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Genetic structure of *Ehrlichia ruminantium* based on Multilocus Sequence Typing: particularities of strains from West-Africa

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ABSTRACT

Ehrlichia ruminantium is the tick-borne rickettsia causing heartwater to wild and domestic ruminants. Previously, Multilocus Sequence Typing of this pathogen (MLST) highlighted the co-circulation of two genotypic groups within nearby villages from Burkina Faso where vaccination assays had been previously recurrently performed. In the present study the laboratory strains that had been involved in vaccination assays, which evidenced their close relationships with strains that were later involved in local outbursts, were genotyped. Characterization of the bacterium genetic diversity was extended to four distant Beninese localities where no vaccination assays had ever been performed, through sampling of N=500 specimens of the local tick vector, *Amblyomma variegatum.* This led evidencing an even higher frequency of new multilocus MLST genotypes in Benin than in Burkina Faso, 93% of the Beninese genotypes included at least one new private allele (i.e., no detected elsewhere), while this was only the case of 80% bacteria genotypes sampled in Burkina Faso. Investigating the genetic structure of the bacterium across the Africa continent revealed a reduction of its diversity in the eastern and southern part of the continent relatively to West Africa. As the bacterial genetic diversity currently remains highest in West Africa than anywhere else in the continent, this area remains optimal for the development and/or testing of vaccines against heartwater.

Keywords: MLST analysis, epidemic peak, heartwater, *Amblyomma variegatum*, West Africa, vaccine development.

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INTRODUCTION

Ehrlichia ruminantium is a tick-borne rickettsia causing heartwater in domestic (goats, sheep, cattle) and wild (antelopes, giraffes, buffaloes) ruminants (Dumler et al., 2001). Among domestic animals, sheep and goats are more susceptible to heartwater than cattle, with high lethality (up to 80%) recorded in sheep (Stachurski et al., 2019). As the animals transferred from healthy to enzootic regions are highly susceptible (Adakal et al., 2002), heartwater heavily handicaps the genetic amelioration programs of African livestock. The recent colonization of West Africa by the southern cattle tick, *Rhipicephalus microplus* had rendered more complex the epidemiological dynamics of this pathogen in some West African areas invaded by *R. microplus.* Indeed, both *R. microplus* and the native West-African tick vector, *Amblyomma variegatum*, co-contribute to the bacterial transmission there (Some et al., 2023 a, 2023 b). Aside this very recent situation, *E. ruminantium* is vectored to wildlife and domestic animals by African *Amblyomma* species with non-overlapping distribution, among which *A. variegatum* has got the largest geographical distribution (Stachurski et al., 2019). Preventing the transmission of *E. ruminantium* revealed to be difficult given the limits that were faced by several control approaches, ranging from tick control to livestock vaccination. Vector control programs are facing difficulties caused by the three-host life-cycle of the *Amblyomma* spp vectors, and their ability to enter in diapause when environmental conditions are not suitable (Stachurski et al., 2010). Alive vaccine is commercialized in South Africa; it contains blood infected with virulent *E. ruminantium* organisms of the Ball 3 isolate, and its use is complemented with antibiotics treatments when fever develops in the animal infected with such alive vaccine. This process has got some disadvantages such as the possibility of transmission of a new pathogenic agent, which can recombine with field-circulating strains. The use of alternative vaccination methods, based on inactivated (Adakal et al., 2010a), attenuated (Zweygarth et al., 2008) and recombinant strains (Collins et al., 2003) were developed, but none has been proved highly successful. Irrespectively of the vaccine type, results are convincing in controlled conditions (with protection up to 100% being observed), but their efficiency to protect against field strains appeared limited. For example, in South Africa, Collins et al. (2003) recorded 47% (16 out of 34) surviving animals during field challenge in a heartwater-endemic area (Northern Province) compared to 100% protection in challenges based on laboratory strains during the pre-test period. These failures are related to the genetic and antigenic diversity of *E. ruminantium* field strains (Adakal, 2002). This highlights the alternative choice to engage naturally circulated strains into a vaccination cocktail. In Burkina Faso, the use of the cocktail, formed of a locally circulating *E. ruminantium* isolate and the Gardel strain from Guadeloupe (French Caribbean's), raised the sheep protection against heartwater from 59% to 72% in field situation (Adakal et al., 2010a). This has raised the issue to characterize the geographical scale at which changes and/or diversity in the immune properties of *E. ruminantium* are determined: are co-circulating strains more or less likely to be heterologous than strains sampled in different regions?

The genetic diversity of this pathogen had been characterized using Multilocus Sequence Typing (MLST) (Adakal et al., 2009). In 2010, this approach allowed to evidence the co-circulation of two groups of genotypes, including one characteristic of an on-going epidemic peak, within three neighbouring villages from Burkina Faso (Adakal et al., 2010b). A year later, the same MLST markers concluded to the surprising

lack of genetic structure among *E. ruminantium* isolates driven from diverse part of the African continent (Nakao et al., 2011). Hence the present study aimed at improving our understanding of these phenomena by enlarging the West-African sampling to another country (Benin) and the laboratory strains that had been involved in the assays of inactivated vaccines in Burkina Faso in the years preceding a local outbreak. Moreover, it was necessary to re-analyse the possible genetic structure within the African continent by considering more available MLST genotyped pathogens rather than only sub-sampling as previously performed by Nakao et al. (2011).

MATERIALS AND METHODS

E. ruminantium stains isolated from N= 500 *A. variegatum* collected in Benin

A. variegatum ticks sampling in Benin and screening for *E. ruminantium* as well as related prevalence results have been published in Farougou et al. (2012). In this study MLST analyses were carried out on infected ticks to characterize the *E. ruminantium* field isolates.

Target loci and MLST nested PCR

To target the *gltA, groEL, lepA, lipA, lipB, sodB* and *sucA* loci, primers and PCR conditions previously described (Adakal et al., 2009) were used. PCR products were then sent for sequencing to *BECKMAN COULTER GENOMICS* (Hope End, Takeley, Essex CM22 6TA, United Kingdom). They were sequenced in both directions in order to reduce the possibilities of misreading and to avoid the mistakes caused by Taq polymerase during the elongation step of the PCR. For each PCR product, consensus sequence was retrieved using *Vector NTI* (*Vector NTI Advance10*).

In addition to the *E. ruminantium* isolates from Beninese ticks, the MLST genotyping was performed on isolates that had previously been involved in the inactivated vaccine research in Burkina Faso.

Complementary MLST genotypes retrieved from past studies

Sequences of target loci from field isolates from South-Africa, Zimbabwe, Zambia, Kenya, Uganda, Sao Tome and Principe, Nigeria, Gambia, Senegal and Burkina Faso available on GenBank (Nakao et al., 2011; Adakal et al., 2010b) were downloaded to complete our dataset. Strains ERGA and ERWO respectively isolated in Guadeloupe and South Africa were added as references strains (Collins et al., 2005; Frutos et al., 2006).

Sequence alignment and phylogenetic tree

Multiple alignments of sequences were carried out with *MUSCLE version 3.5*, as described by Edgar (2004), implemented in *MEGA* version 5. Phylogenetic analyses were performed with PhyML according to Dereeper et al. (2008) using the concatenated sequences of the seven genes (*gltA-sucA-lepA-sodB-lipA-lipB-groEL*). The phylogenetic tree was constructed using Approximate Likelihood-Test (aLRT). Further, concatenated sequences were imported into SpliTree4 *version 4.13.1* software for obtaining preliminary network using the neighbour-net algorithm according to Huson and Bryant (2006).

Sequence-type and minimum spanning tree

Let's call sequence-types (ST) the distinct multilocus genotypes, i.e., allelic profiles differing from one another by at least one allele at any of the seven monitored loci. The strain 2_BF_07 from Burkina Faso was used as reference for the attribution of allelic numbers and the allelic profiles using MLSTest *version 1.0.1.17* as described by Tomasini et al. (2013). It is noteworthy that several field isolates can share a same ST. All ST members were included in minimum spanning tree

(MST) generated using BioNumerics software *version 7.1*. The definition of a partitioning corresponds here to the 'clonal complexes' according to epidemiological terminology (i.e., the group of strains connected to a central ST by at most 2 allelic differences across the seven monitored loci) as defined in eBurst software.

Recombination, Genetic and population differentiation

PHI test was performed using SpliTree4 *version 4.13.1* on the concatenated sequences to determine whether recombination events were statistically significant. Then, the evaluation of the recombination events and identification of probable parents was performed with the Recombination Detection Program, RDP3 *version 3*.44 as described previously by Heath et al. (2006). Concatenated sequences were checked for putative recombinant regions using GeneCov, BootScan, MaxChi, Chimaera, SiScan and 3Seq methods implemented in RDP3. Only one representative sequence (one ST member) was involved in this analysis when sequences were identical.

Genetic diversity and genetic differentiation were assessed using DnaSP5 *version 5.10.01*, as described by Librado and Rozas (2009), for each gene based on the haplotype diversity Hd and Fst estimate. These analyses were computed to assess genetic diversity within each group of strains and genetic differentiation between the groups using the seven target loci separately.

Concatenated sequences were finally used to perform Analysis of Molecular Variance (AMOVA) with Arlequin *version 3.5.2.2* according to Excoffier and Lischer (2010) in order to assess population differentiation among either West African isolates or between West Africa isolates and isolates from East and South Africa.

Since strains from Burkina Faso and Benin are largely more numerous than those from others countries, some analyses in this study, were also done considering these West African strains separately.

RESULTS

E. ruminantium strains from Benin: new alleles

MLST analyses were performed on 15 tick isolates for which all seven bacterial loci could be amplified and sequenced. These 15 isolates provided 15 distinct sequences-types, including 14 bearing at least one allele that had never been identified in previous studies (i.e., 'private' alleles; Table 1). Altogether the Beninese dataset encompassed 27 private alleles. The corresponding sequences have been published in GenBank under the following accession numbers: KT279417 to KT279420 (*gltA*); KT315518 to KT315525 (*sucA*); KT315526 to KT315529 (*lepA*); KT315530 to KT315534 (*sodB*); KT315535 to KT315538 (*groEL*) and KT315539 to KT315541 (*lipB*).

Genetic diversity and differentiation

Haplotypic diversity (Hd) was significant higher in the Beninese sample than in those from other African regions at three loci (*sucA, lipB, groEL*) (Table 2). Higher haplotypic diversity relatively to other regions was noticed in West Africa at the *sodB* and *lipA* loci (P=0.006).

The nucleotide diversity π monitored at each of the seven loci was higher in West Africa than in other regions (Table 3). Geographical variations in this estimate were significant at *sucA* (P=0.045) and *lipA* (P=0.024). Considering all sampling regions, *groEL* and *LipA* were the most and least polymorphic loci respectively (Table 1).

The frequencies of private alleles and private sequence-types were highest in the Beninese sample than any other samples. The 15 Beninese bacterial strains provided 15 distinct sequence-types (STs), among which 14

(93%) were private STs. For the sake of comparison, the 49 isolates sampled in Burkina Faso defined 33 ST among which 29 (87%) harboured private alleles. Finally, the 12 isolates sampled in South/East Africa defined 10 STs among which only six (50%) harboured private alleles (Table 1).

Genetic differentiation per individual locus detected strong genetic differentiation between isolates from Benin and Burkina Faso (Fst>0.30) at each of the seven monitored loci (Table 4). Strong genetic differentiation was also observed at each locus between, on the one hand, the West African samples, and on the other hand, samples from either East Africa (Fst>0.20), South Africa (Fst= 0.13-0.38) or from East- and South Africa (Fst=0.15-0.38) (Table 4). AMOVAs confirmed these patterns at the multilocus level, concluding to significant differentiation between Benin and Burkina Faso (P<<0.05; Φst =0.68) as well as between West-African pathogens and those circulating in East and South Africa (P<<0.05; Φst =0.43) (Table 5). Ignoring the strains involved in the 2002-2009 epidemic peak detected in Burkina Faso resulted in an increase in the genetic differentiation estimated within West Africa without affecting other genetic differentiation signals (Table 5).

Minimum Spanning Tree over all dataset

The minimum spanning tree (MST) built from the 83 isolates (corresponding to 66 STs) discriminates two genetic groups (Figure 1). The first one (group A) is mostly formed of isolates from Burkina Faso including those involved in the 2002-2009 epidemic peak. The ST number 25, represented by strain 1795_BK_08, is the predicted founder of this epidemic peak that includes all the laboratory strains used in vaccination assays (Figure 1). The second group (group B) is pan-African since it is formed of isolates sampled in West-, South- and East-Africa. Most of the Beninese isolates (12 out of 15) belonged to group B. The reference strains ERGA (ST 49) and ERWO (ST 54) are related to West African strains from both groups.

Network and Phylogenetic tree

The neighbour-net network detected the same groups A & B previously observed in the MST (Figure 2). Removing of the isolates in the 2002-2009 epidemic peak resulted in an apparent decrease in the between-group genetic distance (Figure 3). Phylogenetic trees slightly modified the two groups previously detected in MST or neighbour-net network; these groups are thus referred to as A' and B' hereafter (Figure 4). The group A' was composed of the isolates involved in the epidemic peak observed in Burkina Faso (West African I) plus one strain from Ghana (Pokoase_GHA_96) (Figure 4). The group B' regroups two clades such as the first regroups only West African isolates while the second is pan-African (including also ERGA and ERWO reference strains Figure 4).

All Beninese strains are in group B' except 2762_BN_10 (ST 46) (Figure 1), which belongs to the epidemic peak in Group A'. West African strains are present in both groups while South-African and East African strains belong only to the pan-African group B'.

Recombination

Only recombination events supported by at least three detection methods were considered. This allowed identifying four recombination events within the entire dataset (Table 6). The most likely parental sequences of one recombinant event (event #2) were identified as similar to pathogens sampled in Benin (2332_OdBn_09) and Burkina Faso (1754_BK_08). The three other recombinant events were detected in genotypes sampled in West Africa, East Africa and South Africa that were assessed as descending from two West-African parents.

Table 1: Allelic composition and sampling characteristics of the 66 Sequence Types; STs [For each geographical area considered, are recalled the number of isolates, the number of distinct STs, the total number of alleles per locus and that of the alleles specific to the area considered. The allelic composition of each ST across the seven loci considered is detailed as well as the sampling characteristics of the STs (rank order, phylogenetic label, ST-ID, ST-size and ST-composition). Framed characters indicate which alleles are specific ("private" alleles) to a geographical sub-division (i.e., Benin, Burkina Faso, other west-African countries, South Africa or East Africa)].

Geographical area		rank order	Phylogenetic label	Sequence Type (ST)	ST size (N)	ST composition	alleles number per locus	*Glta*	*SucA*	*LepA*	*SodB*	*LipA*	*LipB*	*GroEL*
West Africa:	70 isolates defining 53 STs specific to this area						total:	*10*	*16*	*23*	*15*	*7*	*15*	*12*
							specific to this area:	*9*	*15*	*20*	*15*	*7*	*13*	*10*
							haplotype diversity (Hd)	*0.719*	*0.712*	*0.775*	*0.729*	*0.619*	*0.754*	*0.684*
							nucleotide diversity (Pi)	*0.013*	*0.016*	*0.008*	*0.006*	*0.008*	*0.017*	*0.014*
							typing efficiency (TE)	*0.323*	0.444	*0.442*	*0.833*	*0.778*	*0.385*	*0.179*
							discriminatory power (D)	*0.727*	*0.72*	*0.781*	*0.736*	*0.629*	*0.761*	*0.693*
Benin:	15 isolates defining 15 STs specific to Benin (14 STs include new specific alleles)						total:	*7*	*10*	*8*	*8*	*3*	*8*	*8*
							specific to this area:	*4*	*8*	*5*	*5*	*0*	*3*	*4*
							haplotype diversity	*0.724*	*0.924*	*0.79*	*0.733*	*0.514*	*0.876*	*0.79*
							nucleotide diversity	*0.009*	*0.013*	*0.01*	*0.005*	*0.003*	*0.012*	*0.009*
							typing efficiency	*0.28*	*0.345*	*0.242*	*0.727*	*0.5*	*0.5*	*0.444*
							discriminatory power	*0.758*	*0.933*	*0.817*	*0.767*	*0.575*	*0.892*	*0.817*
		1	2207_BN_08	34	1			5	9	16	3	3	3	5
		2	2209_BN_08	35	1			6	1	8	10	1	1	5
		3	2234_BN_09	36	1			1	10	17	1	3	4	6
		4	2313_BN_09	37	1			1	11	8	1	1	6	5
		5	2332_BN_09	38	1			1	12	18	11	1	1	7
		6	2352_BN_09	39	1			7	1	19	1	1	4	5
		7	2378_BN_09	40	1			1	13	8	12	1	10	3
		8	2396_BN_09	41	1			1	3	8	13	3	11	1
		9	2400_BN_09	42	1			7	1	8	1	1	1	8
		10	2405_BN_09	43	1			8	9	8	1	3	3	5
		11	2452_BN_09	44	1			1	3	11	1	1	12	3
		12	2737_BN_10	45	1			1	14	8	1	1	1	5
		13	2762_BN_10	46	1			3	2	1	2	2	13	2
		14	2789_BN_10	47	1			9	15	20	14	1	10	9
		15	2802_BN_10	48	1			1	1	11	1	1	1	5
Burkina Faso:	49 isolates corresponding to 35 ST (33 specific to Burkina Faso & two sampled elsewhere in West Africa; 29 STs include some allele(s) specific to Burkina Faso)						total:	*5*	*9*	*17*	*10*	*6*	*10*	*8*
							specific to this area:	*2*	*6*	*14*	*7*	*3*	*4*	*4*
							haplotype diversity	*0.628*	*0.578*	*0.674*	*0.669*	*0.521*	*0.616*	*0.579*
							nucleotide diversity	*0.011*	*0.013*	*0.005*	*0.005*	*0.007*	*0.015*	*0.013*
							typing efficiency	*0.312*	*0.409*	*0.708*	*0.769*	*0.75*	*0.278*	*0.136*
							discriminatory power	*0.643*	*0.595*	*0.687*	*0.682*	*0.54*	*0.632*	*0.596*

		16	2277_BF_02	25	8	Ghana 1996 + Gambia 2001 + BF 2001-to-08 (S_GHA_96; GAM_01; 2279_BF_01; 2277_BF_02; 2278_BF_02; 2284_BF_02; 2281_BF_03; 1795_BF_08)		2	2	1	2	2	2	2
		20	1270_BF_08	23	5	Senegal 1981 + two BF localities (SEN_1981; 2282_BF_02; 2283_BF_03; 1270_BF_08; 1946_BF_08)		3	2	1	2	2	2	2
		23	2280_BF_03	50	1			2	2	21	2	2	15	10
		24	2285_BF_03	51	1			3	16	1	15	2	2	11
		25	1028_BF_07	52	1			10	3	14	3	1	6	2
		26	1053_BF_07	53	1			1	3	22	3	6	3	12
		27	2_BF_07	1	1			1	1	1	1	1	1	1
		28	19_BF_07	2	1			2	2	2	2	2	2	2
		29	331_BF_07	3	1			2	2	3	2	2	2	3
		30	395_BF_07	4	2	BF: two localities in 2007 (395_BF_07; 476_BF_07)		2	2	4	2	2	2	2
		31	461_BF_07	5	2	BF: one locality in 2007 (461_BF_07; 474_BF_07)		2	2	5	2	2	2	2
		32	463_BF_07	6	1			3	2	6	2	2	2	2
		33	466_BF_07	7	1			2	2	7	2	2	2	2
		34	469_BF_07	8	1			2	2	3	2	2	2	2
		37	623_BF_07	9	1			1	3	8	3	3	3	4
		38	629_BF_07	10	3	BF: once in 1997 & twice in 2007 (BF_97; 629_BF_07; 635_BF_07)		1	1	1	1	1	4	5
		39	630_BF_07	11	1			1	4	1	1	1	4	5
		40	631_BF_07	12	1			1	3	9	3	3	3	5
		42	668_BF_07	13	1			1	5	10	1	1	5	5
		43	708_BF_07	14	1			1	3	11	1	1	5	3
		44	810_BF_07	15	1			2	2	1	2	2	2	3
		45	891_BF_07	16	1			2	6	1	2	2	2	2
		46	903_BF_07	17	1			4	2	1	4	2	2	2
		47	1062_BF_07	18	1			1	7	1	3	1	6	5
		48	1114_BF_08	19	1			3	2	12	2	2	2	2
		49	1210_BF_08	20	1			2	2	13	5	2	2	2
		50	1232_BF_08	21	1			1	8	11	6	1	5	3
		51	1267_BF_08	22	1			1	3	14	3	1	6	1
		53	1754_BF_08	24	1			2	2	1	7	2	7	2
		55	1796_BF_08	26	1			2	2	1	2	2	8	2
		56	1798_BF_08	27	1			2	2	1	7	4	2	2
		57	1799_BF_08	28	1			2	2	15	2	2	2	2
		58	1905_BF_08	29	1			2	2	1	8	2	2	2
		60	1948_BF_08	30	1			2	2	1	2	2	9	2
		61	1951_BF_08	31	1			1	1	1	9	1	1	1
		62	2165_BF_08	32	1			2	2	1	8	5	2	2
		63	2185_BF_08	33	1			2	3	1	2	2	2	2
Other West-African origins:		6 isolates defining 5 STs				(3 new & 2 sampled in Burkina Faso;	total:	*4*	*3*	*4*	*2*	*3*	*4*	*3*
						one ST new specific alleles)	specific to this area:	*0*	*0*	*1*	*0*	*1*	*0*	*0*
							haplotype diversity	*0.867*	*0.733*	*0.8*	*0.533*	*0.733*	*0.8*	*0.6*
							nucleotide diversity	*0.013*	*0.016*	*0.007*	*0.004*	*0.01*	*0.017*	*0.011*

							typing efficiency	*0.4*	*0.273*	*0.571*	*0.667*	*0.5*	*0.333*	*0.3*
							discriminatory power	*0.905*	*0.81*	*0.857*	*0.667*	*0.81*	*0.857*	*0.714*
		65	NIG_83	56	1			7	1	8	1	1	1	5
		66 & 69	GAM-01 & S_GHA_96	25	8	cf 2277_BF_02 above		2	2	1	2	2	2	2
		67	SAP_81	59	1			1	1	25	1	9	5	3
		68	P_GHA_96	62	1			3	3	10	2	1	14	2
		70	SEN_81	23		cf 2282_BF_02 above		3	2	1	2	2	2	2
South- & East-Africa:		12 isolates defining 10 new specific STs				(6 STs include new specific alleles)	total:	*6*	*4*	*6*	*3*	*3*	*4*	*4*
							specific to this area:	*5*	*3*	*2*	*2*	*2*	*2*	*2*
							haplotype diversity	*0.803*	*0.636*	*0.018*	*0.318*	*0.318*	*0.742*	*0.742*
							nucleotide diversity	*0.004*	*0.003*	*0.004*	*8E-04*	*0.004*	*0.005*	*0.007*
							typing efficiency	*1.2*	*1.333*	*0.857*	*1.5*	*0.429*	*0.8*	*0.571*
							discriminatory power	*0.833*	*0.692*	*0.846*	*0.423*	*0.423*	*0.782*	*0.782*
South-Africa:		6 isolates defining 6 new specific STs				(4 STs include new specific alleles)	total:	*5*	*3*	*5*	*3*	*3*	*2*	*3*
							specific to this area:	*4*	*2*	*2*	*2*	*2*	*2*	*1*
							haplotype diversity	*0.933*	*0.733*	*0.933*	*0.6*	*0.6*	*0.533*	*0.733*
							nucleotide diversity	*0.006*	*0.003*	*0.004*	*0.002*	*0.008*	*0.003*	*0.004*
							typing efficiency	*1*	*1.5*	*0.833*	*1.5*	*0.429*	*1*	*0.75*
							discriminatory power	*0.952*	*0.81*	*0.952*	*0.714*	*0.714*	*0.667*	*0.81*
	RSA	72	ERWO_85	54	1			11	17	8	1	1	10	5
		73	K_RSA_84	57	1			13	18	24	17	8	10	5
		74	Z_RSA_79	60	1			14	19	26	1	1	16	3
		75	B_RSA_52	61	1			15	18	26	1	1	10	3
	Zambia	76	ZAM_86	58	1			11	17	10	1	1	10	3
	Zimbabwe	77	ZIM_90	55	1			12	18	23	16	7	16	13
East-Africa:		6 isolates defining 4 new specific STs				(2 STs include new specific alleles)	total:	*2*	*2*	*2*	*1*	*1*	*3*	*2*
							specific to this area:	*0*	*0*	*0*	*0*	*0*	*0*	*1*
							haplotype diversity	*0.333*	*0.333*	*0.333*	*0*	*0*	*0.6*	*0.533*
							nucleotide diversity	*8E-04*	*0.002*	*7E-04*	*0*	*0*	*0.006*	*0.005*
							typing efficiency	*2*	*1*	*2*	*Infini*	*Infini*	*0.6*	*0.5*
							discriminatory power	*0.524*	*0.524*	*0.524*	*0.286*	*0.286*	*0.714*	*0.667*
	Sudan	78	SUD_81	63	1			11	17	11	1	1	10	3
	Uganda	79; 80 & 83	A4_UGA_08-09	64	3	Two localities in 2008-2009: Amuria (A4_UGA_08-09; A6_UGA_08-09 BF) and Tororo (T9_UGA_08-09)		1	17	11	1	1	1	14
		81	P3_UGA_08-09	65	1			1	17	11	1	1	5	14
		82	S13_UGA_08-09	66	1			1	1	8	1	1	1	3
West Indies, Guadeloupe:		one isolate defining one new ST				(no specific alleles at any locus)								
		71	ERGA_82	49	1			1	3	10	1	1	14	1

Table 2: Haplotype diversity; Hd [Significantly higher Hd values appear in bold characters (P=0.006)].

Areas	***gltA***	***sucA***	***lepA***	***sodB****	***lipA****	***lipB***	***groEL***
Benin	0.724	**0.924**	0.790	0.733	0.514	**0.876**	**0.790**
Burkina Faso	0.635	0.649	0.766	0.756	0.584	0.698	0.656
West Africa	0.737	0.787	0.878	**0.801**	**0.652**	0.829	0.754
South Africa	**0.933**	0.733	**0.933**	0.600	0.600	0.733	0.733
East Africa	0.500	0.500	0.500	0.000	0.000	0.833	0.667
All	0.771	0.839	0.881	0.781	0.658	0.865	0.797

Table 3: Nucleotide diversity; π [Significant differences in π values among areas were noticed at *sucA* (P=0.045) and *lipA* (P=0.024) loci].

Areas	***gltA***	***sucA****	***lepA***	***sodB***	***lipA****	***lipB***	***groEL***
Benin	0.00897	0.01328	0.00970	0.00466	0.00318	0.01176	0.00857
Burkina Faso	0.01174	0.01391	0.00642	0.00623	0.00798	0.01689	0.01578
West Africa	0.01401	0.01641	0.00905	0.00661	0.00852	0.01851	0.01595
South Africa	0.00557	0.00283	0.00421	0.00161	0.00802	0.00521	0.00418
East Africa	0.00127	0.00249	0.00105	0.00000	0.00000	0.00838	0.00597
All	0.01370	0.01575	0.00894	0.00609	0.00858	0.01776	0.01510

Table 4: Genetic differentiation; Fst [Subsets within West Africa (using strains from Benin and Burkina Faso) and between West Africa and East/South Africa were used].

Subsets	***gltA***	***sucA***	***lepA***	***sodB***	***lipA***	***lipB***	***groEL***
BENIN/BURKINA FASO	0.417	0.330	0.356	0.293	0.461	0.343	0.325
BENIN/EAST AFRICA	0.014	0.222	0.082	0.033	0.095	0.000	0.262
BENIN/SOUTH AFRICA	0.130	0.167	0.145	0.017	0.061	0.023	0.000
BURKINA FASO/EAST AFRICA	0.584	0.590	0.624	0.502	0.600	0.411	0.308
BURKINA FASO/SOUTH AFRICA	0.539	0.533	0.366	0.445	0.361	0.471	0.437
WEST AFRICA/EAST AFRICA &SOUTH AFRICA	0.377	0.383	0.155	0.319	0.262	0.318	0.227
WEST AFRICA/EAST AFRICA	0.393	0.435	0.388	0.350	0.419	0.242	0.215
WEST AFRICA/SOUTH AFRICA	0.379	0.365	0.130	0.302	0.204	0.302	0.297
SOUTH AFRICA/EAST AFRICA	0.100	0.200	0.483	0.000	0.089	0.000	0.284

Table 5: Analysis of Molecular Variance; AMOVA [Populations ID are as follow: BF: Burkina Faso; BF_epidemicP: Strains from Burkina Faso which belong to the epidemic peak; BF_Others: Strains from Burkina Faso which are not included in the epidemic peak; BUR: Burkina Faso (It refers to one strain from Burkina Faso which sequences were downloaded on GenBank); BK: Bekuy; BN: Benin; EA: East Africa; LB: Lamba; SA: South Africa; SR: Sara; WA: West Africa. *Pvalue of Variance: among groups, among populations within groups, within populations are the same for Φ_{CT}; Φ_{ST} and Φ_{SC} respectively].

Subsets	Groups	Populations	Size	Scale (Source of variation)	Sum of squares	Variance components	P-value*	%Variation	Φ_{SC}	Φ_{ST}	Φ_{CT}
I	West Africa	BF	49	Among groups	149.04	3.846	0.42	17.68	0.30	0.43	0.18
		BN	15	Among populations within groups	242.65	5.402	0.00	24.83			
		Others_WA	6	Within populations	963.01	12.507	0.00	57.49			
	East&South Africa	SA	6								
		EA	6								
II	West Africa	BF_epidemicP	34	Among groups	149.04	2.079	0.47	9.84	0.61	0.65	0.10
		Others_BF	15	Among populations within groups	637.80	11.570	0.00	54.78			
		BN	15	Within populations	567.86	7.472	0.00	35.38			
		Others_WA	6								
	East&South Africa	SA	6								
		EA	6								
III	West Africa	Others_BF	15	Among groups	28.44	0.051	0.30	0.41	0.18	0.18	0.00
		BN	15	Among populations within groups	93.36	2.213	0.00	17.91			
		Others_WA	6	Within populations	434.03	10.094	0.00	81.68			
	East&South Africa	SA	6								
		EA	6								
IV	Others_BF	BK+SR+BUR	7	Among groups	75.86	2.116	0.13	14.97	0.03	0.17	0.15
		LB	8	Among populations within groups	28.21	0.344	0.08	2.44			

	BN	BN_North	5	Within populations	361.82	11.672	0.00	82.59			
		BN_South	10								
	Others_WA	Others_WA	6								
V	BN	BN_North	5	Among groups	221.21	1.192	0.68	5.24	0.67	0.68	0.05
		BN_South	10	Among populations within groups	409.39	14.372	0.00	63.13			
	BF	BF_epidemicP	34	Within populations	432.12	7.202	0.00	31.64			
		Others_BF	15								
VI	BN_North	BN_North1	3	Among groups	14.23	0.119	0.33	0.96	0.03	0.04	0.01
		BN_North2	2	Among populations within groups	26.57	0.419	0.33	3.37			
	BN_South	BN_South1	7	Within populations	130.93	11.903	0.26	95.67			
		BN_South2	3								
VII	BF_epidemicP	BF_epidemicP1	17	Among groups	395.15	18.551	0.34	75.58	0.05	0.77	0.76
		BF_epidemicP2	17	Among populations within groups	19.10	0.316	0.08	1.29			
	Others_BF	BK+SR+BUR	7	Within populations	255.53	5.678	0.00	23.13			
		LB	8								
VIII	South Africa	SA1	3	Among groups	17.50	2.028	0.33	30.8	0.09	0.37	0.31
		SA2	3	Among populations within groups	10.67	0.389	0.25	5.91			
	East Africa	EA1	3	Within populations	33.33	4.167	0.01	63.29			
		EA2	3								

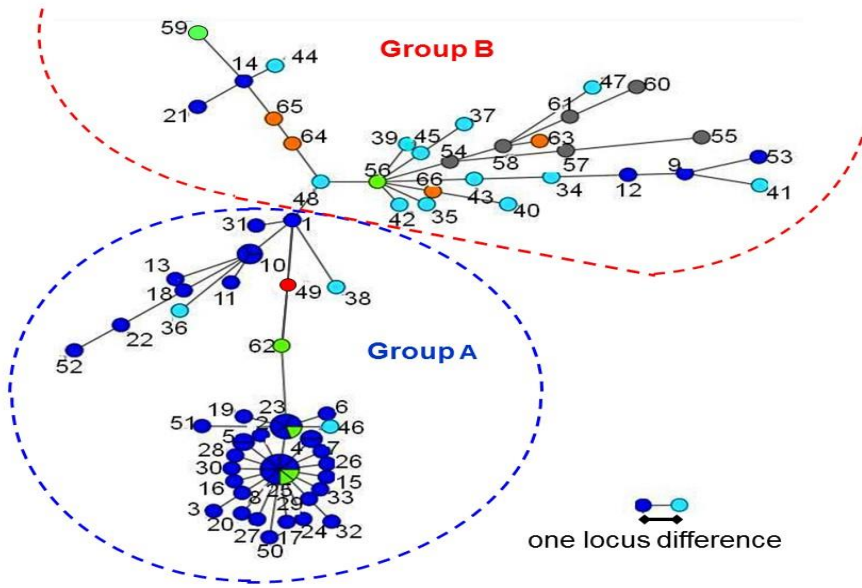

Figure 1: Minimum spanning tree based on allelic profiles.

Each circle represents one sequence-type; i.e., a unique allelic profile over the seven housekeeping genes considered. Circle sizes are proportional to the number of samples sharing the same allelic profile. Figures refer to the sampling ID defined in Table 1. The distance between two connected circles is proportional to the number of loci differing between the two allelic profiles. Colors refer to the sampling geography as follows. The samples collected in Benin appear in light-blue; those collected in Burkina Faso and other West-African countries (Senegal, Ghana, Nigeria, and Gambia) appear in dark-blue or green, respectively. Samples from South-African countries (Republic of South Africa, Zimbabwe, and Zambia) or East-African countries (Uganda and Sudan) appear in grey and orange, respectively. Samples from the Carribean's (Guadeloupe, Sao Tome and Principe) appear in red.

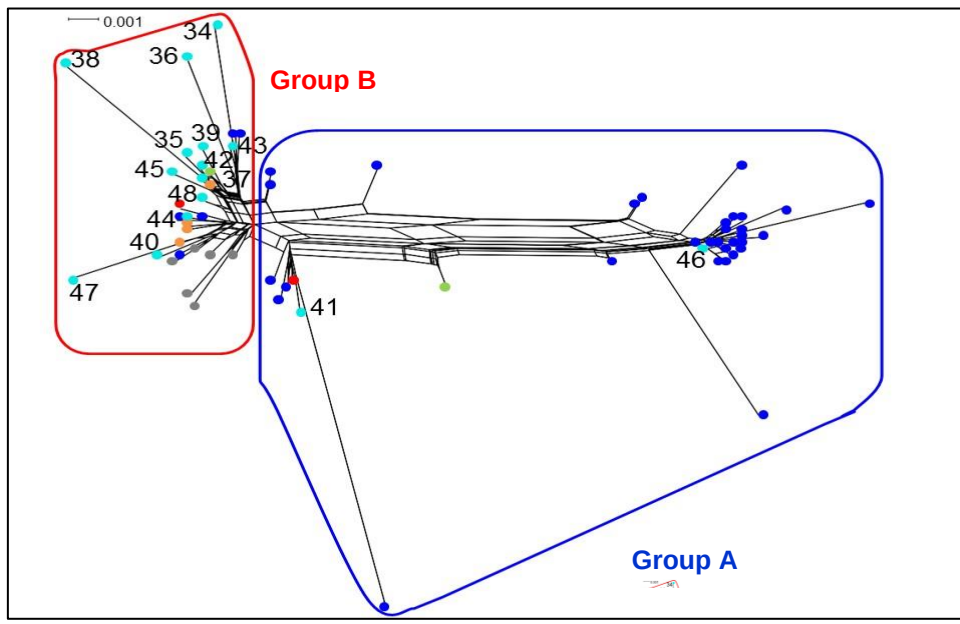

Figure 2: Phylogenetic network computed with all of the sequence types.

The differences between samples are measured in base-pair differences among the concatenated sequences. The sampling geography is color-coded as defined in Figure 1. The figures used as sample-ID are defined in the Table 1. Strains from Benin represented by light-blue circles are followed by their ST numbers.

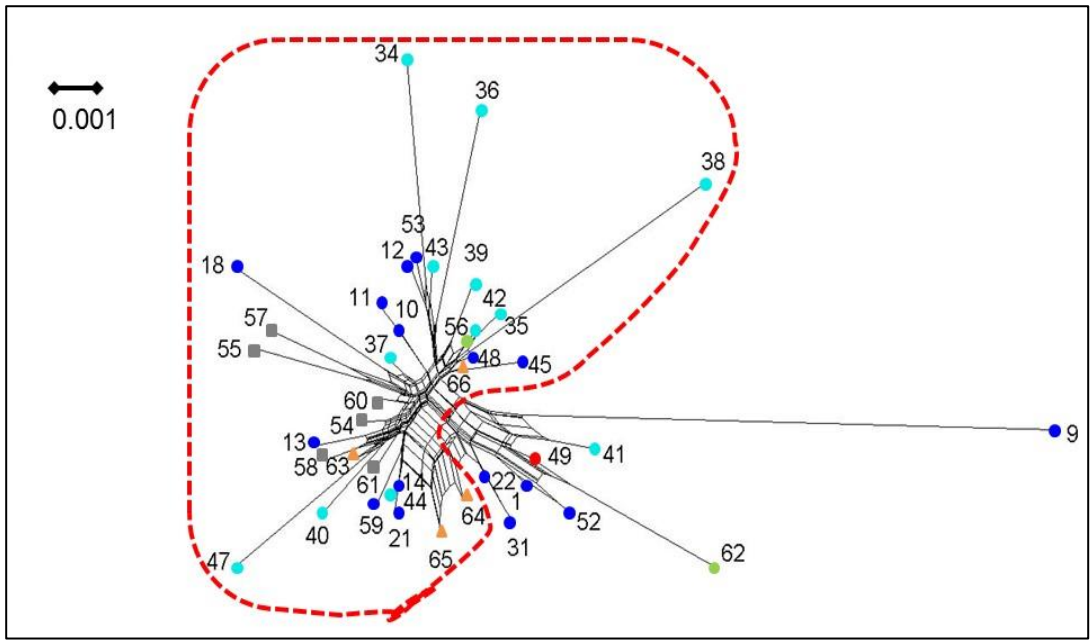

Figure 3: Phylogenetic network based on strains without those included in epidemic peak.
The differences between samples are measured in base-pair differences among the concatenated sequences. The sampling geography is color-coded as defined in Figure 1. The figures used as sample-ID are defined in the Table 1.

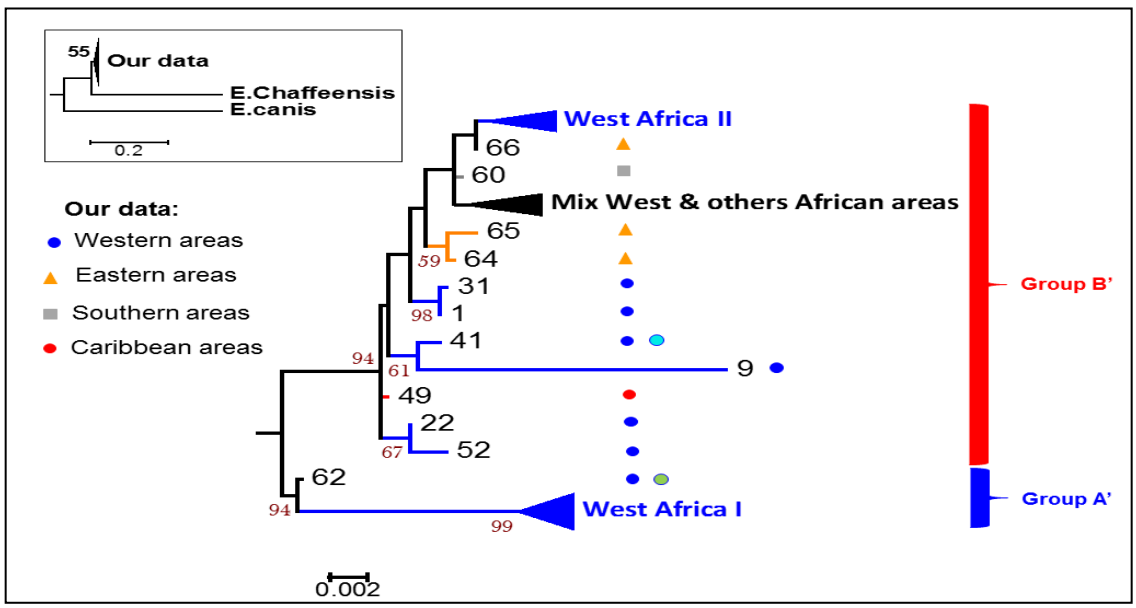

Figure 4: Phylogenetic tree based on concatenated sequences using Maximum Likelihood method.
The group A' defines here is a part of the group A defined in Figures 1 and 2 while the group B' is composed of the strains of group B and some of group A defined in Figures 1 and 2. The confidence in nodes was assessed applying 10 000 permutations. Sequences labels are ST numbers as defined in Table 1. For samples collected in West Africa, a single dark-blue circle is represented whenever these sequences were only detected in Burkina Faso. Otherwise, a second circle appears at right in light-blue, or green to indicate the circulation of this sequence in Benin, or in either Ghana or Nigeria.

Table 6: Recombination events [The size of the concateneted sequences is 2832 bp including 395bp, 401bp, 475bp, 415bp, 341bp, 358bp, 447bp for gltA, sucA, lepA, sodB, lipA, lipB, groEL respectively. + corresponds to detection of significant recombination events (p<0,05). a: Breakpoint position is Undetermined but a proposition of a position is given in bracket. b: Parent of recombination is not clearly determined but an identification which can be a misidentification is done and is in bracket].

Events	Breakpoint		Genes concerned	Recombinant	Major Parent	Minor Parent	Detection method					
	Beginning	Ending					GeneConv	BootScan	MaxChi	Chimaera	SiScan	3Seq
1	2292	2695	LipB(93bp); groEL(310bp)	**623_LB_07**	2_BK_07	Unknown (2280_E008_05)b	+	+	+	+	+	+
2	728	1132	SucA(68bp); lepA(336bp)	**2_BK_07**	2332_PhBn_09	1754_BK_08	+	+	+	+	+	+
	728	1132		**629_LB_07**	2332_PhBn_09	1754_BK_08	+	+	+	+	+	+
	728	1132		**630_LB_07**	2332_PhBn_09	1754_BK_08	+	+	+	+	+	+
	728	1132		**1951_SR_08**	2332_PhBn_09	1754_BK_08	+	+	+	+	+	+
	400	1132	SucA(396bp); lepA(336bp)	**1062_LB_07**	2332_PhBn_09	1754_BK_08	+	+	+	+	+	+
	136	1360	gltA(259bp); SucA(401bp); lepA(475bp); sodB(89bp)	**2207_PhBn_08**	1754_BK_08	2332_PhBn_09	+	+	+	+	+	+
	Undetermined (454)a	1678	SucA(342bp); lepA(475bp); sodB(407bp)	**2737_PhBn_10**	1754_BK_08	2332_PhBn_09	+	+	+	+	+	+
	Undetermined (4)a	704	gltA(391bp); SucA(309bp)	**668_SR_07**	1754_BK_08	2332_PhBn_09	+	+	+	+	+	+
		704		**ERWO_RSA**	1754_BK_08	2332_PhBn_09	+	+	+	+	+	+
		1360	gltA(391bp); SucA(401bp);	**2234_LeBn_09**	1754_BK_08	2332_PhBn_09	+	+	+	+	+	+
		1360	lepA(475bp); sodB(89bp)	**2378_PhBn_09**	1754_BK_08	2332_PhBn_09	+	+	+	+	+	+
		1360		**2789_LeBn_10**	1754_BK_08	2332_PhBn_09	+	+	+	+	+	+
		1360		**2405_TcBn_09**	1754_BK_08	2332_PhBn_09	+	+	+	+	+	+
		1360		**Kwanyanga_RSA**	1754_BK_08	2332_PhBn_09	+	+	+	+	+	+
		1360		**Lutale_ZAM_86**	1754_BK_08	2332_PhBn_09	+	+	+	+	+	+
		1360		**Zeerust_RSA_79**	1754_BK_08	2332_PhBn_09	+	+	+	+	+	+
		1360		**Ball3_RSA**	1754_BK_08	2332_PhBn_09	+	+	+	+	+	+
		1360		**Umbanein_SUD**	1754_BK_08	2332_PhBn_09	+	+	+	+	+	+
		1351	gltA(391bp); SucA(401bp); lepA(475bp); sodB(80bp)	**Crystal_Springs_ZIM_90**	1754_BK_08	2332_PhBn_09	+	+	+	+	+	+
3	388	798	gltA(7bp); SucA(401bp); lepA(2bp)	**2185_LB_08**	891_SR_07	631_LB_07	+		+	+	+	+
	453	771	SucA(318bp)	**2285_BK_433S_07**	891_SR_07	631_LB_07	+		+	+	+	+
	Undetermined (388)a	Undetermined (2367)a	gltA(7bp); SucA(401bp); lepA(475bp); sodB(415bp); lipA(341bp); lipB (340bp)	**Pokoase_GHA_97**	891_SR_07	631_LB_07	+		+	+	+	+
4	Undetermined (1781)a	Undetermined (2408)a	lipA(246bp); lipB (358bp); groEL (23bp)	**2285_BK_433S_07**	Undetermined (1754_BK_08)b	2165_SR_08		+	+	+		+

DISCUSSION

Particular epidemic peak in Burkina Faso

The present study failed to detect in Benin a phenomenon similar to the epidemic peak previously reported in Burkina Faso (Adakal et al., 2010). This might result from sampling bias given the much lower number of genotyped pathogens considered per Beninese region (N < 15) than within three neighbouring villages from Burkina Faso (49 multilocus genotypes within <400 km²). An alternative hypothesis would be that the 2002-2009 epidemic peak reported in nearby villages from Burkina Faso resulted from the vaccination assays previously performed there. This issue was addressed by genotyping the laboratory strains that had been involved in the vaccination assays. This evidenced a high genetic similarity between laboratory strains and those later involved in local outbursts. For instance, the genotype of BK242 strain (2277_BK242) used in vaccination cocktails is a member of the sequence-type #25, which is also the predictive founder of the epidemic peak (Figure 1). It is noteworthy that adding the BK242 strain to the Gardel strain in the vaccination cocktail drastically improved the sheep protection against heartwater under field conditions (Adakal et al., 2010a). Conversely, It is also worthy of note that the sequence-type #25 includes isolates that circulated elsewhere in West Africa in 1996 (Sankat_GHA_96) and 2001 (Kerr_GAM_01). Moreover, the sequence-type 23, genetically similar to ST25 and involved in Burkina Faso epidemics, included the genotype of a pathogen isolated in Senegal in 1981 (Senegal_SEN_81). Similarly, an isolate from Benin (2762_GgBn_10; ST46) and another sampled along the 2002-2009 epidemics in Burkina Faso share the same sequence type n°46. It is thus possible that the vaccination campaigns performed in Burkina Faso had disturbed the local evolution in *E. ruminantium* immune diversity, simply through its impact on immunological interferences in cattle herds between strains that have a long history of co-circulation throughout West Africa. These two alternative hypotheses are simpler than the previously assumed acceleration in the accumulation of newly arisen mutations resulting from vaccination assays (Frutos et al., 2007; Vachiéry et al., 2008). More extensive sampling campaigns within small areas – coupled or not with vaccinations campaigns- would be required to definitively settle this point.

West Africa: an appropriate area for development and test of vaccine against heartwater

Contrarily to previous analyses (Nakao et al., 2011), the present study evidenced significant differentiation of the *E. ruminantium* MLST genotypes between, on the one hand, West Africa and, on the other hand, the southern and eastern parts of the continent (Table 4, Table 5). This signal is robust when only focusing on private allele frequencies, which is the most appropriate way to control for uneven sampling sizes among African regions. Considering the seven loci at once led identify two genetic groups of MLST, with one (presently referred to as A or A') occurring only in West Africa contrarily of the pan-African distribution of the other (presently referred as group B or B'). The co-circulation of both genetic bacterial groups in West Africa was attested by the bacterial sampling performed on sheep living in Burkina Faso. There, one individual sheep was reported co-infected by the 1267_BF_08 and 1270_BF_08 genotypes that respectively belong to the A and B genotypic group (Figure 1). Further, another sheep from Burkina Faso was sequentially infected seven months apart by 630_BF_07 (genotypic group B) and then by 1114_BF_08 (genotypic group A).

Two of the housekeeping genes used in bacterial MLST may partially evolved in relation with vertebrate's immune responses against *E. ruminantium*. For instance, *groEL*, has been shown to be involved in immune response *Ehrlichia sennetsu* (Hennequin et al., 2001), which is a close parent of *E. ruminantium*. It is noteworthy that the differences between the genotypic groups A and B presently evidenced on *E. ruminantium* MLST remains when only considering the *groEL* locus (data not shown). This is also the

case when only considering the *lepA* locus, which codes for aGTP-binding protein presumably interacting with the host immune responses. However, it is noteworthy that other housekeeping genes are also supporting the significant genetic differentiation between the groups A and B (Table 4).

Complementarily, it is worthy of note that the diversity in immuno-properties and/or virulence of bacterial isolates is even higher that detected on the monitored house-keeping genes. This is for instance illustrated by the genotypes BK242 (2277_BF_02; Bekuy) and BM421 (2278_BF_02; Banankeledaga) bacterial isolates. Both share the same sequence-type (ST25) but induced different level of protection when added to vaccination cocktails: BK242 respectively induced 100% and 50% mortality in control and vaccinated sheep, while BM421 promoted 75% and 25% mortality in control and vaccinated sheep respectively (Adakal et al., 2010a).

Ehrlichia ruminantium biogeography in Africa: parallelism with the history of cattle domestication

In this study *E. ruminantium* strains consisted in two genotypic groups that are respectively either restricted to West Africa or present in all parts of the continent. This pattern reminds the pattern observed in the history of domestic cattle in Africa (Hanotte et al., 2002). There have been three historical steps during this history (Hanotte et al., 2002). The earliest step corresponded to the arrival of domestic *Bos taurus* cattle in West Africa about 10,000 years ago Later on, part of these West African *Bos taurus* cattle had diffused toward the eastern and southern parts of the African continent. The third historical step corresponded to the installation of *Bos indicus* in the horn of Africa and recurrent importing events of Asian *B. indicus* along the eastern African coast, which were followed by frequent hybridization between imported *B. indicus* and African *B. taurus* cattle and dispersal of hybrids across Africa. The genetic diversity of *Amblyomma variegatum* present in French West Indies and across Indian Ocean Isles complementary indicates a role for the dispersal of West African cattle in the extension of the geographical distribution of this West African tick vector (Starchurski et al., 2013). The present study completes the history of heartwater disease by evidencing that two genetic groups of the bacterial pathogen co-circulate in West Africa but only one of those is present elsewhere. Overall, these patterns converge for suggesting that the origin of heartwater diseases traced back to bacterial genotypes belong to genetic group A and *A. variegatum* tick-species, which both initially exploited West African wildlife. The geographical origin of the current bacterial genetic group presently referred as group B remains unclear. However, its presence today in West Africa likely result from the later diffusion of *B. indicus* and/or hybrid African cattle races. The present study, as that of Nakao et al. (2011), evidenced recombinants events between the bacterial genetic groups presently referred as A and B. This is likely to indicate that such recombination events could have recurrently occurred during history of cattle farming practices in the African continent. Such recombination events have likely played an important role in the maintenance of *E. ruminantium* genetic diversity and evolution within African regions (Cangi et al., 2016).

Conclusion

The epidemic pattern previously observed in Burkina Faso is atypical when compared to the bacterial diversity circulating in Benin. This could have resulted from defect in the inactivation phase in the development of vaccination assays using local filed strains, or from the effect of vaccination cocktails, which has disturbed the dynamic equilibrium in the antigenic properties of the bacteria naturally circulating in West Africa. The genetic diversity of *E. ruminantium* is highest in West Africa than in all African regions, suggesting a West African origin of the causal agent of heartwater. As the bacterial genetic diversity currently remains highest in West Africa than anywhere else in the continent, this area remains optimal for the development and/or testing of vaccines against heartwater.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

ASB, HA, SF designed the study. ASB conducted the experiments and performed data analysis. ASB, HA and CC wrote the first draft. All authors contributed to and agreed on the final version.

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