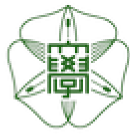
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1 # Genotyping of *Theileria parva* populations in vaccinated and non-vaccinated
2 cattle in Malawi

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16

17

18

19 **Abstract**

20 *Theileria parva* is an apicomplexan protozoan parasite that causes bovine theileriosis (East
21 Coast Fever; ECF) in central, eastern, and southern Africa. In Malawi, ECF is endemic in the
22 northern and central regions where it has negatively affected the development of dairy industry.
23 Despite its endemic status the genetic population structure of *T. parva* in Malawi is currently
24 unknown. To obtain an understanding of *T. parva* in Malawi, we performed population genetics
25 analysis of *T. parva* populations in cattle vaccinated with the Muguga cocktail live vaccine and
26 non-vaccinated cattle using mini- and microsatellite markers covering all the four *T. parva*
27 chromosomes. The *T. parva* Muguga strain was included in this study as a reference strain.
28 Linkage disequilibrium was observed when all samples were treated as a single population.
29 There was sub-structuring among the samples as shown by the principal coordinate analysis
30 (PCoA). Majority of the samples clustered with the *T. parva* Muguga reference strain
31 suggesting that the isolates in Malawi are closely related to the vaccine component which
32 support the current use of Muguga cocktail vaccine to control ECF. The clustering of samples
33 from non-endemic southern region with those from endemic central region suggests expansion
34 of the distribution of *T. parva* in Malawi.

35

36 **Key words:** Genotyping, Malawi, population structure, *Theileria parva*

37

38

39 **Key Findings**

40 - There was sub-structuring among *T. parva* in Malawi into two separate clusters.
41 - Samples from areas with no *T. parva* Muguga cocktail immunisation history clustered
42 with the *T. parva* Muguga reference strain.
43 - The flow of genetic material was likely to occur within population than between
44 populations.

45

46 **Introduction**

47 *Theileria parva,* which causes East Coast fever (ECF), one of bovine theilerioses, is the
48 most important tick-borne pathogen of cattle on the African continent (Lubembe *et al.* 2020).
49 It is estimated that almost half of the cattle population on the African continent are at risk of
50 infection with *T. parva* (Lubembe *et al.* 2020). Although adult indigenous cattle in Africa rarely
51 develop clinical diseases, calves under 6 months old are highly susceptible (Moll *et al.* 1984,
52 1986). However, in exotic and crossbred cattle, the fatality rate may be as high as 80-100% and
53 this has negatively impacted the development of the dairy industry in the endemic countries
54 (Oura *et al.* 2007). The control of ECF in Malawi is mainly based on tick control through
55 acaricide application, livestock movement restriction where cattle from endemic northern and
56 central regions are not allowed to go to the non-endemic southern region. Chemotherapy using
57 buparvaquone is used to manage clinical cases in the endemic areas and immunisation of cattle
58 using the Muguga cocktail live vaccine. The infection and treatment method (ITM) which
59 involves inoculating the animal with a dose of the Muguga cocktail vaccine and simultaneous
60 treatment with long acting tetracycline is currently in use in the northern and central regions
61 where the disease is endemic (DAHLD, 2006). However, in the southern region which has been
62 considered non-endemic, the vaccine has not been deployed although sporadic cases of ECF
63 have been reported (Chinombo *et al.* 1988). The whole genome sequencing of *T. parva* by

64 Gardner et al. (2005) and the identification of *T. parva* specific mini- and microsatellite
65 markers which were first described and applied by Oura *et al.* (2003, 2005) and Katzer *et al.*
66 (2006, 2010) have provided good markers for the genotyping and characterisation of the
67 population structure of *T. parva*.

68 However, the deployment of vaccine that will be effective against the *T. parva* strains
69 circulating in cattle requires genotypic and population genetics data in the endemic countries.
70 Despite *T. parva* being widely spread in central, eastern and southern Africa, there is limited
71 information on the parasite genotypes and population structure available in southern Africa.
72 The information on *T. parva* parasites from cattle available is only from Zambia (Muleya *et al.*
73 2012) and South Africa (Lubembe *et al.* 2020), although the disease is controlled in South
74 Africa. Lubembe *et al.* (2020) investigated the genotypes of *T. parva* from buffaloes in
75 Mozambique and South Africa. The knowledge about the genotypes and population structure
76 of *T. parva* on the African continent is important to assess the diversity of *T. parva* in the
77 continent as these strains may affect the current vaccines being used to control ECF.

78 This study aimed at investigating the genetic population structure of *T. parva* among
79 cattle in Malawi using *T. parva* mini- and microsatellite markers. The specific objectives were
80 1) to determine if genotypes of *T. parva* from vaccinated cattle with *T. parva* Muguga cocktail
81 vaccine will be different from those without vaccination history. 2) to determine if there is
82 presence of sub-structuring among the *T. parva* isolates in Malawi and 3) to determine if the
83 population of *T. parva* in Malawi is panmictic.

84

85 **Materials and methods**

86 *Sample collection and DNA extraction*

87 The cattle blood samples from Kasungu (n = 20), Nkhotakota (n = 20) and Katete (Lilongwe)
88 (n = 19) were obtained from our previous study (Chatanga et al., 2020). The other samples
89 were collected from Likasi farm (33º 17' 05" E; 14º 02' 43" S) (n = 92) and Lilongwe University

90 of Agriculture and Natural Resources (LUANAR) student farm (33º 77' 83" E; 14º 17' 96" S,)
91 (n = 53) in the central region and Mikolongwe (35º 12' 30" E; 15º 51' 49" S) (n = 28) in the
92 southern region (Figure 1). The cattle sampled from Kasungu and Nkhotakota were the
93 indigenous Malawi zebu with no history of *T. parva* immunisation or dipping to control ticks.
94 Furthermore, these animals belong to smallholder farmers who use communal grazing land
95 which allows mixing with herds from other farms.
96 The cattle sampled from Katete farm were Holstein Friesians only, while those from Likasi,
97 LUANAR and Mikolongwe farms were Holstein Friesians, and crossbreeds with the
98 indigenous Malawi zebu, respectively. At Katete, Likasi, and LUANAR farms, cattle are kept
99 under semi-intensive management system and some were immunised with the *T. parva*
100 Muguga cocktail vaccine 2 years and 3 months before the sampling period as previously
101 described (Chatanga et al., 2020) and dipping is done weekly to control ticks. At Mikolongwe
102 farm, which is located in the southern region, cattle are dipped weekly but are not vaccinated
103 against *T. parva* as the farm falls in a non-endemic area. The animals from other farms in
104 Malawi are not allowed into the breeding population at Katete farm for disease control purposes.
105 The farm practices natural breeding and regularly change the bulls used by importing their
106 breeding stock from South Africa where *T. parva* is not endemic. Although the animals at
107 Likasi, LUANAR, and Mikolongwe farms do not mix with cattle from other herds, the
108 introduction of Malawi zebu heifers breeding stock from smallholder farmers in Malawi for
109 crossbreeding with Holstein Friesians is allowed.
110 Approximately 5 ml of cattle whole blood was collected in Ethylene Diamine Tetra
111 Acetic acid (EDTA) vacutainer tubes, aseptically by swabbing the external jugular
112 venepuncture site with methylated spirit swab. The samples were kept on ice before
113 transporting to the laboratory. DNA was extracted from 200 µl of whole blood using Quick
114 Gene DNA whole blood kit S (DB-S) (Kurabo Industries Ltd., Osaka, Japan) according to

115 manufacturer's instructions. The extracted DNA was stored at -20°C until required for use.

116

117 Theileria parva *screening*

118 The samples from Likasi, LUANAR and Mikolongwe were screened in this study for *T. parva*

119 using nested PCR assays targeting the *T. parva*-specific 104-kD antigen gene (*p104*) as

120 described previously by Odongo et al. (2010). The amplification was done using Tks Gflex

121 DNA Polymerase (TaKaRa Bio Inc., Shiga, Japan), the reaction mixture and PCR conditions

122 were set as previously described (Chatanga *et al.* 2020). Approximately, 10 ng of the sample

123 was used as DNA template and molecular grade water instead of genomic DNA was used as

124 negative control in each run for quality control.

125

126 *PCR amplification of mini- and microsatellites*

127 The primers used for satellite amplification and their annealing temperatures are shown in

128 Table 1. The forward primer of each pair was fluorescently labelled with either 6-FAM, / VIC,

129 and/ or PET at the 5' end. The amplifications were conducted in a 10 µL reaction mixture,

130 containing 5.0 µL of 2×Gflex PCR Buffer (Mg^{2+}, dNTP plus), 0.2 µL of Tks Gflex DNA

131 Polymerase, 0.5 µL of 200 nM of each primer, 10.0 ng of the DNA template. The volume was

132 adjusted using distilled water. Negative control containing distilled water instead of DNA

133 template was used for quality control. The cycling conditions were set with an initial

134 denaturation at 94°C for 3 min, followed by 45 cycles of denaturation at 98°C for 10 sec,

135 annealing for 30 sec, and extension at 68°C for 1 min and final extension at 68°C for 5 min.

136 The amplicons were electrophoresed in a 1.5% agarose gel stained with Gel-Red (Biotium,

137 Hayward, CA, USA) and visualized under UV light.

138

139 *Capillary electrophoresis*

140 The capillary electrophoresis was conducted in a 11.5 µL reaction volume comprising of 0.5
141 µL GS-600 LIZ size standard (Applied Biosystems, CA, USA), 10.0 µL ABI HiDi formamide
142 (Applied Biosystems, CA, USA) and 1.0 µL of 10-fold diluted microsatellite PCR product. The
143 mixture was denatured at 95°C for 5 min and immediately cooled on ice before capillary
144 electrophoresis on the ABI 3500xl genetic analyser (Applied Biosystems, CA, USA).
145
146 *Fragment analysis*
147 The DNA fragment sizes were analysed relative to the ROX-labelled GS 600 LIZ size standard
148 using Gene Mapper software version 6 (Applied Biosystems, CA, USA). This facilitated the
149 resolution of multiple products with 1 base pair (bp) difference in a single reaction. Multiple
150 products from a single PCR reaction indicated the presence of mixed genotypes. The output
151 data from the genetic analyser were provided as the area under the peak of each allele
152 (quantitative measurement), with the predominant allele possessing the greatest peak area. In
153 this way, the predominant allele at each locus was identified for each sample, and this data was
154 combined to generate a multi-locus genotype (MLG) representing the most abundant genotype
155 in each sample. Only the alleles with the prescribed base pair range were used to generate the
156 MLG and samples from the same area were electrophoresed and gene scanned on the same
157 plate.
158
159 *Data analysis*
160 An allele sharing co-efficient (Bowcock *et al.* 1994) in Excel microsatellite toolkit
161 (http://animalgenomics.ucd.i.e./sdepark/ms-toolkit/) was used for the similarity comparison of
162 the MLGs (Peakall & Smouse, 2006, 2012). Similarity analysis was determined by constructing
163 a similarity matrix. This was then utilised to conduct principal coordinate analysis (PCoA) and
164 analysis of molecular variance (AMOVA) using the Excel plug-in software GenAIEx6

165 (http://www.anu.edu.au/BoZo/GenAIEx/) (Peakall & Smouse, 2006, 2012). The FSTAT
166 computer package version 2.9.3.2 was used to calculate estimates of F statistics for population
167 genetic analysis (http://www2.unil.ch/popgen/softwares/fstat.htm). LIAN
168 (http://adenine.biz.fh-weihenstephan.de/lian/) was used to test the null hypothesis of linkage
169 equilibrium by calculating a quantification of linkage equilibrium/linkage disequilibrium
170 called the standardized index of association (I_A^S) (Haubold & Hudson, 2000). The statistical
171 independence of alleles at all pairwise combinations of loci under study characterizes linkage
172 equilibrium (LE) and this independent assortment was initially tested by LIAN by determining
173 the number of loci at which each pair of MLGs differs. The mismatch values from this
174 distribution were then used to calculate the variance (V_D) which was then compared to the
175 variance expected (V_E) for LE. Monte Carlo (MC) computer simulation was used to test the
176 null hypothesis that VD = VE. The computer software calculates a 95% confidence limit L.
177 When V_D was greater than critical limit L, the null hypothesis of LE is rejected.
178 Mixed infection of different genotypes was indicated by the presence of several alleles at a
179 locus in one sample. To determine the multiplicity of infection at the nine loci used, the mean
180 number of alleles in each sample was calculated. Finally, the mean for each population as well
181 as the combined population from the index value of each sample was also calculated to show
182 the overall multiplicity of infection for each population and combined population.
183

184 **Results**

185 *Confirmation of* Theileria parva *infection by PCR*

186 Only samples from Likasi, LUANAR and Mikolongwe (n = 173) were screened in this study
187 while samples from Kasungu, Nkhotakota and Katete were screened in a previous study
188 (Chatanga *et al.* 2020). The positive rates for Likasi, LUANAR and Mikolongwe were 18%
189 (17/95), 32% (17/53), and 25% (7/28), respectively. Likasi farm had the least positive rate at

190 18% despite having high number of sampled animals. The findings of this study confirm for
191 the first time, the presence of *T. parva* infection in cattle from the southern region using
192 molecular techniques.
193
194 *Satellite marker diversity and allelic variation*
195 The number of alleles per locus ranged from 3 in marker MS77 to 33 in marker MS14 (Table
196 2), and the average number of alleles observed per locus was 18.67 (Table 3). Markers MS14,
197 MS9, and MS19 had the highest number of alleles (33, 30, and 29 alleles, respectively). Shared
198 alleles were observed at all the 9 loci investigated with no locus with unique alleles for a
199 particular population (Figure 2). The number of shared alleles ranged from 2 at locus MS77 to
200 10 at loci MS14 and MS19 (Figure 2). Similarly, genetic diversity of the 9 loci investigated
201 showed that markers MS48 and MS77 were the least diverse in all the six populations. The
202 populations from Kasungu and Nkhotakota showed low or no diversity at the loci MS48 and
203 MS77 (Table 2). Similarly, the population from LUANAR showed no diversity at locus MS77,
204 but the diversity of marker MS47 could not be determined in this population due to the low
205 number of samples (n = 3) successfully amplified (Table 2).
206
207 *Population diversity structure*
208 The PCoA of *T. parva* for the six cattle populations in Malawi and the *T. parva* Muguga
209 reference strain showed that there were two clusters designated as cluster A and B (Figure 3).
210 Cluster A had the majority of samples (n = 83) from all the six sampling sites and the *T. parva*
211 Muguga reference strain while cluster B, had 17 samples from Katete (n = 14) and Likasi (n =
212 3) farms from the central region. Interestingly, it was observed that even samples from areas
213 without *T. parva* Muguga cocktail vaccination history, clustered together with *T. parva*
214 Muguga strain (Figure 3). The analysis of molecular variance (AMOVA) showed that the

215 genetic variation observed was mainly within populations (99%) while that due to differences
216 between populations was only 1% (Table 4).

217 The analysis of the allelic profile data used to determine the linkage of *T. parva* in
218 Malawi showed that when all the six sub-populations were combined as a single population the
219 standardised index of association (I^S_A) was greater than zero (0.0286). Furthermore, the
220 pairwise variance (V_D) was greater than the 95% critical L value (M_L) which indicated linkage
221 disequilibrium (Table 3). However, when each sub-population was treated separately, it was
222 observed that most populations (4 out of 6) had an I^S_A which was either negative or close to
223 zero and the V_D was less than the M_L indicating linkage equilibrium (Table 3). Katete and
224 Likasi populations showed linkage disequilibrium when treated separately (Table 3). When
225 populations A and B were treated as separate and individual populations as per PCoA clustering
226 (Figure 3), both populations were found to be in LE (Table 3).

227 To determine the differences among the sampled populations, the **estimated**
228 heterozygosity (H_e) and mean number of genotypes/locus were calculated for each of the six
229 population. The **estimated** heterozygosity (H_e) ranged from 0.6127 for Mikolongwe and
230 LUANAR to 0.7900 for Likasi (Table 3). The mean number of genotypes/locus ranged from
231 3.56 to 7.22 for Mikolongwe and Likasi, respectively (Table 3). The overall **estimated**
232 heterozygosity (H_e) and mean number of genotypes/locus for the combined population was
233 0.666 and 18.67, respectively.

234 To determine the degree of genetic differentiation, the Wright's F index was calculated
235 for each of the two clusters based on the PCoA. Cluster A which had majority of the isolates
236 had an F_{ST} value of 0.008 while cluster B with isolates from Likasi and Katete had an F_{ST} value
237 of 0.273 (Table 3). This finding shows significant differences between these two clusters. The
238 combine population had an F_{ST} value of 0.105 (Table 3).

239

240 *Multiplicity of infection*

241 Multiple genotypes of *T. parva* were observed in the majority of the samples regardless of the
242 sampling site and region. The multiplicity of infection in the six sub-populations ranged from
243 1.14 to 1.40 for the population from Mikolongwe and Nkhotakota, respectively as estimated
244 by the calculated mean number of genotypes/locus (Table 5). The standard deviation ranged
245 from 0.21 to 0.36 for Mikolongwe and Kasungu populations, respectively. The combined
246 population had the mean value of 1.32 and a low standard deviation of 0.29 (Table 5).

247

248 **Discussion**

249 Knowledge about the genetic information and population structure of *T. parva* is a pre-requisite
250 to the conception of effective control measures and monitoring of current measures (Lubembe
251 *et al.* 2020). However, this information is not available in most southern African countries
252 including Malawi although it is one of the endemic regions. *Theileria parva* is the most
253 important tick-borne pathogen in Malawi and is endemic in the central and northern regions
254 (DAHLD, 2006). Current control measures in use include immunisation using *T. parva*
255 Muguga cocktail live sporozoite vaccine for exotic, crossbreed cattle and Malawi zebu calves
256 below 6 months in the endemic areas (Perry, 2016; Lawrence *et al.* 1996). To understand the
257 genetic composition and population structure of *T. parva* in Malawi, nine mini- and
258 microsatellite markers were employed to examine 100 *T. parva* samples from six populations;
259 five in the central region where ECF is endemic and one in the southern region which has
260 traditionally been considered as non-endemic until this study. To control the spread of ECF in
261 Malawi, movement of animals from the ECF endemic northern and central regions to the non-
262 endemic southern region is not allowed and vaccination with *T. parva* Muguga cocktail is not
263 permitted in the latter (DAHLD, 2006).

264 Almost all the populations investigated had high mean genetic diversity (**estimated** 265 heterozygosity) that ranged from 0.612 for Mikolongwe and LUANAR to 0.790 for Likasi. 266 The lower **estimated** heterozygosity at Mikolongwe may be due to limited number of samples 267 as it had the least number of samples among the populations examined. Further, this 268 observation may result from the pathogen being newly introduced in this region as reported 269 previously in South Sudan (Salih *et al.* 2018; Marcellino *et al.* 2017). The lower He for 270 LUANAR could be due to reduced exposure of the animals to the parasites as the animal are 271 kept for a short period of time. The animals are disposed after some studies are concluded at 272 the student farm unlike the other farms which keep animals for longer periods. The high 273 **estimated** heterozygosity at Likasi farm is also supported by the PCoA results which showed 274 that the isolates at the farm separated into two clusters and majority were in cluster A while the 275 rest were in cluster B. The higher expected genetic diversity observed at Likasi may result from 276 longer history of genetic recombination than the other populations as it was used as a trial site 277 when the *T. parva* Muguga cocktail live vaccine was being developed for the eastern and 278 southern Africa region (Lawrence *et al.* 1996; Dolan, 1988). The overall **estimated** 279 heterozygosity for Malawi combined population of 0.67 is close to the values reported in 280 Zambia (0.75) (Muleya *et al.* 2012), South Sudan (0.73) (Salih *et al.* 2018) and Tanzania (0.79) 281 (Rukambile *et al.* 2016). However, it is lower than the 0.91 reported in Burundi (Atuhaire *et* 282 *al.* 2021) and the 0.81 from the study combining eastern and southern Africa isolates from 283 cattle and buffaloes (Lubembe *et al.* 2020). This observation may be due to the fact that the 284 study either included samples from buffaloes or involved sampling from a wider geographical 285 area resulting in a higher mean genetic diversity.

286 The observation of shared alleles in most samples from most of the sampled populations 287 at all loci investigated shows that almost similar strains of *T. parva* are circulating among cattle 288 in Malawi. This has also been strongly supported by the PCoA in which the majority of samples

289 from the six populations together with the *T. parva* Muguga reference strain clustered in
290 population A. This finding is in accordance with a previous study based on *Tp1* and *Tp2* genes,
291 which showed that most sequences were either identical or similar to *T. parva* Muguga, *T.*
292 *parva* Kiambu-5 and *T. parva* Serengeti transformed, the components of *T. parva* Muguga
293 cocktail vaccine (Chatanga *et al.* 2020). However, the presence of a separate minority
294 population (cluster B), different from the *T. parva* Muguga reference strain, was also indicated
295 as per PCoA results and the F_{ST} value of 0. 271 between population A and B. The majority of
296 samples from this minority population originated from Katete (n = 17) with Likasi only
297 contributing three (3). The separate population at Katete may result from it being a closed
298 population that does not allow introduction of animals from other farms in Malawi which may
299 introduce other *T. parva* strains circulating in other cattle populations in Malawi. Since some
300 of the sampled animals were only immunised 2 years and 3 months before the sampling, it may
301 help to explain why only a few samples from the farm clustered in majority population The
302 other isolates at Katete farm that clustered in population A may be due to immunisation with
303 the *T. parva* Muguga cocktail vaccine. Although, the other farms also have restricted access to
304 other animals, they allow breeding stock from smallholder farmers in Malawi into their
305 population which is likely to explain the relatedness with those populations from smallholder
306 farmers. The finding of mixed genotypes within a sample shows that mixed infections of *T.*
307 *parva* are common among cattle in Malawi. This may be due to prolonged exposure to ticks
308 and the complex life cycle to *T. parva* which include sexual and asexual stages in the vector
309 tick and host cattle, respectively (Katzer *et al.* 2010). The genetic recombination phenomenon
310 of *T. parva* in the vector tick which it has adopted as a strategy to survive and drive genetic
311 diversity ensures that host cattle can still be infected with new strains (Oura *et al.* 2003).

312 AMOVA showed that *T. parva* isolates in cattle in Malawi have high chance of
313 exchanging genetic material within each population than between populations. However, the

314 population at Katete was exceptional to this observation. This finding may be due to the point
315 that the immunisation with Muguga cocktail vaccine was just done 2 years and 3 months before
316 sampling in some animals which may not have provided enough time for the exchange of
317 genetic material to occur. This was supported by the high genetic variation observed within the
318 population (99%) than between populations (1%). This finding is also in agreement with
319 previous studies which reported similar findings in other countries (Lubembe *et al.* 2020; Salih
320 *et al.* 2018). As reported previously by Salih *et al.* (2018) in South Sudan, the findings of this
321 study do not support the presence of any correlation between genetic structure of a population
322 and the geographical location of the isolates.

323 Linkage analysis showed that the majority of the individual populations were in linkage
324 equilibrium and thus in panmixia, further supporting the results of PCoA and AMOVA that
325 gene flow is likely to occur within a particular population. Although this finding is not in
326 agreement with that reported in Zambia (Muleya *et al*. 2012), it is however supported by the
327 findings by Lubembe *et al.* (2020) that individual populations in southern Africa are in linkage
328 equilibrium. When the populations were combined, linkage disequilibrium was observed. This
329 may be due to Katete farm being a closed population that makes it difficult for random mating
330 to occur even with other populations within a 50 km radius. When the population from Katete
331 was excluded, the combined population was in linkage equilibrium even with those from
332 Mikolongwe with almost 500 km distance from the other population. Currently, there seems to
333 an increase in the detection of infections in the south of the country, this might be attributed to
334 human assisted movement of animals from endemic to no-endemic regions despite having the
335 livestock movement ban in place. Thus, there is a need to revise the current control measures
336 so that this trend can be reversed.

337 In conclusion, this study has for the first time provided molecular evidence of the
338 presence of *T. parva* infection in cattle in the southern region of Malawi. Further, the findings

339 have shown that the *T. parva* population in southern Malawi is closely related to the
340 populations in the central region which is endemic to ECF. The study has also shown that *T.*
341 *parva* genotypes circulating in both the vaccinated and unvaccinated cattle in Malawi are
342 closely related. It has also been shown that there is presence of sub-structuring among the
343 samples investigated. Generally, the population of *T. parva* in Malawi is not panmictic but
344 individual populations are in panmixia. Furthermore, it has been shown that in Malawi there
345 are at least two clusters of *T. parva*, one that is closely related to *T. parva* Muguga reference
346 strain and another that is separate and different from the *T. parva* Muguga cocktail vaccine
347 strain. It is therefore important to carry out a nationwide wide study to provide a comprehensive
348 genetic population structure of *T. parva* in Malawi and to include samples from the
349 neighbouring countries to determine the relatedness of the isolates in the region. This will help
350 to assess the impact of the *T. parva* strains that are different from the *T. parva* Muguga cocktail
351 vaccine strain on the control of ECF in Malawi in particular and southern Africa as a region in
352 general.
353

354 **Authors Contribution**

355 EC, WM & RN conceived and designed the study. EC conducted the experiments. EC, YO &
356 WM performed the population genetic and statistical analyses. EC wrote the original
357 manuscript. WM, KH, CS & RN provided resources. RN & CS obtained funding. KH, CS, KK,
358 NN & RN supervision, writing review and editing. All authors approved the submitted final
359 version of the manuscript.
360

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365 Bishop for providing the *Theileria parva* Muguga reference strain to Professor Chihiro
366 Sugimoto which was used as a reference strain.

367

368 **Ethical Standards**

369 This study was approved by Ministry of Agriculture, Irrigation and Water Development
370 (MoAIWD) in Malawi through the Department of Animal Health and Livestock Development
371 (DAHLD) reference number 10/15/32/D and informed consent was obtained from the
372 owners/custodians.

373

374 **Conflicts of Interest**

375 The authors declare there are no conflicts of interest.

376

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383

384 **Data**

385 All the data generated in this study has been provided in this paper.

386

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492 **Appendix**

493 Table 1. The list of satellite marker primers used in this study.

Marker ID	Primer name	Sequence 5'→ 3'	Chromosome	Annealing temperature (°C)	Amplicon size (bp)	Reference
ms1	ms1F	TGAGGCAGTGTAGAGCGCATAAC	1	60	235-368	Oura et al. 2003
	ms1R	AAATCCGCAACGCTATTGCCGAGG				
MS9	MS9F	CTGGTTCCTCATCTTCACACTA	3	60	230	Katzer et al., 2006
	MS9R	CTTTCCAGAACCTACAATCAC				
MS14	MS14F	ATGCCAATTCGGTAAAGGTCTCCG	2	60	360-600	Katzer et al. 2010
	MS14R	GCATATCTCAGTCAAGCCAACATC				
MS19	MS19F	CCAGACACCTCAAATCCCAAGTA	2	60	304	Oura et al. 2003
	MS19R	CCACACTGCCACCTAATACAAA				
MS39	MS39F	CCAATCAACATCAACTACTCC	4	60	263	Katzer et al. 2010
	MS39R	CGAACTCCAAACGATCTAAAC				
ms47	M47F	GTCACAAGGGAAATCATGTCACTC	1	60	398	Katzer et al. 2006
	M47R	GAGCCTTGAGTAGGTCTAAATTTG				
MS48	M48F	CTACTTCTGGATCAGGTGTGGTGG	1	60	223	Katzer et al. 2006
	M48R	GATTGAGACGATCCCGGTAGTCCT				
MS68	M68F	TCACATCGGGTAACAAGAA	1	60	469	Katzer et al. 2010
	M68R	TATTTATCGACCCCAAATCG				
MS77	MS77F	GGTAACCAACAACCACATTT	2	60	270	Katzer et al. 2010
	MS77R	TGCTTATGAACTCAATCATCTC				

494 ms = minisatellite, MS = microsatellite, F = forward, R = reverse, and bp = base pair.

495 Table 2. Allelic variation among *Theileria parva* from cattle in Malawi.

	Study site	N	Satellite markers								
			MS1	MS9	MS14	MS19	MS39	MS47	MS48	MS68	MS77
Alleles within population	Kasungu	20	3	11	13	12	6	9	1	5	1
	Nkhotakota	20	4	7	13	12	8	8	2	5	1
	Katete	19	4	8	5	8	6	2	2	7	2
	Likasi	17	6	11	11	11	8	6	4	6	2
	LUANAR	17	4	7	10	9	3	1	4	7	1
	Mikolongwe	7	3	5	6	4	3	4	2	3	2
	Overall	100	9	30	33	29	22	20	7	15	3
Genetic diversity	Kasungu	20	0.511	0.860	0.963	0.971	0.542	0.945	0.000	0.660	0.000
	Nkhotakota	20	0.616	0.858	0.949	0.961	0.641	0.886	0.118	0.625	0.000
	Katete	19	0.654	0.808	0.405	0.669	0.758	0.154	0.425	0.857	0.118
	Likasi	17	0.794	0.949	0.941	0.952	0.848	0.893	0.467	0.848	0.400
	LUANAR	17	0.714	0.802	0.895	0.936	0.700	NA	0.495	0.818	0.000
	Mikolongwe	7	0.667	0.905	1.000	0.900	0.700	1.000	0.571	0.600	0.571

496 N = number of samples, NA = not determined

497 Table 3. Population genetic analyses of *Theileria parva* in Malawi.

Population	N	H_e	Mean number of genotypes/ locus	I_A^S	V_D	L	P-value	Linkage	F_{ST}
Kasungu	20	0.666	6.78	-0.0177	1.2222	1.7936	8.20×10^{-01}	LE	
Nkhotakota	20	0.6947	6.67	0.0038	1.3644	1.6290	3.00×10^{-01}	LE	
Katete	19	0.6368	4.89	0.0988	3.1507	2.4566	$< 1.00 \times 10^{-02}$	LD	
Likasi	17	0.7900	7.22	0.0762	2.0248	1.6100	$< 1.00 \times 10^{-02}$	LD	
LUANAR	17	0.6127	5.11	0.0082	1.2739	1.5109	3.30×10^{-01}	LE	
Mikolongwe	7	0.6127	3.56	-0.0334	1.0000	2.2000	8.70×10^{-01}	LE	
Pop A	83	0.7019	16.33	0.0034	1.4721	1.5777	3.20×10^{-01}	LE	0.008
Pop B	17	0.7019	3.89	0.0034	1.4721	1.6322	3.90×10^{-01}	LE	0.273
Combined population	100	0.666	18.67	0.0286	1.6642	1.4900	0.067	LD	0.105

498 N= number of samples, H_e = estimated heterozygosity, I_A^S = standard index of association, V_D = mismatch variance (linkage analysis), L = upper

499 95% confidence critical limits of Monte Carlo simulation, LD = linkage disequilibrium, LE = linkage equilibrium, F_{ST} = Wright's fixation index

500

501 Table 4. The analysis of molecular variance (AMOVA) of *Theileria parva* in Malawi.

Source	*df*	Sum of squares	Mean Sum of squares	Variance components.	% of variation
Among Populations	5	456442.9	91288.58	835.277	1%
Among Individuals	94	6002033	63851.41	0	0%
Within Individuals	100	9308005	93080.05	93080.05	99%
Total	199	15766480		93915.32	100%
	Fixed indices				
	F_{SC}	0.011			
	F_{ST}	-0.186			
	F_{CT}	-0.174			

502 df = degress of freedom, F_{SC} = variation among populations within groups, F_{ST} = measure of population differentiation due to genetic structure,

503 F_{CT} = Variation among groups of populations.

504 Table 5. Multiplicity of infection of *Theileria parva* in Malawi.

Population	Number of samples	Multiplicity of infection				Total number of alleles identified on all loci
		Mean	SD	Min.	Max.	
Kasungu	20	1.34	0.36	0.78	2.22	61.00
Nkhotakota	20	1.40	0.27	0.89	1.89	60.00
Katete	19	1.31	0.33	0.44	1.88	44.00
Likasi	17	1.39	0.30	0.67	1.89	65.00
LUANAR	17	1.14	0.28	0.67	1.56	46.00
Mikolongwe	7	1.31	0.21	1.00	1.56	32.00
Overall	100	1.32	0.29	0.74	1.83	51.33

505 SD: standard deviation, Min. and Max. refer to the minimum and maximum, respectively for the number of alleles identified per locus per sample.

506 The summary of the number of the alleles per locus for each population is provided in Table 2.

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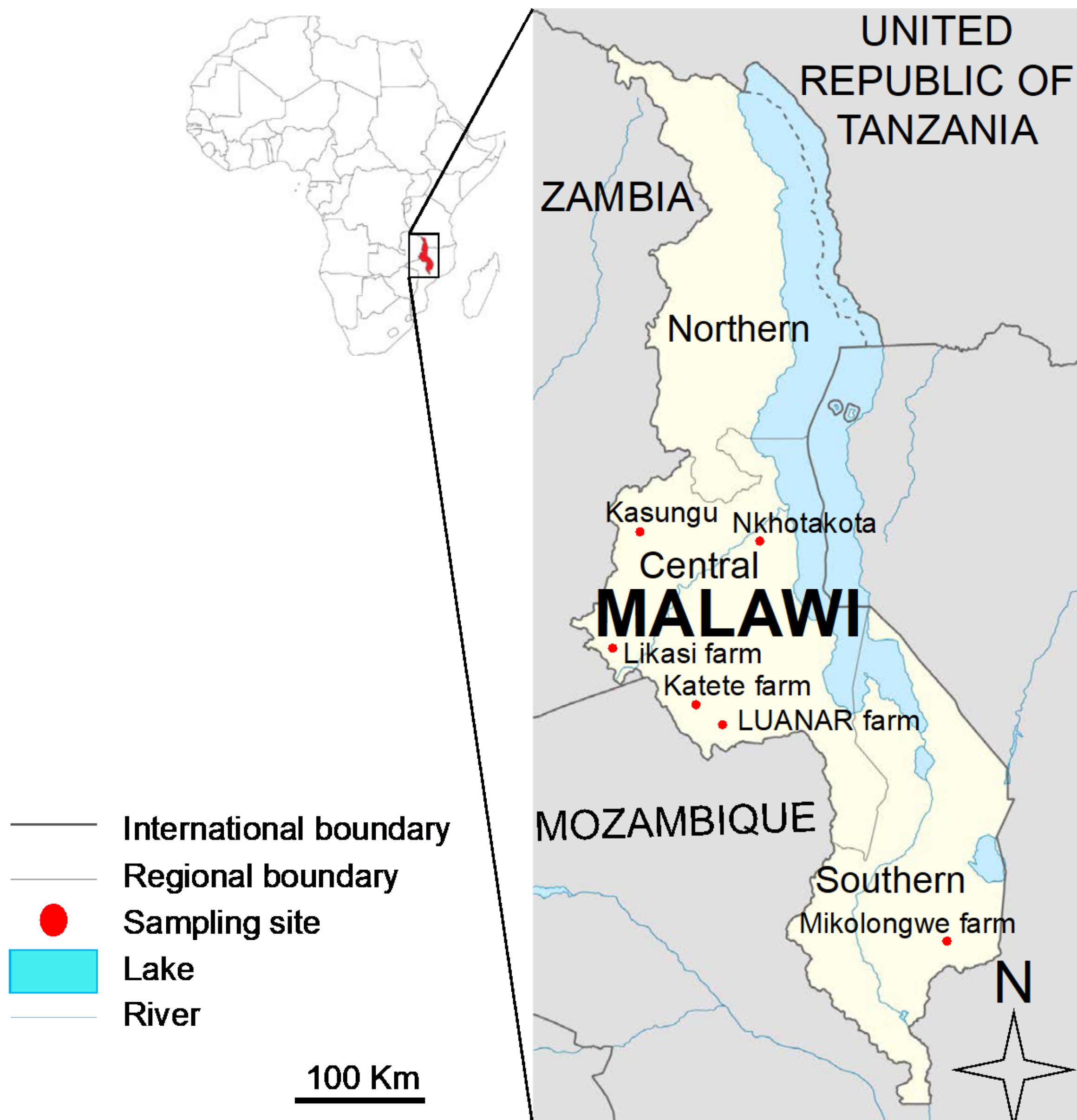

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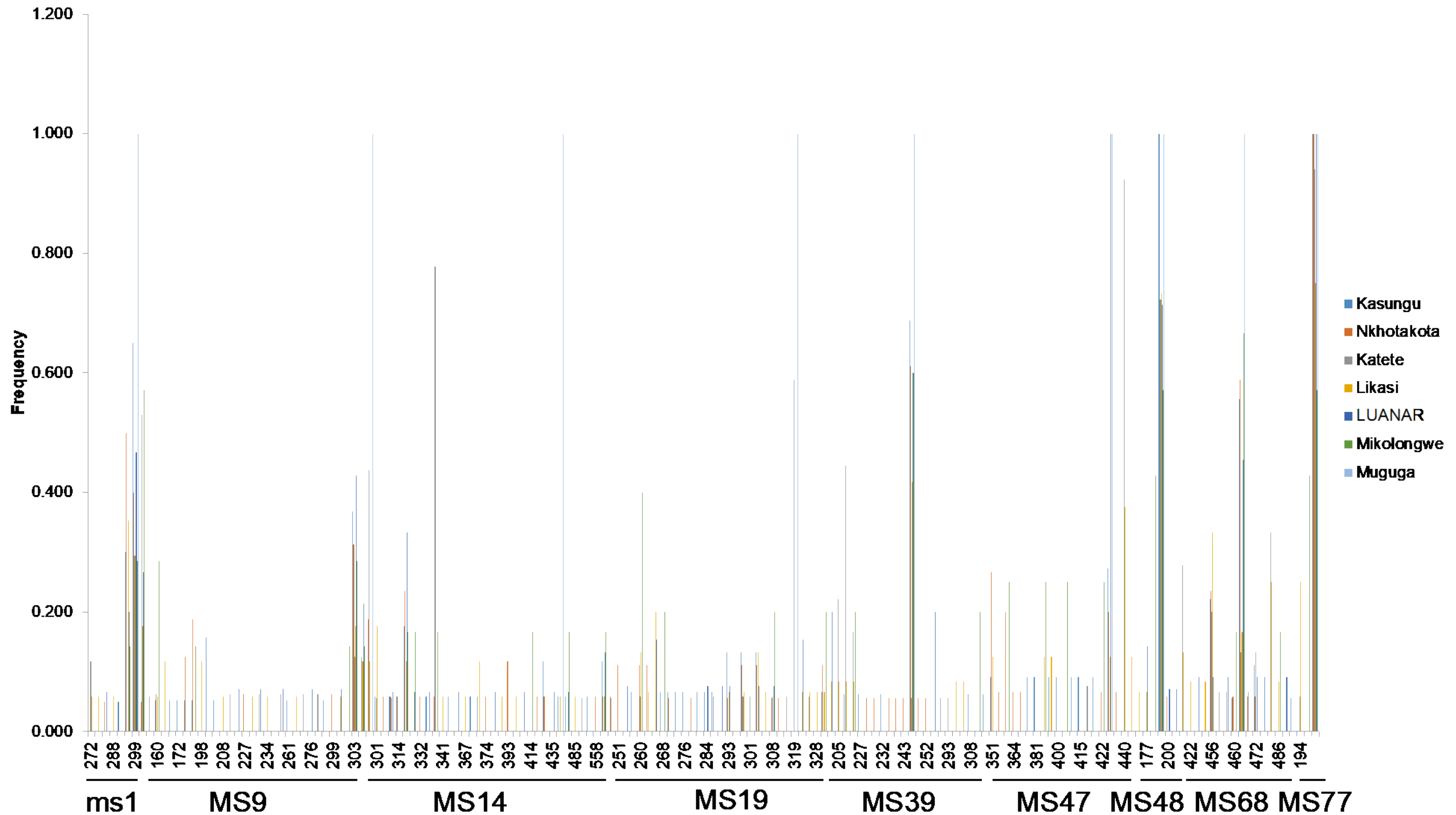
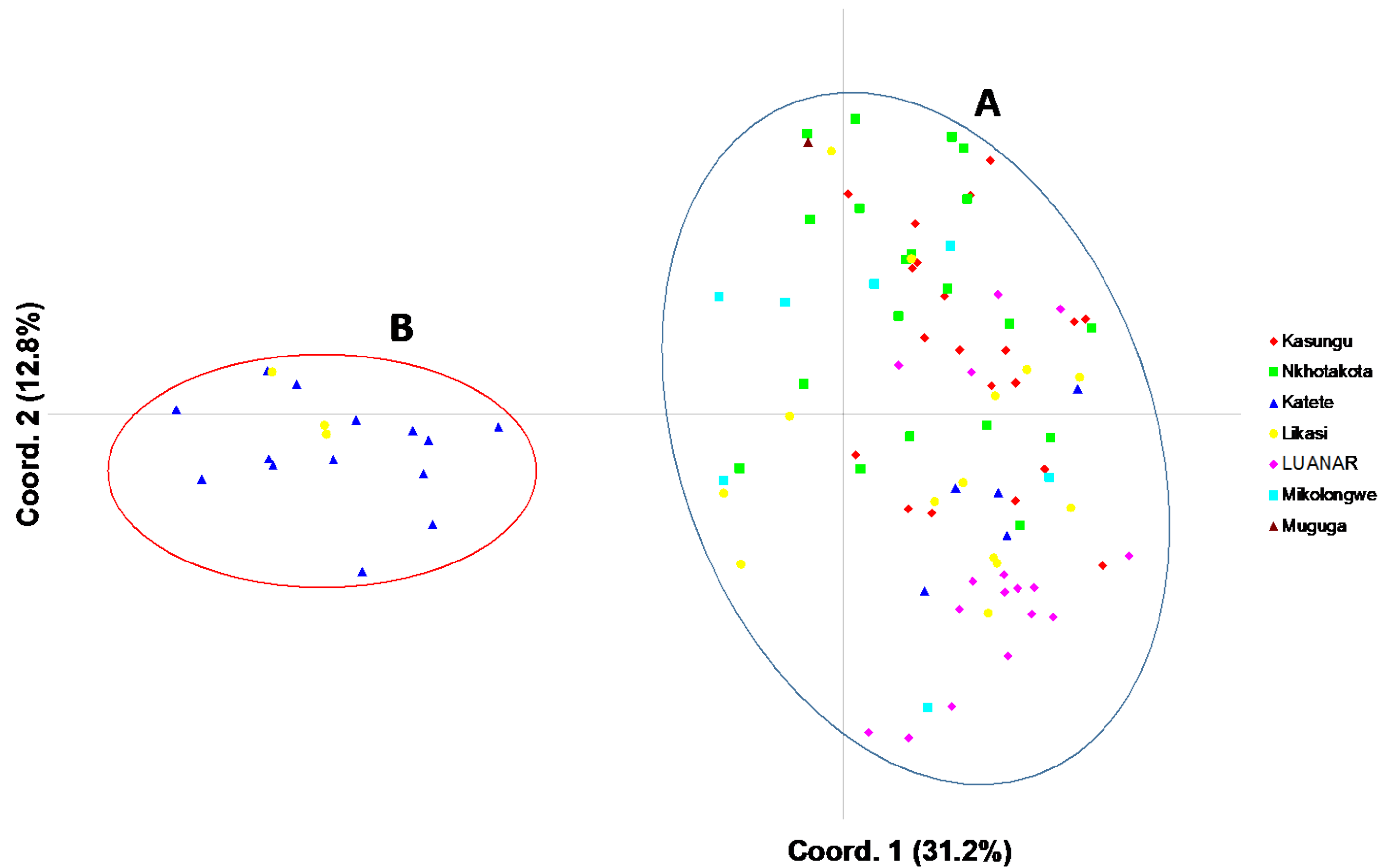
518 **Figure 1**. Map of Malawi showing the sample collection sites.



519

520 **Figure 2**. The allele frequencies of alleles in field populations of *Theileria parva* from Kasungu, Nkhotakota, Katete, Likasi, LUANAR and
521 Mikolongwe and the Muguga reference laboratory strain. Shared alleles were observed at all the loci investigated. Multi-locus genotype (MLG)
522 data was used to generate the histogram. The frequency of each predominant allele was calculated as a proportion of the total of each mark



523

524 **Figure 3**. Principal coordinate analysis (PCoA) of *Theileria parva* populations from Malawi and the Muguga reference laboratory strain. The

525 proportion of variation in the population dataset explained by each axis is shown in parentheses. The PCoA was performed using multi-locus

526 genotype data from Kasungu, Nkhotakota, Katete, Likasi, LUANAR and Mikolongwe and Muguga laboratory reference strain.