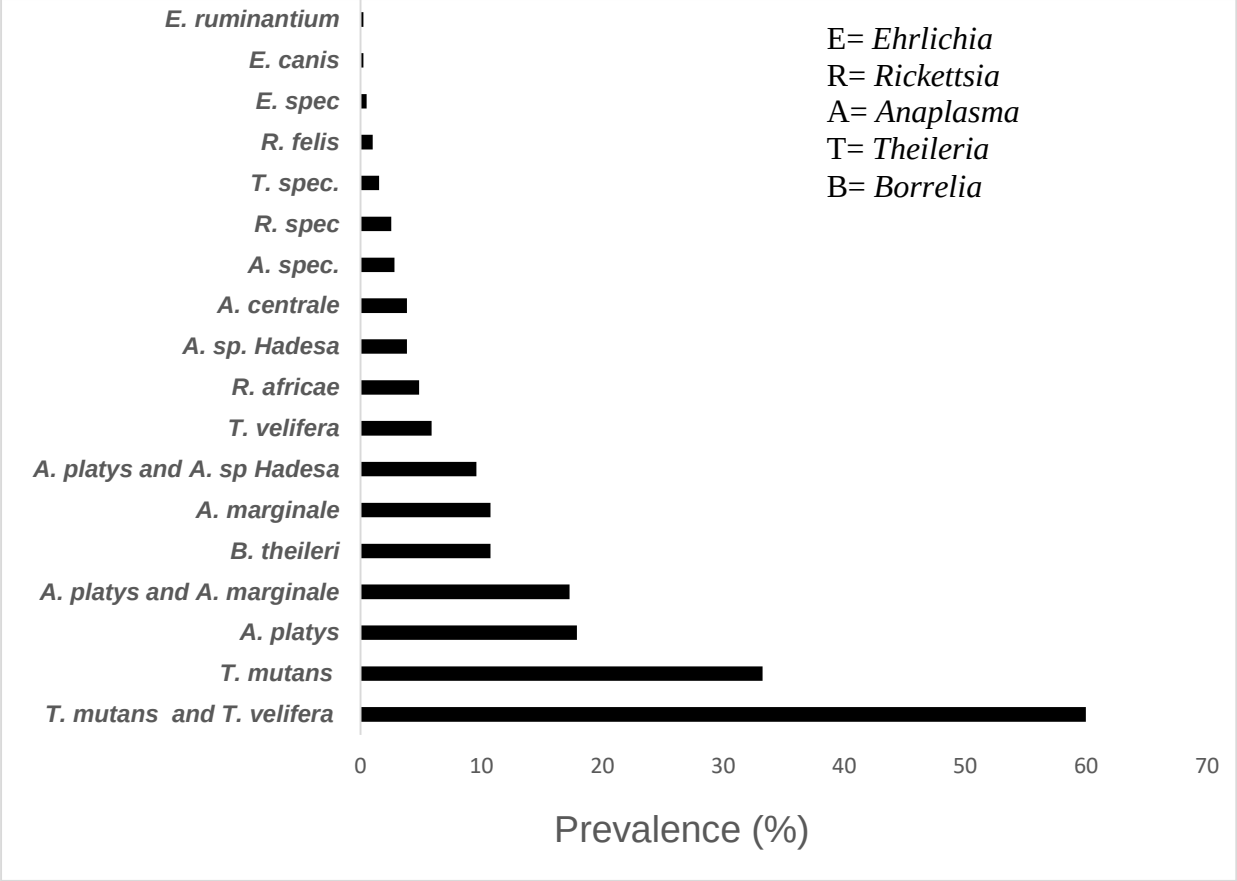


Figure 11. Diversity of species and community structure of Tick-born pathogens in Northern Cameroon (adapted from Abanda *et al.***,** 2019b).

Typically, a high serological prevalence of TBDs, for example *Th. parva* (70%), occurs in calves between the second and the sixth month after birth (Okello-Onen *et al*., 1995). After recovery from infection (mortality is high without treatment), cattle develop a lasting immunity (premunition) even in the absence of an apparent infection (De Vos *et al*., 2004). This premunition of adult animals maintains the endemic stability of TBDs in local cattle and prevents the introduction of European taurine breeds in the endemic areas. The latter one rapidly dies after being infected with *Th. parva* (Jonsson *et al*.,2008).

4.1.3. Blood-dwelling filarial parasites (*Setaria*)-unpublished

The prevalence of blood microfilariae detected by microscopy was 2.8% (27/953). The microfilarial density ranged from 14 to 243 mff per ml of blood. On average 35 mff/ml were found per infected animal. Molecular identification of *Setaria*

spp. adult specimen showed the species to be closely related to *S. labiatopapillosa* (Eisenbarth and Renz, personal communication). *Aedes* species appear to act as vectors of *S. labiatopapillosa*. Canacrini *et al.* (1997) demonstrated the implication of *Anopheles caspicus*, *An. claviger* and *An. maculipennis* as natural vectors in Europe. In Cameroon, *Aedes aegypti* and *Ae. albopictus* are common across the country (Tedjou *et al*., 2019). However, their vectorial implication in *Setaria* spp. transmission has never been addressed so far. *Aedes aegypti* are the vectors of different filarial, virus and bacterial species of human and animals. Therefore, the low prevalence observed in our study might be due to the competition of filarial species on the one hand and between filarial and arboviruses within the vector on the other hand.

4.2. Component community of gut helminths

4.2.1. Susceptibility and diversity of gastro-intestinal helminths (Unpublished part)

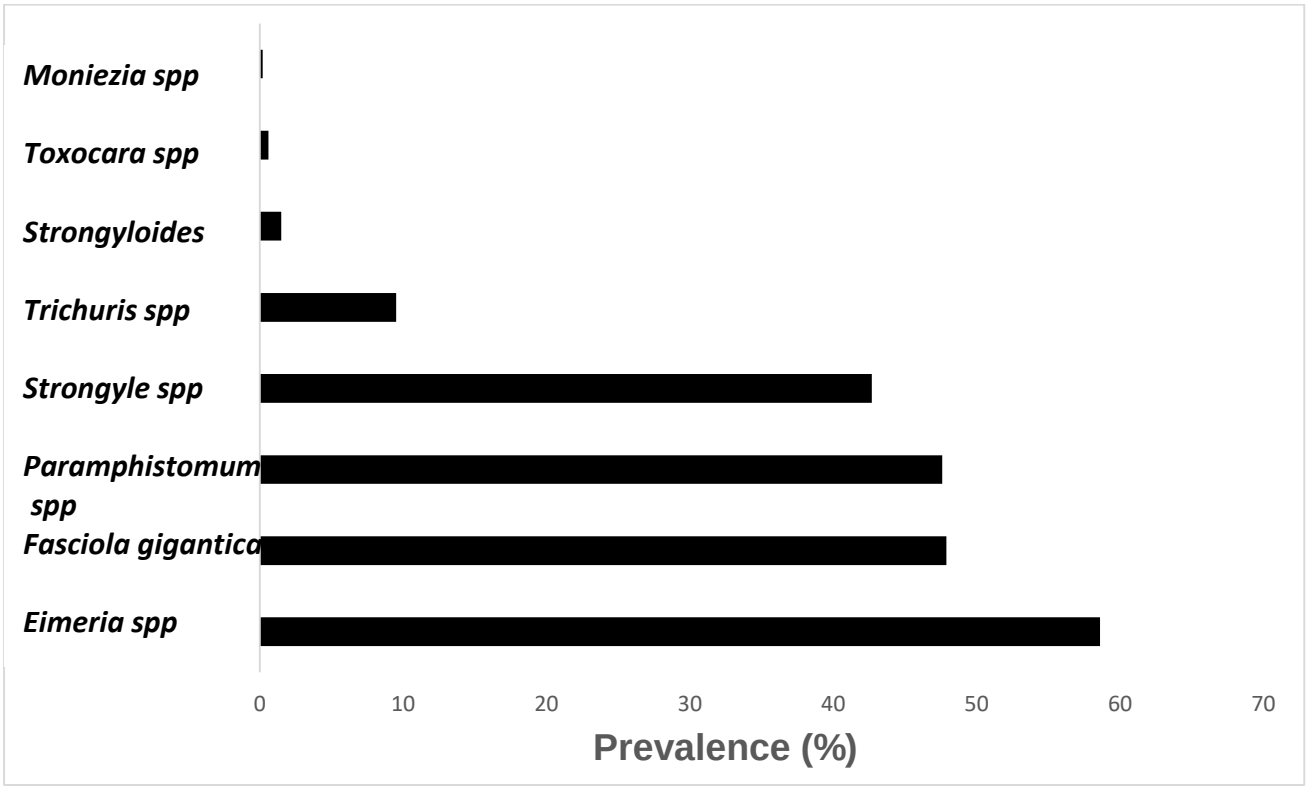
Gut parasites have been identified from faecal samples along with their prevalence and intensities (Table 2 and Figure 12). 73 % of cattle shared eggs of gastrointestinal helminths and occyst of *Eimeria* spp. 57% of cattle were co-infected with at least two groups of parasites. The common nematodes observed included members of the *Strongyle*s group (43 %), *Strongyloides* spp., *Trichuris* spp. and *Toxocara* spp.

Trematodes present included *Fasciola gigantica* (48%) and *Paramphistomum* spp. (48%), one species of cestodes (*Moniezia* sp.) and the protozoon *Eimeria* spp. (59 %). *Strongyles* eggs (0-1750 eggs/gram) were predominant in the study areas.

Table 2. Prevalence and intensity (eggs/gram) of gastro intestinal parasites detected in faeces.

	Prevalence		Intensity (eggs per gram)	
Gut parasites	N	Infected (%)	Min-Max	Mean ±SD
Fasciola	530	47.9	0-463	6.53±21.0
Paramphistomun	527	47.6	0-680	11.76±35.1
Toxocara	7	0.6	0-100	0.36±4.7
Strongyles	473	42.7	0-1750	101.51±190.8
Strongyloides	17	1.5	0-1150	2.35±37.2
Trichuris	105	9.5	0-750	13.10±56.2
Moneizia	2	0.2	0-250	0.27±7.7
Eimeria	649	58.6	0-2250	197.38±334.0

A



B

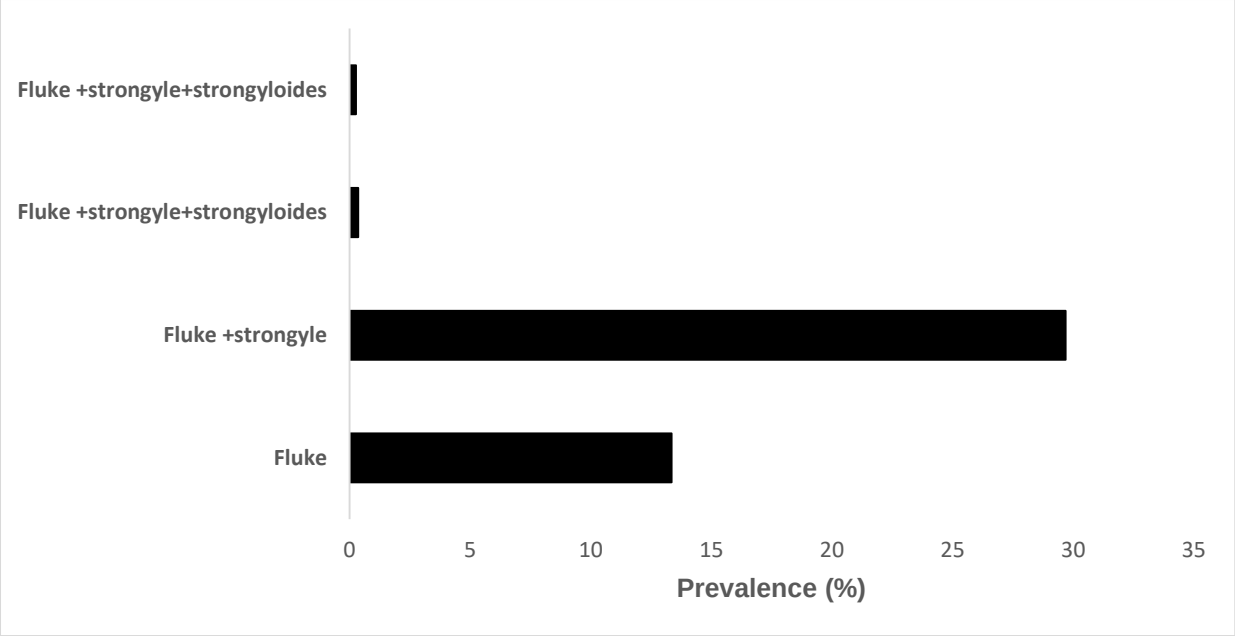


Figure 12. Prevalence of gastro-intestinal parasites **(A)** and co-infections **(B)** in cattle in Northern Cameroon. Adapted from Paguem *et al*., unpublished.

Three groups of helminths were overrepresented in the Guinea and Sahel savannah's zones: liver flukes (*Fasciola gigantica*), stomach flukes (*Paramphistomum* spp.) and *Strongyle* nematodes (Figure13). *Fasciola gigantica* and *Paramphistomum* are trematodes transmitted by snails (Roberts and Suhardono, 1996), while *Trichostrongylides* (viz. *Haemonchus spp., Bunostomum phlebotomum, Cooperia* spp*. and Oesophagostomum radiatum*) are the major soil-transmitted nematodes. 13.4% and 30% of the animals were co-infected with only flukes (liver and stomach) and flukes and strongyles respectively. Trichostrongylid nematodes were reported to induce severe anaemia, emaciation, and hypoproteinaemia (Kaufmann and Pfister, 1990). The prevalence of liver flukes (47,9%) and Strongyles (42,7%) was significantly higher in Mayo-Tsanaga (36%; 32%, X2 =116,60, p =2.2e-16) than in the Vina division (22%; 17%) and in the Mayo-Rey (16%; 21%), Faro (23%;13%) and Faro et Deo division (29%; 15 %).

The highest rate of *Paramphistomum* spp. was recorded in the Vina division (31%).

When looking at the breed level, Kapsiki (72.8%;64.1%) and Gudali (47.3%;46.6%) cattle breeds were significantly highler infected with liver flukes (X^2=79.19, P=2.585e-16) and strongyles (X^2= 98.47, P= 2.2e-16) as compared to

Doayo (33%;18.5%), Red Fulani (24.6%; 24.6%) and White Fulani (44.1%;32.2%). In general, females were more infected with helminths than male cattle and this difference was highly significant (X^2= 32.29; p=1.8e-0). Old cattle (51.9%; 55.9%) were more infected with liver and stomach flukes than mature (47.5%; 48.2%) or young (40%; 26.9%) cattle. There were no age group differences in the infection with *Strongyles* and *Eimeria* spp.

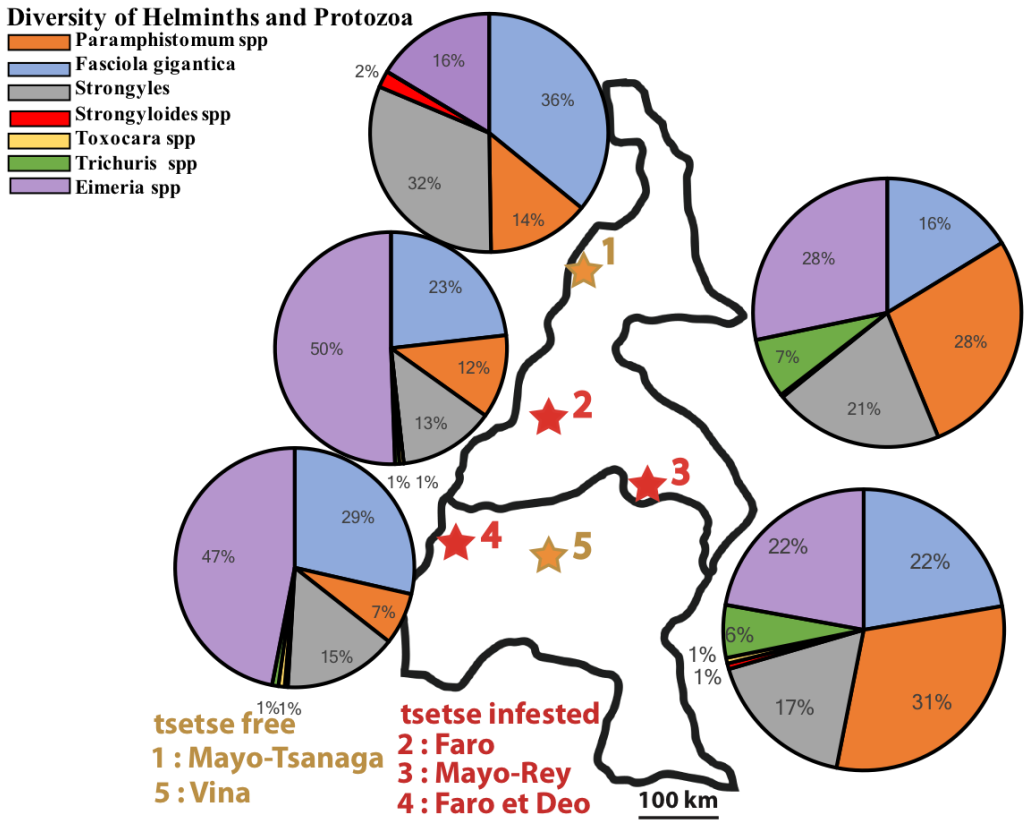


Figure 13. Distribution of helminths and protozoan parasites in the Northern Cameroon. Adapted from Paguem *et al*., unpublished**.**

This high level of mixed infections indicates the high diversity of parasite species infecting cattle in Cameroon. A recent study in Adamawa reported Trichostrongylidae as the most prevalent gastro intestinal helminthosis (69.6%), followed by *Toxocara* (13.6%) and *Trichuris* (12.1%), respectively, among the young cattle stock (Mamoudou *et al*., 2016).

Several risk factors contribute to the observed high prevalence, like the favourable tropical climate, the extensive grazing system and transhumance practices and consistent dependence on standing water, marshy areas, rivers and lakes as a source of drinking water and fresh grass for cattle which harbour divers infective stages of these parasites. The high prevalence of bovine fasciolosis and *Paraphistomum* rumen flukes recorded in this study may be explained by the abundance of the intermediate snails in the study areas. In Nigeria and the rest of Africa, *Bulinus globosus* and *Lymnaea natalensis* are common water snails which are intermediate hosts of *Fasciola* spp. and *Paramphistomum* spp. (Elelu and Eisler, 2018).

However, most of the studies were limited on egg-counts per gram faeces, which may not correctly represent the number and species-composition of adult worms living in the host. Understanding the population structure and diversity of parasitic worms is particularly important for the study of anthelmintic resistance and associated genes (Gilleard, 2006). Therefore, the molecular identification of prevailing Trichostrongylidae will be discussed in the next section.

4.2.2. Molecular diversity of major Trichostrongylids (Paguem *et al*., submitted (a))

In order to identify the common species hidden behind these MacMaster faecal egg counts,33 gastro-intestinal tracts (GI) were collected from cattle, goats and sheep (Paguem *et al*., submitted a)**.** A total of 28,284 worms were recovered from the abomasa, small and large intestines. Five genera of Trichostrongylidae were identified by their morphology. In cattle *Haemonchus* spp., *Trichostrongylus* spp., *Cooperia* spp. and *Oesophagostomum* spp. were the most abundant nematodes, while in goats and sheep, *Haemonchus* spp. and *Trichostrongylus* spp. predominated (Paguem *et al*., submitted a (loc. cit.).

The 18S rDNA and ITS-2 nuclear markers were used to assign the taxonomy of *Haemonchus* spp., *Trichostrongylus* spp. and *Cooperia* spp.

We found three *Haemonchus* species (*H. placei*, *H. contortus* and *H. similis*), two *Trichostrongylus* species (*T. axei and T. colubriformis*) and three *Cooperia*

species (*C. punctata, C. pectinata/C. oncophora)* (Table 4). *H. placei* and *H. similis* were restricted to cattle while *H. contortus* was only observed in goats and sheep. These three *Haemonchus* species have been reported to occur sympatric in the savannah of Northern Ivory Coast, with *H. similis* and *H. placei* being found in cattle and *H. contortus* in sheep and goats (Achi *et al.*, 2003). This observation suggested an interspecific competition between *H. similis* and *H. contortus* leading to the elimination of one species as referred to Gause's law of competitive exclusion (Gause, 1934). In this scenario, under natural local conditions cattle infected with *H. similis* may probably induce immune responses or concomitant immunity to protect against *H. contortus* infection (loc. cit.). Similar phenomena may occur in small ruminants to prevent *H. similis* infection. Armante *et al*. (1997) in Brazil had shown that cattle experimentally co-infected with *H. placei* and *H. similis* more quickly eliminated *H. contortus* as compared to animals infected with *H. placei* alone. Furthermore, no lambs infected with *H. contortus* have acquired *H. similis*.

Trichostrongylus colubriformis were only found in goats and sheep, while *T. axei* were shared between cattle, sheep and goats. These data are indeed new for Central Africa.

Table 3. Molecular identification and abundance of Trichostrongylid species in the abomasum and small intestines of domestic ruminants in Cameroon

Species	Cattle	Goat	Sheep
*H. placei**	**++++**		
*H. similis**	**++**		
*H. contortus **		**+++**	**+++**
*T. axei**	**+++**	**+**	**+**
*T. colubriformis**		**++**	**++**
C. punctata, C. pectinata/C. oncophora**	**++**		

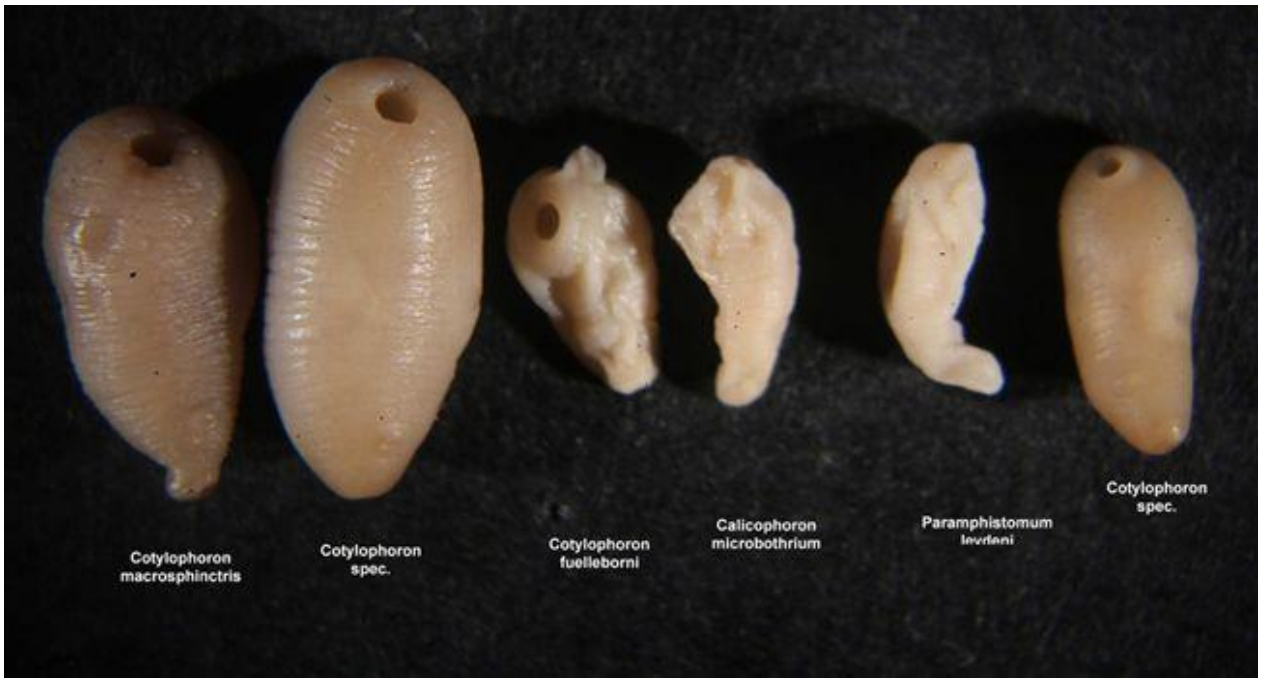
* Identified by ITS2 sequences

Note: abundance ++++ = more than 1000 adult worms found in Gut samples; +++ = 300 - 1000; ++ = 50 - <300; + = 1 - <50; based on morphological classification

4.2.3. Diversity of rumen flukes (Unpublished part)

The population of the *Paramphistomum* spp. infesting Zebu cattle was examined using classical microscopy, scanning electron microscopy (SEM) and ITS2 nuclear genomics sequences. All the *Paramphistomum* spp. found belonged to the family of Paramphistomoidea Fischoeder, 1901. Based on adult morphology and SEM, six different species were identified, including unknown species. Three genera were found: *Calicophoron* spp., *Cotylophoron* spp. and *Paramphistomum* spp. Six species were identified by morphology, namely *Calicophoron calicophoron, Calicophoron microbothrium, Paramphistomum leydeni, Cotylophoron macrosphinctris, Cotylophoron fuelleborni, Cotylophoron sp* (Figure 14). ITS2 nuclear markers analysis supported the presence of at least four clades; clade I, *Calicophoron* spp., clade II *Paramphistomum* spp., clade III *Cotylophoron* spp. and clade IV (Figure 15). A clade IV subgroup with yet unknown species was found. This high diversity may be explained by the abundance of intermediate hosts. In our study, almost 50 % of cattle were infected. We know from literature that snails belonging to *Biomphalaria, Lymnaea* and *Bulinus* are intermediate hosts. Little is known about their abundance in our study areas. Based on our data set, co-infections of all the six species are common in cattle. This study is the first attempt to molecularly identify rumen flukes in Central Africa.

(a)



(b)

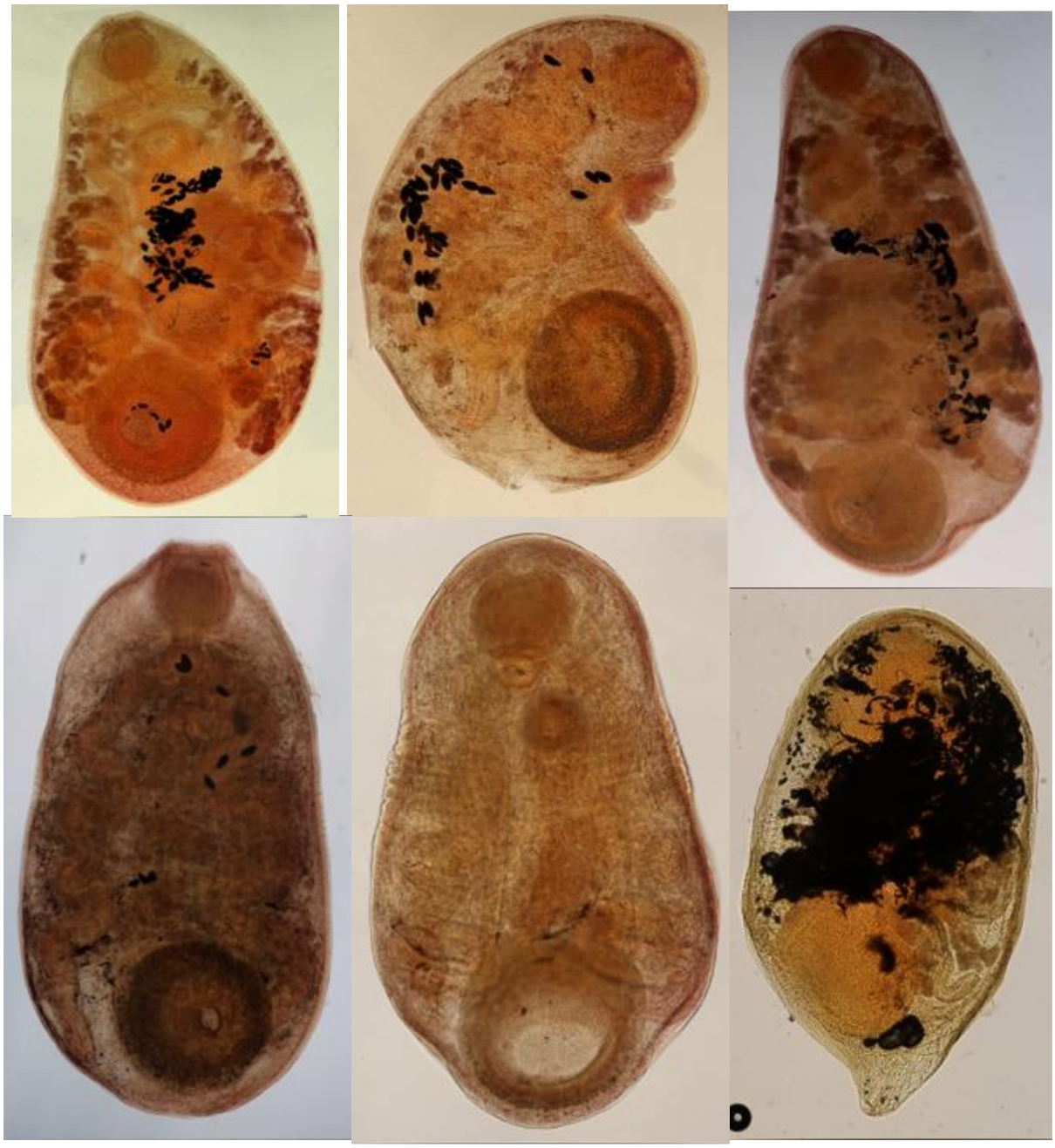
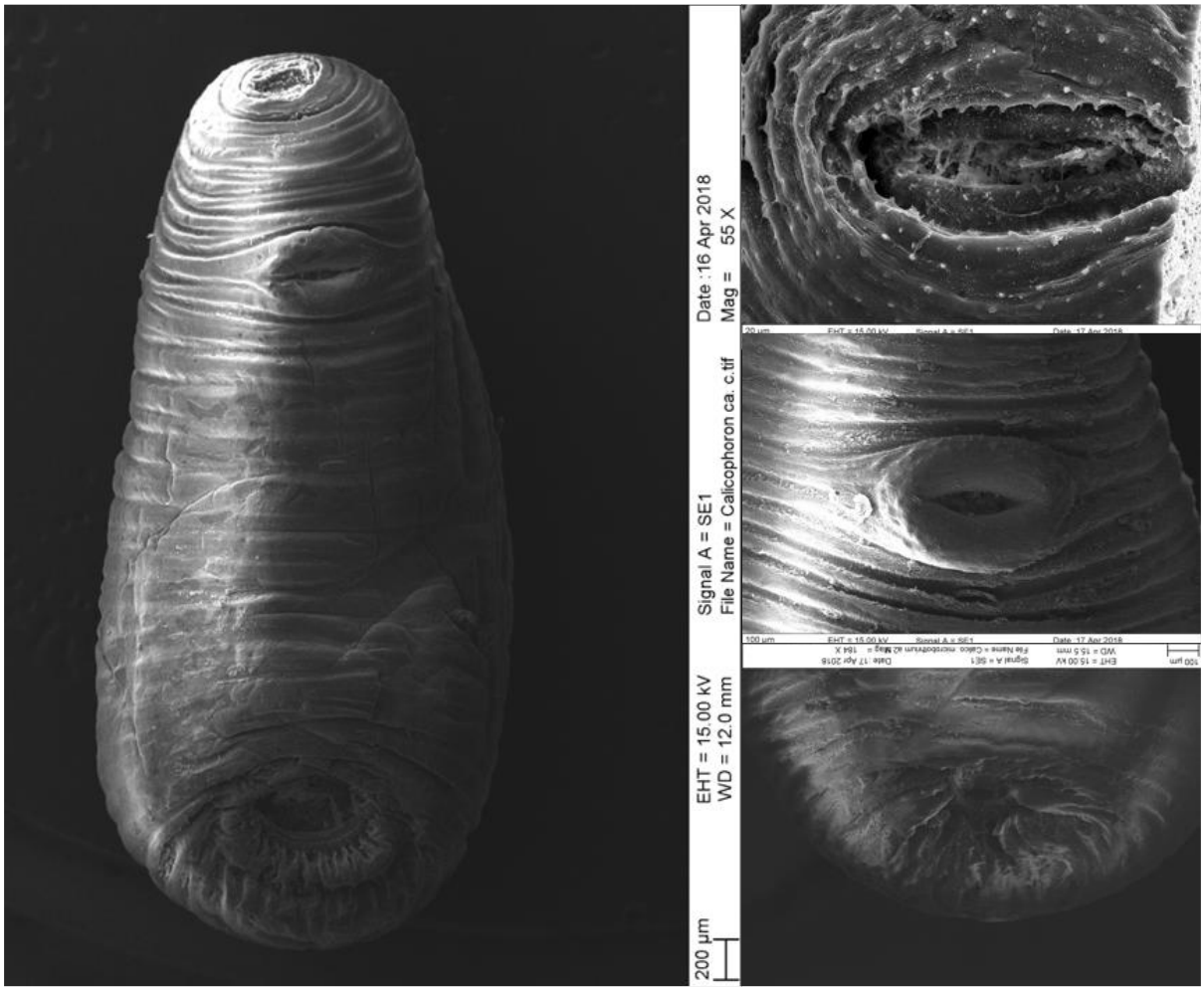


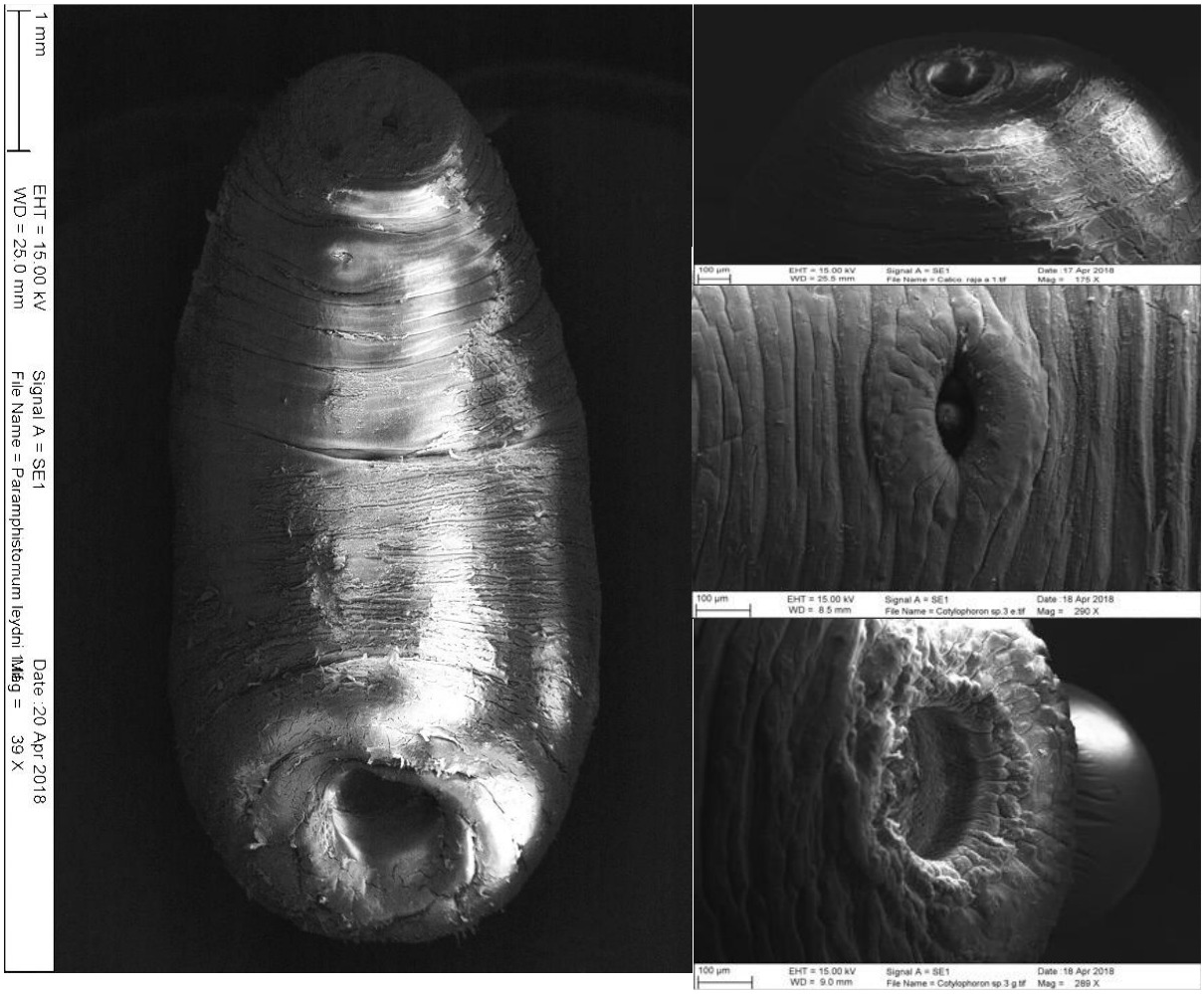
Figure 14 (a+b). Selection of whole adults of different species **(a)** and Whole mounted adults from different species **(b)** showing the anterior sucker, esophagus, caecal bifurcation, caecum, uterus, eggs within the uterus anterior testis, posterior testis and posterior sucker.

(c)



Calicophoron spp

(d)



Paramphistomum leydni

Figure 14 (c+d). Diversity of *Paramphistomum* spp. infecting cattle in Cameron (Photo by A. Paguem 2019). SEM of the Whole-body topography of the adult Calicophoron **(c)** and Paramphistomum **(d)** on the ventral surfaces showing the anterior sucker, posterior sucker and genital canal.

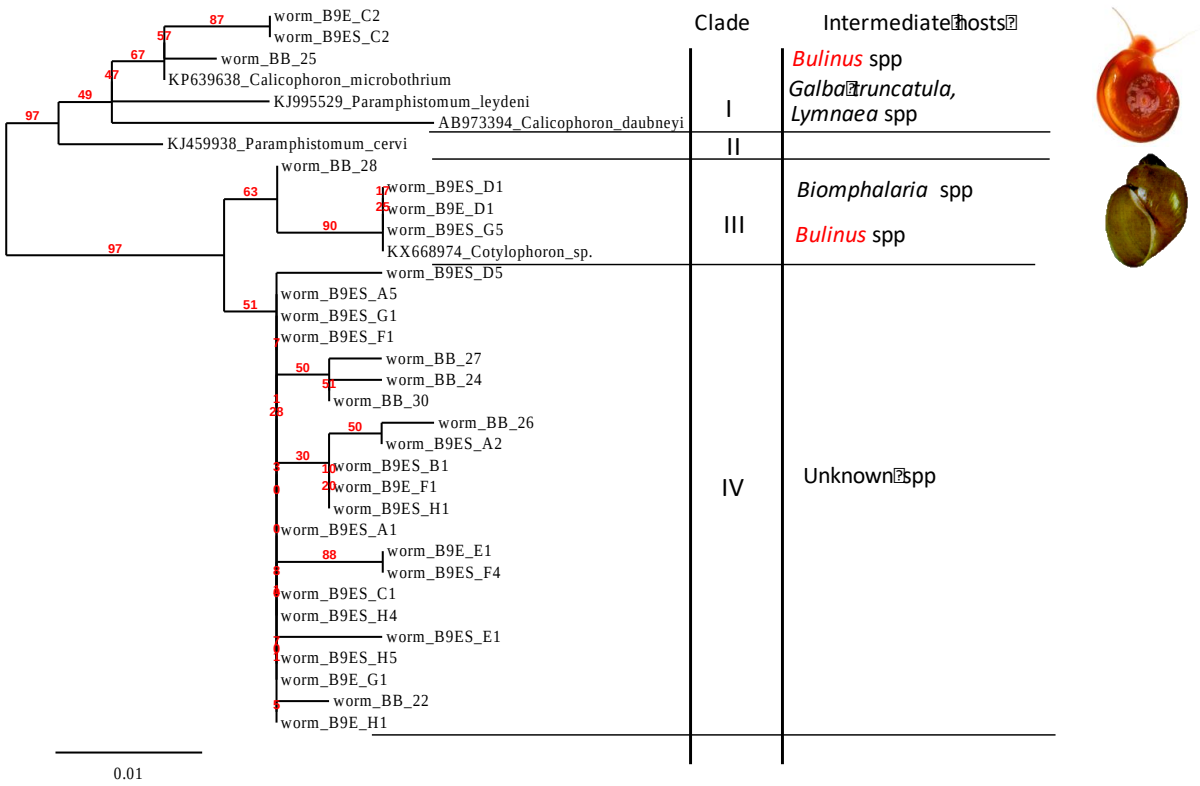


Figure 15. Molecular diversity of *Paramphistomum* spp. in Northern Cameroon with indication of the respective intermediate hosts. Molecular phylogenetic analysis by Maximum Likelihood Method based on partial ITS2 sequences. Four clades are of note: Clade I, clade II, clade III and clade IV. Adapted from Paguem *et al*., unpublished

4.3. Component community of skin dwelling microfilariae (Unpublished part)

Four *Onchocerca* species were found in cattle; *O. ochengi, O. gutturosa, O. dukei* and *O. armillata* with skin-dwelling microfilariae and one filarial parasite, *Setaria labiatopapillosa,* living in the abdominal cavity which has blood-dwelling microfilariae (Figure 16). *O. ochengi* and *O. gutturosa* were the predominant species found in our study area. This confirms previous findings by Wahl et al. (1994) some 25 years ago

Among 1,042 cattle examined for palpable *O. ochengi* nodules, 717 (69%) were infested. The nodule load on the infested animals ranged from 1 to >400 (Table 4). The distribution of *O. ochengi* nodules in different cattle breeds, gender, age groups and sites (Guinee Savannah and Sahel AEZ) is summarised as follows: There were significant differences in the nodule load number in the five sampled areas. The mean nodule load per animal in the Vina (11.74 ±44.12) and Mayo-Rey (13.05±38.05) region was significantly higher (F=6.96; P<.0001) than the mean nodule load per animal in Faro (2.27±7.77) and Mayo Tsanaga (0.94±2.36). All the cattle breeds were susceptible to the infestation with *O. ochengi*. However, the mean nodule loads in Zebu cattle Gudali (11.70 ±40.79) and White Fulani (7.5±32.71) were higher than those in taurine Namchi (2.27±7.77) and Kapsiki cattle (0.94±2.36). The difference observed between the breeds was statistically significant (F= 6.32; P<.0001). The mean nodule load of female cattle (8.37±32.60) was higher than in males (4.28±27.35). This difference between gender was close to significance (F=3.32, P= 0.072). Older animals (8.12±26.41) had a higher nodule load as compared to young animals (5.37±32.93). These findings suggest that nodules accumulate in older animal, and certain cattle breeds are more susceptible to *O. ochengi* infestations.

Table 4. Distribution of *O. ochengi* nodules in different cattle breeds, gender, age groups in the Guinee Savannah and Sahel of Northern Cameroon.

		Nodule count						
Factors		N	Min	Max	Total	Mean±SD	F	P-value
Areas	Vina	180	0	400	2114	11.74 ±44.12^{ab}	6.95	<.0001
	Faro et Deo	186	0	370	951	5.14±31.31^{bc}		
	Mayo-Rey	309	0	400	4032	13.05±38.05^{a}		
	Faro	173	0	58	394	2.27±7.77^{c}		
	Mayo-Tsanaga	195	0	15	195	0.94±2.36^{c}		
Breeds	Kapsiki	195	0	15	195	0.94±2.36^{b}	6.32	<.0001
	Namchi	173	0	58	394	2.27±7.77^{b}		
	Gudali	558	0	400	6527	11.70 ±40.79^{a}		
	White Fulani	59	0	200	443	7.5±32.71^{ab}		
	Red Fulani	57	0	60	127	2.23±9.03^{ab}		
Gender	Male	253	0	370	1082	4.28±27.35^{a}	3.23	0.072
	Female	790	0	400	6593	8.37±32.60^{a}		
Age group	Young	170	0	370	913	5.37±32.93^{a}	0.045	0.639
	Mature	512	0	400	3835	7.5±34.12^{a}		
	Old	361	0	300	2927	8.12±26.41^{a}		

[a, b and c] letters were used indicate the significance of different variables. Variables with the different letters are different.

The prevalence of *O. ochengi* microfilariae (mff, 290/956 = 30.33%) and of *O. gutturosa* (460/956 = 48.11%) were higher as compared to those of *O. armillata* (61/956 = 6.38%) and *O. dukei* (16/959 = 1.69%). The highest prevalence of *O. armillata* was recorded in Mayo-Tsanaga 20.49% (42/205) and the lowest in Mayo-Rey (14/320 = 4.37%) and Faro (5/175 = 2.86%).

In the Savannah and Sudano-Sahelian AEZs more than 50% of the cattle examined were concurrently co-infected with four *Onchocerca* species. This result reflects the abundance of the breeding sites of the *Simulium* and other vector flies. The over-dominance of *O. gutturosa* and *O. armillata* mff in Kapsiki cattle are due to the abundance of populations of ceratopogonid vectors of O*. gutturosa* and the yet unknown vectors of *O. armillata*. Doayo (Namchi) and Kapsiki cattle were less infected with *O. ochengi* than Zebu breeds located in the areas were *S. damnosum* populations of *O. ochengi* vectors are highest (Renz *et al*., 1987; Achukwi *et al.*, 2000; *Eisenbarth et al.*, 2016). This observed difference is presumably due to the abundance of vectors rather than the susceptibility of the various cattle breeds. From the longitudinal survey of our own DFG-COBE cattle herd (data yet unpublished) and previous studies (Achukwi *et al*., 2000), it was evident that the acquisition of palpable *O. ochengi* nodules and skin mff varies between individuals from putative immune (or resistant) to highly susceptible. Populations of filarial parasite are regulated by complex interactions between the filarial parasites themselves (Hildebrandt *et al.,* 2014; Eisenbarth *et al*., 2013), the host and the vector's immune system (Yordanova *et al.,* 2018) and competition between other skin and gut nematodes (this study). Molecules secreted into the host tissue or expressed on the surface of filarial parasite are involved in the establishment and maintenance of the parasite within the host (Eberle *et al*, 2015; Djafsia *et al*., 2018; Manchang *et al*., 2015; Hoch *et al*. 1993).

Intensive studies have been made on Excretory-Secretory products (ESPs) from parasites which play a modulatory function in this interaction (reproduction, nutrition, self-defence etc.). Some ESPs like *O. volvulus* superoxide dismutase

(Ajonina-Ekoti *et al*., 2012) or migration inhibitory factors (Ajonina-Ekoti *et al*., 2013) have been found to exhibit immune-stimulatory effects. However, in co-infected hosts by several nematodes species cross-reactivity has often been reported. Wanji *et al*. (2016) demonstrated that the standard serological test based on the detection of *W. bancrofti* circulating filarial antigen (CFA) cross reacts with *Loa loa*, *O. ochengi* and *O. volvulus* and this question the utility of such tests in co-endemic regions.

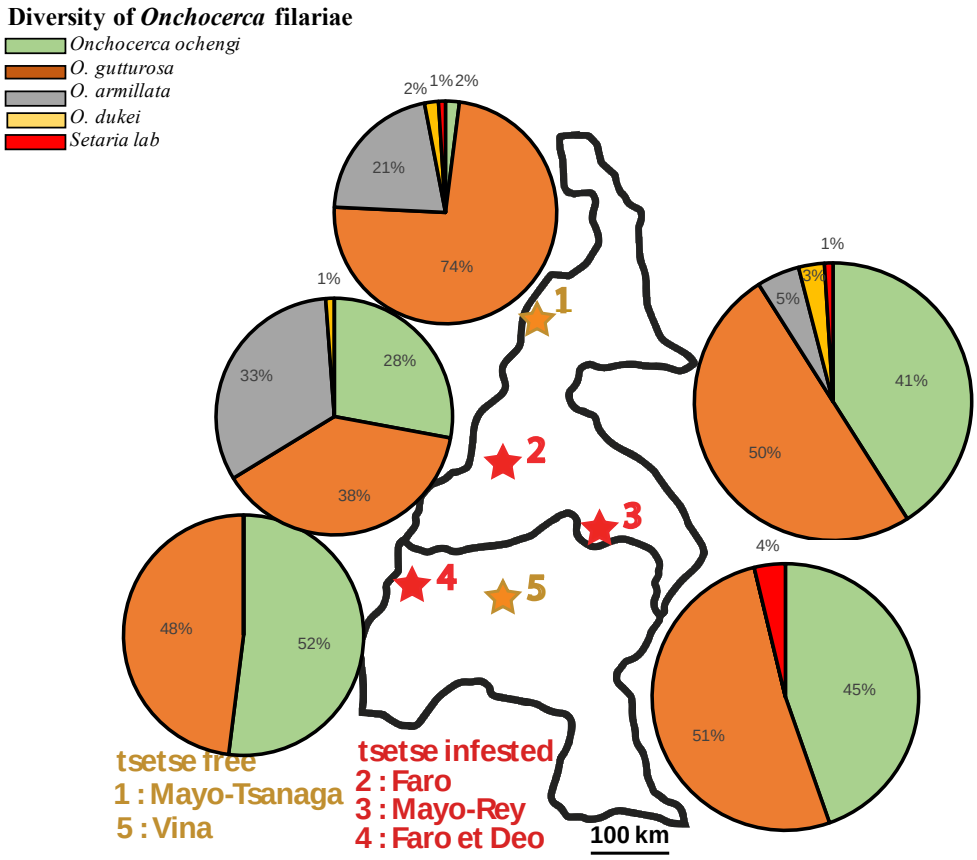


Figure 16. Bovine onchocercosis in Northern Cameroon**.** Adapted from Paguem et al., unpublished.

The high prevalence of bovine filariae combined with the abundance of cattle has been shown to have a protective impact for human onchocerciasis (Renz *et al*., 1994; Wahl *al.,* 1998). Zooprophylaxis describes the protective traits of animals against the transmission of anthroponotic diseases to man (Garrett-Jones, 1964). For instance, cattle divert blood-seeking flies to bite them instead of humans, therefore reducing the vector population biting on humans and thus the risk of

onchocerciasis transmission (Renz *et al*., 1994). Furthermore, cross-reactive immune responses caused by non-human filarial parasites transmitted onto man also diminish the risk for onchocerciasis in humans in the vicinity of cattle and presumably also of game animals (Wahl *et al*., 1998). In an experiment, cross-protecting vaccination has been demonstrated by inoculating live *O. volvulus* infective larvae to naive Zebu cattle. These animals were better protected from *O. ochengi* infection as compared to control animals (Achukwi *et al*., 2007).

4.4. Co-infections of helminths, *Eimeria* oocysts and trypanosomes (Unpublished part)

Three patterns of infections were recorded throughout this study. First, single infections with *Trypanosoma* spp. (3.3%), trematodes (9.9%), nematodes (2.8%) and *Eimeria* spp. (8.9%). Then, the second pattern observed was co-infections two groups of distinct parasites: trypanosomes & trematodes (4.2%), trypanosomes & nematodes (0.4%) and trypanosome & *Eimeria* spp. (4.4%). The third pattern seen was multiple-infections with more than two groups: Trematodes & nematodes & *Eimeria* (41.5%) and trypanosomes & trematodes & nematodes & *Eimeria* (14.9%) (Figure 17).

Doayo (Namchi) cattle were less susceptible to infection than Zebus and Kapsiki cattle. The mean PCV of animals infected with trypanosomes (30.54±5.06) and nematode (31±5.33) was significantly lower if compared to that of non-infected animals (32.81±5.6). The dual combination of trypanosomes and nematodes (*H. placei* and *H. similis*) significantly reduced the PCV (25.25±7.45) of the animals (Figure 18). More surprising, only few animals (less than 1%) were observed within this group, as the majority of these co-infected animals may probably have died. It was previously shown that immune suppression caused by trypanosome infection increased the pathogenicity of nematodes with blood sucking activity like *Haemonchus* spp., which resulted in progressive severe anaemia (Kaufmann *et al*., 1992). The second combination that decreased PCV was trypanosome and coccidian co-infection. This result showed that there is an interaction between these two groups of protozoans which may increase the virulence of trypanosome. Of the animals infected with multiple parasites more than 50% had a PCV similar to those

infected with single parasites. This may be explained by the synergic effect of multi-parasite infection to neutralize their pathogenicity.

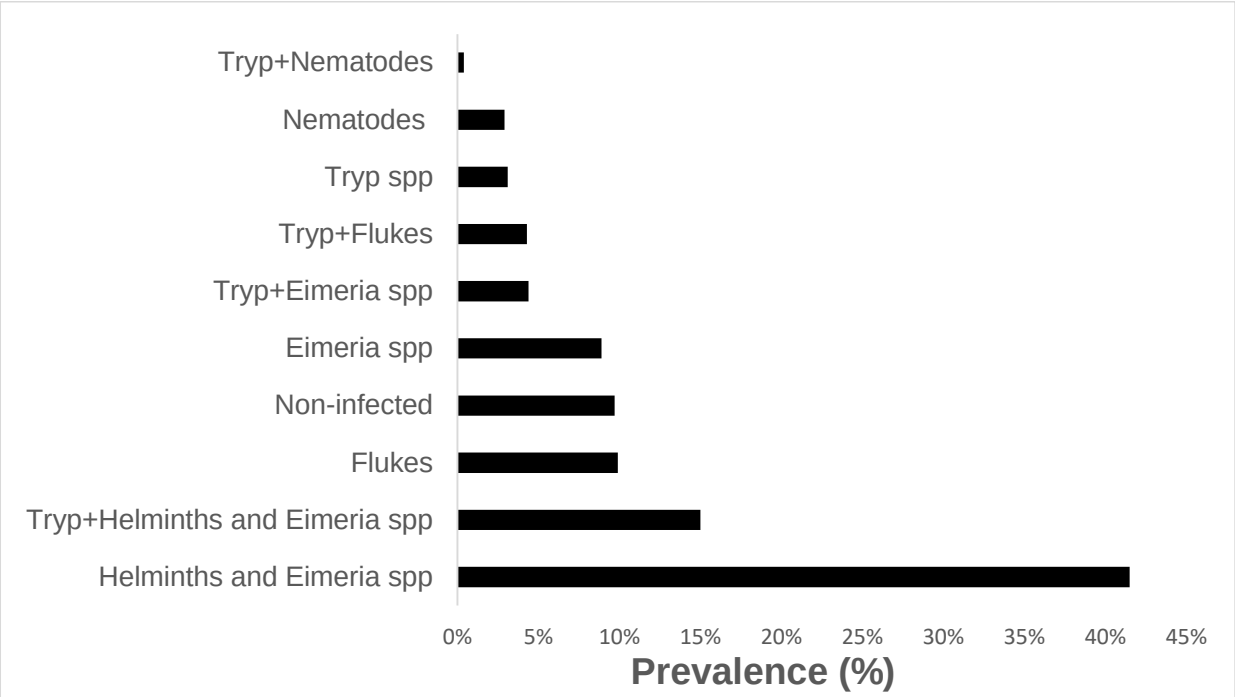


Figure 17. Distribution of helminths, *Eimeria* oocysts and trypanosome infections in Northern Cameroon. Adapted from Paguem *et al*., unpublished.

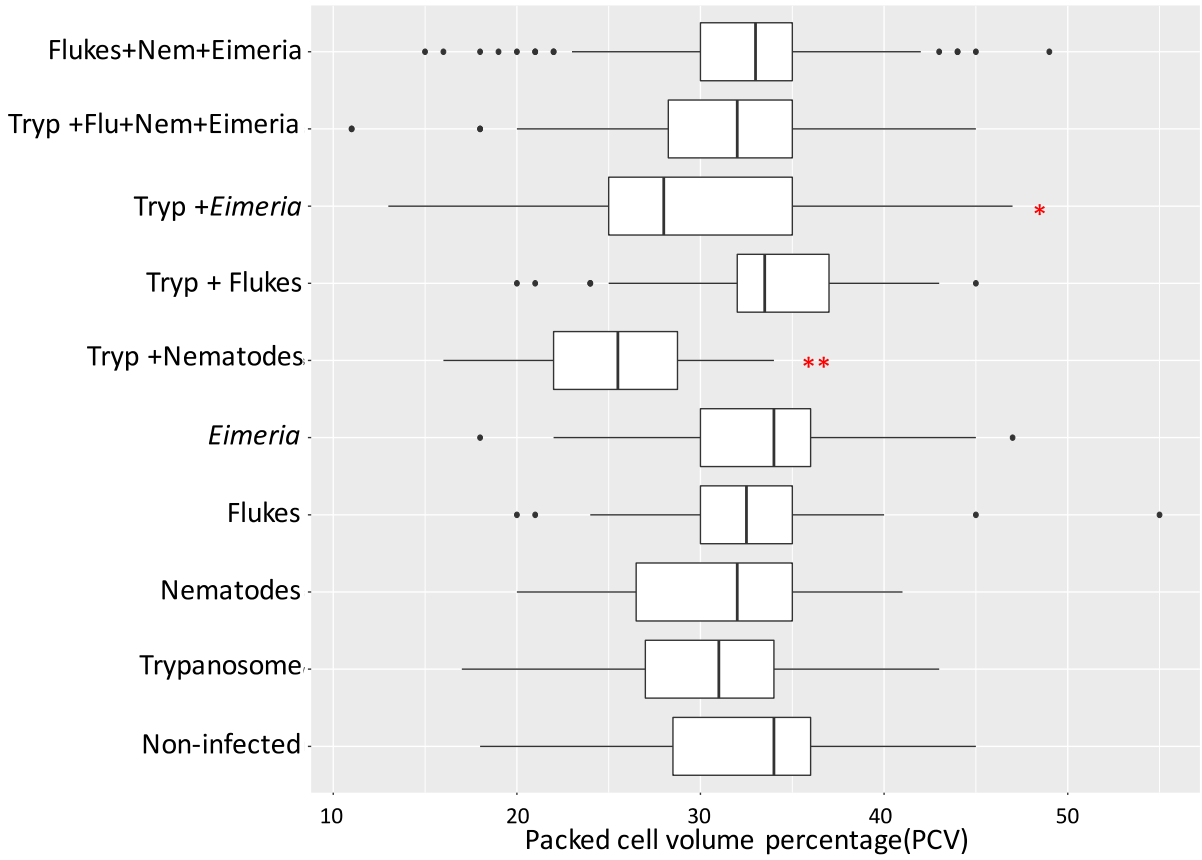


Figure 18. Effects of parasite (co-)infections on the packed cell volume (PCV) of cattle in Northern Cameroon. Unpublished.

4.5. Parasite-parasite associations (Unpublished part)

Trypanosomes, gastrointestinal parasites and *Onchocerca* filariae were examined for their interspecific interactions using a Principal Component Analysis (PCA; Figure 19) and generalized linear mixed models (Figure 20). The first-dimension (Dim1) of the PCA separated parasites into two clusters. The negative cluster 1, which comprised five parasites (*Trichuris sp*, *O. ochengi*, *Eimeria* spp., *T. brucei* and *T. vivax*) and the positive cluster 2 which comprised eight parasites (*Paramphistomum* spp., Strongyles, *Fasciola gigantica*, *Strongyloides* spp., *O. gutturosa*, *O. armillata*, *T. congolense* and *T. theileri* /*T. grayi*).

The second-dimension (Dim2) separated parasites in two clusters. The negative cluster 1 consists of three to four trypanosome species (*T. theileri*/*T. grayi,*

T. congolense and *T. vivax*) whereas the 10 remaining parasites form the positive cluster 2.

Taken together those results form four distinct parasitic groups, two with negative and two with positive interaction. The first negative cluster A consists of *T. vivax* alone whereas the second cluster B consists of *Trichuris sp*, *O. ochengi*, *Eimeria* spp., *T. brucei* and *T. vivax.* The first positive cluster C comprises two parasites *T. theileri* /*T. grayi and O. armillata* the second positive cluster D is made of *Paramphistomum* spp., strongyle, *Fasciola gigantica*, *Strongyloides* spp., *O. gutturosa*.

Figure 20 summarizes the results of the different statistic models of interaction between those parasites and supports the difference observed in the PCA analysis. There were positive (synergistic) and negative (antagonistic) statistically significant associations. Interestingly, there were antagonistic associations between the stercoraria (*T. theileri/T. grayi*), Salivaria (i.e. *T. brucei*), strongyles and *Eimeria* spp. This was also observed between *Fasciola gigantica* and *Eimeria* spp. and between *Paramphistomum* spp. and *O. armillata*. I found a synergistic association between salivarian trypanosomes (*T. brucei* and *T. vivax*), Strongyloides, *O. ochengi* and *O. gutturosa* as well as between *Fasciola gigantica*, *Paramphistomum* spp., strongyles*, Eimeria* spp. and *O. ochengi*. The negative interaction between protozoan and Helminths can be explained by polarization of the immune system of the host (Th1/Th2). Protozoan immune responses supress the Th1 cell of the host which facilitates the proliferation of helminths and often results in the death of co-infected animals (Kaufmann *et al*., 1992).

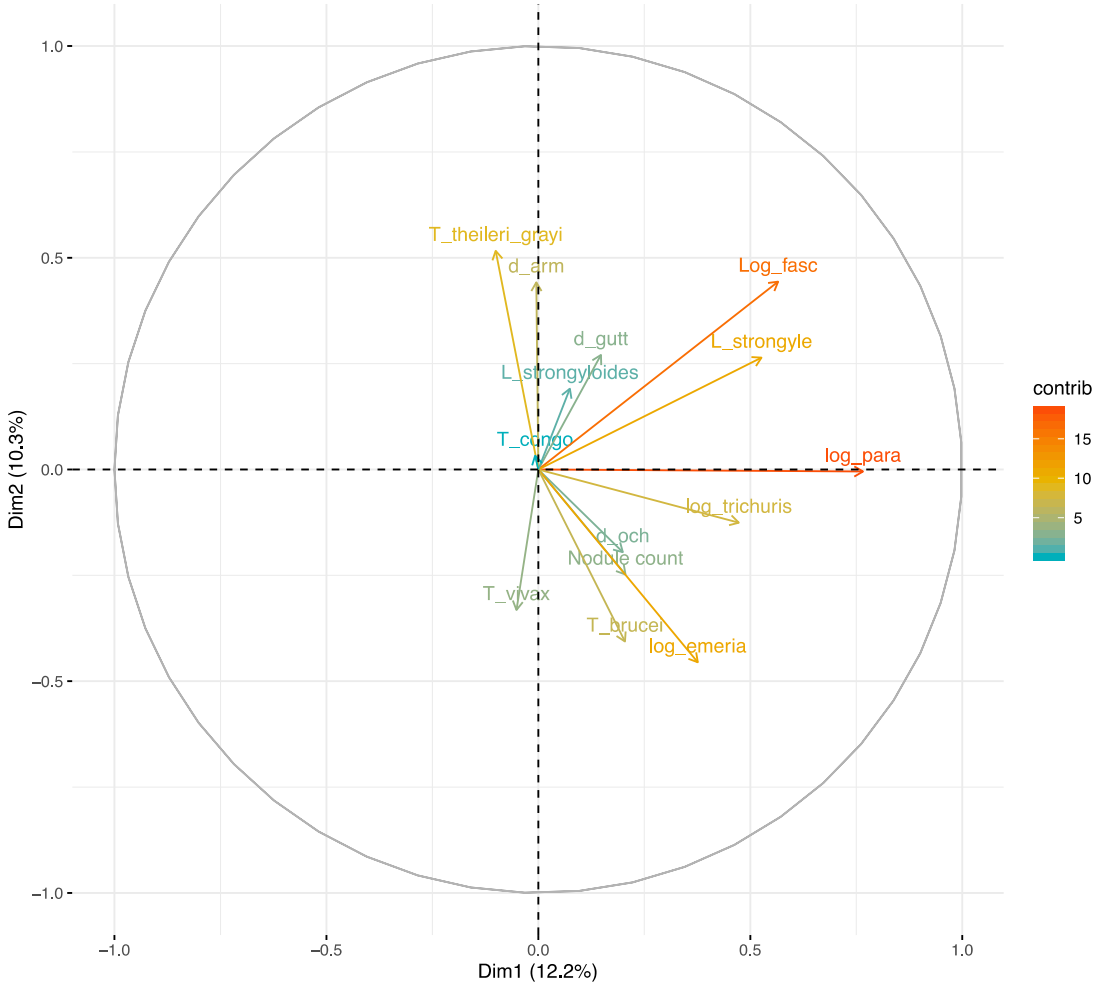


Figure 19. Interactions between trypanosomes, gastrointestinal parasites and *Onchocerca* filariae**.**

The PCA shows the relationships between all parasites: Positively correlated parasites are grouped together. Negatively correlated parasites are positioned on opposite sides of the plot origin (opposed quadrants). The distance between parasites and the centre measures the quality of the parasites variables on the factor map. There are four distinct groups of parasites. Adapted from Paguem et al., Manuscript in preparation.

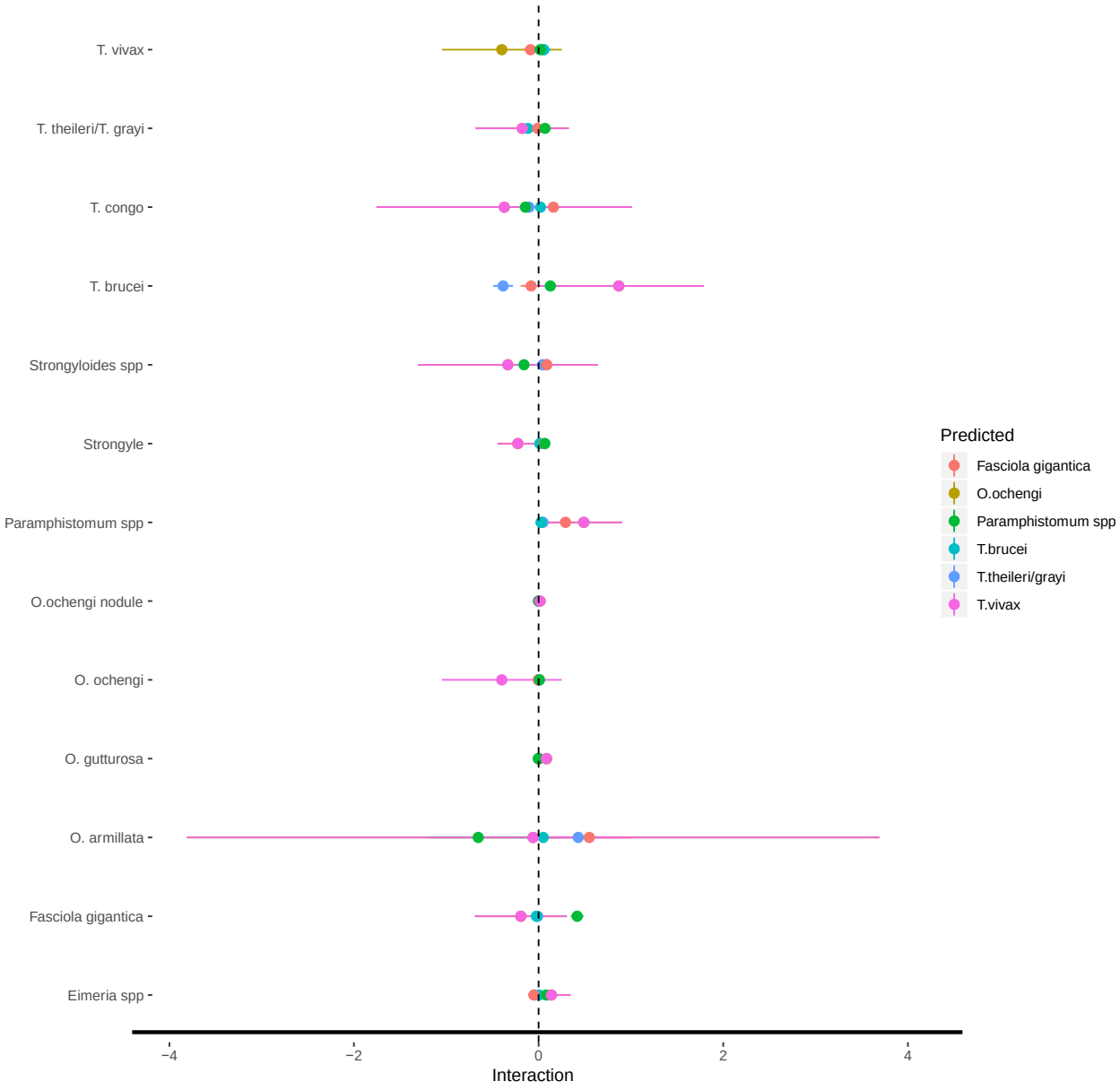


Figure 20. Predicted probabilities with 95% confidence intervals of parasite-parasite interactions**.** Probabilities were estimated from a GLM models where each parasite was predictor (n=6) and all the remaining parasites (13-n) were explanatory variables. The horizontal line represents 95% confidence intervals of predictor. The blue colour indicates positive association (synergistic) and the red colour negative association (antagonistic). Unpublished.

4.6. Host genetic factors of susceptibility and resistance to multi-parasitism

4.6.1. Genetic diversity landscape of different cattle breeds using WGS (Paguem et al., submitted (b))

In the previous chapters, I have shown that cattle breed genetics is one of the key factors of trypanosome susceptibility. African taurine and Zebu cattle are known

to be well adapted to the tropical climate and infectious diseases in contrast to European taurine cattle that are highly susceptible. Within African cattle breeds, taurine are more tolerant to multiple infectious diseases. This unique phenotype of autochthonous African cattle is the result of both natural and artificial selection in the shaping of functional diversity (Mwai *et al*., 2015). Alterations at the genomic level such as insertions, deletions, duplications, inversions, translocations, or other complex rearrangements of large genomic segments have a great impact on gene structure and function. Therefore, the genomic difference of those cattle breeds may explain the observed variations in susceptibility and pathogenicity.

For the first time, we sequenced the genomes of Gudali, White Fulani, Red Fulani, Namchi (Doayo) and Kapsiki cattle breeds. Out of 835 gigabases raw reads were generated. On average, a proportion of 65% were mapped to the *Bos taurus* reference genome (Hereford breed genome UMD3.1). The 35% unmapped reads were examined and were shown to be orthologues to bovidae families (90%), but not present in the European cattle used as a reference genome. This result is probably due to the evolutionary divergence of African cattle from Hereford cattle breed. The phylogenetic tree reconstruction with all autosomal SNPs from the different cattle breeds (Figure 20) indicate the far evolutionary distance of African cattle breeds to Eurasian cattle. Therefore, this supports the hypothesis of evolutionary divergence, which may have occurred several thousand years ago from the common *Bos primigenius* ancestors of domestic cattle in the near Orient and Northern Africa (Decker *et al*., 2014; Mwai *et al*., 2015). The positon of the Namchi cattle closer to Zebu Red Fulani points to a high introgression of Zebu genes. Nonetheless, all African Zebu and taurine have a unique mitochondrial haplotype (T1) believed to be of taurine origin different from actual modern European taurine and Indian Zebu cattle breed (Bradley *et al*., 1996). Taken together, a reference genome of Pan African cattle breeds is urgently needed not only to improve the quality of African cattle genomic assemblage but also to validate the Commercial SNPs chips used in population genetic studies, in selection programs and to identify genetic markers of diseases susceptibility.

I found 50.05 million Single Nucleotides Polymorphisms (SNPs) and 580,000 small insertions and deletions in all five genomes. 2.68 million new variants were discovered for the first time (Paguem *et al*, submitted(b)). A high proportion of

variants (approx. 1 million SNPs) were shared across all breeds. The highest proportion of breed-specific SNPs were found in *Bos indicus*; Brahman (759,804), Red Fulani (473,688), Gudali (461,043) and White Fulani (420,114), respectively, and the lowest breed-specific-SNPs were found in taurine breeds: N'Dama (220,302), Holstein (328,560), Kapsiki (370,074) and Namchi (402,114), respectively (Figure 21).

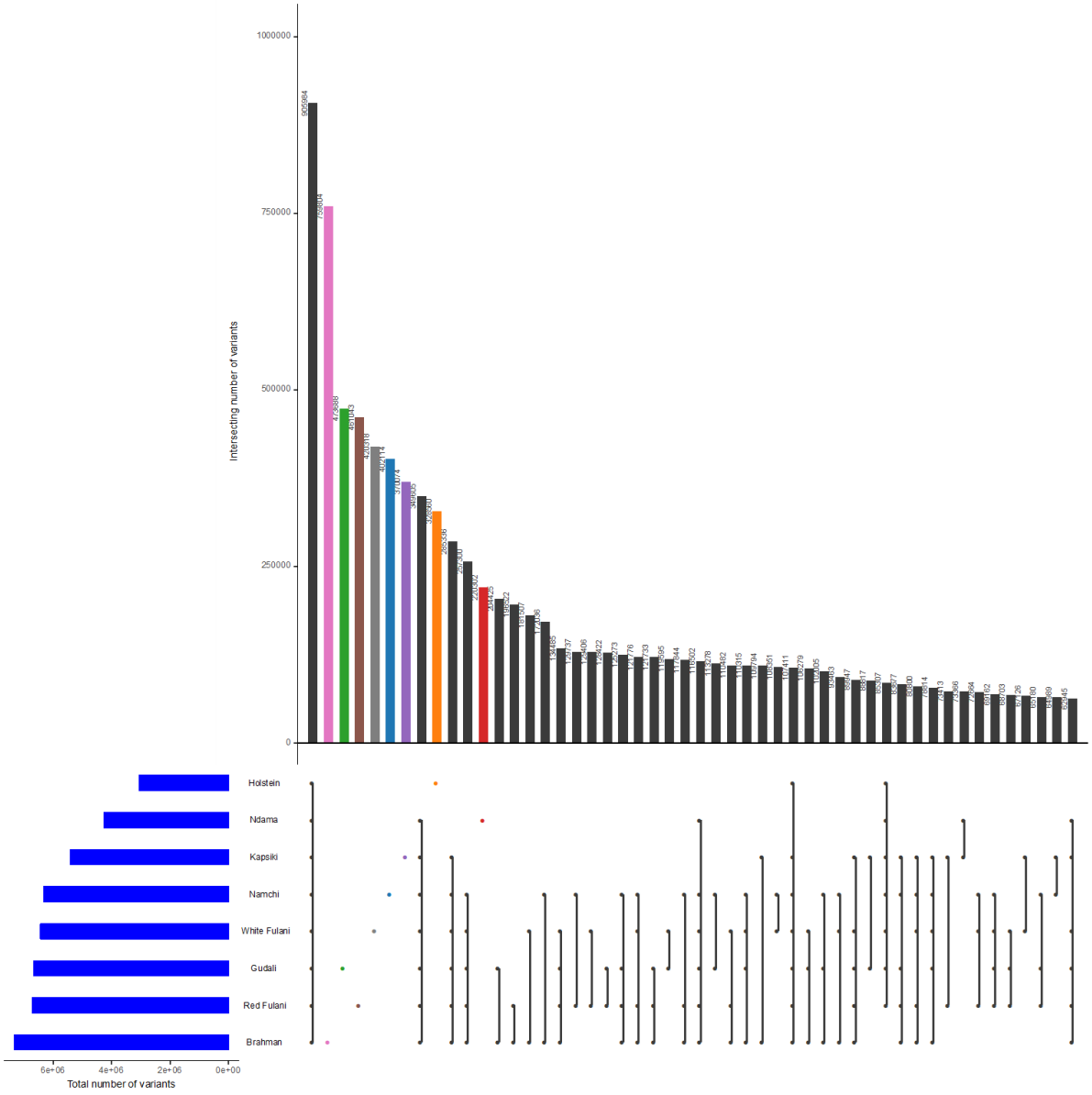


Figure 21. Upset plot showing the SNPs relationship between eight different cattle breeds. Black colour indicates the number of SNPs shared between different cattle breeds and other colour indicates the breed-specific SNPs. Adapted from Paguem *et al*., submitted (b).

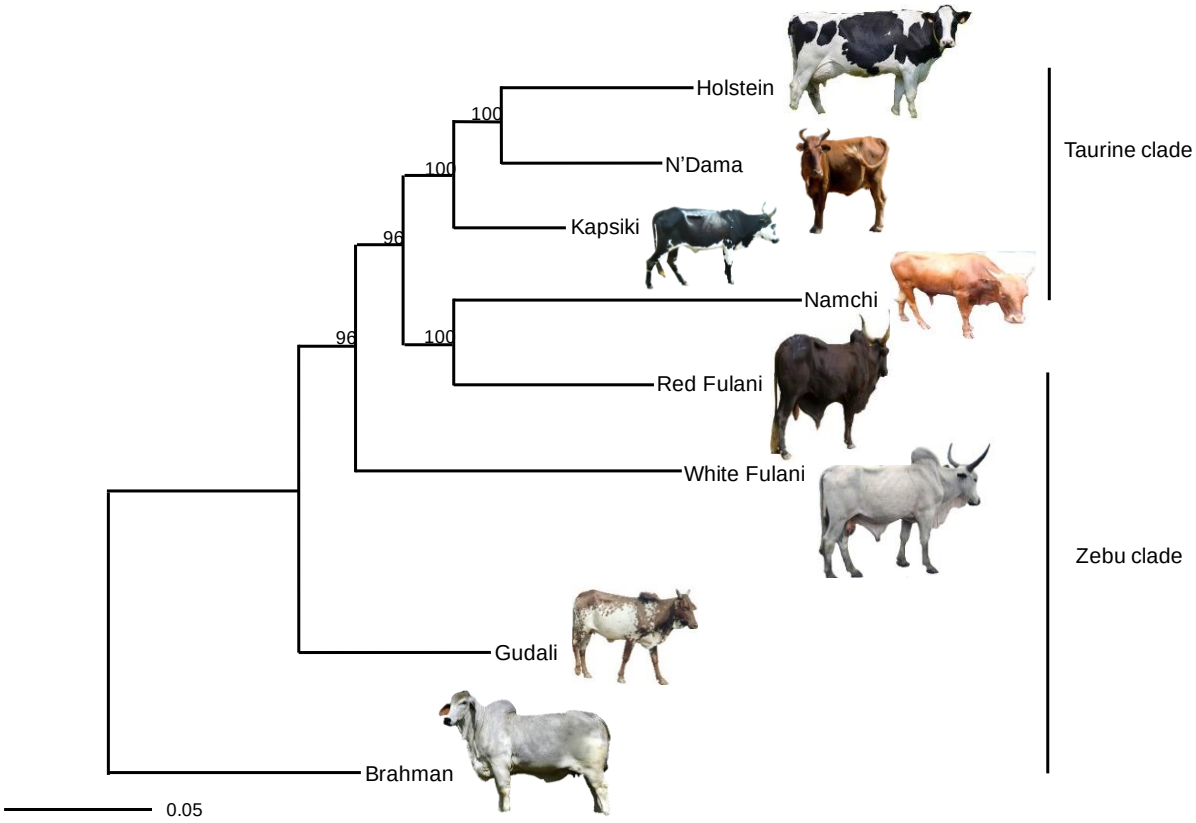


Figure 22. Maximum likelihood tree showing the phylogeny of African taurine and Zebu cattle using whole genomic sequences. The numeric number indicates the pair-wise genetic distance. From Paguem *et al*., submitted (b)

I identified 373 genes carrying breed-specific variants with high impact that may putatively change amino-acids codons, such as frameshift, splice acceptor, splice donor, start lost and stop gained, namely. 88, 82, 72, 66 and 65 in Red Fulani, Gudali, White Fulani, Kapsiki and Doayo (Namchi), respectively. Two novel frameshift variants in BoLA-DQB were identified in Namchi and Gudali.

Polymorphisms in BoLA class II genes have been associated with viral, bacterial and parasite resistance in cattle (Takeshima *et al*., 2006). IRAK1BP1, sialic acid-binding Ig-like lectins (SIGLECs), MYO1H and Heat shock protein family genes were found carrying mutational SNPs. MYO1H have roles in cell motility, phagocytosis, and vesicle transport. Gene Ontology (GO) enrichment and KEGG pathway analysis provides abundant evidence supporting the involvement of these genes on heat stress ("response to decreased oxygen levels, GO: 0036293", "response to hypoxia, GO: 0001666" and "cellular response to stress, GO: 0033554") and immune function ("acute inflammatory response, GO: 0002526", "inflammatory

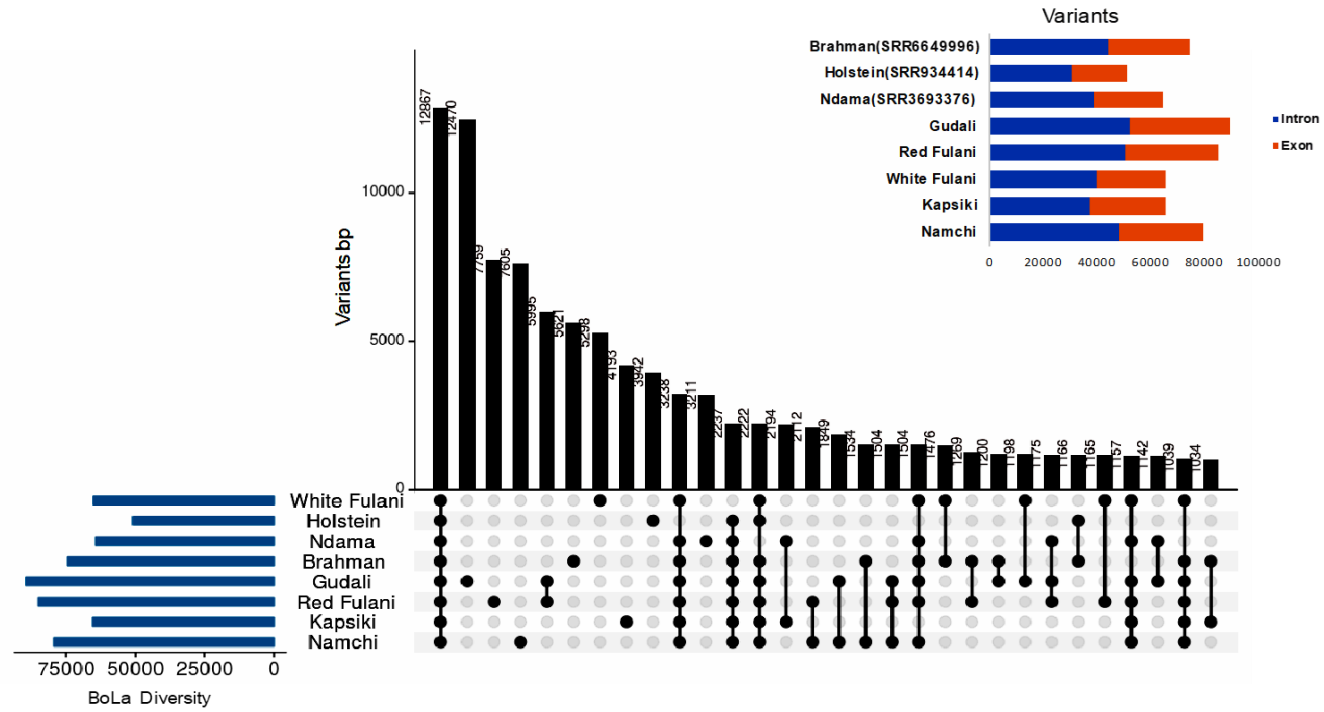
response, GO: 0006954", "antigen processing and presentation of peptide antigen, GO: 0048002").

In Namchi cattle, 4 frameshifts (rs448373338, rs721512537, rs724126999, rs518575055) and one-stop gained (rs208021401) mutation were located in the BTA 1 region associated with Bovine tuberculosis susceptibility QTL (96157) and one variant in BTA 10 (rs524374275) located in the QTLs region associated with tick resistance QTL (101167). Whereas in Gudali cattle one variant (rs516544521) in BTA 11 was located in the Qtls region associated with bovine tuberculosis susceptibility QTL (96344).

4.6.2. Genetic diversity of BoLA (MHC) of different cattle breeds (Unpublished)

The major histocompatibility complex (MHC) includes a set of genes expressing cell-surface glycoproteins that bind pathogen-derived peptides and presents them on the surface of nucleated cells for recognition by specialized T-cells (Takeshima *et al*., 2006; Sette and Sidney, 1999). As a result of pathogen recognition *via* MHC presentation, T-cells and B-cells are activated, expanded, and differentiated into effector cells, which help defend the body against pathogens. One of the key features of the MHC gene region is that it is highly polymorphic (Sette *et al*., 2001; Sidney *et al*., 2016). This high polymorphism allows individuals with diverse MHC genes to bind and recognize peptides derived from numerous different pathogens (Sette and Sidney 1999). BoLA has been associated with health status, vaccine responsiveness, and resistance and susceptibility to a wide range of diseases (Takeshima *et al*., 2006). I retrieved the full-length data of BoLA regions (BTA23: 7,013,913–28,998, 760) from the whole genome sequences of the 8 cattle breeds to test the hypothesis that multi-parasite susceptibility or resistance of cattle breeds is correlated with MHC polymorphism. Such a correlation has been found in sticklebacks *Gasterosteus aculeatus* L. with view to multiple infections with 15 parasite species belonging to ciliata, digenea and cestoda (Wegner *et al*., 2003). The high MHC polymorphism observed in Zebu cattle suggests a strong selection pressure of multi-parasites on Zebu MHC.

A total of 574,932 SNPs was identified from the eight cattle breeds. 234,677 SNPs were located in exon regions and 340,255 SNPs were located in introns (Figure 23). Zebu cattle had a higher number of SNPs as compared to taurine cattle. A high proportion of SNPs (12867) were shared between all cattle breeds. Gudali cattle have higher numbers of specific SNPs (12,470) as compared to White Fulani (5,298 SNPs) and Red Fulani (7,759 SNPs, Figure 23). In taurine cattle breeds, Namchi has a higher number of specific SNPs (7,605) as compared to Kapsiki (4,193 SNPs) and N'Dama (3,211 SNPs). The BoLA polymorphisms arise from point mutations, gene duplication or deletion and intra-locus recombination (Codner *et al*. 2012; Schwartz and Hammond, 2015). I identified 329 genes located in the MHC carrying high impact variants among these 5 cattle genomes. These genes encoded the BoLA classes I, II & III as well as large families of immunological, thermoregulatory and perception genes which include olfactory receptors, zinc-finger genes, tRNA genes, heat shock protein genes, complement factors, lymphocyte genes, interleukin genes, tumour necrosis factor genes and histone genes. BoLA class I and II both play a role in antigen presentation, whereas the function of BoLA class III has been associated with components of the complement system (Ellis and Hammond, 2014). Interestingly the patterns of SNPs in the BoLA were breed-specific. These breed-specific patterns may be linked with the susceptibility and evolutionary divergence.



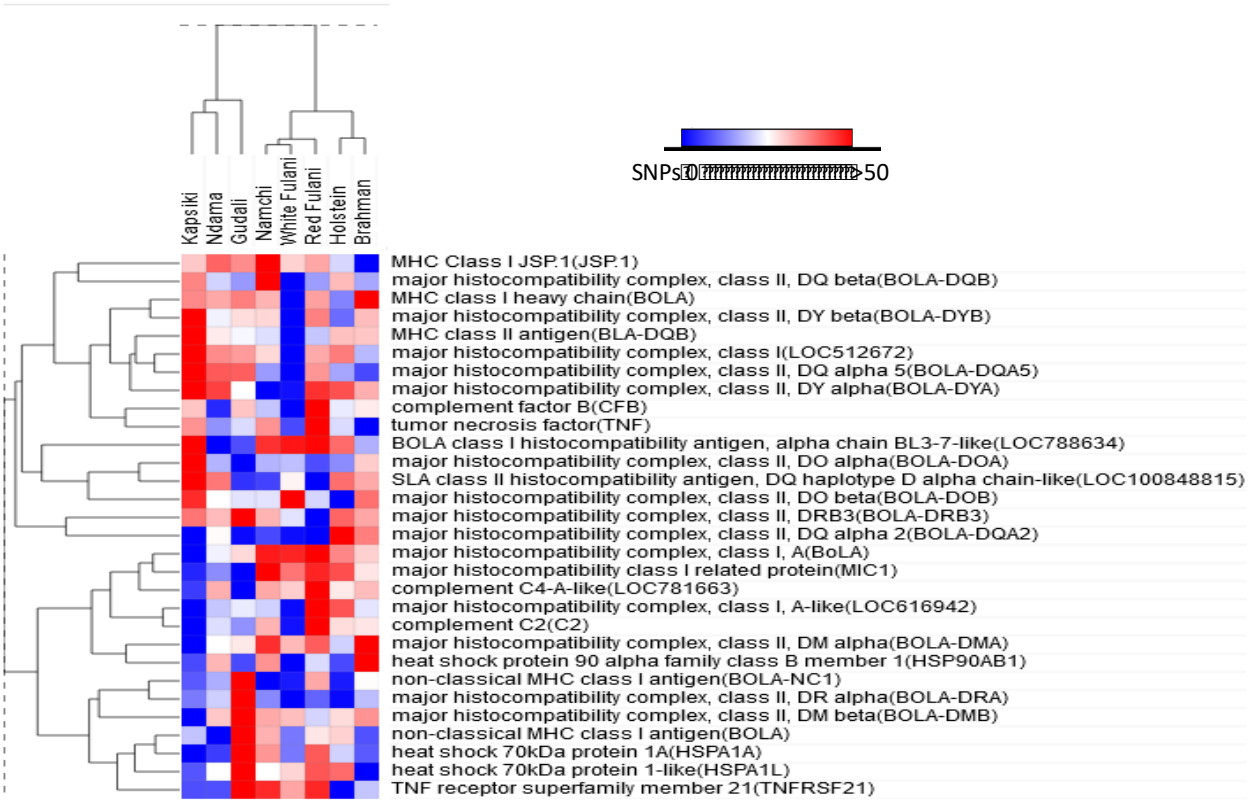


Figure 23. Bovine Leucocyte Antigen Single Nucleotide Polymorphisms (SNPs) Diversity of eight cattle breeds.

On the top right are the upset plots of breed specific and variants shared between different cattle breeds. The numbers are SNP variants per breed. On the top right is a bar plot showing the distribution of exon variants (red) and intron variants (blue) among different cattle breeds. The heat map on the bottom right shows the variants located in different BoLA coding genes and the comparison of cattle breeds relatives.

Blue gradient: one to few variants. **Red gradient:** few to many variants. **White:** 0 variants. Adapted from Paguem et al., unpublished

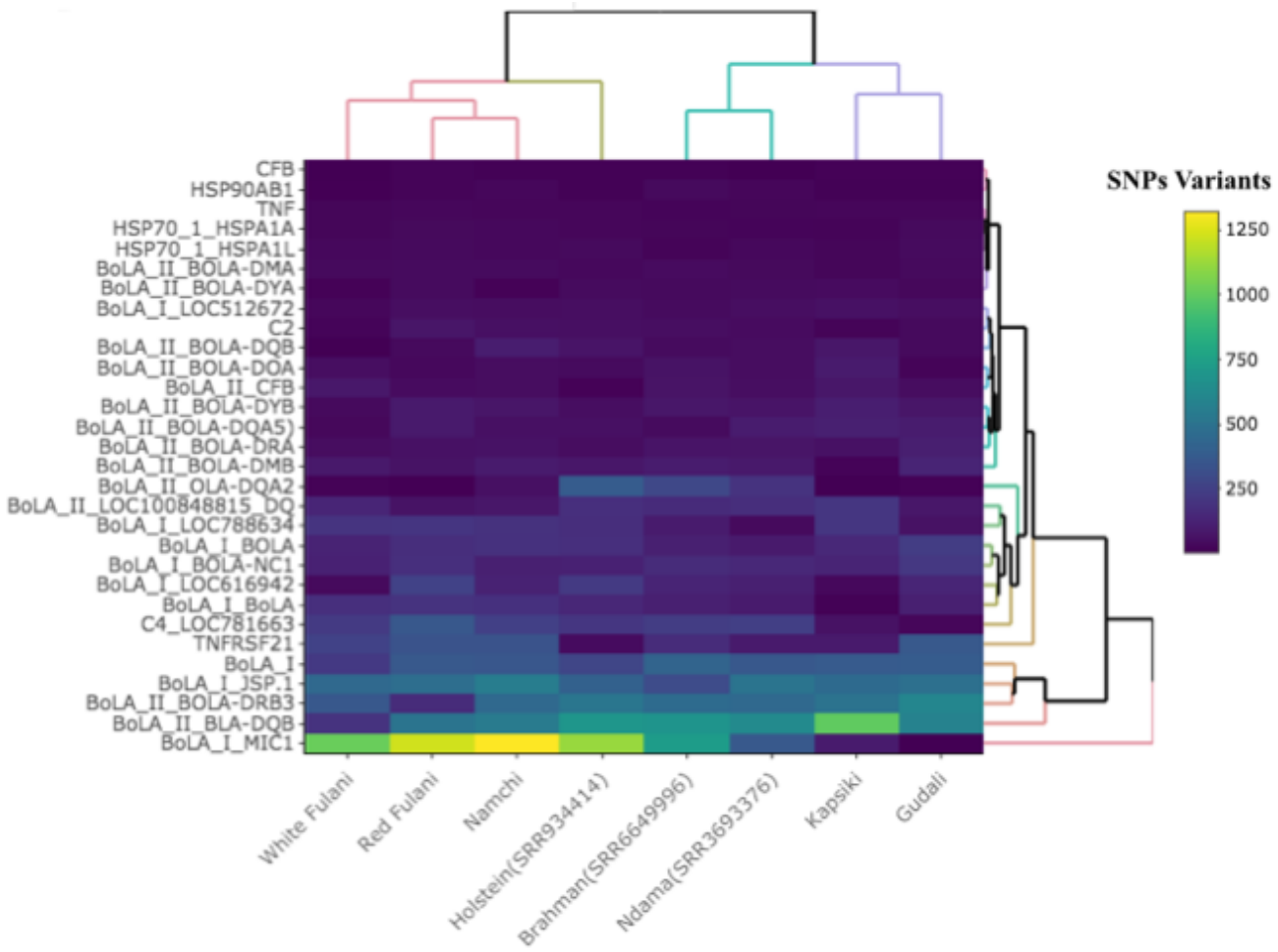


Figure 24. Variant diversity on selected BoLA-I, BoLA-II and BoLA-III.
Heat map plot showing the diversity of SNPs variants on the BoLA class I, II and II across different cattle breeds. Adapted from Paguem *et al*., unpublished.

We further compared the SNPs diversity of 31 genes coding for BoLA class I, II and HSP 90, HSP70, C2, C4 and TNF for Bola class III (Figure 24). The high SNPs diversity was recorded in Doayo (Namchi) and Red Fulani for taurine and Zebu cattle breeds respectively. In class I, out of 9 identified genes, 8 were highly polymorphic and one gene (LOC512672) was less polymorphic (Figure 23 and Figure 24). The most polymorphic gene recorded was the BoLA class I (MIC1). MIC1 encodes for polymorphic class I-like molecules that are stress-inducible by heat shock, infection with viral or bacterial pathogens and malignant transformation. It constitutes one of the ligands of the activation of natural killer cell receptor NKG2D (Brich *et al*., 2008). Doayo (Namchi) cattle harboured the highest number of SNPs (1320) in MIC1 compared to the other cattle breeds. In contrast, Gudali (3 SNPs) and Kapsiki (97 SNPs) genomes were less polymorphic in this gene. Interestingly, in this and previous studies we never reported any case of dermatophilosis (a disease

caused by the Gram-positive bacterium *Dermatophilus congolensis*) and the viral Foot and Mouth disease (FMD) in Namchi cattle. Gudali and other Zebu-related cattle are highly susceptible to these diseases (Maillard *et al*., 2003). Therefore, polymorphism in MIC1 might be involved in resistance. In our DFG-COBE cattle herd, 8 out of 30 animals of Gudali breed died from dermatophilosis proving that the disease is prevalent in the study area.

The BoLA class II cluster comprises three classical class II genes: BoLA-DP, HLA-DQ, and BoLA-DR, each encoding one α and one or two β chains; three non-classical, non-polymorphic class II genes; HLA-DM, HLA-DN, and HLA-DO; and some pseudogenes (Shiina *et al*., 2009). The BoLA-DQB and BoLA-DRB3 genes were the most polymorphic genes. Kapsiki (999 SNPs) and Gudali cattle breeds (607 SNPs) were more polymorphic in BoLA-DQB and BoLA-DRB3 genes. The majority of the MHC variations lies within the peptide-binding region of class I and II molecules (Parham *et al*., 1995). The maintenance and generation of MHC diversity is quite complex; however, it seems the high level of diversity ensures a broad range of immune responses to a variety of different pathogens (Takeshima *et al*., 2006).

4.6.3. Genomic diversity of BoLA-DRB3-genes (Unpublished part)

We retrieved the genomic sequences of BoLA-DRB3 (CH23:25,438,304-25,560,711) corresponding to 52,498616bp from our whole genome sequence data set. BoLA-DRB3 code for two proteins of the MHC II beta domain (74 amino acids) and immunoglobulin domain (Ig domain; 94 amino acids), which are complex multigene families of antigen-binding receptors that function in adaptive immunity. Genetic polymorphisms and the diversity of BoLA-DRB3 are shown in Figure 25. DRB3 genes play a role in resistance (“innate immunity”) and are associated with infectious diseases resistance or tolerance in cattle (Takeshima *et al*., 2006; Maillard *et al*., 2003). 9,728 SNPs were identified from 8 cattle breeds; 426 insertions and deletions (Indels) and 340 SNPs were newly recognized. A higher nucleotide diversity was recorded in taurine as compared to Zebu cattle breeds. 496 SNPs were shared between all cattle breeds. Holstein cattle (235) had a higher number of private SNPs followed by Gudali (163) and Namchi (128). The vast majority of SNPs were located in intergenic (6082) and intron (1648) regions. In exons, almost all the

SNPs were located in the upstream (725) and downstream (846) genes. N'Dama (260 SNPs), Namchi (265 SNPs) and Kapsiki (259 SNPs) cattle had a higher nucleotide diversity in the exon genes than Holstein (246 SNPs) and Brahman (229 SNPs) breeds. Furthermore, we found the highest nucleotide diversity in exon regions in the Gudali cattle breed (295 SNPs). Out of 8 exons identified in the BoLA-DRB3, Exon 2 was the most polymorphic. Exon 2 has been associated with susceptibility and resistance of various pathogens (Takeshima *et al*., 2006; Kaufman, 2018, Takeshima *et al*., 2018; Maillard *et al*., 2003). Exon 2 encodes for a protein of 72 amino acids. 27 missenses and non-synonymous (SNPs) polymorphic sites were identified. In Fulani cattle, all the 27 alleles were homozygote whereas 15 and 12 of the 27 alleles where heterozygote in Gudali and Brahman cattle, respectively. In taurine cattle, N'Dama (18/27) had more heterozygote alleles as compared to Holstein cattle (7/27). Doayo (Namchi) and Kapsiki possessed similar heterozygote allele frequencies (12/27).

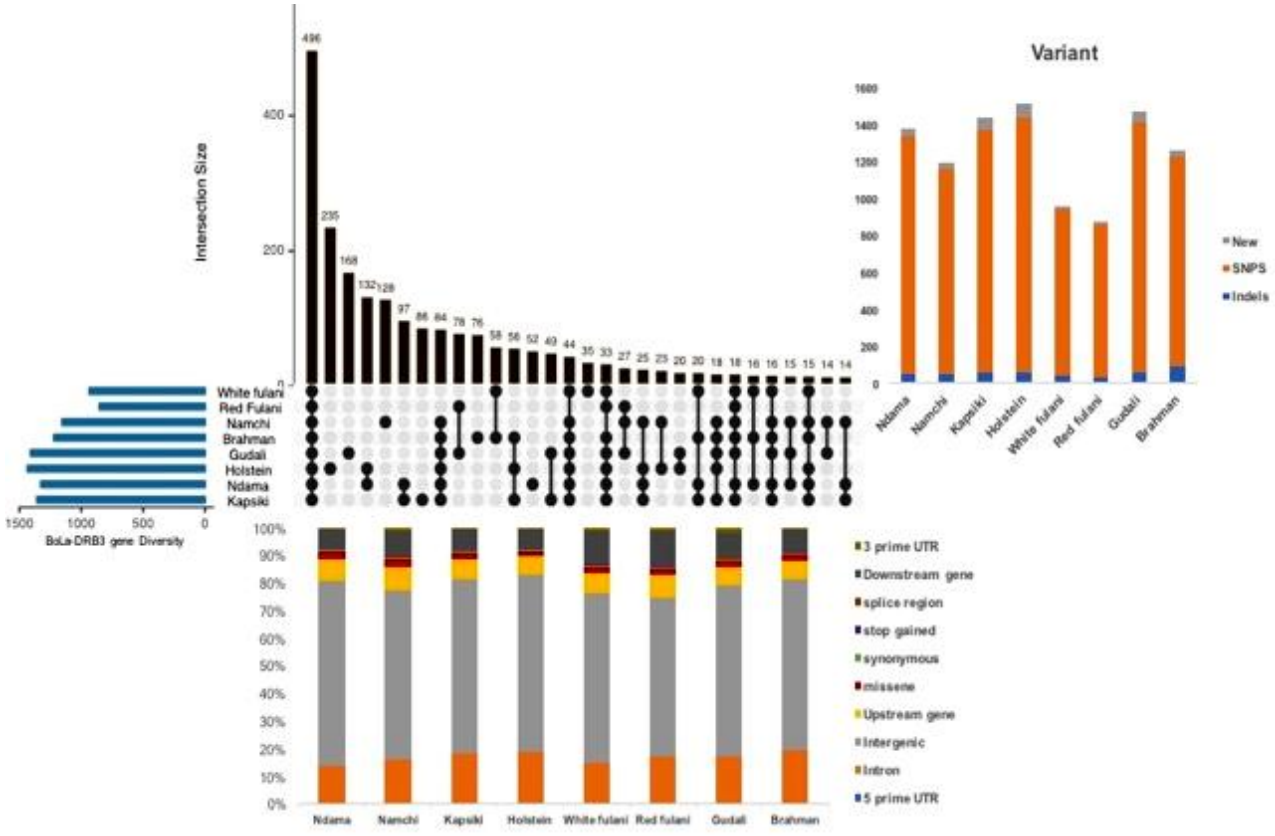


Figure 25. BoLA-DRB3 single nucleotide polymorphism (SNPs) diversity of eight cattle breeds. On the bottom rigth are the upset plot of specific and shared variants between different cattle breeds. On the top left bar plot showing the distribution of new variants (grey), single nucleotide polymorphisms variants (orange) and

insertions and deletions variants (blue) among different cattle breeds. The bottom right bar plot is showing the functional enrichment of variants from different cattle breeds. Adapted from Paguem et al., unpublished.

African taurine cattle (being trypanotolerant) are heterozygous at BoLA-DRB3 loci in contrast to Holstein and Fulani breed. The “heterozygote advantage” hypothesis, which gives an advantage to heterozygote animals by the ability to respond to a greater range of pathogen peptides than homozygotes agrees with our observations (Spurgin and Richardson, 2010; Wegner *et al*., 2003). The excess of heterozygosity is interpreted as a consequence of over dominance (Takeshima et al., 2018), and is further supported by evidence of a positive association between heterozygosity and infectious disease resistance which was observed in humans, laboratory animals, and domestic species. For example, human leukocyte antigen class II heterozygosity is associated with resistance to infections with hepatitis B and hepatitis C virus (Kaufman, 2018). Takeshima *et al*. (2006), showed a significant deviation from Hardy-Weinberg-Equilibrium in the bovine class II BoLA-DQA1 gene in cows with mastitis caused by *Escherichia* or *Streptococcus* bacteria.

5-General Discussion

The aim of this thesis was to investigate the evidence of multiple parasite co-infections in indigenous Cameroonian cattle. A better understanding of the abundance, interaction and community structure of blood, gastro-intestinal and skin dwelling parasite should help to better class the symbiotic nature of such antagonistic or mutualistic multi-parasitism. Subsequently, an attempt was made to identify different factors favoring or limiting these coinfections.

I have shown that coinfection is complex with positive and negative interactions between co-infecting parasites. Furthermore, several factors such as environment, abundance of vectors, seasonal transmission and cattle phenotype (susceptibility or resistance) favor or limit the risk of co-infections. Consequently, the coinfections influence the health parameters of the animals like PCV, weight and body condition in different ways.

5.1. Diversity of parasites infecting cattle

Cattle living in the High Guinea and Sudano-Sahelian savannah carry a high diversity of parasites. More than 90% of the cattle population were infected with at least one parasite. At least 40 parasite species were found co-infecting cattle. Recent research on co-infections in East African shorthorn Zebu calves found more than 50 different parasites, including bacteria and viruses (Bronsvoort *et al*., 2013). Our study was limited to five groups of parasites: trypanosomes, bacteria and piroplasmidae, helminths and protozoan and *Onchocerca* filarial parasites.

We found more than five *Trypanosoma* spp., 15 species of TBDs, 15 species of gut helminths and five species of *Onchocerca* filariae in cattle. Out of the 40 different parasites, nine species or genera were overrepresented in the cattle population with the prevalence found between 40% to 90%. Among these nine, two were trypanosome species (*Trypanosoma theileri* and/ or *Trypanosoma grayi*), two piroplasmidae (*Theileria mutans* and *Theileria velifera*), three gastro-intestinal parasites (*Paramphistomum* spp., *Fasciola gigantica* and *Strongyles*) and two *Onchocerca* filariae (*Onchocerca gutturosa* and *O. ochengi*).

New and unexpected parasite species were discovered, such as *T. grayi* and free-living flagellate *Bodo* spp., *Borrelia theileri* and *Anaplasma platys*. Furthermore, the molecular examination of *Paramphistomum* population revealed the presence of six species (*Calicophoron calicophoron*, *Calicophoron microbothrium*, *Paramphistomum leydeni*, *Cotylophoron macrosphinctris*, *Cotylophoron fuelleborni*, *Cotylophoron* spp.) and the strongyles population were over-represented by *Haemonchus* (*H. placei* and *H. similis*), *Trichostrongylus axei*, *Cooperia* (*C. punctata*, *C. pectinata*/*C. oncophora*) and *Oesophagostomum* spp.

5.2. Co-infections and parasite-parasite associations

At least 40% of cattle population were co-infected with all studied parasites and this rate may reach 60% for T*. theileri* / *T. grayi* and *Th. mutans* / *Th. velifera*. There was evidence of parasite-parasite interactions in our study (see Figure 20). For example, the positive association of *Paramphistomum* and *Fasciola gigantica* and the synergic effect of *Th. mutans* and *Th. velifera* co-infection that presumably protected animals against the pathogenic effects of *Th. parva*. A second example of antagonistic effects is the coinfection of pathogenic trypanosomes (*T. brucei*, *T. vivax* and *T. congolense*) and *Haemonchus,* which increased the risk of animal death. The third example is the antagonistic association of salivaria and stercoraria. A fourth observation of antagonistic association was made between *O. ochengi* filariae (mff and nodules) and *O. armillata* and *O. gutturosa* mff.

These findings provide the first evidence of micro-parasite and macro-parasite interspecific interactions in the fauna in High Guinea savannah and Sudano-Sahelian in Cameroon. These associations can be explained by competition between parasites for their resources and habitat. The mechanism of parasite communication might be by direct contact by secretion of soluble molecules. These molecules can be attached or fixed on the specific receptors of parasite-density regulation and consequently, they can accelerate the development of the second parasite and increase their population or induce cell apoptosis which may result in the death of parasites and reduce the population density.

Another mechanism of parasite communication is through modulation of host-immunity. In this scenario, parasites divert the immune system of the host to fight against its competitor and protect their territory.

Multiple infections are restricted or favoured by several factors such as tropical climate, the abundance of vectors and parasites and hosts' genetic background.

5.3. Facilitating and limiting factors of multi-parasitism

Several environmental factors like climatic condition, seasonality (dry and rain) and temperature in High Guinea savannah and Sudano-Sahelian favour the development of ticks, mosquitos, snails, and tsetse flies, which are vectors of many parasites. The soil moisture additionally favours the development and transmission of helminths. Another factor is the effect of the host genotype. Young animals were more infected with multiple parasites with noticeable negative effect on their PCV and body condition. Doayo (Namchi) were less infected with all kind of parasites than the other taurine breed Kapsiki and all Zebu breeds.

Several alleles and mutations in the BoLA-DRB3 genes were associated with resistance against multiple infections. In addition, I found four major mutations in the genome of Namchi. These mutations were located in the chromosome 1 regions associated with bovine tuberculosis susceptibility and in the chromosome 10 region associated with tick resistance.

5.4. The concept of host territorial defense by parasites

The ability of endemic parasitic fauna of indigenous and /or invasive species to protect their habitat have been intensively study (review by Poulin *et al*., 2011).

In this study, we find 90% individuals infected with bacterial and piroplasmidae parasites transmitted by hard ticks. We however did not find any evidence of pathology. This suggests a stable relationship balance between bacteria and piroplasmidae on one side and the cattle host on the other site (endemic stability). This observation can be extended to a certain level to other parasites.

The adaptation of indigenous African cattle to local prevailing parasites allows them to survive and reproduce under this harsh environment. However, several attempts to introduce European exotic cattle breeds in order to improve meat and milk product have dramatically failed. This was due to all the introduced animals rapidly succumbing due to parasite infections. The second example of such phenomena is the Human African Trypanosomiasis caused by *T. brucei gambiense* and *T. brucei rhodesiense.* Wildlife and livestock are infested but they do not develop any clinical signs of the diseases and therefore act as animal reservoirs. Human or livestock are getting infected when they enter the national park or wildlife habitat and suffer from disease.

5.5. Practical implications

The results of my thesis have practical implications to livestock health campaigns. The data generated and analyzed in this thesis can be used to design and efficiently manage infectious diseases in cattle by prioritizing treatments of harmful parasites, and, at the same time, avoiding to disturb the endemic stability of parasites. In addition, our data provided a broad overview of the epizootiology of major parasitic disease in in High Guinea and Sudano-Sahelian savannah regions.

6- Conclusions

These findings provide, by the use of modern molecular tools, the first evidence of the high diversity of the micro- and macroparasite fauna in High Guinea and Sudano-Sahelian savannah of Central Africa. These multiple infections are favoured by the tropical climate, the abundance of vectors, the poor sanitation and the host genetic background. The increases of the temperature due to climate change may favour the increased of the abundances of vectors like mosquitos and ticks and the emergence and re-emergence of vector borne diseases like *Zika virus* and Rift Valley fever (RVF).

Parasite-parasite interactions occur either within their host or inside the vectors or both. These parasite interactions might either be symbiotic like infections with *Theileria mutans* and *Theileria velifer*a or thereby help to protect animals against the pathogenic effect of *Theileria parva*. In contrast, they can be antagonistic by increasing the virulence of parasites such as *Trypanosoma* spp. and *H. placei*.

Our studies have shown that multiple parasite co-infections have to be taken into consideration during epidemiological studies rather than focussing on single pathogens. The present data provide the basis to strategically design and implement a new approach of parasite management in livestock and game animals. Molecular tools help to identify those parasites, which maintain enzootic stability and premunition at a very low prevalence. Co-infections must be detected in order to reduce the burden of those parasite co-infections that are negatively associated with animal health, production, and reproduction.

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8-Appendix

1. accepted
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RESEARCH ARTICLE Open Access

Widespread co-endemicity of *Trypanosoma* species infecting cattle in the Sudano-Sahelian and Guinea Savannah zones of Cameroon

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Abstract

Background: African animal trypanosomosis remains the major constraint of livestock production and livelihood of pastoral communities in Cameroon. Despite several decades of vector and parasite control efforts, it has not been eradicated. Alternative and sustainable control strategies require a sound knowledge of the local species, strains and vectors. In the Sudano-Sahelian and Guinea Savannah of Cameroon the prevalence and genetic diversity of trypanosomes infecting cattle was investigated by microscopy of cattle blood buffy coat and molecular methods using generic primers targeting parts of the internal transcribed spacer 1 (ITS-1) and encoded glycosomal glyceraldehyde 3-phosphate dehydrogenase-gene (gGAPDH).

Results: A total of 1176 randomly chosen cattle from five divisions in the Sudano-Sahelian and Guinea Savannah of Cameroon were examined. The overall prevalence of trypanosomes by microscopy was 5.9% (56/953) in contrast to 53.2% (626/1176) when molecular tools were used. This indicated a limited sensitivity of microscopy in subclinical infections with frequently low parasitemia. Three trypanosome species were identified by light microscopy: *T. vivax* (2.3%), *T. brucei* (3.7%) and *T. congolense* (3.0%), whereas five were identified by PCR, namely *T. grayi/T. theileri* (30.8%), *T. vivax* (17.7%), *T. brucei* (14.5%) and *T. congolense* (5.1%). Unexpected cases of *T. grayi* ($n = 4$) and *T. theileri* ($n = 26$) were confirmed by sequencing. Phylogenetic analysis of the gGAPDH revealed the presence of *T. vivax,* clade A and *T. vivax* clade C, which were co-endemic in the Faro et Deo division.
T. grayi/T. theileri were the predominant species infecting cattle in tsetse free areas. In contrast, *T. vivax*, *T. brucei* and *T. congolense* were more abundant in areas where the *Glossina*-vectors were present.

Conclusions: The abundance of pathogenic trypanosomes in tsetse infested areas is alarming and even more, the occurrence of *T. vivax*, *T. brucei*, *T. congolense*, *T. theileri* and *T. grayi* in tsetse-free areas implies that tsetse control alone is not sufficient to control trypanosomosis in livestock. To implement control measures that reduce the risk of spread in tsetse free areas, close monitoring using molecular tools and a thorough search for alternative vectors of trypanosomes is recommended.

Keywords: Trypanosomosis, ITS-1, gGAPDH, *T. grayi*, *T. Theileri*, Co-endemic trypanosomes, Cameroon

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Background

In tropical Africa and South America, hemoparasitic flagellates of the genus *Trypanosoma* cause severe to fatal diseases in wild and domestic mammals, including the human host. Trypanosomes infecting mammals are divided into two major families: *Salivaria* and *Stercoraria* [1]. Members of the *Salivaria* include human and veterinary medically important pathogens *Trypanosoma vivax, T. congolense* and *T. brucei* spp. They develop as mammalian infective forms in the mouthparts, e.g. proboscis and salivary glands of tsetse (*Glossina* spp.). Transmission to the vertebrate host occurs during the blood meal of an infective tsetse [2]. In contrast, the *Stercoraria* comprise the South American parasite *T. cruzi* and the worldwide-distributed *Megatrypanum,* e.g. *T. theileri,* where the final stages of the parasite develop in the posterior digestive tract of the arthropod vectors. These species are transmitted by contamination of the bite puncture with infectious excreta from the vector [3]. Trypanosomes can also be transmitted by mechanical vectors, like tabanid and stomoxine horse flies and by hard-ticks [4, 5].

In Cameroon, 90% of the population of the estimated six million cattle are at risk of trypanosome infection [6]. The Adamawa highland plateau in North Cameroon is the country's main area of cattle rearing supplying animal products to all neighboring countries. This was made possible through the control of tsetse on this up to 1000 m high plateau [7], whilst *Glossina morsitans*, *G. fuscipes fuscipes* and *G. tachinoides* still occur in high numbers in the savannah pastures of the Eastern and Northern regions making cattle rearing problematic [7]. However, conventional operations employed over the last three decades have not eradicated the *Glossina* vectors so that pasture lands previously cleared and declared free of *Glossina* have recently been re-invaded [7, 8]. Disease control in these areas depended on continuing diagnosis and treatment of suspected cases with the few trypanocidal drugs available on the market [9]. Isometamidium, diminazene and homidium bromide are the only drugs widely used during more than four decades for trypanosome control. Furthermore, there are reports of drug resistance coming from North Cameroon [10] and elsewhere [11, 12]. Therefore, the unequivocal identification of the prevailing trypanosome species and strains has gotten more attention to prevent unnecessary treatment of non-pathogenic parasites and thereby promoting the development of resistance.

In Northern Cameroon, little is yet known about the genetic diversity of trypanosomes infecting cattle. Most epizootiological data available were based on microscopy, such as phase-contrast or dark field examination of the buffy coat, thin or thick blood smears, and to a lesser extent also serological analyses [13–15]. These investigations indicated *T. congolense*, *T. brucei* and *T. vivax* as the only prevalent species in these areas [7–10]. Microscopy, albeit easy to perform in a fieldwork setting, needs a high investment in time and training, risks to misinterpret rare, emerging or in other ways unexpected specimens and fails to detect immature infections during the first stages of infection [16]. Advances in molecular biology have expanded the limits of the traditional methods in sensitivity and specificity. Generic and specific primers have been designed to amplify the internal transcribed spacer 1 (ITS-1) region of the ribosomal RNA gene locus of trypanosomes, chosen because of its high copy number and inter-species length variation [17–19]. Thus, trypanosome species are recognizable by the fragment length of their PCR-amplified ITS-1 region [17]. This method has evolved to improve sensitivity and detection of trypanosomes in animal blood [18–20]. In addition, the glycosomal glyceraldehyde 3-phosphate dehydrogenase gene (gGAPDH), an ubiquitous and essential glycolytic enzyme, has been used for the species differentiation of trypanosomes because of its lower rate of molecular evolution [21]. Despite the fact that it has no band size separation among different *Trypanosoma* species, it has been a marker of choice for phylogenetic analysis [22, 23].

A recent study in two restricted areas in Northern Cameroon relying on molecular tools for parasite detection [24] revealed active foci of AAT on the Adamawa region in the Faro et Deo close to the border with Nigeria and in the North region near the town of Gamba. The results revealed the crucial need of molecular tools to monitor the diversity of trypanosomes together with their vectors in hyper-endemic foci. A higher diversity of trypanosomes was seen in cattle and tsetse vectors than previously known. Those observations were however based only on a few *Glossina*-infested localities and on less than four hundred cattle examined. Therefore, this study has investigated the epizootiological picture of bovine trypanosomosis in the northern regions of Cameroon comparing tsetse infested areas in the high Guinea savannah and the Sudano-Sahelian zone with areas cleared of tsetse in both agro-ecological zones. Furthermore, the different susceptibilities of the various indigenous cattle breeds found in these zones have been addressed.

Gudali, White Fulani and Red Fulani are the major local zebu cattle breeds [25]. They are claimed to be more susceptible to trypanosomiasis than the autochthonous taurine cattle breed called Namchi (Doayo) [26], which nevertheless is at high risk of becoming extinct [27]. Only few located herds of Doayo cattle remain in the Faro division. The Kapsiki, another taurine cattle breed, with a higher introgression of Zebu genes, found mainly in the Mayo Tsanaga (Rhumsiki) area of the Far North region and also being on the verge of

becoming extinct, were earlier shown to be trypanosusceptible [26].

The present research used both microscopy and molecular methods to study the occurrence and genetic diversity of trypanosomes in cattle from two agro-ecological zones (AEZ), focusing on areas with and without tsetse vectors [28].

Results

Body condition and packed cell volume in relation to breed and age

A total of 1176 animals were randomly sampled. These comprised more female (907; 77.1%) than male (269; 22.9%). Examined animals were from five divisions as follows: Vina ($n = 283$), Faro et Deo ($n = 196$), Mayo Rey ($n = 316$), Faro ($n = 176$) and Mayo-Tsanaga ($n = 205$). In the Faro and Mayo-Tsanaga divisions, only the indigenous taurine breeds, Namchi (Doayo) and Kapsiki, respectively, were examined. Here, the mean PCV of Namchi (Doayo) was significantly higher ($F = 13.88$; $P < 0.001$) than that of Kapsiki (Fig. 1a, Additional file 3: Table S1). Overall, animals with poor body condition had PCVs (average 29.66 ± 6.68) significantly lower ($F = 22.062$, $P < 0.001$) than that of animals in good (32.82 ± 4.99) and very good (34.26 ± 5.46) condition (Fig. 1b). Young cattle aged between 0 to 2.5 years had significantly lower PCVs (31.22 ± 6.82) than the other age groups ($F = 5.38$, $P = 0.005$, Fig. 1c, Additional file 3: Table S2). 97.6% of the Kapsiki cattle had the best body condition score (4 or higher, Additional file 3: Table S1) as compared to those of other cattle breeds. Comparing the different regions, animals in the Faro et Deo division had a mean PCV (28,13 ± 5.76) that was significantly lower ($F = 49.13$, $P < 0.001$) than those found in the Faro division (34.74 ± 5.35; Additional file 3: Table S2).

Parasitological and molecular detection of trypanosomes

Microscopic detection of motile trypanosomes showed that 56 blood samples (5.9% of 971 cattle) carried at least one trypanosome species (Table 1). The highest prevalence was recorded in Faro et Deo (15.8%), followed by Faro (4.5%), Mayo- Rey (3.5%) and Vina (2.3%). In the Mayo-Tsanaga region no microscopy was carried out due to insecure work environment.

The most frequently identified trypanosome species was *T. brucei* spp., followed by *T. vivax* and *T. congolense* (Table 1). However, 7.1% of trypanosomes were not clearly identified according to their motility and morphological characteristics and were recorded as *T. brucei*-like trypanosome species.

In contrast, out of 1176 samples examined by ITS-1 nested PCR, 626 samples showed the presence of one or more trypanosome species, giving an overall prevalence of 53.2% (Table 2). The highest prevalence was recorded in Mayo-Tsanaga (67.8%), followed by Faro et Deo (59.2%) and lowest in Faro (34.1%). From the 56 samples classed positive by microscopy, 41 (71.9%) were also detected by nested PCR (Table 3).

ITS-1 sequences analysis

Samples were identified according to ITS-1 amplicon size as described previously [19, 24] (Table 4). Three representative samples with a product size of 426 bp considered to be *T. brucei* spp. were sequenced and the results aligned to sequences retrieved from databases searches. The results showed that all sequences belonged to *Trypanozoon*, either to *T. brucei* spp. or *T. evansi*. They differ only in their maxi-circles DNA and additional species-specific markers are needed to distinguish these species. Additionally, six PCR amplicons in the range of 645 bp and considered to be *T. congolense* savannah or forest types were sequenced and showed a similarity of 73 to 85% with *T. congolense* isolates from South Africa and Gabon, respectively [GenBank: KX870079, KX452163].

Interestingly, the PCR products of 180 bp and 250 bp ($n = 6$) both corresponded to *T. vivax* sequences isolated from Ethiopia ([GenBank: KM391818, KM391825], 91 to 93% identical). For PCR products in the range of 320 bp, out of 30 samples analyzed, 26 (87%) corresponded to *T. theileri* sequences published in Genbank (98 to 100% identical). The other four sequences (13%) matched with entries of *T. grayi* (90 to 96% identical) with closest similarity to *T. grayi* ANR4 isolated from a tsetse in The Gambia [TriTrypDB: JMRU01000589] and 94 to 99% identical with sequences [NCBI Blastn: MG255201, MG255205] obtained from cattle and tsetse in North Cameroon in Gamba and Kontcha, respectively [24].

Genetic diversity of trypanosome species

In total, five different trypanosomes were identified: *T. congolense, T. brucei* spp., *T. theileri, T. grayi* and *T. vivax*, respectively, using ITS-1 makers and sequencing analysis (Table 4). Due to the inability to discriminate between *T. theileri* and *T. grayi* just on the basis of the ITS1 amplicon size, samples with amplicons in the range of 320 bp were considered as *T. theileri/T. grayi*. Molecular analysis showed these to be the most prevalent species in all five study areas (30.8%, $n = 362/1176$). *T. theileri/T. grayi* was also the species most often missed or misidentified for *T. brucei* or *T. congolense* by microscopic observation, followed by *T. vivax* (Tables 1, 2 and 3). The overall prevalence of mixed infections was 11.4% ($n = 134/1176$). Co-infections of *T. brucei* spp. and *T. vivax* were the most common ($n = 91/1176$), followed by triple infections with *T. congolense*, *T. vivax* and *T. theileri/T. grayi* ($n = 23/1176$). We found eight animals co-infected by *T. brucei* spp., *T. vivax* and *T. congolense*

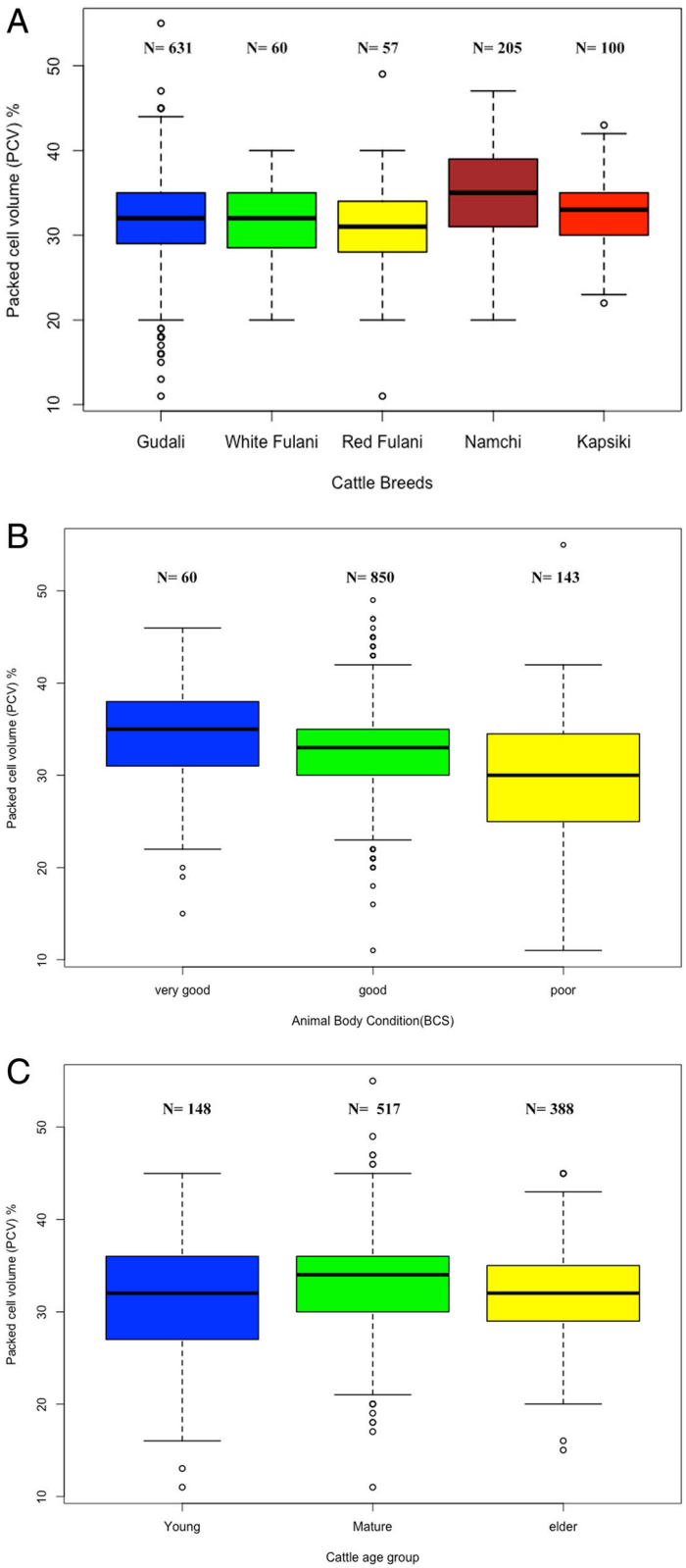

Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 Effect of cattle breed on packed cell volume (**a**). Comparison of the mean of PCV of five indigenous cattle breeds examined. Effect of body condition score on packed cell volume (**b**). Animals were grouped as described in the section "Materials and Methods" without breed distinction and the PCVs were compared. Effect of age group on body condition score (**c**). Animal were grouped by age as described in in the section "Materials and Methods" and PCV was compared. Details of sample collections and processing are indicated in the section "Materials and Methods"

savannah/forest-type, and four animals co-infected by *T. brucei* spp., *T. vivax, T. congolense* savannah/forest and *T. theileri/T. grayi* (Table 2).

The effect of study site, breed and age on the prevalence of trypanosomosis and correlation with the body condition score

Doayo (Namchi) cattle from Faro were significantly less infected (34.6%; $X^2 = 51.78$, $p < 0.000$) with any trypanosome species than the other taurine cattle Kapsiki (67.8%) and the Zebu breeds Gudali (54.1%), Red Fulani (58.1%) and White Fulani (54.1%). There was also a significant difference between the five sampled study sites. The overall trypanosome infection rate was higher in Mayo-Tsanaga (67.8%) than in Faro et Deo (59.2%). However, 56.2% of the infected animals in Mayo-Tsanaga were infected with *T. theileri/T. grayi*, compared to only 7.5% in Faro et Deo. In contrast, when looking only at the species classically considered to be pathogenic such as *T. congolense*, *T. brucei* spp. and *T. vivax,* these were most prevalent in Faro et Deo (44.9%), followed by Mayo-Rey (42.7%), Faro (15.3%) and Mayo-Tsanaga (11.7%). The area with lowest prevalence was Vina (7.8%) (Table 5), a former tsetse-cleared area.

Comparison of areas with or without *Glossina*-vectors

The overall prevalence of trypanosomes was similar or even higher in the tsetse free areas (Vina 53.7% and Mayo Tsanaga 67.8%, Tables 2 and 5) than in the *Glossina*-infested zones (Mayo-Rey 50.3%, Faro et Deo 59.2% and Faro 34.1%). *T. theileri/T. grayi* were the most abundant trypanosome species in the tsetse-free zones. In contrast, in the *Glossina*–infested areas *T. vivax*, *T. brucei* and *T. congolense* were the predominant species (Table 2).

Some *T. congolense, T. brucei and T. vivax* cases were even detected in the areas of Vina and Mayo-Tsanaga, although these areas have been declared tsetse-free (Table 2).

Phylogenetic analysis of gGAPDH

Two main clusters were observed in the 37 gGAPDH sequences examined, comprising the stercorarian *T. grayi* and *T. theileri* on the one hand, and the salivarian *T. congolense*, *T. brucei brucei* and *T. vivax* on the other (Fig. 2). Interestingly, two clades of *T. theileri* were observed (IIB and IA/IB) as previously described [29]. Furthermore, the occurrence of two lineages was also observed in the main group of *T. vivax*, cluster C and cluster A [30]. Cluster C had previously been reported in various regions in Africa and America, while cluster A was described only in Tanzania [FM164789; FM164787]. *T. vivax* C and A were found co-infecting cattle in the Faro et Deo region.

Correlation of packed cell volume with infection status

Animals with single or mixed infections had lower PCV values when compared to those with no infection (Fig. 3). When comparing the mean PCV with the type of infection, animals with single-infections of *T. vivax* (31.68 ± 5.40) and *T. congolense* (31.29 ± 6.92) showed no significant differences from uninfected. Animals carrying *T. theileri* had a mean PCV of 31.9 ± 4.5 ($n = 16$) for clade IIB while that for clade IA and IB it was 35.8 ± 3.4 ($n = 8$) (Additional file 3: Table S3). The observed difference was close to significance ($F = 2.043$, $p = 0.056$). Animal infected with *T. grayi* had the lowest PCV (29 ± 5.5, $N = 4$) of all the groups. However, because of the small sample size of the *T. grayi* group we could not test for statistical significance.

Table 1 Distribution of trypanosome species detected by microscopy in the study area

		Trypanosome species									
Sites	N	Negatives	*Tb*	*Tb*-like	*Tv*	*Tc*	*Tc* + *Tv*	*Tc* + *Tb*	*Tv* + *Tb*	*Tc* + *Tb* + *Tv*	*T. spp.* prevalence (%)
Vina	265	259	4	2	0	0	0	0	0	0	2.3
Faro et Deo	196	165	7	0	2	4	5	7	1	5	15.8
Mayo-Rey	316	305	1	0	5	2	1	1	0	1	3.5
Faro	176	168	1	2	2	0	0	3	0	0	4.5
Total	953	897	13	4	9	6	6	11	1	6	5.9

Tb: *T. brucei*, *Tb*-like: *T. brucei*-like, *Tc*: *T. congolense*, *Tv*: *T. vivax*. Animals from Mayo Tsanaga area were not considered because microscopy data collection was not carried out at this location and one positive animal suspected to be hybrid was not included in this table

Table 2 Distribution of trypanosome species detected by ITS-1 PCR in the study areas

Sites	N	Trypanosome species												
		Negatives	*Tb*	*Tv*	*Tc*	*Tth*	*Tc* + *Tv*	*Tc* + *Tb*	*Tb* + *Tv*	*Tc* + *Tb* + *Tv*	*Tc* + *Tb* + *Tth*	*Tc* + *Tth* + *Tv*	*Tc* + *Tb* + *Tth* + *Tv*	prevalence (%)
Vina	283	131	8	5	0	130	0	0	5	0	0	4	0	53.7
Faro et Deo	196	80	4	29	6	28	2	2	32	3	0	7	3	59.2
Mayo-Rey	316	157	36	39	6	28	2	0	42	4	0	1	1	50.3
Faro	176	116	8	4	1	33	1	1	8	0	1	3	0	34.1
Mayo Tsanaga	205	66	7	1	4	115	0	0	4	0	0	8	0	67.8
Total	1176	550	63	78	17	334	5	3	91	7	1	23	4	53.2

Tb: *T. brucei*, *Tc*: *T. congolense*, *Tth*: *T. theileri* / *T. grayi*, *Tv*: *T. vivax*

Discussion

The present study was carried out to determine the prevailing species and genetic diversity of trypanosomes infecting cattle in five divisions located in two agro-ecological zones of northern Cameroon, using both microscopy and molecular methods. The overall prevalence using microscopy is in agreement with previously reported prevalences of 3.7 to 20%, which were also determined by microscopy only [10]. However, infection rates determined by molecular analysis with ITS-1 nested PCR (53.2%) were much higher. This underpins the difficulty of microscopy to detect parasites at low levels of parasitemia in subclinical infections.

On the other hand, out of 56 trypanosome-positive cases by microscopy, only 41were detected by nested PCR giving the concordance rate of 73.2% between both techniques. This discrepancy has already been reported by Takeet et al. [31] and Adams et al. [19], the latter developing the primers used in our study. They also failed to amplify 56% of samples previously detected positive by microscopy and attributed this failure to the quality and quantity of the extracted parasite DNA. It is also possible that the primers do not amplify all trypanosome parasites [32, 33] or that *Borrelia* bacteria present in the blood are misinterpreted as trypanosomes, since based on their shape, size and movement, under the microscope they appear similar to *T. brucei* in buffy coat slide preparations [34]. Actually, recent molecular studies showed that 17.7% of cattle in the northern Cameroon are infected with *Borrelia theileri* (B. Abanda, A. Paguem, M. Abdoulmoumini, TK. Manchang, A. Renz and A. Eisenbarth. personal communications).

We distinguished only three species of trypanosomes by microscopy, namely *T. congolense, T. vivax* and *T. brucei* spp., while others, which we named *T. brucei*-like, could not be identified beyond doubt. By using PCR, we were able to identify five species of trypanosomes in the study area. This can be explained by the high sensitivity of the generic primers (ITS-1), which can detect traces of DNA up to one parasite per mL of blood of both pathogenic and non-pathogenic species [17, 18]. In contrast, microscopy of the Buffy-coat extracted from a microcapillary tube can reliably detect motile parasites only at a concentration being higher than 1.25×10^3 parasites/mL of blood [15–17]. Such high parasite titers in blood are more typical for trypanosomes causing pathology, like *T. brucei* spp., *T. congolense* and *T. vivax* at the acute clinical stage, and chronic infections are likely to be missed.

Table 3 Comparison of the diagnostic test results obtained by parasite microscopy and molecular (ITS-1 PCR) methods

	PCR									Total	*T. spp.*
	Tb	*Tv*	*Tc*	*Tth*	*Tc* + *Tv*	*Tb* + *Tv*	*Tth* + *Tv*	*Tc* + *Tb* + *Tv*	Negatives		overlap (%)
Tb	1	1	0	6	0	0	1	0	7	16	43.8
Tv	0	3	0	3	0	2	0	0	1	9	88.9
Tc	0	0	2	1	0	0	0	0	3	6	50.0
Tc + *Tv*	1	1	1	0	0	0	3	0	0	6	100.0
Tc + *Tb*	1	3	1	2	0	0	1	0	3	11	72.7
Tc + *Tb* + *Tv*	0	1	1	1	0	0	2	0	1	6	83.3
Tv + *Tb*	0	0	0	0	0	1	0	0	0	1	100.0
Negatives	47	72	8	206	3	14	64	3	468	885	47.1
Total	50	81	13	219	3	17	71	3	483	940	48.6

Tb: *T. brucei*, *Tc*: *T. congolense*, *Tth*: *T. theileri* / *T. grayi*, *Tv*: *T. vivax*. *T. spp*: *T. all species* Animals from Mayo Tsanaga region were not considered because microscopy was not carried out at this location. Only the animals with parasitological and molecular data were considered

Table 4 Trypanosome ITS-1 amplicon sizes of different *Trypanosoma* spp.

Trypanosoma species	Amplicon size (bp)
T. congolense savannah[a]	640
T. congolense forest[a]	**640**
T. congolense kilifi	562
T. brucei brucei[a]	**426**
T. brucei rhodesiense	426
T. brucei gambiense	426
T. evansi[a]	**426**
T. vivax[a]	**180 and 250**
T. theileri[a]	**320**
T. grayi[b]	**318**

The bold lettered species were found in this study
[a]source: Adams et al. [19]
[b]Ngomtcho et al. [24]

Surprisingly, the stercorarian parasites *T. theileri/T. grayi* were the most prevalent species (30.5%) in our study. These two parasites cannot be distinguished by ITS-1 size estimation, but only by sequence analysis, because they have a similar band size of 320 bp on the gel. Four out of 30 samples analyzed by sequencing were identified as *T. grayi* whereas the other 26 were *T. theileri*.

Trypanosoma grayi was found in two of 7 cattle from Mayo-Tsanaga and in one of 6 from Vina and yet another one from 12 cattle examined at Mayo-Rey. Previously, this species was known only to be a parasite of reptiles [1]. However, recently this parasite has been detected in a White Fulani cattle in Faro et Deo [24] and has now also been found in Kapsiki and Gudali cattle. By extrapolation on our 358 *T. theileri*/*T. grayi* cases we could expect almost 50 animals to be infected with *T. grayi*. This observation raises concerns whether these parasites may represent a strain undergoing a change of host range [24]. Further investigations are essential to characterize those *T. grayi* strains and evaluate their pathogenic potential for cattle and/or other livestock. In our study areas animals infected with this parasite correlated with lower PCV which may be an indicator of potential pathogenic effects on animal health. In this context, it is noteworthy that a recent study in Nigeria has observed a high frequency of tsetse colonised with *T. grayi*-like parasites (J. Weber. personal communication). Furthermore, these parasites revealed a high genetic diversity suggesting a dynamic evolution in this region. The 320 bp amplicon representative for the stercorarian parasites of *T. theileri/T. grayi* was most prevalent in the tsetse-free Vina (47.3%) and Mayo-Tsanaga (60.0%) regions and much less frequent in the tsetse-infested areas Faro (21.0%), Faro et Deo (19.4%) and

Table 5 Effect of age, breed, study areas and body condition score on trypanosome prevalence

Factors	Prevalence by PCR		X^2	*P*-value	Prevalence by PCR	X^2	*P*-value
	N	overall (%)			Pathogenic (Tc + Tv + Tb) (%)		
Age							
Young (0–2.5)	171	92 (53.8)			54 (31.6)		
Mature (3–5)	574	332 (57.8)	11.93	0.003	159 (27.7)*	13.68	0.001
Old (6–12)	431	202 (46.9)*			83 (19.3)*		
Body condition							
Poor (0–2)	148	80 (54.1)			52 (35.1)		
Good (3–4)	967	516 (53.4)	0.449	0.799	220 (22.8)*	17.31	0.000
Very good (5)	61	30 (49.2)			24 (39.3)		
PCV							
PCV < 25	109	64 (58.7)	1.931	0.165	49 (45.0)	18.476	0.000
PCV > 25	944	488 (51.7)			241 (25.5)*		
Sex							
Male	283	147 (22.4)			84 (29.7)	5.76	0.018
Female	968	510 (77.6)	0.048	0.439	220 (22.7)*		
Breed							
Gudali	649	351 (54.1)			185 (28.5)*		
White Fulani	60	35 (58.3)			30 (50.0)		
Red Fulani	57	30 (52.6)	46.79	0.000	23 (40.4)*	58.19	0.000
Namchi (Doayo)	205	71 (34.6)*			34 (16.6)*		
Kapsiki	205	139 (67.8)*			24 (11.7)*		
Areas							
Vina	283	152 (53.7)*			22 (7.8)*		
Faro et Deo	196	116 (59.2)			88 (44.9)		
Mayo Rey	316	159 (50.3)*	47.28	0.000	135 (42.7)	166.41	0.000
Faro	176	60 (34.1)*			27 (15.3)*		
Mayo Tsanaga	205	139 (67.8)			24 (11.7)*		

Symbols: (*) indicates difference between variables

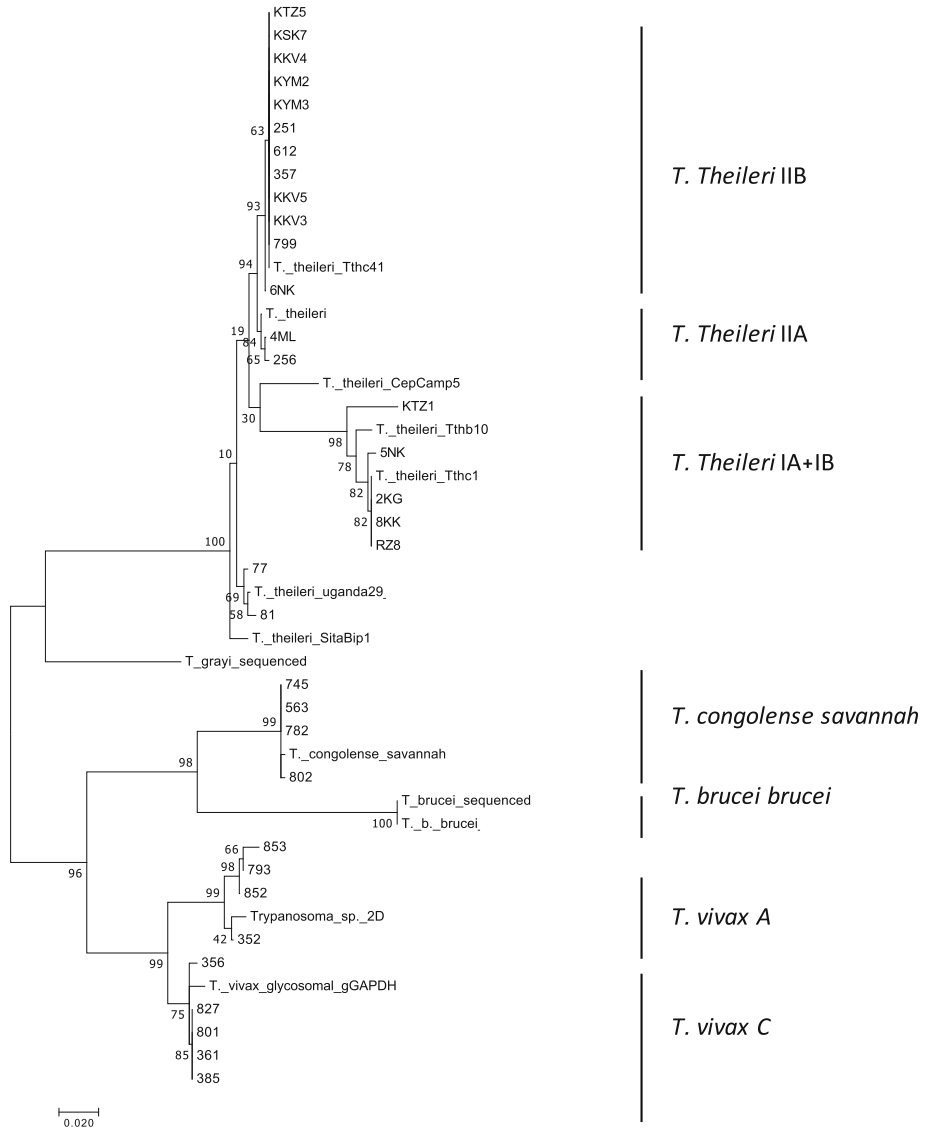

Fig. 2 Molecular phylogenetic analysis by Maximum Likelihood method based on the gGAPDH–encoding gene sequence as detailed under "Material and Methods". It contains an alignment of 535 bp stretches of 37 sequences obtained in this study plus reference sequences [HQ664796; FM164792; HQ664805; HQ664784, HQ664792; HF545654; FM164789; XM_840453; FN400713] retrieved from Garcia et al. [29] and Hamilton et al. [23]. The bootstrap support values (> 70% in 1000 replications) are shown for the nodes

Mayo-Rey (8.2%) (Fig. 4). This observation suggests that abundant mechanical vectors are the drivers of transmission of Stercoraria in the presumably tsetse-free areas [10, 35]. The entomological survey by Lendzele et al. [36] in the Vina and Mayo-Rey division identified seven species of tabanids as potential mechanical vectors: *Tabanus gratus*, *Ta. par*, *Ta. taeniola*, *Ta. biguttatus*, *Ta. sufis* and *Chrysops distinctipennis*. Furthermore, four prevailing species of tabanids were found in the Far North region: *Atylotus agrestis*, *Ta. taeniola*, *Ta. par* and *Ancala spec* [37].. Desquesnes and Dia [38, 39] have proved experimentally the mechanical transmission of *T. vivax* and *T. congolense* by tabanids (*Atylotus agrestis*). In addition, *Ta. par* and *Ta. taeniola* were tested PCR positive for the presence of *T. congolense*, *T. theileri, T. evansi* and *T. brucei* in South Africa and the Gambia [40]. Additionally, ixodid ticks were identified as vectors of *T. theileri* in Germany and in Sudan [4, 5]. However, to our knowledge no detailed studies on mechanical vectors have been performed in the study areas until now.

Infections with *T. brucei* spp. (5.0%) and *T. vivax* (6.7%) were the most prevalent classical pathogenic trypanosomes found in our study areas. They were significantly more prevalent in Faro et Deo and Mayo-Rey (Tables 1 and 2) compared to the other locations. This observation was expected, because Faro et Deo is

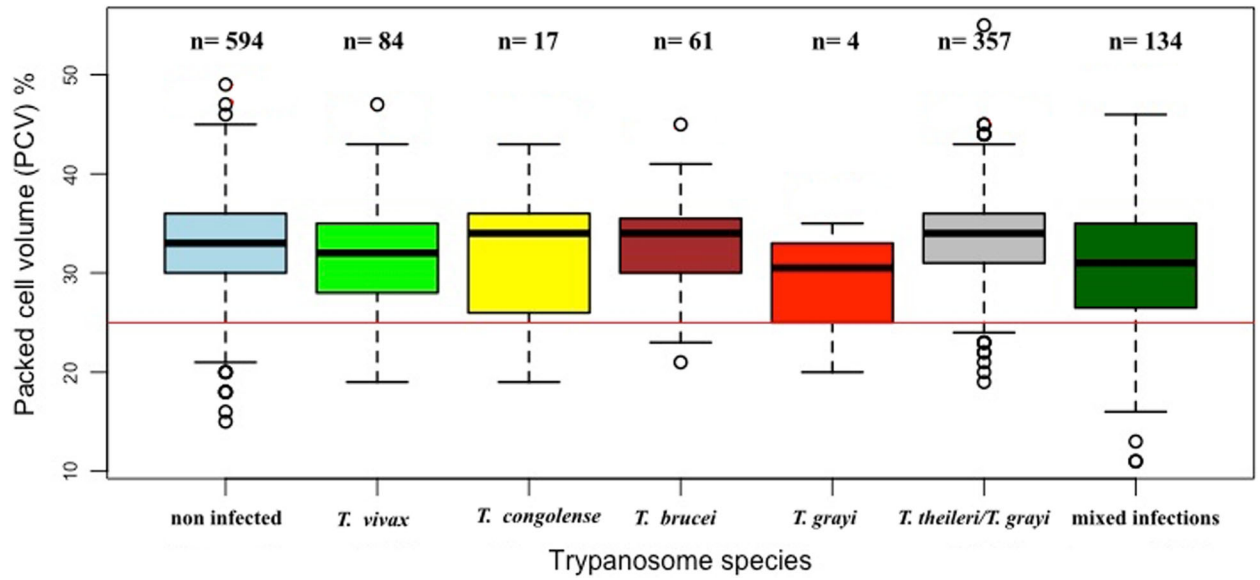

Fig. 3 Effect of the species of trypanosomes detected by PCR on the Packed Cell Volume (PCV). Mixed infection is defined as the combination of at least two trypanosome species identified in the same animal. Details of sample collections and processing are indicated in the section "Materials and Methods"

situated between the tsetse-infested Gashaka Forest Reserve on the Nigerian border and the Faro Game Reserve, and Mayo-Rey between the hunting zones and the Bouba Ndjida National Park, which both harbor a large population of known reservoir species for trypanosomes (antelopes, buffalos, etc.) with particularly abundant tsetse populations [9, 41]. The high infection rate observed in Faro et Deo is in agreement with prevalences of 10 to 41%, obtained in earlier studies [7, 9, 10, 24]. In this area *Glossina morsitans submorsitans* and *G. palpalis palpalis* are the main prevailing vector species [24, 41]. In Mayo-Rey, *G. tachinoides* was also abundant, together with *G. m. submorsitans* [9].

Lower prevalences of *T. vivax*, *T. congolense* and *T. brucei* spp. were observed in Mayo-Tsanaga (11.7%) and Vina (7.8%), both considered tsetse free. However, the presence of these trypanosomes may indicate presence of tsetse in these areas, perhaps due to re-infestation of tsetse from the nearby wildlife reserves which had not been subject to tsetse control. It can be as well due to the introduction of infected animals from tsetse infested zones.

Trypanosoma congolense was detected in the Vina ($n = 4/283$; 1.4%) and in Mayo-Tsanaga ($n = 12/205$; 5.8%) only by molecular methods, a status which does not exclude the activities of tsetse in these areas. In the Adamawa plateau prevalence of 3% by microscopy and 21% by serological tests were previously reported [42]. For the Mayo-Tsanaga division this is the first report of *T. congolense* in cattle. However, since no molecular confirmation was done before, it is possible that these infections were misidentified previously. Or they may have been recently introduced by infected tsetse or infected Fulani animals coming from transhumance through tsetse infested areas of neighboring countries like Nigeria.

Trypanosoma vivax sequence analysis revealed the occurrence of two phylogenetically distinct strains: *T. vivax* type C [30], previously described to be distributed across Africa and America, and *T. vivax* type A, which was isolated so far only in Tanzania [FM164789; FM164787]. In our study areas, we found both strains sympatric with other trypanosomes in the Faro et Deo division. The type A has been reported to be responsible for several outbreaks of bovine trypanosomosis in East Africa [30]. This raises the concern for potential outbreaks in the Faro et Deo region, and the potential to spread further throughout the country.

When looking at the PCV values, animals carrying mixed infections had significantly lower values than the non-infected and single-species infected animals. Furthermore, when comparing the sampling areas, Faro et Deo had the lowest PCV values both in infected and uninfected cattle. It has also to be considered that anaemia may be the result of other hemoprotozoan and/or helminths infections [43]. Infected Kapsiki cattle showed the lowest PCVs when compared to the other indigenous *Bos taurus* breed Doayo (Namchi). It has been previously reported [44] that the Doayo cattle were trypanotolerant while the Kapsiki were trypano-susceptible and this was associated with higher introgression of zebu alleles in the Kapsiki [26]. In a previous study, it was

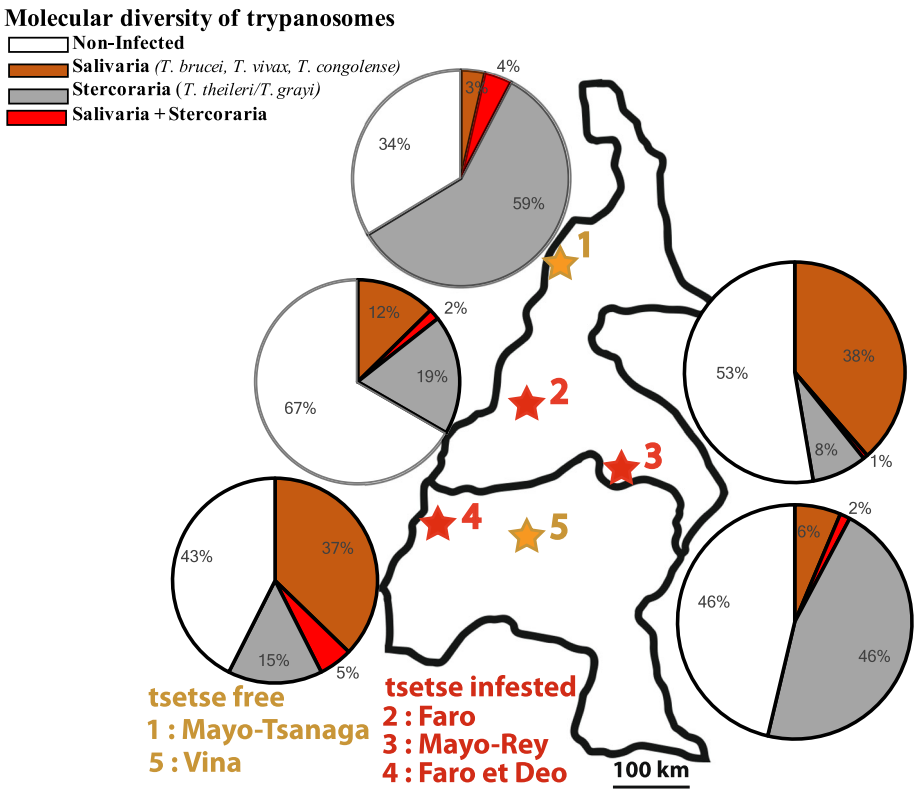

Fig. 4 Distribution of Salivaria (*T. brucei, T. vivax* and *T. congolense*) and Stercoraria (*T. theileri/T. grayi*) in tsetse free and tsetse infested areas in Northern Cameroon. Details of sample collections and processing are indicated in the section "Materials and Methods".(map depicted in Fig. 4 is from our own)

observed that *T. theileri* clade IIB, though considered non-pathogenic in cattle, correlated with low PCV in infected animals [24]. This tendency to become pathogenic was attributed to the genetic association to a previously described clade [29]. Comparing the PCV values of all animals in this study in which DNA of *T. theileri* was found, the mean PCV of cattle infected with clade IIB (31.9 ± 4.5) was slightly lower than those of animals with clade IA and IB (35.8 ± 3.4, $p < 0.057$). This implies that infections with clade IIB may be pathogenic to cattle and should be further investigated and considered during clinical control operations for cattle kept under local husbandry conditions. Once more, this underlines the importance to further investigate the development and evolution of trypanosome species, especially as these two clades of *T. theileri* are found worldwide. The prevalence of *T. brucei spp*, *T. vivax* and *T. congolense* in the tsetse-free areas of Mayo-Tsanaga and Vina raised questions whether the areas are still free and if tsetse control is sufficient enough to eradicate bovine trypanosomosis. Therefore, an entomological survey is urgently needed to check whether these previously tsetse-free areas have been re-infested by *Glossina* or whether these parasites are transmitted by non-tsetse vectors. Both scenarios call for close monitoring of the situation including molecular tools as used in this study as well as a thorough search for alternate vectors.

Conclusions

Bovine trypanosomosis is more prevalent in the two ecological zones of northern Cameroon than previously thought. Five trypanosome species and subtypes were identified. Unexpectedly several cases of *T. grayi* were detected in cattle. Therefore, it may not be excluded that this parasite is already adapted to the cattle host. *Trypanosoma vivax*, clade A, which had previously only been identified in Tanzania was found to be co-endemic with *T. vivax* clade A and *T. vivax* clade C in the Faro et Deo region. Furthermore, the presence of two strains of *T. theileri,* clades IIB and IA/IB, was confirmed. This high diversity of *Trypanosoma* species makes monitoring and local control more complex than previously thought. Finally, the abundance of pathogenic trypanosomes in tsetse infested areas is alarming and even more, the occurrence of *T. vivax*, *T. brucei*, *T. congolense*, *T. theileri* and *T. grayi* in tsetse-free areas implies that tsetse control alone is not sufficient to control trypanosomosis in livestock.

Methods

Study areas

This study was carried out in the Far North, North and Adamawa region of Cameroon (Fig. 5: Additional file 3: Table S4). These three regions are localized in two large Agro-Ecological Zones: the Sudano-Sahelian (Far North region and a larger part of the North region) and the Guinea savannah of the Adamawa plateau (Adamawa region with a little part of the North region). Cattle rearing is most abundant in the Guinea savanna of the Adamawa plateau with suitable climate and pasturelands for extensive cattle rearing. Overall, this plateau contributes to about 38% of beef production in the country [45]. The sampling sites were located in five divisions lying between latitudes 7 to 10°N and 11 to 15°E and covered an area of 164,000 km^2 [46]. A strong climatic gradient runs through the wet high Guinea savannah in the Adamawa up to the dry Sudano-Sahelian zone in the Far North region. The rainy season in the Guinea savannah zone is from April to October, whereas in the Sudano-Sahelian zone it is from June to September. Annual rainfall ranges from 1400 to 1700 mm in the Guinean savannah and 800–1400 mm in the Sudano-Sahelian zone (Fig. 5).

Experimental design and animal selection

A cross-sectional survey was carried out between April 2014 and June 2015. For each herd visited, about 10% of the animals were sampled using a systematic random method described by Dohoo et al. [47]. In the Faro and Mayo Tsanaga divisions only the indigenous taurine cattle breeds Doayo (Namchi) and Kapsiki, respectively, were examined and sampled. From each animal, physical examinations were made and the following variables recorded: breed, sex, body condition score (BCS) using the method described by Pullan for White Fulani [32], on a scale from 0 to 5 (0–2: poor condition, 3–4: good condition and 5 very good condition or fat), and age by dentition categorized as young (< 2.5 years), mature (> 2.5–5 years) and older (> 5 years). In many farms only very few males were present in the herds causing the random selection to be applied on the animals found in the herd without balancing for sex proportions.

Assessment of packed cell volume (PCV) and trypanosome detection

Approximatively 5 mL of blood were collected from the jugular vein of each animal, using a vacutainer tube containing potassium ethylenediaminetetraacetic acid

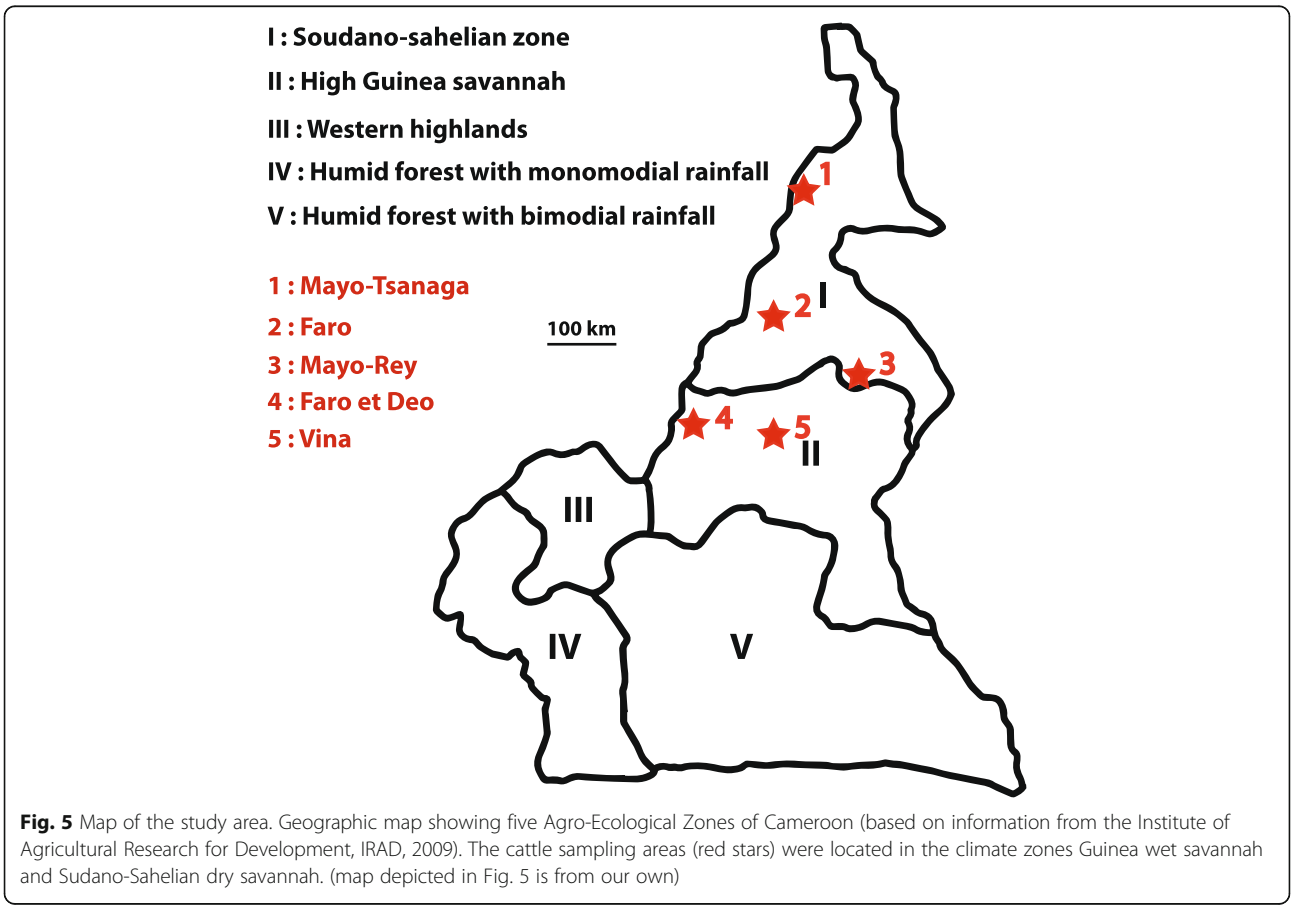

Fig. 5 Map of the study area. Geographic map showing five Agro-Ecological Zones of Cameroon (based on information from the Institute of Agricultural Research for Development, IRAD, 2009). The cattle sampling areas (red stars) were located in the climate zones Guinea wet savannah and Sudano-Sahelian dry savannah. (map depicted in Fig. 5 is from our own)

(EDTA) anticoagulant (VACUETTE® K3 EDTA). The samples were stored in a cooler box until processing within 6 h after collection either at a stationary or mobile laboratory in the field. Plasma was separated from blood by centrifugation at 3000 rpm for 15 min. Then the buffy coat was carefully collected and stored at 4 °C for subsequent DNA extraction. To determine the PCV, blood was introduced into capillary tubes (approx. 70 μL), and after sealing one end of the capillary tube with cristoseal (Sigma Aldrich, Germany) it was centrifuged at 12,000 rpm for 5 min using a microhaematocrit centrifuge (Hawksley, UK). The PCV was measured with a haematocrit reader (Hawksley Limited, UK). Animals that had a PCV value equal or less than 25% were considered anaemic. Subsequently, the capillary tube was cut with a diamond cutter 0.5 mm below the buffy coat to transfer the layer of white blood cells containing accumulated haemoparasites [16, 17] on to a clean microscope slide. After applying a coverslip over the buffy coat, approximately 200 fields of the preparation were examined for the presence of motile trypanosomes with a compound light microscope using 400x magnification [15]. The trypanosome species were classified according to previously described morphological criteria [14].

Genomic DNA extraction, purification, PCR amplification, sequencing of ITS-1 and gGAPDH

Genomic DNA from buffy coat was extracted using the Wizard Genomic DNA Purification Kit (Promega, Germany) according to the manufacturer's instructions, and then stored at.

– 20 °C. Generic primers were used in a nested PCR targeting kinetoplastid ITS-1 as described previously [19, 24]. Briefly, the first reaction (25 μL final volume) contained 2 μM of each outer primers (Table 6), 0.2 mM dNTP mix, 0.5 U Dream *Taq* DNA polymerase (Thermo Scientific, Dreieich, Germany), 1× Dream *Taq* buffer, and 1 μL of extracted DNA. Nuclease-free water and genomic DNA of *T. brucei*, *T. congolense* or *T. grayi* were used as negative and positive controls, respectively. PCR amplification was carried out as follows: initial denaturation step at 95 °C for 60 s, followed by 30 amplification cycles at 94 °C for 60 s, at 52 °C for 60 s, at 72 °C for 30 s, and final extension at 72 °C for 5 min. Thereafter, the second PCR reaction was carried out with 1 μL of first PCR product diluted 80-fold as template under the same cycling conditions as described above, except for an annealing temperature of 54 °C, and using the inner primer pairs (Table 6). 20 μL of the resulting PCR product was loaded onto a 2% TBE agarose gel stained with 0.5 μg/mL of DNA Stain G (SERVA, Heidelberg, Germany). Positive PCR amplicons of variable fragment sizes representing different trypanosome species (Table 4, Additional file 1: Figure S1) were randomly selected for Sanger sequencing. For these samples, the second reaction was carried out in a total volume of 50 μL with 2 μL of 80-fold diluted first PCR product.

Table 6 Generic Primers used for PCR amplification

Primers	5′- 3′ sequence	Sequence length (bp)
ITS1-OutF[a]	CTTTGCTGCGTTCTT	660–180
ITS1-OutR[a]	TGCAATTATTGGTCGCGC	
ITS1-InF[a]	TAGAGGAAGCAAAAG	
ITS1-InR[a]	AAGCCAAGTCATCCATCG	
gGAPDH-OutF[b]	TTYGCCGYATYGGYCGCATGG	900
gGAPDH-OutR[b]	ACMAGRTCCACCACRCGGTG	
gGAPDH-InF[b]	CGCGGATCCASGGYCT YMTCGGBAMKGAGAT	
gGAPDH-InR[b]	GTTYTGCAGSGTCGCCTTGG	

Primer. In: inner primer, Out: outer primer, F: forward, R: reverse,
[a]Adams et al. [19]
[b]Hamilton et al. [23]

An approximately 900 bp region of the gGAPDH gene was amplified by nested PCR and sequenced using the primers described by Hamilton et al. [23]. Nested PCR was carried out using 2x Red Mastermix (Genaxxon Bioscience, Ulm, Germany) to generate PCR products for direct sequencing. Briefly, the first PCR reaction with a final volume of 25 μL contained 1x mastermix, 0.5 μM of outer primers (Table 6), and 2 μL of genomic DNA template under the following conditions: initial denaturation at 95 °C for 3 min, 30 cycles at 95 °C for 1 min, annealing at 55 °C for 30 s, elongation at 72 °C for 1 min, followed by a final elongation step at 72 °C for 10 min. The first PCR products were diluted 80-fold and 2 μL transferred to the second PCR reaction with the inner primers (Table 6, Additional file 2: Figure S2) under the same conditions as the first reaction. Amplified products were subjected to electrophoresis on 2% agarose gels. The selected positive PCR products were sent for sequencing (Macrogen, Netherlands).

A subset of positive amplicons was excised from the gel and purified using GeneJet Gel Extraction Kit (Thermo Scientific, Dreieich, Germany) according to the manufacturer's instructions. DNA concentrations were determined by photometry on a Nanodrop 1000 (Thermo Scientific, Dreieich, Germany) before submitting them to a commercial sequencing provider (Macrogen).

Statistics and phylogenetic analysis

The results from the parasitological and molecular approaches were compared by Chi-Square tests to assess the association between prevalence, breed, BCS, sampled area and age group. Fisher's Exact Test was done to

compare mean PCV values. Since only 269 (23%) samples were collected from male, no sex-differentiating analysis was performed.

Differences were tested for significance at $p < 0.05$ using the statistical software program SPSS v.25.0.0 (IBM, USA). Obtained sequences were analyzed using Geneious (Biomatters, Auckland, New Zealand) and aligned to sequences retrieved from data bases searches (GenBank, NCBI, https://blast.ncbi.nlm.nih.gov/genbank/), and TriTrypDBv.6.0 (http://tritrypdb.org) using nucleotide BLAST.

To investigate the genetic diversity of trypanosomes present in the study area, and to analyze their phylogenetic relationship in order to detect subpopulations of trypanosomes restricted to respective study areas, gGAPDH was used as a marker locus. Phylogenetic trees were aligned and constructed by MEGA7 software [48], and the evolutionary history was inferred using the Maximum Likelihood method (ML) based on the Kimura 2-parameter model [49]. Confidence in branching relationships was assessed using bootstrap re-sampling over 1000 replicates. The final construct nucleotide length used in this analysis was 535 bp.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12917-019-2111-6.

Additional file 1: Figure S1. PCR amplicons of *Trypanosoma* species in northern Cameroon. ITS amplicon sizes of different *Trypanosoma* species in the range of 200 to 650 bp. The amplicons were resolved on a 2% TBE agarose gel. The first lane shows the marker (M), the second lane shows the ITS-1 fragment for *T. vivax* (Tv) at 200 bp and a faint band at around 180 bp. The third and fourth lanes show the presence of two species, *T. theileri* and *T. grayi* (Tth/Tg) at 380 bp, the fifth line *T. brucei* spp. (Tb) at 400 bp and the sixth lane the presence of *T. congolense* forest type (Tcf) at 640 bp. C-1: Water control of 1st reaction, C-2: Water control of 2nd reaction.

Additional file 2: Figure S2. gGAPDH amplicons of different *Trypanosoma* species gave one band size of 900 bp. The first lane shows the marker (M), the second (852) and third (853) lanes are positives, the fourth (884), fifth (895), sixth (898) and seventh (849) lanes are negative samples. The eighth lane is the amplicon of *T. grayi* genomic DNA used as a positive control and C is double distilled water as a negative control.

Additional file 3: Table S1. Effect of cattle breed on animal body condition. **Table S2.** Effect of study area, cattle breed and age group on packed cell volume. **Table S3.** Packed cell volume of animals infected with *T. theileri* clade IA, IB and IIB. **Table S4**. GPS Coordinates of study areas and sampled herds.

Abbreviations

AEZ: Agro Ecological Zones; EDTA: Ethylenediaminetetraacetic acid; gGAPDH: glycosomal glyceraldehyde 3-phosphate dehydrogenase gene; ITS-1: Internal transcribed spacer 1 region of the ribosomal RNA gene locus; PCV: Pack cell volume; SPP: Subspecies

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Author's contributions

Conceptualisation: AP, BA, AR, AE, MDA. Laboratory analysis: AP, JSW, SCHN, MDA. Investigation: AP, BA, MDA, TKM, MA, DN. Project administration: AP, AE, AR. Resources: AP, BA, MDA, SK, AR, AE, Supervision: MDA, A R, SK. Writing – review & editing: AP, BA, MDA, AR, AE, SCHN, JSW, SK, DN, TKM, MA. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated and analyzed during this study are included in this published article and its supplementary information files or available from the corresponding author on reasonable request. The sequences generated during the present study are available in the NCBI Genbank repository under the accession numbers MK674001-MK674048, MK656901-MK656904.

Ethics approval

Permission for the study and ethical approval were obtained from the Ethics committee of the Institute of Agricultural Research for Development (IRAD) in Cameroon, which is the country's government institution for animal health and livestock husbandry improvement. Furthermore, verbal consent was given by the cattle owners and herdsmen and approved by the same ethics committee. All cattle owners and herdsmen participating in the study were contact by telephone and the purpose of the study was explained. When the oral consent was obtained from participant. The veterinarians were sent for biological material collection.

Consent for publication

Not applicable

Competing interests
The authors declare that they have no competing interests.

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Development of a Low-Density DNA Microarray for Detecting Tick-Borne Bacterial and Piroplasmid Pathogens in African Cattle

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Abstract: In Africa, pathogens transmitted by ticks are of major concern in livestock production and human health. Despite noticeable improvements particularly of molecular screening methods, their widespread availability and the detection of multiple infections remain challenging. Hence, we developed a universally accessible and robust tool for the detection of bacterial pathogens and piroplasmid parasites of cattle. A low-cost and low-density chip DNA microarray kit (LCD-Array) was designed and tested towards its specificity and sensitivity for five genera causing tick-borne diseases. The blood samples used for this study were collected from cattle in Northern Cameroon. Altogether, 12 species of the genera *Anaplasma*, *Ehrlichia*, *Rickettsia* and *Theileria*, and their corresponding genus-wide probes including *Babesia* were tested on a single LCD-Array. The detection limit of plasmid controls by PCR ranged from 1 to 75 copies per µL depending on the species. All sequenced species hybridized on the LCD-Array. As expected, PCR, agarose gel electrophoresis and Sanger sequencing found significantly less pathogens than the LCD-Array ($p < 0.001$). *Theileria* and *Rickettsia* had lower detection limits than *Anaplasma* and *Ehrlichia*. The parallel identification of some of the most detrimental tick-borne pathogens of livestock, and the possible implementation in small molecular-diagnostic laboratories with limited capacities makes the LCD-Array an appealing asset.

Keywords: tick-borne pathogen; low-cost and low-density-array; Reverse Line Blot; *Anaplasma*; *Ehrlichia*; *Rickettsia*; *Theileria*

1. Introduction

Tick-borne pathogens (TBP) are of high veterinary and medical importance worldwide. To evaluate the risk of exposure of TBPs in a livestock or human population, effective surveillance and monitoring practices are needed. For cattle and other livestock, the published literature highlights the importance of protozoa of the genera *Babesia* and *Theileria*, bacteria of the genera *Anaplasma*, *Ehrlichia* and *Rickettsia*, and arboviruses as etiologic agents of many diseases, of which a number of them have zoonotic potential [1]. Especially in developing countries, routine diagnostic approaches for the identification of TBPs are generally based on microscopic examination of blood smears [2,3] or serological assays [4,5].

While those techniques require only moderate investments for equipment and infrastructure, they have limitations regarding specificity and sensitivity (microscopy) [6–8], or tend to cross-react with closely related species (enzyme-linked immunosorbent assays) [9]. Furthermore, commercially available kits of the former are often not financially affordable for veterinary laboratories in low income endemic countries. Molecular tools based on PCR [10] and nowadays NGS are becoming more widespread, with NGS being economically viable when used for large sample sizes [11].

The DNA microarray technology of PCR-amplified products combines high throughput, sensitivity, specificity and reproducibility [12]. Its function is based on the reverse line blot (RLB), in which specific oligonucleotide spots (probes) are immobilized on a solid surface (Figure 1). When a target sample with complementary DNA sequence is added, it hybridizes with the probe where it is detected by a fluorescent, chemiluminescent or biotinylated label. The synchronous detection of a multitude of species in the same genetic material has contributed to its popularity in infectious disease diagnostics [10,13]. Low-density DNA microarrays such as the LCD-Array are designed to detect much lower numbers of pathogenic agents than high-density microarrays [14]. However, they are optimized for minimal input of equipment, workflow, costs and expenditure of time, and therefore suitable for small diagnostic laboratories in low and middle income developing countries [14,15].

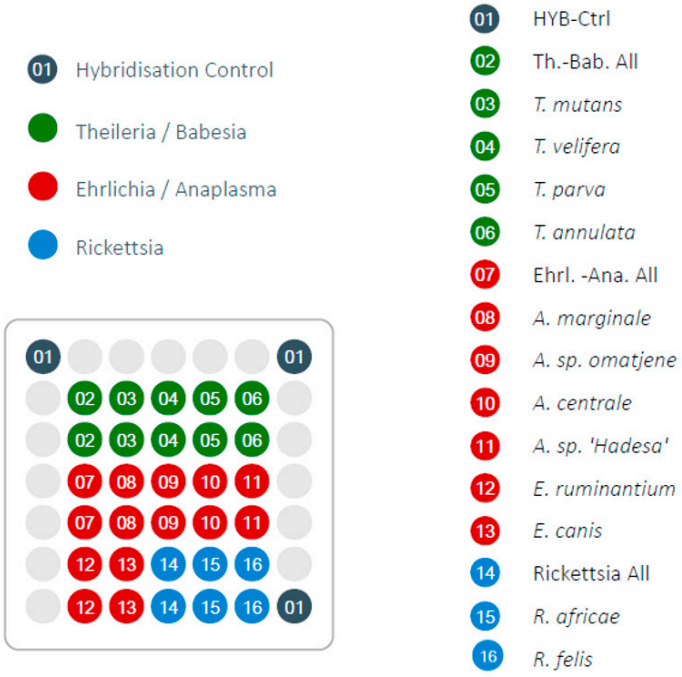

Figure 1. Design of LCD-Array for tick-borne pathogens indicating the screened species and genera. Light grey circles are blank positions.

In TBP epizootiology, the mostly used RLB application has been a mini-blotter coupled with a membrane where the probes of interest have been priorly linked to [10,13]. Although any desirable probes can be attached to the membrane prior to testing, the setup necessitates a high skill level in handling and optimization. Hence, for routine TBP identification a "ready to use" array or biochip for low to medium sample numbers with standardized protocol and reagents would be highly desirable.

In this paper we describe the development and testing of a novel LCD microarray for TBP, based on an already established biochip platform from a commercial provider (Chipron, Berlin, Germany). The same platform has been adapted for the detection of human mycobacteria [16], viruses [14,17], fungi [18] and in food safety [12]. In the field of TBP, this array has been tested once for the two piroplasmidae genera *Babesia* and *Theileria* [19]. In our study, the PCR and LCD-Array also detect ribosomal RNA fragments (18S) of the genera *Babesia* and *Theileria*, and additionally bacterial 16S fragments of the genera *Anaplasma*, *Ehrlichia* and *Rickettsia*. The array design, protocol specifications

and performance in comparison to PCR with Sanger sequencing are described and tested on a naturally exposed cattle population from North Cameroon.

2. Materials and Methods

2.1. Sample Origin, DNA Extraction, PCR and Sanger Sequencing

The tested blood samples ($n = 31$) were collected from cattle in Northern Cameroon. Blood samples (5 mL in EDTA tubes) were taken from the jugular vein of animals and tested by PCR and agarose gel electrophoresis. Briefly, blood samples were centrifuged at 3000 rpm using the Z380 laboratory centrifuge (Hermle Labortechnik, Wehingen, Germany) for 15 min and 300 µL of the erythrocyte and buffy coat was used for DNA extraction according to the manufacturer's instructions of the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Published primer pairs were used for the identification of the genera *Babesia/Theileria* [20] and *Rickettsia* [10]. Based on sequence alignments of the target species and mitochondrial regions in GenBank, a new primer pair was designed for the detection of *Anaplasma/Ehrlichia*. The primer sequences and corresponding annealing temperatures are given in Table 1. To identify TBP-positive samples, a PCR reaction was done in 25 µL total volume combined as followed: 12.5 µL of the 2× RedMaster Mix (Genaxxon BioScience, Ulm, Germany) or 1 mM $MgCl_2$, 0.5 mM 5× buffer, 200 µM nucleotides mix and 1 U GoTaq DNA polymerase (Promega, Madison, WI, USA). To the master mix, 10 pmol of each primer was added per reaction. One microliter of template DNA was added to 24 µL of mastermix reagents, and HPLC-grade water (Sigma Aldrich, Taufkirchen, Germany) was used as PCR negative control. Temperature cycles were programmed on a MasterCycler EPS 96-well thermocycler (Eppendorf, Hamburg, Germany): initial denaturation at 95 °C for 3 min, 35 cycles of 95 °C for 30 s, annealing temperatures (Table 1) for 30 s, 72 °C for 30 s, followed by a final elongation step of 72 °C for 10 min. Five microliter of the amplified products with 1 µL of loading buffer (Genaxxon BioScience, Ulm, Germany) were loaded on a 1.5% agarose gel with Tris Borate EDTA buffer (TBE) stained with Midori Green (Nippon Genetics Europe, Düren, Germany), run for about 40 min at 100 V, and photographed under UV light. The selected specimens with visible PCR product in the gel were prepared and submitted for DNA sequencing according to the provider's recommendation (Macrogen Europe, Amsterdam, Netherlands). The retrieved sequence data was edited manually, MUSCLE aligned and analyzed with Geneious v9.1 (Biomatters, Auckland, New Zealand) and the GenBank nucleotide database (National Center of Biotechnology Information, Bethesda, MD, USA).

Table 1. Primer pairs used for identification of tick-borne pathogens.

Genus	Gene Target	Primer Sequence	Annealing Temp.	Amplicon Size [bp]	Reference
Babesia/Theileria	18S rRNA	GAC ACA GGG AGG TAG TGA CAA G	57 °C	460–500	[20]
		b-CTA AGA ATT TCA CCT CTG ACA GT			
Anaplasma/Ehrlichia	16S rRNA	AGA GTT TGA TCM TGG YTC AGA A	55 °C	460–520	This study
		b-GAG TTT GCC GGG ACT TYT TC			
Rickettsia	16S rRNA	GAA CGC TAT CGG TAT GCT TAA CAC A	64 °C	350–400	[10]
		b-CAT CAC TCA CTC GGT ATT GCT GGA			

b- biotin label at 5′ end.

2.2. LCD-Array Specification and Validation

To allow the detection on the array, a similar PCR reaction was done with one of the paired primers being biotinylated at the 5'-end (Table 1) at a concentration 10-times higher than the corresponding non-biotinylated primer. Moreover, 10 more temperature cycles were added to increase template amplification for hybridization. For sensitivity tests, twelve constructs on the plasmid vector pUC57 (Baseclear, Leiden, Netherlands) with inserts of the following gene loci and species were used as positive controls: For 16S rRNA *Anaplasma centrale*, *A. marginale*, *A. platys* (*A. sp.* 'Ommatjenne'), *A. sp.* 'Hadesa', *E. canis*, *Ehrlichia ruminantium*, *Rickettsia africae* and *R. felis*. For 18S rRNA *Theileria annulata*, *T. mutans*, *T. parva* and *T. velifera* was used. The concentration of plasmid constructs was measured by the Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and the number of copies calculated from the amount of DNA in ng and the length of the template in base pairs using the formulae described on the webpage http://cels.uri.edu/gsc/cndna.html (URI Genomics and Sequencing Center). Ten-fold serial dilutions in HPLC-grade water (Sigma Aldrich, Taufkirchen, Germany) as solvent were prepared and used as PCR templates, resulting in target concentrations ranging from 1 to 75 plasmid copies per reaction. Those dilutions of plasmids were amplified by PCR and loaded on gel electrophoresis, as well as tested on the LCD-Array using the first dilution with no detectable PCR product in the agarose gel, respectively for each of the species amplicons.

The LCD-Array consists of a transparent, pre-structured polymer support, with 50 by 50 mm dimensions. Each array had eight individually addressable sample wells where the probes are spotted on the surface as 19 to 28-meres of oligonucleotides using contact-free piezo dispensing technology [14]. The array presently used contained 33 probe spots of which three are proprietary kit controls ('hybridization controls'), and 30 genera- or species-specific probes in duplicates as controls in case of mechanical failure (Figure 1). Altogether, 12 TBP species and 3 genera or groups of genera ("catch all") were included. The probes were selected according to highest genus or species coverage in GenBank. Parameters of selection were the exclusion of unintended hybridization with other genera or species, melting temperature optimum for the LCD-Array, and distance of the hybridization site to the biotinylated primer.

2.3. LCD-Array Workflow

Single amplicons produced by each of the generic primer pairs or mixtures of the three species groups—each containing one biotinylated primer—were added at a final volume of 10 µL (for single product) and in equal proportions (3.3 µL for the mixture) to the LCD-Array according to the manufacturer's protocol (Chipron, Berlin, Germany). Briefly, 10 µL of the mixture was added to 24 µL Hybridization Mix (Chipron), and 28 µL thereof was applied per sample well. The chip was placed in the kit's humidity chamber and incubated in a 35 °C water bath for 30 min. Afterwards, washing steps were conducted with the supplied washing buffer for about 2 min successively in three small tanks filled with about 200 mL of 1× washing buffer. The slide was dried by spinning in the Chip-Spin centrifuge (Chipron, Berlin, Germany) for 15 s. Then, 28 µL of the previously combined horseradish peroxidase—streptavidin conjugate (Chipron) was added to the array for labeling, and incubated for 5 min. Subsequently, the array was washed and dried as previously indicated. Finally, 28 µL of the staining solution tetra methyl benzidine was added to each sample well. After 5 min incubation at room temperature, the staining process was stopped by washing once for 10 s and drying as described before. All tanks were filled with new washing buffer after each step. The LCD-Array was analyzed using the SlideScanner PF725u with the software package SlideReader V12 (Chipron, Berlin, Germany) for automated identification. By default, the cut-off value for positive detection was 2000 pixel values.

To test the specificity and the sensitivity of the assay, 10 µL of the PCR amplification products of each recombinant positive control plasmid was submitted to the array. The template concentrations were one order below the limit of detection by agarose gel electrophoresis as described above. For cross hybridization tests, PCR products of all three genera/groups of genera were mixed at equal volume.

Cattle field samples (n = 31) were PCR amplified and tested on the LCD-Array for analogy with previously obtained sequencing results.

The statistical analysis was done using R v.3.4.2 (www.R-project.org). Data produced from both tests (sequencing and LCD-Array chip) were considered as paired data. The paired t-test was used to assess the difference between both diagnostics. A statistical p-value below 0.05 was considered significant.

3. Results

3.1. LCD-Array Performance of Synthetic Inserts (Plasmids)

All twelve plasmid constructs hybridized only with their respective probes, including "catch all" on the LCD-Array (Figure 2). The tested concentration of plasmid template on the array was 10 to 1000 times lower than on agarose gel (Table 2). Onagarose gel electrophoresis the product was still visible at 10^{-8} dilution for Theileria and Rickettsia, and for dilutions between 10^{-5} and 10^{-7} for Anaplasma and Ehrlichia (Figure 3).

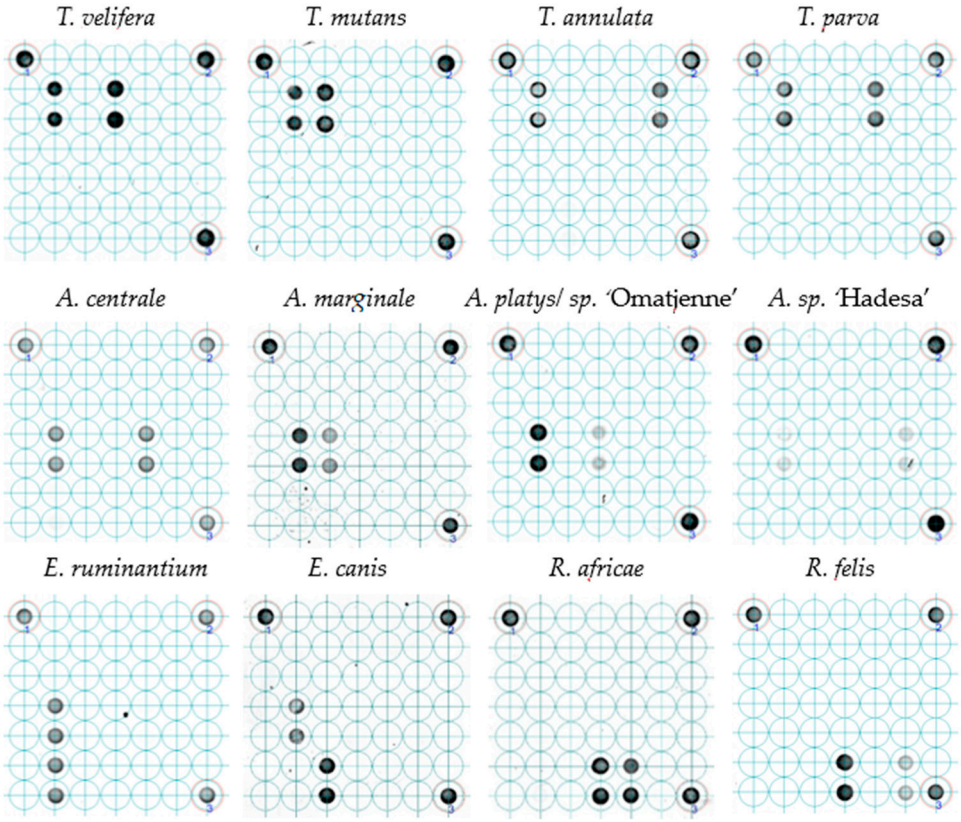

Figure 2. Probe hybridization of LCD-Array of tick-borne pathogens. The dark spots indicate hybridization of plasmids with species-specific inserts to the probe spotted on the array in duplicates. The faint spots indicate lower concentrations in the respective PCR products. The three spots in the corners are internal kit controls. For each of the tested positive controls (plasmids), the concentration came from the first dilution not producing a visible product in agarose gel.

Table 2. Limit of detection (LOD) of LCD-Array for tick-borne pathogens measured in the lowest detectable dilution of the PCR product.

Species	Copies/µL Pre-PCR *	LOD Post-PCR *	LOD LCD-Array
Anaplasma centrale	75	10^{-5}	10^{-8}
Anaplasma marginale	31	10^{-7}	10^{-8}
Anaplasma platys	28	10^{-7}	10^{-8}
Anaplasma sp. 'Hadesa'	34	10^{-7}	10^{-8}
Ehrlichia canis	60	10^{-6}	10^{-8}
Ehrlichia ruminantium	40	10^{-7}	10^{-8}
Rickettsia africae	3	10^{-8}	10^{-9}
Rickettsia felis	2	10^{-8}	10^{-9}
Theileria annulata	6	10^{-8}	10^{-9}
Theileria mutans	3	10^{-8}	10^{-9}
Theileria parva	7	10^{-8}	10^{-9}
Theileria velifera	1	10^{-8}	10^{-9}

* Detected on agarose gel electrophoresis.

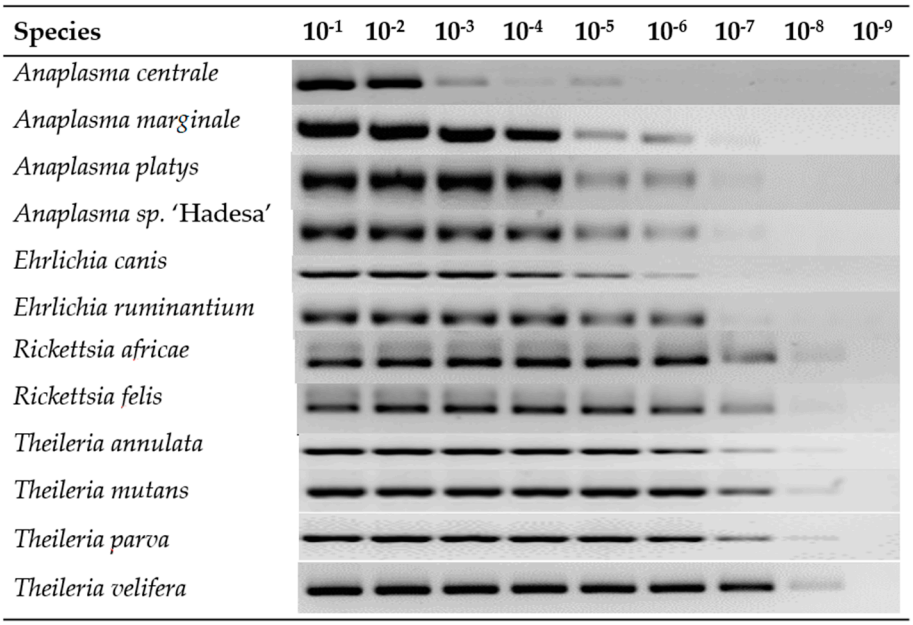

Figure 3. Serial dilution of plasmid amplicons in a 1.5% agarose gel electrophoresis. The last visible band determines the limit of detection which is the lowest dilution detectable on the agarose gel.

3.2. LCD-Array Performance of Cattle Blood Samples from North Cameroon

All pathogens identified by Sanger sequencing in the field-collected blood samples were also detected on the LCD-Array. Furthermore, the array revealed co-infections of more TBPs which were not detected by the sequencing (Figure 4). Statistical comparison showed significant lower detection rates by sequencing as compared to the LCD-Array.

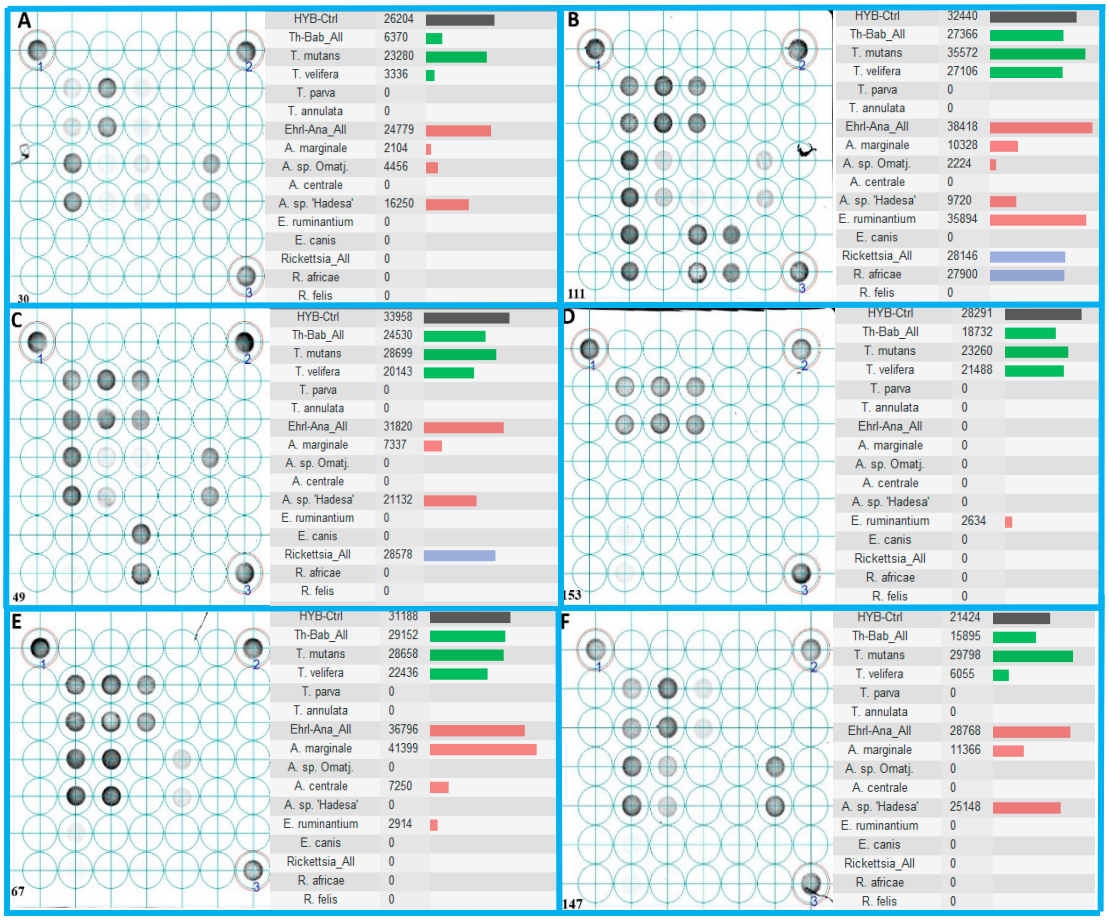

Figure 4. Probe hybridization of six field-collected blood samples (**A**–**F**) on LCD-Array detecting tick-borne pathogens, with 1–3 representing the proprietary kit controls. All shown specimens exhibit co-infections with a minimum of three tick-borne pathogens. The right half of each delimited box shows the hybridization intensity of the corresponding target probe duplicates (Kit control: Black color bar; *Babesia/Theileria*: green color bar; *Anaplasma/Ehrlichia*: red color bar; *Rickettsia*: blue color bar). Results below the cut off value of 2000 are considered negative.

3.2.1. *Anaplasma*

Of the 31 blood samples tested, *A. marginale* was detected in 61.3% (19/31), followed by *A. platys* 41.9% (13/31), *A. sp.* 'Hadesa' 41.9% (13/31), and *A. centrale* 41.9% (13/31). Sanger sequencing had consistently lower detection rates of 12.9%, 29.0%, 6.5% and 12.9% for the same species, respectively. In 26 of 29 positive cases (89.7%) both the species-specific and genus specific ("catch all") probes were hybridizing. The remaining 3 of 29 positive cases reacted only with the *Anaplasma/Ehrlichia* "catch all" probe. From the 31 screened samples, 12 from the *Anaplasma/Ehrlichia* could not be sequenced. Of those unsuccessfully sequenced samples the LCD-Array identified 8 species.

3.2.2. *Ehrlichia*

Ehrlichia species were detected in 17 (54.8%, 17/31) of the screened samples being significantly higher ($p < 0.001$) than the prevalence detected by Sanger sequencing (3.2%, 1/31). Among the unsuccessfully sequenced samples screened under the LCD-Array, *E. ruminantium* was found in co-infection with *A. centrale* and *A. marginale*. In another case *E. ruminantium* was found in co-infection with *A. marginale*. *E. canis* was found by sequencing and hybridized by its specific probe on the array in only one sample, however below the threshold of 2000 pixel values. From the 17 positive cases for *E. ruminantium*, 16 were also positive for the "catch all". From the 31 screened samples, 12 from

the *Anaplasma/Ehrlichia* primers could not be sequenced. The LCD-Array detected 8 of those samples being positive for *A. marginale* ($n = 3$), *E. ruminantium* ($n = 3$) and each co-infected specimens of *A. sp.* 'Hadesa', *A. marginale* and *A. platys*; *A. centrale*, *A. marginale* and *E. ruminantium*, and *A. marginale* and *E. ruminantium*.

3.2.3. *Rickettsia*

Rickettsia africae and *R. felis* were detected on the LCD-Array in 16/31 (51.6%) and 4/31 (12.9%) of cases, respectively, being higher than the detection rates by Sanger sequencing 8/31 (25.8%) and 1/31 (3.2%) of cases, respectively. Eighteen of 20 cases positive for *Rickettsia* species (90%) were also hybridizing with the *Rickettsia*-"catch all" probe. The other two out of 20 samples (10%) were only positive for *Rickettsia* "catch all". PCR amplicons identified by sequencing as bacteria related to *Klebsiella* or *Brevundimonas* did not hybridize with any probe on the LCD-Array. From the 21 PCR-positive samples with negative sequencing results 8 *R. africae* were detected by the microarray, 3 co-infected with *R. africae* and *R. felis*, and one with *R. felis*.

3.2.4. *Babesia*

None of the samples was positively tested and confirmed for *Babesia spp*. Hence, the present LCD-Array did not include probes specific to *Babesia*. However, the *Babesia/Theileria* "catch all" probe is complementary to the 18S loci of the bulk of *Babesia spp*.

3.2.5. *Theileria*

In accordance with the sequencing results, *Theileria mutans* and *T. velifera* were detected in high numbers (90.3%, 28/31, and 77.4%, 24/31, respectively). Detection by sequencing produced unknown *Theileria sp.* in 3 cases, *T. velifera* in one case, *T. mutans* in 17 cases, and *T. mutans* co-infected with *T. velifera* in 3 cases. In 85.7% (24/28) of the cases, *T. mutans* was found in co-infection with *T. velifera* which is significantly higher than recorded by Sanger sequencing of the PCR-product (13.6%; 3/22; $p < 0.001$). 26 of 28 positive animals (92.8%) were also signaling by the "catch all" probe. Both *T. annulata* and *T. parva* were not found neither by sequencing nor by LCD-Array. All PCR-positive samples with no outcome by sequencing ($n = 5$) were identified with the LCD-Array as *T. mutans* and co-infected with *T. velifera* ($n = 3$) and without ($n = 2$).

4. Discussion

The current LCD-Array based on the RLB method has been developed and used to test samples collected from cattle in the northern part of Cameroon. These samples have previously been screened for TBPs using conventional PCR and Sanger sequencing, and a subset of these samples is now being tested by the novel LCD-Array. Co-infection with up to six TBP per animal was common [20], yet difficult to detect by PCR and sequencing alone [13]. In such a scenario, utilization of generic primers poses the problem of correct allocation to the respective species or species complex. DNA sequencing without prior cloning of the less prevalent amplicons is often unsuccessful or distorts the whole readout making it at times incomprehensible [21]. Furthermore, the pathogen concentration in the host blood varies dramatically depending on the animal's state of infection, making the identification challenging when present in very low concentrations. For *Theileria spp.* it is known that carrier animals persist with a low number of infected erythrocytes [22]. Moreover, competition for multiple PCR templates are further limiting factors for the detection of pathogens in low concentrations. In this study, the sensitivity tested on the LCD-Array was between 10 and 1000 times higher than by PCR and Sanger sequencing (Table 2).

The hybridization in some cases of only the "catch all" probe (Figure 4C for *Rickettsia*) suggests the presence of bacteria or parasite species not addressed by the LCD-Array. If DNA sequencing of the PCR product cannot unveil the species responsible for the hybridization, alternative gene loci generally used for molecular taxonomy (e.g., *cox*-I, GAPDH, etc.) could pave the way. The highly pathogenic

piroplasmids *T. annulata* and *T. parva* were not confirmed in the blood samples, although three samples reacted with the corresponding hybridization spots below the cut-off value. Attempts to sequence those inconclusive specimens using primer pairs of species-specific target regions did not bring light to the effective presence of those pathogens. So far, outbreaks with high fatalities are only known in East Africa for *T. parva*, and North Africa for *T. annulata* [23]. By Sanger sequencing of the positively tested animals only *Theileria* species of low pathogenicity were discovered.

Specific probes for the genus *Babesia* were not included in the array because their presence could not be confirmed by PCR in our dataset. Previous infections of *Babesia* spp. may not be detectable by molecular tools as the pathogen can be completely cleared from the blood stream and even from organs [24]. The evidence of *Babesia* in a study from Northern Cameroon [2] could indicate current or very recent infection event in the sampled individuals, allowing its identification on Giemsa stained blood smears.

Reportedly more reliable than the real-time PCR for the detection of new pathogen strains [25], the LCD-Array for TBP can also detect unknown strains or species through conserved oligonucleotide "catch all" probes, representing a whole genus or family. Such amplicons hybridizing with "catch all" probes can be subjected to cloning and DNA sequencing to elucidate their origin. Most generic primer, however, are not able to amplify every variant and/or mutant of the species, genus or family of interest. This limits the detection of all available and yet undetected pathogens [26]. The current microarray was optimized for coverage of as many strains possible of its species or genus reported and deposited in the GenBank repository. Furthermore, the reliance of a species-specific and a genus group-specific probe minimizes the likelihood of false negatives at least on genus level. Since "catch all" probes are efficiently hybridizing with complementary amplicons, a depleting effect can occur if the DNA concentration of the respective pathogen is relatively low (Figure 4). Related to the tested concentration, the species-specific probes were able to hybridize in all cases, sometimes with a weaker intensity (Figure 2: *A. sp.* 'Hadesa'), however with a relatively high copy number. The reason of this discrepancy in comparison to other controls with the same copy number (Figure 2: *T. mutans*) which produce a stronger signal may be optimization issues for the amplification of the *Anaplasma/Ehrlichia* template.

In most of the cases the pathogen in the field-collected sample produced a hybridization signal above the cut-off value hence recognized by the software as a positive pathogen identification. Pathogens showing hybridization with a lower than the cut-off value were considered negative, even if in conformity with the previously obtained Sanger sequencing result. Such cases are better understood when used in a larger sample size. Therefore, recurrent appearance on the LCD-Array below the cut-off value of a doubtful pathogen and its distribution can be an indicator of its presence in the area.

In our sample subset, the inconclusive appearance of *E. canis* below cut-off may be due to the degradation of DNA in the original sample. The cattle samples were collected from April 2014 to June 2015, originally preserved in trehalose solution for transportation [27] and stored at −20 °C between analyses.

No cross reactivity among probes and plasmids were observed in the LCD-Array during testing. A number of the negative samples by gel electrophoresis and Sanger sequencing did not show probe hybridization. Some of the negative samples by PCR show hybridization on the array above the valid cut-off threshold. All field samples tested positive by PCR were confirmed by the LCD-Array being infected with TBPs.

One of the most critical aspects in epidemiological surveillance to avoid false positives and negatives relies on the workflow upstream the LCD-Array or sequencing. From the sampling to the DNA/RNA extraction, appropriate management of the samples is mandatory as inaccurate handling may lead to loss of DNA or contamination [28]. Amplification with Uracil instead of Thymine nucleotides and the addition of Uracil N-glycosylase is one approach to prevent carryover amplicon contamination [29]. Whereas the LCD-Array provided one false negative (*E. canis*), no false positives were confirmed. Optimization of calculation of the cut-off value could reduce the error rate further.

The addition of all three PCR products per sample at the same ratio helped the follow up of the sensitivity and possible cross contamination in case of high copy numbers. Tests using different ratios showed *Anaplasma* being the least sensitive followed by *Rickettsia* and *Theileria* having a higher sensitivity (Figure 2). Consequently, pathogens in low concentration may be overlooked, particularly of *Anaplasma*. This could be improved by protocol optimization or by starting the amplification using a higher template volume (2 or 5 µL) increasing the final concentration. Touch-down PCR program prior to hybridization have showed outstanding results in increasing sensitivity and yield which is of great value as long as the specificity is not hampered [30].

5. Conclusions

The presence of some of the most important non-viral TBPs for livestock on this LCD-Array, including those with zoonotic potential is a valuable asset. In the future, more groups of TBPs including arboviruses or helminths can be added. Although, the production of microarrays with species coverage of 100 and more is possible, the implementation of a running pipeline for diagnostic analyses is more challenging and herein not addressed. With the novel LCD-Array, a sequencing facility which is often lacking in developing countries is not compulsory. Additionally, post-PCR processing times are as short as 45 min, making immediate reporting and response after TBP outbreaks possible. Low- or non-pathogenic species must be incorporated for subsequent identification. Moreover, the better prospect to find endemic or newly introduced species can contribute to the understanding of possible heterologous reactivity responsible of the host health state.

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RESEARCH

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Molecular identification and prevalence of tick-borne pathogens in zebu and taurine cattle in North Cameroon

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Abstract

Background: Public interest for tick-borne pathogens in cattle livestock is rising due to their veterinary and zoonotic importance. Consequently, correct identification of these potential pathogens is crucial to estimate the level of exposition, the risk and the detrimental impact on livestock and the human population.

Results: Conventional PCR with generic primers was used to identify groups of tick-borne pathogens in cattle breeds from northern Cameroon. The overall prevalence in 1260 blood samples was 89.1%, with 993 (78.8%) positive for *Theileria/Babesia* spp., 959 (76.1%) for *Anaplasma/Ehrlichia* spp., 225 (17.9%) for *Borrelia* spp., and 180 (14.3%) for *Rickettsia spp*. Sanger sequencing of a subset of positively-tested samples revealed the presence of *Theileria mutans* (92.2%, 130/141), *T. velifera* (16.3%, 23/141), *Anaplasma centrale* (10.9%, 15/137), *A. marginale* (30.7%, 42/137), *A. platys* (51.1%, 70/137), *Anaplasma* sp. 'Hadesa' (10.9%, 15/137), *Ehrlichia ruminantium* (0.7%, 1/137), *E. canis* (0.7%, 1/137), *Borrelia theileri* (91.3%, 42/46), *Rickettsia africae* (59.4%, 19/32) and *R. felis* (12.5%, 4/32). A high level of both intra- and inter-generic co-infections (76.0%) was observed. To the best of our knowledge, *B. theileri, T. mutans*, *T. velifera*, *A. platys*, *Anaplasma* sp. 'Hadesa', *R. felis* and *E. canis* are reported for the first time in cattle from Cameroon, and for *R. felis* it is the first discovery in the cattle host. *Babesia* spp. were not detected by sequencing. The highest number of still identifiable species co-infections was up to four pathogens per genus group. Multifactorial analyses revealed a significant association of infection with *Borrelia theileri* and anemia. Whereas animals of older age had a higher risk of infection, the Gudali cattle had a lower risk compared to the other local breeds.

Conclusion: Co-infections of tick-borne pathogens with an overall high prevalence were found in all five study sites, and were more likely to occur than single infections. Fulani, Namchi and Kapsiki were the most infected breed in general; however, with regions as significant risk factor. A better-adapted approach for tick-borne pathogen identification in co-infected samples is a requirement for epidemiological investigations and tailored control measures.

Keywords: Tick-borne pathogen, Cattle, Cameroon, *Anaplasma*, *Borrelia*, *Ehrlichia*, *Rickettsia*, *Theileria*

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Background

Tick-borne pathogens (TBPs) have severely impaired livestock productivity worldwide, with an increasing risk for the human population due to their potential zoonotic character [1]. In tropical Africa, ticks are vectors for a large variety of diseases, such as piroplasmoses caused by the protozoans *Babesia* and *Theileria*, bacterial infections with species of the genera *Anaplasma* (anaplasmosis), *Borrelia* (relapsing fever), *Ehrlichia* (heartwater), *Rickettsia* (spotted fever), and also many viral diseases, like Crimean-Congo hemorrhagic fever [2]. These infectious diseases cause considerable losses and diminish the economic value of livestock where the enzootic status remains unstable [2].

In Cameroon, which is one of the main regional providers of beef and other products derived from cattle, the population is dominated by zebu and crossbreeds (European taurine × zebu and African taurine × zebu), with the taurine cattle population at risk of extinction due to widespread and uncontrolled admixture [3]. The main local vectors for TBPs are hard ticks of the genera *Amblyomma*, *Haemaphysalis*, *Hyalomma* and *Rhipicephalus* [4]. Pure *Bos taurus indicus* cattle have been reported less susceptible to TBPs than pure *Bos taurus taurus* cattle, based on attractiveness for the respective tick vectors and/or due to more effective immunological responses [5].

The prevalence of the various TBPs and their interdependences in Cameroon are not well investigated. Most of the studies used conventional microscopy of blood-smears, serology, or *post-mortem* analyses [6, 7] which all have considerable limitations. Identification of individual species of pathogens is almost impossible without the intervention of molecular tools, like PCR. Moreover, studies on the prevalence of the locally available TBPs in Cameroon and in particular on the level of co-infection is scarce. The present study aims to investigate the occurrence of TBPs in the cattle population, including "mild" and "non-pathogenic" conspecifics and their level of co-infection. Furthermore, the level of exposition and infection of different cattle breeds in Cameroon to TBPs, and the potential risk of exposure for the human population is highlighted.

Methods

Study sites and location

The sampling took place from April 2014 (end of the dry season) to June 2015 (middle of the rainy season). A total of 1260 cattle were examined in three different bioclimatic zones in the northern part of Cameroon. The corresponding sites (Fig. 1) were the Adamaoua highlands with 64,000 km^2 of surface, representing the subhumid Guinea savannah biotope, the North with 67,000 km^2, representing the semi-arid Sudan savannah, and the Far North with 34,000 km^2, representing the arid Sahel region. Sampling time was generally in the morning and mostly during the rainy season (April until October). Five sites were visited in the three regions: Vina (n = 396 cattle examined) and Faro et Deo (n =198) in the Adamaoua; Faro (n = 175) and Mayo-Rey (n = 310) in the North; and Mayo Tsanaga (n = 181) in the Far North.

Field work, sampling procedure and DNA isolation

For each herd visited, approximately 10% of the cattle were sampled. Parameters of age in years, sex, breed [Gudali; White and Red Fulani grouped as Fulani; Bokolodji (= Zebu *Bos taurus indicus*); Namchi/Doyao; Kapsiki (= autochtonous *Bos taurus taurus*); Charolais (= European *Bos taurus taurus* and cross-breed)], weight and body condition score (BCS) were taken from each animal. The BCS varied from 1 to 5 according to the fat and muscle appearance: 1–2, poor; 3–4, good; and 5, very good (convex look or blocky). The weight was standardized as recommended by Tebug et al. [8] using the formula LW = 4.81 HG–437.52 (where LW is live weight and HG is thoracic girth measurement in cm). The age was assessed by the dentition [9] and by the information of the herd keeper. Sampled animals were grouped as weaners (1–2.5 years-old), adults (2.5–4.5 years-old), old (4.5–8 years-old) and very old (> 8 years-old).

Approximately 5 ml of blood per animal was collected from the jugular vein in 9 ml ethylene diamine tetra acetic acid (EDTA) treated vacutainer tubes (Greiner Bio-One, Frickenhausen, Germany) and analyzed for packed cell volume (PCV) [10]. Briefly, approximatively 70 µl of collected whole blood was transferred into heparinized micro-hematocrit capillaries and centrifuged for 5 min at 12,000× *rpm* in a hematocrit centrifuge (Hawksley & Sons Limited, Lancing, UK). The solid cellular phase in relation to the liquid serum phase was measured using the Hawksley micro hematocrit reader (MRS Scientific, Wickford, UK). A PCV below the threshold level of 26% was considered anemic. The remaining whole blood was centrifuged at 3000× *rpm* for 15 min. Plasma was collected for immunological studies (not applicable here) and the remaining fraction (red blood cells and buffy coat) was used for DNA isolation.

Samples of 300 µl of the erythrocyte and cellular fraction were purified using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer's instruction. For sample preservation, 50 µl of trehalose enriched 0.1× Tris EDTA (TE) solution (c = 0.2 M, Sigma-Aldrich, Taufkirchen, Munich, Germany) was added as DNA stabilizing preservative in the tube containing the extracted DNA [11], vortexed and spun down. All samples were stored at room temperature

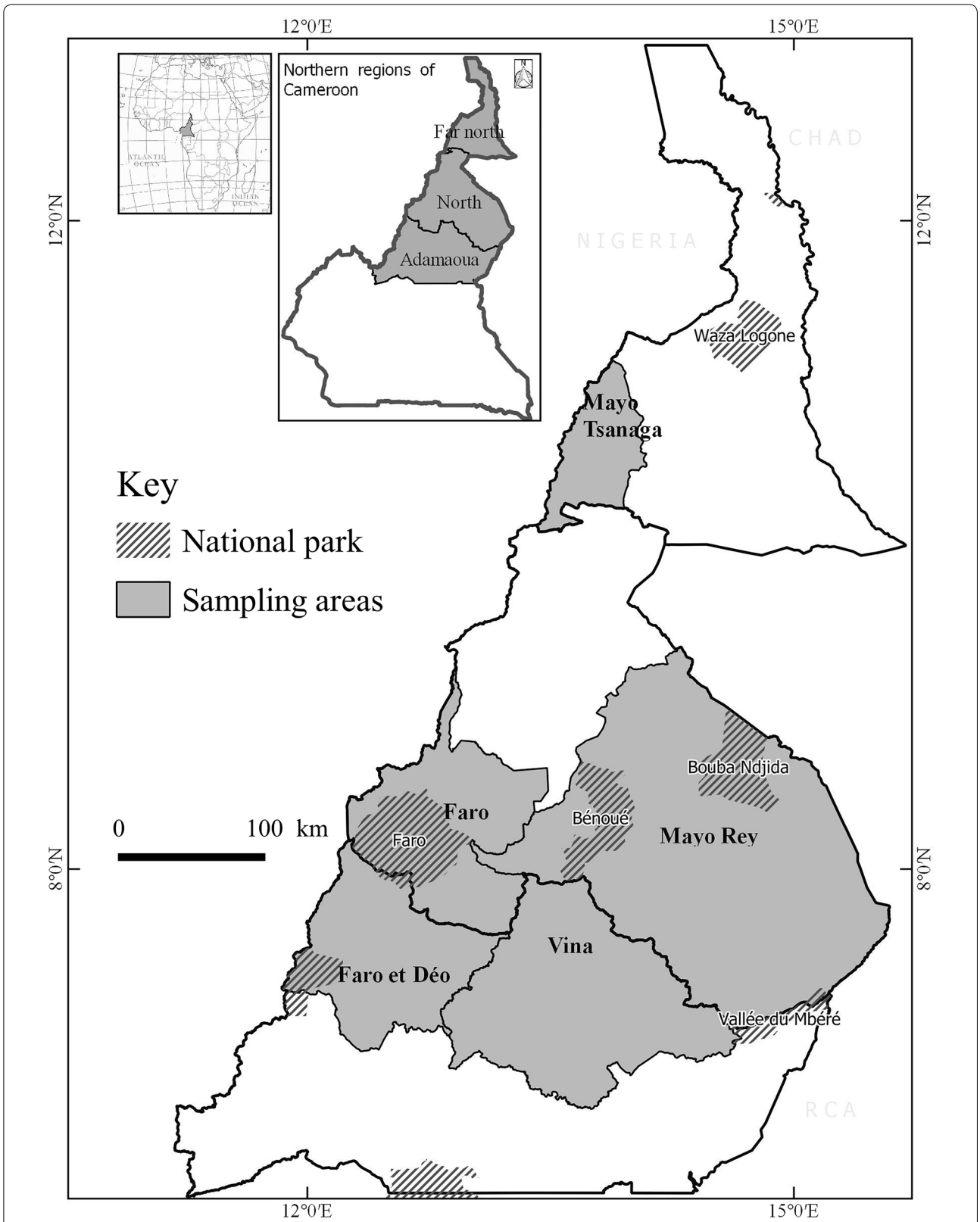

Fig. 1 Sampling areas in the northern part of Cameroon. The Vina and Faro et Deo sites are located in the Adamaoua region, the Faro and the Mayo-Rey in the North and the Mayo Tsanaga in the Far North region. The shaded zones represent the sampling areas and the zones with stripes the national parks

in a dry and light-protected environment after being left to dry at 37 °C. Rehydration was done in the laboratory in Tübingen using 75 µl 0.1× TE buffer at 35 °C for at least 10 min until the pellet was completely resolved, and immediately stored at − 20 °C.

Polymerase chain reaction for tick-borne pathogens

In 25 µl sample reaction tubes, 12.5 µl of the 2× Red-Master Mix (Genaxxon Bioscience, Ulm, Germany) were mixed with the corresponding primer pairs to the final concentration of 1 pmol/µl. One microliter of template DNA and molecular grade water (Sigma-Aldrich) were added to complete the volume at 25 µl. As a negative control, molecular-grade water (Sigma-Aldrich) was used, and positive controls were kindly shared by colleagues from the Freie Universität Berlin, Germany. For the detection of *Borrelia* spp., 1 µl of the first PCR reaction was used as a template for the second amplification in a nested PCR. The corresponding gene loci, primer pairs and annealing temperatures are shown in Table 1.

The PCR cycling conditions were: initial denaturation at 95 °C for 3 min, denaturation at 95 °C for 30 s and elongation at 72 °C for 30 s repeated 35 times, and final elongation at 72 °C for 10 min (MasterCycler EP S Thermal Cycler®, Eppendorf, Hamburg, Germany). All samples were visualized through electrophoresis on a 1.5% agarose gel stained with Midori Green (Nippon Genetics Europe, Düren, Germany). Selected positive reactions were prepared following manufacturer's recommendations (Macrogen, Amsterdam, Netherlands) and sent for sequencing. Obtained sequences were compared to the non-redundant database GenBank (NCBI) using BLASTN (http://blast.ncbi.nlm.nih.gov/) in the Geneious 9.1 software (Biomatters, Auckland, New Zealand).

Phylogenetic tree

Annotated sequences of the same genus and locus were extracted from the GenBank database, and aligned with the MUSCLE algorithm using standard parameters. Maximum Likelihood trees based on the Tamura-Nei model with 1000 bootstrap replications were generated using the software MEGA6 [15]. Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. Furthermore, a discrete Gamma distribution was used to model evolutionary rate differences among sites. The rate variation model allowed some sites to be evolutionary invariable. *Babesia bigemina* was selected as the outgroup for the *Theileria* tree, whereas *Wolbachia pipientis* was the outgroup for both *Anaplasma/Ehrlichia* and *Rickettsia* trees.

Statistical analysis

Descriptive statistics were performed to summarize TBP frequency, percentage, and proportion in study sites and co-infection levels according to region and breed. Multivariate logistic regression (MLG) analysis and descriptive statistics were performed using R v.3.4.2 (www.R-project.org) with the *ISLR* package for the MLG. The association between pathogen acquisition and independent variables were examined by computing the odds ratios (OR), 95% confidence intervals (CI) and *P*-value and using the logit equation in the logistic regression model. Each TBP species was used independently as outcome in separate equations. The other variables (PCV, BCS, age, sex, region and breed) were used as baseline predictors. All cattle breeds with less than 10 sampled individuals and all TBP species with less than 10 infected animals were

Table 1 Selected primer pairs and annealing temperature for the detection of mitochondrial target regions for the genera *Babesia/Theileria*, *Anaplasma/Ehrlichia*, *Rickettsia* and *Borrelia*

Genus	Primer	Target gene	Primer sequence (5′-3′)	Annealing T (°C)	Amplicon size (bp)	References
Babesia/Theileria	RLB-F2	*18S* rDNA	GACACAGGGAGGTAGTGACAAG	57	460–500	[12]
	RLB-R2		CTAAGAATTTCACCTCTGACAGT			
Anaplasma/Ehrlichia	AnaEhr16S_f	*16S* rDNA	AGAGTTTGATCMTGGYTCAGAA	55	460–520	This study
	Ana-Ehr16S_r		GAGTTTGCCGGGACTTYTTC			
Rickettsia	Rick-F1	*16S* rDNA	GAACGCTATCGGTATGCTTAACACA	64	350–400	[13]
	Rick-F2		CATCACTCACTCGGTATTGCTGGA			
Borrelia outer	16S1A	*16S* rDNA	CTAACGCTGGCAGTGCGTCTTAAG	63	1205	[14]
	16S1B		AGCGTCAGTCTTGACCCAGAAGTT			
Borrelia inner	16S2A	*16S* rDNA	AGTCAAACGGGATGTAGCAATAC	56	600–720	[14]
	16S2B		GTTATTCTTTCTGATATCAACAG			

Abbreviation: T, temperature

excluded from the logistic regression. A *P*-value below 0.05 was considered statistically significant.

Results

Cattle breeds examined and sampling sites

A total of 1306 cattle were examined in the three administrative regions of North Cameroon (Adamaoua, North, Far North) of which 1260 blood samples were used for molecular analyses. The different categories sex, age group, breed, region, BCS and PCV, together with the population prevalence of TBPs are summarized in Table 2. Data from seven different groups of cattle breed were gathered, including four zebu breeds Gudali (n = 687), White/Red Fulani grouped as Fulani (n = 116) and Bokolodji (n = 6), two indigenous taurine breeds Namchi/Doyao (n = 181) and Kapsiki (n = 200), cross-breeds (n = 37), and Charolais (n = 27). Most examined animals were female (76.9%). The age ranged from 1 to 16 years and the PCV from 11 to 55%.

Prevalence of TBPs by PCR

The blood samples of all 1260 animals were analyzed for TBP detection by conventional PCR with group-specific primer pairs for *Babesia/Theileria* spp., *Anaplasma/Ehrlichia* spp., *Borrelia* spp. and *Rickettsia* spp. The number of PCR-positive cases was 993 (78.8%) for *Babesia/Theileria* spp., 959 (76.1%) for *Anaplasma/Ehrlichia* spp., 225 (17.9%) for *Borrelia* spp., and 180 (14.3%) for *Rickettsia* spp. (Table 2). Nine hundred and three (80.4%, 903/1123) of all infected cattle were found to carry at least two of the screened pathogen groups, and the overall TBP prevalence was 89.1% (1123/1260) with every individual carrying at least one of the groups described above. The Adamaoua region had an overall prevalence of 87.9% (522/594) for all pathogens combined.

Logistic regression of pathogen acquisition with independent variables

Each of the identified pathogens (n = 7) was used as outcome in a logistic regression analysis. The results are reported in Table 3. Logistic regression analyzing the association of all TBPs as outcome to environmental and health factors highlighted the Kapsiki breed and older age as main risk factors (OR: 1.96, CI: 0.8–0.97, P = 0.01 and OR: 8.8, CI: 2.0–6.2, P = 0.002, respectively).

Table 2 Prevalence of TBPs per screened genera according to PCR results, sex, packed cell volume, body condition score, cattle breed, age and region

Variable	Category	Total	*Anaplasma/ Ehrlichia*	*Borrelia*	*Rickettsia*	*Babesia/ Theileria*
	PCR-positive		959/1260	225/1260	180/1260	993/1260
	Sequenced		187/959	46/225	63/180	167/993
	Identified		146/187	42/46	34/63	141/167
Sex	Female		736/959	166/225	139/180	760/993
	Male		223/959	59/225	41/180	233/993
PCV	≤ 25	114/1148	19/114	28/114	17/114	104/114
	≥ 26	1034/1148	107/1034	146/1034	123/1034	793/1034
BCS	1–2	82/1247	18/82	17/82	1/82	69/82
	3–4	1062/1247	111/1062	188/1062	135/1062	847/1062
	5	103/1247	7/103	17/103	15/103	72/103
Breed	Bokolodji	6/6	5/6	2/6	0/6	6/6
	Charolais	24/27	21/27	8/27	5/27	24/27
	Cross-breeds	35/37	29/37	9/37	2/37	35/37
	Fulani	107/109	97/109	22/109	10/109	107/109
	Gudali	480/590	480/590	88/590	103/590	472/590
	Kapsiki	171/180	171/180	54/180	32/180	169/180
	Namchi/Doayo	156/174	131/174	36/174	27/174	156/174
Age group (yrs)	1–2.5	157/175	152/175	48/175	31/175	157/175
	> 2.5–4.5	361/402	359/402	96/402	74/402	361/402
	> 4.5–8	398/462	376/462	68/462	58/462	398/462
	> 8	77/84	72/84	13/84	17/84	77/84
Region	Adamaoua		462/522	123/522	80/522	466/522
	Far North		171/180	54/180	32/180	169/180
	North		326/421	48/421	68/421	358/421

Table 3 Logistic regression model with all independent variables as exposure and their interaction with odds of being infected by the corresponding TBP species. *P*-values below 0.05 and level of significance are shown in bold

TBP	Region	Age	Sex	PCV	BCS	A.cn	A.H	A.mg	A.pl	B.th	R.af	T.mt	T.vl
A.cn OR	1	0.9	8.9	7.4	3.5		2.7	2.4	6.5	3.0	4.7	2.2	1.2
95% CI	−4.7–0.2	0.6–1.1	0.2–3.9	0.1–3.1	1.7–2.3		na	na	0.02–4.7	0.7–11	na	1.1–5.0	0.9–4.4
P	0.07	0.5	0.8	0.6	0.3		0.9	0.9	0.7	0.09	0.2	**0.002****	**0.002****
A.H OR	1.0	0.9	0.2	< 0.0001	4.3	2.7		2.3	1	1.3	6.7	8.5	5.6
95% CI	0.007–0.7	0.6–1.4	0.03–0.9	na	0.3–43.0	na		na	0.1–6.1	na	Na	1.8–3.7	6.7–5.5
P	**0.04***	0.8	0.05	0.99	0.2	0.9		0.9	0.9	0.9	0.9	**0.003****	**0.0001*****
A.mg OR	3.4	0.9	0.3	1.4	0.4	< 0.0001	< 0.001		0.3	2	0.8	14.8	4.2
95% CI	1.3–9.3	0.7–1.0	0.1–0.9	0.3–4.7	0.05–1.8	na	na		0.02–1.2	0.5–6.7	0.1–4.4	6.4–35.3	0.5–24.1
P	**0.009***	0.3	**0.03***	0.5	0.3	0.9	0.99		0.15	0.2	0.9	**< 0.0001*****	0.1
A.pl OR	1.9	0.8	2	0.9	0.3	1.1	1.2	0.2		1.2	0.7	22.4	2.6
95% CI	0.9–3.9	0.7–0.9	0.8–5.2	0.3–2.4	0.08–1.1	0.1–6.1	0.2–6.7	0.05–0.9		0.4–3.3	0.1–3.0	11.6–4.6	0.5–1.1
P	0.06	**0.02***	0.1	0.9	0.1	0.9	0.8	0.05		0.6	0.6	**< 0.0001*****	0.2
B.th OR	3.5	0.8	1.2	2.9	0.6	2.3	< 0.0001	1.8	1.35		2.1	0.5	1.1
95% CI	2.0–6.2	0.7–0.9	0.8–2.0	1.8–4.6	0.3–1.1	0.5–8.1	na	0.4–5.5	0.4–3.3		0.4–7.6	0.2–1.3	0.2–3.8
P	**< 0.0001*****	**0.003****	0.3	**< 0.0001*****	0.1	0.2	0.9	0.3	0.5		0.2	0.2	0.8
R.af OR	1.7	1	0.4	1	1	3.6	< 0.0001	1	1.1	1.9		8.4	2.06
95% CI	0.5–6.0	0.7–1.2	0.1–1.7	0.2–4.3	0.1–4.9	0.1–34.1	na	0.1–5.1	0.2-–.6	0.3–7.1		2.6–27.9	0.08–1.7
P	0.3	0.8	0.2	0.9	0.9	0.3	0.9	0.9	0.8	0.3		**0.0002*****	0.5
T.mt OR	0.8	1	1	0.4	1.5	12.8	9.3	16.4	21.2	0.6	7.9		6.4
95% CI	0.5–1.4	0.9–1.7	0.5–1.9	0.1–1.0	0.7–3.0	2.0–72.9	2.3–37.0	6.9–39.7	11.1–41.6	0.2–1.5	2.3–2.5		1.6–26.8
P	0.5	0.2	0.9	0.08	0.2	**0.004****	**0.001****	**< 0.0001*****	**< 0.0001*****	0.3	**0.0006****		**0.007****
T.vl OR	0.5	0.8	0.9	2.6	3.2	12.4	23.9	4	3.1	0.9	2	5.3	
95% CI	0.08–3.1	0.6–1.1	0.2–3.6	0.7–9.9	0.6–1.3	2.1–62.9	2.6–223	0.4–2.5	0.6–1.3	0.1–3.5	0.07–17.9	1.0–26.9	
P	0.4	0.3	0.8	0.1	0.1	**0.002****	**0.004****	0.1	0.1	0.9	0.5	**0.04***	

Abbreviations: A.cn, *Anaplasma centrale*; A.H, *Anaplasma* sp. 'Hadesa'; A.mg, *Anaplasma marginale*; A.pl, *Anaplasma platys*; B.th, *Borrelia theileri*; R.af, *Rickettsia africae*; T.mt, *Theileria mutans*; T.vl, *Theileria velifera*; na, not available; OR, odds ratio; CI, confidence interval

Pathogen identification and co-infections

For species identification, 296 of the 1123 PCR positive samples (26.4%) were selected for DNA sequencing, of which 240 (81.0%) could be successfully sequenced. Of these, 78.0% were generated for *Anaplasma*/*Ehrlichia* spp. (146/187), 84.4% for *Babesia*/*Theileria* spp. (141/167), 91.3% for *Borrelia* spp. (42/46), and 53.9% for *Rickettsia* spp. (34/63; Table 2). In total, 12 different species or genotypes were identified by matching with the GenBank database. Ranked after the most prevalent species, these were: *T. mutans*, *A. platys*, *A. marginale*, *B. theileri*, *A. centrale*, *Anaplasma* sp. 'Hadesa', *T. velifera*, *R. africae*, *R. felis*, *Theileria* sp. B15a, *E. ruminantium* and *E. canis*. The phylogenetic ML tree compares those genotypes with database entries from GenBank (Fig. 2a–c).

Co-infections with species of the same genus or group of genera were common. The highest percentage of animals with more than three of the five genera of parasites per individual was found in the Far North region (6.1%), followed by Adamaoua (2.8%) and North region (0.8%). The age was significantly associated to the pathogen acquisition ($P = 0.002$) with older animals being more infected. Kapsiki from the Mayo-Tsanaga division were more infected with TBPs (99.4% per region) than Namchi and zebu breeds from other regions ($P = 0.01$).

Single infections were detected in 264 (24.0%) of the 1123 infected cases. Intra-generic double infections that could still be delimitated to the respective species (Table 4), were most frequent for *T. mutans* + *T. velifera* (60.0%), followed by *A. platys* + *A. marginale* (17.3%), and *A. platys* + *Anaplasma* sp. 'Hadesa' (9.6%). In 45 cases (52%) of intra-generic co-infections, only one species could be identified. The most common inter-generic combinations were of *T. mutans* + *A. platys*, *T. mutans* + *Anaplasma* sp. 'Hadesa', *T. mutans* + *R. africae* and *T. mutans* + *A. marginale*. Gudali breed had less co-infections than Namchi and Kapsiki breeds.

Prevalence of *Anaplasma*/*Ehrlichia* species

PCR-positive samples from the *Anaplasma*/*Ehrlichia* group were found mostly in the Vina site on the Adamaoua Plateau (Table 4). Among the 146 positive sequences, 62.0% represented single infections and 38.0% represented co-infections. Single infections of *E. canis* and *E. ruminantium* were found in the sites Mayo Rey and Faro et Deo, respectively (Table 4). According to the proportions of the identified *Anaplasma*/*Ehrlichia* spp. in all study sites the total prevalence was 36.5% for *A. platys*, 21.9% for *A. marginale*, 7.8% for *A. centrale*, 7.8% for *Anaplasma* sp. 'Hadesa', 0.5% for *E. ruminantium*, and 0.5% for *E. canis*. Infection with *Anaplasma* spp. increases the likelihood of *Theileria* spp. infection and *vice versa* (Table 3). The age appeared being a risk factor for the acquisition of *A. platys*, with older animals being more infected (OR: 0.8, CI: 0.7–0.9, $P = 0.02$, Table 3).

Prevalence of *Borrelia* species

Borrelia pathogens were identified in all studied regions with the Adamaoua having significantly higher prevalence (OR: 3.5, CI: 2.0–6.2, $P < 0.0001$). The only identified species by sequencing was *B. theileri* with an overall prevalence of 17.9%. Gudali breeds were the least infected cattle with statistical support ($P = 0.02$). Younger animals were significantly less infected (OR: 0.8, CI: 0.7–0.9, $P = 0.003$). *Borrelia theileri* infection was significantly associated to anemia (OR: 2.9, CI: 1.8–4.6, $P < 0.0001$).

Prevalence of *Rickettsia* species

Rickettsia spp. were found in all the regions with no statistical difference. Cattle breed and age was not significantly associated to corresponding infected and non-infected groups. At least one individual from all examined breeds was positive for *Rickettsia* spp., except for Bokolodji ($n = 6$) which was excluded from the logistic regression analysis. The two species identified by sequencing were *R. africae* (prevalence 2.8%) and *R. felis* (prevalence 0.6%). For *R. africae*, the presence of *T. mutans* was a contributing risk factor (OR: 8.4, CI: 2.6–26.9, $P = 0.0002$).

Prevalence of *Theileria* species

Theileria mutans and *T. velifera* were detected in all screened regions. Furthermore, a closely related sequence of *T. mutans*, *Theileria* sp. B15a (GenBank: MN120896) has been detected (Fig. 2c). The overall prevalence of *Theileria* spp. was 57.3% for *T. mutans*, 2.7% for *T. velifera*, 0.5% for *Theileria* sp. B15a and 18.4% for *Theileria* spp. identified only to the genus level. *Theileria mutans* was highly associated with a number of TBP co-infections, including *A. centrale*, *A. marginale*, *A. platys*, *Anaplasma* sp. 'Hadesa', *R. africae* and *T. velifera*

(See figure on next page.)

Fig. 2 Molecular phylogenetic analysis of selected genera using rDNA markers by Maximum Likelihood method. Evolutionary analyses were conducted in MEGA6. Black stars indicate sequences generated in the present study. Annotations with asterisks indicate likely misidentifications. **a** *Anaplasma/Ehrlichia 16S* rDNA dataset (357 positions in final dataset) with *Wolbachia pipientis* as the outgroup. **b** *Rickettsia 16S* rDNA dataset (330 positions in final dataset) with *W. pipientis* as the outgroup. **c** *Theileria 18S* rDNA dataset (394 positions in final dataset) with *Babesia bigemina* as the outgroup

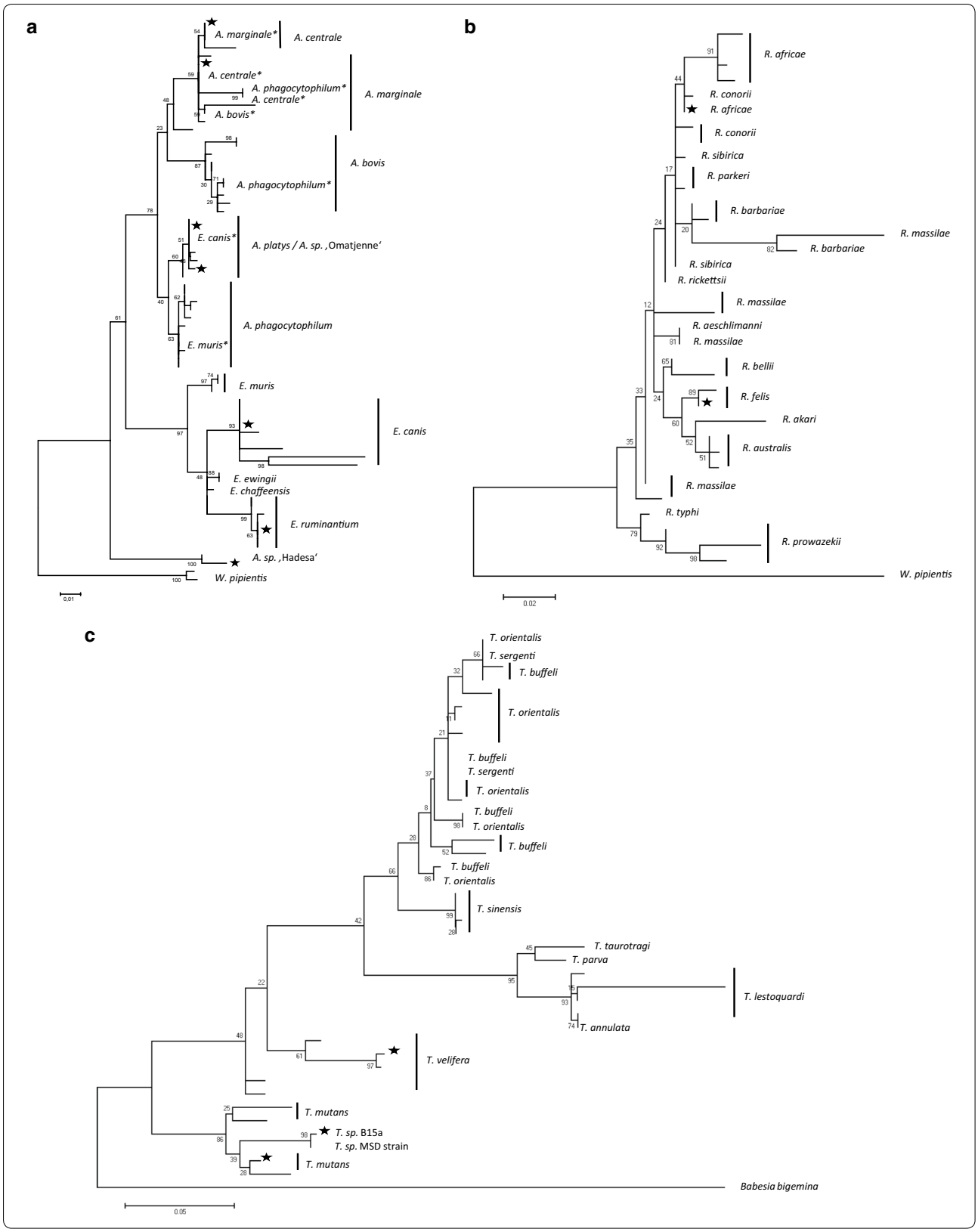
a
A. marginale*
A. centrale
A. centrale*
A. phagocytophilum*
A. centrale*
A. bovis*
A. marginale
A. phagocytophilum*
A. bovis
E. canis*
A. platys / A. sp. ‚Omatjenne'
E. muris*
A. phagocytophilum
E. muris
E. canis
E. ewingii
E. chaffeensis
E. ruminantium
A. sp. ‚Hadesa'
W. pipientis
0,01
b
R. africae
R. conorii
R. africae
R. conorii
R. sibirica
R. parkeri
R. barbariae
R. massilae
R. barbariae
R. sibirica
R. rickettsii
R. massilae
R. aeschlimanni
R. massilae
R. bellii
R. felis
R. akari
R. australis
R. massilae
R. typhi
R. prowazekii
W. pipientis
0.02
c
T. orientalis
T. sergenti
T. buffeli
T. orientalis
T. buffeli
T. sergenti
T. orientalis
T. buffeli
T. orientalis
T. buffeli
T. buffeli
T. orientalis
T. sinensis
T. taurotragi
T. parva
T. lestoquardi
T. annulata
T. velifera
T. mutans
T. sp. B15a
T. sp. MSD strain
T. mutans
Babesia bigemina
0.05

Table 4 Proportion of tick-borne pathogens in cattle blood from North Cameroon determined by DNA sequencing

Species	Positive ($n = 391$)	Proportion (%)[a]	Vina (%)[b]	Faro et Deo (%)[b]	Poli (%)[b]	Mayo-Rey (%)[b]	Mayo-Tsanaga (%)[b]
A. centrale	15	9.8	2 (13.3)	3 (20.0)	1 (6.7)	1 (6.7)	8 (53.3)
A. marginale	42	27.5	6 (14.2)	5 (11.9)	3 (7.1)	21 (50.0)	7 (16.7)
Anaplasma sp. 'Hadesa'	15	9.8	0	5 (33.3)	3 (20.0)	7 (46.7)	0
Anaplasma sp.	11	7.2	4 (36.4)	3 (27.3)	0	6 (54.5)	0
A. platys	70	45.8	20 (28.6)	3 (4.3)	6 (8.6)	33 (47.1)	8 (11.4)
E. canis	1	25.0	0	0	0	1 (100)	0
E. ruminantium	1	25.0	0	1 (100)	0	0	0
Ehrlichia sp.	2	50.0	0	0	0	2 (100)	0
R. africae	19	57.6	4 (21.1)	4 (21.1)	1 (5.3)	8 (42.1)	2 10.5)
R. felis	4	12.1	0	0	2 (50.0)	1 (25.0)	1 (25.0)
Rickettsia sp.	10	30.3	2 (20.0)	3 (30.0)	2 (20.0)	1 (10.0)	2 (25.0)
B. theileri	42	100	22 (52.4)	0	2 (4.8)	7 (16.7)	11 (26.2)
T. mutans	130	81.8	50 (38.5)	16 (12.3)	9 (6.9)	48 (36.9)	7 (5.4)
T. velifera	23	14.5	0	5 (21.7)	5 (21.7)	5 (21.7)	8 (38.1)
Theileria sp.	6	3.8	5 (83.3)	0	0	1 (16.7)	0

[a] Proportion of identified species in the respective group of pathogens

[b] Proportion of pathogen-positive samples per site

(Table 3). Furthermore, the taurine breeds, Namchi and Kapsiki were risk factors for *T. velifera* infection (OR: 9.0, CI: 1.4–64.4, $P = 0.02$) and (OR: 7.4, CI: 1.5–42.3, $P = 0.01$) respectively, as well as for co-infections with *A. centrale* and *Anaplasma* sp. 'Hadesa' (Table 3).

Phylogenetic analysis and genetic distances

Maximum Likelihood trees for the genera *Theileria*, *Rickettsia* and *Anaplasma/Ehrlichia* show the evolutionary relationships of the newly acquired sequences in comparison to published GenBank entries (Fig. 2a–c). Most matched very well with published sequences, but also a new genotype in the clade *A. platys*/*Anaplasma* sp. 'Omatjenne' (GenBank: MN120891), and another unrecorded genotype closely related to *Anaplasma* sp. 'Hadesa' (GenBank: MN124079), were found.

Discussion

Conventional PCR was used to assess the prevalence of circulating tick-borne parasites and bacteria in cattle from Cameroon's most important rearing sites in the northern regions. Four different primer pairs targeting ribosomal RNA loci allowed the identification of six genera of important species of TBPs. To the best of our knowledge, our study provides first molecular proof for the presence of *Borrelia theileri*, *Ehrlichia canis*, *Theileria mutans*, *Theileria velifera*, *Anaplasma* sp. 'Hadesa', *Anaplasma platys* and *Rickettsia felis* in cattle from Cameroon.

Generally, we found a high TBP prevalence, including a high level of co-infection with other TBP species. Many of the identified TBPs in those cattle are of major economic importance in Africa [16], while some are also causing zoonotic infections in humans. The investigated TBPs differed significantly depending on the cattle breed, age and geographical region, where indigenous taurine breeds, older age and the cattle-rich Adamaoua region were the highest risk factors, respectively. Although the detection and identification of co-infections by using generic primers without cloning can be at times challenging, a sample set of the presently identified species was confirmed by a reverse line blot DNA microarray, albeit with a lower detection rate than the microarray [17].

Anaplasma/Ehrlichia group

Anaplasma marginale and *A. centrale* are gram-negative bacteria of the order Rickettsiales, and known to cause bovine anaplasmosis in tropical and subtropical regions [6]. The prevalence in the present study (*A. marginale*: 21.9%, *A. centrale*: 7.8%) was significantly lower than reported in a recent study from North Cameroon with 62.2% and 53.3%, respectively [7], using Giemsa staining. Conversely, our results were higher than reported in the North-West region where the prevalence was 2.2% for *A. marginale* and 0% for *A. centrale*, respectively [6]. The limited mobility of cattle from the 'Centre de Recherche Zootechnique' ranch in the North-West region and possibly better husbandry management [6]

may explain the lower prevalence and TBP diversity in this area. Moreover, transhumance regularly undertaken by cattle holder in the Adamaoua region could explain the diversity of identified *Anaplasma* species, and the observed prevalence variability [18]. Different study results from the same sampling area in the Vina division are best explained by the alternative technical approaches used for identification. In comparison to molecular tools, microscopic analyses of blood smears are used for rapid diagnostic and informative purposes on the animals' health status. In fact, identification by microscopy is prone to errors in species identification, as pathogens may look very similar among and between genera leading to misidentification, or may be missed depending on the animals' patency or developmental status [19]. *Anaplasma marginale* and *A. centrale* are known to be mainly transmitted by ticks of the genus *Rhipicephalus*, in addition to other genera having also been reported as vectors [20]. In Cameroon, *R. appendiculatus* has been identified in the sampling regions as the second most common tick [21], correlating with the high prevalence of these pathogens in the corresponding sites. In our study, sex was significantly associated with the acquisition of *A. marginale*, although with a low odds ratio (OR: 0.3, CI: 0.1–0.9, $P = 0.03$, Table 3).

Anaplasma sp. 'Hadesa' identified in our sample set had been previously identified in blood samples from Ethiopian zebu cattle [22]. The phylogenetic tree grouped our sequence (GenBank: MN124079) to its clade in a relatively high evolutionary distance from other *Anaplasma* and *Ehrlichia* species (Fig. 2a). In our dataset *Anaplasma* sp. 'Hadesa' was inversely correlated with the Adamaoua region, significantly but with low support (OR: 1.0, CI: 0.007–0.7, $P = 0.04$).

Anaplasma platys is known as a canine pathogen, causing cyclic thrombocytopenia in dogs. However, it has also been identified in other mammals including cattle, humans and ticks worldwide [23]. In the present study, it was the most commonly detected *Anaplasma* species (prevalence of 36.5%). Two groups of genotypes were found, one of which had yet no listed entry in GenBank (GenBank: MN120882). The absence of detection of this pathogen in previous studies from Cameroon is very likely due to its misidentification for other TBPs [7]. Furthermore, the clade *A. platys* matched very well with *Anaplasma* sp. 'Omatjenne' (> 99% identity, GenBank: U54806, Fig. 2a), which was first isolated in sheep and *Hyalomma truncatum* ticks from South Africa [24] and later often diagnosed by its corresponding DNA probes used for reverse line blots assay [25]. In the study by Allsop et al. [24], the complete genome of *Anaplasma* sp. 'Omatjenne' (GenBank: U54806) shared 99.9% identity with *Anaplasma* (*Ehrlichia*) *platys* and closely resembled the genome of *E. canis*, most likely due to wrong species annotation [24]. *Rhipicephalus sanguineus* (*sensu lato*) is thought to be the most likely vector of the pathogen which is a tick species already identified in Cameroon [26]. *Anaplasma platys* was identified in 70 specimens of the sequenced subset resulting in a relatively high prevalence (36.5%) in comparison to the records in cattle from Algeria (4.8%) [27], Italy (3.5%) [28] and Tunisia (22.8%) [29]. As a rule, rather than exception, *A. platys* was found in co-infection with other TBPs of the genus *Theileria* with the infection rate increasing with age (Table 3).

Ehrlichia canis is a gram-negative bacterium causing canine monocytic ehrlichiosis in dogs and wild canids; these mammals can serve as a natural reservoir for human infections with *R. sanguineus* ticks as a natural vector in tropical and subtropical areas [30]. *Ehrlichia canis* has also been identified in other *Rhipicephalus* species [31]. Among others, the pathogen has been found in dogs from Cameroon [32], Nigeria, South Africa, Portugal, Venezuela [30]. To our knowledge, the present study provides the first evidence for the ocurrence of *E. canis* in cattle from Cameroon. Only one sample from our sequenced subset ($n = 187$) was identified to be *E. canis*. The infected host was a 2-year-old Gudali female cow from the North region in the Mayo Rey site. In fact, cattle paddocks include space for dogs, chicken and other domestic animals living in close proximity. As for most of the TBPs clinically healthy dogs in the subclinical stage can be carriers of *E. canis* for years [33], facilitating the infection of other susceptible hosts. According to the PCV and the BCS, the animal infected by *E. canis* was not suffering from illness albeit co-infected with *T. mutans*. In our study the *E. canis* strain shared 99.6% identity with the *E. canis* amplicon described in Italy and published under the GenBank accession numbers KY559099 and KY559100 [34] (Fig. 2a).

Ehrlichia (*Cowdria*) *ruminantium* is the etiological agent of heartwater, also called cowdriosis, in domestic ruminants. The evidence of *E. ruminantium* in Cameroon has been clearly demonstrated in cattle carcasses [6] and the tick vector *Amblyomma variegatum* [35]. Only one positive case of *E. ruminantium* could be identified from our samples subset, representing the second molecular evidence of this pathogen in cattle from Cameroon [36]. The prevalence in our data (0.5%), was significantly lower in comparison to the recently published data (6.6%) on cattle blood from the North and Southwest region of Cameroon [36]. The infected animal was a two years old Red Fulani breed from the Faro et Deo division on the Adamaoua plateau. The BCS was within the range characteristic for an asymptomatic animal, and the PCV level (23 %) indicated anemia. The pathogen was found in co-infections with *A. centrale*, *T. mutans*, *B. theileri* and

an unidentified *Rickettsia* sp. The identified strain (GenBank: MN120892) had > 99% sequence identity with the strain 'Welgevonden' as previously described from Cameroonian samples [36].

Babesia/Theileria group

Theileria mutans and *T. velifera* are known as mild to non-pathogenic species in cattle. *Amblyomma variegatum* ticks transmit *T. mutans*, with the vector being endemic in the northern part of Cameroon. Although age has been reported as a risk factor, our study did not show significant associations (OR: 0.1, CI: 0.9–1.7, $P = 0.2$). *Theileria mutans* is known as non-schizont-transforming of the *Theileria* spp. benign group [37]. However, studies have shown that the presence of the piroplasm at high density in red blood cells can cause disease associated to anemia [38]. The present study did not find any significant difference regarding the PCV level (OR: 0.4, CI: 0.1–1.0, $P = 0.08$). The genotype *Theileria* sp. B15a (GenBank: MN120896) detected, formerly isolated from African buffaloes in South Africa, grouped within the *T. mutans* clade (Fig. 2c) indicating it belongs to the same species.

No schizonts have been described for *T. velifera* [37], whose natural host is the African buffalo, found in high numbers in the Waza National park in the Far North region of Cameroon. This may be the reason for the higher *T. velifera* prevalence in the Kapsiki breed, which are the only cattle kept in this area. No highly pathogenic *Theileria* spp. such as *T. parva* and *T. annulata* was detected in the examined animals. This result indicates either its absence in Cameroon, or the presence below detection levels in cattle formerly or presently infected with *T. mutans* and/or *T. velifera*.

Borrelia group

Borrelia theileri is a member of the tick-borne relapsing fever group in contrast to the Lyme borreliosis group [39]. The present study reports for the first time the presence of *B. theileri* in blood samples from cattle in Cameroon. The spirochete bacterium is known to be transmitted to cattle by hard ticks of the genus *Rhipicephalus*, e.g. *R. microplus*, *R. annulatus* and *R. decoloratus* [40]. The pathogen has also been found in *R. geigyi*, however, its capacity as a vector is unknown [40]. Reported cases of tick-borne relapsing fever have been proven responsible for economic losses in livestock [41]. In cattle, *B. theileri* infections have been associated with fever and anemia [41]. In our study area, 17.9% of the studied cattle population was positive for *Borrelia* spp., with *B. theileri* being the only species identified by sequencing.

Furthermore, *B. theileri* was significantly associated with anemia (OR: 2.9, CI: 1.8–4.6, $P < 0.0001$), and present in co-infections with other TBPs in 62% of cases. The highest degree of co-infection comprised *T. velifera*, *T. mutans*, *R. felis*, *A. platys* and *A. centrale.* Similar TBP co-infections excluding *Rickettsia* spp. have been reported [42, 43]. Taurine cattle were significantly more infected than zebu cattle ($P < 0.01$) in line with previously published studies [44], and the difference was significant among age groups with old animals being more infected than their younger counterparts (Table 3). The genotype of *B. theileri* identified in our study (GenBank: MN120889) was 99.9% identical to the strain found in *Rhipicephalus geigyi* from Mali.

Spotted fever *Rickettsia* group

Rickettsia africae is known as the causative agent of African tick bite fever, and has been identified in Cameroon by PCR at a prevalence of 6% from human patients with acute febrile illness without malaria or typhoid fever [35], and at a prevalence of 51% in man from cattle-rearing areas [31]. In previous studies, the pathogen has been identified molecularly in 75% of *A. variegatum* ticks collected from cattle in southern Cameroon [35]. A recent study on ticks collected from cattle in the municipal slaughterhouse of Ngaoundéré in the Adamaoua region in northern Cameroon revealed the presence of *R. africae* among other *Rickettsia* species not identified in our survey [45]. However, the ML tree (Fig. 2b) illustrates the difficulty to clearly distinguish closely related *Rickettsia* spp. when using the *16S* rRNA marker [22]. The genotype of *R. africae* identified in our study (GenBank: MN124096) was 99.7% identical to the strain found in *Hyalomma dromedari* in Egypt and *A. variegatum* in Benin and Nigeria [46].

Rickettsia felis is known as an emerging insect-borne rickettsial pathogen and the causative agent of flea-borne spotted fever [47]. Four out of 34 sequenced *Rickettsia* spp. (11.8%) with a prevalence of 0.6% in the sequenced cattle population were detected. The infected animals were from the North region, more precisely from the Faro, Mayo Rey and Mayo-Tsanaga sites, and were in 75% of cases in autochthonous *B. taurus* breeds. The present study reports for the first time *R. felis* in cattle hosts, with previous identification from fecal samples in chimpanzees, gorillas and bonobo apes from Central Africa, including the southern part of Cameroon at a prevalence of 22% [48]. Furthermore, *R. felis* has been identified in *Anopheles gambiae* mosquitoes [49], and human cases were common in Kenya [50] and Senegal [51]. The strain reported in this study

(GenBank: MN124093) matches at 99.7% identity with the one described in a booklouse from England as rickettsial endosymbiont (GenBank: DQ652592) and in a cat flea from Mexico [52] indicating they are not predominantly transmitted by ticks, even though they have been found before in tick vectors.

Conclusions

In North Cameroon, we identified by sequencing of PCR-amplified rDNA from bovine blood at least 11 species of tick-borne pathogens, some of which are known to be pathogenic to livestock or humans alike. *Anaplasma platys*, *Borrelia theileri*, *Ehrlichia canis*, *Rickettsia felis*, *Theileria mutans* and *Theileria velifera* were identified for the first time in cattle from Cameroon. Furthermore, genuinely new genotype sequences related to *A. platys* and *Anaplasma* sp. 'Hadesa' were discovered. The high pathogen diversity and levels of co-infection in the livestock population is possibly a result from interaction between different host animals (transhumance or contacts between other domestic and wild animals) and their corresponding tick vectors. In addition to the identification of novel TBP species and genotypes, this study shows the necessity of a universally applicable method for TBP identification unbiased by co-infestations with other related pathogens, which appear in more than 75% of the infected cases.

Abbreviations

TBP: tick-borne pathogen; PCR: polymerase chain reaction; PCV: packed cell volume; LW: life weight; GH: thoracic girth; EDTA: ethylene diamine tetra acetic acid; TE: tris-EDTA; NCBI: National Center for Biotechnology Information; BLAST: Basic Local Alignment Search Tool; BCS: body condition score.

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Authors' contributions

BA designed the experiment and method, performed laboratory analyses and drafted the manuscript. BA and AE performed the statistical and phylogenetic analyses. BA, AP, MA and MTK collected samples. BA, AP, MA, MTK, AR and AE contributed to interpretation of the results, wrote and corrected the manuscript. AR and AE supervised and managed the whole study. All authors read and approved the final manuscript.

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Availability of data and materials

The sequences generated during the present study are available in the NCBI GenBank repository under the accession numbers MN120882, MN120888–MN120892, MN120895–MN120896, MN124079, MN124093–MN124096.

Ethics approval and consent to participate

The study has been carried out with the consent of the regional state representatives and traditional authorities from each of the sampling areas. Furthermore, oral consent was given by the cattle owners, herdsmen (who also helped in restraining the animals), and with the participation and approval of the National Institute of Agricultural Research for Development (IRAD) in Cameroon, which is the country's government institution for animal health and livestock husbandry improvement.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Host specificity and Phylogeny of Trichostrongylidae of Domestic Ruminants in the Guinea savannah of the Adamawa Plateau in Cameroon

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Abstract

Gastro-intestinal tracts were examined from thirteen Gudali zebu cattle, ten goats and ten sheep from the Adamawa highland in Northern Cameroon. A total of 28,325 adult helminths were recovered from the abomasa, small and large intestines. Five trichostrongylid genera were identified by their morphology: *Haemonchus*, *Trichostrongylus* and *Oesophagostomum* were predominant in both cattle and small ruminants, whilst *Cooperia* was only found in cattle both in the abomasum and small intestines.

The molecular species identification and the inference of their phylogenetic relationships was based on the analysis of the hypervariable region I of the small subunit 18S rDNA (*SSU*) and the Second Internal Transcribed Spacer (ITS-2) of 408 adult trichostrongylid worms, which were PCR-amplified, sequenced, and compared with available database entries.

Consistent with earlier findings, the *SSU* was invariable within the *Haemonchus* and *Trichostrongylus* genera, confirming the prior classification based on the morphology of the worms, but the ITS-2 was highly inter- and intraspecifically variable and thus allowed to distinguish individual species and to study the haplotype diversity within the different species. In cattle, we report for the first time in Cameroon the presence of two concurrent infesting species of *Haemonchus* (*H. placei* and *H. similis*), together with two species of *Cooperia* (*C. punctata* and *C. pectinata*) and one species of *Trichostrongylus (T. axei*). In goats and sheep, we found one highly polymorphic clade of *Haemonchus contortus* and two *Trichostrongylus* species (*T. axei* and *T. colubriformis*).

When compared with other Trichostrongylidae from different regions of the world and wildlife, the analysis of haplotypes did not indicate any host and geographical isolation, but a very high haplotype diversity among *H. contortus*. These findings illustrate the complexity of trichostrongylid populations in domestic ruminants and suggest grazing overlap between domestic and wildlife hosts.

Key words: 18S rDNA *SSU*, ITS-2, *Haemonchus, Trichostrongylus, Cooperia,* biodiversity, Cameroon, ruminants.

Keys finding

In the guinea savannah of Northern Cameroon we found:

1) *Haemonchus placei* and *H. similis* only in cattle and *H. contortus* only in small ruminants

2) *Trichostrongylus axei* in cattle and in small ruminants but *T. colubriformis* only in small ruminants.

3) *Cooperia punctata* and *C. pectinata* only in cattle.

1. Introduction

Strongylid nematodes represent one of the major radiations of the nematode parasites of vertebrates. Among them, members of the Trichostrongyloidea are of greatest veterinary and economic importance worldwide. This superfamily contains members which live in the abomasum, the small and the large intestine of their artiodactyl hosts and include the genera *Haemonchus*, *Trichostrongylus*, *Cooperia*, *Teladorsagia,* and *Marshallagia* (O'Connor *et al*., 2006). From these five genera, three (*Trichostrongylus*, *Teladorsagia* and *Marshallagia)* can also infect human hosts (Bradbury, 2006).

In Cameroon, *Haemonchus*, *Trichostrongylus, Cooperia* and *Oesophagostomum* are considered to be the most important parasites in domestic animals (Ntonifor *et al*., 2013; Chollet *et al*., 2000; Awa and Achukwi, 2010; Mamoudou *et al*., 2018).

However, species identification, diversity and host specificity have been little addressed. The local livestock production system involves the rearing of sheep and goats, together with cattle in open land pastures often shared by wild animals. This may lead to frequent exposure of these hosts to parasites normally present in the other domestic and game animals.

In our study, we focussed on the genera *Haemonchus*, *Trichostrongylus* and *Cooperia*, found in domestic cattle, sheep and goats in the Guinea savannah of the Adamawa highland in Northern Cameroon. Four species of *Haemonchus*, namely *H. contortus*, *H. placei*, *H. similis* and *H. longistipes*, two species of *Trichostrongylus* (*T. colubriformis* and *T. axei*) and five *Cooperia* spp. (*C. pectinata*, *C. curticei*, *C. oncophora*, *C. spatulata* and *C. punctata*) have been described to infect domestic and wild ruminants worldwide (Achi *et al*., 2003; Amarante *et al*., 1997; Horak *et al.*, 2004).

In Cameroon, *H. placei, H. contortus, T. axei* and *C. pectinata* and C. *punctata* have been reported both in cattle and small ruminants (Ntonifor *et al*., 2013; Chollet *et al*., 2000). The host preferences, specificity, and the degree to which different trichostrongylid species

95 interbreed is still a matter of debate. Furthermore, in the areas where wild and domestic
96 herbivores share grazing land, host switching may occur (Durette-Desset *et al*., 1994, Hoberg
97 *et al*., 2001).
98 The African Guinea savannah harbours the worldwide highest diversity of wild ruminants
99 belonging to the Antilopinae and Bovinae superfamilies of Bovidae (Owen-Smith and
100 Cumming, 1993). All known *Haemonchus* species have been described from these wild
101 animals (Hoberg *et al*., 2004). In particular, *H. contortus* and *H. placei* have been recorded
102 co-infecting domestic ruminants and buffaloes in Brazil (Brazil *et al*., 2012), Pakistan (Ali *et*
103 *al*., 2018) and giraffes in Florida (Garretson *et al*., 2009). Therefore, nematode host-switches
104 and host dispersal of *Haemonchus* spp. and other Trichostrongylidae may occur frequently
105 and contribute considerably to the genetic diversity among the populations globally.
106 This high genetic diversity could also be an indication of an unrecognized assemblage of
107 cryptic species (Hoberg *et al*., 2004). For instance, among the twelve-recognized species of
108 *Haemonchus,* only *H. placei*, *H. contortus*, and *H. longistipes* have been extensively studied.
109 The taxonomy is in part still uncertain because cryptic species may not have been recognized
110 or populations of the same species may have been wrongfully separated. For instance, the
111 separation of *H. horaki* (in grey reedbuck) and *H. okapiae* (in giraffe) from *H. contortus*
112 (from domestic ruminants) is doubtful (Gibbons, 1979).
113 Morphological criteria of adult worms were used to describe the various genera within the
114 trichostrongylid family, and those included the measurement of the male bursa and the two
115 spicula, differences in the synlophe length and pattern, the reproductive system, and the shape
116 of the posterior end of the female worms (Lichtenfels, 1977; Lichtenfels *et al*., 1994; Jacquiet
117 *et al*., 1997). However, interbreeding between the closely-related species clearly mitigates the
118 morphology and makes morphometric identification difficult or impossible (Le Jambre, 1981;
119 Isenstein, 1971; Chaudhry *et al.*, 2015).

120 Therefore, and also with the aim of identifying eggs and juvenile stages, PCR amplification
121 of selected genomic DNA fragments followed by sequencing has proven to be most useful for
122 categorizing closely-related nematodes (van Megen *et al.,* 2009; Floyd *et al.*, 2002). The 18S
123 rDNA (*SSU*) is a highly conserved sequence of the nuclear DNA among eukaryotic
124 organisms coding for the small subunit of the ribosomes and is about 1,700 base pairs in
125 length. Within the *SSU* four hyper-variable regions (HVRI to IV) were described, which in
126 nematodes tend to differ between species and genera but are frequently fairly constant within
127 one species rendering the *SSU* popular for taxonomy and phylogenetic studies (De Ley and
128 Blaxter, 2004; Blaxter *et al.*, 1998; Eberhardt *et al*., 2007).
129 Much in contrast to the rather slowly evolving 18S sequence, the internal transcribed spacers
130 (ITS-1 and ITS-2), which separate the coding units for the three ribosomal RNAs in the
131 nuclear rDNA are not part of the functional ribosome and are therefore subject to rather
132 frequent mutation. Consequentially, these highly variable regions frequently differ within
133 species and are suitable for distinguishing between very closely related species or sub-species
134 (Chilton *et al.*, 1995; Stevenson *et al.*, 1995; Zarlenga *et al.*, 1994; Brasil *et al*., 2012).
135 Control of trichostrongylid parasites is dependent upon the use of broad-spectrum
136 anthelminthic drugs, with the development of resistance threatening its sustainability
137 (Jackson, 1993; Skuce *et al*., 2010). In Cameroon, Ndamukong and Sewell (1992) observed
138 resistance to benzimidazole in sheep and goats in North-West Cameroon (IRAD Mankon).
139 Therefore, correct identification of the prevailing species, as well as understanding their
140 epizootiology, population structure and genetic diversity is particularly important for the
141 study of anthelmintic resistance and associated genes (Gilleard, 2006; Amarante *et al*., 2011).
142 In this study, we isolated individual worms of the genera *Haemonchus*, *Trichostrongylus* and
143 *Cooperia* from zebu cattle, sheep and goats in the Guinea savannah near the city of
144 Ngaoundéré. Their *SSU* HVRI and ITS-2 sequences were analysed to infer the species

infecting domestic ruminants and to determine the most reliable sequence for species identification.

1. Materials and methods

2.1. Study area and samples collection

2.1.1. Study area

The Adamawa highlands are located between 6°-8° N and 11°-16° E and cover an area of approx. 65,700 km^2. Ngaoundéré is situated centrally and is the regional capital. The vegetation is of the Guinea savannah ecotype with a rainy season from March/April until October (average annual rainfall 1,400 - 1,700 mm, Letouzey**,** 1969). The mixture of forest and grassland provides a habitat for a large number of wildlife species. Several national parks and game reserves surround the area: The Mbam and Djerem National Park (121 km to the SW), the Benoue National Park (100 km N), the Bouba Ndjida National Park (200 km NE) and the Faro national park and game reserves (70 km NW).

Adamawa is the cradle of livestock production in Cameroon, especially for cattle. The extensive livestock farming system with some transhumance during the dry season is dominantly practiced by pastoralists. Goats and sheep are kept everywhere in the country under extensive production system where they are allowed to graze freely during the day in the dry season with tethering during the rainy season. The sheep owned by pastoralists are kept with cattle and graze on the same pasture.

2.1.2. Collections of adult trichostrongylid worms

Thirty-three gastro-intestinal tracts (GI) were collected, of which thirteen originate from adult female Gudali short-horn zebu cattle (*Bos indicus*). Ten GI came from West African Dwarf

goats and ten from Djallonke sheep slaughtered in Ngaoundéré**.** These animals originate from different local animal markets of the Adamawa region**.** All animal carcasses were further processed for the local meat consumption. Immediately after slaughtering of the animal, the two ends of the abomasum, small intestine and large intestine were sealed separately by ligation with a thin rope. Each abomasum, small intestine and large intestine was processed separately. The samples were brought to the Programme Onchocercoses laboratory in Ngaoundéré, sliced open and the content washed off with tap water. The mucosa was carefully examined and washed to remove any adhering worms. The collected contents were passed through sieves of 200 and 100 µm diameter, respectively. Collected nematodes were separated under a dissecting microscope into groups according to their length and shape and transferred into clean petri dishes containing phosphate buffered saline (PBS). They were later identified to their genus and/or species as described by Hansen and Perry (1990). A proportion of 20% of the female trichostrongylid worms belonging to the genera *Haemonchus*, *Trichostrongylus* and *Cooperia* were randomly selected for molecular analysis and preserved in 95% ethanol and stored at -20^{o}C until DNA extraction was performed.

2.2. Molecular analysis

2.2.1. Single worm lysis

At the Max Planck Institute for Developmental Biology in Tübingen, the worms were individually placed in 0.2 ml tubes and prepared for PCR analysis as described by Hildebrandt *et al.* (2012). Briefly, single worms were transferred into 0.2 ml PCR tubes containing 10 µl H_2O and three times freeze-thawed using dry ice, with vigorous vortexing in between. 10 µl of 2×lysis buffer (20 mM Tris-HCL pH 8.3, 100 mM KCl, 5 mM $MgCl_2$, 0.9% NP-40, 0.9% Tween 20, 0.02 % gelatine, 240 µg/ml proteinase K) were added and the

194 mixture incubated at 65°C for 8 hours, followed by 95°C for 15 minutes to inactivate the
195 proteinase K. If samples could not be processed immediately, they were stored at -20°C.
196
197 **2.2.2. Single worm genotyping**
198 The HRVI of the *SSU* and *ITS-2* nuclear regions were PCR amplified as described by
199 Eberhardt *et al.* (2007) and Chaudhry *et al.* (2015), respectively, with some modifications.
200 Briefly, PCR was performed in 25 µl final volume composed of 2 µl of DNA template, 2.5 µl
201 of 10x ThermoPol reaction buffer (New England BioLabs), bovine serum albumin (10
202 mg/ml), 0.5 µl of dNTPs mix (2 mM), 0.5 µl of 10 pmol/µl of each primer, 0.2 µl of 0.06 U
203 Taq DNA polymerase (New England BioLabs) and 18.2 µl of nuclease free water. The
204 primers used and the cycling conditions are given in Table 1. The reactions were performed
205 in an automated thermocycler (Biometra T professional gradient Thermocycler, 2013 model).
206 Five µl of each PCR product was loaded on agarose gel and stained with ethidium bromide to
207 confirm the presence of a PCR product prior to sequencing. The sequencing reactions were
208 performed using the BDTv3.1 kit (Applied Biosystems) following the manufacturer's
209 instructions in 10 µl of final volume which consisted of 0.5 µl PCR product, 2 µl of 5x
210 buffer, 0.3 µl of BDT and 1 µl of primer mix (10 pmol/µl). ITS-2 fragments were sequenced
211 from both ends using the PCR primers, whereas the regions around the SSU HVRI was
212 sequenced using the internal sequencing primer RH4503. The samples were submitted to the
213 in-house genome centre at the Max Planck Institute for Developmental Biology for
214 electrophoretic analysis and base calling.
215
216 **2.3. Genotype analysis**
217 Each chromatogram returned from the sequencing facility was visually evaluated for quality
218 and ambiguous positions were manually edited with the corresponding ambiguity code.

Sequences of low chromatographic quality were excluded from the analysis. For the *SSU* HVRI we considered a fragment corresponding to position numbers 57 - 516 in the GenBank entry L04152, and for the *ITS-2* the entire fragment, which is variable in length (in GenBank entry JF680983 *H. contortus* 231 bp, positions 614-844) because these fragments could be reliably determined by PCR and sequencing primers specified above. This resulted in three different *SSU* HVRI sequences and 62 different *ITS-2* sequences. If a sequence contained ambiguous positions, this sequence was considered to be different from sequences with unambiguously one of the two different nucleotides at the respective position. Each *ITS-2* sequence was used as query in a BLASTn search against the non-redundant nucleotide databases. The search was performed at the National Center for Biotechnology Information NCBI (https://blast.ncbi.nlm.nih.gov) in August 2019. For each sequence, the most similar sequence in the databases was retrieved. If multiple sequences were equally similar, one entry was selected unless equally similar sequences were supposed to be derived from different species. In this case one entry for each species was selected. There were two such cases: first, KX829170 [*Haemonchus contortus*] and X78812 [*Haemonchus placei*], which were 100 % identical over the region considered, and with our worm number 30 and second, KY741868 [*Cooperia pectinata*] and KT215383 [*Cooperia oncophora*], which both differed only at one position from our worms in cluster 14.

2.4. Phylogenetic analysis

All alignments and phylogenetic analyses were done using the MEGA 7.0 software package (Kumar *et al*., 2016) using default settings. All figures shown are based on alignments using muscle and tree reconstruction using the neighbour joining method. The trees were evaluated by 1000 bootstrap repetitions. For a more detailed, tree specific description, see figure legends. As a control, alignments were also done with Clustal W and trees were also

reconstructed using the maximum parsimony and the minimal evolution methods. All interpretations described in this paper are also valid for these trees.

Nucleotide sequence data reported in this paper are available in the National Center for Biotechnology Information NCBI GenBank™ databases under the accession numbers: [The submission process is still ongoing but all sequences will be submitted upon acceptance of the manuscript] and are also available in Suppl. File 2.

3. Results

3.1. Morphological identification of gastro-intestinal worms

At total of 28,284 worms were recovered from the abomasa, small and large intestines of cattle, sheep and goats and determined to the genera level by their morphology (Table 2). In cattle *Haemonchus* (n = 9,956), *Trichostrongylus* (n= 4,054), *Cooperia* (n= 3,179) and *Oesophagostomum* (n= 1,563) were the most abundant nematodes, while in goats and sheep, *Haemonchus* (n= 6,102) and *Trichostrongylus* (n= 3,317) predominated.

3.2. Molecular identification of trichostrongylid worms

3.2.1. Molecular analysis of the *SSU* HVRI

Because trichostrongylid worms were consistently found in the abomasa of the three groups of domestic animals, we focussed on worms from this anatomical part only to molecularly identify their species. We selected arbitrarily adult female specimens of 120 *Haemonchus* spp., 40 *Trichostrongylus* spp. and 48 *Cooperia* spp. from cattle for the amplification of a portion of the small subunit 18S rDNA (*SSU*). We used the primers SSU18A (RH5401) and SSU26R (RH5402), previously adopted in a number of nematode molecular taxonomic studies (*i.e.* Blaxter *et al.*, 1998; Dorris *et al.*, 2002; Herrmann *et al.*, 2006; Eyualem and Blaxter, 2003; Floyd *et al.*, 2002) based on their higher reliability.

The amplification was successful for all the 120 *Haemonchus* spp. and the 40 *Trichostrongylus* spp. and unsuccessful for all the 48 *Cooperia* spp. highlighting the unsuitability of the given primer for to the *Cooperia* genus. All *Haemonchus* spp. sequences were 100 % identical with the sequences EU086374, DQ503465 and L04152 describing *H. contortus*, *Haemonchus* spp. and *H. similis*, respectively, which do not differ in the selected *SSU* target region (Smythe *et al*., 2006; Garretson *et al*., 2009; Zarlenga et *al.*, 1994). The sequences of all *Trichostrongylus* worms were 100 % identical with *T. colubriformis* (AJ920350) as previously reported by Chilton *et al*. (2006). There was no *T. axei* sequence available from the databases for comparison. Since these results confirmed that the *SSU* is not suitable for distinguishing different species within the genera *Haemonchus* and *Trichostrongylus*, we did not determine the *SSU* of the sheep and goat derived worms but concentrated on the *ITS-2* sequences.

3.2.2. Molecular analysis of the *ITS-2*

Overall, we identified 17 different *ITS-2* sequences from *Cooperia* spp., 11 different sequences from *Trichostrongylus* spp. and 34 different sequences from *Haemonchus* spp. For each of these sequences we retrieved a highest scoring BLAST hit and included the corresponding sequence in the analysis (for details see Materials and Methods). Neighbour joining grouped these sequences into three very highly supported groups, corresponding to the respective genera, thereby confirming our morphological identification (Suppl. Fig. 1). Below we present and discuss the results for the three genera separately.

3.2.2.1. *Cooperia*

From a total of 58 successfully sequenced *Cooperia* spp. (all from zebu cattle) we obtained 17 different sequences, each present in one to 14 different worms (Fig. 1). The sequences fell

294 into two highly supported groups. One consists of only one sequence present in two worms
295 and its best BLAST hits. Those were multiple identical database entries considered to be
296 derived from *C. pectinata* or *C. oncophora*. We consider the species description in KT215383
297 likely to be false, because when KY741866 was used as query for a BLAST search, six
298 sequences with >99 % identity and a species annotation were identified. Five of them were
299 annotated as *C. pectinata* and the sixth was KT215383 [C. *oncophora*]. There were no hits
300 with identities between 99.1 % and 93.4 %. Other *C. oncophora* annotated entries matched at
301 93.4 % identity or lower. From this we conclude that our two worms in this group belonged
302 to the species *C. pectinata*. All other sequences belonged to the other groups where the best
303 BLAST hits were annotated as derived from *C. punctata* or from *C. spatulata*. A recent study
304 (Ramünke *et al.*, 2018) showed that these two taxa cannot be distinguished using *ITS-2* and
305 proposed that they are actually the same species, namely *C. punctata*. Taken together, of the
306 58 *Cooperia* spp. we isolated from abomasa of zebu cattle two (3.4 %) were *C. pectinata*
307 while 56 (96.6 %) belonged to the species *C. punctata*.
308
309 **3.2.2.2. *Trichostrongylus***
310 From a total of 47 successfully sequenced *Trichostrongylus* spp. (19 from sheep, 20 from
311 goat, 12 from zebu cattle) we obtained 11 different sequences, each present in one to 8 worms
312 (Fig. 2). The sequences fell into two well-supported groups with best BLAST hits annotated
313 as *T. axei* and *T. colubriformis*, respectively. While all worms isolated from zebu fell into the
314 *T. axei* group, small ruminants carried both species. From sheep, 15 were *T. colubriformis*
315 and 4 were *T. axei.* From goats, 14 were *T. colubriformis* and 6 were *T. axei.* Grouping the
316 small ruminants together, 29 (74.4 %) were *T. colubriformis* and 10 (25.6 %) were *T. axei*.
317
318 **3.2.2.3. *Haemonchus***

From a total of 85 successfully sequenced *Haemonchus* spp. (14 from sheep, 4 from goat, 67 from zebu) we obtained 34 different sequences, each present in one to 39 worms (Fig. 3). All sequences from zebu cattle fell into one of two highly supported groups while all sequences from small ruminants fell into a third group with moderate boot strap support. The first group consists of four sequences representing 45 worms isolated from zebu cattle and their best BLAST hits. Except for KX78812 these best hits were annotated as derived from *H. placei*. Given the rest of the tree in Fig. 3, we consider the annotation of KX829170 as *H. contortus* to be likely false. The second group consists of 17 sequences representing 22 worms isolated from zebu cattle and their best BLAST hits, both of which are annotated as *H similis*. Taken together, we found two species of *Haemonchus* in zebu cattle, namely *H. placei* (45 worms = 67.2 %) and *H. similis* (22 worms = 32.8 %). All 13 sequences representing 18 worms derived from small ruminants had best BLAST hits annotated as *H. contortus* indicating that only this species was present among our samples from sheep and goats.

4. Discussion

To the best of our knowledge this data is the first molecular report of the trichostrongylid species prevalent in the Guinea savannah of Central Africa. Our results confirm that the widely used assay of amplification and sequencing of the *SSU* HVRI with the primers RH5401 and RH5402 which are identical with the primers SSU18A and SSU26R (Floyd *et al.,* 2002) is a reliable molecular marker to identify *Haemonchus* spp. and *Trichostrongylus* spp. present in the abomasa of zebu cattle in Northern Cameroon to the genus but not the species level. On the other hand, this primer set does not work for *Cooperia* spp. It is well known for the genus *Haemonchus* and for other nematodes that closely-related species sometimes do not differ in their *SSU* HVRI (Eyualem and Blaxter, 2003; Herrmann *et al.,* 2006; Garretson *et al*., 2009). Therefore, we turned to the *ITS-2* as marker.

In our samples, we identified two species of *Trichostrongylus*, namely *T. colubriformis*, which was restricted to the small ruminants and *T. axei*, which was present in cattle but also in sheep and goats. The most common sequence in cattle was also present in nine out of the ten *T. axei* we found in small ruminants, suggesting that in our study area small ruminants and cattle are exposed to the same population of *T. axei*. Therefore, it would be expected that these animals are also exposed to the same pool of infective larvae of other trichostrongylid parasites. Nevertheless, *T. colubriformis* and all species of *Haemonchus* and *Cooperia* we found were restricted to either cattle or small ruminants and therefore appeared to be host specific. *C. pectinata* and *C. punctata* / *C. spatulata* (according to Ramünke *et al.*, 2018 likely the same species) as well as *H. placei* and *H. similis* were restricted to cattle, while *H. contortus* was found only in small ruminants.

Conclusions

In the present study, sequence variations of 18SSU and ITS-2 genes were used to identify species of *Haemonchus*, *Trichostrongylus* and *Cooperia* infecting domestic animals in the Guinea savannah of the Adamawa highlands, Cameroon. The HVRI containing *SSU* fragment successfully identified the genera *Haemonchus* and *Trichostrongylus*, but failed in the identification of *Cooperia*. The high conservation of this locus within the genera being a reason for not allowing the species separation. The ITS-2 sequence in contrast has more discriminative power. Based on this sequence and by comparison with database entries, we conclude that in cattle of our study area, *T. axei* is the predominant if not the only prevailing species of *Trichostrongylus*. There are also two species of *Haemonchus* present, namely *H. placei* and *H. similis*, and the large majority of *Cooperia* worms are *C. punctata* with at least one additional species, presumably *C. pectinata*. In goats and sheep, *H. contortus, T. axei* and *T. colubriformis* are the common trichostrongylids.

The higher diversity found in *H. contortus* and the lack of geographical and host species isolation suggests that this parasite is freely shared between *Caprinae* and wildlife host species. On the other hand, *H. placei* and *H. similis* are shared between domestic and wild Bovidae. Therefore, it will be important to examine the abomasal parasites in local game animals to get a better insight into the epizootiology of trichostrongylid nematodes in Central Africa.

Ethics statement

This study was approved by the Scientific Directorate of the Institute of Agricultural Research for Development. Abomasa were collected at the local slaughterhouses of Ngaoundéré by veterinarians and well-trained personnel after the animals had been slaughtered as part of the normal operations of the abattoir. The meat of these animals was processed for human consumption. Five of the gastro-intestinal tracts originating from cattle came from the *post-mortem* analysis of animals from a DFG-funded research project (DFG-COBE) which died from severe dermatophilosis infection.

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Declarations of interest:

The authors declare no conflict of interest.

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558 **Table 1**. **Primers and PCR programs**

559

560

Table 2. Morphological identification and distribution of trichostrongylid adult worms in gastrointestinal tracts of cattle, goats and sheep

Figure Legends

Figure 1: Neighbour joining tree of the *Cooperia ITS-2* sequences from the worms isolated in this study and selected data base entries.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 12.93701172 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). 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. The evolutionary distances were computed using the number of differences method (Nei and Kumar, 2000) and are in the units of the number of base differences per sequence. The analysis involved 18 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 241 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016). Nomenclature: sequences retrieved from the data bases are indicated by their accession numbers followed by the species as listed in the corresponding data base entry; sequences obtained in this study are labelled with their worm number (contains the host in which the worm was found) followed by the genus, the worm had been assigned to, based on morphology. If a sequence was found in multiple worms from the same host this is referred to as a cluster, which is defined by one randomly selected worm with this sequence, the cluster number and in the number of worms with this sequence. The following labels were too long to be displayed in the figure:

*Cooperia punctata: KT215380.1, KP150445.1, MH267766.1, MH267767.1; Cooperia spatulata: MH267786.1, MH481606.1, MH481607.1,MH481608.1

°Cooperia pectinata: KY741866.1, MH267780.1, MH267781.1

Figure 2: Neighbour joining tree of the *Trichostrongylus ITS-2* sequences from the worms isolated in this study and selected data base entries.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 8.88720238 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). 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. The evolutionary distances were computed using the number of differences method (Nei and Kumar, 2000) and are in the units of the number of base differences per sequence. The analysis involved 13 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 239 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016). The nomenclature is as in Fig. 1. The following label was too long to be displayed in the figure: +SheepWorm-2F6 Trichostrongylus Cluster 1 (3 worms) and GoatWorm-1F11 Trichostrongylus Cluster 1 (6 worms) and ZebuWorm-79 Trichostrongylus Cluster 3 (6 worms)

Figure 3: Neighbour joining tree of the *Haemonchus ITS-2* sequences from the worms isolated in this study and selected data base entries.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 29.95686680 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to

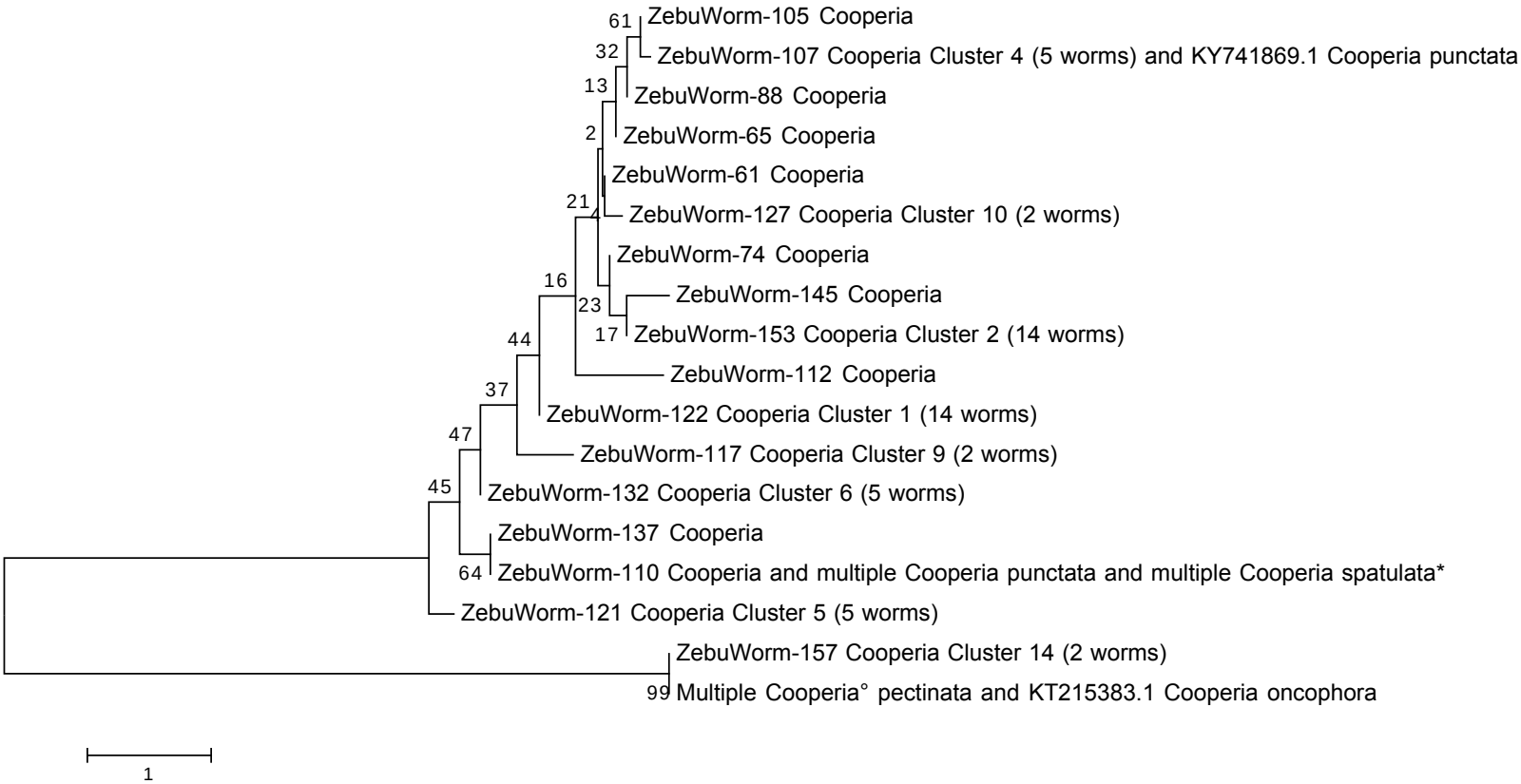
scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method (Nei and Kumar, 2000) and are in the units of the number of base differences per sequence. The analysis involved 36 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 241 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016). The nomenclature is as in Fig. 1.

Suppl. Figure 1: Neighbour-joining tree of all ITS-2 sequences from this study and their highest BLAST hits.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 107.99475850 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). 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. The evolutionary distances were computed using the number of differences method (Nei and Kumar, 2000) and are in the units of the number of base differences per sequence. The analysis involved 67 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 260 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). Nomenclature: sequences retrieved from the data bases are indicated by their accession numbers followed by the species as listed in the corresponding data base entry; sequences obtained in this study are labelled with their worm number (contains the host in which the worm was found) followed by the genus, the worm had been assigned to, based on

morphology. If a sequence was found in multiple worms from the same host this is referred to as a cluster, which is defined by one randomly selected worm with this sequence, the cluster number and in the number of worms with this sequence. The following labels were too long to be displayed in the figure.

Suppl. File 2: FASTA file with all newly described sequences.


61
ZebuWorm-105 Cooperia
32
ZebuWorm-107 Cooperia Cluster 4 (5 worms) and KY741869.1 Cooperia punctata
13
ZebuWorm-88 Cooperia
2
ZebuWorm-65 Cooperia
ZebuWorm-61 Cooperia
21
ZebuWorm-127 Cooperia Cluster 10 (2 worms)
ZebuWorm-74 Cooperia
16
ZebuWorm-145 Cooperia
23
17
ZebuWorm-153 Cooperia Cluster 2 (14 worms)
44
ZebuWorm-112 Cooperia
37
ZebuWorm-122 Cooperia Cluster 1 (14 worms)
47
ZebuWorm-117 Cooperia Cluster 9 (2 worms)
45
ZebuWorm-132 Cooperia Cluster 6 (5 worms)
ZebuWorm-137 Cooperia
64
ZebuWorm-110 Cooperia and multiple Cooperia punctata and multiple Cooperia spatulata*
ZebuWorm-121 Cooperia Cluster 5 (5 worms)
ZebuWorm-157 Cooperia Cluster 14 (2 worms)
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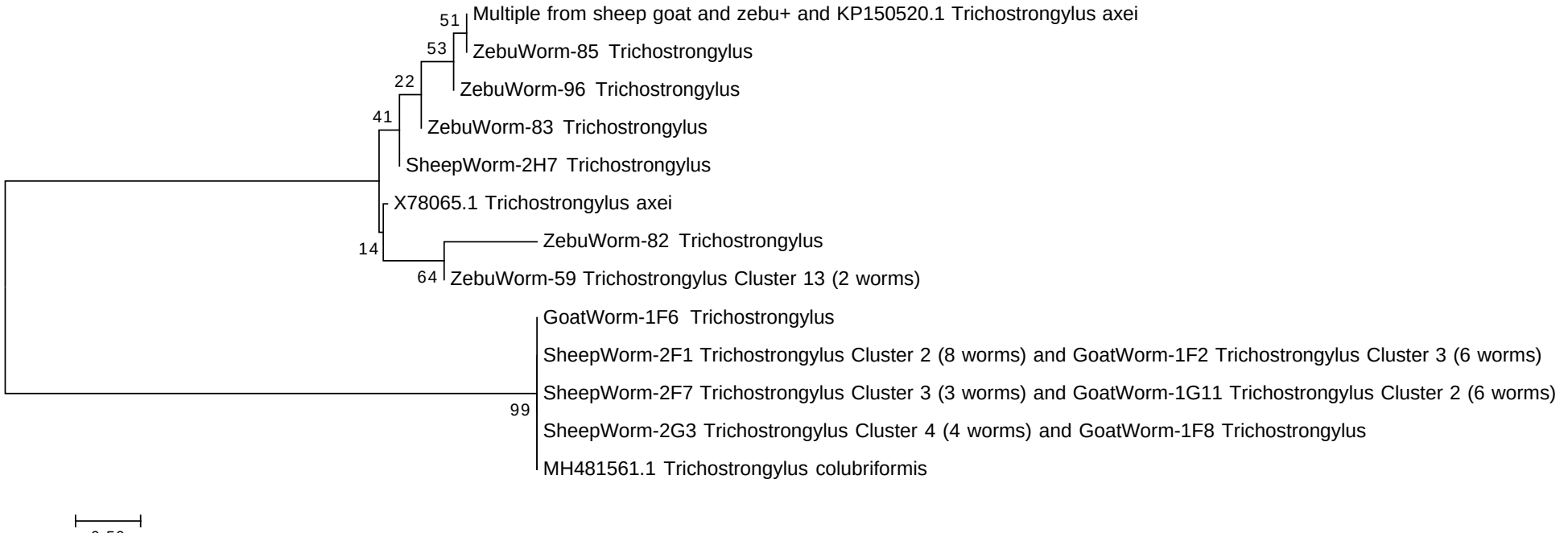
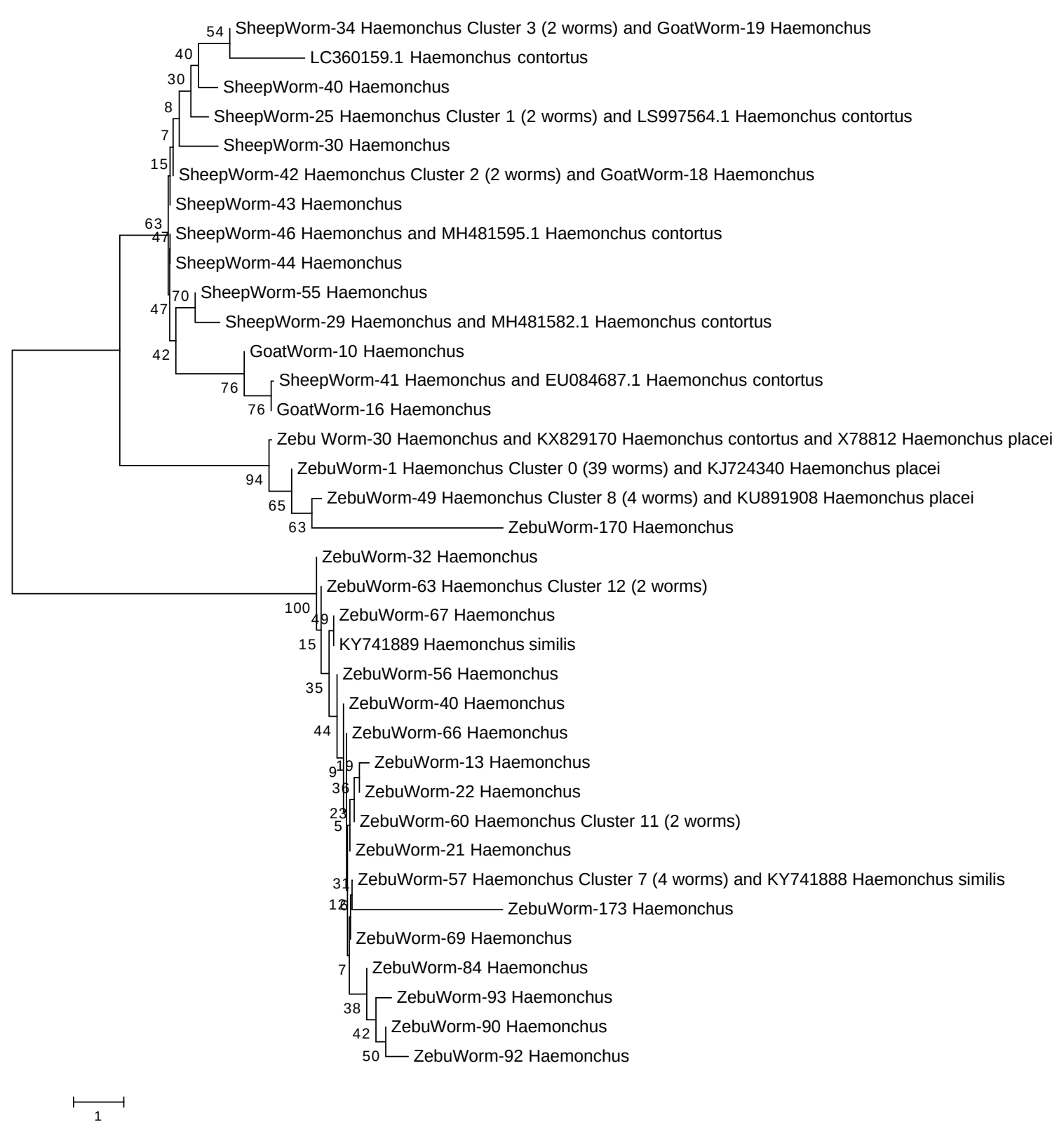
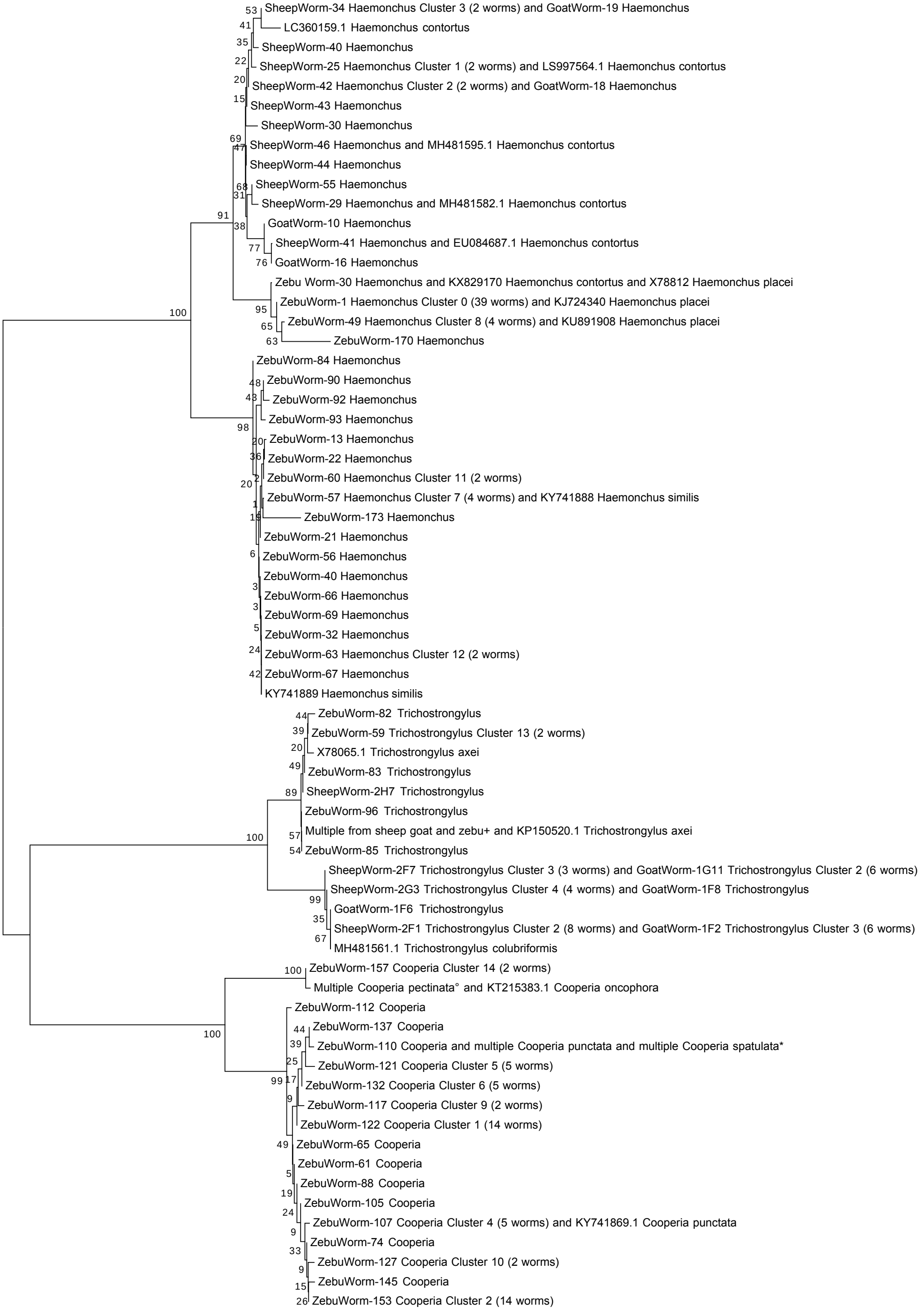
Multiple Cooperia° pectinata and KT215383.1 Cooperia oncophora
1


51
Multiple from sheep goat and zebu+ and KP150520.1 Trichostrongylus axei
53
ZebuWorm-85 Trichostrongylus
ZebuWorm-96 Trichostrongylus
22
41
ZebuWorm-83 Trichostrongylus
SheepWorm-2H7 Trichostrongylus
X78065.1 Trichostrongylus axei
14
ZebuWorm-82 Trichostrongylus
64
ZebuWorm-59 Trichostrongylus Cluster 13 (2 worms)
GoatWorm-1F6 Trichostrongylus
SheepWorm-2F1 Trichostrongylus Cluster 2 (8 worms) and GoatWorm-1F2 Trichostrongylus Cluster 3 (6 worms)
SheepWorm-2F7 Trichostrongylus Cluster 3 (3 worms) and GoatWorm-1G11 Trichostrongylus Cluster 2 (6 worms)
99
SheepWorm-2G3 Trichostrongylus Cluster 4 (4 worms) and GoatWorm-1F8 Trichostrongylus
MH481561.1 Trichostrongylus colubriformis
0.50


SheepWorm-34 Haemonchus Cluster 3 (2 worms) and GoatWorm-19 Haemonchus
LC360159.1 Haemonchus contortus
SheepWorm-40 Haemonchus
SheepWorm-25 Haemonchus Cluster 1 (2 worms) and LS997564.1 Haemonchus contortus
SheepWorm-30 Haemonchus
SheepWorm-42 Haemonchus Cluster 2 (2 worms) and GoatWorm-18 Haemonchus
SheepWorm-43 Haemonchus
SheepWorm-46 Haemonchus and MH481595.1 Haemonchus contortus
SheepWorm-44 Haemonchus
SheepWorm-55 Haemonchus
SheepWorm-29 Haemonchus and MH481582.1 Haemonchus contortus
GoatWorm-10 Haemonchus
SheepWorm-41 Haemonchus and EU084687.1 Haemonchus contortus
GoatWorm-16 Haemonchus
Zebu Worm-30 Haemonchus and KX829170 Haemonchus contortus and X78812 Haemonchus placei
ZebuWorm-1 Haemonchus Cluster 0 (39 worms) and KJ724340 Haemonchus placei
ZebuWorm-49 Haemonchus Cluster 8 (4 worms) and KU891908 Haemonchus placei
ZebuWorm-170 Haemonchus
ZebuWorm-32 Haemonchus
ZebuWorm-63 Haemonchus Cluster 12 (2 worms)
ZebuWorm-67 Haemonchus
KY741889 Haemonchus similis
ZebuWorm-56 Haemonchus
ZebuWorm-40 Haemonchus
ZebuWorm-66 Haemonchus
ZebuWorm-13 Haemonchus
ZebuWorm-22 Haemonchus
ZebuWorm-60 Haemonchus Cluster 11 (2 worms)
ZebuWorm-21 Haemonchus
ZebuWorm-57 Haemonchus Cluster 7 (4 worms) and KY741888 Haemonchus similis
ZebuWorm-173 Haemonchus
ZebuWorm-69 Haemonchus
ZebuWorm-84 Haemonchus
ZebuWorm-93 Haemonchus
ZebuWorm-90 Haemonchus
ZebuWorm-92 Haemonchus
1


SheepWorm-34 Haemonchus Cluster 3 (2 worms) and GoatWorm-19 Haemonchus
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SheepWorm-40 Haemonchus
SheepWorm-25 Haemonchus Cluster 1 (2 worms) and LS997564.1 Haemonchus contortus
SheepWorm-42 Haemonchus Cluster 2 (2 worms) and GoatWorm-18 Haemonchus
SheepWorm-43 Haemonchus
SheepWorm-30 Haemonchus
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SheepWorm-44 Haemonchus
SheepWorm-55 Haemonchus
SheepWorm-29 Haemonchus and MH481582.1 Haemonchus contortus
GoatWorm-10 Haemonchus
SheepWorm-41 Haemonchus and EU084687.1 Haemonchus contortus
GoatWorm-16 Haemonchus
Zebu Worm-30 Haemonchus and KX829170 Haemonchus contortus and X78812 Haemonchus placei
ZebuWorm-1 Haemonchus Cluster 0 (39 worms) and KJ724340 Haemonchus placei
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ZebuWorm-84 Haemonchus
ZebuWorm-90 Haemonchus
ZebuWorm-92 Haemonchus
ZebuWorm-93 Haemonchus
ZebuWorm-13 Haemonchus
ZebuWorm-22 Haemonchus
ZebuWorm-60 Haemonchus Cluster 11 (2 worms)
ZebuWorm-57 Haemonchus Cluster 7 (4 worms) and KY741888 Haemonchus similis
ZebuWorm-173 Haemonchus
ZebuWorm-21 Haemonchus
ZebuWorm-56 Haemonchus
ZebuWorm-40 Haemonchus
ZebuWorm-66 Haemonchus
ZebuWorm-69 Haemonchus
ZebuWorm-32 Haemonchus
ZebuWorm-63 Haemonchus Cluster 12 (2 worms)
ZebuWorm-67 Haemonchus
KY741889 Haemonchus similis
ZebuWorm-82 Trichostrongylus
ZebuWorm-59 Trichostrongylus Cluster 13 (2 worms)
X78065.1 Trichostrongylus axei
ZebuWorm-83 Trichostrongylus
SheepWorm-2H7 Trichostrongylus
ZebuWorm-96 Trichostrongylus
Multiple from sheep goat and zebu+ and KP150520.1 Trichostrongylus axei
ZebuWorm-85 Trichostrongylus
SheepWorm-2F7 Trichostrongylus Cluster 3 (3 worms) and GoatWorm-1G11 Trichostrongylus Cluster 2 (6 worms)
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GoatWorm-1F6 Trichostrongylus
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ZebuWorm-157 Cooperia Cluster 14 (2 worms)
Multiple Cooperia pectinata° and KT215383.1 Cooperia oncophora
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ZebuWorm-121 Cooperia Cluster 5 (5 worms)
ZebuWorm-132 Cooperia Cluster 6 (5 worms)
ZebuWorm-117 Cooperia Cluster 9 (2 worms)
ZebuWorm-122 Cooperia Cluster 1 (14 worms)
ZebuWorm-65 Cooperia
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ZebuWorm-88 Cooperia
ZebuWorm-105 Cooperia
ZebuWorm-107 Cooperia Cluster 4 (5 worms) and KY741869.1 Cooperia punctata
ZebuWorm-74 Cooperia
ZebuWorm-127 Cooperia Cluster 10 (2 worms)
ZebuWorm-145 Cooperia
ZebuWorm-153 Cooperia Cluster 2 (14 worms)
5

Table 1. **Primers and PCR programs**

	Target portion	Primers	Reference	PCR Program
RH5401[a]	*SSU* HRVI	AAGATTAAGCCATGCATG	Eberhardt *et al.*, 2007	95°C for 2 min
RH5402[a]	*SSU* HRVI	TTCTTGGCAAATGCTTTCG		95°C for 30 secs 52°C for 30 secs 40 X 72°C for 2 min 72°C for 10 min
RH5403	*SSU* HRVI	AGCTGGAATTACCGCGGCTG		+4°C
1F	ITS-2	ACGTCTGGTTCAGGGTTGTT	Chaudhry *et al.*, 2015	95°C for 5 min
2R	ITS-2	TTAGTTTCTTTTCCTCCGCT		95°C for 1 min 57°C for 1 min 35 X 72°C for 1 min 72°C for 5 min +4°C

[a] Primers RH5401 and RH5402 are identical with primers SSU18A and SSU26R (Floyd *et al.,* 2002)

Table 2. Morphological identification and distribution of trichostrongylid adult worms in gastrointestinal tracts of cattle, goats and sheep

Compartiment	Parasites	No worms identified (% of genus)			Total (% worms in compartment)
		Cattle	goats	sheep	
Abomasum	*Haemonchus spp.*	9,956 (62)	2,890 (18)	3,212 (20)	16,058 (61)
	Trichostrongylus spp.	4,054 (55)	2,211 (30)	1,106 (15)	7,371 (28)
	Cooperia spp.	2,896 (100)	0	0	2,896 (11)
	Total (%)				26,325 (100)
Small intestines	*Cooperia spp.*	283 (100)	0	0	283 (90)
	Trichostrongylus spp.	0	17 (60)	11 (40)	28 (10)
	Total (%)				311 (100)
Large intestines	*Oesophagostomum spp.*	1,516 (97)	31 (2)	16 (1)	1,563 (100)
	Total (%)				1,563 (100)

Conflict of interest

The authors declare no conflict of interest.

Ethical Statement

This study was approved by the Scientific Directorate of the Institute of Agricultural Research for Development. Abomasa and gastro-intestinal tract were collected at the local slaughterhouses of Ngaoundéré by veterinarians and well-trained personnel after the animals had been slaughtered as part of the normal operations of the abattoir. The meat of these animals was processed for human consumption. Five of the gastro-intestinal tracts originating from cattle came from the *post-mortem* analysis of animals from a DFG-funded research project (DFG-COBE) which died from severe dermatophilosis infection.

Whole genome characterization of autochthonous *Bos taurus brachyceros* and introduced *Bos indicus indicus* cattle breeds in Cameroon regarding their adaptive phenotypic traits and pathogen resistance

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Abstract

Background:

West African indigenous taurine cattle display unique adaptive traits shaped by husbandry management, regional climate and exposure to endemic pathogens. They are less productive with respect to milk and meat production which has been associated with a number of factors, amongst others small size, traditional beliefs and husbandry practices. This resulted in the severe dwindling of their populations size rendering them vulnerable to extinction.

The Namchi (Doayo) taurine cattle breed has documented resistance traits against trypanosome infection and exposure to tick infestation. Nonetheless, the historically later introduced Zebu cattle are the main cattle breeds in Africa today, even though they suffer more from locally prevailing pathogens.

By using a reference-based whole genome sequencing approach, we sequenced for the first time the genomes of five cattle breeds from Cameroon: the Namchi (Doayo), an endangered trypanotolerant taurine breed, the Kapsiki, an indigenous trypanosusceptible taurine breed, and three Zebu (*Bos indicus indicus*) breeds: Ngaoundere Gudali, White Fulani and Red Fulani.

Results: Approximately 167 Giga bases of raw sequencing data were generated and mapped to the cattle reference genome UMD3.1. The coverage was 22 to 30-fold. The single nucleotide polymorphisms (SNPs) were compared with reference genomes of European *Bos taurus* Holstein and of Asian *Bos indicus* Brahman and the African trypanotolerant N'Dama breeds. Of a total of 50 million SNPs identified, 3.43 million were breed-specific ranging from 0.37 to 0.47 million SNPs in the domestic Cameroonian breeds and approximately 0.58 million constituted of small insertions and deletions. We identified breed specific-non-synonymous variants as genetic traits that could explain certain cattle-breed specific phenotypes such as

increased tolerance against trypanosome parasites in the Namchi (Doayo) breed, heat tolerance in the Kapsiki breed, and growth, metabolism and meat quality in the Gudali breeds. Phylogenetic comparison grouped Namchi (Doayo) to the African Zebu clade indicating a hybrid status of the selected animal with a Zebu breed, albeit it showed the Namchi breed's phenotype.

Conclusions:

The findings provide the first comprehensive set of full genome variant data of the most important Cameroonian cattle breeds. The genomic data shall constitute a foundation for breed amelioration whilst exploiting the heritable traits and support conservation efforts for the endangered local cattle breeds.

Keywords: Whole genome sequencing, Zebu gene introgression, trypanotolerance, *Bos taurus:* Namchi (Doayo), Kapsiki; *Bos indicus: White Fulani, Red Fulani, Gudali*, Cameroon

Background

More than 150 cattle breeds or distinct populations have been recorded in Africa [1, 2]. Their phenotypes cluster into the humpless taurine, the humped Zebu, and the anciently fixed taurine-Zebu crossbreeds known as Sanga in East Africa [3].

In Sub-Saharan Africa, trypanosomiasis (Nagana), dermatophilosis, tick-borne diseases and gastrointestinal helminthiasis are the major endemic diseases affecting cattle productivity [4, 5]. Indigenous local taurine breeds like Doayo (also known as under the Fulani word Namchi) are more resistant or tolerant to most endemic diseases than Zebu cattle [5]. They originated from ancestral aurochs populations *Bos primigenius primigenius* and *B. primigenius opisthonomus* from two centers of domestication, namely the Middle East and North Africa, respectively [6, 7].

Today Namchi (Doayo) and Kapsiki are geographically restricted to endemic areas of human and animal trypanosomiasis in Northern Cameroon. Whereas N'dama and Kuri cattle are grouped as residual longhorn *Bos taurus longifrons* introduced already 10,000 years ago [5, 9], Baoulé, Namchi (Doayo) and Kapsiki belong to the West African Shorthorn (WAS) *Bos taurus brachyceros* domesticated on the continent some 6,500 years ago [6, 7].

The Kapsiki cattle form a population of approximately 5000 animals that are found mainly in the Mayo Tsanaga (Rhumsiki) area of the Far North region [8]. In contrast, the Namchi (Doayo) cattle have a population size of only 1000 to 2000 heads in the Poli mountains, which are up to1,900-meter-high and surrounding savannah low lands in the Faro division of Cameroon’s North region [9, 10]. They are well adapted to the local environment including endemic parasites like trypanosomes and ticks [9, 11], but of small size and weight, thus economically not interesting for milk and meat production. The usually small herds of 5 to 50 animals which are kept semi-wild, are neither milked nor exploited commercially. They rather play an important role in the traditional culture of local tribes, like dowries, special feasts and rituals.

Decades of uncontrolled crossbreeding with Zebu cattle have severely dwindled the gene pool of this taurine cattle population [9]. In 1992, these breeds have been classified by the Food and Agricultural Organization (FAO) as being at risk of becoming extinct [10], hence the conservation of their genetic resources has been highly prioritized. The continuous influx of Zebu genes into the WAS breeds stands to threaten the innate characteristics of trypanotolerance and other disease resistance [3].

Bos indicus Zebu cattle in Africa fall into two distinct groups, the West African Zebu (WAZ) and East African Zebu (EAZ). In Cameroon, 99% of the estimated population of six million cattle are WAZ breeds. They consist of two major sub-types of the Sokoto and Adamawa Gudali [17]. In Central Africa, they have the highest potential for beef and dairy production in comparison to other regional WAZ breeds, like White Fulani and Red Fulani. These Fulani cattle are long-horned and long-legged Zebu cattle and are mainly kept by the nomadic Bororo people [18]. All Zebu breeds were introduced through the Nile-valley and the Horn of Africa around 2,000 years ago. They started to become more widespread about 700 years ago with hamitic migrations in North and East Africa [7, 13] and throughout the Sahel zone south of the Sahara. They arrived in Northern Cameroon, coming from the Bornu (Nigeria today) some 200 years ago. This relatively short time span for evolutionary adaption is reflected by a higher susceptibility to locally endemic diseases and vectors making reliance on veterinary drug interventions essential for their survival.

Better knowledge of unique adaptive traits for locally prevailing pathogens is needed not only for breed conservation, but also for future genetic amelioration of cattle breeds to mitigate food insecurity problems in Africa. Long-term selection pressure has operated on the genomic architecture and on regions that control traits for adaptive fitness [1]. For example, autosomal and Y-chromosomal microsatellites indicate a high level of genetic diversity in African cattle breeds as a consequence of repetitive introgression of Zebu genes into autochthonous taurine

genome across the continent. Genome research initiatives, like Bovine Genome Sequencing, HapMap and 1000 Bulls have fostered our understanding of bovine evolution and the complex formation of genetic variants [14-16]. The free availability of cattle reference genomes facilitates whole genome re-sequencing approaches, which are steadily expanding [14-16].

In this study, we report and characterize for the first time the complete genomes of five cattle breeds in Cameroon, namely the endangered taurine trypanotolerant Namchi (Doayo), the trypanosusceptible Kapsiki taurine, and the three Zebu breeds Gudali, White Fulani and Red Fulani, which are all trypanosusceptible. Using the genomic data, 50 million (M) SNPs were identified in this study of which 2.68 M (18.7%) were considered as novels variants. Lower genetic diversity was also found in African taurine cattle breeds than in the Cameroonian *Bos indicus* breeds. Furthermore, specific-non-synonymous variants were detected such as trypanotolerance in Namchi (Doayo), heat tolerance in Kapsiki, and growth, metabolism and meat quality in Gudali.

Results and discussion

Whole genome sequencing, assembly and variant identification

Genomic DNA from the cattle breeds Gudali, White Fulani, Red Fulani, Namchi (Doayo) and Kapsiki were sequenced with the Illumina HiSeq4000 sequencing platform and libraries were sequenced using 150-bp paired-end reads. This generated a total of 835 Gb of raw reads with an average of 167 Gb per sample which provides, to the best of our knowledge, the first comprehensive set of full genome variant data of these breeds. The average genome-wide sequence coverage from the mapped reads ranged from 22.8× for Namchi (Doayo) up to 30.8× for Red Fulani (Table 1). This lies in the range of other cattle re-sequencing studies published [14, 19, 20] whereas the depth of coverage is fairly high in comparison to 10.8 and 15.8-fold

coverage obtained by Kim *et al.* [15] and Kawahara-Miki *et al.* [19], respectively. Taylor *et al.* [21] have suggested that about 95% of the total variants within the genome of cattle are discovered at an average sequence depth of 23.3x which implies that the data obtained in this study is sufficient to detect SNPs and Indels variants with high confidence.

The chosen approach of high depth sequencing yielded approximately 10^9 reads per sample (Table 1) which allowed us to obtain a high coverage per animal tested. However, it also resulted in a relatively low percentage of uniquely mapped reads when aligned to the reference genome (Hereford breed UDM3.1) that was subsequently used for variant calling (ranging from 63% to 65% mapped reads for the Cameroonian cattle breeds, Table 1). This result is consistent with the 60% of uniquely mapped reads by Kawahara-Miki *et al.* on Japanese Kuchinoshima-Ushi bulls [19]. However, while using the same UMD3.1 cattle reference genome, our mapping rates were markedly lower than the 98.5% reported by Kim *et al.* [15] from other indigenous East African cattle breeds (Ankole, Boran and Ogaden). Our rather low mapping rate could be explained either by the PCR-free preparation of sequencing libraries in our case which implies that bovine DNA and non-bovine DNA such as blood microbes and parasites could have been sequenced at similar rates or that the African cattle breed samples chosen are evolutionarily more distant compared to the reference genome and therefore contain sequences of genomics regions not present in the UMD3.1 cattle reference genome.

Variant calling results

A total of 50 million (M) SNPs were identified in this study of which 2.68 M (18.7 %) were not found in dbSNP and considered as novels variants (Table 1; Fig. 1A, Supplemental file Fig. S2). Similar results were obtained by Stafuzza *et al*. [22] on Gyr, Girolando, Gruzerat and Holstein cattle breeds from Brazil. The ratio of the number of heterozygous to homozygous

SNP variants were different across the cattle breeds. Brahman and Namchi (Doayo) had the lowest rate, whereas Kapsiki had the highest (Table 1, Supplemental file Fig. S1). The low ratio of heterozygous to homozygous in Brahman and Namchi (Doayo) cattle could mean that they experience admixture, as reported by Freemann *et al.* [23] in African taurines from Cameroon. On average, 0.58 M (8%) of the detected variants had small insertions and deletions (Indels, Table 1, Fig. 1B).

***De novo* Assembly and analysis of unmapped genomic sequence reads**

In order to better understand the low mapping rate, unmapped reads were assembled into contigs using the *de novo* sequence assembler ABySS and compared against the NCBI Blastn database. These results did not support the hypothesis of microbial and parasitic DNA contamination that could be sequenced at similar rates as the host DNA using the PCR free library preparation protocol. Rather, it supported the idea that the breeds analyzed here are evolutionary more distant compared to the reference genome. *Bos mutus* was found as a best scoring Blast results in 65% of the unmapped Blastn alignments in all samples, followed by *Ovis canadensis* with 17% of the Blastn alignments [Supplemental file 3 Figure S 3, Supplement file 5 Table Supplemental S1]. These findings indicate that the most common sequences of the unmapped read contigs were those of other Bovidae. The mean sequence identity for the *Bos mutus* Blastn hits was at 98% with an average coverage of 700bp, and 92% sequence identity with an average coverage of 650bp for *Ovis canadensis* indicating that these reads are derived from Bovidae but have not been found in the reference genome used for read mapping. *Bos taurus* and *Bos indicus* were only found in ~3% and 1% of the Blastn hits of the unmapped reads, respectively, which demonstrates that most of the reads originating from *Bos taurus* and *Bos indicus* were correctly mapped. We postulate that this high percentage of reads deriving from other Bovidae~~,~~ might arise due to the evolutionary divergence of Cameroonian

cattle breeds to the other investigated breeds. There were no obvious differences in Blastn results found when comparing African Zebu cattle with Namchi (Doayo) and Kapsiki [Supplemental file 3 Figure S3, Table Supplemental S1] although it seems conceivable to expect Namchi (Doayo) and Kapsiki breeds rather distinct to the reference genome when compared to the Zebu cattle. Further investigation using tools that can measure levels of hybridization is needed in order to solve this in the future. Furthermore, the construction of an African breed reference genome or an African pan-genome might help to optimize genome research on African cattle breeds.

Among the species that cover at least 0.5% of the total scoring Blast results, most were of vertebrate origin. Exceptions of the invertebrate kingdom were *Trichogramma pretiosum* in the Brahman control sample, and the bacteria *Lelliottia nimipressuralis* and *Enterobacter spec* in the White Fulani sample (see Supplemental Table S1), albeit all at very low levels (2.4%, 0.8% and 0.6% of the total Blast Scoring results. At even lower rates also *Babesia* spp., a blood-borne parasite known to cause Texas fever in cattle and *Theileria spp.*, a cosmopolitan blood parasite of cattle and blood-invading bacteria of the *Anaplasma* genus were also detected in Namchi (Doayo) [see Supplement file 5 Table Supplemental S1]. Although these finding are only supported by a very low number of alignments of assembled contigs to the blastn database, this data is still in line with a recent epizootiological survey in the same indigenous Cameroonian cattle breeds which revealed that nearly 90% of animals were infected with tick-borne bacterial, piroplasmid and protozoan pathogens [24, 25].

Genetic variability and similarity across breeds

The largest number of SNPs was found in Zebu breeds Brahman, Red Fulani, Gudali and White Fulani, respectively. When looking at the SNP distribution across the taurine breeds the lowest numbers were found in Holstein and N'Dama as compared to Kapsiki and Namchi (Doayo)

cattle (Table 1). A total of 1,013,395 SNPs were common across all breeds, and 121,776 SNPs were Zebu-specific, distributed between Brahman, Red Fulani, White Fulani and Gudali cattle breeds. More surprisingly, there were no SNPs exclusively shared between the European taurine Holstein and WAS taurine (N'Dama, Kapsiki and Namchi (Doayo), Fig. 2), apart from 73,366 SNPs which were shared between N'Dama and Kapsiki only. Furthermore, 85,307 SNPs were common between all tested cattle breeds except Brahman cattle. The highest proportion of breed-specific (bs) SNPs were found in *Bos indicus*: Brahman (759,804), Red Fulani (473,688), Gudali (461,043) and White Fulani (420,114), respectively, and the lowest breed-specific SNPs were found on taurine breeds N'dama (220,302), Holstein (328,560), Kapsiki (370,074) and Namchi (Doayo) (402,114), respectively (bs SNPs are color labelled in Fig. 2). This apparently lower genetic diversity in African taurine breeds was already earlier argued by Kim *et al*. [15], who linked it to the low effective population size and/or population bottlenecks following fatal disease outbreaks such as the Rinderpest. In contrast, indicine Zebu cattle and composites with larger effective population size exhibit a higher level of nucleotide diversity. Furthermore, the higher nucleotide diversity of taurine Namchi (Doayo) and Kapsiki as compared to N'Dama and Holstein may be due to the long history of *Bos indicus* introgression [23].

The density of variants per chromosome was proportional to the chromosome length, except for the X chromosomes which showed a lower number of variants identified (Supplemental file Fig. S2). These findings were expected because the DNA of X chromosomes is subject to an increased natural selection, which leads to less genetic diversity.

Breed clustering and relationships

The cluster relationship between breeds was analyzed by a principal component analysis (PCA) using all autosomal SNPs (Fig. 3A). The first two principal components explained 22% and

16% of the total variance, respectively. Except for Namchi (Doayo), the other WAS breeds N'Dama, and Kapsiki form a separate cluster from WAZ breeds. The WAS breeds N'dama, and Kapsiki are also closer to European taurine Holstein than WAZ breeds and both, WAS and WAZ are clearly separated from Zebu Brahman. This indicates the possibility of admixture events between the West African cattle breeds. To further understand the genetic network among those breeds a phylogenetic tree analysis (Fig. 3B) was carried out with the same autosomal SNPs data as for PCA analysis by using Randomized Accelerated Maximum Likelihood models (RAxML). Again, except for Namchi (Doayo), the *Bos taurus* breeds Kapsiki, N'Dama and Holstein cluster together while the *B. indicus* breeds White Fulani, Gudali, Brahman clustered on a separate clade. The WAS Kapsiki and Namchi (Doayo) cattle are closer to WAZ cattle as compared to European taurine Holstein. In addition, the WAZ are evolutionary distant to Indian Zebu Brahman. This observation concords with previous studies of WAS indicating they possess admixture with indicine ancestry between 22.7% and 74.1% in Central Africa [26, 27]. Gudali are more closely related to Indian Brahman cattle than White Fulani and Red Fulani (Fig. 3B). The Indian Zebu genes introgression into African Zebu breeds has been reported based on autosomal microsatellite markers, between 55 and 83% [3, 27]. The PCA and RAxML findings presented here show that the evolution of Cameroonian cattle breeds is distant both to Indian Zebu Brahman and European taurine Holstein. The higher number of heterozygous to homozygous variant ratio in Kapsiki (2.5) than in Namchi (Doayo) (1.5) was unexpected, because Kapsiki has been regarded as an indigenous taurine population with highest Zebu gene introgression over the last three decades based on microsatellite data [11, 23]. Namchi (Doayo) and Kapsiki have been classified by Freeman *et al.* [23] as hybrids rather than pure breeds. The phylogenetic position of Namchi (Doyao) more closely related to Red Fulani than WAS indicated recent Zebu introgression into the genome of Namchi (Doayo). Although the selected Namchi (Doayo) was not different in appearance to the other animals in

the region, we cannot exclude whether it has been a product of a recent cross-hybridization with another cattle breed, and thus not representing the pure-breed genome. It is reported that there are still some isolated herds of purebred Namchi (Doayo) cattle in the Poli area, but the present study did not have the tools to screen hybridization levels in the selected animal for whole genome data generation. Such screening would be necessary in the present context where traditional husbandry systems face numerous challenges towards maintaining purely taurine breeds due to rampant cross breeding.

SNPs and Indels functional annotations

The SNPs and Indels were annotated in order to identify the location of the variant in terms of genomic features using snpEFF [28]. In general, all the eight breeds exhibited similar distributions of SNPs and Indels in various genomic annotation categories. Most annotated variants were located in intergenic regions (62%) and introns (27%). The remaining SNPs (11%) were found on downstream genes (4.4%), upstream genes (4.7%), untranslated regions (UTR) (0.5%), missense (0.6%), frameshift (0.02%) and other areas (0.7%) (Fig. 4A).

Breed-specific variants with high impact such as frameshift, splice acceptor, splice donor, start lost and stop gained that may putatively change amino-acids codons are located in and/or close to genes that may lead to functional changes were examined in each chromosome. Overall, 607 genes were identified; 98, 90, 85, 73 and 62 in Red Fulani, Gudali, White Fulani, Kapsiki and Namchi (Doayo), respectively (Additional file 6, Table S1-11). The majority of these genes were widely involved in olfactory receptors, carbohydrate metabolism, transcription regulation, ion binding, nucleotide binding, protein transport, fatty acid metabolism, stress response, regulatory elements, proteolysis and immune responses (Additional file 6, Table S8).

Gene ontology and pathway enrichment analysis of high and moderate impact breed-specific SNPs and Indels variants

Based on the Gene ontology (GO) enrichment analysis of bs-ns SNPs with high and moderated impact, we identified 162 significantly enriched GO terms. The majority of the enriched GO terms were associated with biological processes (n=90, Fig. 4B). “Serine-type endopeptidase inhibitor activity, GO:0004867” terms were shared across all N'Dama, White Fulani and Namchi (Doayo) cattle breeds. “Negative regulation of coagulation, GO:0050819” was shared between the two taurines, Kapsiki and Holstein.

The analysis of GO enrichment from bs-Indels from different cattle breeds identified 50 significantly enriched terms (Fig. 4C), and 41 GO enrichments were associated with biological processes.

The GO terms related to adaptation to the high-altitude environment and heat tolerance were enriched in Namchi (Doayo) and Kapsiki. Also, the GO terms **“**response to decreased oxygen levels, GO:0036293”, “response to hypoxia, GO:0001666”, “localization of cell, GO:0051674” were enriched in Kapsiki whereas in Namchi (Doayo) the GO terms **“**cellular response to peptide hormone stimulus, GO:0071375”, “cellular response to peptide, GO:1901653”, “cellular response to stress, GO:0033554” and “cellular response to hormone stimulus, GO:0032870” were evident.

In the African Zebu cattle, GO terms associated with the adaptation to infectious diseases were enriched on immune responses. In Gudali these were the terms “antigen processing and presentation, GO:0019882” and “plasma membrane protein complex, GO:0098797”. In Red Fulani these were the GO terms “acute inflammatory response, GO:0002526”, “inflammatory response, GO:0006954” whereas in White Fulani the GO term “antigen processing and presentation of peptide antigen, GO:0048002” was enriched.

The KEGG pathway analysis identified 31 pathways with at least one SNP in the gene that may explain individual attributes per breed (Fig. 5). Two pathways which carry at least 10 SNPs were discovered in Namchi (Doayo): phagosome and antigen processing and presentation. In Kapsiki: cell adhesion molecules (CAMs) and vascular smooth muscle contraction were found.

Adaptation to tropical climate and high altitude

Adaptation to local environment is multifactorial involving several genes located on different chromosomes and selection [1-3]. To cope with heat, poor feed and high altitude, African cattle have developed behavioral, cellular and physiological mechanism involved in the intensive responses to the mechanical stress, oxygen, food deprivation and homeostasis [29]. During the evolution of Zebu cattle, they have acquired genes for heat-tolerance at the physiological and cellular levels [30]. The superior ability for regulation of body temperature during heat stress is the result of lower metabolic rates as well as increased capacity of heat tolerance. Heat stress also leads to lightening of the coat, because light colored hair coats have a sleek and shiny reflection [30]. However, the lower metabolic rates under heat stress condition are related to reduction in feed intake, milk yield, thyroid hormone secretion, and growth. This finding may explain the lower performance of meat growth in African Zebu cattle as compared to taurine breeds of European descent. Four heat shock factor (HSF) genes (HSF1, HSF2, HSF3, and HSF4) have been isolated in vertebrates, and HSF1, located on chromosome 14, is a master regulator of Heat Shock Protein (HSP70) expression during stress, including heat shock [31]. European taurine Holstein, WAS, WAZ and Indian Zebu Brahman cattle possess distinct patterns of homozygosity and heterozygosity for the SNPs alleles of HSF1 (n= 37 SNPs), HSPA1A (n=22 SNPs), HSPA12B (n= 32 SNPs) and HSPA13 (n=54 SNPs). The heterozygosity alleles in these genes were over represented in WAS and WAZ as compared to

Brahman and Holstein. The increased heterozygosity among the African cattle breeds (WAS and WAZ) indicates the combined effects of genetic isolation and long selection history.

Adaptation to tropical pathogens

Stress response, olfactory receptors and immune responses play a critical role in adaptation to the tropical environment and diseases [15, 16]. Mammalian olfactory receptors (ORs) are encoded by the largest mammalian multigene family with more than 1000 genes organized in clusters on 26 cattle chromosomes [32]. They are essential for avoiding danger, food search, reproduction, and behavior [32].

Chemokines play a role in the inflammation that enables the phagocytic leukocytes of the immune system to be the first line of defense against infectious agents like protozoa and helminth parasites [33]. The tolerance of Namchi (Doayo) cattle against trypanosomiasis (trypanotolerance) caused by the protozoan parasites *Trypanosoma congolense*, *T. vivax* and *T. brucei* is actively driven by the innate immune response. IL-12, INF-γ and TNF-α that are primarily produced by cells of the innate immune system would trigger phagocytic cell activation and inflammation, thus contributing to the control of parasites growth [34]. Furthermore, SIGLEC-1 and BOLA are key molecules involved in regulations of the chemokines and cells of innate and adaptive immune responses. Genetic polymorphisms have been linked to resistance and susceptibility to various pathogens. For instance, polymorphisms in BOLA-DRB3 stands for resistance to bovine virus bacteria and parasites infections [35, 36, 40]. Two novel frameshift variants in BoLA-DQB were identified in Namchi (Doayo) and Gudali [Additional file 4, Figure S4]. Such polymorphisms in BoLA class II genes have been associated with viral, bacterial and parasites resistance [35-37]. We found twenty alleles located in BoLA-DQB1 (n=8), BoLA-DQB2 (n=8), BoLA-DQB3 (n=2), BoLA-DQB5 (n=2). Three genotypes were observed as two homozygous (reference and alternative) and one

heterozygous. The Namchi (Doayo) cattle carried the highest alternative homozygous alleles in the BoLA-BQB region whereas the Kapsiki possessed the highest heterozygous alleles. IRAK1BP1, sialic acid binding immunoglobuline-like lectin (Siglec), MYO1H and Heat Shock Protein family genes were found carrying mutational SNPs. MYO1H plays roles in cell motility, phagocytosis, and vesicle transport [38] and Siglecs are expressed on various white blood cells of the immune system and are involved in the regulation of innate and adaptive immunity [39]. Studies have shown that many coated sialylated viruses, bacteria and parasites are capable to mimic self-recognition and thus dampen or evade an immune response [39].

We found also one frameshift variant on SIGLEC-1, SIGLEC-11 and SIGLEC-14 genes on Kapsiki, Namchi (Doayo) and White Fulani cattle breeds, respectively.

Breed specific variants with high impact were associated with the quantitative trait loci database CattleQTLdb (http://www.animalgenome.org/cgi-bin/QTLdb/BT/index). In Namchi (Doayo) cattle we found four frameshift (rs448373338, rs721512537, rs724126999, rs518575055) and one stop codon gained (rs208021401) variants, on chr 1 regions associated with “Bovine tuberculosis susceptibility QTL (96157)”, one variant in chr 10 (rs524374275) located in the QTLs region associated with Tick resistance QTL (101167) and two variants (rs716221069 and rs458413320) in the regions associated with *Longissimus* muscle area QTL (56136) and Marbling score QTL (10919). In contrast one new splice donor and one splice acceptor variant (rs523455261) on chr 5 were found in Gudali cattle, and one New start loss on chr 8 associated with Carcass weight QTL (chr 5:11314779-11314819, chr 8:85937078-85937118) and on chr 11 one variant (rs516544521) was located in the QTLs region associated with ovine tuberculosis susceptibility QTL (96344).

Taken together these findings indicate that both, Gudali and Namchi (Doayo) cattle possess genotypes and phenotypes associated with disease susceptibility/resistance, and meat and carcass production. This is in line with previous findings and therefore the high impact variants

found in this study are potential markers for genome-wide association studies (GWAS) and should be further investigated.

Conclusions

The whole genome of five indigenous Cameroonian cattle Namchi (Doayo), Kapsiki, Gudali, White and Red Fulani was re-sequenced and analyzed for the first time, and compared to the reference genomes of European *Bos taurus* Holstein, African *Bos taurus* N'Dama and one Asian Zebu *Bos indicus* Brahman. A number of gene pathways were identified as potential candidates for improved adaptation to drought and growth. Moreover, the co-identification of growth-related Gene Ontology terms in Gudali and Holstein is of economic importance, which may indicate the potential of Gudali cattle for genetic improvement for milk and fertility traits. This will need several decades of selection experiments using the purebred Gudali. Heat tolerance and trypanotolerance traits are complex mechanism involving several gene pathways located on different chromosomes. In the trypanotolerant breeds Namchi (Doayo) we have identified eight potential gene ontology terms, including glucose-related genes involved in the control of trypanosome proliferation in the bloodstream. All these candidate genes constitute a valuable resource for development and genetic amelioration of tropical cattle breeds particular in Africa. Furthermore, the full sequence data widens our knowledge on the value of native breeds as genetic resources for future cattle breeding, and the power of selection signature analyses,

Methods

Sampling, library construction and sequencing

One representative individual of each of the five different cattle breeds was selected (Table 2). Blood samples of 5 ml volume per animal were collected in ethylene diamine tetra acetic acid (EDTA)-coated vacutainers. The blood was centrifuged at 3000 rpm for 15 minutes. Then, genomic DNA was extracted from the buffy coat (cellular layer including leucocytes, erythrocytes and blood-dwelling parasites like *Anaplasma* bacteria, piroplasmids, microfilariae of *Setaria*, trypanosomes and *Borrelia* spp.(see Additional file 7 Table S1 for trypanosome, *Anaplasma* bacteria, piroplasmids, *Onchocerca* filarial and gastro intestinal parasites detected on those animals) using the Wizard Genomic DNA Purification Kit (Promega, Germany) according to the manufacturer's instructions. DNA isolation and concentration was verified by fluorescent methods using Picogreen (Life Technologies). Libraries were generated from 2 µg of genomic DNA per specimen using the Illumina TruSeq DNA PCR-Free Library Prep Kit (Illumina, San Diego, CA, USA) following the manufacturer's protocol. 2x 150bp paired-end libraries sequencing was conducted on the Illumina HiSeq4000 platform with the manufacturer's proprietary TruSeq SBS Kit V3-HS.

Short read mapping, variant calling and annotation

The quality of the generated raw Illumina reads was determined using FastQC software (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). Adaptor read sequences were removed using SeqPurge from ngs-bits4 (https://github.com/imgag/ngs-bits, version 0.1-4-gaed0c94). For comparison with other cattle breeds, whole genome raw sequencing data from NCBI Sequence Read Archive SRA was extracted for the breeds Holstein (SRR934414), N'Dama (SRR3693376) and Brahman (SRR6649996). Paired-end reads from the five samples along with these three controls from the SRA archive were mapped against the reference *Bos taurus* Hereford breed genome UMD3.1 using BWA-MEM version 0.7.10-r789 [41]. Reads that mapped to a single location in the genome (uniquely mapped reads) were selected, and

those with multiple region mapping were excluded using the MarkDuplicates tool of Picard5 v.1.137 (http://broadinstitute.github.io/picard). After sequence alignment, the resulting SAM files format were converted to BAM files using Samtools v.1.3 [41]. Then BAM files were sorted and local realignment of reads was performed to correct misalignment due to the presence of small Indels using Genome Analysis Tool Kit 3.1 (GTAK). SNPs and Indels calling were performed using Freebayes v.0.9.21-19-gc003c1e [42]. SNPs and Indels were annotated using snpEFF [28] and Bcftools [41]. To have many of these processes parallelized and automated, a workflow written in the workflow language Snakemake from QBiC was used which is freely available at Github (https://github.com/qbicsoftware/exomseq).

The variants that were identified in only one cattle breed and have no corresponding entries in the dbSNP database were classified as breed-specific novel variants. The average ratios of homozygous versus heterozygous SNPs were calculated for each breed. This ratio is expected to be 1:2 in a freely mating population; therefore, any departure from this condition such as the presence of admixture in the population will be manifested by an increase in the homozygous/heterozygous ratio [43].

Unmapped read analysis

Reads that were not mapped to the reference genome UMD3.1 were extracted from alignment BAM files and sorted by name using Samtools. The sorted BAM files were given as input to ABySS (version 2.1.5) and assembled using the parameter “k=25” indicating k-mer size = 25 in standard de Bruijn graph mode. Resulting contigs.fa files were subdivided into contigs with a length > 500bp. Then the remaining contigs were searched against Blastn database using Nucleotide-Nucleotide BLAST (version 2.8.1+) with the parameters “-num_alignments 1”and “-num_descriptions 1” to show alignments and descriptions for the top 1 matching database match only. The BLAST output was then parsed using the R language (version 3.4.0) to

determine for each sample the species of the BLAST hit, the percent identity, length of match and query, and BLAST e-value. Mean values of these statistics were calculated for each species in each sample.

Gene enrichment and functional analysis

Breed-specific non-synonymous (bs-ns) SNPs, Indels with moderate and high impact in the genome and new variants not found in any publicly available database were extracted from WAS and WAZ using the data repositories Ensembl release 76, dbSNP138, Entrez Gene, NCBI and Uniprot. Gene pathway networks analysis was performed using the R (v3.5.2) package clusterProfiler and the Kyoto Encyclopaedia of Genes and Genome (KEGG) database [44]. The variant carrying genes were functionally characterized based on different gene ontology (GO) terms using clusterProfiler (v3.12) R package(v3.5.2) package. To investigate whether bs-ns SNPs and Indels genes were associated with economic traits, the quantitative trait loci database CattleQTLdb (http://www.animalgenome.org/cgi-bin/QTLdb/BT/index) was screened using an integrated data warehouse of the bovine genome database web server BovineMine v1.4.

Phylogeny of bovine-related species

To understand the genetic relationships between indigenous cattle breeds and other subfamilies of Bovidae, a principal component analysis (PCA) was performed with EIGENSTRAT. For the phylogenetic tree reconstruction, the variant files were converted to FASTA format with Vcf-kit8 (https://vcf-kit.readthedocs.io/en/latest/). Multiple sequence alignment (MSA) was generated using Muscle with default options [45]. Prottest3 [46] was used to find the best substitution model for the MSA, and Raxml was used to generate the Maximum Likelihood

(ML) tree with Blossum62 as best substitution model along with Gamma distribution for rate heterogeneity, estimation for proportion of invariable sites and 100 non-parametric bootstrap replicates using Brahman as out group [47]. Visualization of the tree was generated using ape (v5.3) R-package [48].

List of Abbreviations

KEGG: Kyoto Encyclopaedia of Genes and Genome **GO**: gene ontology **PCA**: principal component analysis **WAS**: West African Shorthorn **WAZ**: West African Zebu **bs-ns**: Breed-specific non-synonymous **HSPA**: Heat Shock 70 KDa protein **HSF**: heat shock factor **ORs:** olfactory receptors **BoLA**: Bovine leucocyte antigen **SNPs:** single nucleotide polymorphism variants **InDels**: Insertions and Deletions variants **Gb:** Giga base pairs

Declarations

Ethics approval and consent to participate

Permission for the study and ethical approval were obtained from the Scientific Directorate of the Institute of Agricultural Research for Development (IRAD) in Cameroon, which is the country's government research institution for animal health and livestock husbandry improvement. Furthermore, verbal consent was given by the cattle owners and herdsmen.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files are available from the corresponding author on reasonable request. The five newly sequenced African cattle genomes in this study are publicly available

from GenBank with the Bio project accession number (will be uploaded after acceptance of the manuscript).

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure file 1.

Figure 1. Distribution of variants per breed. **a**) Bar plot showing the proportion of common SNPs found in at least two breeds (green), breed-specific SNPs (blue) and Indels (orange) across all the examined breeds. **b)** Bar plot showing the number of variants per breed.

Figure file 2.

Figure 2. Relationship between the different cattle breeds showing the number of SNPs that are common across different breeds along with the total number of variants (blue) and the number of breed-specific SNPs are as follow: Brahman (pink), Red Fulani (brown), Gudali (green), White Fulani (grey), Namchi (Doayo) (blue), Kapsiki (purple), N'dama (red) and Holstein (orange). The first bar (black) shows the number of SNPs that are found in all eight breed samples.

Figure file 3.

Figure 3. Genomic relationship among cattle breeds. **a)** Principal component analysis using autosomal SNP data only, which shows the distribution of different cattle breeds across the first two principal components. **b)** Phylogenetic maximum likelihood tree of autosomal SNPs variants.

Figure file 4.

Figure 4. Variant genome annotation and Gene Ontology (GO) of novel, missense and breed-specific variants. **a)** Bar plot showing numbers in million and proportion of variant types and functional consequences. **b**) Heat map of gene ontology terms of different cattle breed-specific SNPs. **c**) Heat map of gene ontology terms of different cattle breed-specific Indels. The GO terms belonging to biological processes (BP), cellular components (CC) and molecular functions (MF) are shown in red, green and blue, respectively. The color of each cell indicates the number of variant carrying genes.

Figure file 5.

Figure 5. KEGG pathway enrichment. Hierarchical cluster and heat map of KEGG pathways enriched by breed-specific SNPs. The color of each cell indicates the number of variant carrying genes. From dark blue color to light blue color (0 to 9 SNPs), from yellow color to orange color (10 to 19 SNPs) and from red light color to red dark color (20 and more SNPs)

Table 1. Summary of sequencing results of the genomes of five Cameroonian cattle breeds including the number of total reads and variants called in million (M) reads.

The reference genome breed was Hereford (UMD3.1). Whole genome data of the breeds N'Dama, Brahman and Holstein were retrieved from the NCBI archive SRA [Holstein (SRR934414), N'Dama (SRR3693376) and Brahman (SRR6649996)]. Hom = homozygous, Het= heterozygous, Het/ Hom = heterozygous to homozygousratio, W. Fulani = White Fulani; R. Fulani = Red Fulani.Bs-SNPs= breeds specific SNPs

Table 2. Information of the selected animals of Cameroonian cattle breeds for whole genome re-sequencing.

W. Fulani = White Fulani; R. Fulani = Red Fulani. LW: Live weight

Additional files

Additional file 1:

Figure S1. Distribution of homozygous and heterozygous SNPs per cattle breed. Bar plot illustrates the number of homozygous (turquois) and heterozygous (red) SNPs per cattle breed.

Additional file 2:

Figure S2. Distribution of SNPs, Indels and breed-specific SNPs per chromosomes and breeds. Bar plot illustrates the number of SNPs found in at least two breeds (green), breed-specific SNPs (blue) and Indels (orange) across all the breeds for each chromosome.

Additional file 3:

Figure S3. Pairwise alignment of contigs assembled from unmapped reads to the non-redundant nucleotide database from NCBI. Each bar represent an individual cattle breed and contained the twenty most common species with significant alignments to the *de novo* assembled contigs.

Additional file 4:

Figure S4. Distribution of SNPs per cattle breed of chromosome 23 between location 25350340 and 25593072 containing the BoLA gene. The X axis represents genomic location and y-axis represents ratio of non-reference base. Value 1 indicates that all reads carry the non-

reference base at a given location whereas a value of 0.5 and 0 indicates half and none of the reads carry the non-reference base, respectively.

Additional file 5:

Table S1. Pairwise alignment of contigs assembled from unmapped reads to the non-redundant nucleotide database.

Additional file 6:

Table S1, trypanosome, *Anaplasma* bacteria, piroplasmids, *Onchocerca* filarial and gastro-intestinal parasites detected from five animals of each cattle breed detected by microscopy and molecular diagnostics using ribosomal nuclear makers.

Table 1. Summary of sequencing results of the genomes of five Cameroonian cattle breeds including the number of total reads and variants called in million (M) reads.

Breeds	Mapped Reads	Total Reads	Mapping rate (%)	Coverage [x]	SNPs	Indels	Bs- SNPs	Hom	Het	Het/ Hom
Namchi	596.3	935.3	63.7	22.8	6.31	0.53	0.40	2.51	3.80	1.5
Kapsiki	743.7	1160.6	64.1	28.6	5.40	0.47	0.37	1.55	3.85	2.5
W. Fulani	707.6	1103.1	64.1	27.2	6.42	0.55	0.42	2.29	4.13	1.8
R. Fulani	716.3	1102.2	65.0	27.6	6.70	0.57	0.47	2.15	4.55	2.1
Gudali	804.9	1271.1	63.3	30.8	6.65	0.57	0.46	2.17	4.49	2.1
N'Dama	154.5	282.1	54.8	4.7	4.26	0.35	0.22	1.53	2.73	1.8
Brahman	146.4	177.0	82.7	5.1	7.31	0.60	0.76	2.96	4.36	1.5
Holstein	255.7	460.6	55.5	7.6	3.05	0.26	0.33	1.19	1.87	1.6

The reference genome breed was Hereford (UMD3.1). Whole genome data of the breeds N'Dama, Brahman and Holstein were retrieved from the NCBI archive SRA [Holstein (SRR934414), N'Dama (SRR3693376) and Brahman (SRR6649996)]. Hom = homozygous, Het= heterozygous, Het/ Hom = heterozygous to homozygousratio, W. Fulani = White Fulani; R. Fulani = Red Fulani.Bs-SNPs= breeds specific SNPs

Table 2. Information of the selected animals of Cameroonian cattle breeds for whole genome re-sequencing.

Breed	Age [years]	Sex	Sampling sites		GPS Coodinates		Altitude	LW [kg]	Subspecies
			Region	Village	N	E			
Namchi (Doayo)	6	male	Faro	Herko	8°30'05.1"	13°08'28.7"	520m	252	*Bos taurus brachyceros*
Kapsiki	5	female	Mayo-Tsanaga	Rhumsiki/Kila	10°27'45.5"	13°38'22.9"	956m	252	*Bos taurus brachyceros*
W. Fulani	5	female	Mayo-Rey	Bini	07°37'29.6"	14°32'10.1"	780m	240	*Bos indicus indicus*
R. Fulani	5	female	Mayo-Rey	Bini	07°37'29.6"	14°32'10.1"	780m	313	*Bos indicus indicus*
Gudali	7	female	Vina	Galim	07°12'2.39"	13°34'49.70"	1050m	400	*Bos indicus indicus*

W. Fulani = White Fulani; R. Fulani = Red Fulani. LW: Live weight

Figure 1 A

Click here to access/download;Figure;FIGURE1_A_Distribution of

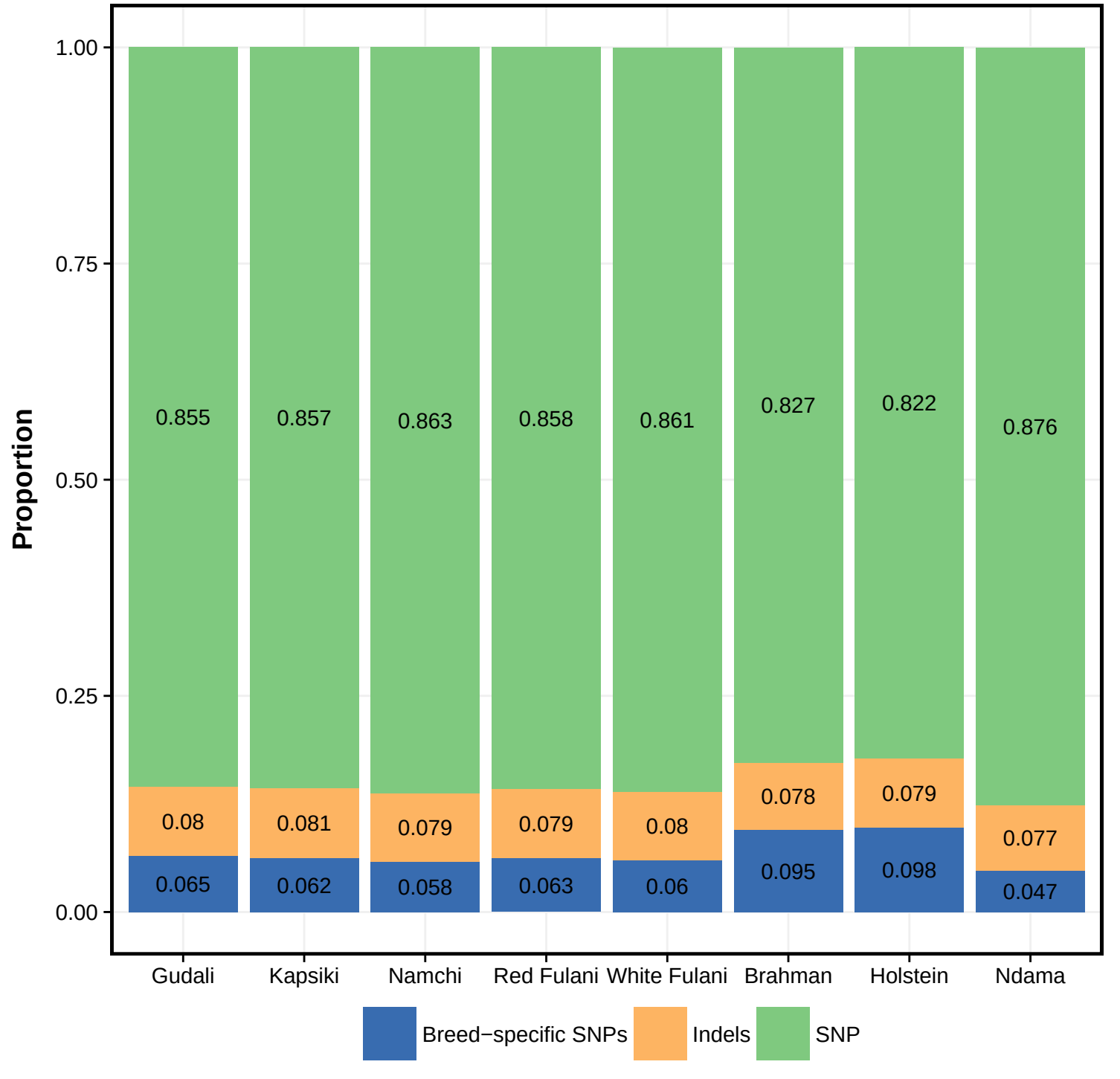

Figure 1 B

Click here to access/download;Figure;FIGURE1_B_distribution of variants

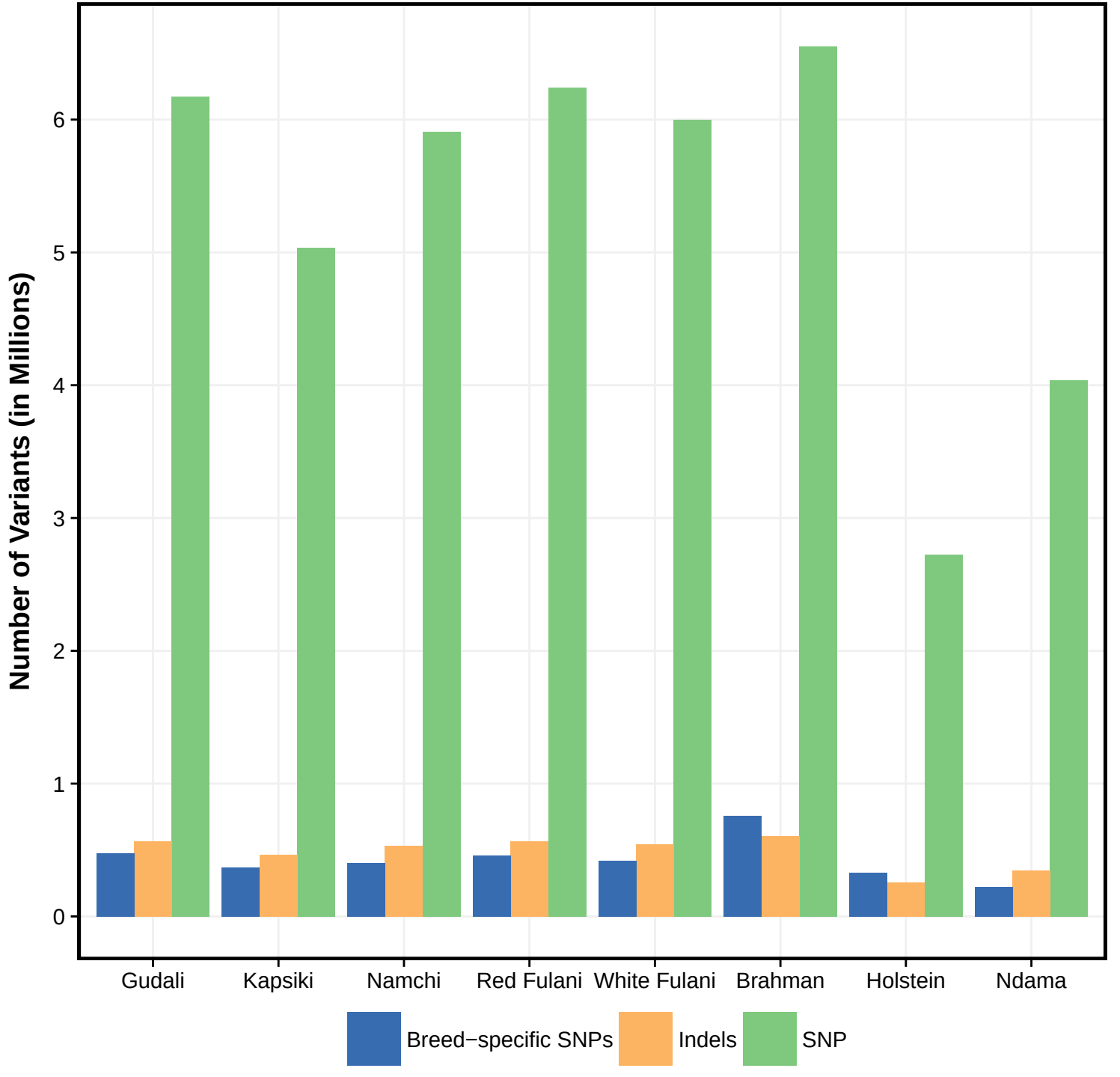

Figure 2

Click here to access/download;Figure;FIGURE2_Relationship_upsetRplot_only_snps.pdf

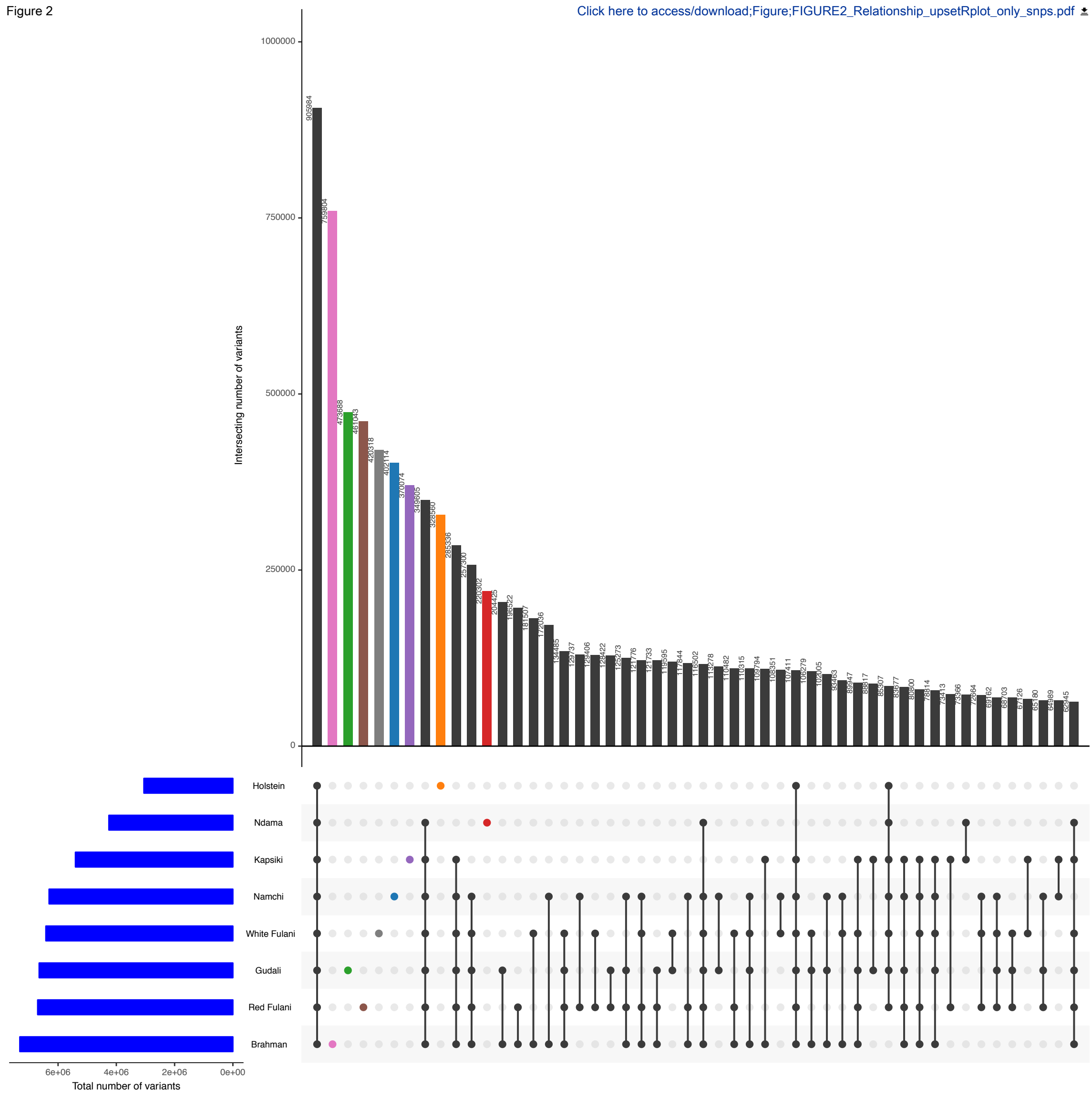

Figure 3 A

Click here to access/download;Figure;FIGURE3_A_Genomic_PCA.png

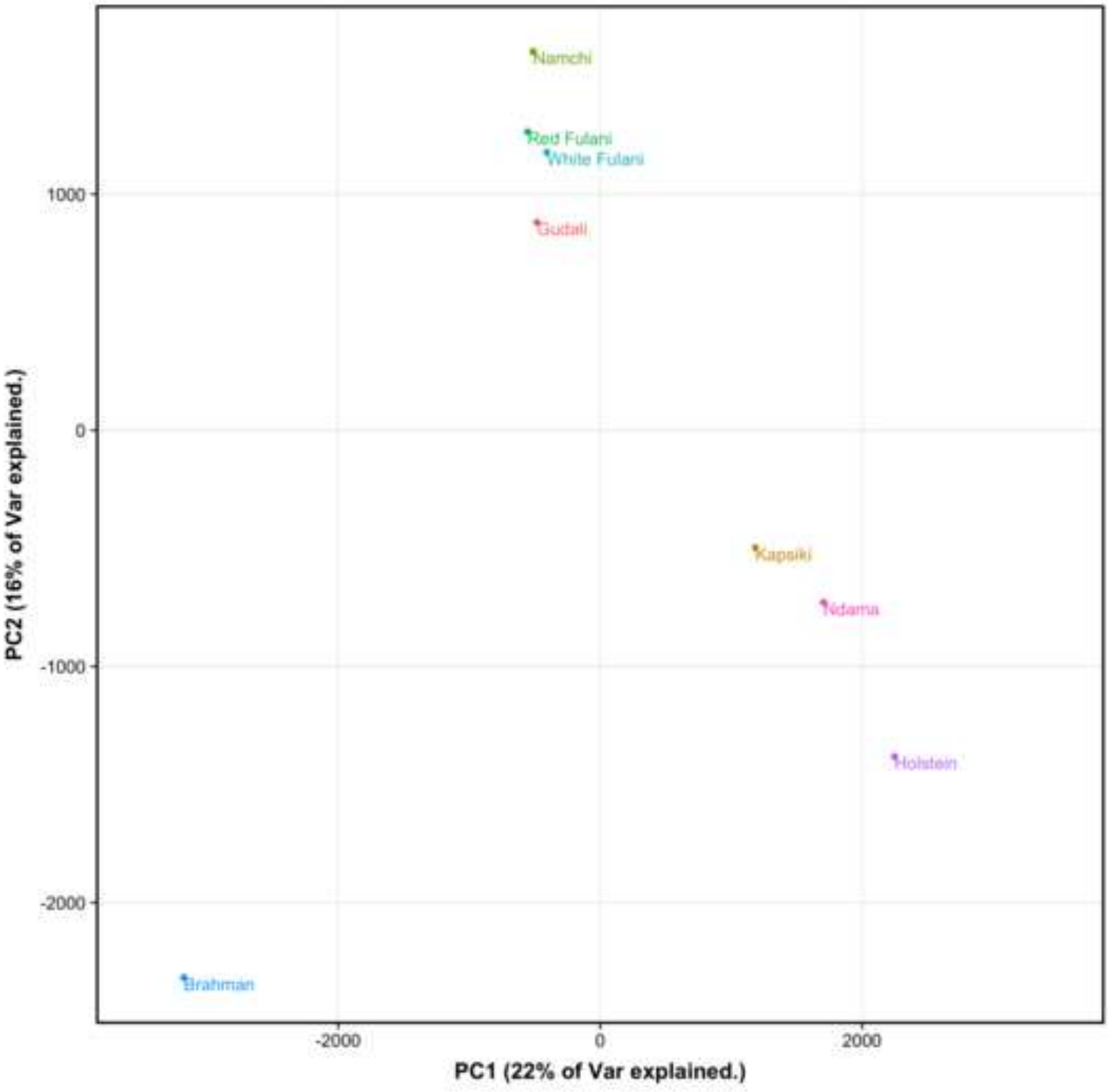

Figure 3 B

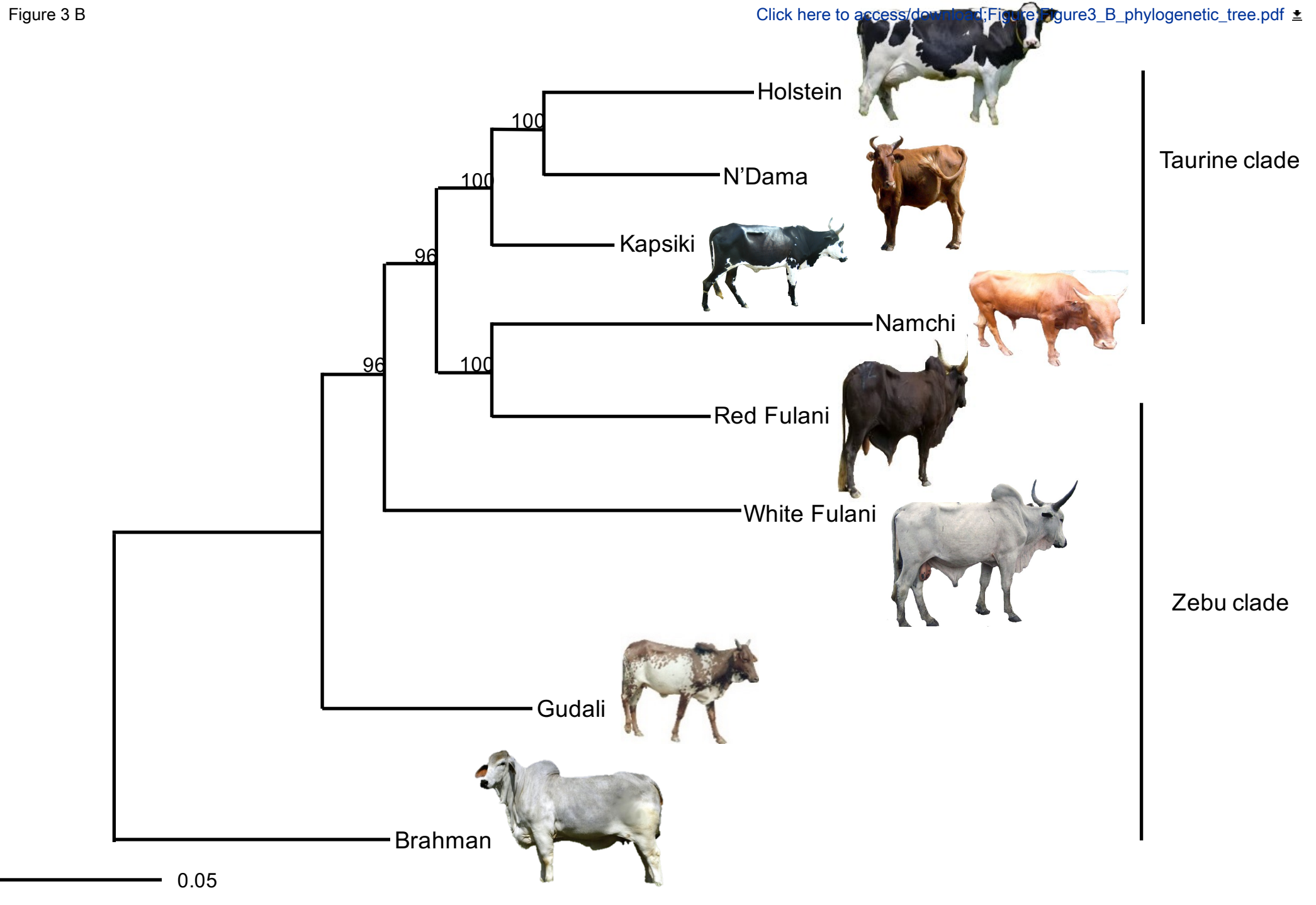

Figure 4 A

Click here to access/download;Figure;FIGURE4_A_proportion_variants_SNPs.pdf

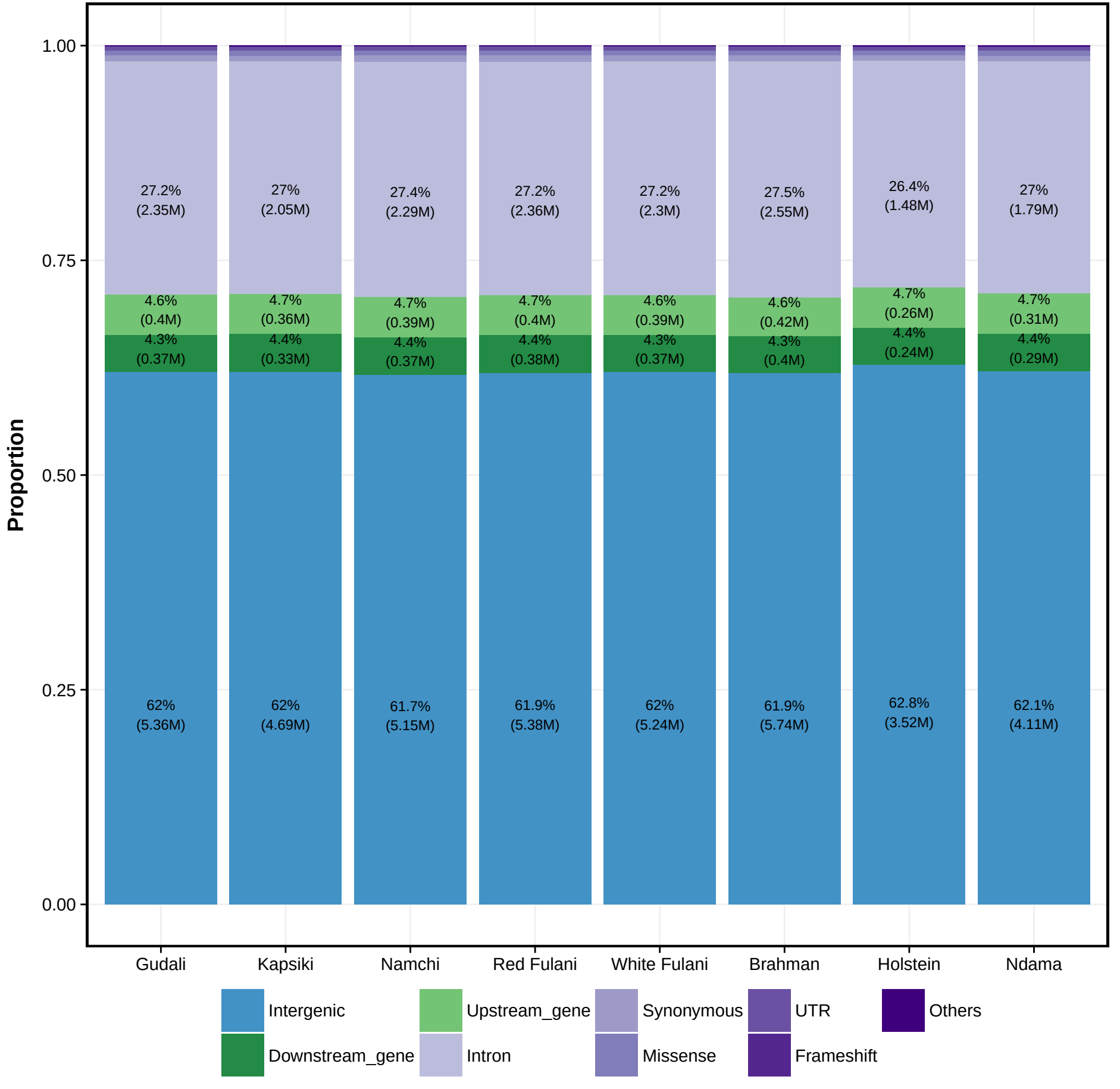

Figure 4 B

Breed specific SNPs

Click here to access/download;Figure;FIGURE4_B_Heatmap_GO_term.pdf

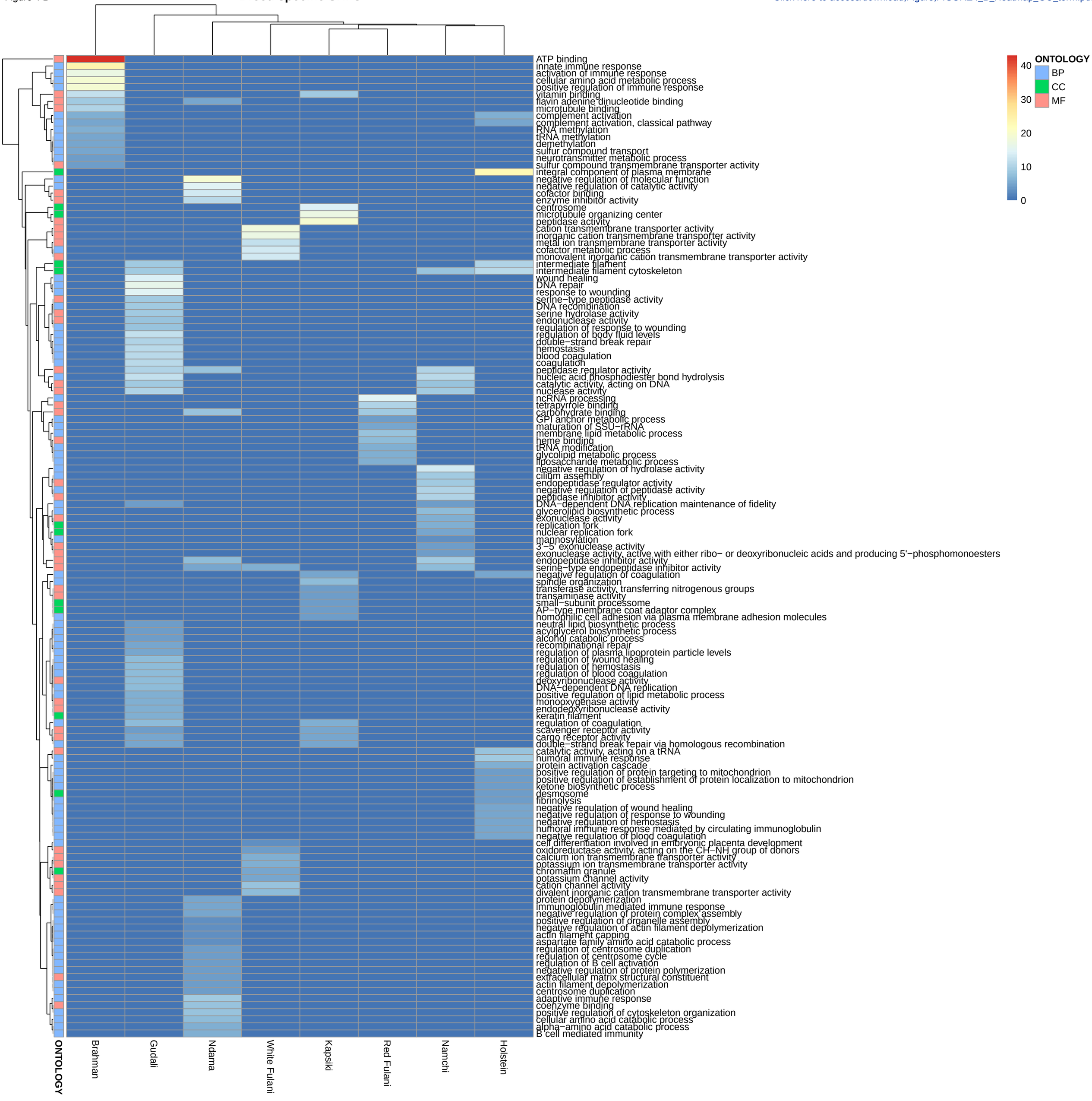

Figure 4 C

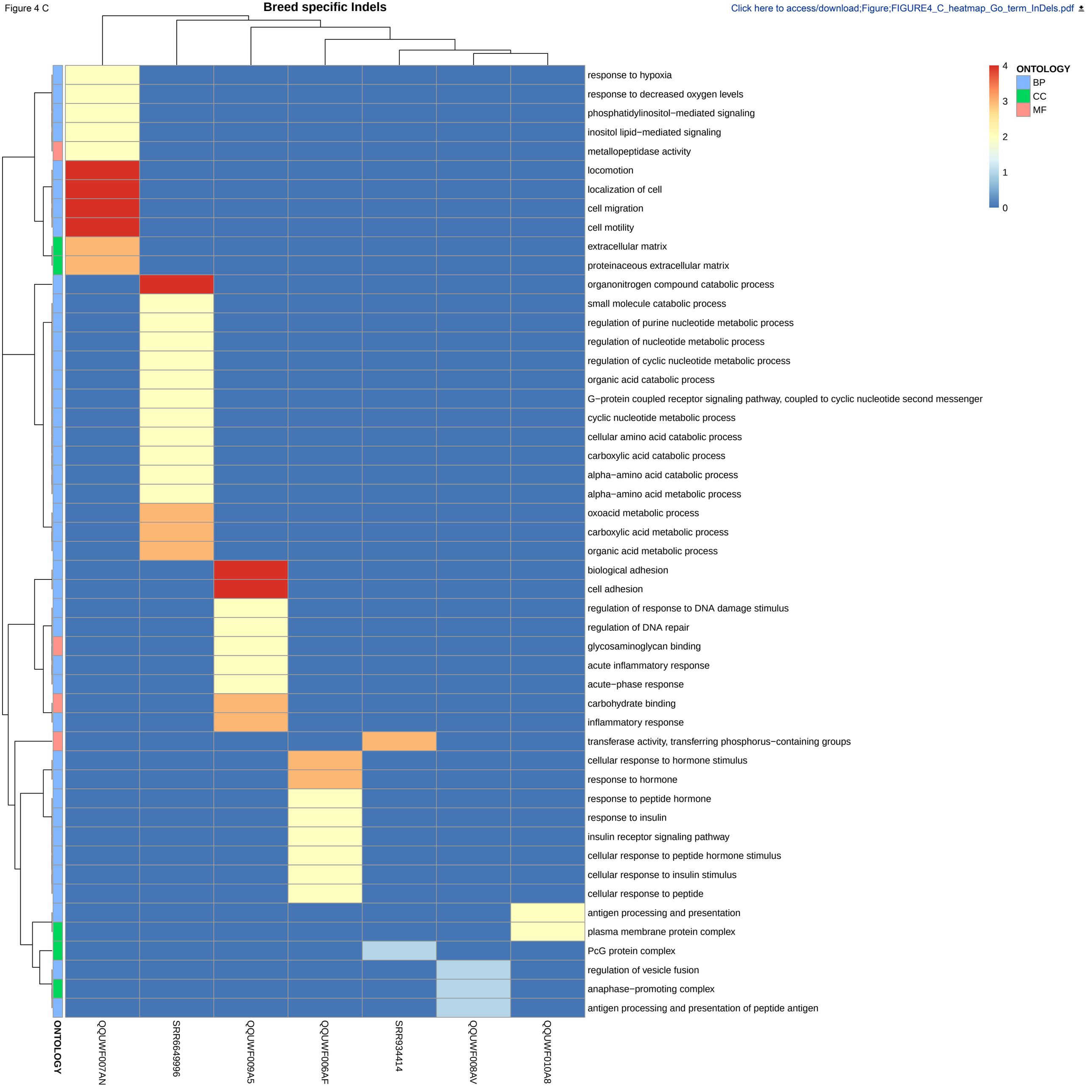

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Figure 5

Breed specific SNPs