ORIGIN, GENETIC DIVERSITY AND HSP 70 GENE POLYMORPHISMS OF DOMESTICATED RABBITS OF KENYA

.

SHARON AUMA OWUOR

MASTER OF SCIENCE

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JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

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Origin, Genetic Diversity and HSP 70 Gene Polymorphisms of Domesticated Rabbits of Kenya

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Sharon Auma Owuor

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature…………………………. Date…………………...

Sharon Auma Owuor

This thesis has been submitted for examination with our approval as University Supervisors

Signature…………………………… Date……………………

Dr. Remmy W. Kasili

Louisiana State University, Baton Rouge, Louisiana, USA

Signature………………………………… Date……………………

Prof. Edward G. Mamati

JKUAT, Kenya

DEDICATION

This work is dedicated to my loving and supportive parents, Mr. Raphael Owuor Goroh and Mrs. Emilly Owuor Goroh, for empowering me to believe in myself and obtain a higher degree. Indeed, you are my pillars. I also dedicate this work to my siblings; Lillian Adawo Goro, Eve Goro, Diana Agnes, Habil Dave Owuor and James Philip Sewe. This family unit stood by me through this entire duration.

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ABSTRACT

Rabbit production in Kenya offers great opportunity for food security among households as a cheap alternative source of animal protein and as a source of income. Rabbits have received limited research, and information on their origins and genetic diversity is scarce. Rabbits in Kenya are neglected and could become extinct, yet they have important traits like heat tolerance and disease resistance. They are also well adapted to the local environmental conditions including high tropical temperatures. Knowledge on local rabbits kept by farmers will shed light on the extent of diversity and adaptation across the country which will facilitate improvement and development of conservation strategies. This study had two objectives. The first objective was to characterize the origins, population structure and genetic diversity of rabbits in Kenya using the mtDNA D-loop hypervariable region. The second objective was to characterize the genetic polymorphisms in Heat Shock Protein 70 gene. A 263-base pair region of mtDNA D-loop region and a 359-base pair region in the gene coding for heat shock protein 70 of 118 rabbits sampled from Kakamega, Vihiga and Bungoma counties in the western region, Laikipia and Nyandarua counties in the central region and Kitui, Machakos and Makueni in the eastern regions were amplified. Sequence analysis revealed 8 mtDNA haplotypes with 5 of these being reported for the first time by this study and 6 HSP70 haplotypes. Network asessment of these haplotypes on a phylogenetic tree and median joining network revealed that the haplotypes and reference sequences obtained from the NCBI database clustered in lineage B and that they diverged from an ancestral sequence; the wild rabbits. The average haplotype (0.40702) and nucleotide (0.01494) diversities observed were low, indicating low genetic diversity within these regions of domesticated rabbits in Kenya. These results suggest that the Kenyan domesticated rabbits originated from Europe. AMOVA revealed a higher variation within populations (85.3%) and low variation among the groups (eastern, central and western) (1.59%), implying a lack of population structure. Nine SNPs sites at positions 277 (C/T), 302 (C/T), 362 (A/G), 365 (T/C), 386 (T/G), 401(T/C), 430(A/G), 431(T/C), 506 (G/C) were identified in the gene coding for heat shock protein 70. 4 out of these SNPs were found in haplotype 4 which occurred in Machakos county spanning agro climatic zones IV and V with higher environmental temperatures. AMOVA revealed a higher within population variation (100%) and low among group variation (1.59%) implying lack of population structure and random occurrence of these SNPs. There was low genetic diversity (Hd 0.09436) in the HSP70 gene. The genetic polymorphisms in the HSP70 gene are not associated with heat tolerance. The results from this study can be built upon by rabbit breeders to enhance rabbit production in Kenya in ways such as informing the breeds that can help combat climate change effects.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Rabbit rearing is increasingly becoming attractive and offers unique advantages over cows, goats, sheep and chicken (Zotte, 2014). Rabbits are small, occupying limited space as they can be reared in the same space with crops. Rabbits have inherent desirable traits such as high fecundity and a rich source of white meat considered as a healthy option given the increase in lifestyle diseases. They are also increasingly becoming popular to the farmer as their waste can be used as manure for the farm and hence complement well with vegetable growing and kitchen gardens (Moreki, 2007). Moreover, rabbits are increasingly becoming a popular delicacy in Kenyan restaurants, a notion that was earlier on considered otherwise a taboo (Mailu et al., 2017).

Food security remains a concern, especially in low and middle-income countries. With increasing over reliance by farmers on other small livestock such as chicken as a source of food and livelihood, the resource-poor Kenyan farmer is increasingly diversifying his/her options and rabbit has proven to be a good alternative. There has been a growing interest in rabbit rearing, which was traditionally considered a hobby among the youth and a preserve of certain social groups, especially women and children (Serem et al., 2013). Before the upsurge in rabbit rearing, rabbits were mainly kept at the house-hold levels on a small-scale for family consumption. However, rabbit rearing is now one of the fastest growing enterprises in Kenya. Thika (Kiambu county), Nakuru (Nakuru county) and Nyeri (Nyeri county) town in Kenya have the highest numbers of rabbit producers, owing to their proximity to the capital, Nairobi county (Mutsami & Karl, 2020).

Both exotic and local breeds are available with the former being the most predominant because of desirable market traits such as size and a source of meat. The local adapted breeds are preferred due to adaptive traits such as disease and heat tolerance (Borter and Mwanza, 2011). Breeding involves selection of animals and combining unique sets of desirable traits. Selection and identification of animals resulted in the

formulation of the ‘modern concept of a breed’ in the 1800s (Othman, 2012). Breeding and selection of a breed results in a drastic reduction in diversity and therefore endangers the survival of the species. In Kenya, exchange of rabbit breeding stocks is common among farmers. It is a lucrative venture with a mature rabbit selling at an average price of Kshs 525 (Mutsami & Karl, 2020). In breeding, the motivation is to obtain rabbits with desirable traits such as disease-resistance and more meat that fetch higher prices (Borter & Mwanza, 2011). The practice of rearing pure breeds has further led to the drastic reduction in the locally well-adapted rabbit breeds.

Climate change is impacting the livestock species negatively; for instance heat stress and decrease in biological functions such as water uptake and daily feed intake (Functions et al., 2007). This not only affects productivity but also survival of the species. Therefore, preservation of the well-adapted species is a step towards maintaining the species diversity and therefore adaptation to challenges associated with climate change. It is imperative therefore that livestock species are evaluated for breeding purposes and future exploitation. Development of molecular biology therefore offers tools for evaluation of variation and diversity in the species. For instance, indices of heat tolerance evaluated using genetic markers are becoming increasingly used in evaluation of genetic diversity in livestock species.

Molecular tools such as mitochondrial DNA (mtDNA) sequencing have been used to elucidate the origin and genetic diversity in cows, pigs and goats (Othman, 2012). Genetic polymorphism in selected genes has been associated with heat tolerance in animal species (Liu *et al*., 2010). Therefore, this study set out to identify the origin and genetic diversity of domesticated rabbits with the aim of informing breeding decisions by farmers and genetic resource conservation. It would be equally informative to evaluate if the gene coding for HSP70, a candidate gene for adaptation to heat tolerance, would contribute to identification and selection of rabbits for resilience to climate change and adaptation to different climatic conditions.

1.2 Statement of the research problem

Climate change has resulted in heat stress, unpredictable weather patterns, emergence and increase of diseases impacting a wide range of livestock species (Okab & Koriem,

2008). This results in decreased productivity of livestock, increased cost of production and differentiated adaptation of different breeds of the livestock. In increasing and sustaining rabbit productivity, there is an increase in the use of exotic germplasm, with the government's effort to promote rabbit production using imported rabbit breeds more likely to result in genetic erosion (Borter& Mwanza, 2011). In addition, valuable genes in local rabbits have neither been identified nor characterized and documented. Animal Genetic Resources are inadequately and ineffectively managed (GoK, 2008). Rege & Gibson (2003) suggested that use of exotic germplasm, environmental and disease stresses and changes in production systems are the major causes of genetic erosion. It is likely that important traits such as heat tolerance and disease resistance that are found in the adapted indigenous rabbits are being lost, yet they need to be conserved for use in future breeding programs and to address emerging challenges such as climate change. In the absence of genetic characterization data, economic valuation of the beneficial traits that animal genetic resources hold becomes a chellenge, so does the economic loss that results from the erosion of these important traits. This calls for the studying of the existing genetic diversity in the domesticated rabbits being reared in Kenya, an aspect that this study seeks to evaluate.

1.3 Justification of the study

Knowledge of genetic diversity will provide the status of Kenya's rabbit genetic resources, potential for improvement and inform the strategies for breeding and guide on how to utilize and conserve animal genetic resources that are at risk. To develop a conservation management strategy, it is necessary to undertake characterization, documentation and develop a complete inventory of the available animal genetic resources. It has been suggested that the information gained from genetic rescue which includes utilization of local breeds with important traits such as disease resistance and selected breeds with important markers for traits of interest will be beneficial in livestock management (Kristensen et al., 2015).

The data that this project sought to generate aimed at informing and guiding the government and other stakeholders in creating national programs and policies that facilitate effective management, sustainable use and utilization of rabbit genetic

resources. Rabbit breeds that have desirable traits need to be conserved for future breeding programs, especially with apparent climate change effects, and food and security needs. Results from this study will also inform the farmers in the agricultural sector on the rabbit breeds that could be selected for thermotolerance and remain productive and help with the challenge of food security, especially in developing countries

1.4 Objectives

1.4.1 General objective

To evaluate origins using mtDNA and genetic diversity of domesticated rabbits in Kenya and polymorphisms in heat shock protein (HSP) 70 gene.

1.4.2 Specific Objectives

1. To characterize the origins, population structure and genetic diversity of rabbits in Kenya using the mtDNA D-loop hypervariable region.
2. To characterize the genetic polymorphisms in Heat Shock Protein 70 gene for heat tolerance.

1.5 Alternative Hypotheses

1. Domesticated rabbits in Kenya have a distinct genetic background
2. There are genetic polymorphisms in the HSP70 gene of domesticated rabbits in Kenya

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin and domestication of rabbits

Rabbits belong to the Kingdom Animalia, Superphylum Chordata, Phylum Vertabrata, Class Mammalia, Order Lagomorpha, Family Leporidae and eight different genera namely: Pentalagus, Bunolagus, Nesolagus, Romerolagus, Brachylaus, Sylvilagus, Oryctolagus and Poelagus (Bose, 2014). Currently, the genus Oryctolagus is represented byonly one species, Oryctolagus cuniculus; European rabbit, which is thought to havedescended from Oryctolagus laynesis (Lopez-Martinez, 2008). Two subspecies, Oryctolaguslacosti and Oryctolagus laynesis, have been identified from fossil remains in the middlePliocene, with the former being found predominantly in southern France and north westernItaly and the latter in the Iberian Peninsula. Both the wild and domestic rabbit belong to asingle species; Oryctolagus cuniculus (Long et al., 2003).

The Iberian peninsula is recorded to be the origin of domesticated rabbits, and two sub species, *Oryctolagus cuniculus cuniculus (clade A)* and *Oryctolagus cuniculus algirus (clade B) coexist*, with the former occurring in the north eastern part and the latter in the south west (Flux, 1983, Bolet et *al.*, 2011). Branco *et al.* (2000), also studied the phylogeography of the European rabbit, *Oryctolagus cuniculus*, in the Iberian Peninsula and found that geographically, clade A was predominant in South West Iberia and clade B north East of Iberia. Another possible center of domestication is South of France, where human-mediated efforts such as migration are said to have introduced rabbits to this locality (Alves *et al*., 2015). Bolet *et al.* (2011), reported that indigenous domesticated rabbits descended from the sub-species *Oryctolagus cuniculus cuniculus* in France. Clade A has thus been associated with *Oryctolagus cuniculus algirus* and clade B with *Oryctolagus cuniculus cuniculus* (Biju-Duval *et al*., 1991).

2.2 Rabbit rearing in Kenya

Humans have played an important role in the spread of rabbits to various parts of the world by way of migration and trade. In least developed countries, such as Kenya, rabbits were introduced less than 100 to 150 years ago by missionaries in the 19th century. A bilateral agreement between the Government of Kenya and German International Development Agency, GTZ, in 1980, led to a deliberate promotion effort of rabbit rearing in Kenya. The government sourced breeding stock from the then West Germany. A National Rabbit Breeding Centre was established at Ngong, Kajiado County, alongside several multiplication centers in various parts of the country, and later other sources of rabbit multiplication centers were established country-wide in areas such as Kilifi, Embu, Mtwapa and Machakos with an objective of providing breeding stocks to farmers. These breeding centers became vital in training of farmers on good rabbit-keeping practices, whereas 4K-clubs in schools played a key role in promoting rabbit rearing among the youth Ministry of Livestock development (MoLD, 2006).

The American Rabbit Breeders Association (ARBA) estimates that there are 47 distinct rabbit breeds (ARBA, 2018). Out of these, the most common breeds being reared in Kenya include French Lop, Giant Flemish, New Zealand White, Chinchilla, Californian White, Dutch, Checkered Giant and Flemish Angora. The breeds in breeding stations are considered pure; continually selected for their desirable performance. The Giant Flemish, Checkered Giant and French Lop are especially desired by breeders because of their large size (Borter & Mwanza, 2011). Despite the rapid growth of rabbit rearing in Kenya, the industry has faced major challenges. Diseases, in-breeding leading to decrease in diversity and loss of desirable traits and the emerging negative effects of climate change have had a negative impact on rabbit rearing (Chesterman & Neely, 2015). Climate change is an emerging challenge in the livestock industry and is not limited to rabbit rearing (Van De Steeg *et al*., 2009). This has necessitated development of approaches to combat these challenges, and these include identification of diverse breeds with desirable traits such as heat tolerance. Domestic animal diversity forms an integral component of global diversity (FAO,

2011). Many well-adapted local breeds in various livestock species are facing a risk of extinction (Belew *et al.*, 2016).

FAO reported that approximately between eight and twenty-one percent of the reported livestock breeds are extinct or at risk of extinction respectively (FAO 2011). Genetic improvement of livestock has been reported as one of the strategies to both mitigate climate change and improve livestock adaptation to climate change effects. Genes underlying adaptation have now been identified, with genomic regions in cattle on BTA6, BTA10, BTA19 and BTA20 being associated with annual mean reduction (Cassandro, 2020).

2.3 Use of markers in studying genetic diversity in rabbits

There are various methods of measuring genetic diversity, with the most widely used being phenotypic characters, biochemical traits and molecular markers (Msoffe *et al.*, 2004). Commonly used markers in diversity studies include major histocompatibility complex (MHC), protein polymorphisms and DNA markers. A molecular marker is a locus in the DNA that is detectable and whose inheritance can be monitored. Some properties of a good marker include transmission from one generation to another (Mendelian inheritance), ease of genotyping, polymorphism (the presence of several alleles in the locus being investigated), co-dominant (allowing for the discrimination between heterozygotes and homozygotes), neutral (having the same fitness in a population) and specificity (Al-Samarai & Al-Kazaz, 2015). Some of the DNA markers used in genetic diversity studies include randomly amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), restriction fragment length polymorphisms (RFLPs), microsatellites, single nucleotide polymorphisms (SNPs) and mitochondrial DNA (mtDNA).

2.3.1 Single Nucleotide Polymorphisms

SNPs are single-nucleotide variations in the sequence of the DNA, often representing a neutral of functional genetic diversity Figure 2.1 illustrates a SNP. SNPs often result in alleles of a gene .

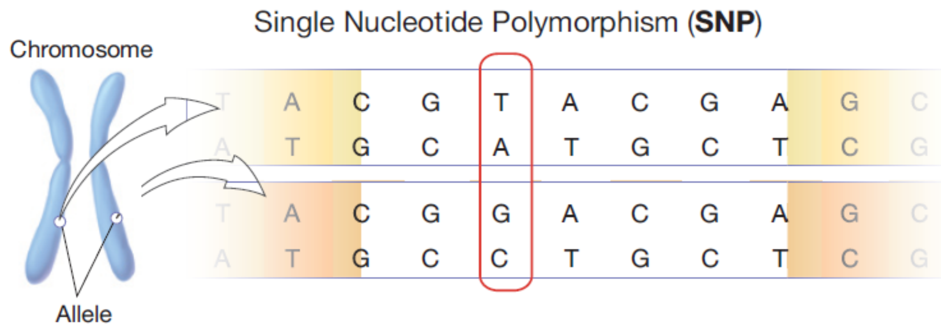

Figure 2.1: SNP T to G and A to C at a locus in 4 "genomes"

Ren *et al*. (2019) utilized the restriction-site-associated DNA (RAD-Seq) approach in the analysis of genetic diversity and population structure among four Chinese rabbit breeds namely Sichuan White, Tianfu Black, Fujian Yellow and Fujian Black. This study obtained 7, 055, 440 SNPs and from these, 113, 973 high-confidence SNPs were detected for purposes of the study of genetic diversity and population structure. This study showed that the Fujian Yellow and Tianfu Black had the least inter-breed differences (F_{ST} = 0.037) whereas Fujian Black and Sichuan White possessed higher inter-breed differences (F_{ST} = 0.0504). A Principal Component Analysis (PCA) revealed a clear distinction of these four breeds. In another study, El-aksher et al. (2017) utilized SNP markers in the analysis of a new synthetic rabbit line, 'Moshtohor' rabbits (M-line) and its parental population. Using the PCR-RFLP method, a SNP, G/A_{2464} was found in the promoter region of the progesterone receptor gene. This SNP was used in the identification of three genotypes: GG, GA and AA.

2.4 Lineage marker

A lineage marker is a marker that is passed down across generations without changing, making them attractive for the tracing of evolution. The Y chromosome and mitochondrial DNA markers are examples of lineage markers (Buckleton *et al*., 2011). While the former determines paternal lineage of only males, mitochondrial DNA markers determine maternal lineage of both males and females. Mitochondrial DNA (mtDNA) possesses many desirable features, making it one of the most frequently used markers in diversity studies. Its abundance in the cell and existence within a double membrane organelle, the mitochondrion gives mtDNA advantages over nuclear DNA

as a lineage marker. Other advantages of mtDNA marker include; mtDNA possesses a low probability of paternal leakage, so in the event that the individual is not available for making direct comparison with a biological sample, a reference sample can be obtained from any maternally-related individual (Galtier *et al.*, 2009). The hyper variable control region whose hyper-variability is mainly through mutation rather than recombination, allows approximation of domestication localities and discrimination among individuals. Mitochondrial DNA mutates 5-10 times faster than nuclear DNA. Disadvantages of mtDNA include its lack of uniqueness as an identifier, as unrelated individuals can share an unknown maternal relative. Another disadvantage that mtDNA markers pose is the rigor and time – consumption that such analyses entail, especially in the event of degraded DNA that may require numerous rounds of amplification to generate sufficient template for sequencing (Bruford *et al.*, 2003).

The utilization of more specific DNA markers such as mtDNA for diversity studies, with less specific ones such as protein polymorphisms, MHC, RAPDs and AFLPs being recommended for answering specific questions by FAO (Food and Agriculture Organization, 2011). mtDNA has been used to explain the origins of many modern domestic livestock species. Luikart *et al.*, (2001), using D-loop hyper variable region and cytochrome b of mtDNA sequence variations observed that domesticated goats have multiple maternal centers of origin and that the lineages diverged prior to domestication.

2.4.1 Structure of mtDNA

Mitochondrial DNA is found within the mitochondria, which is the organelle responsible for cellular respiration, converting sugar into energy. Mitochondrial DNA is abundant in the cytoplasm. Mitochondrial DNA is double stranded, with two complementary strands termed heavy and light based on their Guanine (G) content. The Guanine-rich; heavy strand, encodes 28 of the 37 genes whereas the light strand encodes the remaining genes (Osellame *et al.*, 2012). The structure of mitochondrial DNA (mtDNA) as is shown in Figure 2.2, consists of two main regions: the non-coding region which controls the mtDNA and the coding region which contains the 37 genes. The coding region contains the tRNA, rRNA and protein-coding regions; control

region spans the 3^I end of the gene that code for cytochrome b, to the 5^I end of the 12S rRNA gene. The control region which is non-coding, is organized into 3 main regions: a large central block that is highly conserved and contains approximately 250 nucleotides, hypervariable region I flanked by the central conserved region and the $tRNA^{Pro}$ gene and a third region bound by the central conserved region and $tRNA^{Phe}$ gene. The origin of heavy strand replication (OH) is found in the third region. The control region spans the 3^I end of the gene that code for cytochrome b, to the 5^I end of the 12SrRNA gene. Within the hypervariable segments, there are tandem heterogenous repeats; short repeats of 20 nucleotides and long repeat of 153 nucleotides as is shown in Figure 2.2 .

The D-loop is a triple-helical structure in which a short daughter DNA strand has base-paired with the light strand and effectively displaced a portion of the heavy strand. D-loop spans the two hypervariable regions as shown in Figure 2.2 The D-loop is a mutational hotspot of the mitochondrial genome, making it particularly attractive in studying the origin and diversity of animal species because relatively short sequences can still resolve differences between closely related sequences.

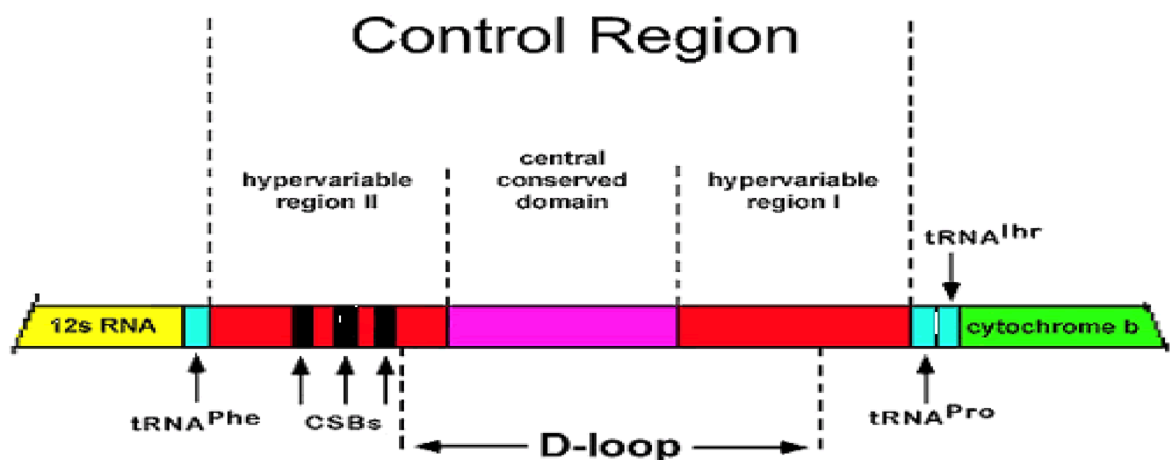

Figure 2.2: Non-coding (control) and coding regions of the mtDNA.

2.4.2 Rabbit mtDNA

SNPs in mtDNA have been widely employed in the assessment of population structure, genetic diversity, maternal ancestry and phylogeography of animals. By comparing the patterns of SNPs, scientists can group SNPs (haplotypes) together hence trace maternal ancestry. Thus various animals can be grouped into haplotypes and these inferred to their respective origins (Morin et al., 2004).

Rabbit mtDNA is a double-stranded DNA molecule that has 17,245 base pairs and 37 genes. Thirteen of these genes are essential in the synthesis of enzymes involved in oxidative phosphorylation, while 22 are essential in the synthesis of transfer RNA, (tRNA) and two in ribosomal RNA, (12s rRNA and 16s rRNA) synthesis (Mignotte *et al.*, 1990). The 13 genes that are essential in the synthesis of oxidative phosphorylation proteins include: seven Complex 1 (ND 1-6 and ND4L) subunits, one complex III (Cyt b) subunit, three complex IV (COI -III) subunits and two complex V (ATPase 6 and 8) subunits (Yang *et al.*, 2014).

The rabbit mtDNA has been extensively used to infer the origin and diversity of rabbits. Paleontological, historical and archaeological data suggest that wild rabbits originated from Southern Spain 6-6.5 million years ago, while their domestic counterparts originated from Northern Spain and other parts of Europe (Lopez-Martinez, 2008), with man playing a key role in the migration of rabbits from their original geographical locations to other worldwide locations such as Western Europe, Morocco and Western Algeria. Although the presence of rabbits in Spain was first reported by Phoenicians, it remains unknown whether they actively transported these animals there (Hardy *et al.*, 1992).

Lineages A and B are the only known recorded maternal lineages to which rabbits belong (Biju-Duval *et al.*, 1991). Lineages A and B have been characterized using mtDNA 16S rRNA and found to have seven nucleotide differences between them namely: five transitions, one transversion and one insertion (Hardy *et al.*, 1992). A study of nuclear genes has equally identified the two maternal lineages, A and B, in the rabbit population (Ferrand and Branco, 2007).

Branco *et al.* (2000), performed a phylogeography of *Oryctolagus cuniculus* in the Iberian Peninsula using RFLP markers of cytochrome b gene of mtDNA. They found 38 distinct haplotypes from 526 rabbits sampled from 20 different locations in the Iberian Peninsula and these haplotypes clustered into two distinct lineages, A and B..Moreover, RFLP analysis of the whole mtDNA molecule by Biju-Duval *et al.* (1991), Monnerot et al. (1994), and Branco *et al.* (2000) revealed two maternal lineages in the European rabbit, A and B. All domesticated rabbits belong to the second lineage, B (Monnerot, 1996) based on phylogenetic studies.

Analyses based on antibody immunoglobulin gene constant region of rabbits revealed a clear structure into two mitochondrial clades, A and B (van der Loo *et al.* 1997). Long *et al.* (2003) studied the diversity and origins of the Chinese rabbit breeds using a 700-bp fragment of mtDNA control region of 140 individuals from 20 rabbit breeds; three Chinese domestic breeds, 12 introduced breeds and five breeds that were derived from their study. Their study resolved four new haplotypes and reported that the so-called Chinese rabbits were introduced from European rabbits, hence share the same centre of origin; Europe, with European rabbits. Mitochondrial DNA has been widely utilized in the establishment of the origin and diversity of rabbits (Mougel *et al.*, 2002; Long *et al.*, 2003; Nguyen *et al.*, 2018; Emam *et al.*, 2020).

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    16sar--------------->
  1 cgcctgttta tcaaaaacat CACCTCTAGC ATTACTAGTA TTAGAGGCAC

                    RsaI
 51 TGCCTGCCCA GTGACATACG TTCAACGGCC GCGGTATCCT GACCGTGCAA
                    G

101 AGGTAGCATA ATCACTTGTT CCTTAATTGG GGACTAGCAT GAATGGCAAC

151 ACGAGGGTTA AACTGTCTCT TTCTTCCAAT CAGTGAAATT GACCTCCCCG
                                     16S3-------------
201 TGAAGAGGCG GGGATAAAAT AATAAGACGA GAAGACCCTA TGGAGCTTTA
    ---->                                           StyI
251 ATTATTTAAC CCAACACTTC CTTTATTCTA CTCTACAACG AGCCTAACTC
                                             T         -
301 AAGGAAATCC CTGGGTTAAA AATTTTGGTT GGGGTGACCT CGGAGTATAA
                                HinfI
351 ATCAACCTCC GAATGATTTT AGCCTAGACT CAACAAGTCA AAGCAATTAT
                                 C                  StyI
401 AATCATAAAT TGACCCAAAA AATTTGATCA ACGGAACAAG TTACCCTAGG
                        T
                          HinfI <-----------------16S5
451 GATAACAGCG CAATCCTATT TTAGAGTCCC TATCGACAAT AGGGTTTACG
                                      BpvI
501 ACCTCGATGT TGGATCAGGA CATCCTAATG GTGCAGCCGC TATTAAAGGT
                               C         T
                              <-------------------16sbr
551 TCGTTTGTTC AACGATTAAA GTCCtacatga tctgagttcag accgg
```

Figure 2.3: Sequence of portion of rabbit mtDNA 16S-rRNA gene. (Hardy *et al.*, 1992).

In Figure 2.3, using the 16S - rRNA of mtDNA obtained from fossil bones of rabbits located on Zembra Island in Tunisia, Hardy *et al*. (1995), sought to determine the geographical origin of the rabbits on the island and the time they were introduced there. The mtDNA 16S-rRNA gene was amplified using two universal primers; 16sar and 16sbr, while rabbit-specific primers 16S3 and 16S5 were also included to confirm the presence of the 16S-rRNA gene. The study identified 7 nucleotide differences present in lineage B mtDNA and six restriction sites namely Rsa I, Sty I (two), HinfI (two), and BpvI (Hardy *et al*., 1995). Using 16s rRNA All the bones that were studied showed an mtDNA 16s rRNA restriction pattern of the maternal lineage B, suggesting their geographical origin to be Northern Spain and Southern France. The time of introduction of these rabbits is suggested to have been between third Millennium B.C and third century A.D.

In a study on the phylogeography of the European rabbit in the Iberian peninsula, Branco et al., (2000) confirmed the partitioning of the rabbits into two mtDNA clades; A and B. Using a 1120 base pair size of the cytochrome b gene amplified from the

mtDNA, RFLP fragments were generated using eight restriction enzymes and analysed. The two clades showed a nucleotide divergence of 11.9% and were separated by 14 mutations. No recorded study has utilized mitochondrial DNA to evaluate diversity and origin of rabbits in Kenya and this present study sought to use the mtDNA molecular marker to evaluate the origins and diversity of rabbits in Kenya.

2.5 Effects of climate change on livestock production

Livestock production remains a key contributor to the global economy, with human population being projected to increase from 7.2 to 9.6 billion by 2050 (United Nations, 2019). A direct impact of increase in human population is the increase in demand for food. The livestock sector remains an attractive source of food security for many reasons. Livestock have been shown to account for 33% of global protein consumption and 17% of global kilocalorie consumption (Andersen *et al*., 1999).

Climate change results in unpredictable and increased livestock diseases both directly and indirectly. Increased temperatures increase the chances of death and morbidity in livestock, whereas the indirect effects are attributed to the negative impact that climate change has on microbial communities such as pathogens and parasites. Microbial communities experience accelerated growth rates outside of their host, which has a negative effect on livestock since these same microbes are the causative agents of diseases in livestock (Van De Steeg *et al*., 2009).

Heat stress of an animal is experienced in its body as a physiological response to high ambient atmospheric temperature (Abdel-Hamid and Dawod, 2015). Livestock tend to keep their body temperature within a range of ±0.5 ^{o}C, which is termed as the thermal comfort zone (Charoensook *et al*., 2012). Upper critical temperature ranges vary depending on the type of species. Heat stress is temperature increase beyond the upper critical temperature of the thermal comfort zone. Animals have varying responses to heat stress, with the main adaptation being acclimation of the livestock's endocrine, excretory, respiratory, circulatory and nervous system responses. As a result of acclimation to heat stress, the livestock species experience reduced performance due to decreased feed intake, increased water intake and a change in the physiological functions such as low milk production and decreased efficiency in feed conversion

(Nardone and Valentini, 2000). The degree to which these animals adapt determines their tolerance to the environment and this adaptation has a genetic basis.

2.5.1 Effect of heat stress on rabbits

While climate change is a global phenomenon, its negative impacts are more severely felt by poor people living in developing countries who, for their survival, rely greatly on agriculture and livestock keeping. Agriculture is the most climate-sensitive economic sector (Van De Steeg et al., 2009). High environmental temperatures induces physiological stress in rabbits, leading to losses in productivity (Bani *et al.*, 2005). The "thermal-neutral zone" for rabbits is between 15-25 ^{o}C (Cervera and Carmona, 1998). Rabbits have been shown to tolerate low temperatures better than high temperatures. Typically, at temperatures above 35^{o}C, rabbits experience heat stress. Frangiadaki *et al.* (2003), compared the performance of does under high and moderate temperatures. In this study, they showed that ambient temperatures higher than rabbit's thermos-neutral zone lead to an increase in still-born rabbits and a decrease in total and born live rabbits. High ambient temperatures also led to a decrease in the number of weaned rabbits and an increase in pre-weaning mortality. This study was able to also show that high ambient temperatures negatively affected rabbit's reproductive systems, led to low feed intake resulting in impairment of milk production.

Piles *et al.* (2012), assessed lactating doe and the impact of high environmental temperature on doe live weight, daily milk yield, average daily feed, average daily water consumption and milk composition namely crude protein, dry matter and gross energy. They grouped lactating rabbits into two environmental conditions. In one room, the temperature was within the range of rabbit thermo-neutral zone of 18-22 ^{o}C, and the second room simulated heat stress at a minimum temperature of 24 ^{o}C and maximum of 30 ^{o}C for at least two hours. This study showed no significant differences for average daily water consumption, daily milk yield and milk consumption in these two groups of lactating rabbits. However, higher environmental temperatures resulted in decreased average daily feed consumption (-9.4%) and a decrease in live weight by 6.2%. The findings from this study suggested that these rabbits acclimatized to

temperature conditions beyond their thermoneutral zones by reducing feed intake rather than water intake.

Abdel-Hamid and Dawod, (2015) investigated the effect of long-term heat stress of temperatures ranging between 30 - 32 °C on the growth performance and carcass traits in 3 rabbit breeds; New Zealand White, Californian and Rex, with an aim of assessing the ideal breed to be utilized in the hot Egyptian climatic conditions. In this study, three growth parameters; body weight, body weight gain and average daily gain, alongside carcass traits were assessed among the two sexes over a ten-week 'fattening period'. New Zealand White and Rex rabbit breeds of both sexes experienced high weight gain and average daily weight gain at 10 weeks' duration of 303 ± 15.8 and 346 ± 15.4 grams respectively, as compared to Californian rabbits that had 249 ± 15.8 grams over the entire fattening period under heat stress. This was despite Californian rabbits having higher body weight at the beginning of the fattening period until the eighth weeks, that is, 1334 ± 36.2 versus 1289 ± 24.3 and 1332 ± 24.7 grams in New Zealand White and Rex rabbit breeds respectively. The gain in weight was attributed to compensatory growth. In assessing the Carcass traits, the dressing out, which is the percentage difference in the warm carcass weight and the shrunk live weight, was assessed. Californian rabbits had a significantly higher dressing out percentage of 60.03 ± 0.37 as compared to New Zealand White with 58.46 ± 0.33 and Rex with DO% of 58.43 ± 0.35.

2.5.2 Climatic conditions in study areas

Kenya is a tropical country lying along the equator with diverse geographical and environmental conditions and is divided into seven distinct agro-ecologic zones (Sombroek *et al*., 1982). Agro-ecologic zone I is humid, II sub humid, III semi-humid, IV semi humid to half arid, V semi-arid, VI arid and VII very arid. Eighty percent of Kenya's landmass is categorized as arid and semi-arid land (ASAL), and various agro-ecologic zones support livestock farming in Kenya. Arid counties experience 85-100% aridity, whereas semi-arid counties are categorized as two, with one category experiencing 30-84% aridity and the other 10-29% (WFP, 2018). MoLD (2010) reports that the rabbit population is approximately 600, 000 with rabbits being

abundant in Central, Western and Rift Valley regions. Rabbits for this study were sampled from both ASAL and non ASAL counties in the Eastern, Western and Central regions of Kenya. Kitui, Machakos, Makueni and Laikipia counties, occurring in the eastern and central regions of Kenya are semi arid counties, whereas Vihiga, Kakamega, Bungoma, Kitui and Nyandarua are found in non ASAL regions of the country. Because of climate change consequences, species and breeds of livestock that are well adapted may become more widely used. Several adaptation and mitigation measures to combat the effects of climate change on livestock have been adopted. These include modification of livestock management systems, breeding strategies, policy and institutional changes and changing the perception of farmers towards climate change (Rojas-Downing *et al*., 2017). Traits associated with thermal tolerance and disease resistance are among the desirable characteristics recommended for breeding purposes (Hoffmann, 2010).

Agricultural diversification systems include diversifying animals and crops and the integration of livestock systems with forestry and crops. In the diversification of animals and crops, livestock production can be increased when the animals are exposed to heat stress and this is an equally effective way of combating climate-change related diseases by utilization of crops that can better withstand the various extremes of temperatures (Waha *et al*., 2018). Agroforestry has also been shown to aid greatly in maintaining the balance between the protection of the environment, agricultural production and carbon sequestration. This has been shown to result in an improvement in the quality of water, soil, biodiversity and modification of pests and diseases, while in the long run improving nutrient recycling (Jose, 2009).

The effects of high temperatures on highly productive breeds which are usually unable to withstand extremes of temperatures such as heat stress can be alleviated by the utilization of high external inputs. These inputs include cooling systems and the adjustment of the diets of such animals to reduce production of metabolic heat (Pilling and Hoffman, 2011). In a study of how climate control strategies can be optimized in rabbit houses, Estellés *et al*. (2012). identified the factors that need to be controlled; air velocity, temperature, humidity and gas concentrations (carbon dioxide and ammonia). In this study, four structures that were considered representative of the

usual rabbit buildings were assessed for air velocity patterns and their effects on the temperature in the buildings. It is well established that temperature ranges exceeding the optimal limits for animals affect rabbits' reproductive and productive abilities, resulting in reduced litter size, and increases in mortality (Frangiadaki *et al*., 2003). Air distribution in rabbit buildings was found to be affected by two factors namely; location of the inlets in the building and the orientation of the building with respect to the wind direction.

Heavy external inputs are applicable where there are enough resources to aid in their implementation. However, in instances where these are not available, such as in resource – poor settings as low and middle income countries such as Kenya, well-adapted animals are likely to be adopted to aid in combating effects of climate change (van de Steeg and Tibbo, 2012).

2.6 Livestock adaptation measures to climate change

2.6.1 Genetic approach to combat heat stress in livestock

Understanding the genetic basis of physiological response to heat is critical for improved production efficiency and welfare of livestock (Coble *et al*., 2014). In cows, the variation in productive traits has been linked to variation in genetics. Pritchard et al. (2013) estimated the heritability in milk yield in cows under heat stress to be 30%. In another study, Dikmen *et al*. (2012), determined the heritability of rectal temperature in cows under heat stress, which ranged between 0.13-0.17; implying that 13-17% of the variation in the rectal temperature of these cows under heat stress was because of variations in genetics of the cows. There is a genetic association between heat stress and rectal temperature, where under the same dry bulb temperature of 32.2 °C, rectal temperatures vary from 38.3 °C to 40.05 °C an indicator that some cows regulated their rectal temperatures more efficiently than others (Dikmen and Hansen, 2009).

A major disadvantage of selecting cows for heat stress is that these cows poorly tolerate cold stress. As a result, in areas where cold temperatures are more extreme, such cows would not be able to survive. Heat-stressed cows also have low milk yields.

This would essentially mean selection against high milk yield, in the selection of cows that are heat tolerant (Hansen, 2013).

Research areas on the impact of genetics in beef cattle have mainly focussed on three areas namely: genetic by environment interactions, mating systems and identification and utilization of animals that are well adapted to environments of extreme temperatures either using traditional genetic approaches or using genomic technologies (Rolf, 2015). Using this technique, animals that can be stable across all environments have been preferred. The *Bos indicus* cattle are well adapted to humid environments with high heat due to the fact that they have low levels of milk production and slow growth rates, resulting in low metabolic rates and less internal heat. However, in crosses of *Bos Taurus* and *Bos indicus*, the *Bos indicus* cattle have been shown to outperform *Bos Taurus* cattle. The superiority of Bos indicus can be helpful in the breeding industry by utilization of cross breeds of cows (Gaughan, 2009).

Environmental factors such as temperature has been shown to influence gene expression in rabbits (Jung and Sang, 2007). The C gene is responsible for pigmentation in rabbits fur, skin and eyes. This gene has been shown to be inactive above 30 ºC, leading to white rabbit fur. Below 20 ºC, rabbits are pigmented on their ears, nose and feet (Lobo, 2008).

2.6.2 HSP 70 gene as a candidate gene for heat tolerance

Under conditions of environmental stress, a cell stops or slows down most of its biological functions such as the synthesis of DNA, RNA and proteins, and transport processes. However, one unique set of proteins; the heat shock proteins (HSPs) are rapidly and preferentially expressed by all animals in response to stresses such as extremes of temperature, cellular energy depletion, extreme ionic concentration and toxic substances (Schlesinger, 1990). Cellular stress response was first reported in *Drosophila busckii* salivary glands which were exposed to high temperature. Larvae salivary glands that were exposed to a temperature shock of 30 °C or more showed a distinct change in the puffing pattern (Ritossa, 1962). Other stressors that have been shown to induce the expression of heat shock proteins include chemical stressors such

as water. Land snail *Codringtonia species* showed a positive correlation between the rate of water loss and HSP70 protein levels (Kotsakiozi *et al*., 2015). This study found that harsh environmental conditions enabled the animals to adapt by maintaining high levels of HSP 70.

The expression of heat shock proteins in response to stress is known as heat response. Heat shock response is characterized by cessation of normal protein synthesis, transcription of heat shock genes, translation of mRNA-encoding heat shock proteins and selective synthesis of heat shock proteins (Velichko *et al*., 2013). Living organisms have been shown to respond to environmental stresses, including elevated temperatures, by synthesizing a set of proteins known as heat shock proteins (Verghese *et al*., 2012). Other biochemical and cellular features that accompany this heat shock response include the overall decrease in ongoing protein synthesis, the nuclear translocation of heat shock proteins and the collapse of cytoskeleton elements (Blagosklonny, 2001).

Heat shock proteins are highly conserved and categorized as families based on their molecular weight and sequence homology, ranging from 15 – 110 kilo Daltons (Sorensen *et al*., 2003). These families are classified as HSP 110, HSP 100, HSP 90, HSP 70, HSP 60, HSP 40, HSP 10 and other small HSP families. Among the family of heat shock proteins, HSP70 family has been shown to have a significant role in thermotolerance (Beckham et al., 2004). This family of proteins has been shown to be the most phylogenetically conserved and to have the highest performance in response to heat stress. HSP 70 is commonly utilized in most studies as an indicator of stress (Manzo *et al*., 2012). The major families of heat shock proteins and their major roles are presented in Table 2.1**.**

Table 2.1: Major families of molecular chaperones

Chaperone families	Major functions of chaperone families
Small heat shock proteins (HSP27)	Prevent aggregation of other proteins by collecting protein garbage
HSP 60 family	Assistance in protein folding and refolding
HSP 70 family	Assistance in protein folding and refolding
HSP 90 family	Stabilize substrate proteins and maintain their active, or inactive state, prevent aggregation of other proteins by collecting protein garbage
HSP 100 family	Desegregation of proteins

HSPs mainly function as molecular chaperones. The chaperoning role entails binding to and stabilization of unstable proteins, facilitation of folding, assembly and translocation of newly synthesized proteins, disposal of other proteins and the prevention of aggregation of protein intermediates. Molecular chaperones also prevent the improper folding and denaturation of proteins during cellular stresses hence protecting cells from toxic effects of heat and other stressors (Kregel, 2002). The protein-folding processes in most cellular compartments is performed by the cellular machinery comprising HSP 70 chaperones and co-chaperones (Miller and Fort, 2018). HSPs have also been shown to play a dual role in apoptosis by either promoting or inhibiting apoptosis (Kennedy *et al.*, 2014). Heat shock factors act as transcription factors, turning the heat shock protein genes on or off.

HSPs, specifically HSP70, have also been shown to have negative impacts on *Drosophila*. *Drosophila* engineered to produce extra copies of HSPs show slower growth rates compared to control cells, with the growth rate accelerating when the cells pump out the HSP70 from their cytoplasm (Xiao *et al.*, 2019). Furthermore, following heat shock, *Drosophila* embryos have been shown to rapidly remove HSP70 from their cells because at high concentrations, heat shock proteins can be toxic and alter the functions of cells (Jagla *et al.*, 2018).

A family of transcription factors known as heat shock factors (HSF) mediate the expression of heat shock protein messenger RNA. In resting cells that are not under heat stress, HSF-1 forms a complex with some heat shock proteins such as HSP 70 and

90. Upon encountering a stressor, there is a rapid increase in damaged proteins and this destroys the HSF-HSP complex, liberating the HSF. HSF-1 then undergoes trimerization, nuclear translocation and phosphorylation prior to binding to nucleotide segments in the promoter region of the heat shock protein gene called heat shock elements. This binding initiates the transcription of the heat shock protein RNA and is facilitated by a polymerase enzyme. This mechanism is illustrated in Figure 2.4 below.

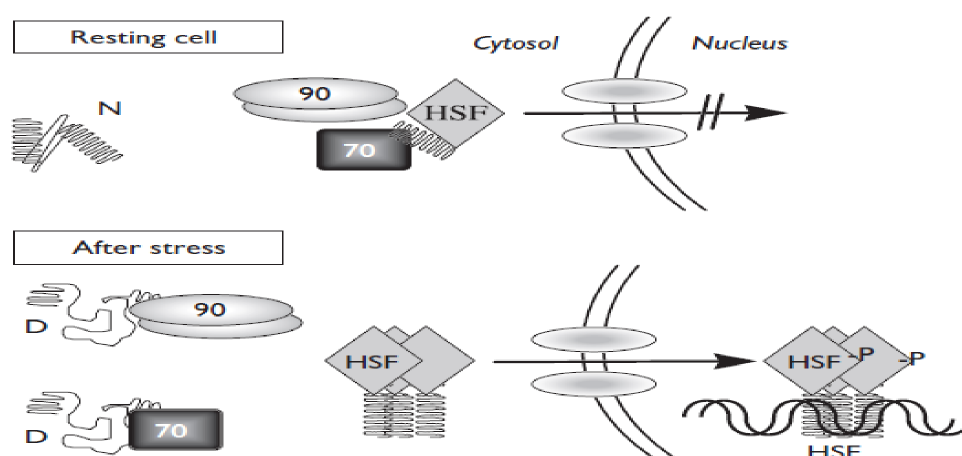


Figure 2. 4 Mechanism of induction of heat shock factors during stress.

Abbreviations: 70, HSP 70; 90, HSP 90; D, denatured protein; HSE, heat shock element, HSF, heat shock factor; N, native protein; P, phosphorylation of heat shock factor during stress. (Adapted from Schlesinger, 1990)

Molecular markers such as SNPs related to livestock adaptability are now commonly being utilized in marker-assisted selection. It has been suggested that the selection of animals with superior genetic potential enables them better adapt to climate change helping minimize problems associated with heat stress (Baena *et al*., 2018). HSF 1 and HSPA6 are functional candidate genes that have been associated with heat stress in Angus cattle. Baena *et al*., (2018) sequenced and characterized SNPs related to adaptation to heat stress in Angus cattle. In this study, 72 Angus cows in a wet sub-tropical climate experiencing mean annual temperatures of 19.9 C and raised in a feedlot system were left under the sun for the maximum possible duration of time. Twenty of the 72 cows were selected as phenotypic representatives of adaptability traits namely respiratory rate and coat temperature. HSF1 and HSPA6 genes were

amplified and assessed for the presence of SNPs. This study identified 12 SNPs in the HSF1 gene, with 8 being mapped to coding regions and silent, and 4 being in the intron with no characterized function. HSPA6 was found to have 6 SNPs all located in the exon, with two resulting in synonymous mutations and four having non-synonymous mutations where SNP1 changed amino acid alanine to serine, SNP2 histidine to glutamine, SNP3 replaced isoleucine with phenylalanine and SNP5 changed proline to alanine. The eight silent SNPs in HSF1 gene failed to change the HSP70 protein amino acid sequence, and this study concluded that even synonymous mutations can affect protein expression levels through mechanisms such as modification of the open reading frame and promotion of alternative RNA splicing (Curi, 2004). SNPs mapped to intergenic regions have been suggested to be regulatory mutations that do not alter amino acid sequences. Overall, the study concluded that the SNPs that were identified could have an important role in function, formation and expression of heat shock proteins.

Charoensook *et al*. (2012), in a study of polymorphisms in the bovine HSP90AB1 gene in Thai indigenous cattle subjected to heat stress, identified breed-specific physiological responses to heat stress. Forty-seven healthy and non-lactating female cows of *Bos indicus* breeds White Lamphun; Mountain cattle; and cross breeds of Holstein Friesian * Thai native breed were kept in a farm under natural conditions and assessed for physiological observations of heat stress namely respiratory rate, rectal temperature, pack cell volume and heat tolerance coefficient during the morning, and afternoon hours. From this study, they proposed that HSP90AB1 gene is a candidate gene for heat tolerance, proposing its use as a genetic marker in the selection of appropriate breeds for hot climate.

HSP70 gene polymorphisms have also been associated with semen characteristics in Iraqi Holstein bulls during summer and winter (Habib *et al*., 2017). The semen of 30 Holstein bulls was collected using an artificial vagina and evaluated for quality by assessing their mass motility, abnormalities and live-to-dead sperms. The HSP70 gene was amplified, sequenced and analysed. Three haplotypes, A, B and C were identified. Haplotype A was a silent mutation at position 6 C < G, haplotype B had mutations at 114 G < A, 1451 C < A, 1590 A < G, 1695 C < T and 1719 G < T, with all mutations

being silent except for the 1451 C < A which was a missense mutation. Haplotype C was identical to haplotype B except for the absence of the missense mutation. In associating these SNPs to sperm quality, the study resolved that the missense mutation had a positive effect on sperm characteristics, with all haplotypes experiencing low quality semen during the hot seasons, while haplotype B had better semen mass motility (P <0.05) as compared with haplotypes C and A. The study suggested that the missense mutation can be an effective marker in the selection of bulls to be used in breeding programs and artificial insemination.

HSP 70-1 has been characterized in goat (*Capra hircus*) with the gene and protein sequences compared to other mammalian species namely cattle, buffalo, yak, sheep, pig, horse and human (Gade *et al*., 2010). This study revealed a high percent homology in the heat shock protein genes of these animals and identified the role of the amino and carboxyl termini in the chaperoning, folding and unfolding of improperly folded proteins.

Another member of the heat shock protein 70 family, heat shock 70kDa protein (HSPA6) and heat shock transcription factor 1 (HSF1) genes which encode for the heat shock proteins have been associated with the reduction of cell damage due to heat stress. These genes have been mapped to bovine chromosomes 3 and 14 respectively (Zimin *et al*., 2009). Bhat *et al*., (2016) sequenced a 295 bp fragment of HSP70 gene and sequenced the same in 64 Tharparkar cattle. Their study identified a SNP with G > T substitution, which was responsible for a change in amino acid aspartate to tyrosine in allele A. The study concluded that this polymorphism was a potential determinant for heat tolerance in cattle.

2.6.3 Rabbit heat shock protein 70 gene

The rabbit (*Oryctolagus cuniculus*) heat shock protein 70 is encoded by the gene HSPA2 of family A, member 2. This gene is in chromosome 12; 2306 base pairs and has 1 exon. The promoter spans the first 138 base pairs of the gene, with the coding sequences spanning 138 – 2060 base pairs; XM_008262601.2. Figure 2.5 below shows the location of the gene coding for heat shock protein 70 in the rabbit genome (NCBI).

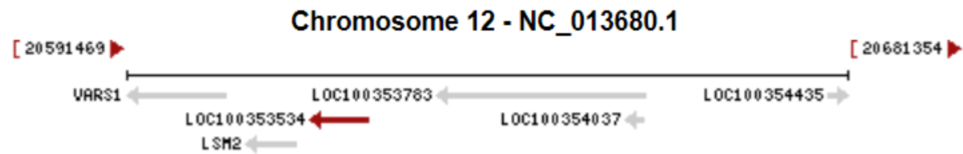


Figure 2. 5: Location of heat shock protein 70 gene (LOC100353783) on chromosome 12. Source (NCBI)

Rex rabbits exposed to chronic heat stress were shown to have elevated levels of HSP 70 in their testis, compared to control rabbits that were not subjected to heat stress, evidence that the HSP70 was induced by heat stress. In the same study by Manzerra *et al*. (1996), the HSP70 levels were partially restored to normal levels after an additional 9-week recovery period. This study suggested that elevated expression of HSP70 in heat stress was because of the protective role this protein plays in preventing cellular damage (Pei *et al*., 2012).

Heat shock cognate 70 (HSC70) is a heat shock protein from the 70kDa stress protein family (Calabrese *et al*., 2000). Both heat shock protein 70 and heat shock cognate 70 belong to the molecular chaperone family and are co-expressed under stressful conditions, with Hsc70 being a constitutively-expressed protein and Hsp70 a stress-inducible protein. In a study that confirmed that Hsc70 and Hsp70 contribute to antimony – tolerance in *Leishmania* protozoan parasite, both Hsc70 and Hsp 70 were shown to be co-induced in cosmid banks transfected with two copies of Hsp 70 and 1 copy of Hsc70 (Brochu *et al*., 2004). High HSC70 expression-levels has been observed in neurons, specifically the hipoccampus and Purkinje neurons of the cerebellum (Belay and Brown, 2006). New Zealand rabbits have a body temperature of 39.6 ± 2ºC, with hyperthermic rabbits showing an elevated increase in this temperature by 3.4 ± 2ºC. Hyperthermic rabbits were shown to have different levels of the stress-inducible HSP70 and the constitutively expressed HSC70 in different tissues following heat stress. New Zealand white rabbits were exposed to elevated temperatures so their body temperatures were elevated by 2-3 °C and assessed for expression of HSC70 and HSP70. Both HSP70 and HSC 70 were elevated in elevated temperature conditions, with HSP70 induction being greatest in non-neural compared to tissues of the nervous

system, while HSC70 was greater in neural tissues, suggesting that high levels of HSC70 in neural tissues plays a role in the modulation of levels of HSP induction. Overall, this study confirms the induction of heat shock proteins during cellular stress response (Manzerra *et al*., 1996).

Climate change remains a problem in the livestock sector and livestock that are well adapted to the extremes of temperature will greatly improve the productivity in the livestock sector. Considering the importance of HSP 70 in conferring thermotolerance to animals, this present study sought to evaluate the adaptation to climate change by assessing the potential role of heat shock protein 70 gene in conferring heat tolerance among the rabbits found in areas with elevated temperatures. This was done by assessing polymorphisms that are present in HSP 70 gene in domesticated rabbits that could be associated with particular geographic regions based on the temperature profiles of those regions.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

Clearance for this research was granted by NACOSTI under permit number NACOSTI/P/15/62961/8524 (Appendix 1). Local rabbits used for the study were sampled from farmers practicing backyard small-scale rabbit farming in the households in selected counties in Kenya. The regions sampled are based on the climate profiles and are listed in Figure 3.1.

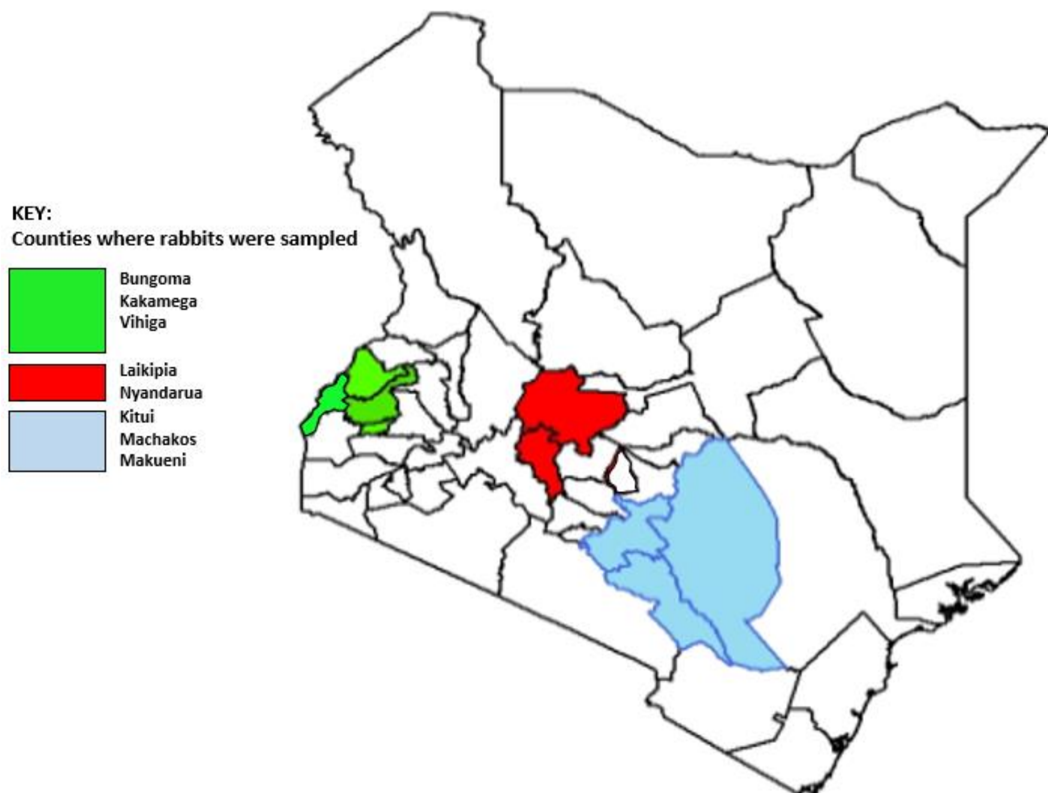


Figure 3.1: Map of Kenya showing the study areas

3.2 Study design

Random sampling by eyeballing was adopted during the field surveys conducted during this study. Two mature rabbits more than 6 months old; a male and a female whenever possible were selected for sampling from each homestead. In addition, five exotic rabbit breeds were also sampled from Ngong breeding station as shown in Table 3.1. Population genetic studies require sampling of 25 -30 individuals per population for the accurate determination of allele frequencies and based on this criterion, blood samples were collected from 25 individuals per population which were the sampled regions namely Machakos, Makueni, Kitui, Nyandarua, Likipia, Vihiga , Bungoma and Kakamega (Hale *et al*., 2012).

Table 3. 1: Number of rabbits that were sampled from each county

County	Sample size
Vihiga	12
Kakamega	16
Bungoma	12
Machakos	18
Makueni	12
Kitui	8
Nyandarua	16
Laikipia	16
Forest wild	2
Ngong breeding station	6
Total	**118**

3.3 Data collection

The selected rabbit was restrained, and blood was drawn from the marginal vein of the ear. The needle was jabbed, and blood drawn to a 75% filling of the 10mls syringe. All the blood was applied on four spots on a labelled FTA classic card (Whatmann Biosciences) (Gutiérrez-Corchero *et al*., 2002) for each rabbit and allowed to dry under a shade. The FTA cards were packaged in paper envelopes with silica gel desiccant, transported to the laboratory at Jomo Kenyatta University of Agriculture and Technology, and stored in a dark room at room temperature until when used for DNA extraction.

3.4 Molecular analysis

3.4.1 DNA extraction for mtDNA and HSP70

DNA extraction was carried out following the chelex protocol (Walsh et al,. 1991). Three 5mm discs were punched out of the FTA filter paper (Whatman Bioscience) using a Harris uni-core tool (Gutiérrez-corchero, 2002). The discs were placed in a labelled 1.5ml eppendorf tube labeled according to the rabbit identity. Five percent chelex (wt: vol) was prepared by weighing 5grams of chelex and adding it to 100mls distilled water followed by vortexing. DNA extraction from the chelex was performed by measuring 180µl of the chelex using a pipette and dispensing into each Eppendorf tube. The tubes were heated to 99°C on a heat block for ten minutes with centrifugation. The tubes were spun at a high speed for a minute, ensuring that all the contents were at the bottom of the tube and the supernatant was drawn from each tube and placed into clean Eppendorf tubes. The supernatants were used as DNA template. Prior to utilization for PCR, the DNA concentration was determined on a nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) and the working concentrations were standardized to 50ng/µl using the C1VI formula in a total volume of 25 µl using distilled water as the diluent.

3.4.2 mtDNA PCR amplification, purification and sequencing

A 450-base pair region of mtDNA D-loop region, flanking the tRNA – Pro gene control region was amplified using forward 5’CCACCATCAGCACCCAAAGCTG3’ and reverse 5’TGGGCCCGGAGCGAGAAGAG3’ primers (Rane and Barve, 2011). PCR amplifications were carried out in 25 µl reaction volume containing 2.5 µl of 125ng DNA, 5 µl 5X Q5 buffer, 2 µl 10mM dNTPs, 1.25 µl each10µM forward and reverse primers, 12.4 µl PCR grade water and 0.6 µl (2U/µl) Q5High Fidelity DNA polymerase (New England Biolabs). Amplification conditions were set as follows: - Initial denaturation at 95ºC for five minutes, 35 cycles of 95ºC for 15 seconds, 60ºC for one minute and 72ºC for two minutes, and a final extension at 72ºC for seven minutes on a PEQLAB thermocycler. A 1.5% agarose gel was then prepared by weighing 1.5 grams agarose powder and dissolving in 100mls 1X Tris Acetate EDTA (TAE) buffer. The agarose was heated in a microwave for approximately 3 minutes,

until complete dissolution of the agarose powder. The dissolved agarose was cooled briefly under room temperature and the gel was stained using 2 µl (10mg/ml) ethidium bromide by pipetting the ethidium bromide into the dissolved agarose and swirling for complete dissolution. The gel was cast on a tray pre-fixed with combs and allowed to solidify. Upon complete solidification, the gel was placed in a gel tank and flooded with 1X TAE buffer. The combs were then gently removed, and the amplicons loaded using loading dye 1X New England Biolabs. The gel was run at 80 volts for 1 hour and visualized under UV light. The amplicons were purified using Zymogen DNA & Concentrator ™ - 25 kit protocol (ZYMO RESEARCH) following the manufacturer's instructions. The concentrations of the final purified products were standardized to at least 50 ng/ µL using the C1VI formula in a total volume of 25ul using distilled water as the diluent, packaged and sent for sequencing at Macrogen Company, (Amsterdam, Netherlands). mtDNA sequencing was done using a single primer only in one orientation.

3.4.3 HSP 70 PCR amplification, purification and sequencing

A 359-base pair region of HSP70 gene, located in exon 1, chromosome 12, was amplified by PCR. PCR amplifications were carried out in 40µl total reaction volumes containing 150ng of template DNA, 8µl 5X Q5 buffer, 8µl 10 mM dNTPs, 1µl 10µM forward 5'AACCGCACCACGCCCAGCTACG3' and reverse 5'TGCGAGTCGTTGAAGTAGGCC3' primers (inqaba biotec South Africa), 25.8µl PCR grade water and 0.4µl Q5 High Fidelity DNA polymerase (2U/µl). Amplification was carried out in PEQLAB thermo cycler. Thermo-cycling conditions were as follows: - Initial denaturation 95ºC (5min), 40 cycles of 95ºC (15seconds), 60ºC (30 seconds) and 72ºC (30seconds) and a final extension at 72ºC (7minutes). The amplicons were run on a 1.5% agarose gel containing 1 µg/ml ethidium bromide and visualized under UV light using gel documentation system (SynGene). The amplicons were purified using Zymogen DNA & Concentrator ™ - 25 kit protocol (ZYMO RESEARCH) following the manufacturer's instructions. The concentrations of the final purified products were standardized to at least 50 ng/ µl using the C1VI formula in a total volume of 25 µl using distilled water as the diluent, packaged and sent for

sequencing at Macrogen Company, (Amsterdam, Netherlands). HSP70 gene sequencing was done using both forward and reverse primers.

3.5 Data analysis

3.5.1 mtDNA data analysis

3.5.1.1 Sequence editing, haplotype generation and sequence alignment

The raw sequences for the amplified fragments from Macrogen were edited using CLC Main Workbench 7.9.1 (www.clcbio.com) by inputting the raw sequences into the software. This was followed by viewing the chromatograms and base qualities and manual trimming of the poor reads based on their quality scores, resulting in a final consensus sequence. Sequence editing resulted in a sample size of 118. The poorly resolved sequences were discarded. Subsequent comparisons and analyses were restricted to a 263-basepair region incorporating the hyper variable segment I (HVSI), from position 15,595 to 15, 858. Using DnaSP version 5.10.01 (Librado & Rozas, 2009), haplotypes were derived from the 118 sequences. Haplotype distribution in the various regions was tabulated. The haplotypes were aligned using Clustal W in MEGA 7.0.14 (Tamura *et al*., 2013) against *Oryctolagus cuniculus* mitochondrion complete genome (accession number NC_001913.1) to find regions of similarity. For comparison, 17 mtDNA sequences of wild and domesticated rabbits (Appendix 2) from several locations (Long *et al*., 2003) were included in data analysis.

3.5.1.2 Phylogenetic analysis of mtDNA haplotypes and network profiles

A phylogenetic tree was constructed using the maximum likelihood tree algorithm as implemented in MEGA 6.0 (Tamura *et al*., 2013) with 1000 bootstrap replications to provide for confidence in branching order. The model used for mtDNA was Hashegawa Kishino Yano with five discrete gamma categories. The affinity of the domesticated rabbits to other rabbits was revealed by the maximum likelihood tree incorporating 17 rabbits, both wild and exotic species, downloaded from GenBank. The final tree was viewed and edited by FigTree version 4.1.2. To test the robustness of the phylogenetic analyses, sequence clusters were detected by the analysis of

phylogenetic networks using uncorrected p-distances on the pylogenetic splits decomposition network on SplitsTree4 version 4.17.1 (Huson and Bryant, 2006). The resultant phylogenetic networks formed the basis of validation of the haplotypes.

To investigate possible relationships among the haplotypes and link the haplotypes to presumed domestication centers, a median joining network was constructed using the Network version 5.0.0.0.0 software package (Bandelt, Foster & Rohl, 1999). Network analysis included the reference sequences downloaded from Genbank and the ancestral sequence, wild rabbits from Iberian Peninsula.

3.5.1.3 mtDNA population variability and structure

Diversity was determined by calculating number of polymorphic sites, haplotype diversity and nucleotide diversity using DNaSP 5.10.01 (Librado & Rozas, 2009). Population genetic structure was tested using analysis of molecular variance (AMOVA).The groupings used for AMOVA were among groups, among populations within groups and within populations. The groups are eastern, western and central while populations are the various counties listed in Table 3.1 The statistical significance of the AMOVA results was based on 1000 permutations (Excoffier and Lischer, 2010).

3.5.2 HSP70 data analysis

3.5.2.1 Sequence editing, haplotype generation and alignment of HSP70 sequences

The raw sequences of the amplified fragments were trimmed using CLC Main Workbench 7.9.1 (www.clcbio.com) and edited on CLC Sequence Viewer 5.1.1 program (www.clcbio.com) by inputting the raw sequences into the software. This was followed by viewing the chromatograms and base qualities and manual trimming of the poor reads based on their quality scores, resulting in a final consensus sequence. Subsequent analyses were restricted to a 253-basepair region located in the coding sequence and exon 1, from position 261-513. Using DnaSP version 5.10.01, haplotypes were derived from the 83 sequences generated on CLC using DnaSP

Version 5.10.01 (Librado & Rozas, 2009). Frequency of the various haplotypes in the different populations was tabulated. The haplotypes were aligned using Clustal W in MEGA 7.0.14 (Tamura et al., 2013) against the reference sequence, the predicted *Oryctolagus cuniculus* heat shock 70 kDa protein 1 like mRNA (accession number XM_008262601.2). The haplotypes were then translated in the 6 reading frames using Expert Protein-Analysis System (ExPASy) software (Gasteiger et al., 2003) and the most biologically informative frame was used to perform a multiple sequence alignment of the HSP70 protein sequences.

3.5.2.2 Phylogenetic analysis of HSP70 haplotypes and network profiles

A multiple sequence alignment of the haplotypes using the MUSCLE algorithm in MEGA was first done. A phylogenetic tree involving the HSP70 haplotypes that were obtained in the study regions, the *Oryctolagus cuniculus* HSP70 reference sequence (accession number XM_008262601.2) and *Oryctolagus cuniculus* HSP70 gene paralog was constructed using the maximum likelihood tree algorithm as implemented in MEGA 7.0.14 (Tamura *et al.*, 2013) following 1000 bootstrap replications. The model used for inference of this evolutionary history was Hashegawa Kishino Yano with 5 discrete gamma categories. The affinity of domesticated rabbits to other mammalian species was revealed by the maximum likelihood tree incorporating one HSP70 paralog downloaded from GenBank. To test the robustness of the phylogenetic analysis sequence clusters were detected using uncorrected p-distances on the pylogenetic splits decomposition network on SplitsTree4 version 4.17.1 (Huson and Bryant, 2006). The resulting phylogenetic network diagrams were used for haplotype validation.

3.5.2.3 Population genetic diversity of HSP 70

Genetic variation (number of polymorphic sites, haplotype diversity (H) and nucleotide diversity) for each population were calculated using DnaSP. The population genetic structure was assessed by calculation of the nested analysis of molecular variance (AMOVA) based on among groups, among populations within groups and within populations AMOVA groupings using Arlequin 3.5.22 software. The statistical

significance of the AMOVA results was based on 1000 permutations (Excoffier & Lischer, 2010)

CHAPTER FOUR

RESULTS

4.1 Characterization of the origins, population structure and genetic diversity of rabbits in Kenya using mtDNA D-loop

4.1.1 mtDNA PCR amplification

The amplified DNA gave good quality amplicons using the primer pairs that targeted the D-loop region. All the 118 samples were successfully amplified. The amplicons were run on a 1.5% agarose gel. The target amplified fragment size was 450 base pairs. Water was used as a negative control. The gel images below are from representative regions. In Figure 4.1, amplicons 1-14 represent selected rabbit populations from Bungoma county, while 15-18 represent selected rabbit populations from Kitui county In Figure 4.2, amplicons 146-156 represent selected rabbit populations from Kakamega , amplicons 100, 119 and 120, Vihiga, while 122-145 represent selected rabbit populations from Nyandarua. In Figure 4.3, amplicons 51-72 represent selected rabbit populations from Machakos, while 73-90 represent selected rabbit populations from Kirinyaga.

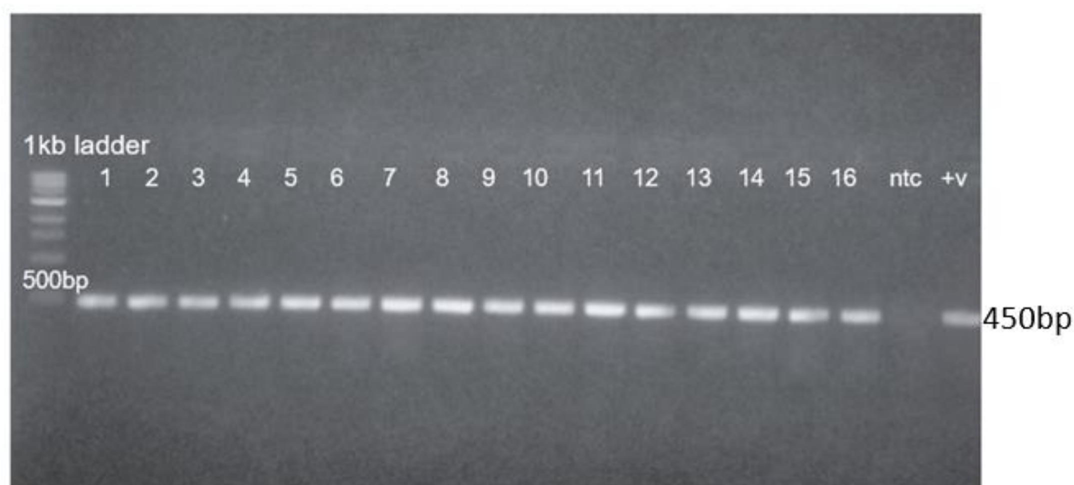


Figure 4.1: Amplified products of mtDNA D-loop region from Bungoma and Kitui

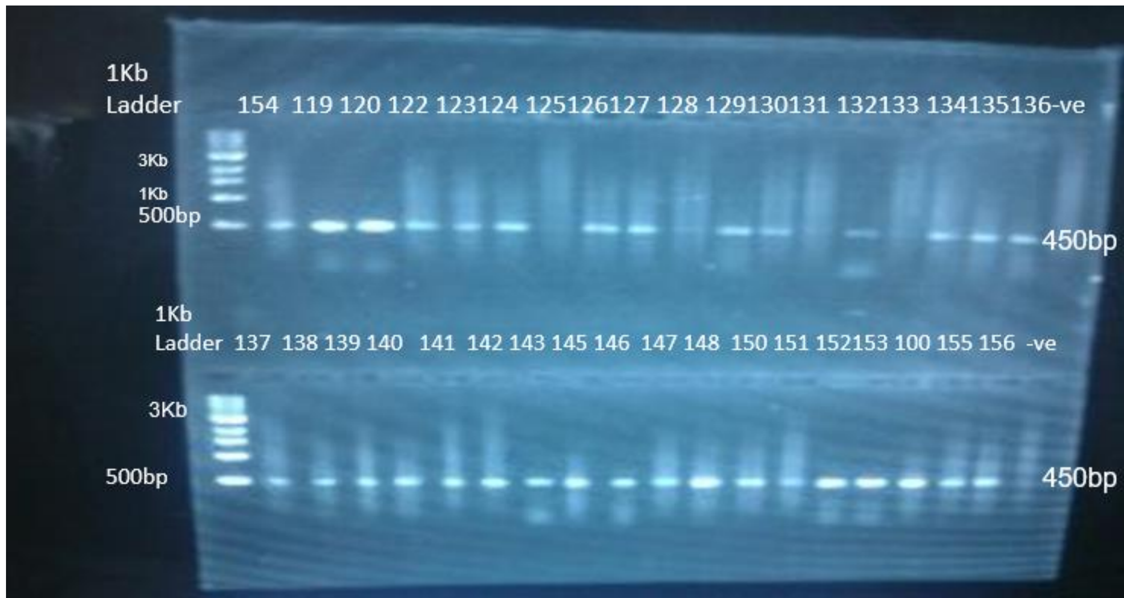


Figure 4. 2: Amplified products of mtDNA D-loop region from Kakamega, Vihiga and Nyandarua

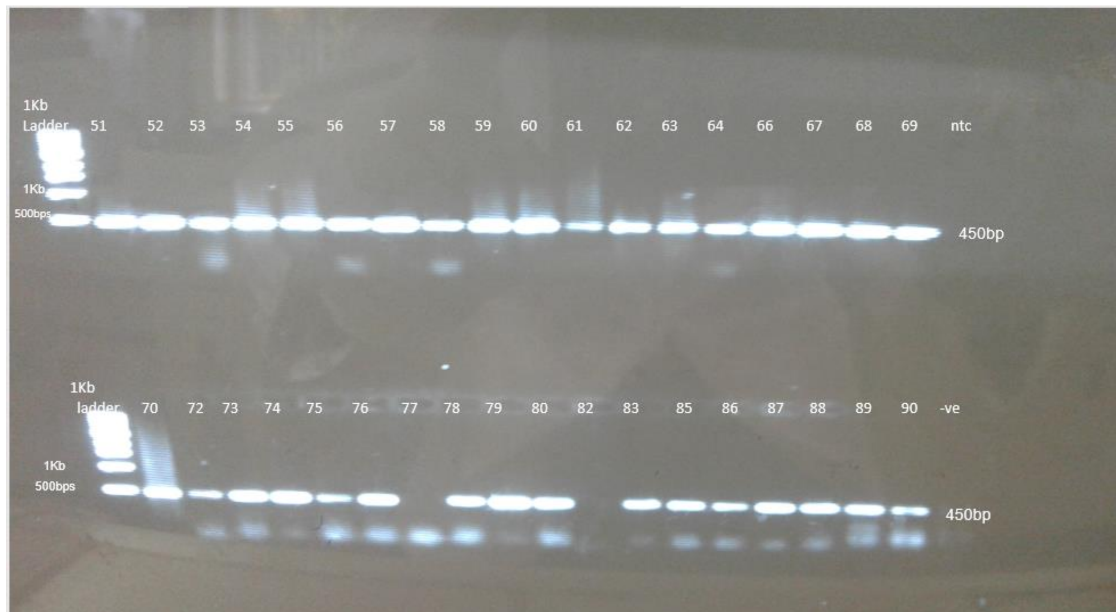


Figure 4.3:Amplified products of mtDNA D-loop region from Machakos and Kirinyaga

4.1.2 Sequence editing

mtDNA sequences were edited using CLC Sequence Viewer 5.1.1 program (www.clcbio.com). Figure 4.4 shows sequence chromatograms. The upper

chromatogram is of haplotype 7, being represented by a rabbit sample from Makueni, whereas the lower chromatogram is of haplotype 1, being represented by an exotic breed, Flemish Giant White, from Ngong breeding station. Both haplotypes show variation at position 317 where haplotype 1 has a Cytosine, whereas haplotype 7 has a Tyrosine, at the same position.

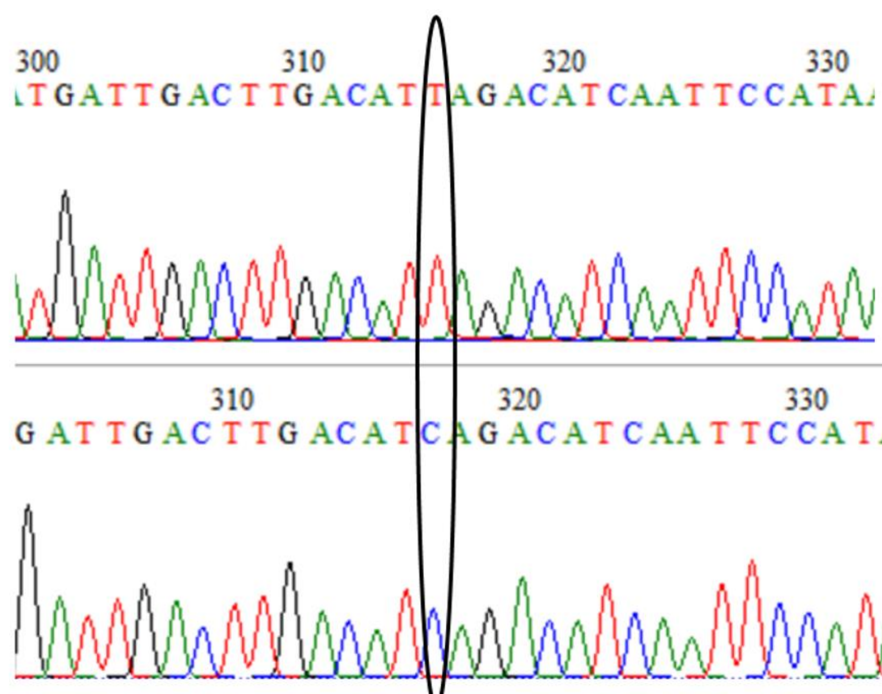


Figure 4. 4: Sequence chromatogram showing variable regions

4.1.3 Distribution of mtDNA haplotypes in the agroecologic zones

A total of 8 haplotypes were detected, with the haplotype distribution per agroecologic zone being presented in the Table 4.1. Haplotype 1 was found in the pure breed from Ngong, Flemish Giant White US which was sampled from Ngong breeding station. Haplotype 4 was found in 3 counties namely Kakamega (agroecologic zone II), Nyandarua (agroecologic zones II and IV) and Laikipia (agroecologic zone V and VI respectively), whereas haplotype 8 was found in 2 counties; Bungoma (agroecologic zone II) and Machakos (spanning agroecologic zones IV , V and VI). Haplotypes 2, 5 and 6 were the least frequent haplotypes and occurred in Vihiga (agroecologic zone II), Nyandarua and Machakos counties respectively. Haplotypes 3 and 7 occurred in all the sampled agroecologic zones.

Table 4.1: Haplotype distribution in the sampled rabbits from agroecologic zones

County/ Haplotype	Vihiga	Kakamega	Bungoma	Kitui	Machakos	Makueni	Nyandarua	Laikipia	Stock	Total
H1	0	0	0	0	0	0	0	0	1	1
H2	2	0	0	0	0	0	0	0	0	1
H3	6	12	2	2	7	1	3	7	0	8
H4	0	1	0	0	0	0	1	1	0	3
H5	0	0	0	0	0	0	1	0	0	1
H6	0	0	0	0	1	0	0	0	0	1
H7	5	3	7	6	8	11	12	9	0	8
H8	0	0	2	0	1	0	0	0	0	2
Total	**3**	**3**	**3**	**2**	**4**	**2**	**4**	**3**	**1**	**25**

4.1.4 Sequence alignment of reported haplotypes and reference sequences

Alignment of the 8 haplotypes and the reference sequences (Appendix 2) is shown in the Figure 4.5. The accession numbers for the reference sequences are tabulated in Appendix 2. The alignment confirmed the first 5 SNPs that distinguished the 8 haplotypes, with SNP 1 being located at position 182 having an Adenine in all the haplotypes except haplotype 1 that had a Guanine at that position. Haplotype 1 is the exotic breed from Ngong breeding station, Flemish Giant White, and it is identical to the reference sequences with accession numbers AF003190, AF003195, AJ293838, AJ293842 and AJ293840. The second SNP was at position 196, Adenine. It distinguishes haplotype 8 from all the other haplotypes and reference sequences, with the other haplotypes and reference sequences having a Thymine base. The third SNP at position 235 had haplotypes 2, 7 and 8 having Thymine bases and the rest of the haplotypes having Cytosine bases. Haplotypes 2, 7 and 8 were identical at this position to the reference sequences; AF003189, AF003192, AF003193, Z83346, Z83365, U62924, U62926, AJ293843, AJ293832, AJ293841, AJ293844, AJ293836, AJ293835, AJ293834, AJ293839, AJ293833 and AJ293837. The remaining haplotypes 1, 3, 4, 5 and 6 had a Cytosine base at this position and were identical at this position to the remaining reference sequences. The fourth SNP at position 252, a Cytosine, was unique to haplotype 1 corresponding to the reference sequences from the pure breeds; AF003190, AJ293840, AJ293842 and AJ293838. The rest of the haplotypes had a Thymine base at this position. The fifth SNP at position 256 was a Cytosine base unique to haplotypes 7 and 8, with the rest of the haplotypes having a Thymine base at this position. Overall, alignment of the eight haplotypes and published sequences of the European rabbit revealed 24 unique haplotypes. This study has obtained five new haplotypes (H3, H4, H5, H6 and H8) whereas the other three haplotypes (1, 2 and 7) are identical to previously published haplotypes (Long et al., 2003).

Figure 4.5: Variation among the 8 haplotypes with reference sequences from GenBank.

4.1.5 Phylogenetic analysis of mtDNA haplotypes

4.1.5.1 Maximum likelihood tree of mtDNA and reference populations

Inference of the rabbit origins on the unrooted Maximum-likelihood tree in Figure 3.4 incorporating the eight haplotypes , reference sequences (Appendix 2) and an outgroup identified two lineages, A and B, with lineage A composed of wild rabbits in Iberian Peninsula and one in Spain and lineage B comprising domestic rabbits. Lineage B was identified based on the clustering of all the sampled rabbits with reference populations that were identified as belonging to lineage B (Long et al., 2003). This study obtained 4 new haplotypes, H3, H4, H5 and H6. Haplotypes 1, 2 and 7 were reported in studies by Long et al. (2003), while haplotype 8 was highly identical with English (AJ293835) It can be observed that haplotypes 3, 4, 6 and 5 clustered uniquely on the phylogenetic tree, forming polytomies (Fig 4.6). Overall, all the sampled rabbits belong to lineage B.

4.1.5.2 A splits decomposition network of mtDNA with reference populations

Figure 4.7 shows a splits decomposition network of the 8mtDNA haplotypes, reference sequences and the wild rabbits outgroup. The algorithm that was utilized was uncorrected_P Neighbornet with equal angle. The distantly related wild rabbit from Iberian Peninsula is the farthest on the network, and the new haplotypes that were discovered by this study, namely haplotypes 3, 4, 5 and 6 cluster together, validating the phylogenetic tree groupings

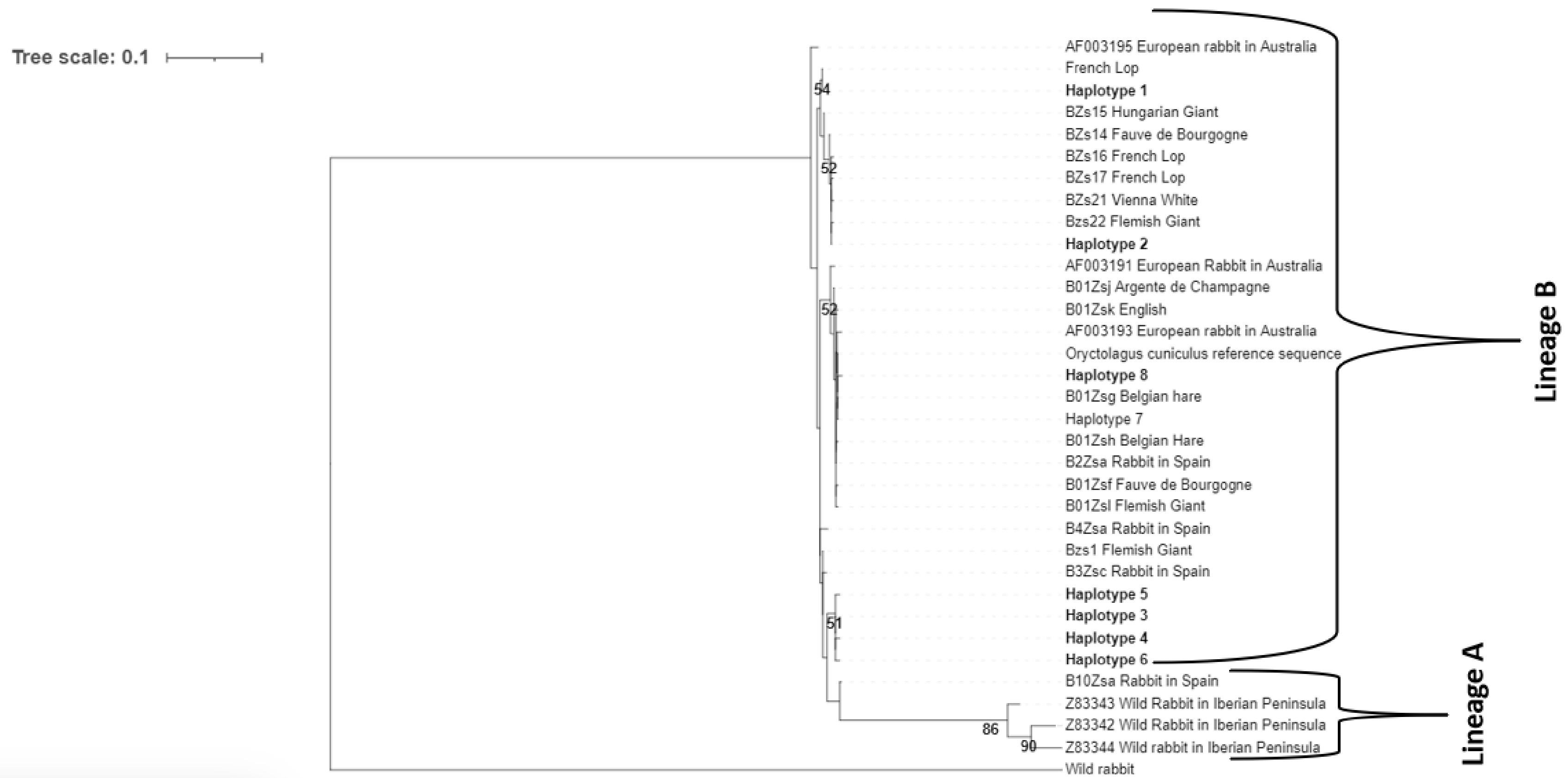


Figure 4.6: Classification of the evolutionary relationships on a Maximum Likelihood (ML) tree

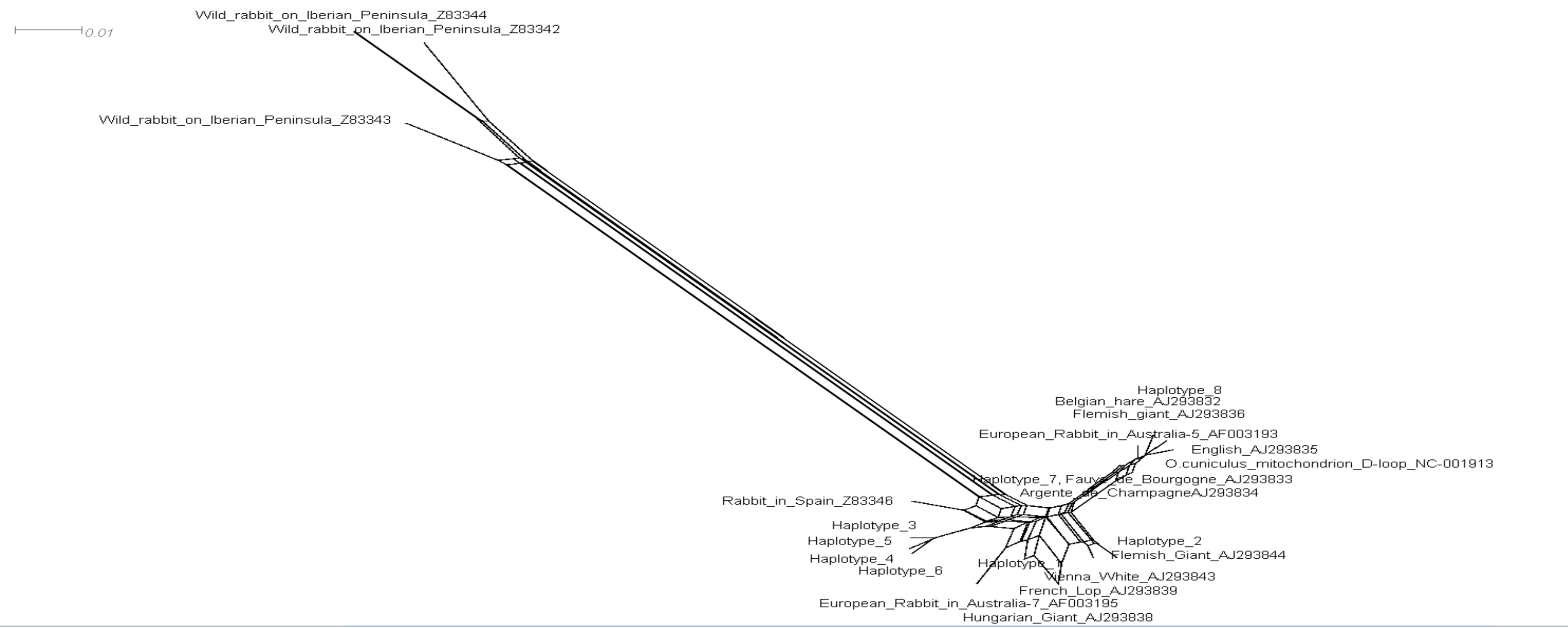


Figure 4.7: A splits decomposition network showing phylogenetic analysis of the 8mtDNA haplotypes , the reference sequences and the outgroup derived from GenBank.

4.1.5.3 Relationships of mtDNA haplotypes with reference populations

In the network profile shown in Figure 4.8, each circle represents one haplotype with the size being proportional to the number of individuals Branched between the haplotypes represent substitutions (mutation steps). The study rabbits, reference sequences and ancestral sequences comprising wild rabbits from Iberian peninsula generated a total of 17 haplotypes. Haplotypes 15, 16 and 17 represent the 3 wild rabbits samples from Iberia. The largest haplotypes were haplotype 2 and 3, comprising 66 and 40 rabbits.respectively and occurred in all the agro-climatic zones. The third largest haplotype was haplotype 1 comprising 5 rabbits, three exotics and two from western region followed by haplotypes 5 and 6 each with 3 rabbits from central and western and eastern and western regions respectively. All the other haplotypes each comprised one rabbit. The rabbits from the 8 haplotypes inferred from this study occurred in the largest haplotypes inferred on the haplotype network.

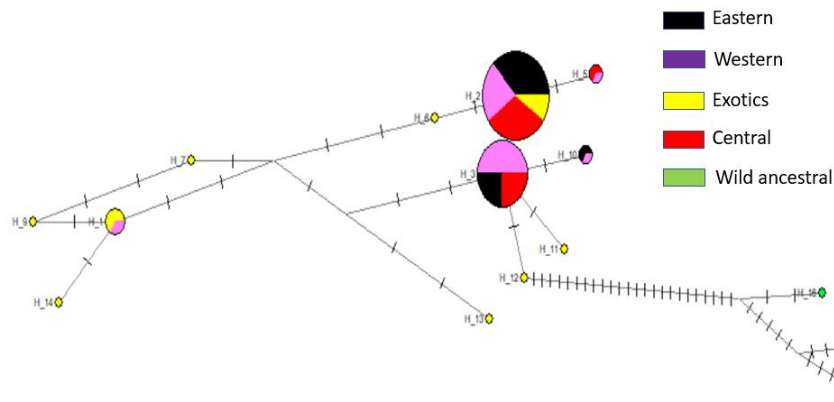


Figure 4. 8: Network analysis illustrating relationships of the mtDNA haplotypes

4.1.6 Genetic variation of rabbit mtDNA

Overall, a total of 17 variable sites; 16 transitions and 1 transversion, were identified in all the sampled rabbits. The number of polymorphic sites that were observed, number of haplotypes per region, haplotype diversity (Hd) and nucleotide diversity (π)

as calculated using DnaSP revealed that Machakos and Nyandarua counties had the highest number of haplotypes, 4, whereas Vihiga, Kakamega, Bungoma and Laikipia counties each had 3 haplotypes. Makueni and Kitui counties had the least number of haplotypes, 2 each. Haplotype diversity was least in Makueni county (0.16667) and the same had the least nucleotide diversity of 0.00577. the highest haplotype (0.66667) and nucleotide (0.02041) diversities were in Vihiga county. Generally, the regions with the least haplotype diversities had a corresponding low nucleotide diversity, and those with the highest haplotype diversities had corresponding high nucleotide diversities as shown (Table 4.2).

Table 4.2: Number of polymorphic sites and haplotypes, haplotype and nucleotide diversities in the various sampled regions.

County	Sample size	Number of polymorphic sites	Number of haplotypes	Haplotype diversity	Nucleotide Diversity
Vihiga	12	11	3	0.66667	0.02041
Kakamega	16	10	3	0.42500	0.01173
Bungoma	12	10	3	0.58182	0.01259
Machakos	18	11	4	0.63971	0.01923
Makueni	12	9	2	0.16667	0.00577
Kitui	8	9	2	0.42857	0.01484
Nyandarua	16	11	4	0.49265	0.01618
Laikipia	16	10	3	0.58088	0.01878

4.1.7 Molecular variation of the mtDNA haplotypes

The variations in mtDNA haplotypes among groups, among populations within groups and within populations as assessed using analysis of molecular variance (AMOVA) revealed -2.39% variation among groups and 85.3% variation within populations. There was 17.1% variation among populations within groups. The most significant variation existed within populations P value 0.00196 ± 0.00136; followed by among populations within groups; P value 0.00391 ± 0.00185 and finally among groups; P value 0.52688 ± 0.01673 (Table 3.6).

Table 4.3: Variation accounting for population genetic structure of rabbit mtDNA haplotypes

Source of variation	d.f	Sum of squares	Variance components	Percent of variation	P value
Among groups	2	11.647	-0.05599	-2.39	0.52688±0.01673
Among populations within groups	5	36.740	0.39994	17.10	0.00391±0.00185
Within populations	103	205.541	1.99554	85.30	0.00196±0.00136

4.2 Characterization of the genetic polymorphisms in Heat Shock Protein 70 gene for heat tolerance.

A total of 83 DNA samples gave successful amplification of the HSP70 gene 359-base pair segment using gene-specific forward and reverse primers. The amplicons were run on a 1.5% gel.

4.2.1 HSP70 PCR amplification

The target fragment size was 359 base pairs. Water was used as a negative control. The gel images below are from representative regions. In Figure 4.9, amplicons 1-14 represent selected rabbit populations from Vihiga county. The gel image in Figure 4.10 has amplicons from Kitui county while Figure 4.11 shows amplicons of representative samples from Kakamega county.

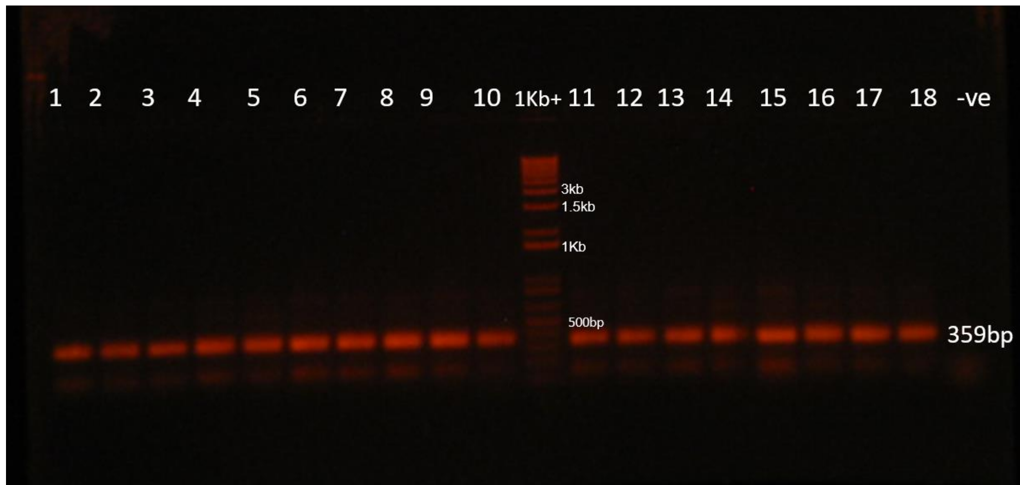


Figure 4.9: Amplified products of HSP70 gene samples obtained from Vihiga county.

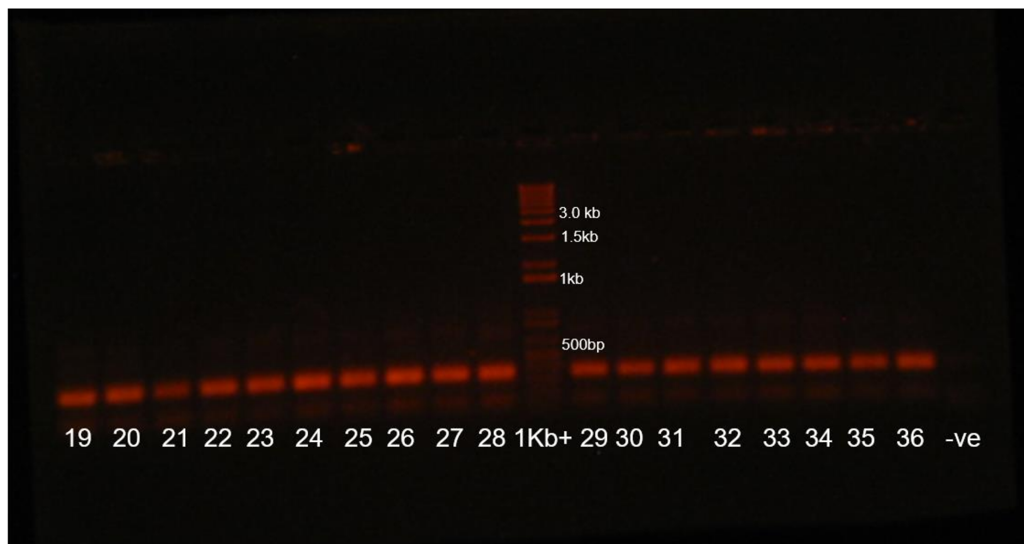


Figure 4.10: Amplified products of HSP70 gene samples obtained from Kitui county.

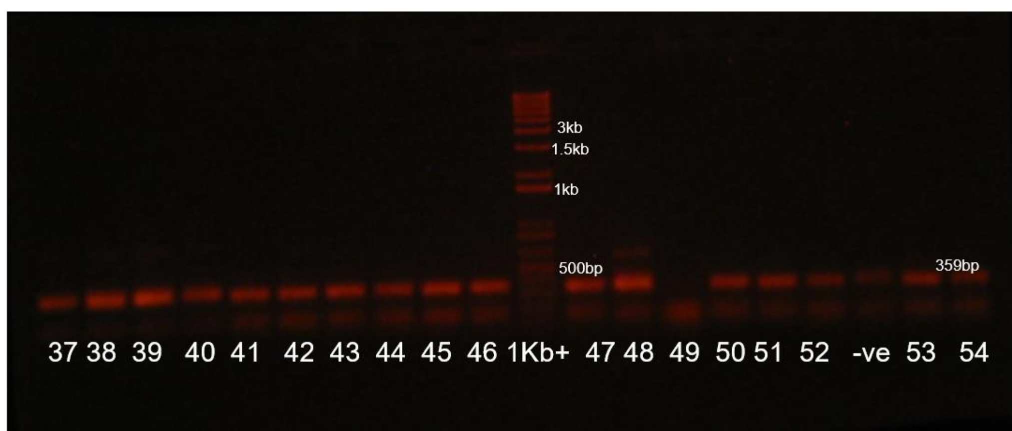


Figure 4.11: Amplified products of HSP70 gene samples obtained from Kakamega county.

4.2.2 Sequence editing and haplotype generation

The raw forward and reverse sequences were viewed and edited on CLC sequence viewer 5.1.1 program (www.clcbio.com), resulting in a 253-base pair sequence. A total of 6 haplotypes were identified. Haplotype 1 (CCATTTAG), Haplotype 2 (TCATTTAG), Haplotype 3 (TTATTTAG), Haplotype 4 (CCGGCCGG), Haplotype 5 (CCATTCAG), Haplotype 6 (CCATTTAC).

4.2.3 Distribution of HSP70 haplotypes in the various sampling regions

A comparison of haplotype distribution across all populations showed that only haplotype 1 occurred across all populations and it was the most abundant, present in 78 rabbits. Haplotypes 2 was present in 1 female rabbit from Bungoma. Haplotype 3 occurred in one male rabbit from Bungoma, whereas haplotype 4 was present in 1 male rabbit from Machakos county. Haplotype 5 was present in 1 female rabbit from Machakos county, whereas haplotype 6 was present in 1 male rabbit from Laikipia county (Table 4.4).

Table 4. 4: Haplotype distribution across agroecologic zones

Haplotype	Vihiga n=8	Kakamega n=6	Bungoma n=8	Kitui n=4	Machakos n=15	Makueni n=4	Nyandarua n=16	Laikipia n=9	Kirinyaga n=11	Breeding Stock n=2	Total
Hap 1	8	6	6	4	13	4	16	8	11	2	78
Hap 2	0	0	1	0	0	0	0	0	0	0	1
Hap 3	0	0	1	0	0	0	0	0	0	0	1
Hap 4	0	0	0	0	1	0	0	0	0	0	1
Hap 5	0	0	0	0	1	0	0	0	0	0	1
Hap 6	0	0	0	0	0	0	0	1	0	0	1
Total	8	6	8	4	15	4	16	9	11	2	83

4.2.4 Sequence alignment of reported haplotypes and reference sequences

An alignment of the six reported haplotypes and the reference sequence obtained from GenBank is shown in Fig 4.12. The alignment shows the first two SNPs that distinguished the 6 haplotypes, with SNP 1 located at position 277 and having a Cytosine base in all the haplotypes except for haplotypes 2 and 3 with a Thymine base at this position. The second SNP is at position 302 where all haplotypes have a Cytosine base at this position except haplotype 3 with a Thymine base. Haplotype 1 was the most abundant and occurred in all the agro climatic zones. This suggests that haplotype 1 could be the haplotype conserved across all the agro-climatic zones. The haplotypes containing the mutation 277 (C/T) were haplotypes 2 and 3, whereas the mutation 302 (C/T) occurred in haplotype 3 only. All these haplotypes were found in agro climatic zone II. Haplotype 4 occurred across agro climatic zones IV and V and contained the mutations 362 (A/G), 365 (T/C), 386 (T/G), 401(T/C) and 430(A/G). The mutation 431(T/C) occurred in haplotype 5, which was also found in agro climatic zones IV and V, while 506 (G/C) occurred in haplotype 6, occurring across agro climatic zones V and VI. Overall, these results suggest that the 9 mutations observed in this study occurred in all the haplotypes except haplotype 1, with haplotype 4 containing most of the mutations.

4.2.5 Multiple sequence alignment of HSP70 haplotype and reference sequence protein sequences

An alignment of the translated nucleotide sequences of the 6 haplotypes showed variation at two positions, with haplotypes 2 and 3 having an Isoleucine substitution for Threonine in the rest of the haplotypes at the exact position, while haplotype 4 had a Serine substitution for Asparagine in the exact location in the rest of the haplotypes (Fig 4.13).

Species/Abbrv	Sequence
1. XM 008262601 (GenBank Reference sequence).	A G C T A C G T G G C C T T C A C C G A C A C G G A G C G G C T G A T C G G C G A C G
2. Haplotype 1	A G C T A C G T G G C C T T C A C C G A C A C G G A G C G G C T G A T C G G C G A C G
3. Haplotype 2	A G C T A C G T G G C C T T C A T C G A C A C G G A G C G G C T G A T C G G C G A C G
4. Haplotype 3	A G C T A C G T G G C C T T C A T C G A C A C G G A G C G G C T G A T C G G C G A T G
5. Haplotype 4	A G C T A C G T G G C C T T C A C C G A C A C G G A G C G G C T G A T C G G C G A C G
6. Haplotype 5	A G C T A C G T G G C C T T C A C C G A C A C G G A G C G G C T G A T C G G C G A C G
7. Haplotype 6	A G C T A C G T G G C C T T C A C C G A C A C G G A G C G G C T G A T C G G C G A C G

Figure 4. 12: Multiple sequence alignment of the 6 haplotypes with a reference sequence from GenBank

Species/Abbrv	Sequence
1. RABBIT (XM 008262601.2) REFERENCE SEQUENCE	S Y V A F T D T E R L I G D A A K N Q V A M N P Q N T V F D A K R L I G R K F N D P V V Q S D M K L W P F Q V I N
2. Haplotype1 HSP70	S Y V A F T D T E R L I G D A A K N Q V A M N P Q N T V F D A K R L I G R K F N D P V V Q S D M K L W P F Q V I N
3. Haplotype2 HSP70	S Y V A F I D T E R L I G D A A K N Q V A M N P Q N T V F D A K R L I G R K F N D P V V Q S D M K L W P F Q V I N
4. Haplotype3 HSP70	S Y V A F I D T E R L I G D A A K N Q V A M N P Q N T V F D A K R L I G R K F N D P V V Q S D M K L W P F Q V I N
5. Haplotype 4 HSP70	S Y V A F T D T E R L I G D A A K N Q V A M N P Q N T V F D A K R L I G R K F N D P V V Q S D M K L W P F Q V I S
6. Haplotype 5 HSP70	S Y V A F T D T E R L I G D A A K N Q V A M N P Q N T V F D A K R L I G R K F N D P V V Q S D M K L W P F Q V I N
7. Haplotype 6 HSP70	S Y V A F T D T E R L I G D A A K N Q V A M N P Q N T V F D A K R L I G R K F N D P V V Q S D M K L W P F Q V I N

Figure 4. 13: Multiple sequence alignment of HSP70 amino acid sequences with a reference sequence from GenBank

4.2.6 Phylogenetic analysis of HSP70 haplotypes

4.2.6.1 Maximum likelihood tree showing variation of HSP70 and reference populations

A phylogenetic tree inferring the relationship between the HSP70 haplotypes, rabbit HSP70 reference sequence and HSP70 homologs selected mammalian HSP70 sequences was constructed. The six haplotypes clustered together with the rabbit HSP 70 reference sequence obtained from the database, confirming that the haplotypes had sequence and evolutionary closeness to the reference sequence. The HSP70 gene of the Oryctolagus cuniculus HSP70 Paralog 4 retrieved from Genbank was used to root the tree.The tree is based on Maximum Likelihood algorithm with 1000 bootstrap values based on Hashegawa Yano Kishino model as shown in Figure 4.14.

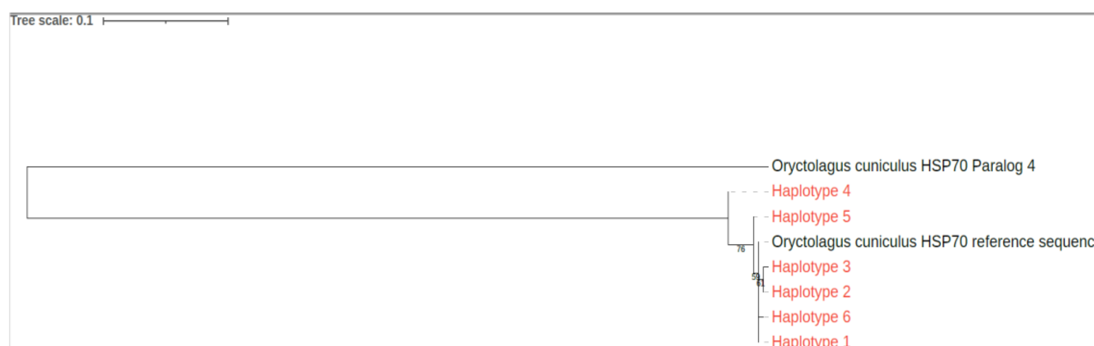
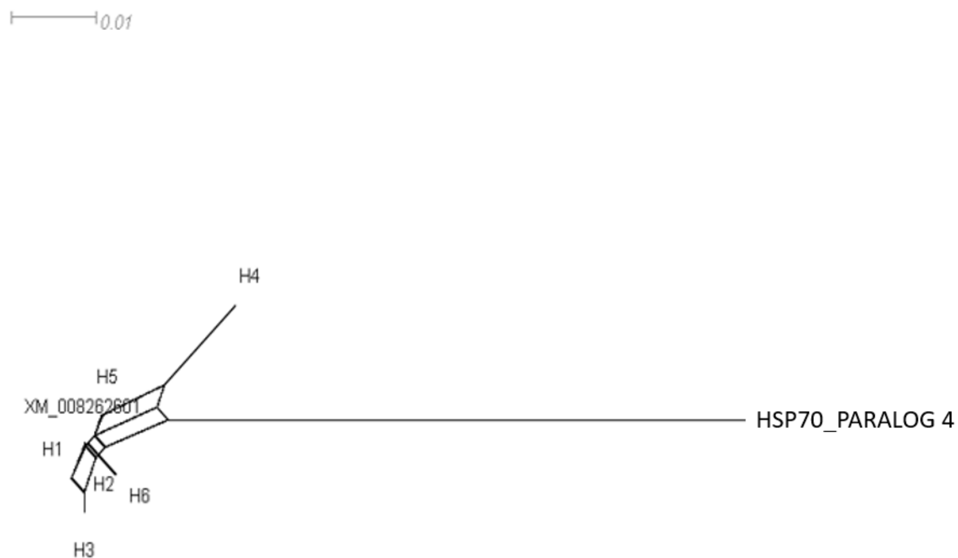


Figure 4. 14: Maximum likelihood tree showing evolutionary relationships of *Oryctolagus cuniculus* HSP70 gene

4.2.6.2 A splits decomposition network of HSP70 with reference populations

The 6 HSP70 haplotypes clustered with the reference sequence (XM_008262601) and were distant from the HSP70 paralog as shown in Figure 4.15.



Algorithm Fit=97.802 Taxa=22 Chars=468 [Characters >Uncorrected_P>NeighborNet>EqualAngle]

Figure 4. 15: A splits decomposition network showing phylogenetic analysis of the HSP70 haplotypes and HSP70 homolog

4.2.7 Genetic variation of rabbit HSP70

4.2.7.1 Genetic diversity and haplotype diversity

Based on the assessment of haplotype and nucleotide diversity in the gene coding for heat shock protein 70 (HSP70)., Kitui, Makueni, breeding stock rabbits sampled from Ngong breeding station, Kakamega, Vihiga, Kirinyaga and Nyandarua populations were identical Laikipia reported a nucleotide diversity of 0.00088 and Bungoma had the highest diversity of 0.00268 (Table 4.6). Neutrality tests, Tajima's D and Fu and Li's test was negative and not significant for all the individual populations and in the populations grouped regionally. Out of the six haplotypes that were reported, a total of 9 polymorphic sites were identified. The polymorphic sites were at positions 277 (C/T), 302 (C/T), 362 (A/G), 365 (T/C), 386 (T/G), 401(T/C), 430(A/G), 431(T/C), 506 (G/C).

Table 4. 5: HSP 70 diversity in rabbits from all the sampling regions

Region	n	No of haplotypes	Hd	Pi (nd)	Tajima's D	Tajima's Significance
Bungoma	8	3	0.4643	0.00268	-0.4479	P > 0.10 (NS)
Kakamega	6	1	0.0000	0.000	N/A	N/A
Vihiga	8	1	0.0000	0.000	N/A	N/A
Kirinyaga	11	1	0.0000	0.000	N/A	N/A
Laikipia	9	2	0.2222	0.00088	-1.0882	P > 0.10 (NS)
Nyandarua	16	1	0.0000	0.000	N/A	N/A
Kitui	4	1	0.0000	0.000	N/A	N/A
Machakos	15	3	0.2571	0.00361	-1.76624	P > 0.10 (NS)
Makueni	4	1	0.0000	0.000	N/A	N/A
Breeding stock	2	1	0.0000	0.000	N/A	N/A

4.2.7.2 Molecular variation of the HSP70 haplotypes

The source of variance at the molecular level was calculated among the groups (sampled regions, Eastern, Western and Central), among the populations within the groups (sampled counties within the regions) and within the populations (within the individuals in the various populations). The low F_{ST} of 0.00003 suggested a lack of population structuring in this population, and these results support the F_{ST} results as is shown in Table 4.7.

Table 4. 6: Analysis of molecular variance of HSP70 in the populations

Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation Indices	P value
Among groups	3	0.466	0.00211Va	1.59	FCT=0.01593	0.372±0.0162
Among populations within groups	6	0.690	-0.00211Vb	-1.59	FSC = -0.01616	0.448±0.01401
Within populations	73	9.664	0.13238Vc	100.00	F_{ST}=0.00003	0.394±0.00894

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Origins, population structure and genetic diversity of rabbits in Kenya using mtDNA D-loop

5.1.1 mtDNA D-loop sequence variability and haplotype distribution patterns

This study resolved a total of 8 haplotypes. The unique haplotypes identified in this study; 3, 4, 5 and 6 have not been previously reported in literature. Sequence alignment of the eight haplotypes alongside the reference sequences revealed that haplotypes 5 and 6 each had one rabbit that were from Nyandarua and Machakos counties respectively. These results suggest that eastern region, specifically Machakos county, had the new haplotypes identified in this study. This could be attributed to breeding for desired traits among the rabbit farmers that could have led to the emergence of these unique haplotypes. Another possible reason could be that these new haplotypes are adapted to the high annual temperatures in Eastern region where Machakos county is located compared to the other counties.

Haplotypes 3 and 4 were identical to the rabbits in Spain used as the reference sequences. These two haplotypes could be genetically identical breeds as those rabbits in Spain and could have the same domestication origins. The haplotype with the most number of rabbits, haplotype 7, being the most common haplotype that had equally been reported in earlier studies Long *et al.* (2003), could have been introduced to Kenya as a result of the programme with the German government that saw Kenya popularize rabbit keeping in the 19th century (MoLD, 2010). Haplotype 1 had only 1 rabbit; Flemish Giant White, which was obtained from Ngong breeding station, and was identical to the reference haplotype, French Lop. These are both exotic breeds and confirmed that the rabbits in Ngong breeding station are pure breeds. The four rabbits in haplotype two were from International Livestock Research Institute, Vihiga and 1 exotic breed, California White. This suggests that this haplotype consists of mainly pure breeds, and that the rabbit that was sampled from Vihiga could be a pure breed. It is equally important to note that the rabbits that were designated as wild rabbits from

Kenya belonged to haplotype 7, and this is an evidence of domestication by the Kenyan rabbit farmers.

Two indices for evaluation of mtDNA variation and genetic diversity of populations or breeds are haplotype diversity (Hd) and nucleotide diversity (Pi) were determined. Overall, the genetic diversities observed were low, with Vihiga having the highest nucleotide diversity (0.02041) and Makueni county having the least nucleotide diversity (0.00577), indicating an overall low genetic variation of domesticated rabbits in Kenya. This could be attributed to crossbreeding between the domesticated and exotic breeds by farmers. To confirm an expected effect of variation among populations and low genetic diversity, it was observed that the number of rabbits that shared haplotype 3 were 40 whereas 61 individuals shared haplotype 7. Similar results were reported by Long et al. (2003),where a low genetic diversity was observed in the three Chinese domestic and introduced breeds. Haplotypes A1 and A2 have been reported in previous studies by Long et al., (2003). In this study, 19 breeds shared haplotype A1, which was identical to the European rabbit in Australia, and three breeds shared haplotype A2, which was found to be identical to wild rabbit in Australia. Intrabreed sequence analysis revealed 16 transitions and 1 transversion with no insertions or deletions. The transition type of mutation has been reported to be more common among very close relatives such as species within genus (Kocher et al., 1989). In this present study, transitions were the most frequent mutation, suggesting genetic closeness.

Other causes of low genetic diversity include founder effects during population expansion because of domestication and high selection pressure during commercial animal production which leads to an inherent decrease in strain variability (Long et al., 2003). Reduced breed variability directly results in reduced livestock genetic variability which equally applies to rabbits, as reported by this study.

Low genetic diversity is undesirable for sustainable livestock and crops' improvement since loss in diversity could lead to loss in some lineages that could be harboring desirable traits. A possible mitigation strategy is the establishment of pedigree records followed by deliberate breeding strategies to further diversify or preserve desirable

traits that are becoming extinct. This study has revealed rare haplotypes that can be conserved to be exploited in future breeding strategies. The low genetic diversity limits genetic improvement efforts. This observation calls for genetic improvement to generate genetic diversity and avoid the risk of genetic erosion by adopting measures such as complete characterization of breeds that are available in the country and their associated production environments, a compilation of complete rabbit breed inventories, adaptation of genetic improvement programs that target desirable adaptive traits such as heat tolerance, and an increased support of developing countries by the more developed counterparts in management of animal genetic resources (Hoffman, 2010).

5.1.2 Relationship of haplotypes

In the network connecting all the haplotypes belonging to lineage B, the largest haplotypes, haplotype 2 and 3, were centered with most of the sequences emanating from them and the rest of the sequences being less than 10 mutation steps away The outgroup was 35 mutation steps away from the haplotype 3 and 45 mutation steps away from haplotype 2. Similar results were reported by Long et al. (2003) in which a network representing 32 haplotypes derived from Chinese rabbits and other domesticated rabbits showed the so-called Chinese rabbits clustering together with the European rabbits The extent of haplotype sharing in the network which is illustrated by clustering of the sampled rabbits in two main haplotypes and scattering of the individual exotics around the main haplotypes indicated a lack of population structuring which was also supported by low genetic diversity.

5.1.3 The population structure of rabbits in Kenya

AMOVA analysis at the 3 hierarchical levels revealed that genetic variation of 2.39% among groups and 85.3% variation within populations. This means that 2.39% of the variation occurred among the regions with 85.3% of the variation occurring within the respective counties in the regions. This shows an independent arrival of the various haplotypes in the sampled regions. The significant genetic differentiation observed can be attributed to the fact that the regions are geographically distant, therefore chances of exchange of breeding stock among farmers from the various regions are

lower than exchange of breeding stock among farmers in the same region. Populations that are isolated undergo high differentiation, as observed by Perez *et al.* (2018), and in this study. Population genetic structure of domesticated rabbits is important in informing loss of genetic diversity that results from two processes: breed formation and domestication (Alves *et al.*, 2015)

5.1.4 Phylogeographic relationships of mtDNA haplotypes with European haplotypes

The study established that all the sampled rabbits belonged to lineage B, the known progenitor of domesticated rabbits (Long *et al.*, 2003) and originated from Europe, which is identified as the geographical center of origin of all domesticated rabbits Rabbit domestication has been attributed to a single origin in France (Bolet *et al.*, 2011). Biju Dival *et al.* (1991), reported two maternal lineages, A and B, in the European rabbits. Lineage A mainly consists of wild rabbits belonging to sub-species *Oryctolagus cuniculus algirus* whereas Lineage B is predominantly the sub-species *Oryctolagus cuniculus cuniculus* (Long *et al.*, 2003). All domestic rabbits belong to the second lineage (Monnerot *et al.*, 1996). Phylogenetic analyses in this study revealed that the sampled rabbits belonged to lineage B, which originated from Europe (Hardy *et al.*, 1992). This suggests that the domesticated rabbits in Kenya were introduced from Europe.

Abdel-kafy *et al.*, (2018) reported that native rabbits from middle Egypt belong to lineage A. Their study agree with Surridge *et al.*, (1999) who reported that the European wild rabbit originated in South Spain and North Africa. In the current study, domesticated rabbits in Kenya were found to belong to lineage B and originated in Europe. Network analysis of the phylogenetic relationship of the eight haplotypes detected in this study and sequences retrieved from GenBank revealed associative patterns with the European rabbits, herein referred to as European exotics, such as rabbit in Spain and Argente de Champagne, whose origin is Western Europe (Bolet *et al.*, 2011). The results obtained here confirm that Kenyan domesticated rabbits originated from European rabbits.

5.2 Characterization of the functional polymorphisms on the HSP70 gene in domesticated rabbits in Kenya

5.2.1 HSP70 haplotype distribution across agro-climatic zones in Kenya

Multiple sequence alignment of the HSP70 gene haplotypes revealed a total of 9 polymorphic sites. All these SNPs were located within the coding sequence. The protein sequence alignment revealed that in these SNPs, Isoleucine replaced Threonine in haplotypes 2 and 3, while Serine replaced Asparagine for haplotype 4. In a study that characterized goat HSP 70-1 gene, amino acid threonine replaced isoleucine at position 9 of the amino acid alignment, I>T (Gade et al., 2010). This study observed the converse, isoleucine replaced threonine at position 6, T>I Gade et al. (2020) characterized HSP 70- 1 in goats and found that at nucleotide level the goat HSP 70-1 to be 96 – 99% similar to that of cattle, buffalo and sheep, whereas at the amino acid level, similarity was 95 – 100%.

Haplotype 4 contained 5 out of the 9 mutations that were observed. Haplotype 4 was in Machakos county, which spans agro-climatic zones IV and V with higher environmental temperatures than the other regions. This could mean that these SNPs were associated with the elevated environmental temperatures of 22–40 ºC in this region. These results can be ascertained by characterizing the expression of HSP 70 across the sampled populations.

5.2.2 HSP70 haplotype phylogeny

The maximum likelihood tree comprising all the HSP70 haplotypes, reference sequence and paralog showed that all haplotypes clustered together with *Oryctolagus cuniculus* reference sequence (XM 008262601.2), with the paralog froming the root. This could mean that all the HSP70 haplotypes belong to 1 clade and are highly conserved. Similar results can be seen in the splits decomposition network with the 6 HSP70 haplotypes, reference sequence and the paralog. He et al. (2010) characterized HSP70 genes in the liver of warm freshwater fish and reported high conservation which was associated with its function, molecular chaperone The closeness of these halpotypes infers lack of ancestral haplotypes for the gene coding for HSP70, and the

paralog seems to have evolved rapidly as is shown by its position relative to the rest of the sequences.

5.2.3 HSP70 haplotype diversity

Computations of haplotype diversity and nucleotide diversity revealed that Bungoma had the highest haplotype diversity in HSP70 gene (0.4643), with a high corresponding nucleotide diversity of 0.00268. Machakos county which reported the highest number of SNPs also had a haplotype diversity of 0.2571 in the HSP70 gene, with a nucleotide diversity of 0.00361. The low haplotype diversity in Laikipia of 0.2222 and a corresponding low nucleotide diversity of 0.00088 suggest high levels of inbreeding in these populations. This high level of inbreeding is farther confirmed by the lack of diversity in the remaining populations, namely Kakamega, Vihiga, Kirinyaga, Nyandarua, Kitui and Makueni. Inbreeding is associated with selection of mates which occurs when individuals mate with relatives, including sibling mating, parent-offspring and first-cousin mating. Isolation distances also result in inbreeding, where populations that are geographically and physically close to each other are prone to mate easily, resulting in inbreeding (Holsinger, 2012). Non-random mating results in reduced differentiation within populations. Other known causes of reduced genetic variations in populations include genetic drift, selection, mutations, low gene flow and natural selection. Another possible cause of non–random mating in populations is geographical proximity. Populations that are close to one another mimic an inbred population since they tend to mate with each other. It is highly likely that the geographical proximity of these sampled areas resulted in the low genetic diversities observed Sánchez and Woolliams (2004).

5.2.4 HSP70 population genetic structure

AMOVA revealed a higher within population variation (100%) and low among group variation (1.59%). This suggested only 1.59% of the variation in the HSP 70 gene occurred between the geographically sampled regions while all the molecular variation in the HSP70 gene occurred among the populations within the sampled regions. This is farther supported by the minimal population differentiation based on the sampled regions (1.59%).

F_{ST} calculates the variation in the subpopulations relative to the variation that exists in the total population. Quantification of genetic differentiation based on allele frequencies as determined by the F_{ST}, showed a low genetic differentiation 0.00003 that was not significant (P >0.05). This implies lack of population structure in HSP70 gene within the populations. The variation among groups FCT=0.01593 was also not significant., further supporting a lack of population structuring in the three regions. Pairwise F_{ST} values also revealed no distinct populations, with low differentiation of less than 0.1 being observed. Ottewell et al., (2014), using microsatellite markers, categorize F_{ST} values of <0.1 as low differentiation, 0.15 – 0.2 moderate, and >0.25 as strongly differentiated. This lack of population structure implies that the haplotypes observed could be occurring at random and may not be associated with specific or adaptation to climate change. This could be attributed to the high levels of inbreeding, resulting in a homogenous population. From these findings, it is highly likely that the mutations that were observed in this gene which led to the 6 haplotypes were random amd may not be associated with adaptations to climate change as is suggested by their abundance in Machakos county spanning agro-climatic zones IV and V which contained 4 out of the 9 mutations and has higher environmental temperatures. Moreover, rabbits are kept under controlled temperatures, mostly caged, and it is highly unlikely that they could have adapted to the various climatic conditions in the various regions where they are bred. The genetic polymorphisms in the gene coding for HSP70 are not associated with heat tolerance.

5.3 Conclusion

This study demonstrated a presence of low genetic diversity as observed in the low nucleotide and haplotype diversities in the studied rabbit populations using mtDNA molecular marker. The low genetic diversity observed could be attributed to the exchange of breeding stock among farmers, which is a common practice in regions where the government does not have well defined programmes to supply farmers with breeding stocks. Phylogenetic analyses showed that the haplotypes that were identified in this study clustered together with other haplotypes belonging to mitochondrial type lineage B, where all domesticated rabbits belong. Domesticated rabbits have been shown to have originated from Europe.

This study was able to show that rabbits sampled from the various regions in Kenya all have a common center of origin, Europe.

The study identified a total of 9 polymorphisms resulting in 6 haplotypes in the HSP70 gene, with extremely low genetic diversity and lack of structuring in the HSP70 gene. The variation observed in the gene coding for heat shock protein 70 is not associated with the varying climatic conditions in these regions, hence no haplotypes could be associated with specific regions.

5.4 Recommendations

1. Improvement programmes should focus on supplying farmers with a wide range of breeding stocks to enhance diversity of the available gene pool. Unique mtDNA haplotypes should be identified, conserved and assessed for beneficial traits. The influence and significance of these SNPs could be evaluated to establish their functional characteristics.

2. Further research should focus on gene expression studies to assess mechanisms through which the HSP70 gene facilitates adaptation of rabbits to heat stress.

3. Other underlying causes for the implied heat tolerance in the rabbits in Machakos county, which spans agro – climatic zones IV and V should also be assessed using approaches such as Genome Wide Association Studies.

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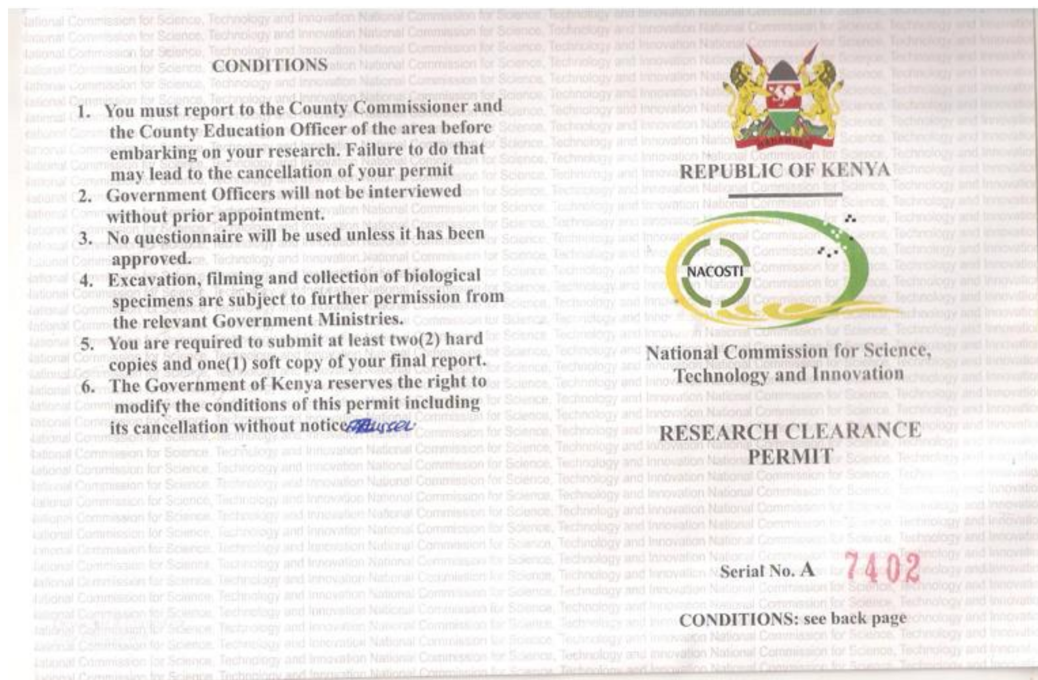
APPENDICES

Appendix I: Research permits

NACOSTI/P/15/62961/8524



CONDITIONS

1. You must report to the County Commissioner and the County Education Officer of the area before embarking on your research. Failure to do that may lead to the cancellation of your permit
2. Government Officers will not be interviewed without prior appointment.
3. No questionnaire will be used unless it has been approved.
4. Excavation, filming and collection of biological specimens are subject to further permission from the relevant Government Ministries.
5. You are required to submit at least two(2) hard copies and one(1) soft copy of your final report.
6. The Government of Kenya reserves the right to modify the conditions of this permit including its cancellation without notice

REPUBLIC OF KENYA

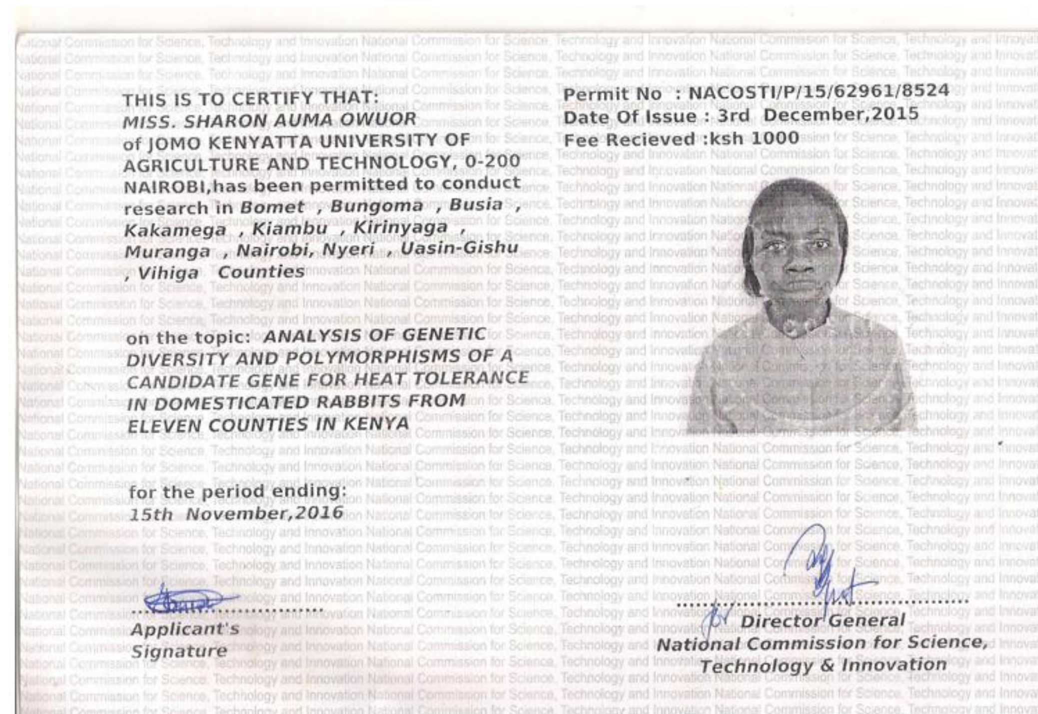
NACOSTI

National Commission for Science, Technology and Innovation

RESEARCH CLEARANCE PERMIT

Serial No. A 7402

CONDITIONS: see back page



THIS IS TO CERTIFY THAT:
MISS. SHARON AUMA OWUOR
of JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY, 0-200 NAIROBI,has been permitted to conduct research in *Bomet , Bungoma , Busia , Kakamega , Kiambu , Kirinyaga , Muranga , Nairobi, Nyeri , Uasin-Gishu , Vihiga Counties*

on the topic: *ANALYSIS OF GENETIC DIVERSITY AND POLYMORPHISMS OF A CANDIDATE GENE FOR HEAT TOLERANCE IN DOMESTICATED RABBITS FROM ELEVEN COUNTIES IN KENYA*

for the period ending:
15th November,2016

Permit No : NACOSTI/P/15/62961/8524
Date Of Issue : 3rd December,2015
Fee Recieved :ksh 1000

Applicant's Signature

Director General
National Commission for Science, Technology & Innovation

Appendix II: Selected mtDNA sequences used for comparison with sample mtDNA in the study

	Sequence	Accession number	Identity
1.	O. Cuniculus D-loop	NC-001913	Oryctolagus cuniculus mtDNA
2.	Aus – 3	AF003191	European rabbit in Australia-3
3.	Aus – 5	AF003193	European rabbit in Australia-5
4.	Aus – 7	AF003195	European rabbit in Australia-7
5.	B10Zsa	Z83346	Rabbit in Spain
6.	Bzs1	Z83354	Rabbit in Spain
7.	B2zsa	Z83364	Rabbit in Spain
8.	B4zsa	Z83350	Rabbit in Spain
9.	B3zsc	Z83351	Rabbit in Spain
10.	Z83342	A10zsa	Wild rabbit in Iberian Peninsula
11.	Z83343	A1zsa	Wild rabbit in Iberian Peninsula
12.	Z83344	A9zsa	Wild rabbit in Iberian Peninsula
13.	Bzs15	AJ293838	Hungarian Giant
14.	B01zsk	AJ293835	English
15.	B01zsj	AJ293834	Argente de Champagne
16.	Bzs16	AJ293839	French Lop
17.	B01zsg	AJ293833	Fauve de Bourgogne
18.	Bzs14	AJ293837	Fauve de Bourgogne
19.	Bzs17	AJ293840	French Lop
20.	B01zsl	AJ293836	Flemish Giant
21.	Bzs22	AJ293844	Flemish Giant
22.	Bzs19	AJ293841	French Lop
23.	B01zsh	AJ293832	Belgian Hare
24.	Bzs21	AJ293843	Vienna White
25.	B01zsf	AJ293831	Fauve de Bourgogne

Appendix III: GenBank Accession numbers and identities of reference sequences used in the HSP70 multiple sequence alignment and networks

Accession number	Identity
XM 008262601.2	Rabbit
NC_013681.1:c31481592-31478857	HSP70 Paralog4