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Contrasting population genetics of cattle- and buffalo- derived *Theileria annulata* causing tropical theileriosis

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47 **Abstract**

48 The present study was designed to improve understanding of *Theileria annulata* in sympatric

49 water buffalo and cattle in the Punjab province of Pakistan. The prevalence of tropical theileriosis

50 is high, buparvaquone resistance is widespread, and vaccine protection is poor in the field. Better

51 understanding is, therefore, needed of the factors that influence the genetics of *T. annulata*

52 populations both within its hosts and in its overall populations. Here we utilise a panel of six

53 satellites and a mitochondrial cytochrome b marker to explore the multiplicity of *T. annulata*

54 infection and patterns of emergence and spread of different parasite genotypes. Parasite materials

55 were collected from infected animals in defined regions, where water buffalo and cattle are kept

56 together. Our results show that *T. annulata* is genetically more diverse in cattle- than in water

57 buffalo-derived populations (the mean numbers of unique satellite alleles were 13.3 and 1.8 and

58 numbers of unique cytochrome b locus alleles were 65 and 27 in cattle- and water buffalo- derived

59 populations, respectively). The data show a high level of genetic diversity among the individual

60 host-derived populations (the overall heterozygosity (H_e) indices were 0.912 and 0.931 in cattle,

61 and 0.874 and 0.861 in buffalo, based on satellite and cytochrome b loci, respectively). When

62 considered in the context of high parasite transmission rates and frequent animal movements

63 between different regions, the predominance of multiple *T. annulata* genotypes, with multiple

64 introductions of infection in the hosts from which the parasite populations were derived, may have

65 practical implications for the spread of parasite genetic adaptations; such as those conferring

66 vaccine cross-protection against different strains affecting cattle and buffalo, or resistance to

67 antiprotozoal drugs.

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69 Key Words: Tropical theileriosis*, T. annulata*, buparvaquone resistance, antiprotozoal drugs.

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1. Introduction

Vector-borne haemoprotozoa impact globally on the health, welfare, and production of livestock (Jabbar et al., 2015). The genus *Theileria* comprises of two important species, *Theileria annulata* and *Theileria parva* (Lawrence, 1979). *T. parva* typically causes East Coast Fever, Corridor Disease, or January Disease in southern and eastern Africa (Uilenberg et al., 1982), whereas, *T. annulata* causes tropical theileriosis in North Africa and South Asia (Nourollahi-Fard et al., 2015). Tropical theileriosis amongst the most important neglected tropical parasitic diseases of livestock (Sivakumar et al., 2014). Cattle and buffalo become infected with *T. annulata* following the transmission of sporozoites through ixodid ticks of the genus *Hyalomma*. These stages invade lymphocytes and develop by schizogony. Micromerozoites released from the lymphocytes enter erythrocytes and multiply by binary fission to produce merozoites. Piroplasms ingested during tick feeding, migrate to the arthropod gut where gametogeny and production of zygotes occurs. These enter the haemolymph and are carried to the salivary gland, where further replication by binary fission gives rise to sporozoites. The life cycle is completed when the next instar of the tick feeds (Gharbi and Darghouth, 2015). Clinically infected cattle and buffalo show high fever and lymphadenopathy, sometimes accompanied by haemolytic anaemia, respiratory and ocular lesions (Al-Hosary et al., 2010; Mahmmod et al., 2011).

Avoidance, removal or suppression of *Hyalomma* ticks or prophylactic use of antiprotozoal drugs are generally impractical in the control of tropical theileriosis. The unsustainability of these disease control measures highlights the potential for effective *T. annulata* vaccines. Attenuated vaccines against *T. annulata* have been used in many countries where tropical theileriosis is endemic (Brown, 1990; Darghouth et al., 1999; Tait and Hall, 1990). However, there are practical constraints to the widespread use of live *T. annulata* vaccines in control programmes: (i) a requirement for distribution in liquid nitrogen, which accounts for approximately 30% of the cost (Bouslikhane et al., 1998); (ii) the need to use the vaccine immediately after thawing; (iii) the difficulty and cost of quality control to ensure consistent efficacy and the absence of other pathogens; (iv) problems with post-vaccination reactions, which have been recorded in 3% of animals immunised with Chinese, Moroccan, Iranian or Tunisian stocks (Darghouth, 2008); and (v) the possibility of reversion to virulence. To overcome these problems, research for the development of a killed subunit vaccine against *T. annulata* has focused on antigens present on the surface of sporozoite and merozoite stages of the parasite. High levels of genetic diversity are a major hurdle for the development of subunit vaccines for use in different host species; for example,

114   where cross-protection between cattle- and buffalo-derived *T. parva* strains may be incomplete
115   (Young et al., 1973). Studies of *T. parva* in African buffalo have shown high genetic diversity
116   when compared to cattle (Oura et al., 2005); but while *T. annulata* can infect both cattle and Asian
117   buffalo (Nourollahi-Fard et al., 2015), genetic comparisons between strains infecting sympatric
118   host populations have not been reported. Understanding of allelic variations of buffalo- and cattle-
119   derived *T. annulata* and their circulation is needed to inform antigenicity and immunogenicity in
120   vaccine development.

121   Control of *T. annulata* is heavily dependent on the prophylactic and therapeutic use of a single
122   anti-protozoal drug, buparvaquone, but is now compromised by the emergence of resistance
123   (Mhadhbi et al., 2015). Buparvaquone resistance has been reported with increasing frequency and
124   now represents a serious challenge to efficient livestock production (Mhadhbi et al., 2010).
125   Current understanding of the mechanisms and genetic basis of buparvaquone resistance in *T.*
126   *annulata* is limited (Chatanga et al., 2019; Mhadhbi et al., 2015; Sharifiyazdi et al., 2012), with no
127   functional link between mutations in the candidate cytochrome b locus and resistance phenotype
128   having been demonstrated. Although previous studies have described multiple genotypes and high
129   levels of genetic diversity (Al-Hamidhi et al., 2015; Weir et al., 2011), there is a lack of
130   information on changes in response to drug selection pressure, fitness costs of mutations, and gene
131   flow. It is, therefore, important to understand the genetic diversity and population sub-structure of
132   *T. annulata*, with reference to the emergence and spread of buparvaquone resistance mutations.
133   There is a need for a better understanding of the transmission of different *T. annulata* genotypes
134   present in individual cattle and buffalo hosts as a proxy for multiplicity of infection when
135   considering sustainable control strategies.

136   Population genetic studies of *T. annulata* have previously been performed using panels of
137   satellite makers (Weir et al., 2007), but there are no reports based on mitochondrial cytochrome b
138   sequence analysis using a post-genomic next-generation sequencing approach. Deep amplicon
139   sequencing of metabarcoded mtDNA affords a practical and rhigh-throughput method when
140   compared to conventional Sanger sequencing, to investigate genetic diversity between and within
141   parasite populations. The Illumina MiSeq platform can provide 100,000 or more reads of up to
142   600 bp of loci of interest, depending on primer design. Recently, we have used these methods to
143   study the population genetics of *Calicophoron daubneyi* (Sargison et al., 2019) and *Fasciola*
144   *gigantica* infection in livestock keep in the United Kingdom and Pakistan.

145   In this paper, we describe the use of a mitochondrial cytochrome b locus and six polymorphic
146   satellite markers investigate the population genetics of buffalo- and cattle-derived *T. annulata*.
147   The results may help to: inform future rational vaccine design for the control of tropical
148   theileriosis; predict the likely emergence and spread of genetic adaptations such as buparvaquone
149   resistance; and identify patterns of infection with different genotypes.
150

151   **2. Materials and Methods**
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153   *2.1. Parasite resources and gDNA isolation*
154   Blood samples were collected from the piroplasm-positive cattle and buffalo in veterinary
155   clinics throughout the Punjab province of Pakistan between 2017 and 2019. The procedures
156   involved jugular venipuncture and withdrawal of 5 ml of intravenous blood into EDTA tubes,
157   followed by storage at -20°C. Samples were collected by para-veterinary staff under the
158   supervision of local veterinarians following consent from the animal owners. The study was
159   approved by the Institutional Review Board of the University of Veterinary and Animal Sciences
160   (UVAS-24817). Peripheral blood smears were prepared and stained with 4% Giemsa, then
161   visualised under 1000x oil immersion to detect piroplasm infection. 'Haemoprotobiome'
162   sequencing was performed on piroplasm-positive blood samples to confirm the presence of *T.*
163   *annulata* (Chaudhry et al., 2019). Genomic DNA was isolated from *T. annulata* positive samples
164   by lysis with GS buffer and proteinase K as described in the TIANamp Blood DNA Kit
165   (TIANGEN Biotech Co. Ltd, Beijing) and stored at -20°C.
166   Four cell-line stocks of *T. annulata* positive controls originally derived from Turkey, India,
167   Tunisia, and Morocco (Katzer et al., 1994) were available at the University of Edinburgh Roslin
168   Institute, UK.
169

170   *2.2. Adapter and barcoded PCR amplification of T. annulata cytochrome b*
171   A 517 bp region of the cytochrome b locus was selected for deep amplicon sequencing. One μl
172   gDNA of each of 31 buffalo, 54 cattle and the 4 positive control derived *T. annulata* samples was
173   used as templates for the 1st round adapter PCR amplification. A *de novo* primer set
174   (Supplementary Table S1) was used under the following conditions: 13.25 μl ddH$_2$O, 1 μl gDNA,
175   0.75 μl of 10 mM dNTPs mix, 5 μl of 5X HiFi Fidelity Buffer, 0.5 μl of 0.5U DNA polymerase
176   enzyme (KAPA Biosystems, USA), and 0.75 μl of 10 μM forward adaptor primers and reverse

177   adaptor primers. The thermocycling conditions were: 95°C for 2 min, 35 cycles at 98°C for 20 sec,

178   55°C for 15 sec, and 72°C for 2 min, followed by a final extension of 72°C for 2 min. The PCR

179   product was purified with AMPure XP Magnetic Beads (1X) (Beckman coulter Inc., USA). A

180   barcoded primer set was used in the 2nd round of PCR amplification to add a fragment of unique

181   sequence into each purified product (Supplementary Table S2) under the following conditions:

182   13.25 µl ddH$_2$O, 2 µl bead purified PCR products, 0.75 µl of 10 mM dNTPs mix, 5 µl of 5X HiFi

183   Fidelity Buffer, 0.75 µl of 0.5U DNA polymerase enzyme (KAPA Biosystems, USA), and 1.25 µl

184   of 10µM forward and reverse primers. The thermocycling conditions were: 98°C for 2 min, 7

185   cycles at 98°C for 20 sec, 63°C for 20 sec, and 72°C for 2 min. The PCR products were purified

186   with AMPure XP Magnetic Beads (1X) (Beckman coulter Inc., USA).

187

188   *2.3. Illumina Mi-Seq run and data handling*

189   A pooled library was prepared with 10 µl of barcoded bead purification PCR product from each

190   *T. annulata* sample and sent to Edinburgh Genomics, UK for deep amplicon sequencing. The size

191   of the amplicon was measured by qPCR library quantification (KAPA Biosystems, USA), before

192   running on an Illumina Mi-Seq sequencer using a 600-cycle pair-end reagent kit (Mi-Seq Reagent

193   Kits v2, MS-103-2003) at a concentration of 15 nM with addition of 15% Phix control v3

194   (Illumina, FC-11-2003). The Illumina Mi-Seq post-run processing uses the barcoded indices to

195   split all sequences by sample and generate FASTQ files. These were analysed using Mothur

196   v1.39.5 software (Schloss et al., 2009) with modifications in the standard operating procedures of

197   Illumina Mi-Seq (Kozich et al., 2013) in the Command Prompt pipeline. Briefly, the raw paired

198   read-ends were run into the 'make.contigs' command to combine the two sets of reads for each

199   sample. The command extracted sequence and quality score data from the FASTQ files, creating

200   the complement of the reverse and forward reads, and then joining the reads into contigs. After

201   removing the too long, or ambiguous sequence reads, the data were aligned with the *T. annulata*

202   cytochrome b reference sequence library prepared from the positive controls (for more details

203   Supplementary Data S1 and section 2.1) using the 'align.seqs' command. Any sequences that did

204   not match with the *T. annulata* cytochrome b reference library were removed and the

205   'summary.seqs' command was used to summarise the 517 bp sequence reads of the *T. annulata*

206   cytochrome b locus. The sequence reads were further run on the 'screen.seqs' command to

207   generate the *T. annulata* cytochrome b FASTQ file. Once the sequence reads were classified as *T.*

208   *annulata*, a count list of the consensus sequences of each population was created using the

209   'unique.seqs' command. The count list was further used to create FASTQ files of the consensus
210   sequences of each population using the 'split.groups' command (for more details Supplementary
211   Data S2).

212

213   *2.4. Bioinformatics data analysis*

214   The consensus sequences of *T. annulata* cytochrome b locus were aligned using the MUSCLE
215   alignment tool in Geneious v10.2.5 software (Biomatters Ltd, New Zealand) and then imported
216   into the FaBox 1.5 online tool (birc.au.dk) to collapse the sequences that showed 100% base pair
217   similarity after corrections into a single genotype. The genotype frequency of each sample was
218   calculated by dividing the number of sequence reads by the total number of reads. A split tree was
219   created in the SplitTrees4 software (bio-soft.net) by using the neighbour-joining method in the
220   JukesCantor model of substitution. The appropriate model of nucleotide substitutions for
221   neighbour-joining analysis was selected by using the jModeltest 12.2.0 program (Posada, 2008).
222   The tree was rooted with the corresponding cytochrome b sequence of *T. parva*. The branch
223   supports were obtained by 1000 bootstraps of the data. The genetic diversity of cytochrome b was
224   calculated within and between populations by using the DnaSP 5.10 software package (Librado
225   and Rozas, 2009), and the following values were obtained: Heterozygosity (H_e), the number of
226   segregating sites (S), nucleotide diversity (π), the mean number of pairwise differences (k), the
227   mutation parameter based on an infinite site equilibrium model, and the mutations parameter
228   based on segregating sites (S_θ).

229

230   *2.5. Satellite genotyping and bioinformatics data analysis of T. annulata*

231   Six satellite markers (TS6, TS8, TS20, TS31, TS12, TS16) were selected for use on each
232   population as previously described by Weir et al. (2007). A summary of primer sequences and
233   allele ranges is given in Supplementary Table S3. Eighteen buffalo and 35 cattle derived *T.*
234   *annulata* populations were successfully analysed from six markers. PCR amplification was
235   performed using a master mix containing 17.2 µl ddH$_2$O, 0.3 µl of 100 µM dNTPs, 2.5 µl of 1X
236   thermopol reaction buffer, 0.3 µl of 1.25U Taq DNA polymerase (New England Biolabs) and 0.3
237   µl of 0.1µM forward and reverse primers. The thermo-cycling parameters were 94°C for 2 min
238   followed by 35 cycles of 94°C for 1 min, staying at the annealing temperatures of 60°C (TS6,
239   TS20), 55°C (TS8, TS12, TS16), or 50°C (TS31) for 1 min and 65°C for 1 min, with a single final
240   extension cycle of 65°C for 5 min. The forward primer of each microsatellite primer pair was 5'

241 end labelled with fluorescent dye (IDT, UK), and the ROX 400 internal size standard was used on

242 the ABI Prism 3100 genetic analyser (Applied Biosystems, USA).

243 Individual chromatograms were analysed using Peak Scanner software version 2.0 (Thermo

244 Fisher Scientific, USA) to determine the size of the alleles. These were combined across six

245 markers to generate a multilocus genotype (MLG). From the MLG data, allelic variation (A) and

246 heterozygosity (H_e) for individual populations were calculated using Arlequin 3.11 software (Guo

247 and Thompson, 1992). Significance levels were calculated using the sequential method of

248 Bonferroni correction for multiple comparisons in the same dataset (Rice, 1989). Analysis of

249 molecular variance (AMOVA) was estimated through the partition of satellite diversity between

250 and within populations (Excoffier et al., 1992) and fixation index (pairwise F_{ST}) values were

251 calculated using Arlequin 3.11 to provide a measurement of population genetic sub-structure.

252 Principal coordinate analysis (PCoA) was performed using GenALEx software to illustrate the

253 extent of genetically distinct features of individual populations with plot coordinates (Peakall and

254 Smouse, 2012).

255 The likelihood ratio test statistics (G-test) were calculated using Arlequin 3.11 software

256 (Excoffier et al., 2005) to estimate genetic linkage equilibrium and deviations from Hardy-

257 Weinberg equilibrium. There was no evidence to support linkage disequilibrium for any

258 combination of satellite loci in the individual *T. annulata* populations of buffalo and cattle,

259 indicating that alleles at these loci were randomly associating and not genetically linked (data on

260 file). There was some significant departure from Hardy-Weinberg equilibrium, even after

261 Bonferroni correction, in addition to relative P values for 108 loci combinations for buffalo and

262 210 loci combinations for cattle *T. annulata* populations. The bottleneck software (version 1.2.02)

263 was, therefore, used to search for the evidence of heterozygosity excess and mode-shift. The

264 Wilcoson signed-rank test was used to evaluate the statistical significance of any possible genetic

265 drift equilibrium (Lefterova et al., 2015). The data showed that there was no heterozygosity excess

266 according to the Sign Test and Wilcoxon Test (data on file). The mode shift analysis established

267 most populations had a normal L-shaped distribution.

268

269 **3. Results**

270

271 *3.1. Allelic diversity between cattle- and buffalo- derived T. annulata populations*

272   The allelic diversity data show that *T. annulata* is more diverse in cattle as compared to water

273   buffalo. The mean numbers (±SD) of satellite alleles in the cattle- and buffalo- derived

274   populations were 34.7 (±9.1) and 23.7 (±5.2), respectively. The number of alleles (A_n) for each

275   satellite marker in the cattle- and buffalo-derived populations ranged from 22 to 46 and 17 to 32,

276   respectively. The mean numbers (±SD) of cattle- and buffalo-specific *T. annulata* satellite alleles

277   (A_u) per marker were 13.3 (±6.3) and 1.8 (±2.04), respectively (Table 1 and Fig. 1). Seventy-nine

278   and 41 cytochrome b locus alleles (A_n) were identified in the cattle- and buffalo-derived *T.*

279   *annulata* populations. The numbers of cattle- and buffalo-specific *T. annulata* alleles (A_u) were 65

280   and 27, respectively (Table 1 and Fig. 1).

281

282   *3.2. Genetic diversity within T. annulata populations derived from cattle and buffalo*

283   Overall, the mean heterozygosities (H_e) (±SD) of the cattle- and buffalo-derived *T. annulata*

284   populations were 0.912 (±0.056) and 0.874 (±0.028) respectively (Table 2). The heterozygosity

285   (H_e) data reveal high levels of genetic diversity within each population of *T. annulata*; with mean

286   values (±SD) for the six satellite markers ranging from 0.8 (±0.082) to 0.959 (±0.017) in

287   individual cattle- and 0.738 (±0.196) to 0.956 (±0.034) in buffalo-derived populations (Table 2).

288   High levels of heterozygosity (H_e) were seen at the *T. annulata* cytochrome b locus, ranging

289   from 0.556 to 0.923 (overall mean value 0.931) in cattle-derived populations and from 0.545 to

290   0.900 (overall mean value 0.861) in buffalo-derived populations (Table 3).

291

292   *3.3. Genetic sub-structure between T. annulata populations derived from cattle and buffalo*

293   Genetic differentiation was shown by comparing the fixation indices (F_{ST}) of two populations

294   with each other in a pairwise manner. The F_{ST} values indicated a low level of genetic

295   differentiation between *T. annulata* populations ranging from 0.001 to 0.114 in cattle and 0.003 to

296   0.132 buffalo, respectively (Supplementary Table S4).

297   An AMOVA was conducted using the panel of six satellite markers to estimate the genetic

298   variation within and between the populations. This showed that genetic variation was distributed

299   97.91% and 97% and within; and 2.09% and 3% between cattle- and buffalo-derived *T. annulata*

300   populations, respectively (data on file). PCoA was performed to illustrate as a two-dimensional

301   plot the extent to which populations are genetically distinct. The two axes accounted for 72.5%

302   (25.67 + 46.83) and 73.93% (27.6 + 46.33) of the variation in the cattle- and buffalo-derived

303 populations, respectively; and showed that populations from different regions formed overlapping

304 clusters, hence were not geographically sub-structured (Fig. 2).

305

306 *3.4. Genotype distribution of T. annulata populations derived from cattle and buffalo*

307 *T. annulata* satellite data were found to be highly polymorphic in each population, with overall

308 numbers of genotypes per population ranging from 2 to 26 in cattle and 2 to 17 in buffalo. The

309 mean numbers (±SD) of *T. annulata* genotypes in cattle- and buffalo-derived populations were

310 8.60 (±2.52) and 6.25, respectively (Table 4).

311 Seventy-nine *T. annulata* cytochrome b genotypes from individual cattle-derived populations

312 were analysed separately. Fifteen populations had a single genotype at high frequencies ranging

313 from 90 to 100%. These comprised of ten populations containing only one genotype; two

314 populations containing two genotypes; one population with three genotypes; one population with

315 four genotypes; and one population with seven genotypes (Fig. 3A). Thirty-nine populations had

316 multiple genotypes at high frequencies. These comprised of five populations containing two

317 genotypes; eight populations containing three genotypes; ten population containing four genotypes;

318 seven populations with five genotypes; four populations with six genotypes; one population with

319 seven genotypes; two populations with eight genotypes; one populations with 12 genotypes; and

320 one with 14 genotypes (Fig. 3A). Phylogenetic analysis of the 79 cytochrome b genotypes of the

321 cattle-derived *T. annulata* populations, showed that two genotypes for 22.9% and 10.8% of the

322 total number of the sequence reads, being present in 36 and 8, populations, respectively. Seven

323 genotypes accounted for between 5.0% and 7.0% of the sequence reads and 45 genotypes

324 accounted for less than 1.0% of the sequence reads (Fig. 4A). The split tree shows that eight

325 genotypes are shared between populations derived from cattle holdings in multiple locations of

326 Lahore, Chakwal, Gujranwala, Okara, and Sahiwal. Seven genotypes are shared between

327 populations derived from cattle holdings in any two locations of Lahore, Gujranwala, Okara, and

328 Sahiwal. Sixty-one genotypes are present in a single location (Gujranwala=24; Qadirabad=12;

329 Okara =14; Lahore=11 genotypes) (Fig. 4A).

330 Forty-one *T. annulata* cytochrome b genotypes from individual buffalo-derived populations

331 were analysed separately. A single genotype predominated in 15 populations at a frequency of

332 between 90 and 100%. These comprised of eight populations containing only one genotype; three

333 populations containing two genotypes; one population with three genotypes; one population with

334 five genotypes; one population with six genotypes; and one population with seven genotypes (Fig.

335   3B). Sixteen populations had high frequencies of multiple genotypes. These comprised of seven

336   populations contained 2 genotypes; two populations contained 3 genotypes; one population

337   contained 4 genotypes; three populations had 5 genotypes; two populations had 7 genotypes and

338   one population contained a maximum of 8 genotypes (Fig. 3B). Phylogenetic analysis of the 41

339   cytochrome b genotypes of the buffalo-derived *T. annulata* populations, showed that three

340   genotypes accounted for 39.1%, 10.8%, and 5.5% of sequence reads being present in 26, 15 and 3

341   populations, respectively. Eighteen genotypes accounted for between 1.0% and 3.0% of sequence

342   reads and 20 genotypes accounted for less than 1.0% of sequence reads (Fig. 4B). The split tree

343   shows that two genotypes are shared between *T. annulata* populations derived from buffalo

344   holdings in multiple locations of Gujranwala, Hafizabad, Okara, and Sahiwal. Five genotypes are

345   present in the populations derived from buffalo holdings in Gujranwala and Okara. One genotype

346   is present in the populations derived from buffalo holdings in Hafizabad and Okara and one

347   genotype from Okara and Sahiwal. Thirty-two genotypes are present in a single location (16

348   genotypes in Gujranwala; 13 genotypes in Okara; and 3 in Hafizabad) (Fig. 4B).

349

350   **4. Discussion**

351   *Theleria annulata* is considered to be the most economically important protozoan parasite of

352   water buffalo and cattle in Asia and North Africa, causing high mortality and morbidity. The

353   annual economic impact of the pathogen is estimated to be billions of USD, with a significant

354   impact on both meat and milk production (Jabbar et al., 2015). Our results reveal a high level of

355   genetic diversity within *T. annulata* infecting individual hosts, and between different host-derived

356   populations; being greater in cattle- than in water buffalo-derived populations. *Theileria annulata*

357   genotype distribution in the Punjab province of Pakistan is consistent with high parasite

358   transmission rates and frequent animal movements between different regions. These results may

359   have practical implications for the spread of parasite genetic adaptations.

360

361   *4.1. Potential impact of T. annulata allelic diversity on immunisation cross-protection*

362   The satellite and cytochrome b data reported in the present study show that *T. annulata* is

363   genetically more diverse with more circulation of alleles in cattle as compared to water buffalo.

364   The satellite data were informative due to the numbers of alleles present, showing more unique

365   alleles in cattle-derived than in Asian water buffalo-derived *T. annulata* populations. The

366   cytochrome b locus provided further evidence of allelic diversity and differences between cattle-

367 and buffalo- populations. Similarly high levels of parasite heterogeneity have been reported in *T. parva*, albeit with higher allelic diversity in African buffalo- than in cattle-derived parasite populations (Oura et al., 2011). Such allelic variation may reflect the capacity of parasite to undergo transmission. For example, the differences between *T. annulata* and *T. parva* could have arisen if the buffalo-derived *T. annulata* transmission is more restricted than transmission between cattle-derived populations in the Punjab province of Pakistan; while *T. parva* transmission may be more efficient between infected buffalo than cattle in endemic east coast fever regions of Africa (Oura et al., 2011).

375 The high level of allelic variation described in *T. annulata* might have practical implications for the development of a sporozoite and merozoite antigen subunit vaccine. Immunisation of cattle with different subunit versions of the sporozoite stage surface protein (SPAG1, TaSP) has provided a degree of protection against cattle-derived *T. annulata*, probably through a reduction in the level of parasitaemia (Schnittger et al., 2002). However, little is known about the antigenic diversity of *T. annulata* candidate loci, which may be important in vaccine development (MacHugh et al., 2011), or about the impact of differences between cattle- and buffalo-derived populations. Our results highlight the need for better understanding of antigenic diversity in the development of a subunit vaccine for tropical theileriosis, capable of providing cross-protection against cattle- and buffalo-derived *T. annulata* genotypes across different regions.

4.2. Potential impact of T. annulata genetic diversity on the emergence of buparvaquone resistance

388 Our satellite and cytochrome b locus data show high levels of genetic diversity in both cattle- and buffalo-derived *T. annulata* populations, reflecting high effective population sizes (Al-Hamidhi et al., 2015) and implying high mutation rates across the *T. annulata* genome. The 8.35 Mb nuclear genome sequence of *T. annulata* spans four chromosomes that range from 1.9 to 2.6 Mb, with 3,792 putative protein-coding genes. In addition, a total of 49 transfer RNA and five ribosomal RNA genes were identified (Pain et al. (2005). The high level of genetic diversity in *T. annulata* may practical implications for the emergence of antiprotozoal drug resistance mutations. Only one drug, buparvaquone, is available for the treatment of tropical theileriosis: Pakistani livestock are treated frequently with buparvaquone and anecdotal evidence suggests that resistance is widespread.

399   *4.3. Potential impact of the multiplicity of T. annulata infection on control strategies for tropical*
400   *theileriosis*

401   The presence of a single cytochrome b genotype in 15 cattle and 9 buffalo- derived populations
402   suggests a single emergence and subsequent spread of *T. annulata* infection. The satellite data
403   provide further evidence of low levels of genetic differentiation among cattle-and buffalo-derived
404   populations and show overlapping clusters that are not geographically sub-structured, consistent
405   with the high levels of gene flow due to livestock movements or translocations of ticks to a new
406   region. This could influence the spread of drug-resistant alleles. Studies of the global genetic sub-
407   structure of *T. annulata* have shown a high level of genetic differentiation between Turkey and
408   Tunisia (Weir et al., 2007). Studies have also shown a low level of genetic differentiation in *T.*
409   *annulata* within-countries including Turkey, Tunisia, Oman, China and Portugal (Al-Hamidhi et
410   al., 2015; Gomes et al., 2016; Weir et al., 2011; Yin et al., 2018), reflecting high levels of animal
411   movement, or translocation of ticks.

412   The predominance and the high proportions of multiple cytochrome b genotypes in 39 cattle-
413   and 16 buffalo-derived populations implies multiple emergence of infection. The numbers of
414   satellite genotypes per population provide further evidence for this pattern of emergence of
415   different genetically adapted strains. Random cross mating of gametes and genetic recombination
416   of *T. annulata* in ticks gives rise to the formation of new genotypes (Al-Hamidhi et al., 2015). The
417   multiplicity of infection of *T. annulata* may be influenced by variations in the intensity of
418   transmission due to levels of different tick species infestation, or prevalence of tick infection (Yin
419   et al., 2018). *Hyalomma scupense* is the most common and economically important species and
420   the major vector of *T. annulata*, but *Hyalomma marginatum* and *Hyalomma anatolicum* are also
421   found in Tunisia (Bouattour et al., 1996). There are four *Hyalomma* species responsible for the
422   transmission of *T. annulata* in Turkey, of which *H. anatolicum* is the major vector for *T. annulata*
423   (Aktas et al., 2004). Two tick species, *Hyalomma lusitanicum*, and *H. marginatum* are the vectors
424   of *T. annulata* in Portugal (Estrada-Pena and Santos-Silva, 2005). There are a few reports that *H.*
425   *anatolicum* may be a vector of *T. annulata* in the Punjab province of Pakistan (Karim et al., 2017).
426   The multiplicity of *T. annulata* infection may also be influenced by population bottlenecking
427   effects, arising from seasonal effects of climatic conditions on tick transmission and completion of
428   the parasite's lifecycle. The *T. annulata* transmission season in Turkey is between May and
429   September, with the peak of clinical cases occurring in mid-summer (Sayin et al., 2003). In a
430   region of endemic stability of tropical theileriosis in Tunisia, there is a high level of multiplicity

431   of *T. annulata* infection, but clinical disease is rare; while in a region of endemic instability, a
432   proportion of the population becomes infected all year round, and the clinical disease occurs
433   particularly in adult cattle (Gharbi et al., 2011).

434   Control strategies for tropical theileriosis need to consider factors such as: the reproductive
435   isolation of parasite populations; management of host movements; control of tick vectors;
436   mitigation of the impacts of climate change; and consequences of parasite exposure to
437   antiprotozoal drugs. Understanding of the multiplicity of infection and of high levels of gene flow
438   is, therefore, important in the educational dissemination and implementation of advice on
439   sustainable parasite control.

440
441
442   **Acknowledgement**

449

450   **Conflict of interest**

451   None
452

453   **References**
454
455   Aktas, M., Dumanli, N., Angin, M., 2004. Cattle infestation by Hyalomma ticks and prevalence of
456   Theileria in Hyalomma species in the east of Turkey. Vet Parasitol 119, 1-8.
457   Al-Hamidhi, S., M, H.T., Weir, W., Al-Fahdi, A., Johnson, E.H., Bobade, P., Alqamashoui, B.,
458   Beja-Pereira, A., Thompson, J., Kinnaird, J., Shiels, B., Tait, A., Babiker, H., 2015.
459   Genetic Diversity and Population Structure of Theileria annulata in Oman. PLoS One 10,
460   e0139581.
461   Al-Hosary, A., Abdel-Rady, A., Ahmed, L.S., Mohamed, A., 2010. Comparison between Using of
462   BUPAQUONE ® and Other Compounds in Treatment of Bovine Theileriosis.
463   International Journal for Agro Veterinary and Medical Sciences 4, 3-7.
464   Bouattour, A., Darghouth, M.A., Ben Miled, L., 1996. Cattle infestation by Hyalomma ticks and
465   prevalence of Theileria in H. detritum species in Tunisia. Vet Parasitol 65, 233-245.
466   Bouslikhane, M., M., K., H., O. 1998. La theilériose bovine au Maroc, investigations
467   épidémiologiques et étude de l'impact sur la productivité des élevages. In: Résumé de
468   communication orale, 15 ème congrès vétérinaire maghrébin.

469 Brown, C.G., 1990. Control of tropical theileriosis (Theileria annulata infection) of cattle.
470   Parassitologia 32, 23-31.
471 Chatanga, E., Mosssad, E., Abdo Abubaker, H., Amin Alnour, S., Katakura, K., Nakao, R., Salim,
472   B., 2019. Evidence of multiple point mutations in Theileria annulata cytochrome b gene
473   incriminated in buparvaquone treatment failure. Acta Trop 191, 128-132.
474 Chaudhry, U., Ali, Q., Rashid, I., Shabbir, M.Z., Abbas, M., Numan, M., Evans, M., Ashraf, K.,
475   Morrison, I., Morrison, L., Sargison, N.D., 2019. Development of a deep amplicon
476   sequencing method to determine the proportional species composition of piroplasm
477   haemoprotozoa as an aid in their control. bioRxiv.
478 Darghouth, M.A., 2008. Review on the experience with live attenuated vaccines against tropical
479   theileriosis in Tunisia: considerations for the present and implications for the future.
480   Vaccine 26 Suppl 6, G4-G10.
481 Darghouth, M.A., Bouattour, A., Kilan, M., 1999. Tropical theileriosis in Tunisia: epidemiology
482   and control. Parassitologia 41 Suppl 1, 33-36.
483 Estrada-Pena, A., Santos-Silva, M.M., 2005. The distribution of ticks (Acari: Ixodidae) of
484   domestic livestock in Portugal. Experimental & applied acarology 36, 233-246.
485 Excoffier, L., Laval, G., Schneider, S., 2005. Arlequin (version 3.0): an integrated software
486   package for population genetics data analysis. Evolutionary bioinformatics online 1, 47-50.
487 Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from
488   metric distances among DNA haplotypes: application to human mitochondrial DNA
489   restriction data. Genetics 131, 479-491.
490 Gharbi, M., Darghouth, M.A., 2015. Control of tropical theileriosis (Theileria annulata infection in
491   cattle) in North Africa. Asian Pacific Journal of Tropical Disease 5, 505-510.
492 Gharbi, M., Touay, A., Khayeche, M., Laarif, J., Jedidi, M., Sassi, L., Darghouth, M.A., 2011.
493   Ranking control options for tropical theileriosis in at-risk dairy cattle in Tunisia, using
494   benefit-cost analysis. Revue scientifique et technique (International Office of Epizootics)
495   30, 763-778.
496 Gomes, J., Salgueiro, P., Inacio, J., Amaro, A., Pinto, J., Tait, A., Shiels, B., Pereira da Fonseca, I.,
497   Santos-Gomes, G., Weir, W., 2016. Population diversity of Theileria annulata in Portugal.
498   Infect Genet Evol 42, 14-19.
499 Guo, S.W., Thompson, E.A., 1992. Performing the exact test of Hardy-Weinberg proportion for
500   multiple alleles. Biometrics 48, 361-372.
501 Jabbar, A., Abbas, T., Sandhu, Z.U., Saddiqi, H.A., Qamar, M.F., Gasser, R.B., 2015. Tick-borne
502   diseases of bovines in Pakistan: major scope for future research and improved control.
503   Parasites & vectors 8, 283.
504 Karim, S., Budachetri, K., Mukherjee, N., Williams, J., Kausar, A., Hassan, M.J., Adamson, S.,
505   Dowd, S.E., Apanskevich, D., Arijo, A., Sindhu, Z.U., Kakar, M.A., Khan, R.M.D., Ullah,
506   S., Sajid, M.S., Ali, A., Iqbal, Z., 2017. A study of ticks and tick-borne livestock pathogens
507   in Pakistan. PLoS neglected tropical diseases 11, e0005681.
508 Katzer, F., Carrington, M., Knight, P., Williamson, S., Tait, A., Morrison, I.W., Hall, R., 1994.
509   Polymorphism of SPAG-1, a candidate antigen for inclusion in a sub-unit vaccine against
510   Theileria annulata. Mol Biochem Parasitol 67, 1-10.
511 Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of
512   a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence
513   data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. 79, 5112-5120.
514 Lawrence, J.A., 1979. The differential diagnosis of the bovine theilerias of Southern Africa.
515   Journal of the South African Veterinary Association 50, 311-313.

516 Lefterova, M.I., Budvytiene, I., Sandlund, J., Farnert, A., Banaei, N., 2015. Simple Real-Time
517   PCR and Amplicon Sequencing Method for Identification of Plasmodium Species in
518   Human Whole Blood. J Clin Microbiol 53, 2251-2257.
519 Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA
520   polymorphism data. Bioinformatics 25, 1451-1452.
521 MacHugh, N.D., Weir, W., Burrells, A., Lizundia, R., Graham, S.P., Taracha, E.L., Shiels, B.R.,
522   Langsley, G., Morrison, W.I., 2011. Extensive polymorphism and evidence of immune
523   selection in a highly dominant antigen recognized by bovine CD8 T cells specific for
524   Theileria annulata. Infection and immunity 79, 2059-2069.
525 Mahmmod, Y.S., Elbalkemy, F.A., Klaas, I.C., Elmekkawy, M.F., Monazie, A.M., 2011. Clinical
526   and haematological study on water buffaloes (Bubalus bubalis) and crossbred cattle
527   naturally infected with Theileria annulata in Sharkia province, Egypt. Ticks Tick Borne
528   Dis 2, 168-171.
529 Mhadhbi, M., Chaouch, M., Ajroud, K., Darghouth, M.A., BenAbderrazak, S., 2015. Sequence
530   Polymorphism of Cytochrome b Gene in Theileria annulata Tunisian Isolates and Its
531   Association with Buparvaquone Treatment Failure. PloS one 10, e0129678.
532 Mhadhbi, M., Naouach, A., Boumiza, A., Chaabani, M.F., BenAbderazak, S., Darghouth, M.A.,
533   2010. In vivo evidence for the resistance of Theileria annulata to buparvaquone. Vet
534   Parasitol 169, 241-247.
535 Nourollahi-Fard, S.R., Khalili, M., Ghalekhani, N., 2015. Detection of Theileria annulata in blood
536   samples of native cattle by PCR and smear method in Southeast of Iran. Journal of
537   parasitic diseases : official organ of the Indian Society for Parasitology 39, 249-252.
538 Oura, C.A., Asiimwe, B.B., Weir, W., Lubega, G.W., Tait, A., 2005. Population genetic analysis
539   and sub-structuring of Theileria parva in Uganda. Mol Biochem Parasitol 140, 229-239.
540 Oura, C.A., Tait, A., Asiimwe, B., Lubega, G.W., Weir, W., 2011. Haemoparasite prevalence and
541   Theileria parva strain diversity in Cape buffalo (Syncerus caffer) in Uganda. Vet Parasitol
542   175, 212-219.
543 Pain, A., Renauld, H., Berriman, M., Murphy, L., Yeats, C.A., Weir, W., Kerhornou, A., Aslett,
544   M., Bishop, R., Bouchier, C., Cochet, M., Coulson, R.M., Cronin, A., de Villiers, E.P.,
545   Fraser, A., Fosker, N., Gardner, M., Goble, A., Griffiths-Jones, S., Harris, D.E., Katzer, F.,
546   Larke, N., Lord, A., Maser, P., McKellar, S., Mooney, P., Morton, F., Nene, V., O'Neil, S.,
547   Price, C., Quail, M.A., Rabbinowitsch, E., Rawlings, N.D., Rutter, S., Saunders, D., Seeger,
548   K., Shah, T., Squares, R., Squares, S., Tivey, A., Walker, A.R., Woodward, J., Dobbelaere,
549   D.A., Langsley, G., Rajandream, M.A., McKeever, D., Shiels, B., Tait, A., Barrell, B.,
550   Hall, N., 2005. Genome of the host-cell transforming parasite Theileria annulata compared
551   with T. parva. Science 309, 131-133.
552 Peakall, R., Smouse, P.E., 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic
553   software for teaching and research--an update. Bioinformatics 28, 2537-2539.
554 Posada, D., 2008. jModelTest: phylogenetic model averaging. Molecular biology and evolution 25,
555   1253-1256.
556 Rice, W., 1989. Analyzing tables of statistical tests. Evolution 43.
557 Sargison, N.D., Shahzad, K., Mazeri, S., Chaudhry, U., 2019. A high throughput deep amplicon
558   sequencing method to show the emergence and spread of Calicophoron daubneyi rumen
559   fluke infection in United Kingdom cattle herds. Veterinary parasitology.
560 Sayin, F., Dincer, S., Karaer, Z., Cakmak, A., Inci, A., Yukari, B.A., Eren, H., Vatansever, Z.,
561   Nalbantoglu, S., 2003. Studies on the epidemiology of tropical theileriosis (Theileria
562   annulata infection) in cattle in Central Anatolia, Turkey. Tropical animal health and
563   production 35, 521-539.

564 Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski,
565   R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., 2009. Introducing mothur: open-source,
566   platform-independent, community-supported software for describing and comparing
567   microbial communities. Appl. Environ. Microbiol. 75, 7537-7541.
568 Schnittger, L., Katzer, F., Biermann, R., Shayan, P., Boguslawski, K., McKellar, S., Beyer, D.,
569   Shiels, B.R., Ahmed, J.S., 2002. Characterization of a polymorphic Theileria annulata
570   surface protein (TaSP) closely related to PIM of Theileria parva: implications for use in
571   diagnostic tests and subunit vaccines. Molecular and biochemical parasitology 120, 247-
572   256.
573 Sharifiyazdi, H., Namazi, F., Oryan, A., Shahriari, R., Razavi, M., 2012. Point mutations in the
574   Theileria annulata cytochrome b gene is associated with buparvaquone treatment failure.
575   Vet Parasitol 187, 431-435.
576 Sivakumar, T., Hayashida, K., Sugimoto, C., Yokoyama, N., 2014. Evolution and genetic
577   diversity of Theileria. Infect Genet Evol 27, 250-263.
578 Tait, A., Hall, F.R., 1990. Theileria annulata: control measures, diagnosis and the potential use of
579   subunit vaccines. Rev Sci Tech 9, 387-403.
580 Uilenberg, G., Perie, N.M., Lawrence, J.A., de Vos, A.J., Paling, R.W., Spanjer, A.A., 1982.
581   Causal agents of bovine theileriosis in southern Africa. Tropical animal health and
582   production 14, 127-140.
583 Weir, W., Ben-Miled, L., Karagenc, T., Katzer, F., Darghouth, M., Shiels, B., Tait, A., 2007.
584   Genetic exchange and sub-structuring in Theileria annulata populations. Mol Biochem
585   Parasitol 154, 170-180.
586 Weir, W., Karagenc, T., Gharbi, M., Simuunza, M., Aypak, S., Aysul, N., Darghouth, M.A.,
587   Shiels, B., Tait, A., 2011. Population diversity and multiplicity of infection in Theileria
588   annulata. Int J Parasitol 41, 193-203.
589 Yin, F., Liu, Z., Liu, J., Liu, A., Salih, D.A., Li, Y., Liu, G., Luo, J., Guan, G., Yin, H., 2018.
590   Population Genetic Analysis of Theileria annulata from Six Geographical Regions in
591   China, Determined on the Basis of Micro- and Mini-satellite Markers. Frontiers in genetics
592   9, 50.
593 Young, A.S., Brown, C.G., Burridge, M.J., Cunningham, M.P., Kirimi, I.M., Irvin, A.D., 1973.
594   Observations on the cross-immunity between Theileria lawrencei (Serengeti) and Theileria
595   parva (Muguga) in cattle. Int J Parasitol 3, 723-728.
596

597 **Figure Legends**

598 **Fig. 1.** The mean number of alleles in *T. annulata* derived from buffalo and cattle based on a panel
599 of six satellite and and a cytochrome b marker. The bar of each marker shows the proportion of
600 alleles in buffalo and cattle. The Y-axis shows the number of alleles of each marker. The unique
601 and shared alleles of buffalo and cattle are represented by a different colour.
602
603 **Fig. 2.** Principal coordinate analysis using a panel of six satellite markers to represent 35 cattle-
604 and 18 buffalo-derived *T. annulata* populations.
605
606 **Fig. 3.** Relative genotype frequencies of the cytochrome b locus of 54 cattle-derived (2A) and 31
607 buffalo-derived (2B) *T. annulata* populations, collected from the Punjab province of Pakistan.
608 Each genotype (CHA and BHA) is represented by a different colour in the individual population
609 (CY). The population distribution and the frequency of sequence reads generated per population

610   for each of the 79 cattle-derived and 41 buffalo-derived *T. annulata* genotypes is shown in the
611   insert table.
612
613   **Fig. 4.** Split tree analysis of 79 cattle-derived (3A) and 41 buffalo-derived (3B) *T. annulata*
614   genotypes collected from the Lahore (A), Gujranwala (B), Chakwal (C), Qadirabad (D), Okara (E),
615   Sahiwal (F), and Hafizabad (G) regions of the Punjab province of Pakistan. Each population (CY)
616   is represented by a different colour in the individual genotype (CHA and BHA). The pie chart
617   circles represent the distribution and percentage of sequence reads generated per genotype
618   identified in 54 cattle- and 31 buffalo-derived populations, as indicated in the insert table.
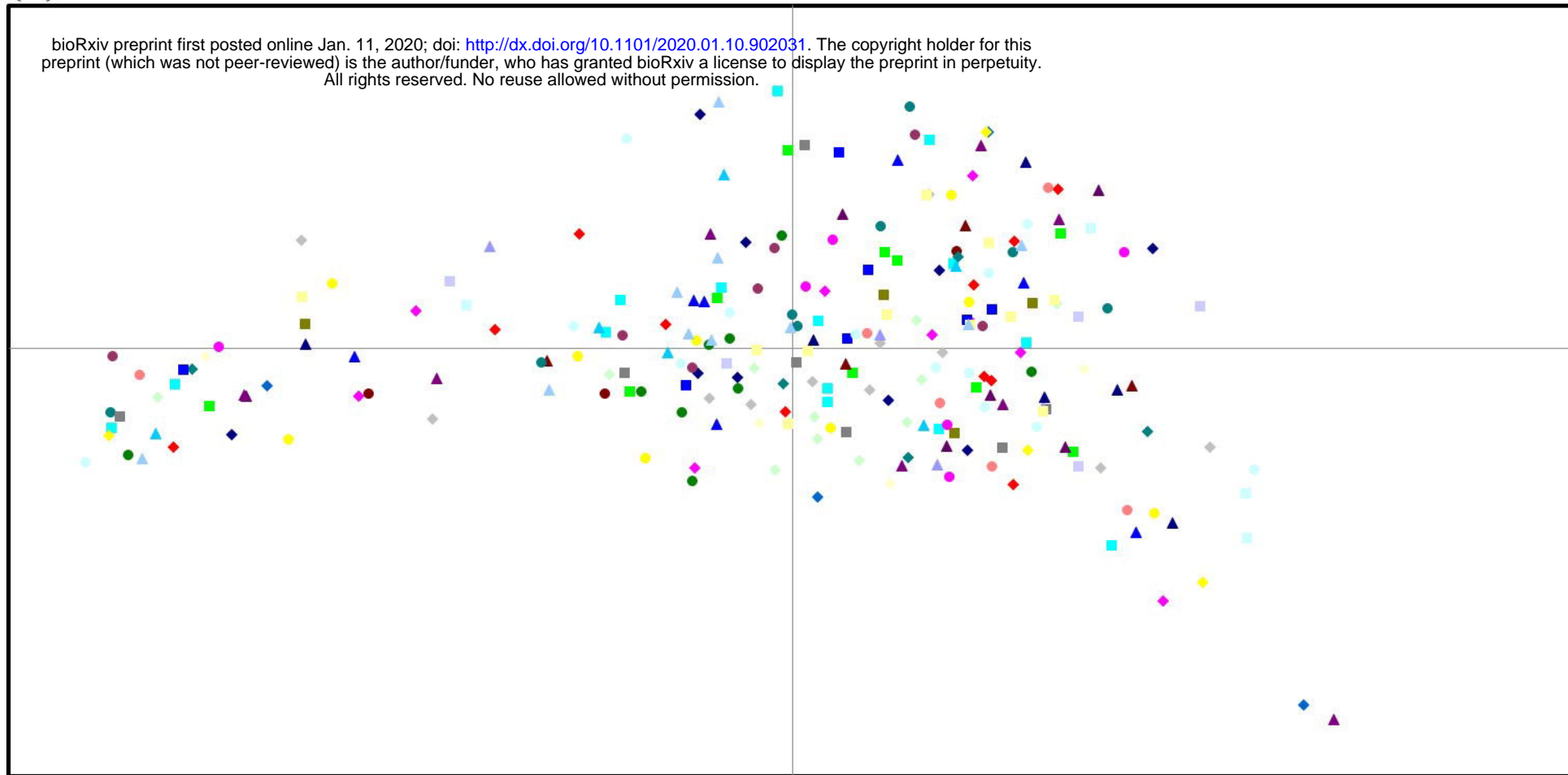619
620
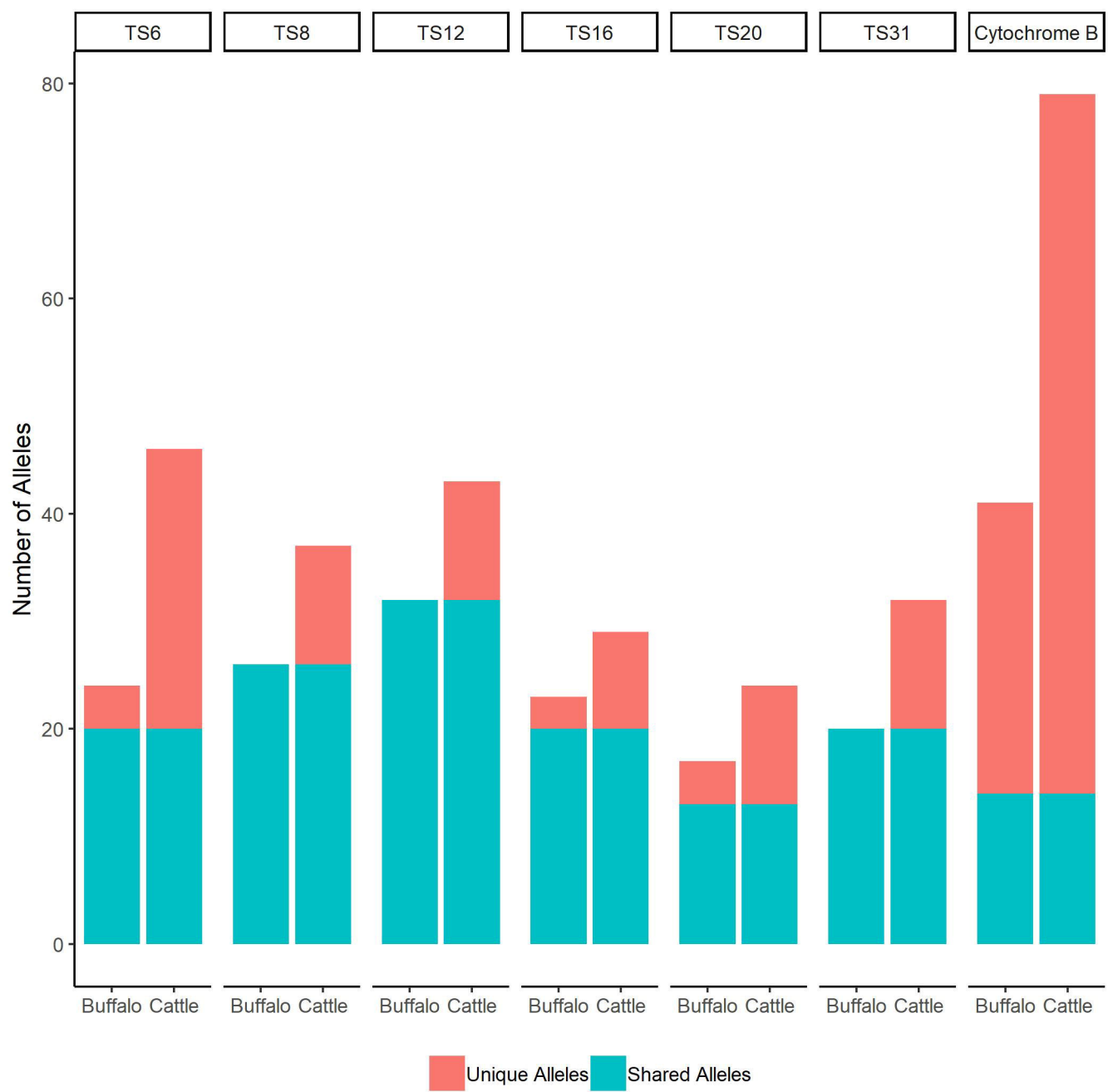
Principal Coordinates Analysis
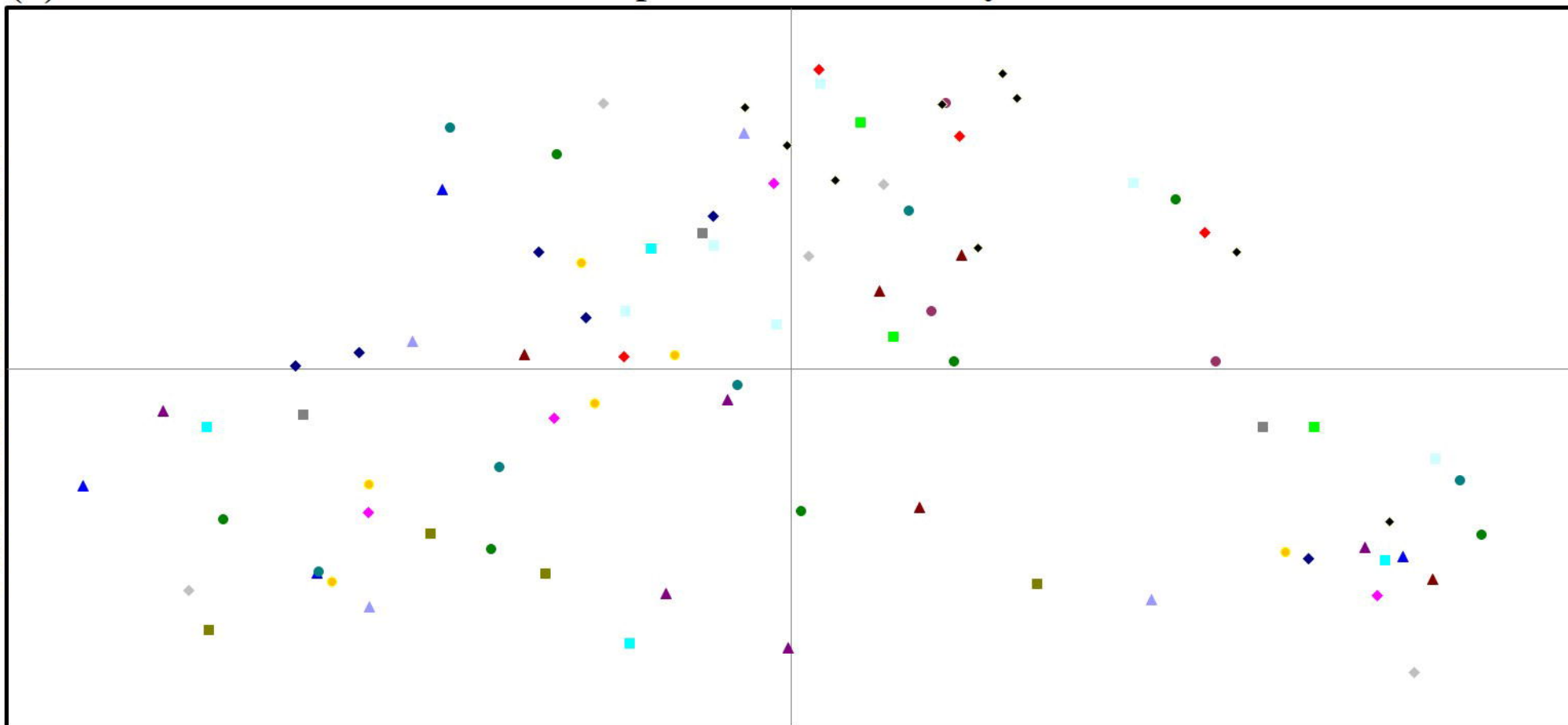
(A) Cattle

Coordinate 2 varience: 25.67

Coordinate 1 varience: 46.83

Legend:
- pop = CY2
- pop = CY6
- pop = CY79
- pop = CY82
- pop = CY89
- pop = CY84
- pop = CY20
- pop = CY90
- pop = CY26
- pop = CY27
- pop = CY28
- pop = CY30
- pop = CY34
- pop = CY39
- pop = CY38
- pop = CY40
- pop = CY41
- pop = CY37
- pop = CY160
- pop = CY162
- pop = CY154
- pop = CY131
- pop = CY130
- pop = CY120
- pop = CY119
- pop = CY134
- pop = CY150
- pop = CY173
- pop = CY124
- pop = CY175
- pop = CY177
- pop = CY136
- pop = CY151
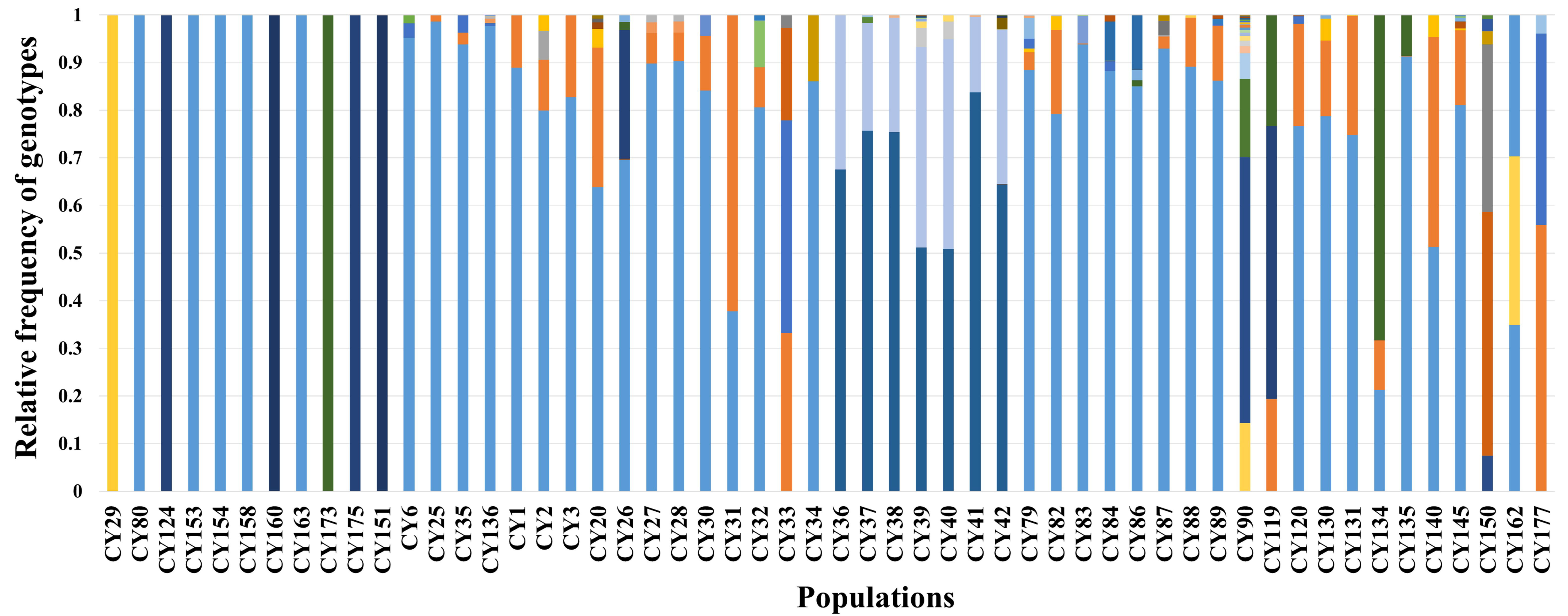- pop = CY145
- pop = CY33

Principal Coordinates Analysis

(B) Buffalo

Coordinate 2 varience: 27.60

Coordinate 1 varience: 46.33

Legend:
- pop = CY7
- pop = CY16
- pop = CY92
- pop = CY109
- pop = CY157
- pop = CY103
- pop = CY15
- pop = CY98
- pop = CY129
- pop = CY142
- pop = CY141
- pop = CY138
- pop = CY148
- pop = CY126
- pop = CY149
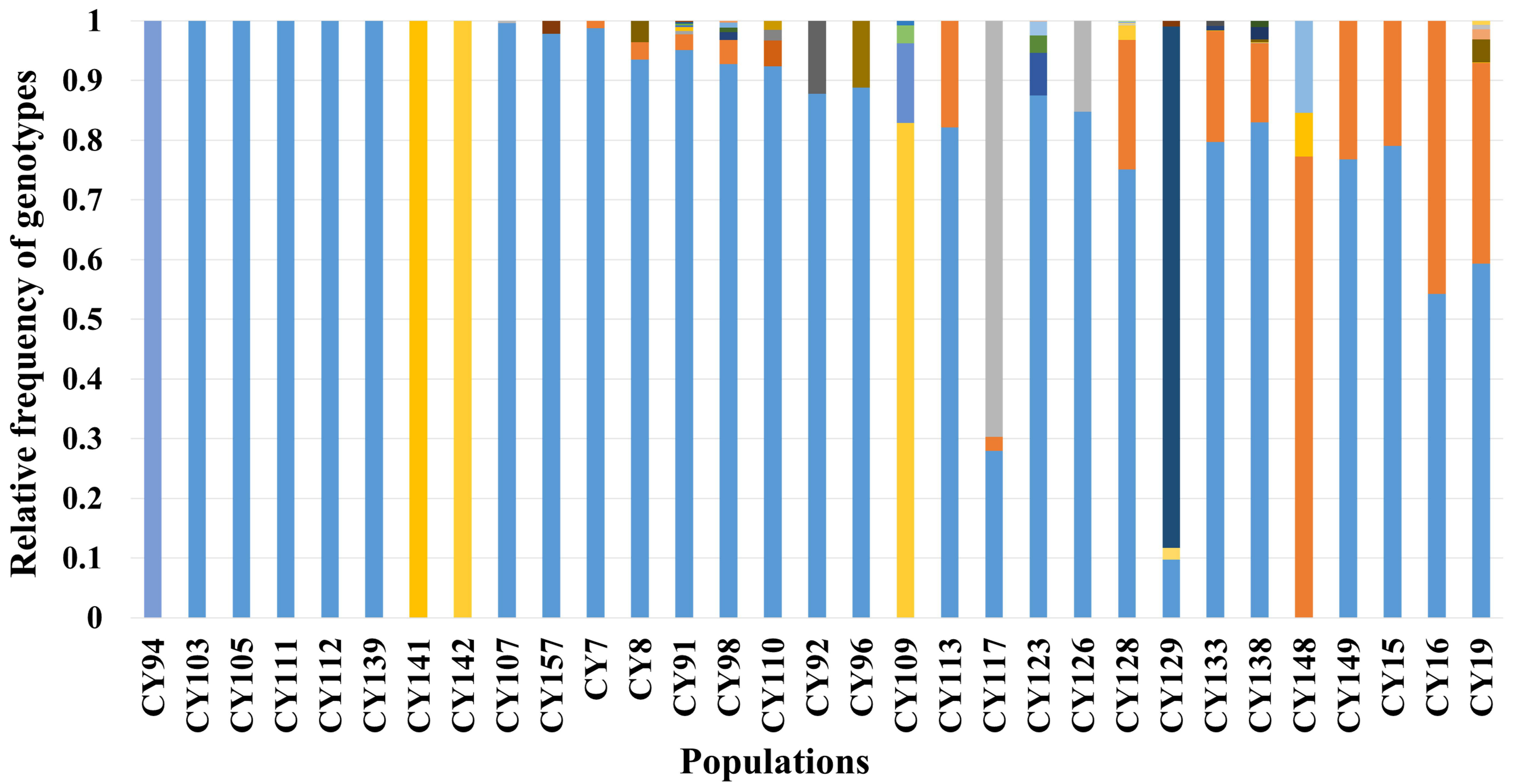- pop = CY149
- pop = CY139
- pop = CY133

(A) Cattle

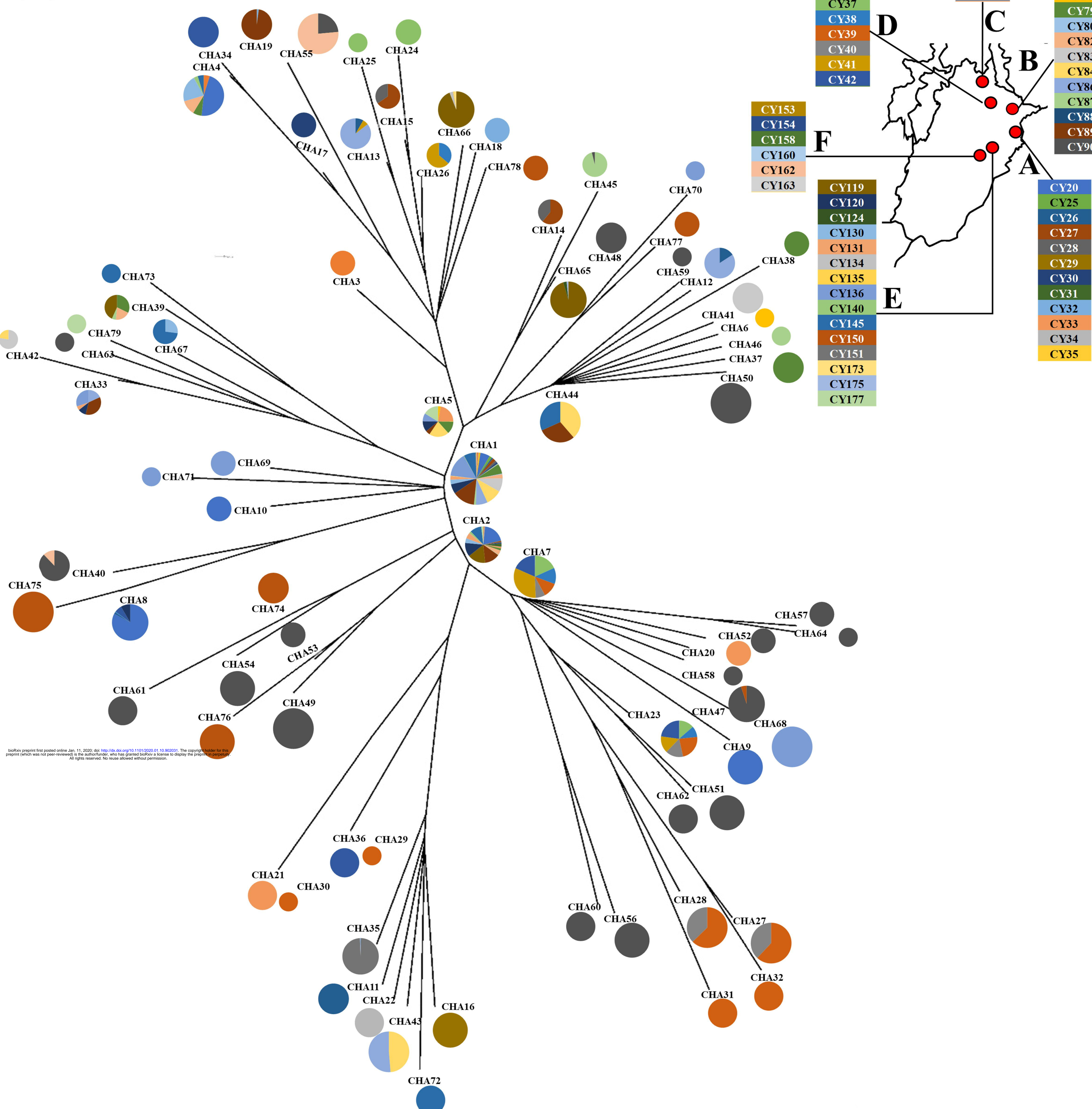| Populations	Sequence reads	Genotypes (%)
CY29	6442	CHA16(99.2),CHA4(0.2)
CY80	2997	CHA1(100)
CY124	3450	CHA65(100)
CY151	2032	CHA35(100)
CY153	4391	CHA1(100)
CY154	3483	CHA1(100)
CY158	1514	CHA1(100)
CY160	3621	CHA35(100)
CY163	2842	CHA1(100)
CY173	3393	CHA66(100)
CY175	3798	CHA65(1 00)
CY6	7361	CHA1(95.2), CHA5(3.0), CHA6(1.7), CHA7(0.1)
CY25	12422	CHA1(98.7), CHA2(1.2)
CY35	3780	CHA1(90.8), CHA2(3.4), CHA5(4.7)
CY136	109536	CHA1(97.6), CHA68(0.9), CHA69(0.4), CHA5(0.6), CHA33(0.1), CHA70(0.1), CHA71(0.1)
CY1	3055	CHA1(89), CHA2(11)
CY2	8032	CHA1(77.9), CHA2(10.6), CHA3(6.0), CHA4(5.3)
CY3	3573	CHA1(82.7), CHA2(17.2)
CY20	66615	CHA1 (60.8), CHA2(29.2), CHA4(6.9), CHA8(1.3), CHA9(0.8), CHA10(0.7)
CY26	8670	CHA1(69.6), CHA11(27.0), CHA12(1.6), CHA13(1.4), CHA8(0.1)
CY27	15741	CHA1(89.7),CHA2(6.4), CHA14(2.2), CHA15(1.5), CHA4(0.6)
CY28	9559	CHA1(90.3),CHA2(5.9), CHA14(2.3), CHA15(1.3)
CY30	8168	CHA1(81.1), CHA2(11.5), CHA17(6.3)
CY31	4219	CHA2(62.2), CHA1(37.7)
CY32	2304	CHA1(80.6), CHA2(8.4), CHA18(9.7), CHA19(1.1)
CY33	2162	CHA2(33.2), CHA5(43.6), CHA20(19.4), CHA21(4.5)
CY34	13252	CHA1(86.1), CHA22(13.8)
CY36	4812	CHA7(67.6), CHA23(32.3)
CY37	47941	CHA7(75.6), CHA23(22.6), CHA24(1.2), CHA25(0.4)
CY38	4812	CHA7(75.3), CHA23(24.0), CHA26(0.5), CHA8(0.2)
CY39	45065	CHA7(51.1), CHA23(42.0), CHA27(4.0), CHA28(1.4), CHA29(0.4), CHA30(0.2), CHA31(0.2), CHA32(0.2)
CY40	29245	CHA7(48.8), CHA23(44.0), CHA27(5.8), CHA28(1.2), CHA33(0.3)
CY41	77853	CHA7(83.7), CHA23(15.7), CHA13(0.1), CHA26(0.3)
CY42	57452	CHA7(62.4), CHA23(32.4), CHA34(4.3), CHA35(0.3), CHA36(0.2), CHA8(0.1)
CY79	48294	CHA1(85.4), CHA37(5.3), CHA2(4.6), CHA5(3.1), CHA4(0.7), CHA38(0.4), CHA39(0.1), CHA40(0.4)
CY82	23106	CHA1(79.2), CHA2(17.6), CHA4(2.8), CHA39(0.2)
CY83	64110	CHA1(90.8), CHA41(8.8), CHA2(0.1), CHA42(0.1)
CY84	77806	CHA1(86.3),CHA43(10.2), CHA5(2.0), CHA44(1.3), CHA40(0.3), CHA42(0.3)
CY86	60995	CHA1(85.0), CHA43(11.4), CHA13(2.1), CHA12(1.2), CHA33(0.1)
CY87	9526	CHA1(90.9), CHA45(4.1), CHA2(3.5), CHA39(0.1), CHA46(1.2)
CY88	5518	CHA1(89.1), CHA2(10.2), CHA40(0.5)
CY89	114872	CHA1(86.2), CHA2(11.5), CHA19(1.0), CHA44(0.7), CHA5(0.3), CHA33(0.1)
CY90	64051	CHA47(55.7), CHA48(16.4), CHA40(14.3), CHA49(5.4), CHA50(1.5), CHA51(1.1), CHA52(0.9), CHA53(0.7), CHA54(0.5), CHA55(0.5), CHA56(0.4), CHA57(0.3), CHA58(0.2), CHA59(0.2), CHA60(0.2), CHA61(0.2), CHA62(0.2), CHA63(0.2), CHA64(0.1), CHA45(0.1)
CY119	80952	CHA65(57.2), CHA66(23.2), CHA2(19.3), CHA39(0.1)
CY120	55003	CHA1(76.6), CHA2(21.4), CHA5(1.5), CHA33(0.7), CHA8(0.1)
CY130	26053	CHA1(78.8), CHA2(15.8), CHA67(0.7)
CY131	20896	CHA1(74.8), CHA2(24.9), CHA66(0.4), CHA4(0.1), CHA33(0.1)
CY134	3929	CHA66(68.3), CHA1(21.3),CHA2(10.3)
CY135	2083	CHA1(90.3), CHA66(9.5), CHA2(0.9)
CY140	4043	CHA1(50.3), CHA2(43.1), CHA4(6.5)
CY145	63270	CHA1(81.1), CHA2(15.7), CHA44(1.3), CHA67(0.8), CHA4(0.4), CHA72(0.3), CHA73(0.2)
CY150	25039	CHA74(48.1), CHA75(32.2), CHA47(7.4), CHA76(5.7), CHA77(5.4), CHA78(0.8)
CY162	3576	CHA1(34.8), CHA40(35.4),CHA55(29.6)
CY177	3917	CHA2(55.8), CHA5(40.1), CHA79(3.9)

(B) Buffalo

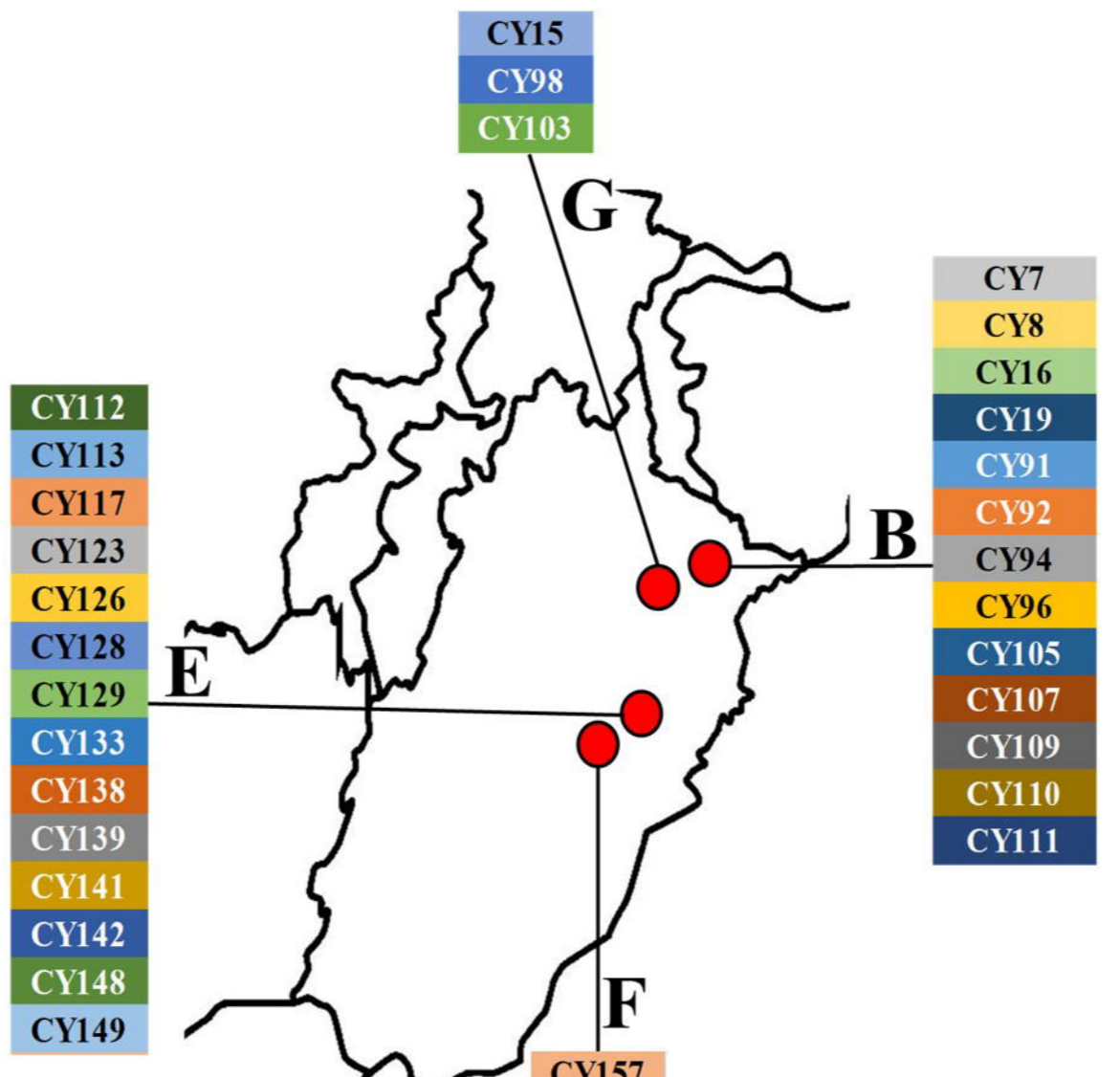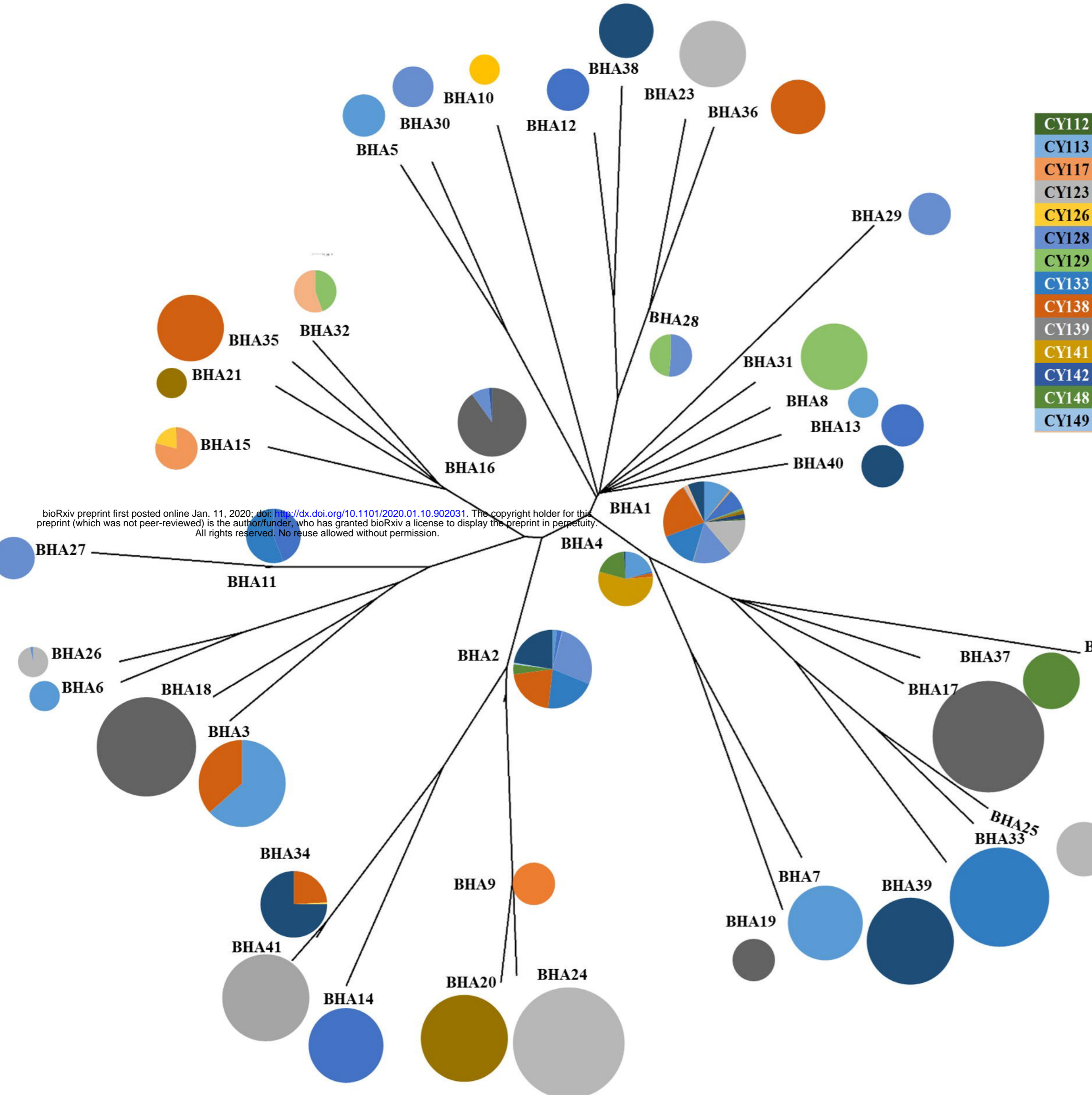| Populations	Sequence reads	Genotypes (%)
CY94	22831	BHA41(100)
CY103	14412	BHA1(100)
CY105	12823	BHA1(100)
CY111	51382	BHA1(100)
CY112	15822	BHA1(100)
CY139	18352	BHA1(100)
CY141	14892	BHA41(100)
CY142	12272	BHA16(100)
CY107	12712	BHA1(99), BHA15(0.3)
CY157	13062	BHA1(97.8), BHA32(2.1)
CY7	14892	BHA1(98.7), BHA2(1.2)
CY8	13682	BHA1(93.4), BHA2(2.9), BHA2(3.5)
CY91	29533	BHA1(95.1), BHA2(2.6), BHA3(0.5), BHA4(0.6), BHA6(0.2), BHA7(0.2), BHA8(0.1)
CY98	23295	BHA1(92.7), BHA2(4.0), BHA11(1.2), BHA12(0.8), BHA13(0.8), BHA14(0.2)
CY110	38681	BHA1(92.3), BHA20(4.3), BHA21(1.7), BHA22(1.4)
CY92	12361	BHA1(87.7), BHA9(12.2)
CY96	16971	BHA1(88.8), BHA10(11.9)
CY109	16723	BHA16(82.9), BHA17(13.3), BHA18(2.9), BHA19(0.7)
CY113	14081	BHA1(82.1), BHA2(17.8)
CY117	13502	BHA1(28), BHA15(69.8), BHA2(2.2)
CY123	43438	BHA1(87.5), BHA23(7.1),BHA24(2.9), BHA25(2.3), BHA26(0.1)
CY126	14262	BHA1(75.1), BHA15(15.2)
CY128	55010	BHA1(75.1), BHA2(21.7), BHA16(2.3), BHA26(0.3), BHA27(0.2), BHA28(0.2), BHA29(0.2), BHA30(0.1)
CY129	15470	BHA31(87.3), BHA1(9.7), BHA2(0.1), BHA28(1.9), BHA32(0.9)
CY133	49042	BHA1(79.7), BHA2(18.6), BHA4(0.2), BHA11(0.7), BHA33(0.8)
CY138	70250	BHA1(83.0), BHA2(13.2), BHA3(0.1), BHA4(0.2), BHA34(0.5), BHA35(2.0), BHA36(1.0)
CY148	12369	BHA2(77.2), BHA37(15.4), BHA4(7.3)
CY149	10771	BHA1(76.7), BAH2(23.2)
CY15	12621	BHA1(79.0), BHA2(20.9)
CY16	15922	BHA1(54.2), BHA2(45.7)
CY19	29321	BHA1(59.3), BHA2(33.6), BHA4(0.3), BHA34(3.9), BHA38(1.6), BHA39(0.7), BHA40(0.6)

(A) Cattle

Genotypes	Sequence reads	Number of populations
CHA1	306152 (22.9)	36 (CY1, CY2, CY3, CY6, CY20, CY25, CY26, CY27, CY28, CY30, CY31, CY32, CY34, CY35, CY79, CY80, CY82, CY83, CY84, CY86, CY87, CY88, CY89, CY120, CY130, CY130, CY131, CY134, CY135, CY136, CY140, CY145, CY153, CY154, CY158, CY162, CY163)
CHA7	144834 (10.8)	8 (CY6, CY36, CY37, CY38, CY39, CY40, CY41, CY42)
CHA2	93391 (6.9)	27 (CY1, CY2, CY3, CY20, CY25, CY27, CY28, CY30, CY31, CY32, CY33, CY35, CY79, CY82, CY83, CY87, CY88, CY89, CY119, CY120, CY1 CY131, CY134, CY135, CY140, CY145, CY177)
CHA23	80938 (6.0)	7 (CY36, CY37, CY38, CY39, CY40, CY41, CY42)
CHA35	70229 (5.2)	3 (CY42, CY151, CY160)
CHA65	84581 (6.3)	3 (CY119, CY124, CY175)
CHA66	80068 (5.9)	5 (CY119, CY131, CY134, CY135, CY173)
CHA43	12608 (0.9)	2 (CY84, CY86)
CHA74	11807 (0.8)	1 (CY150)
CHA48	10559 (0.7)	1 (CY90)
CHA40	10464 (0.7)	5 (CY79, CY84, CY88, CY90, CY162)
CHA75	13825 (1.0)	1 (CY150)
CHA5	7836 (0.5)	9 (CY6, CY33, CY35, CY79, CY84, CY89, CY120, CY136, CY177)
CHA4	6560 (0.4)	10 (CY2, CY20, CY27, CY29, CY79, CY82, CY130, CY131, CY140, CY145)
CHA41	5724 (0.4)	1 (CY83)
CHA49	6472 (0.4)	1 (CY90)
CHA27	7923 (0.5)	2 (CY39, CY40)
CHA44	6823 (0.4)	3 (CY84, CY89, CY145)
CHA11	4346 (0.3)	1 (CY26)
CHA37	5130 (0.3)	1 (CY79)
CHA13	4519 (0.3)	3 (CY26, CY41, CY86)
CHA55	4388 (0.3)	2 (CY90, CY162)
CHA34	5381 (0.4)	1 (CY42)
CHA19	5243 (0.3)	2 (CY32, CY89)
CHA28	5018 (0.3)	2 (CY39, CY40)
CHA68	4006 (0.2)	1 (CY136)
CHA50	3964 (0.2)	1 (CY90)
CHA12	3922 (0.2)	2 (CY26, CY86)
CHA20	4758 (0.3)	1 (CY33)
CHA51	4746 (0.3)	1 (CY90)
CHA67	3729 (0.2)	2 (CY130, CY145)
CHA76	4689 (0.3)	1 (CY150)
CHA77	5621 (0.4)	1 (CY150)
CHA52	3594 (0.2)	1 (CY90)
CHA24	4588 (0.3)	1 (CY37)
CHA14	4674 (0.3)	2 (CY27, CY28)
CHA9	3536 (0.2)	1 (CY20)
CHA10	4516 (0.3)	1 CY20)
CHA3	5489 (0.4)	1 (CY2)
CHA53	3476 (0.2)	1 (CY90)
CHA26	4474 (0.3)	2 (CY38, CY41)
CHA69	5449 (0.4)	1 (CY136)
CHA16	4441 (0.3)	1 (CY29)
CHA33	3397 (0.2)	6 (CY40, CY86, CY89, CY120, CY131, CY136)
CHA15	3373 (0.2)	2 (CY27, CY28)
CHA17	4354 (0.3)	1 (CY30)
CHA54	5347 (0.4)	1 (CY90)
CHA56	4315 (0.3)	1 (CY90)
CHA45	5308 (0.3)	2 (CY87, CY90)
CHA39	4273 (0.3)	4 (CY79, CY82, CY87, CY119)
CHA18	5225 (0.3)	1 (CY32)
CHA78	4223 (0.3)	1 (CY150)
CHA38	3210 (0.2)	1 (CY79)
CHA57	3510 (0.2)	1 (CY90)
CHA25	2206 (0.1)	1 (CY37)
CHA70	2200 (0.1)	1 (CY136)
CHA72	1994 (0.1)	1 (CY145)
CHA29	1888 (0.1)	1 (CY39)
CHA58	2186 (0.1)	1 (CY90)
CHA59	2985 (0.1)	1 (CY90)
CHA60	2185 (0.1)	1 (CY90)
CHA22	1674 (0.1)	1 (CY34)
CHA61	1873 (0.1)	1 (CY90)
CHA73	2162 (0.1)	1 (CY145)
CHA62	2459 (0.1)	1 (CY90)
CHA63	2647 (0.1)	1 (CY90)
CHA42	2143 (0.1)	2 CY83, CY84)
CHA36	2539 (0.1)	1 (CY42)
CHA30	1835 (0.1)	1 (CY39)
CHA31	1933 (0.1)	1 (CY39)
CHA6	2132 (0.1)	1 (CY6)
CHA71	2627 (0.1)	1 (CY136)
CHA79	2526 (0.1)	1 (CY177)
CHA32	2845 (0.1)	1 (CY39)
CHA46	1515 (0.1)	1 (CY87)
CHA64	1408 (0.1)	1 (CY90)
CHA21	1805 (0.1)	1 (CY33)

(B) Buffalo

Genotypes	Sequence reads	Number of populations
BHA1	278937 (39.1)	26 (CY91, CY92, CY96, CY98, CY103, CY105, CY107, CY110, CY111, CY112, CY113, CY117, CY123, CY126, CY128,CY129, CY133, CY138, CY139, CY149, CY157, CY7, CY8, CY15, CY16, CY19)
BHA2	77171 (10.8)	15 (CY19, CY98, CY113, CY117, CY128, CY129, CY133, CY138, CY148, CY149, CY7, CY8, CY15, CY16, CY19)
BHA16	39389 (5.5)	3 (CY109, CY128, CY142)
BHA31	14779 (2.0)	1 (CY129)
BHA23	19109 (2.6)	1 (CY123)
BHA17	18232 (2.5)	1 (CY109)
BHA34	19535 (2.7)	3 (CY138, CY8, CY19)
BHA35	19437 (2.7)	1 (CY138)
BHA24	20260 (2.8)	1 (CY123)
BHA25	10998 (1.5)	1 (CY123)
BHA4	12882 (1.8)	6 (CY91, CY133, CY138, CY141, CY148, CY19)
BHA36	14721 (2.0)	1 (CY138)
BHA11	13670 (1.9)	2 (CY98, CY133)
BHA18	15501 (2.1)	1 (CY109)
BHA38	12485 (1.7)	1 (CY19)
BHA33	14428 (2.0)	1 (CY133)
BHA37	12365 (1.7)	1 (CY148)
BHA15	8310 (1.1)	3 (CY107, CY117, CY126)
BHA41	7283 (1.0)	1 (CY94)
BHA3	8268 (1.1)	2 (CY91, CY138)
BHA28	6217 (0.8)	2 (CY128, CY129)
BHA39	7212 (1.0)	1 (CY19)
BHA40	5199 (0.7)	1 (CY19)
BHA12	4192 (0.5)	1 (CY98)
BHA13	5192 (0.7)	1 (CY98)
BHA20	4169 (0.5)	1 (CY110)
BHA9	5151 (0.7)	1 (CY92)
BHA27	3925 (0.5)	1 (CY128)
BHA19	3024 (0.4)	1 (CY109)
BHA32	3117 (0.4)	2 (CY129, CY157)
BHA29	4110 (0.5)	1 (CY128)
BHA5	3102 (0.4)	1 (CY91)
BHA30	2790 (0.3)	1 (CY128)
BHA10	2078 (0.2)	1 (CY96)
BHA6	2378 (0.3)	1 (CY91)
BHA7	2477 (0.3)	1 (CY91)
BHA21	2869 (0.4)	1 (CY110)
BHA14	1264 (0.1)	1 (CY98)
BHA26	1864 (0.2)	2 (CY123),
BHA22	1657 (0.2)	1 (CY128)
BHA8	1954 (0.2)	1 (CY91)