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Genetic polymorphisms on heat-shock protein 70 gene and mitochondrial DNA d-loop variations: implications on selection for heat stress in indigenous chickens in Kenya

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Background: Climate change resulting in heat stress, is one of the most challenging environmental conditions affecting poultry. Studying thermotolerance is crucial in the prevention of heat stress in chickens because it may lead to the identification of genetic lines that can withstand adverse effects of heat stress. This study aimed at investigating polymorphisms in heat shock protein 70 (HSP70) gene in indigenous chicken ecotypes. We also analyzed the mitochondrial DNA (mtDNA) D-loop of indigenous chickens to assess their origins and genetic diversity.

Methods: We collected samples From Turkana basin, Mt. Elgon catchment, Lake Victoria basin and Lamu chicken ecotypes in Kenya. Genomic DNA was extracted from 280 chicken samples. The first 360 bp region of HSP70 and the first 760 bp region of the mtDNA were then amplified via PCR. These were later sequenced using Sanger ABI 3730 method.

Results: We reveal through a detailed analysis of the HSP70 gene fragment in 20 indigenous chickens the presence of three HSP70 haplotypes (*GC, AC*, and *AG*) and 28 mtDNA haplotypes. Phylogenetic analysis of HSP70 revealed the presence of the ancestral haplotype *GC* which dominated in Turkana basin ecotype. The 28 mtDNA haplotypes clustered in haplogroups A, B, C, D, E and I. Haplogroup E which has never been reported in commercial chickens dominated in Turkana basin ecotype indicating no admixture with commercial chickens. mtDNA haplogroups were shown to have originated from various parts of South and Southeast Asia. Lack of population structure in indigenous chicken ecotypes could be an indication of genetic admixture. The mtDNA nucleotide and haplotype diversity indices were low for Turkana basin ecotype and high for Lamu ecotype. High HSP70 nucleotide diversity indices were recorded in Turkana basin ecotype, while low values were recorded in Lamu ecotype. Most of the mtDNA genetic variations occurred within individuals for the three hierarchical categories considered while most variations in HSP70 gene occurred within populations.

Conclusions: This is the first study to analyze the HSP70 polymorphisms in indigenous chickens in Africa and results obtained should pave the way for further in-depth studies on heat stress.

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1 **Genetic polymorphisms on heat-shock protein 70 gene and mitochondrial DNA d-loop**
2 **variations: implications on selection for heat stress in indigenous chickens in Kenya**
3

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19

20 **ABSTRACT**

21 **Background:** Climate change resulting in heat stress, is one of the most challenging
22 environmental conditions affecting poultry. Studying thermotolerance is crucial in the prevention
23 of heat stress in chickens because it may lead to the identification of genetic lines that can
24 withstand adverse effects of heat stress. This study aimed at investigating polymorphisms in heat
25 shock protein 70 (HSP70) gene in indigenous chicken ecotypes. We also analyzed the

26 mitochondrial DNA (mtDNA) D-loop of indigenous chickens to assess their origins and genetic
27 diversity.

28 **Methods:** We collected samples from Turkana basin, Mt. Elgon catchment, Lake Victoria basin
29 and Lamu chicken ecotypes in Kenya. Genomic DNA was extracted from 280 chicken samples.
30 The first 360 bp region of HSP70 and the first 760 bp region of the mtDNA were then amplified
31 via PCR. These were later sequenced using Sanger ABI 3730 method.

32 **Results:** We reveal through a detailed analysis of the HSP70 gene fragment in 20 indigenous
33 chickens the presence of three HSP70 haplotypes (*GC, AC*, and *AG*) and 28 mtDNA haplotypes.
34 Phylogenetic analysis of HSP70 revealed the presence of the ancestral haplotype *GC* which
35 dominated in Turkana basin ecotype. The 28 mtDNA haplotypes clustered in haplogroups A, B,
36 C, D, E and I. Haplogroup E which has never been reported in commercial chickens dominated
37 in Turkana basin ecotype indicating no admixture with commercial chickens. mtDNA
38 haplogroups were shown to have originated from various parts of South and Southeast Asia.
39 Lack of population structure in indigenous chicken ecotypes could be an indication of genetic
40 admixture. The mtDNA nucleotide and haplotype diversity indices were low for Turkana basin
41 ecotype and high for Lamu ecotype. High HSP70 nucleotide diversity indices were recorded in
42 Turkana basin ecotype, while low values were recorded in Lamu ecotype. Most of the mtDNA
43 genetic variations occurred within individuals for the three hierarchical categories considered
44 while most variations in HSP70 gene occurred within populations.

45 **Conclusions:** This is the first study to analyze the HSP70 polymorphisms in indigenous chickens
46 in Africa and results obtained should pave the way for further in-depth studies on heat stress.

47 **Keywords**: *candidate gene, climate change, genetic diversity, poultry, thermotolerance, Horn of*
48 *Africa*

49 **INTRODUCTION**

50 Climate change is one of the most significant challenges affecting Africa today. The African
51 continent is vulnerable to climatic variation resulting in multiple stresses such as heat stress
52 which has adverse effects on the agricultural sector since it causes diseases and low productivity,
53 consequently threatening food security. Heat stress also affects the physiological and
54 morphological functions of poultry. Diversity among livestock is crucial because it contributes
55 significantly to livestock development in the face of changing threats such as climate change.
56 Studies to understand diversity in indigenous chickens in developing countries have been done
57 using microsatellite markers (*Hillel et al., 2003*; *Mwacharo et al., 2007*) and mtDNA *(Fumihito*
58 *et al., 1996*; *Liu et al., 2006*).

59 The increasing human population has increased the demand for animal products such as meat
60 and eggs, which has in turn resulted in the erosion of genetic resources due to crossbreeding
61 especially in developing countries (including Kenya) where most diversity exists. While most of
62 the crossbreeding programs aim at improving the production of chickens, they do not consider
63 other factors such as the heat tolerance ability of these improved chickens. Heat tolerance should
64 be considered when coming up with highly productive chickens because climate change may
65 result in heat stress, making chickens to produce minimally which could contribute to their
66 mortality. There are many existing technologies in breeding programs in poultry that consider
67 climate change adaptation, and as such, indigenous chickens which are heat tolerant are bred
68 with exotic breeds to improve on heat tolerance*(Lu, Wen & Zhang, 2007*; *Aengwanich, 2009*;
69 *Hoffmann, 2010*; *Renaudeau et al., 2012*).

70 Certain genes have been found to be beneficial in heat stress tolerance. These genes include the
71 heat shock protein family such as the heat shock protein 70 (HSP70) gene (*Kregel, 2002*;
72 *Fujimoto & Nakai, 2010*). HSP70 is a molecular chaperone required for correct folding of newly

73 synthesized proteins and maintaining protein homeostasis *(Hartl, 1996; Kregel, 2002; Mayer &*
74 *Bukau, 2005*; *Zeng et al., 2013*). It is also involved in functions such as apoptosis (Kregel, 2002),
75 but most importantly in the regulation of heat shock response and the acquisition of
76 thermotolerance (*Mayer & Bukau, 2005*).
77 Some genes such as the naked-neck gene (*Na*), dwarf (*dw*), frizzled (*F*) have been linked with
78 heat tolerance in chickens (*Deeb & Cahaner, 2001a*; *Deeb & Cahaner, 2001b*; *Magothe, Muhuyi*
79 *& Kahi, 2010*). The scaleless mutation which results in featherless chickens has been used to
80 improve thermotolerance (*Cahaner et al., 2008*; *Renaudeau et al., 2012*). Various comb types
81 have also been attributed to heat regulation in indigenous chickens (*Moraa et al., 2015*).
82 Polymorphism studies in the HSP70 gene have shown sequence variations associated with
83 mRNA expression levels. Results from the analysis of HSP70 in birds exposed to heat stress
84 found that birds that were more resistant to heat had only P*st*I HSP70 allele located upstream
85 from the coding region while the other breeds displayed two different alleles for that gene
86 (*Mahmoud & Edens, 2005*). Polymorphisms have been detected in the coding and regulatory
87 regions of HSP70 in chickens with several heat tolerance abilities (*Zhang, Du & Li, 2002*). The
88 beginning of the coding region and the promoter region of HSP70 have been analyzed in
89 chickens with various heat tolerance abilities, and two single nucleotide polymorphisms (SNPs)
90 identified; A+258G and C+276G both of which were silent mutations (*Mazzi et al., 2003*). The
91 different genotypes at polymorphism sites A+258G and C+276G were associated with the
92 mRNA expression level of HSP70 in the liver and leg muscle with the heterozygotes having
93 higher expression levels than the other genotypes (*Zhen et al., 2006*).
94 This study aimed to analyze the functional polymorphism of HSP70 in relation to heat stress in
95 Turkana basin, Mt. Elgon catchment, Lake Victoria basin and Lamu chicken ecotypes in Kenya.

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96 The study also investigated variations in mtDNA to establish their genetic diversity. Our research
97 will therefore play a significant role in future molecular programs primarily in the exploitation of
98 genes deemed useful during heat stress in poultry.
99

100 MATERIALS & METHODS

101 Study clearance

102 This study received a “no objection to the research” from the Directorate of Veterinary Services,
103 Ministry of Agriculture, Livestock and Fisheries in Kenya under permit number
104 RES/POL/VOL.XXVII/162 to sample indigenous chickens in Kenya.

105 Sample collection and DNA extraction

106 We collected our samples at the farms by using the rural participatory approach method which
107 involved explaining to the farmers about our research to get consent from them. We administered
108 questionnaires to the farmers, recording information about their chickens in the process. We also
109 collected blood samples from 280 genetically unrelated indigenous chickens. Two mature birds
110 (above six months) were selected from each flock for study as previously described (*Mwacharo*
111 *et al., 2011*). All blood samples were spotted on FTA® Classic Cards (Whatman Biosciences) and
112 stored at room temperature before DNA extraction. We extracted genomic DNA from the air-
113 dried blood preserved on FTA classic cards according to the manufacturers’ instructions.

114 PCR amplification and sequencing

115 The first 360-bp of the HSP70 gene was amplified via PCR using forward primer HSPF
116 (5’AACCGCACCACACCCAGCTATG-3’) and reverse primer HSPR
117 (5’CTGGGAGTCGTTGAAGTAAGCG-3’) (*Akaboot et al., 2012*). The amplified region

118 corresponds to position 52,784,305-52,784,620 of the galGal4 Chromosome 5 of chicken HSP70
119 (NP_001006686) (www.ensembl.org).
120 PCR amplifications were carried out in 25µl reaction volumes containing 1 X PCR buffer
121 (10mM Tris-HCl pH 8.3, 50mM KCL, 0.1% Triton X-100), 2.5 mM of each dNTP, 10 pM of
122 each primer and 1 unit of Taq DNA polymerase (Promega, Madison WI, USA) and 20ng
123 template genomic DNA. Thermocycling conditions were as follows: 94^0 C (3min), 35 cycles of
124 94^0C (1 minute), 58^0C (1 minute) and 72^0C (2 minutes) and a final extension step at 72^0C (10
125 minutes).
126 The first 760bp of the mtDNA D-loop region was amplified via PCR using primers AV1F2 (5'-
127 AGGACTACGGCTTGAAAAGC-3') and CR1b (5'-CCATACACGCAAACCGTCTC-3')
128 (*Mwacharo et al., 2011*). PCR amplifications were carried out in 10µlreaction volumes
129 containing 3.8µl of double distilled water, 1µl of template genomic DNA, 5µl Dream Taq Green
130 Master Mix (2X), 0.2µl Primer (forward + reverse, 20pmol/µl). Thermocycling conditions were
131 as follows: 94^0 C (3min), 35 cycles of 94^0C (15 seconds), 60^0C (30 seconds) and 72^0C (30
132 seconds) and a final extension step at 72^0C (10 minutes).
133 The PCR products were run on 1% agarose gel using 1X TBE buffer (89Mm Tris, 89mM Boric
134 acid, 2Mm Na_2EDTA) in a voltage of 100V for 25 minutes. The gels were stained with
135 GelRed™ Nucleic acid gel stain and visualized under UV light (BTS-20 model, UVLtec Ltd.,
136 UK). One kb DNA Ladder was used to identify the approximate size of the molecule run on a
137 gel.
138 All PCR products that were positive upon visualization on agarose gel were sequenced at
139 Macrogen in Europe. The products were sequenced in both the forward and reverse directions
140 using the Sanger ABI 3730 method.

141 **Data analysis**

142 We visualized the chromatograms and edited them manually using Chromas Lite version 2.1.1
143 (*Avin, 2012*) (Technelysium Pty Ltd, Australia). We trimmed out the primers and created a
144 consensus nucleotide sequence. We aligned the consensus against reference sequences from
145 GenBank using Clustal X version 2.1 (*Thompson et al., 1997*). We restricted our subsequent
146 analyses to a 316bp promoter region of HSP70 and the first 343 bp mtDNA D-loop incorporating
147 the first hypervariable segment (HSV1)
148 We constructed the haplotypes manually and by the use of DnaSP v 5.10 (*Librado & Rozas,*
149 *2009*). Genetic diversity indices on HSP70 and mtDNA for the ecotypes were calculated using
150 DnaSP v5.10 and ARLEQUIN v3.5.1.2 (*Excoffier, Laval & Schneider, 2005*).
151 We constructed a phylogenetic tree for HSP70 involving the haplotypes observed and the
152 reference sequences downloaded from GenBank using the Maximum Likelihood algorithm as
153 implemented in MEGA v6.06 following 1000 bootstrap replications (*Tamura et al., 2013*). We
154 also constructed a splits decomposition network for both HSP70 and mtDNA haplotypes using
155 splits tree v4.14.2 (*Bandelt & Dress, 1992*). We assessed the population genetic structure by the
156 analysis of molecular variance (AMOVA) (*Excoffier, Laval & Schneider, 2005*).
157

158 **RESULTS**

159 **Genetic Polymorphisms in HSP70 Gene**

160 We obtained good quality sequences of 277 sampled indigenous chicken ecotypes in Kenya. We
161 compared these sequences with other avian HSP70 sequences downloaded from GenBank. We
162 identified three HSP70 haplotypes which we renamed to haplotype *AC*, haplotype *GC* and

163 haplotype *AG*. We deposited the haplotype sequences in GenBank under accession numbers
164 KT948639, KT948640, and KT948641 respectively.
165 We then did an alignment of the three haplotypes generated with the ancestral red jungle fowl
166 (NC_006092.3), using Muscle Version 3 (*Edgar, 2004*). Two polymorphic sites were observed at
167 positions 153 and 171 which correspond to position 52,784,398 and 52,784,416 respectively in
168 the Gallus_gallus-4.0 chromosome 5 from UCSC genome browser.

169 **Phylogenetic analyses**

170 To determine the model of sequence evolution and the rate of heterogeneity of the sequences we
171 used MEGA Version 6 software (*Librado & Rozas, 2009*). Phylogenetic analysis of our HSP70
172 haplotypes with other avian HSP70 sequences downloaded from GenBank (XM_009675580.1,
173 XM_005022658.1, AB259847.1, EU622852.1, XM_009576438.1, XM_005506375.1,
174 AB096696, JJRM01051595) showed clustering of haplotype *GC* to the ancestral red jungle fowl
175 (Fig. 1), while haplotype *AG* and *AC* clustered together. The tree was rooted using the rock
176 pigeon HSP70 sequence.
177
178
179
180 **Fig. 1** A rooted maximum likelihood tree of the three HSP70 haplotypes and other avian HSP70
181
182 We validated these results by constructing a splits decomposition network which shows the red
183 jungle fowl clustering with haplotype *GC* and was genetically distant from haplotypes *AG* and
184 *AC* which clustered together. The other avian species were genetically distant from the chicken
185 haplotypes (Fig.2).

186

187 **Fig. 2** Phylogenetic network tree of the HSP70 haplotypes with other avian HSP70 sequences

188

189 **Distribution of the HSP70 haplotypes in the sampled chicken ecotypes**

190 The relative frequencies of observed haplotypes in the sampled chicken ecotypes are shown in
191 Fig.3. Haplotype *GC* was frequently distributed in all the ecotypes but dominant in Turkana
192 basin chicken ecotype. Haplotype *AG* was equitably distributed in all the ecotypes while
193 haplotype *AC* had the least frequencies in the ecotypes (Fig. 3).

194

195 **Fig. 3** HSP70 haplotype distribution in the sampled indigenous chicken ecotypes

196

197 Green represents haplotype *AG*, Blue haplotype *AC*, and Yellow haplotype *GC*.
198 The various numbers represent the distribution of the haplotypes in the areas sampled. Pie chart
199 1 represents Turkana east, 2: Turkana central, 3: Turkana West, 4: West Pokot, 5: Trans Nzoia,
200 6: Bungoma, 7: Busia, 8: Lake Victoria basin, 9: Lamu north, 10: Lamu central, and 11: Lamu
201 south.
202 We calculated several diversity indices for the indigenous chicken ecotypes (Table 1). Lower
203 estimates of expected heterozygosity (H_E) were calculated for Lamu ecotype compared to the
204 other ecotypes. Turkana basin populations had highest estimates of H_E. Mt. Elgon catchment
205 ecotype displayed the least values for observed heterozygosity (H_O), while Lake Victoria basin
206 ecotype displayed the highest values. Turkana basin ecotype recorded the highest values for
207 nucleotide diversity (Pi) while Lamu ecotype had the least values for nucleotide diversity (Table
208 1).

209

210 **Table 1** Diversity indices of HSP70 gene in indigenous chicken ecotypes in Kenya

211

212 We performed an analysis of molecular variance (AMOVA) to infer the population genetic
213 structure of HSP70 haplotypes of indigenous chicken ecotypes in Kenya. We assessed using
214 1000 replicates the variations in HSP70 haplotypes among the ecotypes, among individuals
215 within the ecotypes and within individuals using AMOVA (*Excoffier, Laval & Schneider, 2005*)
216 of pairwise differences as implemented in Arlequin v.3.5.1.2 (*Schneider, Roessli & Excoffier*,
217 *2000*). Based on pairwise differences in AMOVA, variance among the ecotypes and variance
218 among individuals within the ecotypes accounted faor 7.99% and 2.39% respectively. On the
219 other hand, the variance within individuals was high, and it accounted for 89.62% of the total
220 variance (Table 2).

221

222

223 **Table 2** AMOVA of the HSP70 gene in sampled indigenous chicken ecotypes

224

225 We used a Mantel test to assess the non-random association between genetic differentiation
226 (F_{ST}) and the geographic distances between populations. We plotted a regression graph of the
227 genetic and geographic distances using GenAIEx v6.501 software (*Peakall & Smouse, 2006*).
228 From the results, a slight positive correlation ($p>0.05$) is observed between the genetic variation
229 and the geographic location in indigenous chicken ecotypes in Kenya (Fig. 4).

230

231 **Fig. 4** A regression graph showing the relationship between geographic and genetic distance in

232 mtDNA haplotypes

233 **mtDNA variation and haplotype distribution**

234 We obtained partial 343 bp mtDNA D-loop sequences of 280 sampled indigenous chicken
235 ecotypes in Kenya. These sequences were compared with other chicken mtDNA sequences
236 downloaded from GenBank with accession numbers; AB114069, AB007744, AB114070,
237 AY588636, AB114076, AF512285, AF515588, D82904, AB009434. We identified 28 mtDNA
238 haplotypes which we renamed to Haplotype_1-Haplotype_28. We deposited these haplotypes in
239 GenBank under accession numbers MH681612- MH681639.
240 To determine the model of sequence evolution and the rate of heterogeneity of the sequences we
241 constructed a splits decomposition network. We used our 28 haplotypes and nine reference
242 haplogroup sequences downloaded from GenBank (*Liu et al., 2006*). We included *Gallus gallus*
243 *bankiva* as an outgroup. The decomposition network revealed the presence of a major clade E.
244 Most of the haplotypes in this clade were from Turkana basin chicken ecotype. Three haplotypes
245 clustered around clade C while clade A, B, D and I had single haplotypes. None of the 28
246 haplotypes centered around clade F, G and H (Fig. 5)

247

248 **Fig. 5** A splits decomposition network of the mtDNA haplotypes with reference sequences

249

250 We calculated some diversity indices for the indigenous chicken ecotypes as shown in Table 3.
251 All mtDNA haplotypes from studied populations were polymorphic with the number of
252 haplotypes ranging from 3 to 9 (Table 3). Lamu North ecotype had the highest number of
253 haplotypes while Turkana basin ecotype had the least number of haplotypes. Lamu ecotype had

254 the highest haplotype diversity (0.8895±0.0416), while Turkana basin ecotype had the lowest
255 haplotype diversity (0.1700±0.1025). Lamu ecotype also recorded the highest values for
256 nucleotide diversity (0.160150±0.091934) and Turkana basin ecotype had the lowest values of
257 nucleotide diversity (0.024845±0.021751). The highest variation for the analysis of molecular
258 variance (AMOVA) was recorded within the ecotypes (78.06%) (Table 5).
259
260 **Table 3** mtDNA haplotype diversity indices of sampled Kenyan indigenous chicken ecotypes
261
262 We used DnaSP v5 (*Librado & Rozas, 2009*) to study the historical and population dynamics of
263 the studied indigenous chicken ecotypes. We calculated observed and expected distributions of
264 mismatches under the population growth decline model (*Rogers & Harpending, 1992)*. The
265 mismatch distribution is multimodal as shown in Fig. 6.
266
267 **Fig. 6** mtDNA Pairwise nucleotide difference under the population growth decline model in
268 chicken ecotypes in Kenya
269
270 We calculated the demographic and spatial expansion of the mtDNA haplotypes using Arlequin
271 as shown in Table 4. Harpendings spatial expansion raggedness index ″*r*″ of the mtDNA
272 haplotype is significant for Busia chicken ecotype in Mt. Elgon catchment chicken ecotype
273 (P=0.019). The demographic and spatial expansion raggedness index was not significant
274 (P>0.05) for the other indigenous chicken ecotypes.
275
276 **Table 4** Demographic and spatial expansion indices of mtDNA haplotypes

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277

278 To infer the maternal genetic structure of indigenous chicken ecotypes in Kenya, We assessed
279 using 1000 replicates the variations in mtDNA haplotypes among ecotypes, among populations
280 within ecotypes and within populations using AMOVA (*Excoffier, Laval & Schneider, 2005*) of
281 pairwise differences as implemented in Arlequin v.3.5.1.2 (*Schneider, Roessli & Excoffier,*
282 *2000*). Based on pairwise differences in AMOVA, variation among ecotypes and populations
283 within the ecotypes accounted for 18.8% and 3.14% respectively. On the other hand, the
284 variation within individuals was high, accounting for 78.06% (Table 5).

285

286 **Table 5** Summary of AMOVA of mtDNA haplotypes in indigenous chicken ecotypes in Kenya

287

288 **DISCUSSION**

289 Single nucleotide polymorphisms (SNPs) in the coding and non-coding regions have been
290 shown to play a critical role in regulating their function, and this has led to a better
291 understanding of the gene structure (*Chen et al., 2016*). To the best of our knowledge, this is the
292 first study to provide insights into the genetic polymorphisms in HSP70 among indigenous
293 chicken ecotypes in Kenya in relation to their genetic background. We identified three HSP70
294 haplotypes -haplotype *GC*, *AC,* and *AG*. These haplotypes were shared within and between the
295 chicken ecotypes. The extent of HSP70 and mtDNA haplotype sharing among indigenous
296 chicken ecotypes which can be attributed to extensive inbreeding practices indicates lack of
297 population structure (*Makanjuolal et al., 2010*). The HSP70 mantel test and the mtDNA
298 AMOVA test revealed a lack of population structure within indigenous chicken ecotypes in
299 Kenya. Lack of population structure has also been reported previously (*Muchadeyi et al., 2008*;

300 *Mwacharo et al., 2011*)

301 Haplotype *AG* was the most frequent haplotype in all the ecotypes while haplotype *AC* had the
302 least frequencies in all the ecotypes. Phylogenetic analysis with other avian species including
303 the ancestral red jungle fowl sequences indicated that haplotype *GC* is the ancestral haplotype
304 since it clustered with the ancestral red jungle fowl. This haplotype dominated in Turkana basin
305 chicken ecotype. This result is supported by the mtDNA data which indicates that the ancestral
306 mtDNA haplotype E is dominant in Turkana basin chicken ecotype.

307 Polymorphisms in chicken HSPA70 gene has been shown to be related to resistance to heat
308 stress (*Zhang, Du & Li, 2002*; *Maak et al., 2003*). It is clear from this study that haplotype *AG*
309 and *GC* could be advantageous to heat tolerance in indigenous chickens since haplotype *GC*
310 dominated in Turkana basin chicken ecotype while haplotype *AG* was dominant in Lamu
311 chicken ecotype. Turkana baasin and Lamu are arid and semi-arid lands characterized by high
312 ambient temperatures of up to 42ºC and we therefore hypothesize that the two haplotypes have a
313 crucial role in heat tolerance.

314 The percentage variation of HSP70 was highest within individuals. Therefore, we postulated
315 that there is a clear genetic variation in HSP70 gene within individual indigenous chicken
316 ecotypes in Kenya and that the sequence of the HSP70 gene that changed during the extensive
317 history of evolution, natural and artificial selection could play a significant role in heat stress.

318 It has been shown that individual variations in heat shock responses may be related to DNA
319 polymorphisms in the HSP70 gene in birds (*Enrique et al., 1998*; *Iwamoto et al., 2008*)

320 Genetic changes bring about variations in gene products and their expression levels. This could
321 be one of the factors that contribute to phenotypic variations between various species (*McManus*
322 *et al., 2010*). From our study, Indigenous chickens in Kenya are diverse and seem to have

323 varying levels of thermal tolerance. High ambient temperatures have negative impacts on the
324 poultry industry since it affects the growth and production performance in chickens (*Mack et al.,*
325 *2013*). Various studies have indicated that mutations in gene sequencing alter gene expression,
326 morphology, and physiology (*Deeb & Cahaner, 2001a*; *Maak et al., 2003*; *Mack et al., 2013*).
327 For instance, changes in the *AT* content of the promoter affects the expression of HSP70 which
328 plays a significant role in regulatory evolution (*Chen et al., 2011*). This, therefore, is an
329 indication that other changes in the gene sequence of HSP70 could contribute to the evolution of
330 this gene.
331 The different heat tolerant capability traits in chicken are brought about by the coding regions
332 and polymorphism regulation of HSP70 (*Zhang, Du & Li, 2002*). Therefore, the use of modern
333 molecular breeding technology to identify genetic markers related to thermotolerance could
334 allow for the possibility of direct gene selection. It has been shown that the C.-69A>GSNP in
335 the 5′-flanking region of the HSP70 gene affects chickens' thermotolerance traits in white
336 recessive rock chickens exposed to thermoneutral temperatures and the GG genotype might be
337 advantageous for the prevention of heat stress. Thus, this SNP may be a potential molecular
338 marker for further genetic improvement of thermotolerance in chicken (*Chen et al., 2016*).
339

340 **Origins of indigenous chicken ecotypes in Kenya**

341 Despite the lack of a population structure in indigenous chicken ecotypes, the splits
342 decomposition network revealed distinct maternal lineages that existed in indigenous chicken
343 ecotypes in Kenya. Overall, the 28 haplotypes observed in this study clustered into six
344 genetically distinct clades: Clade A, B, C, D, E and I. These have been reported previously in
345 indigenous chickens in other African countries (*Oka et al., 2007*; *Muchadeyi et al., 2008*;

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346 *Razafindraibe et al., 2008*; *Makanjuolal et al., 2010*; *Mwacharo et al., 2011*). Clade A has been
347 observed in indigenous chickens from South Africa (*Muchadeyi et al., 2008*; *Razafindraibe et*
348 *al., 2008*) . Clades A and D have been reported to be the major haplogroups that occur in the
349 African continent (*Mwacharo et al., 2011*) while clades A, B, and E are widely distributed in the
350 world (*Liu et al., 2006*). Clade D, which is the dominant haplogroup in Africa has been reported
351 previously in East Africa (*Mwacharo et al., 2011*); and West Africa (*Makanjuolal et al., 2010*).
352 Clade A is the second dominant haplogroup in East Africa and has been previously reported in
353 Kenya (*Mwacharo et al., 2011*). It has also been observed in Zimbabwe (*Muchadeyi et al.,*
354 *2008*) and Madagascar (*Razafindraibe et al., 2008*) although it is absent in West Africa
355 (*Makanjuolal et al., 2010*). It is speculated that this clade could have originated from South and
356 Southwest China and/or surrounding areas. It has been suggested that it originates from
357 Southeast Asia (*Muchadeyi et al., 2008*), while Makanjuolal *et al* (*Makanjuolal et al., 2010*)
358 suggests without evidence that it originated from Indonesia following Austronesian arrivals on
359 the Island. The fact that this haplogroup has been reported in South and East African regions
360 suggests either an early trading contact all along the coast of East Africa or an arrival in a single
361 African coastal region and subsequent dispersal along the coastal area of the continent at an
362 unknown point in time (*Mwacharo et al., 2011*).
363 Clades B and C have similar biological history and were reported for the first time in the African
364 village chickens (*Mwacharo et al., 2011*). It has been proposed that this haplogroup originated
365 from Yunnan province and or surrounding areas (Myanmar, and Thailand). This haplogroup has
366 been found to be identical to haplotypes found in the brown and white egg layers. This could be
367 a manifestation of the crossbreeding programme of indigenous chickens with the commercial
368 flocks and this introgression has been shown to have occurred in the Dutch fancy breed of

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369 chicken based on mtDNA analysis (*Dana et al., 2011*). This could be an indication that the
370 exotic commercial genotypes have been successfully introgressed into indigenous chicken
371 ecotypes in Kenya. Clades A and B were observed in Mt. Elgon catchment chicken ecotype.
372 Clade E was observed in all the indigenous chicken ecotypes. Turkana basin chicken ecotype had
373 most haplotypes in this clade, followed by Mt. Elgon catchment ecotype, Lake Victoria basin
374 ecotype then Lamu ecotype. This haplogroup demonstrated a star-like pattern and this is a clear
375 indication of rapid population expansion (*Mwacharo et al., 2011*). Just like clade B, this clade
376 was reported for the first time in African indigenous chickens in Ethiopia and Sudan (*Mwacharo*
377 *et al., 2011*). It is worth noting that this clade has never been reported in commercial chickens
378 indicating that it's a pure indigenous breed (*Muchadeyi et al., 2008*; *Mwacharo et al., 2011*).
379 This clade has a close relationship with mtDNA sequences from Yunnan province in China and
380 this supports the origin of this haplogroup from Yunnan province and/or adjacent regions
381 (*Muchadeyi et al., 2008*). It has been suggested that this haplogroup might have found its way to
382 Africa via the horn of Africa and diffused southwards to Sudan and Ethiopia (*Mwacharo et al.,*
383 *2013*). It can, therefore, be concluded that this haplogroup found its way to Kenya through the
384 Lake Turkana basin as an entry point from Ethiopia and Sudan.
385
386

387 **CONCLUSIONS**

388 Our data indicated that there are still some ancestral haplotypes that exist in Kenyan indigenous
389 chicken ecotypes. It is also evident that indigenous chicken ecotypes in Kenya may genetically
390 harbour different levels of thermal tolerance. This has been demonstrated by the variation in the
391 partial HSP70 gene that was analyzed in this study. Further genomic studies should be
392 conducted to establish whether other beneficial heat stress genotypes that could be utilized in

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393 breeding programs primarily in the heat stressed environments exist. This should then improve
394 on the breeding programs thus leading to improved poultry productivity hence ensuring food
395 security.
396

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401

402 **ADDITIONAL INFORMATION AND DECLARATIONS**

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410 **Competing interests**
411 The authors declare there are no competing interests

412 **Author contributions**

413 • Sheila C. Ommeh conceived the study, designed the experimets, data collection, data
414 analysis, manuscript preparation, interpreted the data, revised and approved the final
415 manuscript.
416 • Grace M. Kennedy did data collection, data analysis, manuscript preparation, interpreted
417 the data, revised and approved the final manuscript.
418 • Philip M. Panyako did data analysis and manuscript preparation
419 • Philip A. Oyier did data analysis, manuscript preparation, interpreted the data, revised
420 and approved the final manuscript.
421 • Emmanuel K. Ndiema did data collection, interpreted the data, revised and approved the
422 final manuscript.
423 • Jacqueline K. Lichoti interpreted the data, revised and approved the final manuscript.

424 **Data availability**

425 GenBank accession numbers of mtDNA haplotypes: MH681612- MH681639 and HSP70
426 haplotypes: KT948639, KT948640, KT948641.

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Figure 1

A rooted maximum likelihood tree of the three HSP70 haplotypes and other avian HSP70

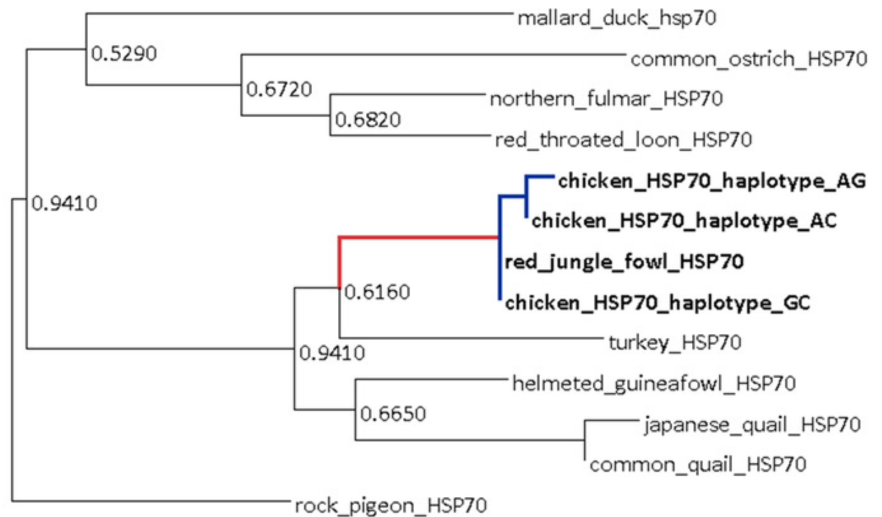

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Figure 2

Phylogenetic network tree of the HSP70 haplotypes with other avian HSP70 sequences

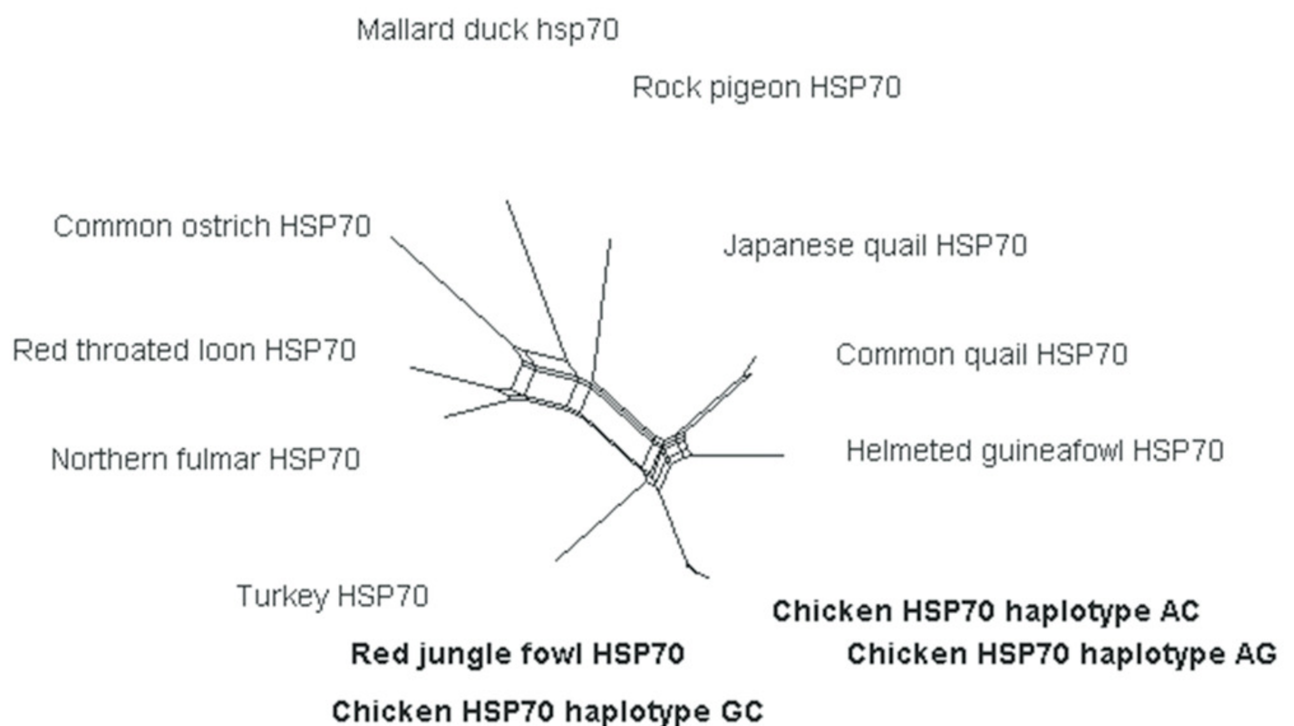

Figure 3

HSP70 haplotype distribution in the sampled indigenous chicken ecotypes

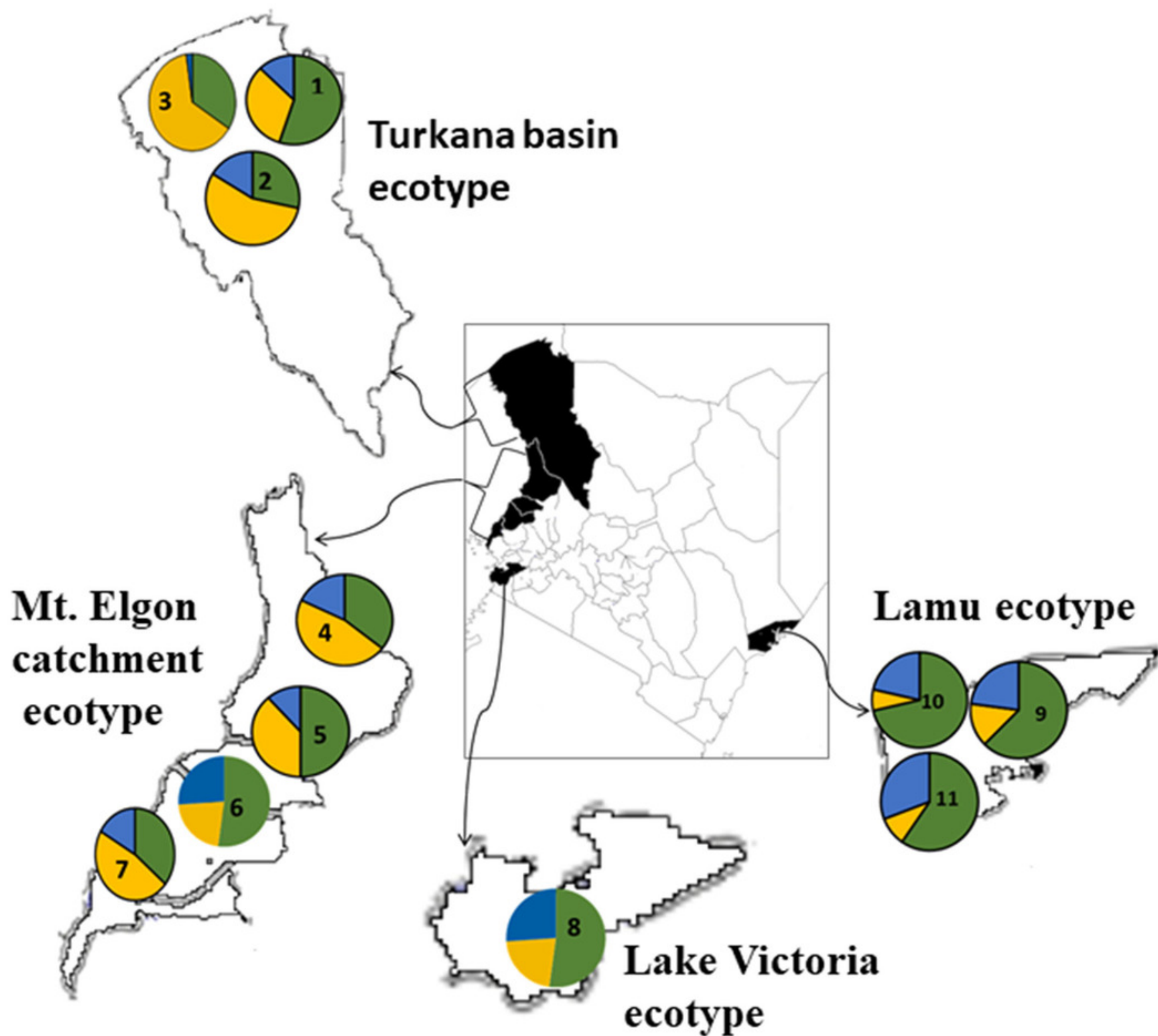

Figure 4

A regression graph showing the relationship between geographic and genetic distance in mtDNA haplotypes

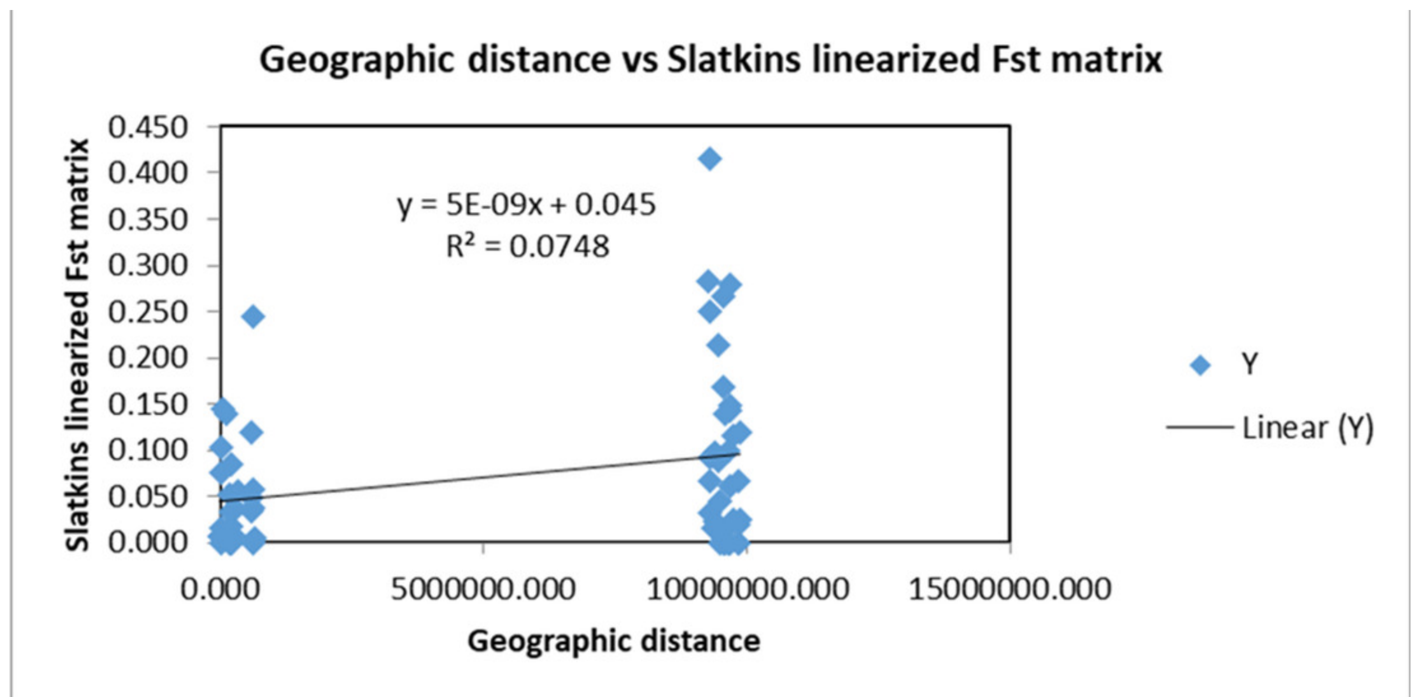

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Figure 5

A splits decomposition network of the mtDNA haplotypes with reference sequences

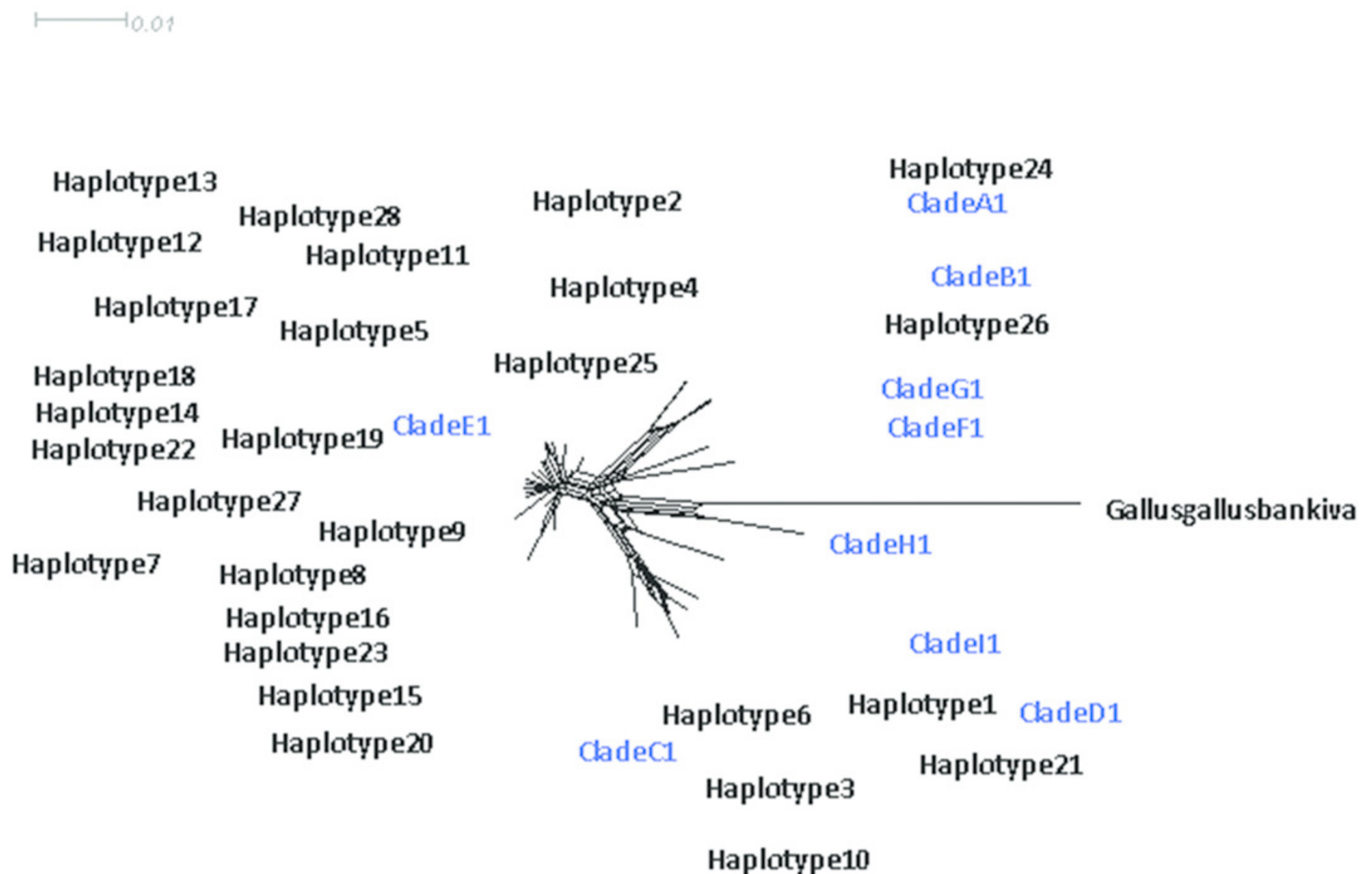

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Figure 6

mtDNA Pairwise nucleotide difference under the population growth decline model in chicken ecotypes in Kenya

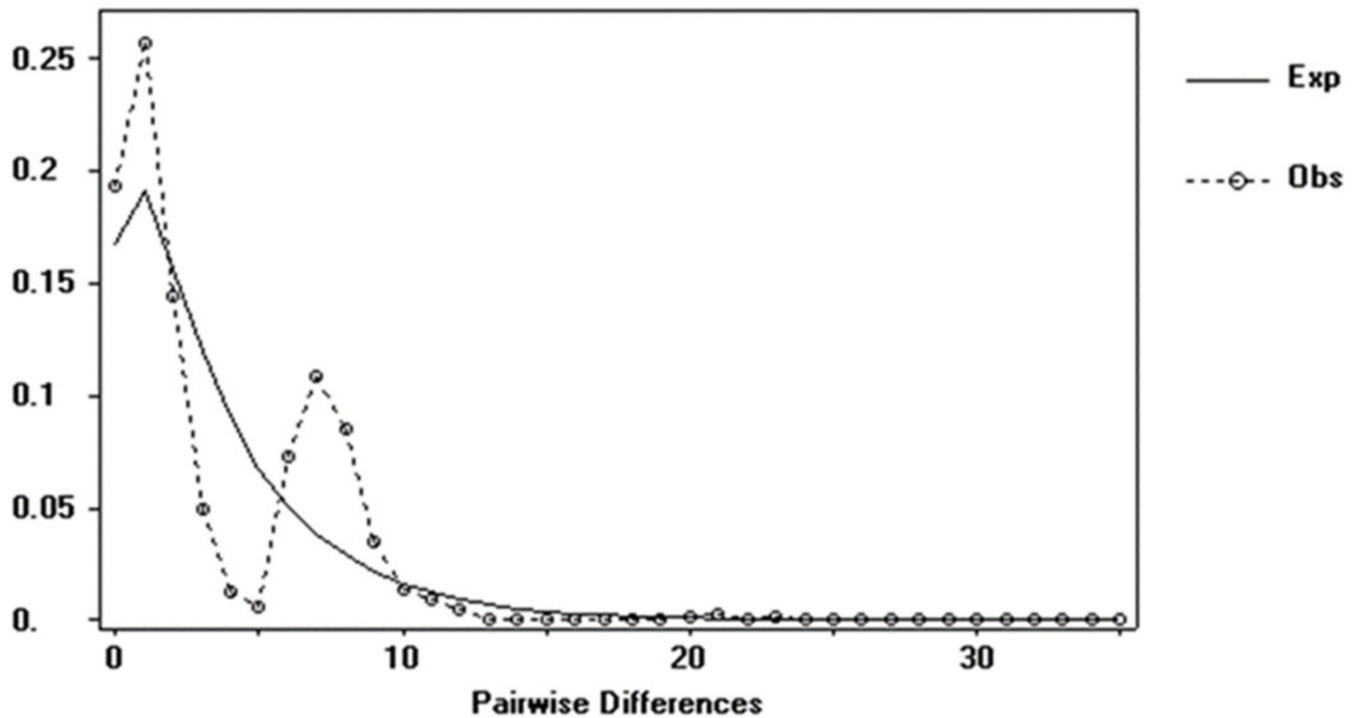

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Table 1(on next page)

Diversity indices of HSP70 gene in indigenous chicken ecotypes in Kenya

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1 **Table 1** Diversity indices of HSP70 gene in indigenous chicken ecotypes in Kenya

2

Ecotypes	No. of homozygotes	No. of heterozygotes	H_O	H_E	P_i
Turkana basin	96	118	0.5514	0.6291	0.003036±0.002297
Mt.Elgon catchment	50	60	0.5455	0.6281	0.00294±0.002243
Lake Victoria basin	12	34	0.7391	0.6192	0.002685±0.002129
Lamu	80	104	0.5652	0.4995	0.001989±0.001730

3

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Table 2 (on next page)

AMOVA of the HSP70 gene in sampled indigenous chicken ecotypes

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1 **Table 2** AMOVA of the HSP70 gene in sampled indigenous chicken ecotypes

2

Variance components	Hierarchy	d.f.	variation	Percentage of variation	*p*-value
Among ecotypes	1	4	22.4	7.9	0.00000+/- 0.00000
Among individuals within ecotypes	2	549	164.9	2.3	0.21970+/- 0.00436
Within individuals	3	554	158	89.6	0.00337+/- 0.00057

3

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Table 3 (on next page)

mtDNA haplotype diversity indices of sampled Kenyan indigenous chicken ecotypes

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1 **Table 3** mtDNA haplotype diversity indices of sampled Kenyan indigenous chicken ecotypes

Ecotype	Sample size	polymorphic sites	No. of haplotypes	haplotype diversity	Nucleotide diversity
Turkana basin					
Turkana north east	37	8	3	0.2868±0.0880	0.028743±0.593763
Turkana north	23	8	3	0.1700±0.1025	0.024845±0.021751
Turkana west	21	13	4	0.6071±0.1640	0.024235±0.023361
Mt. Elgon catchment					
West Pokot	27	13	7	0.8006±0.0506	0.107245±0.064330
Trans Nzoia	12	6	5	0.7576±0.0927	0.048701±0.036333
Bungoma	25	12	6	0.6567±0.0903	0.071786±0.046606
Busia	19	7	8	0.8304±0.0657	0.041771±0.031413
Lake Victoria basin					
Rusinga	12	9	4	0.4545±0.1701	0.053571±0.039012
Mfangano	12	5	6	0.8030±0.0959	0.054654±0.039604
Lamu					
Lamu North	20	11	9	0.8895±0.0416	0.160150±0.091934
Lamu central	46	12	8	0.7662±0.0392	0.146066±0.082310
Lamu South	26	12	7	0.6431±0.0934	0.138242±0.079897

2

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Table 4(on next page)

Demographic and spatial expansion indices of mtDNA haplotypes

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1 **Table 4** Demographic and spatial expansion indices of mtDNA haplotypes

Ecotype	Demographic expansion		Spatial expansion	
	raggedness index	raggedness p-value	raggedness index	raggedness p-value
Turkana basin				
Turkana North East	0.62145	0.485	0.62145	0.599
Turkana North	0.57811	0.665	0.57811	0.757
Turkana West	0.24107	0.314	0.24107	0.291
Mt.Elgon catchment				
West Pokot	0.09531	0.491	0.09531	0.344
Trans Nzoia	0.07071	0.676	0.07071	0.709
Bungoma	0.13966	0.356	0.13966	0.606
Busia	0.24161	0.019	0.24161	0.013
Lake Victoria basin				
Rusinga	0.21579	0.989	0.21579	0.707
Mfangano	0.10445	0.406	0.10445	0.425
Lamu				
Lamu North	0.06097	0.447	0.06097	0.73
Lamu Central	0.08215	0.183	0.08215	0.673
Lamu South	0.12914	0.437	0.12914	0.746

2

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Table 5(on next page)

Summary of AMOVA of mtDNA haplotypes in indigenous chicken ecotypes in Kenya

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1 **Table 5** Summary of AMOVA of mtDNA haplotypes in indigenous chicken ecotypes in Kenya

2

Source of variation	df	variation	percentage variation	p-value
Among ecotypes	4	0.29534	18.8	0.00079±0.00027
Among populations within ecotypes	8	0.04936	3.14	0.02030±0.00147
Within populations	267	1.2261	78.06	0.00000±0.00000

3

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