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Microsatellite and minisatellite genotyping of *Theileria parva* population from southern Africa reveals possible discriminatory allele profiles with parasites from eastern Africa

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ABSTRACT

The control of *Theileria parva*, a protozoan parasite that threatens almost 50% of the cattle population in Africa, is still a challenge in many affected countries. *Theileria parva* field parasites from eastern Africa, and parasites comprising the current live *T. parva* vaccine widely deployed in the same region have been reported to be genotypically diverse. However, similar reports on *T. parva* parasites from southern Africa are limited, especially in Corridor disease designated areas. Establishing the extent of genetic exchange in *T. parva* populations is necessary for effective control of the parasite infection.

Twelve polymorphic microsatellite and minisatellite loci were targeted for genotypic and population genetics analysis of *T. parva* parasites from South Africa, Mozambique, Kenya and Uganda using genomic DNA prepared from cattle and buffalo blood samples. The results revealed genotypic similarities among parasites from the two regions of Africa, with possible distinguishing allelic profiles on three loci (MS8, MS19 and MS33) for parasites associated with Corridor disease in South Africa, and East Coast fever in eastern Africa. Individual populations were in linkage equilibrium ($V_D < L$), but when considered as one combined population, linkage disequilibrium ($V_D > L$) was observed. Genetic divergence was observed to be more within (AMOVA = 74%) than between (AMOVA = 26%) populations. Principal coordinate analysis showed clustering that separated buffalo-derived from cattle-derived *T. parva* parasites, although parasites from cattle showed a close genetic relationship. The results also demonstrated geographic sub-structuring of *T. parva* parasites based on the disease syndromes caused in cattle in the two regions of Africa.

These findings provide additional information on the genotypic diversity of *T. parva* parasites from South Africa, and reveal possible differences based on three loci (MS8, MS19 and MS33) and similarities between buffalo-derived *T. parva* parasites from southern and eastern Africa.

1. Introduction

The causative agent of cattle theileriosis in eastern and southern Africa, *Theileria parva*, is known to adopt diversity as a survival strategy (Katzer et al., 2010), and its presence in the buffalo further complicates its epidemiology. Consequently, the success in control of *T. parva* has been undermined by the diversity of the parasite in the buffalo and cattle hosts resulting from recombination activities in the tick vector. Therefore, establishing the diversity and population structure of *T. parva* field parasites is critical in the design of effective control strategies for this parasite. Several molecular tools including PCR based assays (Iams et al., 1990; Nene et al., 1992; Toye et al., 1995; Skilton et al., 1998; Bishop et al., 2001), restriction fragment length polymorphism (RFLP) analysis of *T. parva* DNA (Bishop et al., 1993) and Southern blotting using *T. parva* repetitive DNA probes (Bishop et al., 2001) were developed to characterize *T. parva* parasites. However, these tools only detect polymorphisms in limited regions of *T. parva* genome, and therefore, not suitable for extensive genome wide characterization.

Subsequently, studies conducted by Oura et al. (2003) and Katzer

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et al. (2010) identified genome-wide microsatellite and minisatellite markers for characterizing *T. parva* parasites. Microsatellite loci are short tandem repeats of 2–8 bps while minisatellites have longer repeat units (8–100 bps) (Oura et al., 2003). Satellite loci show high rates of mutation which result in high levels of polymorphism making them ideal for molecular profiling of *T. parva* parasites (Oura et al., 2003). Hence, characterization of a panel of 11 and 68 polymorphic micro- and minisatellites respectively on *T. parva* genome (Oura et al., 2003; Katzer et al., 2010), as well as 42 PCR-RFLP markers (Katzer et al., 2010) has been a significant advancement in the analysis of genomic changes in *T. parva* parasite populations. Studies on *T. parva* parasites using satellite markers have demonstrated extensive diversity in field parasite from Uganda (Oura et al., 2005, 2011b; Muwanika et al., 2016), Tanzania (Elisa et al., 2015; Rukambile et al., 2016), Sudan (Salih et al., 2018) and Zambia (Muleya et al., 2012), and in cultured *T. parva* parasites from Kenya (Odongo et al., 2006; Katzer et al., 2010), but limited diversity within the Muguga cocktail vaccine components (Patel et al., 2011; Hemmink et al., 2016). Despite *T. parva* infections being of economic importance in southern Africa, reports on the genotypic diversity of the parasite in this region are limited, with the only available data focusing on parasites from Zambia (Muleya et al., 2012). In South Africa, other than sequence typing of genes encoding schizont and sporozoite antigens of *T. parva* (Sibeko et al., 2010; Hemmink et al., 2018; Mukolwe et al., 2020), diversity of the parasite based on satellite loci has not been reported. In the absence of sufficient data especially in South Africa where Corridor disease is important, the current study sort to establish the genotypic diversity of *T. parva* field parasites from southern Africa using satellite markers, and identify allele profiles that could be explored as potential markers to differentiate *T. parva* parasites infecting cattle in southern and eastern Africa.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Animal Ethics Committee of the Faculty of Veterinary Science, University of Pretoria (AEC Certificate # V080-16). Additional approvals including Section 20 and import permits were obtained from the Department of Agriculture, Forestry and Fisheries, South Africa. Other approvals for collection of blood samples were obtained from the relevant authorities in the respective countries (Mukolwe et al., 2020).

2.2. Sample collection and detection of T. parva

Blood samples were collected from cattle and buffalo from South Africa, Mozambique, Kenya and Uganda, in both present and previous studies as indicated in Table 1. DNA was extracted from the blood samples using the DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. However, elution was done in 100 µL instead of the recommended 200 µL to increase the concentration of extracted DNA. *Theileria parva* genomic DNA was detected using the *T. parva*-specific hybridization probe-based real-time PCR assay (Sibeko et al., 2008).

2.3. Selection of samples for satellite loci analysis

Samples positive for *T. parva* on qPCR (n = 176) were selected from the following sample groups; Kenya cattle (n = 15), Kenya buffalo (n = 28), Uganda Mbarara (n = 49), Uganda Karamoja (n = 20), KwaZulu-Natal (KZN) buffalo (n = 23), Corridor disease (CD) clinical cases (n = 14), non-clinical *T. parva*-positive cases (n = 10) and Mozambique buffalo (n = 17). Initially, nested PCR was performed using one of the microsatellite markers (ms2) (Table S2) for selection of samples for analysis with the rest of the satellite markers. Consequently, 96 samples from which ms2 was successfully amplified were selected for downstream analysis. These included; Kenya cattle (n = 10), Kenya buffalo (n = 17), Uganda Mbarara (n = 18), Uganda Karamoja (n = 10), KZN buffalo (n = 14), CD clinical cases (n = 12), non-clinical *T. parva*-positive cases (n = 3) and Mozambique buffalo (n = 12). In order to maintain consistency for comparative analysis on genotyping results, only the selected samples (n = 96) were analyzed with all markers (Table S2), and no other sample(s) were introduced or excluded.

Table 1
The geographical origin of blood samples collected from southern and eastern Africa.

Geographical origin			[a]Host	Number of samples collected
Country	Province/Region	Locality		
Kenya	Rift Valley Region	Nakuru County	Cattle	25[b]
		Laikipia County	Buffalo	40[c]
Uganda	Mbarara Region	Kiruhura District	Cattle	137
	Karamoja Region	Kaabong District	Cattle	99
		Nakapiripirit District	Cattle	123
South Africa	KwaZulu-Natal Province	Hluhluwe-iMfolozi Park	Buffalo	100
		uMkhanyakude District	Cattle	223
	Mpumalanga Province	Bushbuckridge Municipality (Mnisi)	Cattle	24[c,d]
Mozambique	Sofala Province	Marromeu National Reserve	Buffalo	40
Total				**811**

[a] Except for cattle whose clinical status is provided, all other cattle sampled were non-clinical cases.
[b] 10 of these samples were collected from active clinical cases of ECF, and 15 from non-clinical cases.
[c] Samples obtained from previous studies.
[d] 14 of these samples were collected from active clinical cases of Corridor disease, and 10 from non-clinical cases from a herd with previous *T. parva* infections. Detail on these samples is provided in Table S1.

2.4. PCR amplification of mini- and microsatellite loci

Nested PCR was performed using nested primers previously described and designed to amplify three microsatellite (ms) and nine minisatellite (MS) loci on the *T. parva* genome (Oura et al., 2003, 2005; Salih et al., 2018). The inner forward nested primers were labelled at the 5' end with one of the following fluorescent dyes; ATTO-565-red, ATTO-550-orange, ATTO-532-blue, CAL fluoro-green, Cy3-yellow, and 6-FAM-blue (Sigma-Aldrich, USA) (Table S2). The primary PCR amplification was carried out in a 10-µl reaction volume consisting of DreamTaq DNA Polymerase, 2X DreamTaq buffer (10 mM Tris–HCl, 4 mM $MgCl_2$), 0.0024 µmol of each dNTP, 10 pmol of each primer, and at least 100 ng of genomic DNA. The cycling conditions employed were as follows; an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min, and then a final extension step at 72 °C for 10 min. For the secondary PCR, 0.5 µl of the primary PCR product was used as the template in a reaction mix similar to that used for the primary PCR. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 30 s, primer annealing at 58 °C for 1 min, primer extension at 72 °C for 1 min, and then a final extension step at 72 °C for 20 min. The PCR products were resolved by 2% agarose gel electrophoresis in 1X TAE buffer.

2.5. Capillary flow genotyping of T. parva DNA

The reaction mix containing 0.5 − 1 µl of the PCR product of each sample for every marker was loaded in the same well of the 96-well

Table 2
Satellite loci diversity in cattle- and buffalo-derived *T. parva* parasites.

	ms2	MS3	ms5	MS7	ms7	MS8	MS16	MS19	MS25	MS27	MS33	MS34
N	95	86	85	83	64	92	75	81	67	78	84	88
Na	11	23	11	14	6	16	16	18	13	8	10	10
Ne	4.607	9.268	3.193	6.361	3.266	6.739	8.775	9.550	3.969	4.666	7.028	3.530
I	1.776	2.609	1.591	2.147	1.410	2.194	2.362	2.542	1.871	1.749	2.118	1.580
h	0.783	0.892	0.687	0.843	0.694	0.852	0.886	0.895	0.748	0.786	0.858	0.717
uh	0.791	0.903	0.695	0.853	0.705	0.861	0.898	0.906	0.759	0.796	0.868	0.725

N - sample size, **Na** - number of different alleles, **Ne** - number of effective alleles, **I** - Shannon's information index, **h** - diversity, **uh** - unbiased diversity.

plate with 9 µL of Liz-HiDi formamide mixture prepared by mixing 12 µL of Gene Scan 500 Liz size standard and 1000 µL of Hi-Di formamide (Patel et al., 2011). Rapid denaturation was performed at 96 °C for 3 min using GeneAmp® PCR system 9700 thermocycler, followed by rapid cooling on ice-ethanol bath for 1 min. The amplicons were analyzed on the ABI 3730 Genetic Analyzer (Applied Biosystems, USA) at the SegoliP sequencing unit of the BecA-ILRI Hub, Nairobi, Kenya. Allele scoring was performed using the GeneMapper® Software version 5.0 (ThermoFisher Scientific, USA). Alleles with maximum peak heights per marker per sample were scored, and those with the largest area under the curve, were considered predominant alleles, while alleles with at least one third of the predominant allele's height were considered minor (Salih et al., 2018). Two types of data files were generated; the multi-locus genotypes file (MLG file) comprising of only the predominant allele(s) at each locus, which was used to assess the diversity of populations, including analysis of molecular variance (AMOVA) and principal coordinate analysis (PCoA), and the allelic profile file comprising of all alleles (predominant and minor alleles) identified at each locus, which was used to assess multiplicity of infection (MOI) and linkage.

2.6. Population genetic analysis

For this analysis, *T. parva* populations were formulated to correspond to eight sample groups i.e. Uganda-Mbarara, Uganda-Karamoja, non-clinical *T. parva*-positive case, Corridor disease clinical cases, Kenya cattle, Kenya buffalo, KZN buffalo and Mozambique buffalo, hereinafter referred to as individual populations.

In order to test for the hierarchical variance of gene frequencies within and between individual populations, AMOVA was performed using the add-in excel software GenAlEx 6.5 (Peakall and Smouse, 2012) following user's manual. A mantel test of GenAlEx 6.5 (Peakall and Smouse, 2012) was performed to examine the genetic distance between individual populations, where mean Shannon (sHua) values over loci were obtained. The extent of linkage within and between individual populations and the standardized index of association within populations was determined using LIAN 3.7 programme (Haubold and Hudson, 2000). The standardized index of association is a measure of the association between alleles at pairs of loci where a predicted value close to zero or negative indicates panmixia, and greater than zero, a non-panmictic population. LIAN 3.7 programme tests the null hypothesis of linkage equilibrium (LE) by calculating a 95% confidence limit 'L' (L_{MC}), where when the observed mismatch (pairwise) variance (V_D) is greater than L, the null hypothesis of LE is rejected. For linkage analysis, each individual population was analyzed separately to check for geographical sub-structuring, following which the individual populations were analyzed as a combined population to establish whether the combined population was in linkage equilibrium or disequilibrium. Multiplicity of infection (MOI) defined by the number of alleles (> 1) per locus was determined using LIAN 3.7 programme (Haubold and Hudson, 2000), where the mean value per individual population was obtained. The genetic relationship of *T. parva* parasites within and between individual populations was determined by performing PCoA in GenAlEx 6.5 (Peakall and Smouse, 2012).

3. Results

3.1. Confirmation of T. parva positive samples

Out of the 811 DNA samples (buffalo = 180, cattle = 631) extracted from whole blood, 275 consisting of 145 and 130 samples from cattle and buffalo, respectively, were detected as positive for *T. parva*. Buffalo samples from South Africa and Kenya had the highest percentage of positives recording 76% and 75% respectively. South African cattle had the lowest percentage of positives (11.3%) among the cattle samples investigated.

3.2. Satellite loci diversity

Considering both cattle- and buffalo-derived *T. parva* parasites, the satellite loci were found to be highly polymorphic. MS19 showed the highest diversity (uh = 0.906), while ms5 was the least diverse (uh = 0.695) (Table 2). Diversity in the other loci ranged from 0.705 – 0.903. The number of alleles per locus ranged from six (6) in ms7 to 23 in MS3, with an average number of alleles (Na) per locus being 13 (Table 2). The number of alleles identified in each individual population on all the satellite loci ranged from 16 to 55, with the highest number observed in *T. parva* parasites from Mozambique buffalo (Table 3).

3.3. Predominant alleles and multi-locus genotypes (MLGs)

The most frequent predominant alleles were used to tabulate multi-locus genotypes as summarized in Table 4. The number of MLGs identified per individual population ranged from one (1) in *T. parva* parasites from KZN buffalo to four (4) in the rest of buffalo-derived parasites from Mozambique buffalo and Corridor disease clinical cases, and all cattle-derived *T. parva* parasites. Due to the limited number of samples (n = 3) that were genotyped for non-clinical *T. parva*-positive cases, the MLGs from this group could not be computed. Cattle-derived *T. parva* parasites had one common MLG (ms2-179, MS3-239, ms5-172, ms7-150, MS7-152, MS8-165, MS16-273, MS19-162, MS25-199, MS27-209, MS33-175, MS34-248), whereas none was identified in the buffalo-derived parasites (Table 4). Other than a few loci (MS3, ms5, MS16, MS19 and MS27) which had two predominant alleles with equal frequencies in cattle-derived *T. parva* parasites, each of the other loci in these parasites had one most frequent predominant allele that was common for each locus (Table 4). On the contrary, the majority (75%) of the loci in buffalo-derived *T. parva* parasites had two predominant alleles with equal frequencies, and there was no common predominant allele on any locus (Table 4). Some loci had unique common profiles of predominant alleles (MS8-165, MS16-273, MS19-162, MS33-175) in cattle-derived *T. parva* parasites, a feature that was absent in the buffalo-derived *T. parva* parasites (Table 4). When *T. parva* parasites associated with Corridor disease from South Africa, and East Coast fever (ECF) from Uganda and Kenya cattle populations were compared, three loci with unique predominant allele profiles; MS8-155, MS19-127, MS33-160 in Corridor disease parasites, and MS8-165, MS19-162, MS33-175 in ECF parasites, were identified (Table 4). Detail on the

Table 3
Summary of the number of alleles identified in individual populations.

Marker	[a]Number of alleles identified per population							
	Uganda Karamoja cattle (n = 10)	Uganda Mbarara cattle (n = 18)	CD clinical cases (n = 12)	Nc Tp-positive cases (n = 3)	Kenya cattle (n = 10)	Kenya buffalo (n = 17)	KZN buffalo (n = 14)	Mozambique buffalo (n = 12)
ms2	4	3	**5**	1	3	4	2	**5**
MS3	5	**8**	6	1	7	5	3	7
ms5	3	5	**6**	2	5	5	5	4
ms7	1	1	2	1	1	1	1	**3**
MS7	4	5	**7**	2	3	2	2	5
MS8	4	4	3	2	5	1	2	**7**
MS16	2	**6**	5	1	4	3	4	4
MS19	**5**	4	4	1	4	4	4	1
MS25	4	4	3	–	4	4	–	**6**
MS27	2	4	**5**	2	3	4	1	4
MS33	3	**4**	**4**	1	**4**	**4**	1	**4**
MS34	**5**	4	4	2	1	2	1	3
Total	42	52	54	16	44	39	26	55

[a] Numbers in bold indicate the highest number of alleles identified at each locus across populations. CD - Corridor disease. Nc Tp - non-clinical *T. parva*.

frequencies of predominant alleles identified per locus for each individual population is provided in Table S3.

3.4. Multiplicity of infection (MOI)

Theileria parva parasites from all the individual populations in southern and eastern Africa had multiple genotypes. A measure of multiplicity of infection within each population was estimated by the calculated mean number of alleles per locus (Table 5). The mean values ranged from 1.03 to 2.69, with buffalo-derived *T. parva* parasites from Mozambique buffalo population recording the highest, and non-clinical *T. parva*-positive cases the lowest. Among the buffalo-derived parasites, those from Mozambique buffalo recorded the highest mean (2.69), while parasites from Mbarara-Uganda recorded the highest mean (2.00) among the cattle-derived *T. parva* parasites (Table 5). When all individual populations were analyzed as one combined population, the mean value was 2.03 with a high standard deviation (0.61) (Table 5), indicating significant variation in the data from all sites. The standard deviations (SDs) for each individual population was relatively high, with non-clinical *T. parva*-positive cases and KZN buffalo recording the highest and the lowest SDs respectively.

3.5. Linkage

Linkage between and within individual populations from the two regions investigated was evaluated using the allelic profile data. When all the individual populations were analyzed as one combined population, the standardized index of association (I_A^S) was greater than zero (0.0266), characteristic of a non-panmictic population, and the observed mismatch variance (V_D = 3.1233) was greater than the critical value 'L' (L_{MC} = 2.5460), indicating that the combined population is in a state of linkage disequilibrium (LD) (Table 6). When the individual populations were analyzed separately, the standardized index of association for each population was close to zero or negative, indicating panmixia, and the observed mismatch variances (V_D) were less than the corresponding critical values 'L' (L_{MC}), consequently, the null hypothesis of linkage equilibrium within individual populations was accepted (Table 6).

3.6. AMOVA, PCoA and genetic distance

The analysis of molecular variance showed a high percentage of genetic variation (74%) to be within individual populations, with only 26% accounting for the differences between populations. The PCoA plot

Table 4
Summary of the predominant alleles per locus and the multi-locus genotypes (MLGs) per population.

Population	n	[a]Predominant alleles (bp) per locus											[c]No. of MLGs
		ms2	MS3	ms5	ms7	MS7	[b]MS8	MS16	[b]MS19	MS25	MS27	[b]MS33	MS34	
Uganda-Karamoja cattle	10	*179*	*239* 253	*172*	*150*	*152*	***165*** **(3)**	*273*	***162*** **(3)** 264	*199*	*209*	***175*** **(3)**	*248*	4
Uganda-Mbarara cattle	18	*179*	*239*	*172*	*150*	*152*	***165*** **(13)**	*273* 289	***162*** **(3)** 252	*199*	*209*	***175*** **(13)**	*248*	4
Kenya cattle	10	*179*	*239*	153 *172*	*150*	*152*	***165*** **(6)**	*273*	***162*** **(4)**	*199*	*209* 221	***175*** **(5)**	*248*	4
Kenya buffalo	17	202	239 253	172	142	171	191	223	165	199	185	184	182	2
CD clinical cases	12	202 214	219	172	150	249	**155 (7)**	176	**127 (4)**	199	149 161	**160 (7)**	248	4
Non-clinical *T. parva*-positive cases	3	214	248	159 162	150	152 232	155 172	189	127 179	–	149 209	160	201 195	NA
KZN buffalo	14	202	244	172	172	253	155	206	150	190	149	150	182	1
Mozambique buffalo	12	179	228	172	153	171 192	183	189	127	214	161	145 158	182	4

[a] Common MLG in cattle-derived parasites is indicated in italics.
[b] Allele profiles on three loci (MS8, MS19 and MS33) unique to parasites associated with ECF are indicated in italics, bolded and underlined. Allele profiles on three loci (MS8, MS19 and MS33) unique to parasites associated with Corridor disease in South Africa are bolded and underlined. Numbers in parenthesis indicate the number of samples that expressed the respective allele.
[c] NA - sample size too small to compute MLGs.

Table 5
Summary statistics of multiplicity of infection in samples from individual populations, and when all individual populations were analysed as one combined population.

Population	n	[a]Number of alleles per locus				[b]Total number of alleles identified on all loci
		Mean	SD	Min	Max	
Uganda-Karamoja	10	1.14	0.46	0.50	2.00	42
Uganda-Mbarara	18	2.00	0.53	0.58	2.67	52
Non-clinical *T. parva*-positive cases	3	1.03	0.88	0.08	1.83	16
CD clinical cases	12	1.79	0.34	1.25	2.42	54
Kenya cattle	10	1.81	0.36	1.33	2.42	44
Kenya buffalo	17	2.49	0.38	1.67	3.00	39
KZN buffalo	14	2.14	0.26	1.75	2.75	26
Mozambique buffalo	12	2.69	0.29	2.00	3.00	55
Combined population	**96**	**2.03**	**0.61**	**0.08**	**3.00**	

n: number of samples.
[a] SD - standard deviation, Min and Max refer to the minimum and maximum number of alleles identified per locus per sample. Detail on the frequencies of the alleles identified on each locus in each population is provided in Table S3.
[b] A summary of the number of the alleles per locus for each population is provided in Table 3.

Table 6
Linkage analyses of individual populations, and when combined as one population.

Population	I_A^S	V_D	L_{para}	L_{MC}	Linkage
Uganda-Mbarara	0.0079	2.7119	2.9863	3.1330	LE
CD clinicals cases	−0.0006	2.4552	3.1687	3.0399	LE
Kenya cattle	0.0242	3.0828	3.2534	3.3101	LE
Kenya buffalo	0.0081	2.6400	3.0099	3.1289	LE
KZN buffalo	−0.0098	2.2598	3.3262	3.6821	LE
Mozambique buffalo	0.0354	3.1655	3.2030	3.4117	LE
Combined population	**0.0266**	**3.1233**	**2.5324**	**2.5460**	**LD**

I_A^S = standardized index of association, V_D = observed mismatch variance (linkage analysis).
LD = linkage disequilibrium, LE = linkage equilibrium.
L_{MC} and L_{para} = upper 95% confidence limits of Monte Carlo simulation and parametric tests respectively (linkage analysis).
Note: Due to small sample sizes, linkage analysis for Uganda Karamoja and Non-clinical *T. parva*-positive cases populations could not be computed.

illustrating the relationship between *T. parva* populations from southern and eastern Africa is shown in Fig. 1. Genotypes of *T. parva* parasites from clinical cases of Corridor disease from South Africa showed a close genetic relationship with representative genotypes from parasites from non-clinical *T. parva*-positive cases from the same country, East African cattle and Mozambique buffalo, while those from cattle from Kenya and Uganda clustered together. Among the genotypes from parasites from buffalo, those from Hluhluwe-iMfolozi Park in KZN were closely related to representative genotypes from clinical cases of Corridor disease, whereas some from buffalo from Kenya showed a close relationship to those from Mozambique. The genetic divergence between populations was determined by estimating the mean Shannon (sHua) values over loci (Table S4). The genetic distance between cattle- and buffalo-derived *T. parva* parasites ranged from 0.136 − 0.199, and 0.173 − 0.437, respectively (Table S4), which was in congruence to the relationship displayed by PCoA.

4. Discussion

Theileria parva has a complex life cycle involving asexual and sexual phases in the bovine host and the tick vector respectively. Diversity of *T. parva* parasites resulting from recombination activities in the tick vector has compromised the current efforts in controlling the parasite, and complicated its population dynamics in the bovine and wildlife hosts. In order to establish potent control measures and evaluate their success, information on the diversity and population structure of *T. parva* field parasites is important. Thus, the current study evaluated *T. parva* parasites from bovine and wildlife hosts from southern and eastern Africa using mini- and microsatellite markers. The satellite markers used in this study are highly informative based on their polymorphic information component (Rukambile et al., 2016; Salih et al., 2018), and the loci are evenly distributed on the four chromosomes of *T. parva*. For the adjacent pairs of loci occurring on the same chromosome, separation by at least 315 kbp (Katzer et al., 2010) was considered, hence, the probability that the loci were physically linked is negligible.

Among the buffalo-derived *T. parva* parasites, the most diverse were from Mozambique buffalo, and the least were from the non-clinical *T. parva*-positive cases from South Africa. Since sample size is a critical variable of genetic diversity in population studies (Yin et al., 2018), it is highly possible that the limited diversity observed in the non-clinical *T. parva*-positive cases was due to the small sample size. The African buffalo is the natural reservoir of *T. parva,* which is known to harbor multiple genotypes of the parasite (Oura et al., 2011b; Sibeko et al., 2011), therefore, the high levels of diversity observed in parasites of buffalo origin would be expected. Previous studies involving multilocus sequence typing (MLST) of schizont antigen genes in *T. parva* parasites from buffalo from South Africa and Kenya (Pelle et al., 2011; Hemmink et al., 2018), and analysis of the p67 gene in buffalo-derived parasites from eastern and southern Africa (Sibeko et al., 2010; Sitt et al., 2019; Mukolwe et al., 2020) demonstrated high levels of diversity, confirming that parasites of buffalo origin are more diverse than the cattle-adapted *T. parva* parasites. There seems to be no significant differences in the diversity of *T. parva* populations obtained from the buffalo in the current study as depicted by the MOI values. Parasites from Mbarara in western Uganda were the most diverse among the *T. parva* parasites from cattle. The farms from which the samples were obtained are adjacent to Lake Mburo National Park (LMNP) which is not fenced, and the buffalo population in this park is infected with *T. parva* (Oura et al., 2011a, b). It could therefore be assumed that the cattle population sampled share grazing pastures with buffalo from the park, hence, the possibility of transmission of multiple *T. parva* genotypes from the buffalo to cattle. However, analysis of the p67 gene in *T. parva* parasites from the same cattle population in western Uganda revealed limited diversity (Mukolwe et al., 2020), similar to MLST involving schizont antigen genes in *T. parva* parasites from cattle from Kenya (Pelle et al., 2011) and South Sudan (Salih et al., 2017). Buffalo-derived *T. parva* parasites do not establish a carrier state in cattle (Mbizeni et al., 2013; Morrison et al., 2020), and all cattle sampled from western Uganda were non-clinical cases, suggesting that the possibility of this cattle population being infected with buffalo-derived parasites at the time of sampling was unlikely. Therefore, it is probable that the high levels of diversity observed in *T. parva* population from western Uganda was due to the relatively large sample size analyzed, rather than genotypes from the buffalo. All cattle-derived parasites from Kenya and Uganda had a common multi-locus genotype, similar to the p67 gene (Mukolwe et al., 2020), suggesting the presence of a *T. parva* population that is adapted for cattle-to-cattle transmission, and most likely responsible for ECF in eastern Africa.

When the allelic profiles of parasites associated with Corridor disease in South Africa and ECF in eastern Africa were compared, three loci (MS8, MS19 and MS33) had unique profiles, which were not shared between these parasites or other populations, indicating possible discriminatory alleles for the two groups of parasites. Due to the potential differences in the sensitivity of satellite markers, the unique allele profiles were detected in some, but not all sample analyzed. Therefore, a robust investigation of *T. parva* parasites from cattle from the two

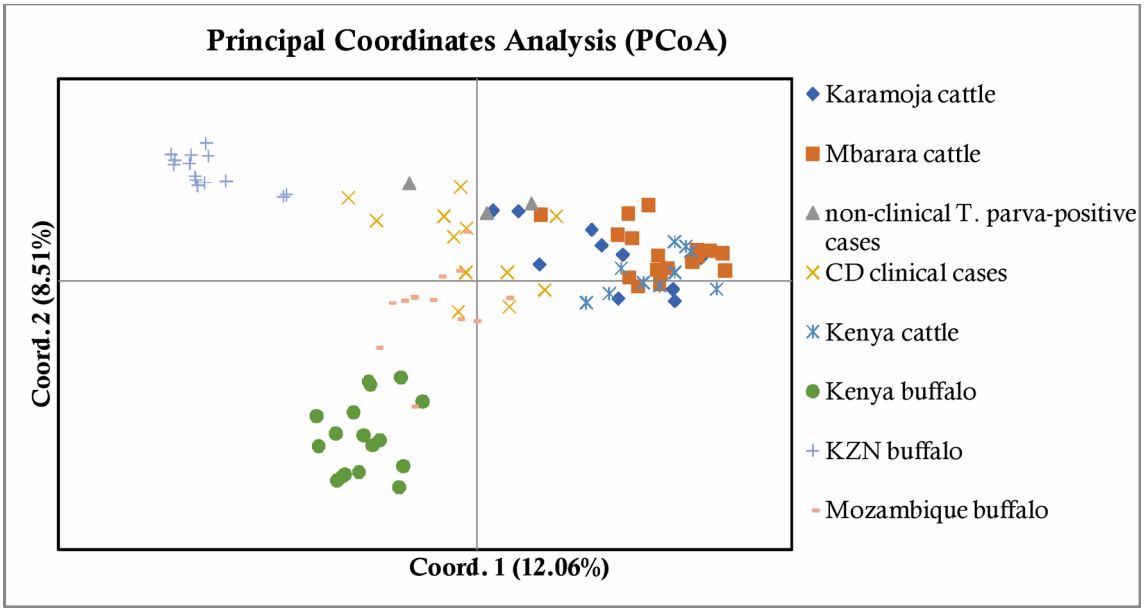

Fig. 1. Principal coordinate analysis (PCoA) scatter plot of genotypes from *T. parva* parasites from eastern and southern Africa generated using multi-locus genotype data. The principal coordinates demonstrating the proportion of variation in the dataset are presented.

regions of Africa on the three loci would be necessary to support or invalidate this preliminary finding. It could be argued that the differences observed on the three loci resulted from artifacts, however, PCoA analysis of the overall data showed similar results (though not specific for the three loci) possibly indicating true genotypic differences with some similarities between these parasites. Analysis of the p67 gene revealed two subtypes of allele type 1 corresponding to buffalo-derived *T. parva* parasites from South Africa, and parasites responsible for ECF and Corridor disease in Kenya (Mukolwe et al., 2020), confirming that despite their similarities, these parasites have some specific genetic differences. Considering that Corridor disease is a controlled disease in South Africa, and sporadic outbreaks occur following unprecedented contact between buffalo and cattle (Mbizeni et al., 2013), the number of *T. parva* positive samples from cattle from South Africa were limited for this study. This being the first report on the genotypic diversity (based on satellite loci) of *T. parva* parasites from South Africa where Corridor disease is the only important *T. parva* infection, it would be significant to further characterize more *T. parva* positive field samples from cattle in Corridor disease designated areas in South Africa to support the current findings.

Similar to other apicomplexan parasites, *T. parva* field parasites are known to possess multiple genotypes as a result of multiplicity of infection (Oura et al., 2005; Odongo et al., 2006; Beck et al., 2009; Muleya et al., 2012; Rukambile et al., 2016; Salih et al., 2018), and ticks infected with a mixture of *T. parva* genotypes present an ideal prerequisite for genetic exchange (Oura et al., 2005). High multiplicity of infection has been shown to have a positive correlation with the parasite transmission intensity and the host age (Weir et al., 2011; Al-Hamidhi et al., 2015; Roy et al., 2019), where the abundance of ticks and/or the intensity of tick infections contribute to the disparities in transmission intensity (Weir et al., 2011). It has also been hypothesized that due to the continuous exposure of cattle to multiple genotypes as a result of continuous tick challenge, there is a progressive increase of co-infecting genotypes as the animals age (Weir et al., 2011), hence, adult animals would be expected to possess a high multiplicity of infection. All the animals sampled in the current study were adults, and the MOI values were found to be high in individual populations as well as when the populations were analyzed as one combined population, indicating a possibility of high transmission intensities of *T. parva* within the host populations from the two regions of Africa.

Among the parasites from buffalo from southern Africa, those from Mozambique had the highest level of variation with a mean value of 2.69 alleles per locus, while from eastern Africa, parasites from the Kenyan buffalo had the highest observed variation (mean = 2.49). Among *T. parva* parasites from cattle, those from western Uganda had the highest variation (mean = 2.00), followed by those from clinical cases of Corridor disease from Bushbuckridge municipality bordering Kruger National Park (KNP) in South Africa. Since the wildlife host harbors multiple strains of *T. parva* (reviewed in Nene et al., 2016), it is possible that the high variation observed in parasites from the buffalo is a natural phenomenon promoted by perpetual transmission by the vector. In addition, recombination and cross-mating activities in the tick vector are also likely to result in increased genetic exchange among buffalo-derived *T. parva* parasites leading to a build-up of mixed *T. parva* genotypes in the buffalo hosts. This could also happen in the bovine host especially in areas where parasite sharing occurs between the buffalo and cattle, hence, the scenario in Bushbuckridge municipality in South Africa, and also probable in western Uganda. Previous studies involving sequence typing of the schizont antigen genes in buffalo-derived *T. parva* parasites from South Africa (Hemmink et al., 2018) and Kenya (Pelle et al., 2011; Sitt et al., 2018) revealed the presence of a high number of alleles per locus than the current study. Although the satellite markers used in the current study are highly informative, it could be possible that some have limited sensitivity in detecting alleles that occur in low frequencies, resulting in the relative divergence observed.

Although the current study did not establish the presence of the principal vector *Rhipicephalus appendiculatus*, in areas where samples were obtained, multi-acaricide resistance among *Rhipicephalus* ticks has been reported in South Africa (Robbertse et al., 2016), Uganda (Vudriko et al., 2016) and Kenya (Gachohi et al., 2012), indicating the possibility of abundance of *R. appendiculatus* ticks. This, coupled with a likelihood of high intensity of *T. parva* infection among these ticks, and the close proximity to the wildlife reservoir, could be promoting the high transmission intensities of *T. parva* in South Africa, and perhaps western Uganda. These results concur with those of Oura et al. (2005) and Muwanika et al. (2016) previously reported in Uganda, although it would be informative to characterize *T. parva* parasites from buffalo from KNP and LMNP to establish the possibility of transfer of buffalo genotypes to the co-grazing cattle population. Parasites from the non-

clinical *T. parva*-positive cases from South Africa had the lowest level of variation (mean = 1.03), contrary to the previous argument, probably due to the small sample size analyzed. Thus, investigation of a larger sample size would be necessary to support or disprove the presumed hypothesis.

Analysis of linkage equilibrium between alleles at pairs of loci within *T. parva* populations further supported the evidence of high genetic exchange among *T. parva* parasites, where all individual populations from southern and eastern Africa were in linkage equilibrium (LE). Although reports on linkage in *T. parva* parasites from southern Africa are not currently available, the finding in the current study indicate that *T. parva* populations in this region are panmictic. The *T. parva* population from Mbarara Uganda was found to be in LE, similar to the previous report in some populations from the same region (Oura et al., 2005), but in contrast to linkage disequilibrium (LD) reported in the expansive western region of Uganda (Muwanika et al., 2016). Contrary to LD reported in *T. parva* isolates from western, central and coastal Kenya (Odongo et al., 2006), parasites from cattle in this country in the current study were in LE. The observed LE in *T. parva* populations from the two regions of Africa could possibly indicate the absence of sub-structuring within populations with frequent genetic exchange promoted by panmixia. Subsequently, the previous findings by Odongo et al. (2006) and Muwanika et al. (2016) could have been due to geographical sub-structuring considering the geographical distance separating the sites from which the parasites were obtained. When all individual populations were considered a single metapopulation in the current study, LD was observed, with the standardized index of association greater than zero, indicating genetic and geographical sub-structuring of *T. parva* populations. However, a more logical explanation to the observed LD in the combined population could be that the parasites were obtained beyond geographical boundaries that could allow random genetic exchange between populations.

Analysis of molecular variance revealed a possible indication that genetic divergence occurs more among parasites within specific regions (geographical areas) than between regional metapopulations. This suggests that cross-mating and recombination between *T. parva* parasites seems to be confined within specific *T. parva* populations unless there are factors that promote intermixing of host populations in the presence of the tick vector, which would facilitate *T. parva* spread. Genetic sub-structuring of *T. parva* parasites was observed on PCoA, where parasites that have adapted for cattle-to-cattle transmission and possibly responsible for ECF in eastern Africa clustered together, separate from those of buffalo origin. Buffalo-derived *T. parva* parasites from buffalo and cattle from South Africa showed a close genetic relationship despite the geographical distance between HluhluweiMfolozi Park in KwaZulu-Natal province, and Bushbuckridge municipality in Mpumalanga province where the samples were collected respectively. This confirms that *T. parva* parasites responsible for Corridor disease originate from the buffalo as a result of cross-infection, and the population of *T. parva* parasites circulating in bovine and wildlife hosts in South Africa have similar genotypes. To further support this hypothesis, it would be important to also characterize *T. parva* parasites from buffalo from KNP.

There was evidence of a very close genetic relationship between *T. parva* parasites from clinical cases of Corridor disease from South Africa, and buffalo from the neighboring country, Mozambique. Although Corridor disease cases have been reported in some areas bordering KNP in Maputo province of Mozambique (Sibeko, personal communication), no case has been reported in areas bordering Marromeu National Reserve (MNR) which is approximately 700 km from KNP. The area proximal to MNR also lies within the tsetse fly belt where trypanosome challenge is very high (Garcia et al., 2018) and grazing of cattle in such areas is restricted, hence chances of contact between cattle and buffalo are minimal. However, the genetic relationship displayed by PCoA, and the similarities previously identified on the p67 gene (Mukolwe et al., 2020) reveal a possible risk of Corridor disease outbreak in areas adjoining MNR should the buffalo population interact with cattle in the presence of the tick vector.

5. Conclusion

The current study reports on the genotypic diversity (based on satellite loci) of *T. parva* parasites from southern Africa. Parasites associated with Corridor disease in South Africa and ECF in eastern Africa are genotypically different with some similarities, and probably share a common ancestor. We report a preliminary finding on possible distinguishing allelic profiles on three loci (MS8, MS19, MS33) in buffalo-and cattle-derived parasites from southern and eastern Africa respectively, however, we recommend further investigation to support this finding.

Supplementary data

Supplementary material related to this article can be found in the online version at https://doi.org/10.17632/k6nb7rpxv3.2.

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CRediT authorship contribution statement

Donald M. Lubembe: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **David O. Odongo:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Diaeldin A. Salih:** Formal analysis, Visualization. **Kgomotso P. Sibeko-Matjila:** Conceptualization, Methodology, Resources, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

None.

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