Marker development and genetic structure of endangered Grevy's zebra *(Equus Grevyi)* in Kenya

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DECLARATION

I declare that this is my original work and that it has not been presented for award of degree at any other university or published anywhere.

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DEDICATION

To my Mother Lucy Anyango Wire and my late father Michael Ouma Odhiambo.

You've been and still are the unseen pillars giving me the impetus to press through life even with challenges. I salute you.

AKNOWLEDGEMENT

My greatest appreciation is to God Almighty for the guidance through this work. Secondly, I thank the University of Nairobi for awarding me scholarship to pursue this program. I also appreciate Prof. Nicholas Oguge while at Earth Watch Institute and Dr. Paul Muoria of African Wildlife Foundation who facilitated my initial field visits to the Samburu ecosystem in preparation for this work. My special appreciation goes to Dr. Siva Sunderesan of Princeton University and the Denver ZOO and my supervisors Prof. Brenda Larison of the University of California, Los Angeles and Dr. Joel Ochieng and Prof. Joseph Owino Junga of University of Nairobi for their support on the ideas, important literature and all resources required for this work. I also take this opportunity to appreciate the Staff of Molecular Genetics Laboratories at National Museums of Kenya (NMK) and Center for Biotechnology and Bio-informatics, University of Nairobi (CEBIB) with special recognition of Dr. Kem Githui and Mrs. Ann Mwaura for their technical guidance during the laboratory work at NMK. Further appreciations go to my late Dad for encouraging me to take on this Masters program and to my entire classmates at this course year, for moral and material support throughout this work. And to all whose participation have been critical but are not mentioned, you are greatly appreciated.

God bless you all.

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LIST OF ABBREVIATIONS

CITES- Convention on International Trade in Endangered Species

DNA- Deoxy-Ribonucleic Acid

IUCN- International Union for Conservation of Nature

KREMU- Kenya Rangeland Ecological monitoring Unit

Mt DNA- Mitochondrial DNA

NMK- National Museum of Kenya

PCoA- Principle Coordinate Analysis

PCR- Polymerase Chain Reaction

PIC- Polymorphism Information Content

UCLA - University of California, Los Angeles

UCSC- University of California, Santa Cruz

IUCN/SSC - International Union for Conservation of Nature/Species Survival Commission (SSC)

ABSTRACT

Grevy's zebra (*Equus grevyi*) has experienced precipitous decline in population size and geographic range and is among the most endangered of mammals. Supposed hybridization with the more abundant plains zebra *(Equus quagga*) is a further threat to its survival due to possible genetic swamping. Its population genetic structure and associated evolutionary processes are poorly understood while such information may be valuable for inferring genetic potential and survival aptitude of the population that is also facing constant habitat loss and general environmental changes. Capacity to study these processes is limited by insufficiently studied, validated or documented genetic tools (markers). We tested 48 tetra-nucleotide microsatellites markers of the domestic horse (*Equus caballus*) for amplification success and polymorphism in Grevy's and plains zebras. Their performance on noninvasive samples was also evaluated and preliminary genetic analysis conducted using seven markers and fecal derived Grevy's DNA. Thirty-three and thirty-eight markers amplified well, were sufficiently polymorphic and moderately to highly informative and thus useful for genetic analysis of the Grevy's and plains zebras respectively. Preliminary genetic assessment indicates a structured population of the Grevy's zebra and likely inbreeding within subpopulation. Enhanced gene flow is recommended to boost subpopulation connectivity and counteract effects of possible genetic drift.

1.0 INTRODUCTION

The genus *Equus* comprises seven species, five of which are listed as vulnerable to critically endangered. Grevy's zebra (*Equus grevyi*), one of the three extant species of zebra, is among the most endangered mammals (Moehlman et al 2013). It has experienced a rapid decline in population size and geographic range primarily due to hunting and competition with people and their domestic livestock (Williams 2002; Williams and Low 2004, Low et al. 2008). Disease and drought have also led to recent declines in the species in northern Kenya (Manyibe et al. 2006; Muoria et al. 2007) and hybridization between Grevy's zebra and the more abundant plains zebra (*Equus quagga*) has recently been observed in at least two locations in Kenya (Cordingley et al. 2009). Understanding the impact of these threats on the underlying genetics of the species can aid in the development of more targeted conservation measures.

Although conservation efforts for Grevy's zebras could benefit immensely from genetic data, relatively few genetic markers have been developed for the study of this species. The objective of this study was to develop and test more tetra-nucleotide microsatellite markers for genetic analysis of Grevy's zebra and related plains zebra and to assess the level of genetic variation and structure of Grevy's population in Samburu and Laikipia regions in Kenya. Microsatellite markers remain important tools for genetic studies of wild populations due to their multi-allelic nature, ease of use and affordability. They are also particularly useful for the study of endangered species for which only non-invasively acquired DNA from sources such as feces is often available. Tetra-nucleotide markers can be particularly valuable for use with non-invasive samples as they are less prone to stuttering than dinucleotide markers and are therefore easier to score accurately (Ellegren 2004). Ito et al (2013 and 2015) recently developed 28 microsatellite

markers for Grevy's zebra (mostly di and tri-nucleotide markers). Analysis of genetic structure and detection of hybrid levels require examination of varying but often large number (12 to 48) of highly polymorphic loci (Boecklen and Howard 1997; Hansen et al. 2000; Vaha and Primmer 2006 and Randi 2008) depending on the specific objective of the study, with low divergence between sub-populations requiring even larger number of loci. This underscores the need for developing more microsatellite markers for the Grevy's zebra genetic analysis.

Bioinformatic search of the horse genome assembly (Wade et al. 2009) was conducted to identify tetra-nucleotide microsatellite markers. Forty-eight tetra-nucleotide microsatellites were selected and tested for their utility for genotyping Grevy's and plains zebra using blood samples. Their utility for genotyping non-invasively obtained Grevy's fecal samples was also evaluated. A sample of seven of the polymorphic tetra-nucleotide microsatellite markers were used to genotype a set of wild Grevy's from nine locations from Samburu and Laikipia regions of Kenya to conduct a preliminary assessment of genetic variation and structure of the population.

2.0: OBJECTIVES

2.1: GENERAL OBJECTIVE

To assess the level of genetic variation and the genetic structure of Grevy's zebra in Samburu and Laikipia regions in Kenya.

2.2: SPECIFIC OBJECTIVES

i. To assess the amplification success rate and level of genetic polymorphism of 48 horse tetra-nucleotide microsatellite markers in Grevy's and Plains zebra using blood samples.

ii. To assess the amplification success rate of these tetra-nucleotide markers in non-invasive samples (feces) from the endangered Grevy's Zebra.

iii. To assess the level of genetic variation and the genetic structure of the wild Grevy's zebra in Laikipia and Samburu Counties in Kenya.

3.0 LITERATURE REVIEW

3.1: Grevy's population

3.1.1: Taxonomy of Grevy's Zebra (Equus Grevyi)

The family Equidae contains only one extant genus (Genus *Equus*) which contains seven living mammalian species including horses, asses and zebras. All members of this genus are referred to as equines which are odd-toed ungulates with slender legs, long heads, relatively long necks, manes (erect in most subspecies) and long tails (Moehlman et al. 2008).They are herbivorous, and mostly grazers and able to survive on lower quality vegetation (Moehlman et al. 2008). Wild equine populations have limited geographical distribution mainly found in Africa and Asia.

The Grevy's zebra is the most endangered of the three species of Zebra (Moehlman et al. 2008).The other two zebra species are the plains zebra (*Equus quagga*) found mainly in Kenya, Tanzania, and Sudan with peripheral populations in Somalia, Uganda, Burundi, and Rwanda – and continued south through Malawi, Mozambique, Zambia, Zimbabwe, northern and eastern Botswana, Swaziland, Lesotho, and South Africa as far south as the Orange River (Hack et al 2002) and the mountain zebra (*Equus zebra*) native to south-western Angola, Namibia and South Africa (Novellie 2008). Grevy's zebra is the only existing member of the subgenus *Dolichohippus*. Its taxonomic classification is summarized as: Kingdom: Animalia, Phylum: Chordata, Order: Mammalia, Class: Perissodatyla, Family: Equidae, Genus: Equus, Species: *Equus grevyi.*

3.1.2: Physical description

The Grevy's zebra, also known as the imperial zebra, is the largest of the extant equids in the wild (Whitaker and Whitelaw 2007). It is also the largest and most endangered of the three species of zebra (Moehlman et al 2013). Grevy's zebras (Figure 1a) have larger heads, larger and rounded ears, thick erect manes, brown muzzle and the neck is thicker and more robust than in other zebra species (Churcher 1993). Its coat has black and white stripes which are narrower than in other zebras. The stripes are shaped like chevrons and wrap around each other in a concentric pattern. They have a black dorsal stripe bisecting all the other stripes on the back of the zebra. The pattern of the chevrons is distinct especially on the limbs and is often used for identification of individuals. On the head, chevrons extend dorsally to the cheek, and the pattern turns to be more linear. The entire belly of this zebra is white unlike in other zebras where black and white stripes extend to the belly (Figure1b) (Churcher 1993).

The males of the Grevy's zebra are slightly larger (usually about 10 percent larger) than the females. Their foals are reddish-brown or russet striped at birth instead of the black stripes acquired as they age. They are also born with a dorsal mane extending from the top of the head to the base of the tail. The mane is erect when the animals are excited and flatten when they get relaxed (Churcher 1993).

Figure 1: a) Grevy's zebra **b) Plains zebra**

3.1.3: Ecology and Habitat

Grevy's zebra are primarily grazers, but are effective browsers in times of drought or in those areas with limited grasses and shrubs. Their habitat is largely the arid and semi-arid grass/shrubland and they prefer areas with permanent sources of water (Moehlman et al. 2008). They eat large quantities of forage just like other ceacal digesters and are constrained to feed on the higher biomass vegetation, regardless of quality when food is limited (Ginsberg 1988; Williams 1998). The adults can go for between two to five days without water but lactating females require water more often and can only go for one or two days without water (Becker and Ginsberg 1990; Rowen 1992).

Breeding males are territorial defending water and food resource territories of 2–12 km² while the non-territorial individuals have expansive home ranges extending up to 10,000 km². They are extremely mobile with movements of distances greater than 80 km for some individuals depending on availability of resources (Klingel 1974; Rubenstein 1986; Rowen and Ginsberg 1992; Williams 2002). Breeding is highly dependent on random patterns of climatic variation, particularly rainfall and on conditions that facilitate oestrus among females (Ginsberg 1988; Williams 1998). Females would go into periods of anoestrus when food and water resources are limited, due to poor body condition (Ginsberg 1989). Foal survival has also been directly related to the extent of movement by their mothers. The survival is low when the mares move long distances or make small frequent movements (Rowen 1992a; Williams 1998).

3.1.4: Grevy's Zebra Distribution and Conservation status

The Grevy's zebra is endangered and listed in both the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Moehlman 2008) and in Convention on International Trade in Endangered Species (CITES) Appendix I. IUCN Redlist is an inventory system by IUCN that provide information and analyses on the status, trends and threats to species in order to inform and catalyze action for biodiversity conservation. It classifies species into nine categories (IUCN Standards and Petitions Subcommittee 2016; Figure 2) depending on extinction risk. CITES works by controlling international trade in specimens of selected species. The species covered by CITES are listed in three Appendices, according to the degree of protection they need. Appendix I include species threatened with extinction and trade in specimens of these species is permitted only in exceptional circumstances.

Grevy's zebra warrants such listing since its global population size has declined rapidly in recent times from estimates of 15,000 in the late 1970s (Grunblatt et al. 1989) to between 1,700 and 2,100 animals around 2003 (Nelson 2003; Williams et al. 2003) and about 2400 currently (Low et al. 2008).

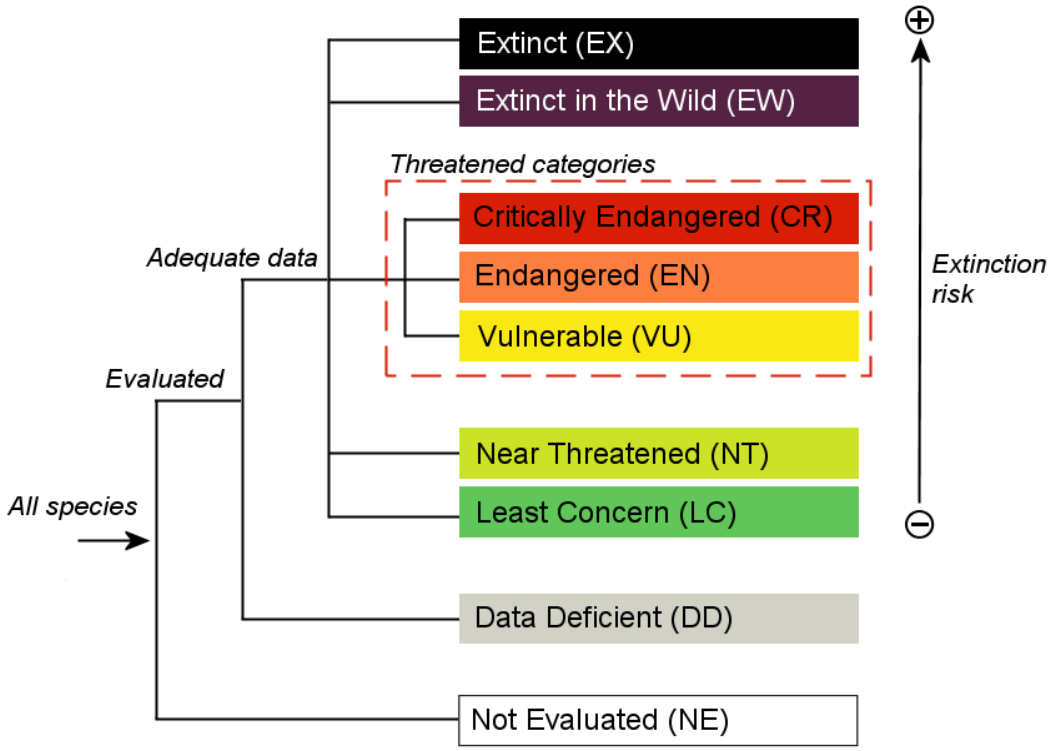


Figure 2: Structure of the IUCN Redlist Categories. (IUCN Standards and Petitions Subcommittee 2016)

From 1988 to 2007, the global population of Grevy's Zebra declined by approximately 55% with approximately 68% decline between 1990 and 2007. The Grevy's zebra population in Kenya declined from an estimated 4,276 in 1988 (KREMU 1989) to an estimated population size of 1468-2135 in 2006.

A population estimate of 1838-2319 in 2007 may indicate that either the population is increasing and slowly stabilizing or observation efforts were more accurate in identifying individuals (Mwasi and Mwangi 2007). Grunblatt et al. (1989) also demonstrates a significant decline in the population range over the years. The most recent estimates put the total population of Grevy's Zebra remaining in the wild in Kenya and Ethiopia at less than 2,000 mature individuals (Rubenstein et al 2016). There were approximately 2,350 individuals in Kenya (1,716 mature animals) in 2016 and about 230 individuals in Ethiopia (168 mature animals) in 2012 with largest regional population numbering around 1,300 individuals (949 mature) and are found in the centre of the Samburu region of central Kenya (Rubenstein et al 2016).

Historically, the range size for these zebras extended from east of the Rift Valley in Kenya to western Somalia, and from northern Ethiopia in Alledeghi Plain through the Awash Valley, the Ogaden, north-east of Lake Turkana in Ethiopia to north of Mt. Kenya and south-east down the Tana River in Kenya (Bauer et al. 1994; Figure 3). Currently the species has a discontinuous range extending from the eastern side of the Rift Valley in Kenya to the Tana River with small isolated populations in the Alledeghi Plains in the northeast of Awash National Park in Ethiopia. Some are found in southern Ethiopia from Lake Ch'ew Bahir extending to just north of Mt. Kenya (Bauer et al. 1994). A few animals are found further southeast along the Tana River with small, introduced population surviving in and around Tsavo East National Park in Kenya. The Somali and Djibouti population is considered to be extirpated with the last confirmed sightings in Somali dating to 1973.There are no records of the species occurrence in Eritrea (Yalden et al. 1986, Bauer et al. 1994).

Today, the species is only found in northern Kenya and southern Ethiopia, 93% of which is occurring within Kenya (Low et al. 2008). The numbers identified and size of the species range fluctuates seasonally as animals move to search for food and water. In dry seasons, the animals tend to be more concentrated around permanent water sources. They are most abundant and most easily observed in the southern portion of their range in southern Samburu and the Laikipia Plateau (Moehlman et al 2008).

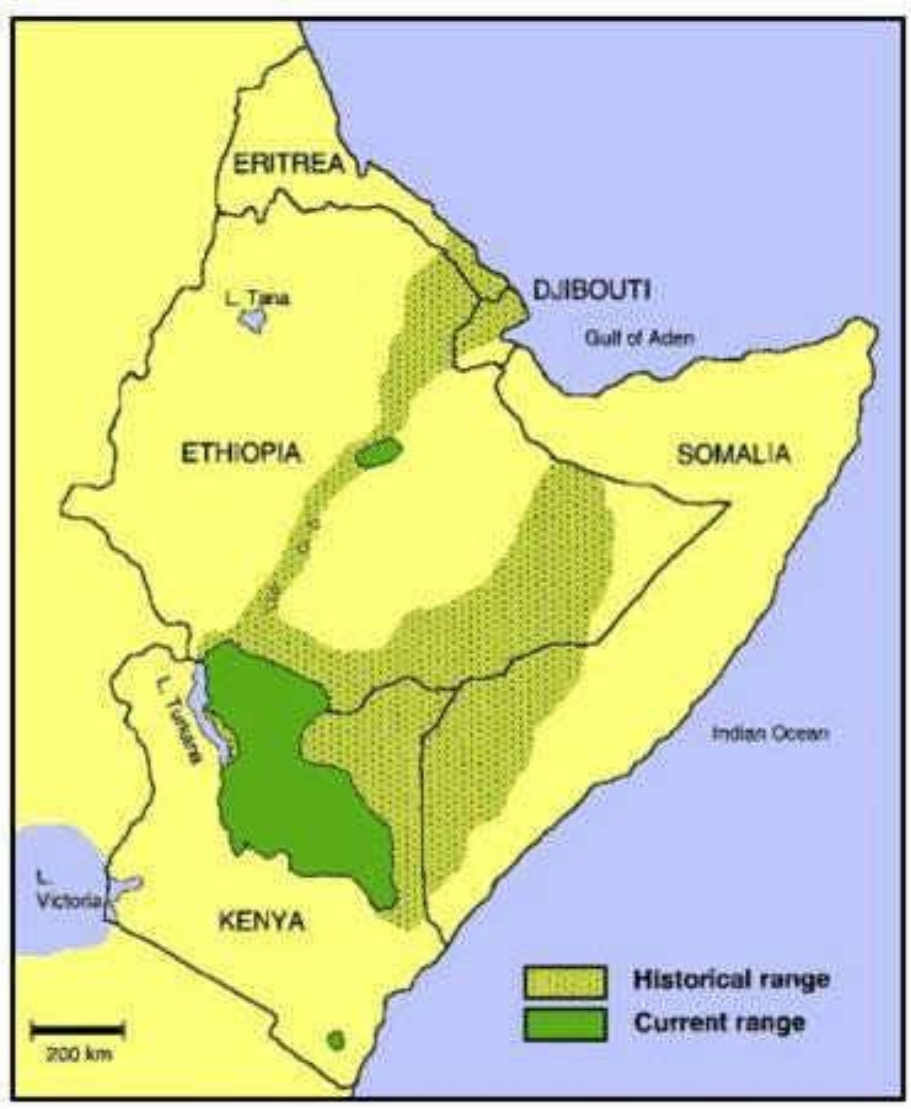


Figure 3: Grevy's zebra historic and current distribution (adapted from Bauer et al. 1994)

3.1.5: Grevy's Zebra threats and Conservation Efforts

The decline in population size and range is attributed to hunting for meat, and/or medicinal purposes; competition with domestic livestock resulting in loss of access to critical resources; and an increasing scarcity of the resources due to over-exploitation (Williams 2002; Williams and Low 2004). Disease and drought have also played significant role in the species decline in northern Kenya (Manyibe et al. 2006). Muoria et al. (2007) recorded an outbreak of anthrax in the Wamba area of southern Samburu in Kenya, during which more than 50 animals succumbed to the disease. Hybridization with the more abundant Plains zebra (Cordingley et al. 2009) may also further threaten the Grevy's existences in some parks due to possible genetic and demographic swamping (Todesco et al. 2016).

The Grevy's population benefitted from the ban on wildlife hunting in Kenya in 1977 but recent data suggest that the continuing decline may be the result of low recruitment due to low juvenile survival (Williams 1998). Low juvenile survival may be the result of competition for food and water resources with pastoral people and domestic livestock (Williams 1998). Further, the frequent droughts experienced in the species areas may be causing frequent movement for lactating females in search of water (Williams 1998; 2002). Also, the water supply in critical perennial rivers like the Ewaso Ng'iro River has declined and dry season river flow decreased by nearly 90% (Williams 2002).

Although the species is legally protected in Ethiopia, official protection has been limited with community-based conservation proving to be more effective. The protected areas in Ethiopia are also insignificant and include Alledeghi Wildlife Reserve, Yabello Sanctuary, Borana Controlled Hunting Area and Chalbi Sanctuary (Moehlman et al 2008). The core and crucial protection for the Kenya's southern population of Grevy's zebra are the Buffalo Springs, Samburu and Shaba National Reserve complex and the private and community wildlife conservancies in Isiolo, Samburu and the Laikipia Plateau (Williams 2002; Moehlman et al 2008). On the Laikipia Plateau in Kenya, Grevy's zebra numbers have increased since they first expanded into this area in the early 1970s due to protection and reduced competition with domestic livestock (Williams 2002). The protected areas in Kenya also form only less than 0.5% of the Grevy's zebra range (Moehlman et al 2008). Protection of water supplies, management of protected areas, community involvement in conservation and monitoring of numbers in the wild are some of the conservation actions focused on the wild Grevy's populations (Williams 2002). Other efforts have included translocations to new locations (e.g Meru National Park in Kenya) and community scouting. The efforts may have yielded some fruit considering marginal increase in individual counts in the wild (Mwasi and Mwangi, 2007). However, as endangered species numbers decrease, genetic variation is lost and may only slowly be restored through the accumulation of mutations over many generations. Endangered species with low genetic variation therefore risk extinction even long after its population size has recovered. Small populations are also more vulnerable to genetic factors, demographical and environmental stochasticity, genetic drift and inbreeding and have increased probability of extinction.

Kenya has recently developed a National Conservation and Management strategy for Grevy's zebras, (the 2nd edition 2012-2016 is now in use) with a vision 'to *have viable populations of Grevy's zebra in their natural habitat functioning in healthy ecosystems and valued locally and globally'* (KWS 2012). Understanding the genetic effect of the past population decline and the current population genetic status is paramount in designing sustainable conservation measures for the species. Analysis of the genetic diversity of the species, the population genetic subdivision and structure and the trend of gene flow within the population are some of the important aspects that need to be investigated for a more comprehensive conservation strategy. This component of investigation is lacking in the conservation efforts of many wild species including the Grevy's zebra.

3.2: Zebra Genetic Diversity Studies

Genetic variation enables adaptation and enhances survival of natural populations in changing environments and the loss of it reduces population fitness due to inbreeding depression (Reed and Frankham 2003; Frankham 2005; Kliman et al 2008). Effect of genetic drift and inbreeding is greater as populations become smaller and genetic variation is lost (Kliman et al. 2008). This includes loss of beneficial alleles and increase in frequency of harmful alleles and such populations often become less fit and are vulnerable to changing environment.

Eisner et al (1995) describes conservation as an attempt to protect the genetic diversity resulting from evolutionary processes. IUCN has identified genetic, species and ecosytem diversity as three levels of biodiversity that must be conserved (McNeely et al. 1990). Because resources for conservation are often limited, it is important to determine conservation priorities by establishing

evolutionarily significant populations/units (ESU) or Management units (MUs) (Moritz 1994; Crandall et al 2000: Schwartz et al. 2007). Identification of MUs has largely been based on population analysis of genetic markers to identify populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the level of divergence (Moritz 1994; DeYoung and Honeycutt 2005). Palsboll et al. (2006) proposes identification of MUs based on the amount of population genetic divergence instead of simply the rejection of panmixia. Both methods require application of molecular markers such as those developed in the current study.

Many genetic studies on the equines including the Horse genome project (www.uky.edu/Ag/Horsemap/) have been based on the domestic horse (*Equus caballus).* Very little is documented about the genetic analysis of the other members of the genus (*Equus*). However, the genetic analyses of these other members are made possible through cross-species amplification (Primmer et al. 1996; Breen et al. 1994; Wallner et al. 2004; Moodley et al. 2006). Microsatellite markers have been employed for genetic analysis including parentage testing, linkage analysis and genetic diversity studies in the horse (Ellegren et al. 1992; Marklund et al. 1994; Georgescus et al. 2005) and have been tested for their applicability to similar studies in the other members of the genus *Equus* (Aranguren- Mendez et al. 2001 and Moodley et al. 2006). Ito et al (2013 and 2015) have recently developed 28 microsatellite markers for Grevy's zebra genetics analysis. Analysis of genetic structure and detection of hybrid levels however require examination of a larger number of highly polymorphic loci (Boecklen and Howard 1997; Hansen et al. 2000) thus the screening of more markers is still required.

3.3: Genetic Markers

A genetic marker is a gene or DNA sequence with a known location on the chromosome that can be used to identify cells, individuals or species. It may be a short DNA sequence, such as sequence surrounding a single base-pair change (Single nucleotide polymorphism, SNP), or a long one, like minisatellites. Examples of commonly used genetic markers are: RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism), RAPD (Random Amplification of Polymorphic DNA), SSLP (Simple Sequence Length Polymorphism), SNP (Single Nucleotide Polymorphism), Microsatellites/SSR (Simple Sequence Repeat) and the control region of mitochondrial DNA (mtDNA).

3.3.1: Allozyme polymorphism

Allozymes are proteins produced by allelic variants at a gene locus, and are useful as markers because of their existence in many forms (structural differences) and can be separated by capillary electrophoresis (Avis 1994). Differences in underlying DNA sequences are expressed as amino acid differences in the polypeptide chains of the different allelic forms of an enzyme. Resulting protein products may migrate at different rates when run through a starch gel subjected to an electrical field due to charge and size differences. These differences in the presence/absence and relative frequencies of alleles distinguish among genetic units and are useful for quantifying genetic variation at populations, species, and higher taxonomic levels (DeYoung and Honeycutt 2005). The use of allozymes is however limited because i) their use is prone to heterozygote deficiencies due to null alleles (enzymatically inactive variants); ii) the amount and quality of tissue samples required for their analysis; iii) some variations at DNA

sequence are not expressed at the protein level, underestimating the levels of detectable variation; iv) Some changes in nucleotide sequence have no effect on the encoded polypeptide (silent substitutions) and v) some polypeptide changes are symonymous (do not alter the mobility of the protein in an electrophoretic gel)

3.3.2: Mitochondrial DNA Sequence (MtDNA Sequence)

Sequence divergence accumulates faster in mitochondrial than in nuclear DNA (Brown 1985) because of faster mutation rate in mtDNA. This may result from a lack of repair mechanisms during replication for mitochondria (Wilson et al. 1985) and smaller effective population size because of strictly maternal inheritance of the haploid mitochondrial genome (Birky et al. 1989). The entire mtDNA molecule is transcribed except for the control region (D-loop) measuring approximately 1- kb. These non-coding segments like the D-loop demonstrate elevated levels of variation compared to coding sequences such as the cytochrome-b gene (Brown et al. 1993), presumably due to limited functional constraints and relaxed selection pressure. Though it has large numbers of alleles per loci, only limited number of markers is available on the mtDNA molecule making its PIC values lower than highly variable nuclear markers such as RAPDs, microsatellites, AFLPs, and SNPs.

3.3.3: Restriction Fragment Length Polymorphism (RFLP)

DNA is digested by specific restriction enzyme (endonucleases) to produce DNA sequences that can be detected by the presence of fragments of different lengths (Beuzen et al 2000). Variation in the DNA sequence exists as a result of difference in restriction sites along the DNA. The basic technique for detecting RFLPs involves fragmenting a sample of DNA by a restriction enzyme,

which can recognize and digest DNA wherever a specific short sequence called restriction enzyme site occurs, in a process known as restriction digestion (Botstein et al 1980: Beuzen et al 2000). The resulting DNA fragments are then separated by length by electrophoresis on agarose gel. RFLP is specific to a single clone/restriction enzyme combination and it occurs when the length of a detected fragment varies between individuals.

3.3.4: Random amplified polymorphic DNA (RAPD)

RAPD (pronounced 'rapid'), for *R*andom *A*mplification of *P*olymorphic *D*NA, is a type of PCR reaction, but the segments of DNA that are amplified are random (Williams et al 1990). PCR is used to randomly amplify segments of nuclear DNA with an identical pair of primers 8–10 bp in length. The primers are short and relatively low annealing temperatures (often 36–40 ^{0}C) are used, resulting in amplification of multiple products, each product (presumably) representing a different locus (Williams et al 1990; Kumar and Gurusubramanian 2011). Most of the amplified loci are assumed to be selectively neutral because most of the nuclear genome in vertebrates is non-coding. Presence or absence of each product represents the genetic variation and divergence within and between the taxa of interest and is dictated by changes in the DNA sequence at each locus. Polymorphisms in RAPD can occur due to base substitutions at the primer binding sites or to indels in the regions between the sites. Primers for RAPD are commercially available and do not require prior knowledge of the target DNA sequence or gene organization. Multilocus amplifications can be separated electrophoretically on agarose but higher resolution of bands has been achieved with discontinuous polyacrylamide gel electrophoresis (dPAGE) and silver staining (Dinesh et al. 1995).

Demonstrating Mendelian inheritance of the loci is difficult with this marker. It is also difficult to distinguish between homozygotes and heterozygotes (Kumar and Gurusubramanian 2011).
In addition, the presence of different DNA regions which have the same lengths and thus appear to be a single locus (paralogous PCR product) limits the use of this marker. They are also subject to low reproducibility due to the low annealing temperature used in the PCR amplification (Kumar and Gurusubramanian 2011).

3.3.5: Amplified Fragment polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) is a PCR-based fingerprinting technique and was first described by Vos et al. 1995. Small amount of purified genomic DNA is digested using two restriction enzymes, one with an average cutting frequency (like *Eco*RI) and the other with a higher cutting frequency (like *Mse*I or *Taq*I) (Paun and Schönswetter 2012). Double-stranded oligonucleotide adapters are then designed to allow simultaneous restriction and ligation with re-ligated fragments cleaved again. Two subsequent PCR amplifications is done on an aliquot under highly stringent conditions with adapter-specific primers with an extension of one to three nucleotides on their 3' ends running into the unknown chromosomal restriction fragment (Paun and Schonswetter 2012). An extension of one selective nucleotide amplifies 1 of 4 of the ligated fragments, whereas three selective nucleotides in both primers amplify 1 of 4,096 of the fragments. The PCR primer which spans the average-frequency restriction site is labeled. A highly informative pattern of 40 to 200 bands is obtained after polyacrylamide gel electrophoresis. Polymorphism on the patterns obtained is due to (i) restriction site mutation, (ii) mutations in the sequences adjacent to the restriction sites (complementary to the selective primer extensions), and (iii) deletions or insertions within the amplified fragments.

The advantage of the AFLP method is that large (over 100) number of polymorphisms is obtained and there is high reproducibility because of high PCR annealing temperatures. It is however expensive than RAPDs, but because large numbers of loci can be analyzed from a single run, the cost per marker is reduced significantly. It does not require prior molecular information and thus is applicable to any species, including species that have less information.

The bands are bi-allelic and have relatively low PIC scores, but are still useful because of the larger number of loci that can be simultaneously scored. Special equipment such as automated gene sequencers for electrophoretic analysis of fluorescent labels are required limiting their use. Traditional electrophoretic methods can also be employed with radioactive labels or special staining techniques such as silver staining.

3.3.6: Microsatellites/SSR (Simple Sequence Repeat)

Microsatellites are tandem repeats of 1–6 nucleotides found at high frequency in the nuclear genomes of most taxa. They are also known as simple sequence repeats (SSR), variable number tandem repeats (VNTR) and short tandem repeats (STR). The mutation rate for the repeat sequences is high due to slippage and proofreading errors during DNA replication. This changes the number of repeats and the length of the repeat string (Eisen 1999). The high rate of mutation results in the high levels of allelic diversity important for genetic studies of processes acting on ecological time scales (Schlotterer 2000). They can potentially provide estimates of migration, distinguishing relatively high rates of migration from panmixia, and estimating the relatedness of individuals (Kimberly et al. 2006).

Microsatellites are useful when working with degraded DNA because they can still be amplified since microsatellites are usually shorter in length than sequenced loci (100– 300 vs. 500–1500 bp) (Taberlet et al. 1999). The chance of successfully amplifying DNA fragment is proportional to its length (Frantzen et al. 1998). This trait allows microsatellites to be used in many genetic analyses involving ancient DNA, or DNA from hair and fecal samples which are prone to degrading factors (Taberlet et al. 1999). Microsatellite are scattered throughout the genome and are highly polymorphic. By assaying several microsatellite loci, a multi locus geneotype can be obtained: a 'genetic finger print', which is unique to individual animals. They are also co-dominantly inherited (alleles from both parents are traceable in the offspring) and useful for studying paternity and kinship, genetic variation, population genetic structure and gene flow (Bruford and Wayne 1993; Queller et al. 1993). Further, the advent of the polymerase chain reaction has made microsatellites prominent in many biological studies including investigation of the genetic structuring of populations and addressing specific questions in evolutionary and conservation biology.

Growing number of reports suggest that changes in repeat numbers might cause quantitative variation in protein function and gene activity (Koreth et al. 1996; Li et al. 2002), however, the exact function of microsatellites is still unclear. Other functions in bacterial pathogenicity have also been demonstrated (Bayliss et al. 2004). Microsatellites have been preferred over other genetic markers in the field of conservation genetics because they are easy to obtain either by direct isolation of species-specific markers or by application of markers originally isolated from related species. They can also be used on non-invasively obtained samples (Ellegren 2000) and are comparatively easy to automate with amplification of many loci possible in a single PCR by

multiplexing. They are generally assumed to be more polymorphic in the species from which they are cloned than in related species and those loci chosen on the basis of polymorphism on one species often exhibit shorter repeats in related species (Ellegren 1995). Their reproducibility is easy and they provide additional information because their mode of inheritance is better understood.

3.4: Genomics in Conservation

Most management recommendations coming from conservation genetic studies are entirely and exclusively based on the analyses of molecular marker data. Identification of genetic erosion, reconstruction of demographic history, and other processes of importance at the landscape level have been successfully done using these markers. Most of the genetic analysis using these markers is based on the assumptions that the levels of marker variation correlate with levels of functionally important variation, and that low levels of functional variation will lead to low average fitness and low potential for evolutionary adaptation. Relevance of marker variation for fitness, and for levels of detrimental and adaptive genetic variation has not been irrefutably determined (Kohn et al. 2006). Existing data suggest low correlation between neutral and detrimental and adaptive variation, largely, because this relationship is dependent upon genomic sampling and population-specific demographic history (Hedrick 2001).

The genomics applications in conservation genetics context may have more relevance into determining levels of functional genetic variation within and between populations, and in the association between neutral and functional variation (Kohn et al. 2006). Bioinformatics and population genomic approaches may address the limitation of the traditional marker assays.

Genomic science and the study of functional genetic variation will improve our understanding of the pattern and processes affecting genetic variation in rare and endangered species and might be used to test the implicit assumption of conservation genetics, that the observed levels of neutral genetic variation can be used to predict the levels of detrimental variation accrued and adaptive variation lost by endangered species owing to population size decline (Kirkpatrick and Jarne 2000; Hedrick 2001).

Next-generation sequencing technologies has also made it possible to apply genomic methods to less studied organisms and to screen large numbers of individuals for large numbers of markers extensively spread in the entire genome at relatively low cost (Cosart et al. 2011; Bi et al. 2012; Lemmon et al. 2012).Genomic resources can also now be applied to genome-enabled endangered taxa like the Grevy's zebra to better understand their underlying evolutionary processes to inform their conservation and management and it has rapidly been in cooperated as a conservation tool to explore evolutionary processes important for long time population survival like inbreeding depression, outbreeding depression, hybridization, introgression and adaptation (Allendorf et al. 2010; Angeloni et al. 2012).

4.0: MATERIALS AND METHODS

4.1: Sampling

Nine Grevy's zebra blood samples collected during collaring efforts in Kenya, seven plains zebra blood samples obtained from Etosha National Park, Namibia, one plains zebra blood sample from the San Diego Zoo and one Grevy's zebra blood sample from the Denver Zoo were used for the used for the marker screening. Eighty two fecal samples were collected from nine different locations in two regions (Laikipia and Samburu) in Kenya's (Figure 4) and used to test the utility of these markers on non-invasively obtained samples, preferred for threatened species.

A herd of grazing Grevy's zebras would be followed from a close distance and observed from a vehicle until one animal defecated. Samples were then collected by slowly approaching the location where the animals defecated and fecal samples collected by scraping the outer layer of the fresh feces. It is widely recognized that the outer most fecal material yields the least degraded DNA and the lowest concentration of PCR inhibitors (Flagstad et al. 1999; Fernando et al. 2003; Wehausen et al. 2004). Feces contain cells from epithelial lining; thus DNA from the host itself can typically be isolated and analyzed (Kohn and Wayne 1997). The fecal samples were stored in 95%ethanol in a refrigerator and DNA was extracted within 3 days of collection.

4.2: DNA Extraction

DNA was extracted from the blood samples using the Qiagen Blood Extraction kit and eluted in TE buffer according to manufactures instructions. For the fecal samples, DNA was extracted using the Qiagen Plant DNA extraction kit (to neutralize plant materials that could inhibit DNA amplification) following manufacturer's recommendations.

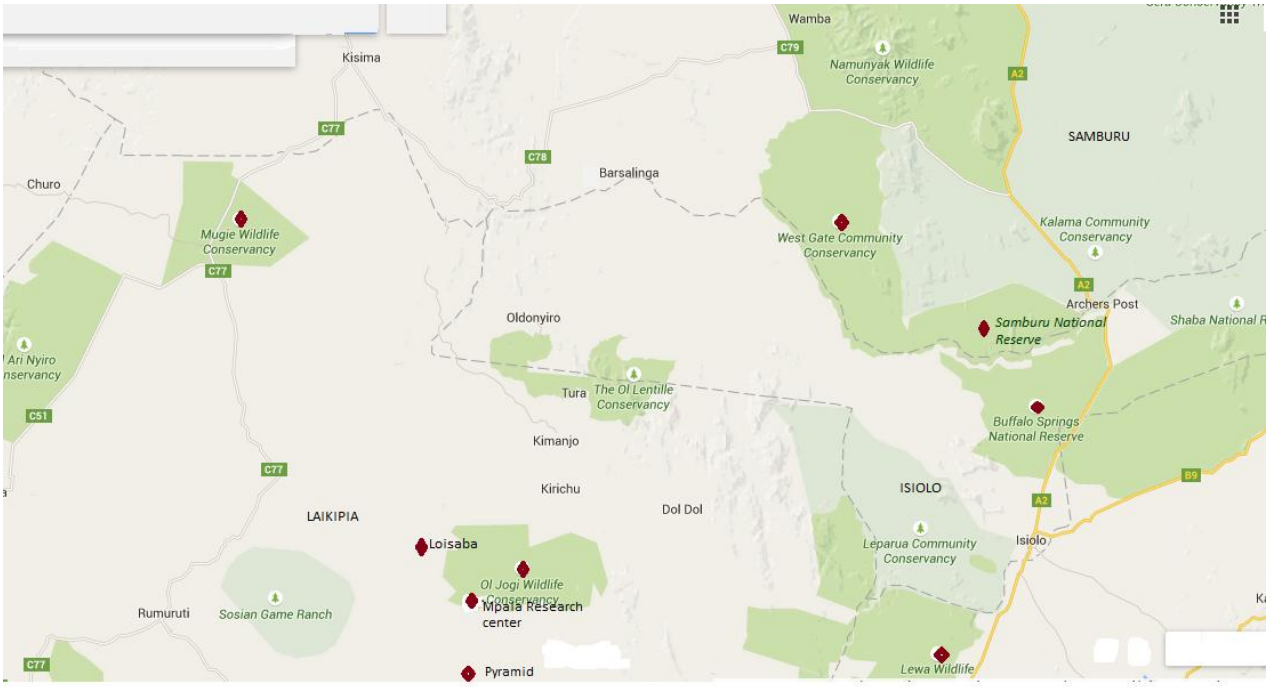


Figure 4: Sampling locations for Grevy's zebra feaces from Laikipia and Samburu and Samburu Counties in Kenya

4.3: Marker Identification and Primer Design

To identify candidate markers, the horse genome was searched using Msatcommander (Faircloth 2008) delimiting the search to include only perfect tetra-nucleotide repeats. Primers were selected using a Primer 3 (Rozen and Skaletski 2000) in Msatcommander. The output was then screened in UCSC genome browser and only markers at least 5 kb away from any RefSeq genes and whose primers are in a well conserved region were selected. BLAT (Kent 2002) was used to check the uniqueness of each of the primers. Finally 48 tetra-nucleotide repeats (Table 1) that fit these initial selection criteria were selected. Primer labeling was done using the cost effective protocol of Boutin-Ganache et al. (2001). For each primer pair, either the forward or reverse primer was designed to contain 17-20bpM13-tail. The length of the primer varied depending on

how much matching sequence was present at the 5' end of the primer sequence. The non-M13 primer was designed with a GTTT pigtail at the 5' end in order to reduce possibility of stutter banding (Brownstein et al. 1996). The M13 primer was then labeled with one of four M13 dye labeled primers during the PCR reaction (6-Fam, VIC, NED, or PET).

4.4: PCR Amplification

PCR and genotyping reactions were conducted at the National Museums of Kenya (NMK) and the International Livestock Institute (ILRI) in Kenya for Grevy's zebra, and at the University of California Los Angeles (UCLA) for plains zebra. At UCLA and NMK, PCR amplification was conducted using the QIAGEN Multiplex PCR Kit in 10 ul reactions containing 1.5 ul of template DNA (40-50ng/ul), 1.0 ul Primer mix, 0.4 ul10uM Bovine serum Albumin (BSA) and 5.0 ul Master Mix (containing Hot StartTaq Polymerase, dNTPs, PCR buffer, 3Mm $MgCl_2$) and the remaining double distilled water. At ILRI reactions were performed using 1.0 ul 10X NEB PCR Buffer, 0.8ul 25mM $MgCl_2$, 0.8 ul of 2mM dNTPs, 0.04 ul 5U Amplitaq, 1 ul primer mix, 0.4 ul10uM BSA., 3.5 ul sterile H_20 1.5 ul DNA template. The primer mix was a cocktail of the three primers: Primer 1(reverse) (100 uM), Primer 2 (Foward-M13 Hybrid) (2.5 uM), M13 dye labeled primer (2.5 uM), and ddH_2O in the ratio 1:2:2:45 respectively. The reaction was set in a Techne TC-4000 (VS 34.11) Thermo-cycler at NMK and an Eppendorf Mastercycler at UCLA and ILRI using the following touch-down PCR protocol: 95°C for 15 min; 30 cycles at 94°C for 30 s, 59°C for 90 s and 72°C for 60 s; then 20 cycles at 94°C for 30 s, 53°C for 90 s, and 72°C for 60 s plus a final extension at 60°C for 30 min.

Table 1: 48 Tetra-nucleotide microsatellites tested

Marker Name	Forward Primer	Reverse Primer	Repeat motif	Chromo-some number	Size (bps)
EC1AAGG13	AATGTTTGAAGTGCGACAG	**GGCTCACCTCTAGCAAATG**	AAGG(13)	1	320
EC1ATCC10	GGTGCCTAGGGTTACTTTC	**AATGTTTGTGACTACCAGTGC**	ATCC(10)	1	448
EC10AAAT10	AGCCCTGAGTGACTACAGC	**GGAAGCCTCTAAATATAAACAAC**	AAAT(10)	10	408
EC10AAAT9	TTCCCTTGGGATATCTAGTG	**AAAGATTCTATGGCCTCAG**	AAAT(9)	10	376
EC10AGAT9	CCCTCTTCCGGCATATAC	**AAAGTAGGAACATGTACAACAATC**	AGAT(9)	10	212
EC15AAGG11	TCCTTCTTCCTTGCTTCAG	**AGGACCACGTGTATTCAGTG**	AAGG(11)	15	236
EC15AAGG9	GGACCACTGAAATGACAAAC	**AATTTCTTGATTGCATGTCC**	AAGG(9)	15	312
EC16AGAT12	GGTGAGCGCAAATTTC	**TTTGCTGTGTAGTCATTTGAAG**	AGAT(12)	16	432
EC16ATCC9	ATTCCTATGTACCATATAATGTAGTG	**CAGGGAACGTATAAAGTGC**	ATCC(9)	16	336
EC17AAAG18	TCCACAGAGAATAAGATTGGTAG	**GCACATATGTCCAGCAGAAC**	AAAG(18)	17	426
EC17AAGG10	TGAGCCTTAGGAGTCTTCAG	**TGGCCAGTCTAGTTTCCTC**	AAGG(10)	17	351
EC17AGAT13	GCAGTTAAATGTATAAGCTTGTTC	**AAGGTCACCGATTGTTCTC**	AGAT(13)	17	404
EC17ATCC9	AAATTAAACCTGGCATAGATG	**TTCAATCCATCCATACAGC**	ATCC(9)	17	296
EC18AGAT11	ATGTTCCAAGAGCAACAAG	**TAGTTCTGCGCTGCTGTAC**	AGAT(11)	18	376
EC18AGAT13	TTTAGATTCTTTACCCATATCTCTC	**TCCCATATGTATCCGTAGC**	AGAT(13)	18	328
EC18AGAT9	AAGTCAAATTCTTCAAACTGG	**CAGCAAGCACAACTCCTC**	AGAT(9)	18	216
EC18ATCC10	CACCAGGGAGTTTATCACC	**ACCAGCCCTAATAGAGAGG**	ATCC(10)	18	436
EC19AAAT9	AAGATAAAGTACGTATTCTGGTTTC	**CAACTCTACTACATACATTCCAGAAG**	AAAT(9)	19	416
EC2AAAG14	AAACCCATTTAGAGGACATG	**GGTCAGATATCCATGCAAAG**	AAAG(14)	2	288
EC2ATCC9	GGCACTGTCAGATTTCCAC	**CTCCTGTTCAAAGTAGTGGATAG**	ATCC(9)	2	240
EC21AGAT12	TTTCTCAATTTGTCAAGTGG	**TCCCTATATTGGTGTCTCTATATTAG**	AGAT(12)	21	224
EC21AGAT9	TGAGTATTAAGATATCATCCTTCCTC	**AATGAACTACTCCATTTGACC**	AGAT(9)	21	324
EC22AAAT9	CAGTTCTTGCATCTGATTTG	**GCCTCTAAATATTGCCTGTC**	AAAT(9)	22	328
EC22AGAT11	AAAGTTTAGGATTGTGTTTAGG	**CCTGATTTCTCCAAGACTTATG**	AGAT(11)	22	256
EC25AAGG11	GCTTAAGTGCCAGTGTTTG	**TTACTGCGTTTCCTTCAAG**	AAGG(11)	25	420
EC25ATCC10	AGCGGTTTCAGCTTACTAC	**GGTTCCTTTGCAAACTTC**	ATCC(10)	25	436
EC27AGAT10	GATCAGTGCAGCAAGGTC	**GTGGCACCTTCTCTGTTTC**	AGAT(10)	27	448
EC27AGAT12	TATGCAGCATGATAACACC	**CATGACCCAGCATATATATCG**	AGAT(12)	27	424
EC27AGAT9	AAACTCAGACAATGCCATTC	**GGTCATTGTGTGTCTGTTCC**	AGAT(9)	27	432
EC28ATCC12	CTTCTCATCCATTCCAACAG	**TTTCTGGGAAGATTACTGC**	ATCC(12)	28	426
EC29ATCC9	GAGTAATAAGAAGGCACGATATC	**GCTCTCTCCTGATGCGTAG**	ATCC(9)	29	344
EC3AAAT10	AACAATATCCCAACCAAAG	**TGCTGGTAAGGTGAGTTTC**	AAAT(10)	3	272
EC3AACC10	GACCTCCTTTCCCAGAGAC	**AATGTATGCCCGAGATTTAC**	AACC(10)	3	328
EC30AGAT10	ACCCTAGAATTTCCCAGTG	**AGCCTTATCACCCTGAATG**	AGAT(10)	30	288
EC30AGAT11	TAAGCTTCTGATGCACACC	**AGGCTGTTAGGGTCTGTTC**	AGAT(11)	30	305
EC31ACAT9	CCCACAGACTGAGAACCTC	**GAAAGCTATCCCTACAATGAAG**	ACAT(9)	31	162
EC4AAAT9	CAGGTTGCCATTATGTTTC	**GAGGATGTAAGCAGGTTTG**	AAAT(9)	4	368
EC5AAAG13	GGGAGAAATATTGCAGAGG	**GACTGCGGAACACCTATTC**	AAAG(13)	5	424
EC7AAAG11	CTAGAAGCATGCACCATTC	**GCAAGACTTGAAGAGGAATG**	AAAG(11)	7	356
EC7AGAT12	TGACCCTCCATTGTTTAAAG	**AGTGGTAGATAAAGCTAGAGTGTG**	AGAT(12)	7	192
EC8AAAG16	CTACATCAACCCAGGAACC	**TACGGTTGGAATGTGACAC**	AAAG(16)	8	336
EC8AGAT10	ATTCCTCCGTTATCAGTGG	**AGCAATGCAAGGTTCTAAG**	AGAT(10)	8	256
EC8AGAT13	TTTCTTCCAGGTTTGAATG	**AGTAATGGCTCAGGGAAAG**	AGAT(13)	8	416
EC9AAAG19	CACCCACTTATTTGACAGC	**TGGTGAGAATGATGCTACC**	AAAG(19)	9	432
EC9AAGG10	CAGGCTCTTCAAATGTCTG	**GGTGACCTGCCTCTTTC**	AAGG(10)	9	192
EC9AGAT12	GGTTAGTATGCCAGCAGTTG	**GCACACATTACCAGAATTATTAATC**	AGAT(12)	9	286
EC9AGAT14	AAAGGCAGCAAGGTGTC	**CTGAGGAGAAATGGAACTTC**	AGAT(14)	9	400
ECXAAGG10	GTTTCAGCTGCTGGAAAG	**AATCCCTCATGTCCTGTTG**	AAGG(10)	X	400

4.5: Fragment Separation and Genotype scoring

A sample of the PCR products was run on 8% Polyacrylamide gel to confirm amplification. The remaining products were diluted with HiDi Formamide and LIZ standard and run on an ABI 3730 capillary sequencer. The output files were analyzed using GeneMapper v3.7 (Applied BiosystemsInc). Genotypes from SSRs were exported to an Excel spreadsheet for statistical analyses.

4.6: Data Analysis

4.6.1 Marker development and testing

Markers were analyzed for levels of successful amplification for blood samples. Those markers amplifying with success rates above 75% on blood were also amplified on fecal DNA to establish their utility on non-invasive samples. Preliminary data processing was done using Microsatellite Toolkit (Add-in for Microsoft Excel: Stephen Park, Trinity College, Dublin (http://animalgenomics.ucd.ie/sdepark/ms-toolkit/) where the data was checked and converted to Genepop format or converted into other formats using CONVERT (Glaubitz 2004) or CREATE (Coombs et al 2008). Mean number of alleles per locus, observed (Ho) and expected (He) heterozygoity were assessed using FSTAT (Goudet 2001) and GenAIEx 6.5 (Peakall and Smouse, 2012) to estimate the level of genetic polymorphism of the markers on the blood samples. The informativeness of the markers was further quantitatively accessed by Polymorphic Information Content (PIC) statistics (Hildebrand et al. 1992) using CERVUS 3.0.7 software (Kalinowski et al. 2007).

PIC measures the value of a marker for detecting polymorphism in a population. It depends on the number of detectable alleles and the distribution of their frequencies, and equals 1 minus the sum of the square of all allele frequencies (Liua and Cordes 2004). Microsatellite marker with two alleles of frequency 0.5 each will for instance have PIC value equals 1- [(0.5)2 + (0.5)2] = 0.5, while PIC for a microsatellite marker of two alleles with allele frequencies of 0.9 and 0.1 is 0.18. Thus, the greater the number of alleles, the greater the PIC; and for a given number of alleles, the more equal the allele frequencies, the greater the PIC (Liua and Cordes 2004). Possible non random allelic associations between pairs of loci were not assessed because the numbers of samples were too low, and pooling different populations would cause a false linkage.

4.6.2 Genetic diversity and structure

Genetic variation and differentiation measures, i.e allelic diversity, heterozygosity, allelic richness, mean number of alleles and F-Statistics were assessed on the fecal DNA derived data using GenAIEx 6.5 (Peakall and Smouse 2012). Analysis of molecular variance (AMOVA) was also conducted as implemented in GenAIEx and genetic structure and population connectivity estimated using STRUCTURE 2.3.4 (Pritchard et al 2000). Output files from STRUCTURE were analyzed using the online based software CLUMPAK (Kopelman et al 2015) that combines the functions of CLUMPP and DISTRUCT to summarize replicated runs from STRUCTURE to generate graphical presentation of the data as well as estimate the probable number of clusters from the data set. The estimated optimum number of clusters, (K) was also assessed using STRUCTURE HARVESTER (Earl and vonHoldt 2012) implementing both the methods of Rosenberg et al (2001) and Evanno et al (2005). Principal Coordinate analysis (PCoA) in GenAIEx was performed to further explore the pattern of variation between the populations.

5.0: RESULTS

5.1 Marker development and testing

Thirty three out of the 48 markers tested amplified well (75% of the time) for Grevy's blood samples and 38 amplified well for Plains zebra blood samples. The 33 markers that had good amplification for Grevy's blood were also tested on 6 to 100 Grevy's fecal samples and 17 amplified well (>75%) with 26 of the markers amplifying at least 50% of the time with the fecal samples. Number of alleles per locus ranged between one and seven for both the Grevy's (average 3.4) and the plains zebra (average 4.0) as assessed using the data from blood samples alone. Heterozygosity Observed (Ho) and Expected (H_e) for this category of samples were 0.421 and 0.47 respectively for the Grevy's and 0.453 and 0.556 for the plains zebra while Allelic richness averaged 3.2 for the Grevy's and 3.7 for the plains zebra.

On the PIC criterion, 17 and 23 markers, were highly informative (PIC Values $\geq$ 0.5) respectively, for Grevy's and plains zebra, while four and eight were moderately informative (PIC values 0.25-0.5) (Botstein et al 1980) (Table 2-in bold, markers at least moderately informative in either species). Average PIC Value for the markers was 0.38 for Grevy's zebra and 0.46 for the plains zebra. Of the 21 Grevy's markers selected as at least moderately informative on PIC criterion, twelve amplified at least 50% of the time and seven amplified at least 75% of the time on DNA derived from Grevy's feces. This outcome could likely be substantially improved by extracting underperforming extracts additional times and combining and concentrating extracts.

Table 2: Amplification success rate %, Heterozygosity (Ho and He), PIC values and Allelic richness (AR) for Grevy's and Plains zebra (n/a means not assessed)(in bold, markers at least moderately informative for either species).

Locus	Grevy's Zebra							Plains zebra							Grevy's fecal trials		
	%	N	Ho	He	PIC	AR	Allelic Range	%	N	Ho	He	PIC	AR	Allelic Range	Samples tested	successes	% success
EC1AAGG13	100	9	0.11	0.11	0.09	1.8	293-301	0	n/a	n/a	n/a	n/a	n/a	n/a	6	6	100
EC1ATCC10	**83**	**8**	**0.5**	**0.74**	**0.64**	**4.7**	**457-469**	50	n/a	n/a	n/a	n/a	n/a	n/a	6	5	83
EC10AAAT10	**100**	**9**	**0.77**	**0.72**	**0.62**	**4.5**	**402-430**	**100**	**8**	**0.75**	**0.73**	**0.64**	**4.6**	**414- 434**	65	43	66
EC10AAAT9	25	n/a	n/a	n/a	n/a	n/a	376	25	n/a	n/a	n/a	n/a	n/a	n/a	6	0	0
EC10AGAT9	**78**	**7**	**0.86**	**0.7**	**0.58**	**3**	**222- 230**	**88**	**7**	**0.43**	**0.48**	**0.41**	**3**	**218- 232**	6	3	50
EC15AAGG11	100	9	0.11	0.11	0.1	1.8	240- 244	**88**	**7**	**0.14**	**0.36**	**0.28**	**2**	**240-244**	6	6	100
EC15AAGG9	**78**	**8**	**0.75**	**0.67**	**0.59**	**4.7**	**317- 336**	**100**	**8**	**0.75**	**0.71**	**0.62**	**4.4**	**301- 340**	91	63	70
EC16AGAT12	100	9	0.11	0.11	0.1	1.8	421- 425	100	8	0	0	0	1	421	6	5	83
EC16ATCC9	**100**	**9**	**0.33**	**0.72**	**0.64**	**5.3**	**333- 365**	**100**	**8**	**0.13**	**0.64**	**0.55**	**3.7**	**352-369**	91	78	86
EC17AAAG18	**100**	**9**	**0.89**	**0.82**	**0.75**	**6.4**	**413- 450**	**100**	**8**	**0.63**	**0.62**	**0.55**	**5**	**401 -492**	87	60	69
EC17AAGG10	100	9	0	0	0	1	341	100	8	0	0	0	1	341	6	5	83
EC17AGAT13	**86**	**8**	**1**	**0.8**	**0.71**	**4.9**	**406 - 431**	**100**	**8**	**0.75**	**0.7**	**0.61**	**3.9**	**419 -431**	6	3	50
EC17ATCC9	0	n/a	n/a	n/a	n/a	n/a	n/a	0	n/a	n/a	n/a	n/a	n/a	n/a	6	0	0
EC18AGAT11	**100**	**9**	**0.44**	**0.6**	**0.52**	**3.8**	**376 - 392**	**100**	**8**	**0.75**	**0.88**	**0.8**	**6.3**	**382 -398**	6	0	0
EC18AGAT13	**100**	**8**	**0.63**	**0.71**	**0.62**	**4**	**208 - 345**	**100**	**8**	**0.75**	**0.81**	**0.72**	**4.9**	**345 - 365**	6	0	0
EC18AGAT9	88	8	0.25	0.23	0.2	2	241- 242	**100**	**8**	**0.25**	**0.84**	**0.76**	**5.6**	**222- 254**	6	1	17
EC18ATCC10	**100**	**9**	**0.78**	**0.5**	**0.36**	**2**	**439- 443**	75	6	0	0	0	1	439	6	5	83
EC19AAAT9	**100**	**9**	**0.33**	**0.43**	**0.32**	**2**	**442 - 450**	75	6	0	0.3	0.24	2	445 -450	6	5	83
EC2AAAG14	78	7	0	0	0	1	308	**100**	**8**	**0.63**	**0.78**	**0.7**	**5.4**	**304 - 328**	6	3	50
EC2ATCC9	100	9	0.22	0.22	0.19	2.6	215 - 253	100	8	0.25	0.23	0.2	2	253 - 257	90	77	86
EC21AGAT12	17	n/a	n/a	n/a	n/a	n/a	224	**100**	**8**	**0.88**	**0.84**	**0.77**	**6.3**	**218 -246**	6	6	100
EC21AGAT9	78	7	0	0	0	1	406	**100**	**8**	**0.5**	**0.61**	**0.5**	**3**	**406 -414**	6	1	17
EC22AAAT9	100	9	0	0	0	1	337	**88**	**7**	**0.57**	**0.47**	**0.39**	**2.9**	**325 -341**	6	1	17
EC22AGAT11	**100**	**9**	**0.89**	**0.81**	**0.74**	**6.3**	**265 - 274**	**100**	**8**	**0.75**	**0.73**	**0.65**	**5.2**	**257 -281**	6	5	83
EC25AAGG11	**75**	**7**	**0.57**	**0.69**	**0.59**	**4**	**434 -449**	**100**	**8**	**0.88**	**0.75**	**0.68**	**5.9**	**426- 458**	6	0	0
EC25ATCC10	67	n/a	n/a	n/a	n/a	n/a	436	**88**	**7**	**0.29**	**0.71**	**0.59**	**3**	**475 - 483**	6	1	17
EC27AGAT10	0	n/a	n/a	n/a	n/a	n/a	448	**100**	**8**	**0.5**	**0.67**	**0.59**	**4.4**	**463 - 479**	6	0	0
EC27AGAT12	**78**	**7**	**0.71**	**0.62**	**0.5**	**3**	**401 - 409**	**100**	**7**	**0.71**	**0.56**	**0.46**	**3**	**406 -414**	6	6	100
EC27AGAT9	11	n/a	n/a	n/a	n/a	n/a	432	0	n/a	n/a	n/a	n/a	n/a	n/a	6	0	0
EC28ATCC12	50	n/a	n/a	n/a	n/a	n/a	426	100	8	0	0.23	0.2	2	420 -425	6	5	83
EC29ATCC9	78	7	0	0.26	0.22	2	279 -359	0	6	0	0	0	1	359	6	2	33
EC3AAAT10	**100**	**9**	**0.56**	**0.62**	**0.5**	**3**	**279 - 341**	**100**	**8**	**0.88**	**0.82**	**0.73**	**5.4**	**267- 302**	97	82	85
EC3AACC10	100	9	0	0.21	0.18	2	307 -341	**100**	**8**	**0.38**	**0.33**	**0.26**	**2**	**314 -341**	6	5	83
EC30AGAT10	**100**	**9**	**0.44**	**0.58**	**0.48**	**3.6**	**295 -321**	**100**	**7**	**0.71**	**0.58**	**0.5**	**3.8**	**300 -316**	6	1	17
EC30AGAT11	**89**	**9**	**0.56**	**0.69**	**0.6**	**4.5**	**295 - 325**	**100**	**8**	**0.13**	**0.64**	**0.52**	**3**	**301 -321**	71	62	87
EC31ACAT9	13	n/a	n/a	n/a	n/a	n/a	162	0	n/a	n/a	n/a	n/a	n/a	n/a	6	0	0
EC4AAAT9	**78**	**7**	**0.43**	**0.76**	**0.66**	**4**	**380 -435**	100	8	0.25	0.24	0.22	2.5	373 -384	6	0	0
EC5AAAG13	**78**	**7**	**0.43**	**0.5**	**0.35**	**2**	**425 -435**	**75**	**6**	**0.5**	**0.56**	**0.48**	**4**	**418 -430**	6	0	0
EC7AAAG11	89	8	0	0	0	1	356	50	n/a	n/a	n/a	n/a	n/a	n/a	6	5	83
EC7AGAT12	44	n/a	n/a	n/a	n/a	n/a	192	**88**	**7**	**1**	**0.89**	**0.8**	**6.6**	**198 - 230**	6	4	60
EC8AAAG16	44	n/a	n/a	n/a	n/a	n/a	336	**88**	**7**	**0.14**	**0.47**	**0.39**	**2.9**	**316 -325**	6	1	17
EC8AGAT10	**89**	**9**	**0.89**	**0.84**	**0.77**	**5.7**	**235 -278**	**75**	**6**	**0.5**	**0.79**	**0.68**	**5**	**262 -278**	70	66	94
EC8AGAT13	22	n/a	n/a	n/a	n/a	n/a	416	0	n/a	n/a	n/a	n/a	n/a	n/a	6	0	0
EC9AAAG19	**88**	**7**	**0.43**	**0.85**	**0.75**	**6**	**408 -440**	**100**	**8**	**0.75**	**0.88**	**0.8**	**6.3**	**413 -449**	6	0	0
EC9AAGG10	71	n/a	n/a	n/a	n/a	n/a	192	**100**	**8**	**0.13**	**0.33**	**0.26**	**2**	**192 -200**	6	6	100
EC9AGAT12	50	n/a	n/a	n/a	n/a	n/a	286	**100**	**8**	**0.75**	**0.86**	**0.78**	**6.2**	**285 -320**	6	4	67
EC9AGAT14	38	n/a	n/a	n/a	n/a	n/a	400	62.5	n/a	n/a	n/a	n/a	n/a	n/a	6	3	50
ECXAAGG10	38	n/a	n/a	n/a	n/a	n/a	400	**88**	**7**	**0.29**	**0.63**	**0.52**	**3.7**	**368 -408**	6	0	0

5.2 Genetic diversity and Structure

From the analysis using Grevy's fecal samples, number of alleles per locus ranged between 11 and 46 with a mean of 22.7. Mean Heterozygosity observed (H_o) and expected (H_e) across loci and populations were 0.489 and 0.661 respectively. F-statistics (Table 3) revealed a considerable level of genetic differentiation (average Fst = 0.162) though the levels of migration across populations was estimated to be quite high (Nm=1.895). Inbreeding, as measured by Fis was highly varied across loci, averaging 0.274. There was excess of homozygotes in all loci except one (EC8AGAT10) as shown by positive values of Fis and the low observed heterozygosities. Analysis of molecular variance (AMOVA) indicated little variation among populations (3%) with more variation partitioned to the within individual differences (46%) and among individual differences (51%) (Table 4)

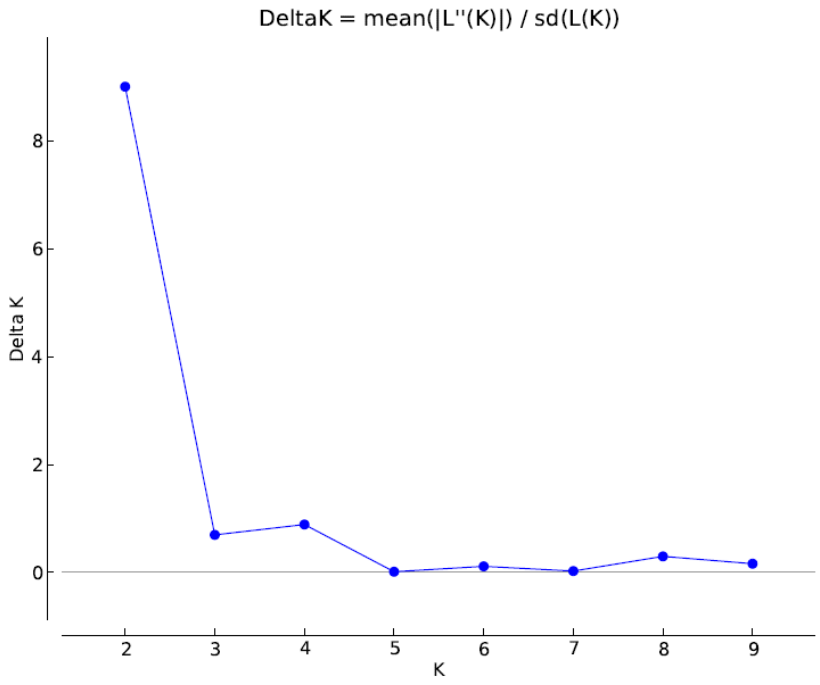
Table 3: Number of alleles, Heterozygosity and F-Statistics for Seven microsatellite markers.

Locus	N	Number of Alleles	Heterozygosity (Observed)	Heterozygosity (Expected)	F_{IS}	F_{IT}	F_{ST}	Nm
EC10AAAT10	82	11	0.444	0.711	0.375	0.412	0.059	4.009
EC15AAGG9	82	46	0.532	0.712	0.252	0.400	0.198	1.011
EC16ATCC9	82	14	0.257	0.458	0.438	0.664	0.402	0.371
EC17AAAG18	82	21	0.521	0.704	0.261	0.318	0.078	2.947
EC3AAAT10	82	29	0.395	0.638	0.381	0.454	0.117	1.878
EC30AGAT11	82	14	0.490	0.632	0.225	0.337	0.144	1.481
EC8AGAT10	82	24	0.785	0.772	-0.017	0.123	0.137	1.570
Mean	82	22.7	0.489	0.661	0.274	0.387	0.162	1.895

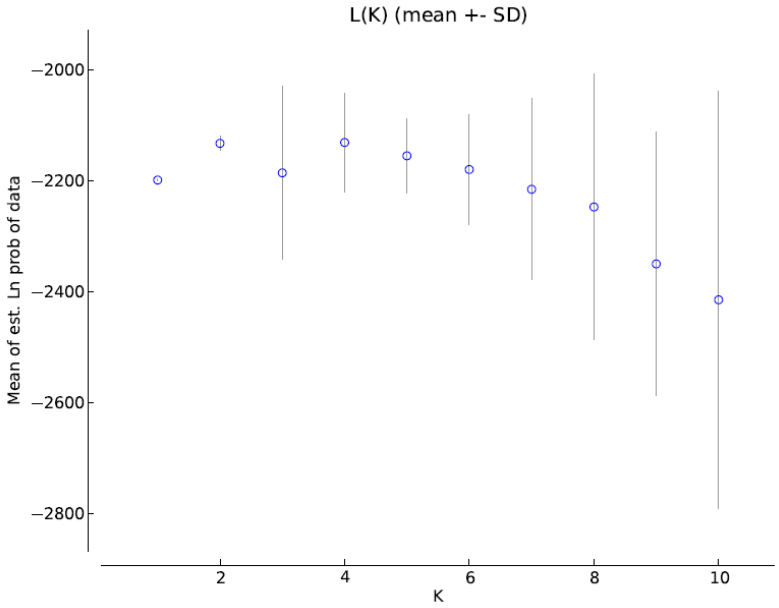
Table 4: Summary of AMOVA analysis

Summary AMOVA Table					
Source	df	SS	MS	Est. Variance	%
Among Populations	8	53.433	6.679	0.094	3
Among Individuals	73	363.012	4.973	1.712	51
Within Individuals	82	127.0	1.549	1.549	46
TOTAL		543.445		3.355	100

Clustering by STRUCTURE indicated very limited genetic structure among populations contrary to the Fst values but in agreement with the high estimates of migrants per generation (Nm) and the results of AMOVA. Structure analysis further supports existence of two main clusters (Optimum K = 2) according to Evanno et al 2005 using Delta K to estimate optimal number of clusters (K) (Figure 5a). Optimal K, according to Rosenberg et al 2001 could be K= 2, 3 or 4 (Figure 5b). Rosenberg et al. 2001 estimates optimal K using the log likelihood for each K, Ln P(D) = L(K) which plateaus (or continues increasing slightly) and has high variance between runs when approaching true K. The true value of K may be difficult to determine, but we aim for the smallest value of K that captures the major structure in the data (Pritchard et al. 2000). All the individuals were admixed to varying degrees (Figure 6).



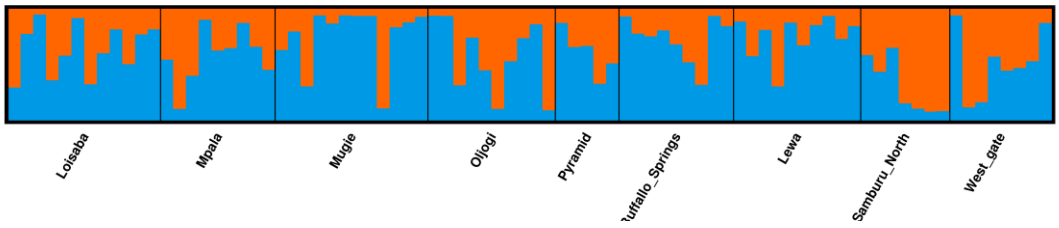
a)



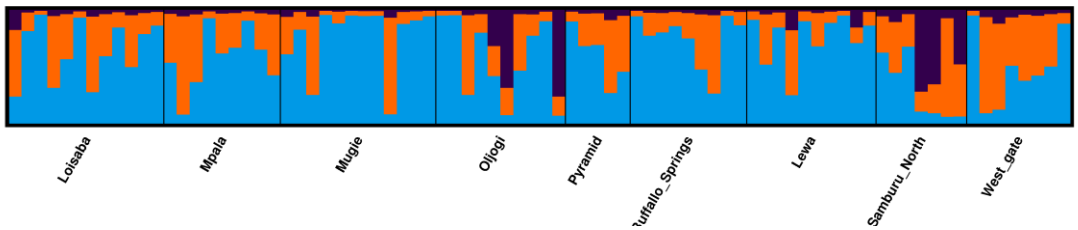
b) _____

Figure 5: Estimating optimum K using the methods outlined in a) Evanno et al 2005 and b) Rosenberg et al 2001 for Grevy's zebra

a) K= 2 (Admixture model)



b) K= 3 (Admixture model)



c) K = 4 (Admixture model)

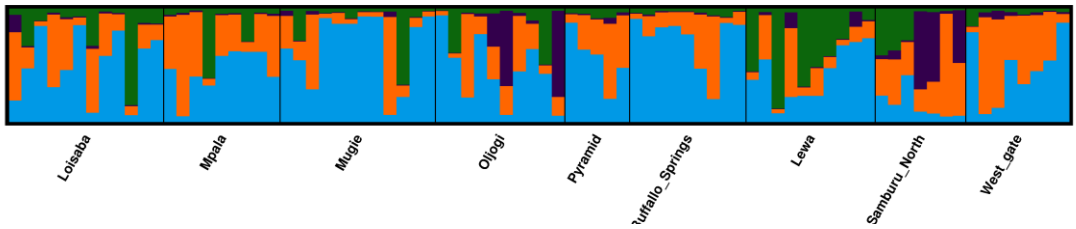


Figure 6: Bar plots showing clustering of individuals in STRUCTURE results from K=2 to K=4

Principal coordinate analysis (PCoA) distinctively separated the Mpala and Pyramid populations from the rest which were clustered together (Figure 7). Percentages of variation explained by the first three axes were 56.7%, 27.52% and 11.45% respectively.

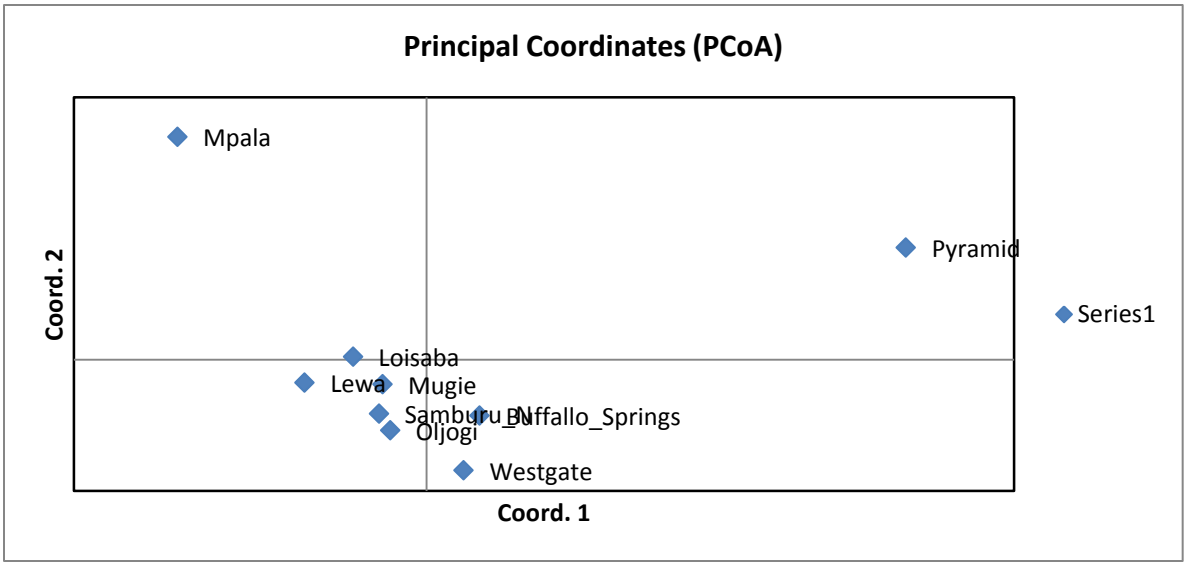


Figure 7: a) First and Second component of Principle coordinate analysis of seven microsatellites in nine populations of Grevys

6.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

Successful amplification for both blood and fecal samples confirm the efficacy of the tested markers for genetic analysis of the two zebra species (Grevy's and Plains). The successful extraction and amplification of DNA from Grevys' fecal samples further confirm that non-invasively obtained samples are equally valuable and can successfully be used for genetic analysis of the wild grevy's zebra population. This is important for endangered species such as the Grevy's zebra whose populations require minimal human interruption. The markers were also sufficiently polymorphic considering the small sample sizes used. This will facilitate comprehensive genetic analysis of the endangered Grevy's population needed to determine its structure and confirm the hybridization threats. These preliminary results further suggest low genetic variation among the endangered Grevy's population compared to the more abundant Plains zebra. This observation however requires confirmation with more comprehensive sampling and the use of a larger number of markers. This current study represents early attempts at understanding the genetic makeup of wild Grevy's population in Kenya that is facing extinction threats yet has little record in literature of its genetic status. Additional markers developed here add to the expanding number of genetic tools for the population analysis of wild equines. Also as a quality measure, comprehensive screening of all markers before application in cross-species studies is important because markers do not work uniformly well across all species in the same genus (Morin *et al*., 1998; Chambers *et al*., 2004). For instance in this study, different amplification success was recorded for Grevy's and plains zebra for markers identified for the domestic horse.

Microsatellites are also generally more polymorphic in the species from which they are cloned than in related species thus loci chosen on the basis of polymorphism on one species often exhibit shorter repeats in related species (Ellegren, 1995). This further emphasizes the need for continued screening of markers.

Preliminary F_{ST} values (F_{ST}= 0.162) are indicative of high divergence between sampled populations (Cavalli-Sforza *et al.,* 1994) and the presence of minimal geneflow. Geneflow is estimated between 1 to 2 migrants per generation following the formulae: $F_{ST} = 1/(Nm +1)$. The actual value from these analysis was Nm = 1.895. Homozygote excess observed could be the result of non-random mating or population sub-division (Wahlund effect) but under circumstances of low quality/quantity DNA as for fecal samples, allele dropouts (one allele of a heterozygous individual to go undetected) may also present as homozygote excess. PCR replicates would help to confirm if observed excess of homozygotes is due to allelic dropout (Taberlet *et al*., 1996) but was not done due to limited resources available for this study. Excess homozygotes may also result from null alleles due to slippage during amplification process, especially with small sample sizes but such excess due to null alleles would be locus specific (De Meeûs *et al*., 2006 ; 2007). The homozygote excess in the current case was spread across loci and populations. Wahlund effect due to apparent population structuring (subdivision) of the Grevy's zebra population is the most probable cause of homozygote excess in the current case.

F_{IS} indices were all non-zero and predominantly positive across loci indicating departure from Hardy-Weinberg proportions indicative of possible non-random mating within the subpopulations and suggestive of inbreeding. Inbreeding increases the frequency of homozygous

loci which increases the potential for expression of recessive deleterious or lethal alleles thus increasing the risk of extinction of species (Kennedy *et al*., 2014). Inbreeding depression also reduces traits associated with early fitness like juvenile survival (Pusey & Wolf, 1996; Keller & Waller, 2002). Data suggest that numbers of Grevy's have also decline because recruitment has been limited by low levels of infant and juvenile survival (Williams 1998) which may be indication of the effects of inbreeding depression.

The results generally suggest that the Grevy's population in the regions sampled is a moderately structured population with possible inbreeding within subpopulation and very limited subpopulation connectivity. AMOVA reveals higher variation within individuals and among individuals within subpopulations as opposed to among sub-populations suggesting higher genetic diversity within subpopulations with minimal genetic differentiation between them. This may be the result of substantial recurrent gene flow as supported by the distribution of admixture in all individuals (Figure 6 a, b and c) further indicating some levels of gene flow among the subpopulations. The amount of gene flow however may be insufficient to facilitate substantive divergence. The minimal differentiation is highly indicative of a recent habitat fragmentation event allowing insignificant time of isolation for accumulation of substantial divergence to occur. This is consistent with the recent history of habitat loss/fragmentation due to overgrazing by livestock and depletion of water and other rangeland resources through completion with livestock and hunting activities throughout the Grevy's range in Kenya (Low *et al.*, 2009;Rubenstein, 2010). The AMOVA emphasizes the nature of population structuring present but does not clearly indicate the level of variation within the subpopulations thus does not contradict the possible inbreeding observation from the F_{IS} results. The reason for the separation

of Mpala and Pyramid populations from the rest by PCoA is not clear. Perhaps analysis with more markers would resolve the fine scale structure and validate these observations. Inclusion of more markers and extensive sampling was not possible in the current study due to limited resources.

This study concludes that;

i) Tetra-nucleotide microsatellite markers available for the domestic horse (*Equus caballus*) amplify well and have sufficient polymorphism to be used for population genetic analysis of Grevy's and Plains zebras.
ii) Kenya's wild Grevy's zebra population may survive short term environmental challenges with the current level of genetic diversity within subpopulations but conservation of this diversity and management of gene flow to increase the diversity is paramount for long term survival. Genetic drift is of concern for Grevy's zebra following the findings of low gene flow, high population divergence and the indicators of inbreeding.

We recommend that;

i) Wildlife management authorities in Kenya should adopt conservation measures to prevent further loss of genetic diversity of the Grevy's and institute measures to increase gene flow to ensure long term survival of the species. As a primary conservation objective, they should prioritize and maintain establishment of connectivity between the sub populations thorough habitat restoration or facilitated genetic exchange. Habitat restoration will facilitate natural dispersal throughout the species range. Where natural dispersal is not possible, genetic and demographic rescue through translocations may be done.

ii) Further analysis with a view to understanding the genetic diversities at each subpopulation should be conducted to inform translocation exercises. Precautionary approaches regarding potential effects of inbreeding depression on the viability of the species is key, hence translocations must be informed by the individual genetic diversity of the source populations.

iii) Analysis with a larger number of markers (from the markers screened in this study plus other appropriate markers) and a larger sample size to be conducted on the Kenyan Grevy's population to resolve its fine scale genetic structure.

iv) All studies employing the markers from the horse genome for analysis of other equines to continuously pretest/screen and report the outcomes of their screening work.

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