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Original article

Population genetic structure of *Theileria parva* field isolates from indigenous cattle populations of Uganda

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ABSTRACT

Theileria parva causes East Coast Fever (ECF) a protozoan infection which manifests as a non-symptomatic syndrome among endemically stable indigenous cattle populations. Knowledge of the current genetic diversity and population structure of *T. parva* is critical for predicting pathogen evolutionary trends to inform development of effective control strategies. In this study the population genetic structure of 78 field isolates of *T. parva* from indigenous cattle (Ankole, $n = 41$ and East African shorthorn Zebu (EASZ), $n = 37$) sampled from the different agro ecological zones (AEZs) of Uganda was investigated. A total of eight mini- and micro-satellite markers encompassing the four chromosomes of *T. parva* were used to genotype the study field isolates. The genetic diversity of the surveyed *T. parva* populations was observed to range from 0.643 ± 0.55 to 0.663 ± 0.41 among the Central and Western AEZs respectively. The overall Wright's *F* index showed significant genetic variation between the surveyed *T. parva* populations based on the different AEZs and indigenous cattle breeds ($F_{ST} = 0.133$, $p < 0.01$) and ($F_{ST} = 0.101$, $p < 0.01$) respectively. Significant pairwise population genetic differentiations ($p < 0.05$) were observed with F_{ST} values ranging from 0.048 to 0.173 between the eastern and northern, eastern and western populations respectively. The principal component analysis (PCA) showed a high level of genetic and geographic sub-structuring among populations. Linkage disequilibrium was observed when populations from all the study AEZs were treated as a single population and when analysed separately. On the overall, the significant genetic diversity and geographic sub-structuring exhibited among the study *T. parva* isolates has critical implications for ECF control.

1. Introduction

Theileria parva is a protozoan parasite which causes East Coast Fever (ECF), a deadly lympho-proliferative disease of mainly exotic but also indigenous cattle (Waladde et al., 1993) in subSaharan Africa (SSA). East Coast Fever (ECF) conflicts with several national plans to improve cattle productivity in the region (Morrison et al., 1987). The disease discourages hybridisation of indigenous cattle with exotic genotypes which are highly susceptible to the infection and yet the latter are desired for higher productivity (Conelly, 1998; Gachohi et al., 2012). Although this disease causes about one million deaths every year, another 28 million are at constant risk of getting infected (Morrison et al., 1987).

The use of acaricides to check tick infestations on cattle is the most common approach for controlling tick-borne diseases, especially ECF. This method has been recently disadvantaged by acaricide resistant tick populations, food-safety concerns and environmental pollution (George et al., 2004). One of the approaches envisaged to curtail the development of acaricide resistant tick populations is the use of infection and treatment method (ITM) vaccination which currently uses the live Muguga cocktail vaccine being popularised for integrated ECF control in eastern Africa (Di Giulio et al., 2009; GALVmed, 2011). The ITM was developed (Radley et al., 1975) to mimic the acquisition of natural immunity developed among indigenous cattle after primary exposure to ECF. It was shown that using a mixture of three East African isolates (Kiambu 5, Muguga and Serengeti transformed), a high degree of protection could be achieved in an area with field strains arriving from many parts of Tanzania (Uilenberg et al., 1977, 1978). Its widespread deployment is nevertheless constrained by concerns of possible introduction of novel parasite genotypes to unvaccinated

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cattle (De Deken et al., 2007; Oura et al., 2007), via vaccine-derived infected ticks. In addition, it has been perceived that *T. parva* can undergo population genetic transformation in the short-term after ITM vaccination (Bishop et al., 2001; Oura et al., 2004, 2007; Mwega et al., 2014). The occurrence of ECF outbreak among vaccinated cattle due to challenge infection with different parasite strains from the vaccine stabilates might therefore demands additional combination of local strains into the vaccine (Sitt et al., 2015).

Since indigenous cattle are reared in all the AEZs of Uganda and are often non-clinical carriers of *T. parva*, they can be a constant source of infection to naïve exotic cattle (Kabi et al., 2014). Additionally, the unlimited movement of *T. parva* infected cattle might introduce novel parasite genotypes to new locations (Geysen et al., 1999; De Deken et al., 2007; Hayashida et al., 2013; Mwega et al., 2014). This will continuously complicate the population genetic structure of *T. parva* and hinder the use of ITM in the control of ECF. Location specific studies of *T. parva* field isolates have demonstrated genetically diverse parasite populations in Kenya (Nene et al., 1992), Zimbabwe (Bishop et al., 1994), Uganda (Oura et al., 2011) and Zambia (Muleya et al., 2012).

Mini and micro-satellite markers have been validated for revealing the genetic diversity, population dynamics and geographic origins of *T. parva* field isolates (Oura et al., 2003, 2005; Katzer et al., 2006; Odongo et al., 2006). Although *T. parva* genetic diversity location specific studies have been conducted in the recent past (Oura et al., 2011), this survey provides additional information on nationwide population genetic structure with comparisons between contrasting agro-ecological zones (AEZs) and indigenous cattle breeds to further allow strategic planning of ECF control integrated with the impending ITM approach in Uganda.

2. Materials and methods

2.1. Sampling, genomic DNA extraction and nested PCR detection of T. parva

Blood samples were collected from indigenous cattle breed groups (Ankole and EASZ) with no apparent clinical signs of ECF in a landscape approach throughout the entire country as previously described in Kabi et al. (2014).

A total of 78 DNA samples, which previously tested positive in a nested polymerase chain reaction (nPCR) assay for *T. parva* using the p104 (Odongo et al., 2010) marker were used for this investigation. The samples were categorised according to their origin including: Central – Lake Victoria crescent ($n = 11$), Eastern – North eastern savannah grasslands and drylands ($n = 18$), Northern – Northwestern savannah grasslands ($n = 19$) and Western – Pastoral Rangelands, South Western Highland Ranges and Western Savannah grasslands ($n = 30$) AEZs of Uganda as shown in Fig. 1.

2.2. PCR amplification and analysis of mini- and microsatellite loci

In this study, a total of eight mini- and two microsatellite loci previously described by Oura et al. (2003), and known to be polymorphic were used. At most, two markers per chromosome were chosen on each of the four chromosomes of *T. parva* as shown in Table 1. A total of 78 samples were genotyped with a total of eight loci. In the nested PCR, the forward primers of each primer pair were fluorescently labelled at the 5′-end with one of the following four standard dyes; 6-FAM (Blue), NED (Yellow), PET (Red) and VIC (Green) (Bioneer, Korea), for detection on an ABI genetic analyser. Genomic DNA (10 ng) were amplified using 5 U *Taq* polymerase (Promega), 1× PCR buffer (10 mM Tris–HCl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$), 0.2 mM dNTPs, and 0.2 μM of each reverse and forward primers, and made up to a final reaction volume of 10 μl with sterile water. The PCR was carried out in a Gene Amp® PCR System 9700 (ABI-USA). The cycling conditions were optimised as follows: denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 1 min and final extension was at 72 °C for 20 min before cooling to 4 °C. The reaction was run for 35 cycles.

To prepare for capillary electrophoresis a reaction mix containing (0.5–2 μl) of PCR product was added into each well of a 96-well plate containing 8 μl of Hi-Di Formamide and GeneScan 500 LIZ size standard (ABI-USA). Rapid denaturation was carried out in a thermocycler at 95 °C for 5 min followed by rapid chilling of the micro-titre plate on ice. The ABI 3730 genetic analyser (Applied Biosystems-USA) was used to analyse the amplicons. The alleles were scored using the Gene-Mapper programme (Applied Biosystems-USA). Amplicons with a maximum peak height were scored, and a predominant peak was defined as that with the largest area under the curve. All data generated from the Gene-Mapper were resized by the allelobin software based on consensus sequence repeats of the markers. A predominant allele at each locus was used to generate allele frequency data and multilocus genotypes (MLGs) in an excel file.

2.3. Data analyses

Summary statistics of allele frequencies per locus within each *T. parva* population was calculated using the genetic analysis package Power Marker V. 3.25 (Liu and Muse, 2005). Major allele frequency, gene diversity (GD) and polymorphic information content (PIC) were used to determine diversity at each mini- and microsatellite locus in each of the *T. parva* populations from the eastern, central, northern and western AEZs zones. The maximum number of alleles identified for each marker, patterns of genotypic distribution in each study populations, genotype diversity and analysis of molecular variance (AMOVA) among and within populations was estimated by GenAlEx V. 6.5 (Peakall and Smouse, 2006) add-in microsoft excel software. The statistical significance of AMOVA was assessed using 999 random permutations. In addition the principal component analysis (PCA) was used for comparison of the different *T. parva* populations based on the different AEZs.

The null hypothesis of linkage equilibrium was tested using LIAN an online software (http://adenine.biz.fh-weihenstephan.de/lian/) which computes the standardised index of association (I_A^S) and quantifies linkage equilibrium (LE) or disequilibrium (LD) as previously defined by Haubold and Hudson (2000). The I_A^S measures the association between alleles at pairs of loci, i.e., I_A^S values close to 0 or negative is indicative of panmixia, while those significantly greater than 0 is indicative of non-panmictic populations. Linkage equilibrium is characterised by the statistical independence of alleles across all loci and is investigated by initially determining the number of loci at which each pair of multi-locus genotypes (MLGs) differs. From the distribution of mismatch values, a variance (V_D) is calculated which is compared to the variance expected for LE, termed V_e. The null hypothesis that $V_D = V_e$ is tested by both a Monte Carlo simulation and a parametric method in order to estimate a 95% confidence limit (95% CI), which are denoted L_{mc} and L_{para}, respectively. When V_D is found to be greater than L, the null hypothesis is rejected and LD is accepted.

2.4. Ethical clearance

This study was ethically cleared by Makerere University Institute of Environment and Natural Resources (MUIENR) and approved by the higher degree committee of Makerere University. Permission to undertake the study was obtained from the Uganda National Council for Science and Technology (UNCST) reference

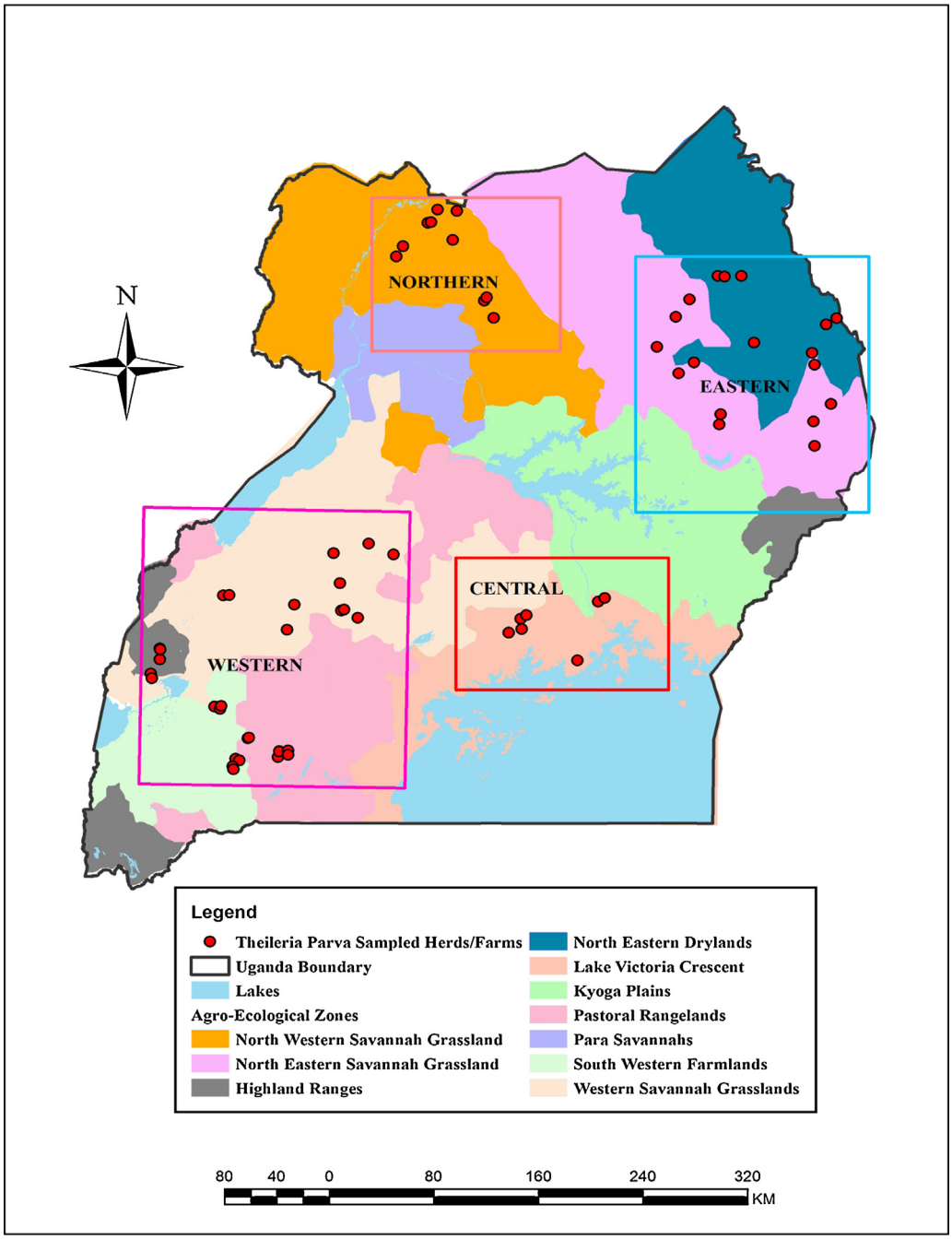

Fig. 1. Location of the *T. parva* population sampled herds/farms from Central, Eastern, Northern and Western AEZs were genotyped isolates.

Table 1
Major allele frequency, gene diversity and polymorphic information content (PIC) obtained from mini and microsatellite markers used to genotype field isolates of *Theileria parva* in this study.

Marker	Consensus repeat sequence (Oura et al., 2003)	Chromosome number	Size range of alleles (bp)	Major allele frequency	Gene diversity	PIC	Allele number
ms 2	tat	1	176–250	0.2949	0.8457	0.84	15.0
ms 5	att	1	152–170	0.2179	0.8207	0.80	9.0
MS 13	tgtgtaaaaa	2	261–290	0.3846	0.6539	0.58	4.0
MS 16	actaatatttgttattt	2	188–393	0.2308	0.8415	0.83	10.0
MS 25	ttatatagttaagt	3	180–340	0.3846	0.7506	0.72	6.0
MS 27	taatcaaattat	3	130–220	0.3333	0.7529	0.72	7.0
MS 35	actattaac	4	120–150	0.4359	0.6902	0.64	4.0
MS 46	tcaaccata	4	170–190	0.4615	0.6309	0.56	3.0
Mean				0.3429	0.7483	0.71	7.0

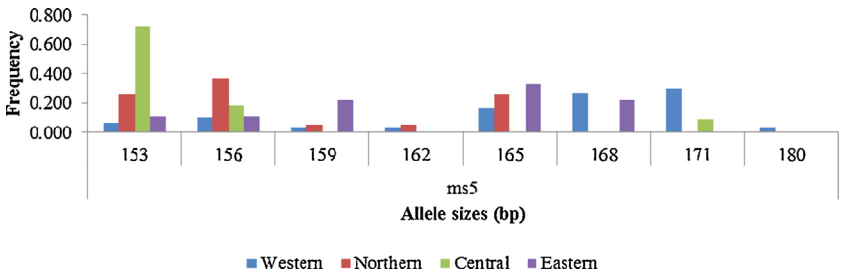

Fig. 2. Representative marker ms 5 locus displaying allele frequencies identified in western, northern, central and eastern population. The size of each allele (bp) is shown on the *x*-axis, shared and private are displayed.

number NS 325. The district veterinary personnel and farmers provided oral consent for the use of their cattle and information for the study.

3. Results

3.1. Allele diversity and distribution

The panel of eight polymorphic mini- and microsatellite markers (Table 1), represented on each of the four chromosomes of *T. parva*, were used to genotype 78 *T. parva* positive samples, obtained from central ($n = 11$), eastern ($n = 18$), northern ($n = 19$) and western ($n = 30$) AEZs of Uganda. Six samples were excluded from the final analysis because most of the markers failed to produce signals during gel electrophoresis. All loci were polymorphic with a polymorphic information content (PIC) varying from 0.56 (MS 46) to 0.84 (ms 2) with an overall mean of 0.71. The number of alleles identified per locus ranged from 3 (MS 46) to 15 (ms 2) with an overall mean number of 7. The gene diversity ranged from 0.63 to 0.84 exhibited by MS 46 and ms 2 respectively with an overall mean diversity of 0.74 as summarised in Table 1.

One representative marker ms 5 displaying allele frequencies identified in western, northern, central and eastern populations are shown in Fig. 2.

3.2. Population genetic diversity and differentiation

The overall number of alleles in the four populations of *T. parva* from central, eastern, northern and western AEZs, and indigenous cattle breeds at each of the loci is shown in Tables 2 and 3. A total of 49 alleles were observed among the Ankole of which 10 were private alleles, while a mean diversity of 0.72 was observed. A total of 47 alleles were observed among the EASZ cattle population of which 8 were private alleles, while the mean diversity of 0.69 was observed. The total numbers of alleles identified within the different AEZs were: Central 31 (private 2), Eastern 35 (private 4), Northern 40 (private 3) and Western 44 (private 6).

To measure the levels of genetic differentiation among the four *T. parva* populations, Wright's *F* index was calculated. The overall F_{ST} indices based on the different AEZs and cattle breeds were ($F_{ST} = 0.133$, $p = 0.001$) and ($F_{ST} = 0.101$, $p = 0.001$) respectively. Results exhibited significant differences ($p \leq 0.05$) when pairwise F_{ST} indices were compared as shown: Eastern: Northern (0.048), Central: Northern (0.085), Eastern: Central (0.101), Northern: Western (0.154), Central: Western (0.160), Eastern: Western (0.173) as shown in Table 4.

Principal component analysis (PCA) was used to provide evidence for geographic sub-structuring among the four *T. parva* populations. The western *T. parva* population clustered mainly to the right-hand side with only 4 samples located across the perpendicular axis, while the northern population clustered on the left-hand side with only two samples to the right of the perpendicular axis. The eastern population clustered on the left-hand side with only 4 individual to the right of the perpendicular axis. The central population clustered in the upper left quadrant with only two samples below the horizontal axis as shown in Fig. 3.

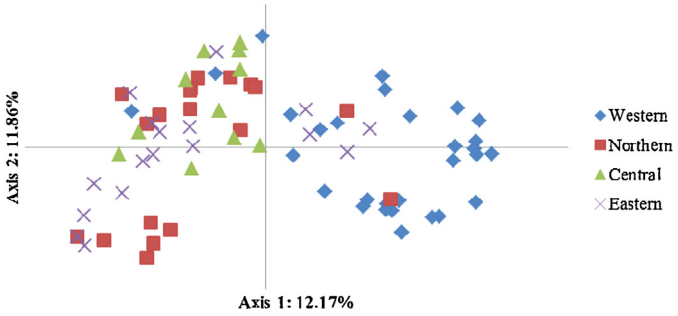

Fig. 3. Principal component analysis (PCA) of *T. parva* populations. PCA was performed using multi-locus genotype data from Western ($n = 30$), Northern ($n = 19$), Central ($n = 11$) and Eastern ($n = 18$) AEZs. The proportion of the variation in the data set explained by each axis is indicated in parentheses. Geographical sub-structuring among the study populations was observed.

To determine whether the study *T. parva* populations within the different AEZs comprised of a single panmictic population with an elevated degree of genetic exchange, the extent of linkage equilibrium (LE) of the alleles at paired loci were measured using the standard index of association (I_A^S). First, the whole data set combining the different AEZs was considered as a single population, the I_A^S value of 0.086 ($p < 0.001$) and a V_D value (2.0591) greater than L (1.4058) was obtained, indicating a state of LD. To test the hypothesis of geographical sub-structuring, the I_A^S, V_D, and L values were calculated separately for each of populations from the different AEZs. The overall I_A^S value for western *T. parva* population was 0.1452 ($p < 0.001$) and the V_D value was greater than L value, indicating a state of LD. The I_A^S values for northern, central and eastern populations were 0.1703 ($p < 0.001$), 0.202 ($p < 0.001$) and 0.143 ($p < 0.001$) respectively, and all indicated a V_D greater than the L value, implying LD as shown in Table 5.

4. Discussion

Regular studies on the genetic population structure of *T. parva* are critical given its unique epidemiology to enable appropriate planning of effective control measures in addition to evaluating the emergence of new genotypes and population structure. The results presented in this study used eight mini- and micro-satellite markers spread throughout the four chromosomes of *T. parva* to elucidate the genetic population structure of *T. parva* isolates obtained from a country wide survey based on non-symptomatic ECF among indigenous cattle populations from the central, eastern, northern and western AEZs of Uganda.

Table 2
Number of alleles and diversity of *T. parva* observed among Ankole and EASZ cattle by variable number tandem repeat (VNTR) markers.

Theileria parva population	VNTR markers	Number of alleles		Diversity
		Total	Private	
Ankole ($n = 41$)	ms 2	11	3.00	0.779
	ms 5	9	3.00	0.822
	MS 13	4	0.00	0.696
	MS 16	8	2.00	0.835
	MS 25	5	1.00	0.795
	MS 27	6	1.00	0.765
	MS 35	4	0.00	0.470
	MS 46	3	0.00	0.652
	Total	49	10	Mean diversity 0.72 ± 0.046
EASZ ($n = 37$)	ms 2	12	4.00	0.884
	ms 5	6	0.00	0.786
	MS 13	4	0.00	0.627
	MS 16	8	2.00	0.812
	MS 25	5	1.00	0.627
	MS 27	6	1.00	0.589
	MS 35	4	0.00	0.701
	MS 46	3	0.00	0.503
	Total	47	8	Mean diversity 0.69 ± 0.045

Table 3
Number of alleles and diversity of *T. parva* from the different cattle populations of detected by variable number tandem repeats (VNTR) markers.

Populations	VNTR markers	Number of		Diversity
		Allele	Private	
Western ($n = 30$)	ms 2	9	2	0.647
	ms 5	8	2	0.793
	MS 13	3	0	0.638
	MS 16	6	1	0.791
	MS 25	5	0	0.769
	MS 27	6	1	0.738
	MS 35	4	0	0.242
	MS 46	3	0	0.620
	Total	44	6	Mean diversity: 0.655 ± 0.06
Northern ($n = 19$)	ms 2	9	2	0.848
	ms 5	5	0	0.720
	MS 13	3	0	0.598
	MS 16	6	0	0.753
	MS 25	4	0	0.593
	MS 27	6	1	0.571
	MS 35	4	0	0.720
	MS 46	3	0	0.499
	Total	40	3	Mean diversity: 0.663 ± 0.41
Central ($n = 11$)	ms 2	5	0	0.793
	ms 5	3	1	0.430
	MS 13	3	0	0.645
	MS 16	6	1	0.793
	MS 25	5	0	0.744
	MS 27	2	0	0.397
	MS 35	4	0	0.727
	MS 46	3	0	0.628
	Total	31	2	Mean diversity: 0.643 ± 0.55
Eastern ($n = 18$)	ms 2	8	2	0.833
	ms 5	5	0	0.765
	MS 13	3	0	0.642
	MS 16	6	1	0.772
	MS 25	4	1	0.543
	MS 27	3	1	0.568
	MS 35	4	0	0.623
	MS 46	2	0	0.494
	Total	35	4	Mean diversity: 0.655 ± 0.04

Numerous alleles were observed at one or more loci analysed, suggesting the occurrence of mixed infections of different genotypes of *T. parva* in all the four study populations from where the samples were obtained. This is commonly encountered among field isolates of *T. parva* obtained from indigenous cattle which live freely with multiple generations of ticks with minimal tick control measures. Earlier studies in Uganda (Oura et al., 2005), Zambia (Muleya et al., 2012) and Tanzania (Mwega et al., 2014) similarly revealed the occurrence of high levels of mixed infections of *T. parva* among the surveyed cattle.

In the current study, possible allele scoring errors were eliminated by using a quantifiable approach to generate MLGs, each being represented by the most dominant genotype allele throughout the entire sample size. Mistakes due to mixed infections among

Table 4
Pairwise comparisons of *T. parva* genetic populations obtained from indigenous cattle based in the Western, Northern, Central and Eastern AEZs of Uganda.

Populations ($n = 78$)	Western	Northern	Central	Eastern
Western ($n = 30$)	–	0.001[b]	0.001[b]	0.001[b]
Northern ($n = 19$)	0.154	–	0.009[b]	0.020[a]
Central ($n = 11$)	0.160	0.085	–	0.002[b]
Eastern ($n = 18$)	0.173	0.048	0.101	–

Above the diagonal: p values for significant differences between populations. Below the diagonal: Wright's F_{ST} values indicating differences between populations.
[a] Significant.
[b] Highly significant.

Table 5
Population genetic analyses of *T. parva* from the western, northern, central and eastern AEZs of Uganda.

Population	V_D	I_A^S	Var $(V_D)_{para}$	Var $(V_D)_{mc}$	L_{mc}	p-Value	Mean genetic diversity ± SD	Linkage
All AEZs ($n = 78$)	2.0591	0.0749	0.0013	0.0010	1.405	<0.001	0.7737 ± 0.02	LD
Western ($n = 30$)	2.9373	0.1424	0.0138	0.0115	1.700	<0.001	0.6981 ± 0.06	LD
Northern ($n = 19$)	3.2166	0.1647	0.0259	0.0298	1.964	<0.001	0.7296 ± 0.03	LD
Central ($n = 11$)	3.0424	0.1846	0.0499	0.0569	1.811	<0.001	0.7576 ± 0.05	LD
Eastern ($n = 18$)	2.8625	0.1302	0.0262	0.0356	1.886	<0.001	0.7259 ± 0.04	LD

I_A^S: standard index of association, V_D: mismatch variance (linkage analysis), L: upper 95% confidence limit of Monte Carlo simulation (linkage analysis) and LD: linkage disequilibrium.

field samples were eliminated by scoring only a single allele for the generation of MLGs as previously described by Muleya et al. (2012) and Mwega et al. (2014).

The mean number of alleles per locus as well as allele frequencies varied between the central, eastern, northern and western zones suggesting the existence of multiple genotypes in all the *T. parva* parasite populations. Parasite population from the central zone showed the lowest mean genetic diversity when compared with those from other zones. This could have resulted from the lower non-clinical disease prevalence (Kabi et al., 2014) associated with reduced cattle exposure to infected ticks as a result of the crop-livestock management system in the central zone. The higher *T. parva* genetic diversity was observed in the western zone which could have resulted from increased interaction of cattle and tick populations associated with wild ruminants such as buffaloes in wildlife protected areas of Lake Mburo National Park (LMNP). However, an earlier study by Oura et al. (2011), established that buffaloes sampled from Kidepo Valley National Park (KVNP in northeastern Uganda), Murchison Falls National Park (MFNP in northwestern Uganda) and Queen Elizabeth National Park (QENP) did not harbour *T. parva.* However, cattle from the neighbouring localities have been shown to carry *T. parva* (Kabi et al., 2014) signifying a low interaction of ticks and a possibility of lack of transmission of *T. parva* between the buffaloes and cattle. A recent study has now shown that *T. parva* associated with buffaloes (corridor disease) is capable of causing acute infection among domestic immunised cattle known to share grazing pastures with the former (Sitt et al., 2015), due to non-homologous parasite genotypes. Additionally, the sexual life cycle of *T. parva* in the ticks enables high genetic exchange between the different genotypes harboured by the ticks (Katzer et al., 2006, 2010).

The observation of private alleles in all populations is an exhibition of genetically distinct populations of *T. parva* circulating among indigenous cattle of Uganda.

Genetic and geographical sub-structuring was also observed on PCA, where samples from western, eastern, central and northern significantly clustered separately. Only a few samples ($n = 4$) from western and eastern, while $n = 2$ from northern and central clustered differently. This state of genetic and geographical sub-structuring indicated by both allele frequencies and PCA were further confirmed by the significant differentiation observed among the populations from the different AEZs and breeds. Related observations of genetic and geographic sub-structuring have been made by (Oura et al., 2005, 2011; Asiimwe et al., 2013). Furthermore, a state of LD with an I_A^S value greater than 0 was also observed when the central, eastern, northern and western populations were treated as a single population, indicating the absence of random mating between *T. parva* from all the AEZs. This further strengthens the reality of existence of a state of genetic and geographical sub-structuring of *T. parva* populations. A more realistic explanation of the current findings is that the study *T. parva* isolates were obtained beyond the boundaries of random genetic exchange between populations.

5. Conclusions

The results of this study have indicated that the study *T. parva* populations from the sampled AEZs are distinctly different, with significant genetic diversity in each population. The *T. parva* populations investigated revealed no evidence of linkage equilibrium. The use of ITM as an integral component of ECF control could be tailored to specific parasite genotype populations within the country, as a way of avoiding the risks of introduction of novel genotypes and further complication of the prevalent parasite population genetic structure.

Competing interests

The authors declare that they have no competing interests.

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