Genetic Variation and Parentage in the Ethiopian Wolf *Canis simensis*

Samantha Alison Hook

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School of Environment and Life Sciences

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Declaration

I declare that, with the exception of any statements to the contrary, the contents of this thesis are my own work, that the data presented herein has been obtained by experimentation and that no part of the report has been copied from previous thesis', book, manuscripts, research papers or the internet.

Signed……

Print name……Samantha Alison Hook……

Date……

Abstract

The Ethiopian wolf (*Canis simensis*) is among the most endangered canids in the world, with only 300-500 individuals left in the wild. The most severe threats for the remaining populations are habitat fragmentation, hybridisation with domestic dogs, and diseases (rabies and canine distemper virus). The present MSc by Research applies DNA fingerprinting (microsatellite genotyping) based on existing faecal samples collected between 2007 and 2010 to (i) determine the standing amount of genetic variation after recent rabies outbreaks, (ii) reveal the family structure within and between packs using genetic parentage analysis, comparing the obtained results with existing data from field observations and (iii) investigate the effects of recent rabies outbreaks on the genetics of the population. In total, 43 individuals were successfully characterised based on seven microsatellite loci, demonstrating that faecal samples collected several years before analysis are a valuable source for DNA. This is approximately between 10% and 20% of the total population of Ethiopian wolves. Parentage through software analysis in Colony found posterior probabilities of no less than 1.00 for all six offspring individuals analysed. The parentage assignments revealed that offspring regularly moved between packs, which may be attributed to the loss of individuals through rabies during the period of investigation. Analysis found through Coancestry determined that four out of six pairs of parents were above the mean pair-wise relatedness coefficient. It was found that inbreeding avoidance was not a contributing factor to producing offspring in the population. The results of this study contribute to our understanding of the social system of the Ethiopian wolf, document the consequence of disease outbreaks to pack structure, and should be useful to devise future in-situ management plans towards stabilising the existing amount of genetic variation.

Chapter One: Introduction

1.1. The Ethiopian Wolf

Endemic to the highlands of Ethiopia, the Ethiopian wolf *Canis simensis* is Africa's rarest and most endangered canid (Gottelli and Sillero-Zubiri 1994; Marino 2003; Marine and Sillero-Zubiri 2011; EWCP 2013). The Bale Mountains contain the largest number of individuals, with approximately half of the estimated population size (Marino 2003; Gottelli et al. 2004; Marino & Sillero-Zubiri 2011). Ethiopian wolves were classified as Endangered in 2004 under the IUCN Red List, as the population is affected by habitat fragmentation combined with a continuing decline in numbers of mature individuals (Marino and Sillero-Zubiri 2011). The species was first described by Rüppell in 1835 (Rüppell 1835; EWCP 2013; Marino & Sillero-Zubiri 2011). It did not receive full protection from Ethiopia's Wildlife Conservation Regulations until 1974 (Marino & Sillero-Zubiri 2011). Ethiopian wolves are one of four species of *Canis* within Africa, and were originally thought to be closely related to African jackals (*C. aureus, C. mesomelas, and C. adustus*). Phylogenetic analyses, however, revealed that Ethiopian wolves are more closely related to the grey wolf (*C. lupus*) and the coyote (*C. latrans,* Gottelli et al. 1992). This charismatic species has captured the attention of many organisations across the globe, encouraging numerous studies and leading to large media attention. As there are no animals in captivity, the only opportunity to document and study the wolves is in their natural environment (Marino and Sillero-Zubiri 2011; EWCP 2013).

1.1.1. Biology and Behaviour of Ethiopian Wolves

Ethiopian wolves are found in packs of between 2 and 18 adult individuals, with well-defined hierarchies (Randall et al. 2004). Each pack is a cohesive social family, with communal territories averaging 6 km² in size (Sillero-Zubiri and Gottelli 1995; EWCP 2013). Its morphology is very distinct, with long legs and muzzle. The main pelage of adult

wolves is bright rufous with a white underbelly. Males are on average 20% larger than females; males weigh up to 20 kg, while females reach no more than 16 kg (Sillero-Zubiri and Macdonald 1994). Typically one pack comprises one dominant male and female, with subordinate wolves who all cooperatively share roles in territory defence and nurturing offspring (Sillero-Zubiri et al. 1996). Females become receptive to mating between August and November (Sillero-Zubiri et al. 1998), with a gestation period that lasts between 60 and 62 days (Sillero-Zubiri et al. 1996). The dominant male and female are the only pair in a pack to copulate and produce young, although dominant females are receptive to visiting males from neighbouring packs if the opportunity arises (Sillero-Zubiri et al. 1996). During the mating season, levels of testosterone and glucocorticoid are higher in dominant males than their subordinates (van Kesteren et al. 2012). Similarly, dominant females' estradiol concentrations increase above that of their subordinate females during the mating season (van Kesteren et al. 2013). Dominant females are typically replaced after death by a subdominant resident daughter, resulting in a high risk of inbreeding (Randall et al. 2007). Dominant females usually produce litter sizes of up to six pups, and all group members participate in parental care (Sillero-Zubiri et al. 2004). Studies in the Bale Mountains have shown that the distribution and abundance of the Ethiopian wolves follows that of its main prey, afroalpine rodents such as the giant molerat *Tachyoryctes macrocephalus* (Sillero-Zubiri et al. 1994; Sillero-Zubiri & Gottelli 1995; van Kesteren et al. 2012; van Kesteren et al. 2013; EWCP 2013).

1.1.2. Geographical Range of Ethiopian Wolves

Ethiopian wolves currently exist in six extant populations at altitudes of 3,000–4,500 m: Simien Mountains, North Wollo and South Wollo highlands, Guassa-Menz, Arsi Mountains and the Bale Mountains (Figure 1, Sillero-Zubiri and Gottelli 1995; Marino

2003; Marino and Sillero-Zubiri 2011). They are endemic to the Ethiopian highlands North and South of the Great Rift Valley. Across their range, rainfall varies between 1,000 and 2,000 mm/year, and the high altitude undergoes one pronounced dry period annually from December to March (EWCP 2013). Due to the greater agricultural pressure in the Northern highlands, wolves are becoming increasingly restricted to elevations above 3,500 m (Yalden et al. 1980; Marino 2003; Haydon et al. 2006). Previous habitat ranges where Ethiopian wolves have recently become extinct include the Gosh Meda (North Shoa) and Mount Guna, while in Mount Choke Ethiopian wolves have been extinct for several decades (Figure 1, Marino 2003; EWCP 2014).

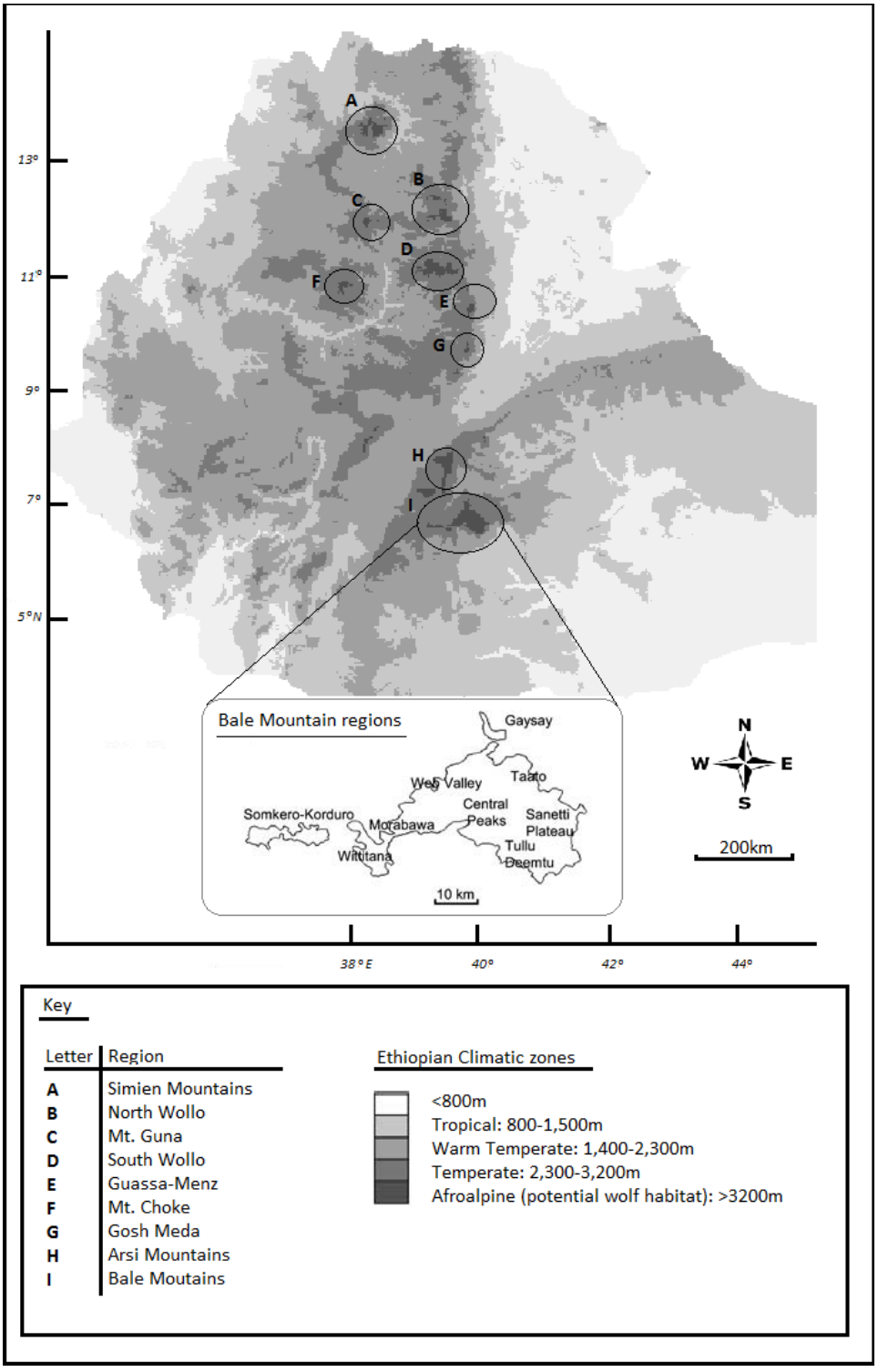

FIGURE 1. Wolf habitat still existing within the Ethiopian Highlands. The Bale Mountains is extended to display the different regions found within the area. Adapted from Marino (2003).

1.1.3. Bale Mountains National Park

The Bale Mountains National Park (BMNP) was proposed in 1960 in order to conserve the largest tract of afroalpine habitat on the continent, together with Ethiopia's two most charismatic mammalian endemics (the mountain nyala *Tragelaphus buxtoni* and the Ethiopian wolf; Hillman 1986; Refera and Bekele 2004). A project to formulate management plans for future conservation efforts did not occur until 1983, funded by Wildlife Conservation Society under the auspices of the Ethiopian Wildlife Conservation Organisation (Hillman 1986; Hillman 1988; Sillero-Zubiri et al. 1994; Marino 2003). This plan enabled the establishment of the BMNP, and today the park conserves 2,200 km² of highlands, mountains and lowland forests (located in the eastern portion of the mountains, Hillman 1988). The BMNP is located in the south-east of the Ethiopian highlands, south of the Great Rift Valley (Figure 1). The park lies 400 km south-east of the capital Addis Ababa, and due to its extreme altitudinal range is characterised by a wide array of different habitat types (Hillman 1986; Hillman 1988; BMNP 2015). These include northern grasslands (located at Gaysay, Figure 1), juniper woodlands (located at the parks headquarters), afroalpine habitats (located at the Sanetti Plateau and Web Valley, Figure 1), the Erica belt of moorlands, forests and the Harenna Forest (located across the BMNP, BMNP 2015). The close proximity of these rich habitats to the capital has resulted in significant human impacts. A government changeover in 1991 created civil unrest which disrupted the management of the BMNP, leading to an increase in encroachment of human settlements and livestock densities within the park boundaries (Stephens et al. 2001; Vial et al. 2010). The expansion and intrusion of the human population into remote areas such as the Bale Mountains often generates pronounced peaks in the threats to, or extinction of, native fauna and flora (Vial et al. 2010).

1.2. Decline of the Ethiopian Wolf

The total number of Ethiopian wolves in the wild is currently estimated at around 300 to 500 individuals (Haydon et al. 2006; Randall et al. 2007; Marino 2003). There are no reliable population estimates prior to 1986, and it is therefore impossible to determine the extent of recent declines (Marino and Sillero-Zubiri 2011). Nevertheless it seems reasonable to assume that current the number of Ethiopian wolves is a small fraction of that which existed before the impact of human activity. Recent declines are the result of direct human-wolf interactions, but perhaps more importantly, due to the effects of rabies which may be caused by a local increase in the number of domestic dogs.

1.2.1. Rabies and the Impact of Human Activity

Rabies is one of the oldest recorded zoonotic infectious diseases and is enzootic for a broad range of mammalian hosts (Tabel et al, 1974, Adamovich 1985; Butzeck 1987; Theberge et al. 1998). Without correct management, rabies can contribute to the decline and even extinction of wild species. Rabies is now the most common disease occurring in wild canids (Cleaveland et al. 2002; Funk et al. 2003; Randall et al. 2004; Woodroffe et al. 2004). The Ethiopian wolf is a prime example of how outbreaks in rabies can dramatically affect a population (Randall et al. 2004). Outbreaks within Ethiopian wolf populations have only been published for the previous 25 years; however, the disease has likely been a threat for centuries (Randall et al. 2004).

Rabies is an acute encephalomyelitis virus that is part of the genus *Lyssavirus* ('lyssa' from the Greek meaning 'rage', Jackson & Wunner 2007). The rabies virus (RABV) is one of fourteen defined strains of the Lyssavirus that are zoonotic, causing progressive encephalomyelitis in humans (Linscott 2012; Jackson 2013). RABV can be found on every continent (with the exception of the Antarctic) due to the large distribution of

mammalian reservoir hosts, consisting of various carnivore and bat species (Jackson 2013). Although it is one of the oldest recorded infectious diseases on the planet, there is still no effective vaccination and treatment which could potentially eradicate the virus (Butzek, 1987; Theberge et al. 1998). It is typically transmitted to humans through bites containing RABV-infected saliva (Jackson & Wunner 2007; Linscott 2012; Jackson 2013). RABV enters the peripheral nerves and travels to the brain for replication. RABV then spreads via the nervous system to tissues, concentrating largely in the saliva glands (Linscott 2012). Once symptoms are present in an individual, the disease is nearly always fatal (World Health Organisation 2013). More than 55,000 people die annually from rabies, 90% of which in Asia and Africa (World Health Organisation 2013) where there is a lack of effective health systems and domestic animal vaccination programs (Sterner & Smith 2006). In Ethiopia approximately 10,000 human deaths per year are caused by rabies (Fekadu 2007; Jemberu et al. 2013). This high mortality rate has made Ethiopia one of the worst affected countries in the world (Jemberu et al. 2013). Unvaccinated domestic dogs have been found to be the main reservoir of RABV, with more than 90% of rabies in humans originating from a domestic dog. However, little research has been done to identify other possible wildlife reservoirs (Randall et al. 2004; Haydon et al. 2006; Sterner & Smith 2006; Jemberu et al. 2013).

Between 35,000 and 40,000 people live within the Bale Mountains National Park boundaries (FZS 2007; Vial et al. 2010; Randall et al. 2011). The large number of settlements within the park boundaries also leads to an increase in the local abundance of livestock and in particular domesticated dogs (Stephens et al. 2001; Atickem et al. 2009). It has been estimated that, in Ethiopia, one in every five households owns a dog (Deressa et al. 2010). Within the Web Valley of the Bale Mountains, domestic dog

densities range from 4 dogs/km² in the dry season to 10 dogs/km² in the wet season (Atickem et al. 2009). The sympatric relationship between domestic dogs and humans has altered the dynamics of infectious diseases and increased the disease susceptibility for Ethiopian wolves. Serious impacts of rabies on susceptible host populations occur when RABV is maintained within populations of abundant reservoir hosts (Haydon et al. 2002; Randall et al. 2004; Woodroffe et al. 2012). Transmission is due to close contact between wolves and dogs, which can cause major outbreaks within susceptible populations (Haydon et al. 2002; EWCP 2014). In the case of Ethiopian wolves, future outbreaks of rabies could have devastating effects. Since 1995, efforts have been made to control the spread of infectious diseases within the Ethiopian wolf population of the Bale Mountains (EWCP 2014). The transmission of rabies is currently being tackled through vaccinations of the domestic dog population (Haydon et al. 2002; FZS 2007; Randall et al. 2011; EWCP 2014).

In Ethiopia, rabies has been recognised for many centuries. The first recordings of rabies within Ethiopian wolves were published by Sillero-Zubiri et al. (1996). The outbreak began in 1988 and ended in 1992. Sillero-Zubiri et al. (1996) studied *C. simensis* in the Bale Mountains, specifically the Web Valley and Sanetti Plateau (all separated by only 15km), from October 1991 to February 1992. Out of the 53 individuals observed, 41 died or disappeared (Sillero-Zubiri et al. 1996). In 2003 and 2004, a further 38 carcasses were recovered in the Web Valley after another outbreak emerged, and 36 individuals were reported to have disappeared (Randall et al. 2004). Recovery of all wolf carcasses is unfeasible in the vast area of the Bale Mountains and therefore it is impossible to determine how many of these disappearances are due to rabies (Randall et al. 2004; Sillero-Zubiri et al. 2006). However, rabies has been identified as the prime candidate for

sudden drop in numbers through death and disappearance (Randall et al. 2004). A further outbreak of rabies was confirmed between October 2008 and May 2009, when five brain samples were recovered from wolf carcasses (Johnson et al. 2010).

Mass reductions from outbreaks of disease in any endangered species can have dramatic effects on the future of a population. Genetic variation is critical for levels of fitness and survival for future generations of a given species. In order to record the effects of RABV outbreaks on the genetic structure of Ethiopian wolves, genetic monitoring from individuals within the population before and after outbreaks is crucial.

1.3. Population Genetic Bottlenecks and Conservation

Genetic variation in a changing environment is a key factor for enabling adaptation and therefore survival. Loss of genetic diversity can be caused by a number of events but ultimately they have the same outcome: lower levels of fitness and higher risks of extinction. A population bottleneck is defined as an event which dramatically reduces the effective size of a population, leading to a sharp temporary increase in genetic drift resulting in low levels of genetic variation (Nei et al. 1975; Wright 1986; Lacy 1987; Nature Ed. 2014). Genetic variation is influenced largely by the number of individuals, which contributes to short and long-term persistence of a given population (England et al. 2003). Most allele frequencies are stable and bettermaintained in larger populations over long periods of time (England et al. 2003). Genetic drift results in infrequently occurring alleles and lower genetic diversity, and after a bottleneck event the small remaining population faces a higher level of inbreeding (Nei et al. 1975; Chevolot et al. 2008; Cohen et al. 2013). The resulting population is susceptible to fixation of mildly deleterious mutations, further decreasing the given quality of a gene pool (Lande 1994; Luikart 2002).

Low genetic diversity can substantially contribute to the risk of extinction by reducing fitness (Hedrick and Miller, 1992; Brakefield and Saccheri, 1994). It has also been hypothesised that the loss of alleles can create populations containing individuals which are genetically distinct from original populations (Avise et al. 1987). Ultimately, a bottlenecked population is at higher risk of being unable to adapt to new selection pressures within its niche environment, such as a shift in available resources or disease (Nei et al. 1975; Lande 1994). A bottleneck event increases the probability that the amount of genetic variation required during a selection process may have previously drifted out of the population (England et al. 2003; Cohen et al. 2013).

Luikart et al. (2002) found that some bottlenecks can occur in a population that are undetectable by traditional demographic monitoring approaches such as capture-mark-recapture. These types of bottlenecks are defined as 'cryptic bottlenecks', and demonstrate the importance of implementing genetic monitoring within management programs for threatened or endangered species (Luikart et al. 2002). A bottleneck can greatly reduce population genetic variation, even if it is short lived (less than two generations long, Hongye and Roossinck 2004). On the other hand, if a bottleneck population accepts immigrants from neighbouring populations, the loss of genetic variation can be reduced or even reversed (Vilà et al. 2003). Many threatened and managed species, such as Ethiopian wolves, are currently suffering from bottlenecks (Randall et al. 2010).

1.3.1. Inbreeding in Small Populations

Inbreeding is defined as the mating of individuals related by ancestry for the production of offspring (Marcovitch 2010). It is measured as the probability that two alleles on a given locus are identical by descent, as measured through the inbreeding coefficient (*F*). *F*

ranges from outbreds at a value of 0 to completely inbred individuals at a value of 1. An increased probability of homozygosity and exposure to recessive/rare deleterious alleles is found within inbred offspring, reducing reproductive fitness (Frankham et al. 2010). These factors are the primary cause of inbreeding depression (Hedrick and Fredrickson 2008). Genetic rescue via the introduction of unrelated individuals from alternative populations (such as captive populations) is often advised to reduce inbreeding depression (Hedrick and Fredrickson 2008). This eliminates deleterious alleles from the population due to natural selection, known as purging (Boakes et al. 2007). Purging has been seen as modest in experimental evidence, and inbreeding depression is likely to continue if an inbred population is exposed to further inbreeding (Boakes et al. 2007).

Inbreeding is often an effect of isolation. It is important to test the levels of inbreeding in isolated population fragments. The knowledge of genetic stability and viability through monitoring the fragmented and isolated population is essential for a species future existence.

Due to their low population size, Ethiopian wolves require a genetic monitoring programme which also takes inbreeding into account. Inbreeding can be measured through genetics by measuring the inbreeding coefficient. The inbreeding coefficient determines the strength of inbreeding by using the fixation indices (F_{IS}). F_{IS} determines the probability that two alleles within an individual are Identical By Descent (IBD). An increase in homozygosity or fixation of alleles results from inbreeding. A higher F_{IS} value indicates a considerable degree of inbreeding. Numerous software applications have been developed to calculate the inbreeding coefficient once individual alleles have been detected. The inbreeding coefficient is becoming a more commonly used method in conservation genetics.

1.4. Conservation Genetics and Genetic Markers

Conservation genetics has been defined as the application of evolutionary and molecular genetics to biodiversity conservation (Frankham 2010). This science has undergone exponential growth over the past 25 years, taking the theory-based concepts of population genetics into an empirical discipline (Ouborg et al. 2010). The use of genetics is now being actively applied to the field of conservation, ultimately improving the genetic viability of wild and captive populations of endangered species (Frankham et al. 2010; Marucco et al. 2011). Conservation genetics is often overlooked in international wildlife policies (Bouzat 2010; Laikre 2010), although molecular techniques could play a vital role in the management of populations. Developments in technology for molecular genetics have generally led to an extensive use of genetic markers in the last decades (Ouborg et al. 2010; Gardner et al. 2011). Genetic markers such as allozymes, microsatellites and mitochondrial and nuclear DNA sequences allow the study of individual relatedness, population size and population dynamics. Molecular markers can also be used to assess genealogical relationships, to determine fine scale genetic structuring and to measure genetic differentiation and gene flow within and between populations (Luikart et al. 1998; Marino et al. 2005; Waits and Paetkau 2005; Selkoe and Toonen 2006; Schwartz et al. 2007; Randall et al. 2009; Munday and Knight 2010; Mullins et al. 2010; Olsen et al. 2012). Recent developments in next generation sequencing, whole genome scans and gene-expression pattern analysis have also begun to play an important role in the growth of conservation genetics (Allendorf et al. 2010; Frankham et al. 2010; Frankham 2010; Davey et al. 2011). Advancement of laboratory techniques has generated more cost effective genetic marker methodologies that can analyse larger numbers of individuals with multiple loci (Guichoux et al. 2011). Improvements in

computer technology have produced software packages that apply statistical approaches to laboratory-generated datasets (Selkeo and Toonen 2006).

1.4.1. Microsatellites in Conservation Genetics

Microsatellites were first used in natural populations more than 20 years ago (Ellegren 1991; Schlotterer et al 1991), and since then they have become an increasingly important part of population genetics and molecular ecology (Gardner et al. 2011). Microsatellite markers can also be termed simple sequence repeats (SSR) or short tandem repeats (STR), and are found in both prokaryotic and eukaryotic genomes (Zane et al. 2002; Bhargave and Fuentes, 2010). They consist of between 1 and 6 nucleotides, which display a high level of allelic polymorphism based on differences in numbers of repeat motifs between individuals (Zane et al. 2002; Selkoe and Toonen, 2006). Although exact mechanisms of polymorphism in microsatellites are unknown, the most likely cause is due to slippage events during DNA replication (Schlötterer & Tautz, 1992). Typically microsatellite loci are amplified by polymerase chain reactions (PCR), using fluorescently-labelled primers for fragment detection. Applications of microsatellite markers include quantifying genetic diversity, loss of heterozygosity, parentage analysis, linkage mapping, and animal and plant species and individual identification.

1.4.2. Methods for Sampling in Conservation Genetics

The application of population genetic tools and theories to practical conservation can ultimately reduce the risk of extinction in threatened species (Frankham et al. 2010). In order to directly access information at DNA level, samples must be collected. There are three different types of sampling methods; destructive, non-destructive and non-invasive. Destructive sampling involves an animal ultimately being destroyed for the collection of tissue for genetic analysis (Taberlet et al. 1999). Non-destructive sampling

requires capture of an animal for the removal of blood or tissue (such as a feather, toe or ear clipping, Taberlet et al. 1999). Lastly, non-invasive sampling, which is becoming more accessible with the improvement of genetic techniques, is restricted to situations whereby DNA is collected from a source left by an animal (Waits and Paetkau 2005). Examples include faeces and urine, shed or snagged fur, skin or feathers, egg shells or skulls found in owl pellet remains (Poulakakis et al. 2005; Schmaltz et al. 2006; Brinkman et al. 2010; Mullins et al. 2010; Olsen et al. 2012).

Non-invasive sampling is attractive for use in conservation genetics as it allows for the genetic study of populations without ever having to observe, physically restrain or capture the animal (Waits and Paetkau 2005; Schwartz et al. 2007). In cases where species are endangered and/or elusive, this technique provides an excellent opportunity to collect conservation-relevant data of individuals without disrupting to their natural behaviour (Adams and Waits 2007; Broquet et al. 2007; Janečka et al. 2008; Pérez et al. 2009; Mondol et al. 2009). The large majority of studies using non-invasive sampling rely on the use of samples collected from hair or faeces (Broquet et al. 2007). New methodologies enable scientists to extract DNA non-invasively and more efficient methods for genetic conservation are continually being developed, making the techniques attractive to researchers (Waits and Paetkau 2005; Broquet et al. 2007). Faecal samples produce a higher yield of DNA than from a single hair sample; however, they contain large amounts of alien DNA from the digestive tracts and external environment during decomposition (Taberlet et al. 1999; Taberlet and Luikart 1999; Brinkman et al. 2010). Faecal samples have also been found to contain inhibitors that can lead to unsuccessful PCR (Broquet et al. 2007; King et al. 2008; Shimatani et al. 2008; Rivière-Dobigny et al. 2009). These vary with diet, and most difficulties are found in

studies where faeces contain remnants of plants (Broquet et al. 2007). Broquet et al. (2007) found that there is no difference between genotyping error rates of hair and faecal samples.

Most studies using microsatellites with non-invasive sampling rely on individual identification based on 5 to 10 hypervariable genotyped loci (Taberlet and Luikart 1999). DNA genotyping must be free from error in order to properly assess the species conservation genetics (Taberlet et al. 1999; Taberlet and Luikart 1999). Non-invasive samples are usually taken from extracts of the individuals targeted, and therefore have a high probability of DNA degradation and/or contamination from alien DNA (King et al. 2008; Shimatani et al. 2008; Rivière-Dobigny et al. 2009). This can reduce the amplification success and also increase genotyping errors; however, by using specific primers, amplification of alien DNA can largely be avoided (Broquet et al. 2007).

The many benefits of combining non-invasive sampling and conservation genetics have attracted an increase in recent research using the methodology. Most monitoring programs researching anthropogenic changes to natural ecosystems have yet to take full advantage of the potential of non-invasive sampling and conservation genetics (Schwartz et al. 2007; Frankham et al. 2010; Marucco et al. 2011). These can provide relevant ecological and evolutionary information, while costing less and maintaining a higher level of accuracy and sensitivity than traditional monitoring approaches (Schwartz et al. 2007). By understand the current level of genetic variation in rare, fragmented and/or endangered populations; monitoring programs can contribute to our understanding on how to further conserve a given species (Luikart et al. 2002; Boakes et al. 2007; Schwartz et al. 2007).

1.5. Contributions of Molecular Techniques to Elucidate the Biology of Ethiopian Wolves

In the last twenty years, DNA analysis has expanded the knowledge of Ethiopian wolf biology (Gottelli et al. 1994; Marino et al. 2005; Marino 2003; Randall et al. 2007; EWCP 2013). Gottelli et al. (1994) determined the relationship of Ethiopian wolves to other wolf-like canids through phylogenetic analysis of mitochondrial DNA (mtDNA sequences). Ethiopian wolves were defined as a distinct species which is more closely related to domestic dogs (*C. lupus*), coyotes (*C. latrans*) and grey wolves (*C. lupus*) than any other African canid (Gottelli et al. 1992). The sympatric relationship that domestic dogs and wolves share has proven to result in hybrids (Gottelli et al. 1992; Randall et al. 2007; Sillero-Zubiri & Macdonald 1998). Gottelli et al. (2004) used mtDNA to study the genetic diversity of the seven isolated populations of Ethiopian wolves. The partitioned mtDNA haplotypes were most likely a result of Pleistocene global climatic changes that occurred with the end of glaciation around 10,000-15,000 years ago, causing habitat reduction and fragmentation (Gottelli et al. 2004).

Randall (2006) optimised microsatellites derived from domestic dogs for non-invasive faecal samples of Ethiopian wolves, demonstrating that there is a 99% overlap in genotypes created based on faecal and tissue samples. Randall et al. (2007) extended this research to elucidate kinship between packs, parentage of pups and inbreeding. Breeding pairs of Ethiopian wolves were often unrelated, although mean pair-wise relatedness within packs was significantly higher than estimated from observations (Randall et al. 2007). This provided evidence for female-biased dispersal, which has resulted in decreasing inbreeding (Randall et al. 2007). Randall et al. (2009) used microsatellite data to assess the fine-scale genetic structure in Ethiopian wolves in the Bale Mountains. The

study evaluated the impact of historical versus recent demographic process on the genetic variation on several small populations within this area. There was a higher genetic variation in the Bale Mountains than previously reported by Gotelli et al. (2004). This was influenced by geography and social structure, showing the importance of population dynamics. Genetic similarity was found between neighbouring packs, indicating gene flow between them (Randall et al. 2009).

Due to the small population sizes of the Ethiopian wolf, they are likely highly susceptible to genetic drift and random fixation or loss of rare alleles (Lacy 1987). Genetic diversity is therefore deemed to avert the negative effective of inbreeding (Randall et al. 2007; Sillero-Zubiri et al. 1996; Loeschcke et al. 1994).

1.6. Aims and Objectives

This thesis sets out to investigate further the genetic variation and parentage analysis of the Ethiopian wolf. As the rarest canid in Africa, with a total population of no more than 500 individuals, the Ethiopian wolf is an ideal species for testing theoretical predictions of the role of genetics in conservation. The mechanisms that are shaping the genetic variation of the species will be strongly influenced by the environmental factors such as behaviour, population dynamics and disease. Multiple projects have been established under the Ethiopian Wolf Conservation Programme (EWCP) which is a current partnership between many research institutes and charities (EWCP 2013). The Ethiopian wolf has become a special case for understanding genetic variation and parentage due to its precarious conservation status. The only existing populations are small and isolated. This may ultimately result in population extinction.

The main aims of this thesis are to:

(i) Document the standing amount of genetic variation of Ethiopian wolves from the population existing between 2007 and 2010 in the Web Valley

(ii) Compare the allelic diversity with studies based on previously collected samples

(iii) Clarify the genetic relatedness between the whole population and individuals within each pack.

(iv) Investigate whether the previous rabies outbreak had putative effects on the genetic structure of the Web Valley population of Ethiopian wolves.

The objective is to use faecal samples collected from the Web Valley region the Bale Mountains to successfully extract and amplify DNA with multiple microsatellite loci. Faecal samples were collected between 2007 and 2010 and stored at -20ºC; however, it is recognised that a certain level of degradation may have occurred over time. Upon successful DNA extractions, samples will be amplified with microsatellite loci originally designed for the domestic dog by Breen et al. (2001) and optimised for Ethiopian wolf DNA by Randall et al. (2006). Microsatellite data will be used to reveal the amount of allelic diversity within the population using population genetics software. Individual multilocus fingerprints from microsatellites will determine the population structure and infer genealogical structure.

Despite the importance of studying genetics for the conservation of the Ethiopian wolf, there has been no published study since 2007, highlighting the great importance of this thesis for the future of the species.

Chapter Two: Methods

2.1. Sampling Techniques

TABLE 1. A summary of the 51 samples collected from the Web Valley (based on the packs from which they were sampled).

		Sex			Age			
Pack Name	**Total number of individuals**	**Males**	**Females**	**Unknown**	**Adult**	**Subadult**	**Juvenile**	**Pup**
Addaa	1	1	0	0	1	0	0	0
Alando	8	6	2	0	6	2	0	0
Darkeena	7	3	4	0	6	1	0	0
Kotera	2	1	1	0	2	0	0	0
Megity	11	6	5	0	9	0	2	0
Mulamo	4	1	2	1	3	0	0	1
Sodota	8	4	3	1	5	1	2	0
Tarura	6	2	4	0	5	1	0	0
Floater	2	0	2	0	2	0	0	0
N/A	1	1	0	0	1	0	0	0
New Sodota	1	0	1	0	0	1	0	0
Total	51	25	24	2	41	5	4	1

All faecal samples were collected by Freya van Kesteren (7°N, 39°40' E, Figure 1), from August to February annually between 2007 and 2010. A total of 51 individuals split between 11 wolf packs (Table 1) were sampled continuously over the study period. Data

including region, pack, age, sex and ID were recorded for each sample collected whenever possible. Individuals were identified by ear tags or individual markings. Individuals were previously tagged by the Ethiopian Wolf Conservation Programme (EWCP 2013). All samples were stored at -20ºC before DNA extractions.

2.2. Selecting Microsatellite Markers

Breen et al. (2001) developed the first fully integrated canine genome map, defining a total of 1800 microsatellite markers covering more than 90% of the dog genome. Based on this source, 17 microsatellites (16 tetranucleotides and 1 dinucleotide) were identified as polymorphic in Ethiopian wolves by Randall et al. (2006). To quantify the applicability of these markers for parentage inferences and to quantify their amount of polymorphism, Randall (2006) used the probability of identity among siblings (PIDsib) based on the equation published by Evett and Weir (1998), and found that only 5 are needed to distinguish between random pairs of individuals with 99.0% certainty (PIDsib = 0.01). By selecting 8 of the most informative markers, a 99.9% certainty (PIDsib = 0.001) to determine between individuals was found (Randall, 2006). For the present study, 9 out of these 17 markers were selected based on PIDsib, total error rate per reaction (Erxn), allelic dropout rate (ADO), false allele rate (FA) and probability of false homozygote (P(FH)), based on Randall (2006), and used here. Only tetranucleotide repeat loci markers were chosen, as they have a tendency to be more polymorphic than dinucleotide repeat markers (Mellersh et al. 2000), and lower incidence of stutter peaks and are easier to score (Murray et al. 1993). A list of the markers chosen is shown in Table 2. Forward primers were labelled with fluorescent dyes FAM, AT550 or HEX. All three dyes were

multiplexed, ensuring the expected allele size range did not overlap for loci with identical dyes.

TABLE 2: Characteristics of the 9 markers selected for this study. Primer sequences 5' to 3', expected allele size ranges are taken from Randall et al. (2006).

Locus	Primer sequence	Length	Dye	Expected Allele Size Range
FH2001	F: TCCTCCTCTTCTTTCCATTGG	21	FAM	129 - 149
	R:TGAACAGAGTTAAGGATAGACACG	24		
FH2054	F: GCCTTATTCATTGCAGTTAGGG	22	AT550	161 – 185
	R: ATGCTGAGTTTTGAACTTTCCC	22		
FH2137	F: GCAGTCCCTTATTCCAACATG	21	FAM	157 – 173
	R: CCCCAAGTTTTGCATCTGTT	20		
FH2138	F: AATGTGCCCAACATTCCACT	20	HEX	313 – 342
	R: AAGTCCCATGTCAGGCTCC	19		
FH2140	F: GGGGAAGCCATTTTTAAAGC	20	AT550	127 – 150
	R: TGACCCTCTGGCATCTAGGA	20		
FH2226	F: GGACTACCCCATTGCATTTG	20	AT550	190 – 211
	R: GAATCGAGTCCCATATCGGG	20		
FH2422	F: TTGCCCGTCCTATACTCCTG	20	FAM	205 – 220
	R: CCACATGATTTCACTTGTATATGG	24		
FH2472	F: ATTGTCTGTTAACCAAACCTGC	22	AT550	264 – 313
	R: AGTCCTTCGAGTGAGTGAGGT	21		
PEZ19	F: GACTCATGATGTTGTGTATC	20	HEX	186 – 202
	R: TTTGCTCAGTGCTAAGTCTC	20		

2.3. DNA Extractions

DNA extractions were carried out using a QIAamp® DNA Stool Mini Kits (Qiagen, 2010). The procedure was conducted in a separate lab from that used for PCR reactions to prevent cross contamination with post PCR products. Negative controls were included in each set of extractions to monitor contamination. The manufacturer's protocol was followed except that 100 mg of faeces was used, compensated by increasing the amount of ASL buffer. Pre-sterilized barrier-filter tips were used during the extractions. DNA was stored at -20ºC.

2.4. Amplification

A Touchdown Polymerase Chain Reaction (TD-PCR) was used to amplify the chosen 9 microsatellites (Table 1), using a Veriti 96 Well Thermal Cycler (Applied Biosystems). Each 10 µl reaction volume contained 1 µl of genomic DNA, 0.1 µl dNTPs (2 mM), 0.1 µl of forward and 0.1 µl of reverse primer (2 mM), 0.1 µl GoTaq® DNA Polymerase 5 u/µl (Promega Ltd., USA), 0.6 µl MgCl2 (50 mM Stock concentration Bioline Ltd, UK), 1 µl of 10x Reaction Buffer (Bioline Ltd, UK, 160 mM $(NH4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25° C, 0.1 % stabilizer) and 7 µl of PCR grade H_2O. Negative controls containing 1 µl of PCR grade H_2O and master mix were included in each experiment. The optimised TD-PCR cycling profile for the standard dye labelled primers included denaturing at 95 ºC for 15 minutes, followed by 12 cycles of 30 seconds of denaturing at 95 ºC, 90 seconds of annealing at 60 ºC, dropping by 0.5 ºC per cycle, and 60 seconds of extension 72 ºC; continued by 33 cycles for 30 seconds at 89 ºC, 90 seconds at 55 ºC and 60 seconds at 72 ºC. A final extension of 30 minutes at 60 ºC completed the TD-PCR.

2.5. Amplification Tests

Amplified TD-PCR products were detected visually by electrophoresis before genotyping. This process was conducted in a different room from that of the PCRs to prevent cross-contamination with post-PCR products and pre-PCR consumables. Gels of a 1.5% agarose concentration were prepared in 100 ml-sized gel trays with 20 x 1.5 mm combs. Gel trays were double taped at both ends and the appropriate number of combs was added to define the gel structure. A solution of 1.5 g agarose (Bioline Ltd, UK) to 100 ml of 0.5x Tris-borate-EDTA (TBE) buffer (89mM Tris-borate, 2mM EDTA, pH 8.3, Severn Biotech, UK) was mixed in a conical flask and heated in a 750 Watt microwave until dissolved (approximately 60 seconds). Precautionary measures were taken to ensure the solution did not boil which would have increased concentration measures due to loss through evaporation. Once cooled to roughly 50 ºC, 100 µl of GelRed™ agent (Biotium, Hayward, CA, USA) was mixed with the solution and poured evenly into the taped gel tray to set over a 20 minute period. GelRed™ is an intercalating agent which binds to the DNA, fluorescing under UV light for visualisation. Between 3 µl and 5 µl of TD-PCR products were mixed within 4 µl of loading dye in separate PCR tubes, ensuring the original TD-PCR products were kept for future genotyping. The comb was removed from the solidified gel to create the defined wells, and the tape was removed from both ends of the tray. The solidified gel and tray was fully submerged in an electrophoresis chamber containing sufficient TBE buffer solution, keeping defined wells located nearest to the negative electrode. A mix of 5 µl of Hyperladder II, 1 kb or 100 bp and 4 µl loading dye was loaded consistently into the first well. The mixed TD-PCR products were then loaded in the following wells in a systematic order. The electrophoresis lid was placed on top of the

chamber with power leads attached to a power supply. The program ran for 1 hour at 110 Volts, between 70 and 100 mA.

A transilluminator was used to view the electrophoretic movement of the DNA molecules within the gels. Photographic evidence was taken using software Genesnap (Syngene) and printed copies of pictures were created with a G-Box Syngene.

2.6. Genotyping

Due to the high sensitivity of the genetic analyser (Applied Biosystems Ltd.), TD-PCR products were diluted using ddH_2O. Band strength and primer dye were used when estimating dilution rates of each TD-PCR product. Bands defined as bright were diluted to the rate of 1/25 (AT550), 1/50 (HEX) and 1/100 (FAM) (with dye AT550 being the weakest dye and FAM dye being the strongest). The dyes defined as faint were diluted to the rate of 1/5 (HEX) and 1/10 (FAM), without dilution for AT550 dye due to the generally poor strength of the dye. Diluted TD-PCR products were then multiplexed in 96 well plates with up to three different dyes (FAM, HEX or AT550). Genotype plate maps of 96 wells were used to record the transfer of samples from labeled TD-PCR tubes. A master mix volume of 9 µl containing 0.1 µl of size standard, 5 µl formamide and 3.9 µl of ddH_2O was loaded into a new 96 well genotyping plate. A multichannel pipette was used to transfer 1 µl of the multiplexed diluted TD-PCR products into corresponding wells. A sterile septa cover was placed over the plate and the products were denatured for 10 minutes at 95°C before being put on ice. A further 10 minutes was used to cool the products. The plate was then loaded into an ABI3130 Genetic Analyser for genotyping (Applied Biosciences 2014).

2.7. Statistical Analysis

2.7.1. Allele Scoring and Filtering the Data

Peak Scanner™ 2 Software Version 1.0 (Applied Biosystems) was used to identify peaks and the fragment sizes of all .fsa files produced by the ABI3130 genetic analyser. Integers of measured fragment sizes based on allele peaks were transferred into Microsoft® Excel format. Genetic markers which did not produce enough conclusive or sufficient data were removed from the data set. Individuals that had failed, or produced little/no conclusive allele peaks during genotyping, were also excluded from the data set.

2.7.2. Formatting for Software Analysis

Generally the genotypes were required in six integer formats to represent the two alleles in their length in basepairs; missing alleles were represented with "000". Each of the software used had its own format requirement of how to identify each individual, population and locus. Locus, population and individual-specific identifiers were generally specified before presenting the allelic data.

2.7.3. Scoring Errors and Deviations

Micro-checker Version 2.2.3 was used to test for scoring errors and to assess the frequencies of null alleles (van Oosterhout et al. 2004). Allele frequencies and observed and expected heterozygozities (*Ho* and *He*) were calculated using Cervus 3.0.7. (Field Genetics Ltd, Marshall 1998-2014, Kalinowski et al. 2007). To test for deviations from Hardy-Weinberg equilibrium and to obtain basic information for each locus, Genepop on the Web Version 4.2 was used (Raymond and Rousset 1995; Rousset 2008).

2.7.4. Inbreeding Coefficient and Kinship

F_{IS} was calculated using *F*-statistics. *F*-statistics are a measure of genetic structure developed by Sewall Wright (1969, 1978). F_{IS} was determined using Genepop on the Web Version 4.2(Raymond and Rousset 1995; Rousset 2008), implementing the methods developed by Weir and Cockerham (1984).

2.7.5. Population Bottlenecks

Bottleneck version 1.2.02 (Piry et al. 1999) was used to test for significant heterozygosity excess compare to the equilibrium expectations for a stable population. This method is based on the assumption that population reductions cause rare alleles to be lost faster than gene diversity, therefore resulting in a transient heterozygosity excess compared to the observed number of alleles. The test uses either a two-phase mutation model (T.P.M.) and a step-wise mutation model (S.M.M.). The T.P.M. is considered the most appropriate for microsatellites (Di Rienzo et al. 1994). In total, 70%, 80%, and 90% stepwise mutations were assumed in the TPM to compare the results with previous findings by Randall et al. (2012). The programme was run for 1000 iterations and the significance was tested with the Wilcoxon signed-rank test as recommended by Maudet et al. (2002). The Wilcoxon signed-rank test assumed that all loci fit the T.P.M. and S.M.M. and mutation-drift equilibrium.

2.7.6. Pack and Parentage Analysis

A Bayesian clustering method as implemented in Structure Version 2.3.4. (Pritchard et al. 2000) was used to compare the obtained genetic structure with the known number of packs. Structure simultaneously estimates allele frequencies to assign individuals to probable populations in order to minimise deviations from Hardy-Weinberg and linkage

disequilibrium. If packs represent family groups, they might represent genetic units identifiable through Structure e.g. based on differential allele frequencies, despite deviations from Hardy-Weinberg equilibrium due to high degrees of relatedness within them. The software run parameters assumed 11 packs 1000000 Reps in total with 100000 Burn-in. Packs are outlined in Table 1. It can be argued that the packs named 'Floater' and 'N/A' can be grouped together. The two individuals within 'Floater' are moving between packs and therefore have unknown origins, which will is also the case of the 'N/A' individual. Secondly, it can be argued that the New Sodota individual female originated from Sodota and had left the pack, but had not yet established a new pack, nor had been seen to nurture young. Taking this into account, it is possible that the Bayesian clustering method will find only 8 packs, rather than 11.

To analyse differences in the genetic structures of packs, private alleles were also visually detected within the genotyped data set. Private alleles are defined as alleles which only occur within a single population (or, in this case, pack) among a broader collection of populations or packs (Szpiech and Rosenberg 2010). As a hypothesis, packs share common alleles which derive from the same ancestry. Private alleles within packs can be defined as rare alleles as they are not commonly found within all individuals within the population. Rare recessive alleles are associated with inbreeding.

Cervus 3.0.7. (Field Genetics Ltd, Tristan Marshall 1998) was used for identity analysis (maternity and paternity, Kalinowski et al. 2007). Identity analysis also can reveal any repetitive genotypes to identify individuals which have been resampled. This is particularly useful in Ethiopian wolves, as samples were collected through faecal matter and a number of individuals were unmarked. The program Colony was used to perform

parentage analysis with the genotypic data (Jones and Wang 2010). Colony assigns sibship and parentage jointly by applying a maximum likelihood method to the individual genotypes at a number of codominant or dominant markers. A simulation of parentage where the number of codominant alleles is used to find the number of potential candidate parents for each offspring was carried out. The simulation took into account all of the potential adult males and females, offspring and errors which may lie within the genotyped data. This likelihood was calculated by Colony with indicating values ranging between 0 to 1. Coancestry v1.0.1.5 was used to estimate pairwise relatedness point estimates between individuals with respect to the entire adult samples (Wang 2011). It is expected that genetically similar packs will have higher mean relatedness point estimates between parental individuals, as they are thought to be closely related family structures. Relatedness point estimate could only be applied on packs with three of more individuals. Coancestry was also used to determine how two parents of one or more offspring as identified by Colony were related to one another with regards to their relatedness point estimate (Wang 2011). The pairwise relatedness coefficient (as calculated by Coancestry pairwise relatedness coefficient) estimated the relatedness point between every possible pair of individuals. These values ranged between -1 and 1; zero is the population mean relatedness coefficient as expected by chance, negative values indicate a lower relatedness coefficient and positive values indicate a higher relatedness coefficient.

Chapter Three: Results

3.1. Amplification Tests through Electrophoresis

A total of 51 individual samples were amplified using TD-PCRs across 9 microsatellites. Not all samples were amplified in the first attempt. TD-PCRs were repeated up to three times. Of the 51 samples, 48 successfully yielded in PCR products. Figure 2 shows an example of a largely successful amplification (locus FH2422).

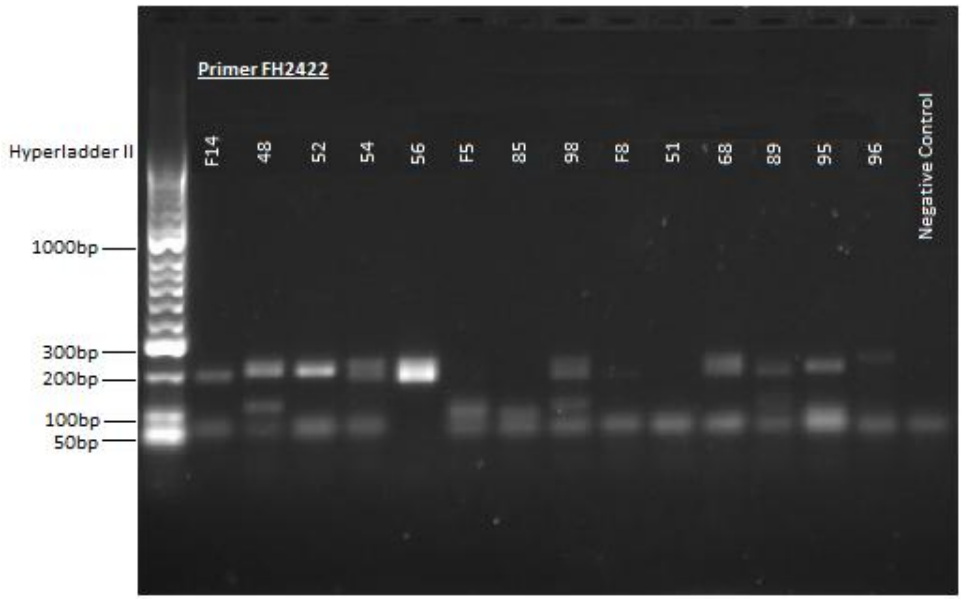

FIGURE 2. Agarose gel image of amplifications of locus FH2422 as an example. Sample labels are displayed across the top of the image. The observed allele sizes are in line with the allele size range of 205 – 220 base pairs as described by Randall (2006). Size variations and homo- and heterozygote genotypes already become apparent. Bands at around 50 to 100 bp were considered primer dimer.

3.2. Analysis of genotypes

3.2.1. Genotyping and Loci Removal

Of the 9 originally chosen microsatellites, only 7 provided sufficient genotyped data. The microsatellites FH2138 and FH2472 (Table 2), which were characterised based on a rather large PCR product (above 300bp), had a lower probability of being scorable. On average, each individual has been amplified with 6 microsatellites. Of the 48 individuals genotyped, 43 successfully amplified for at least four loci and were used for further analyses (Appendix 1.).

3.2.2. Scoring Errors and Null Alleles

Micro-checker did not identify significant evidence for scoring errors due to stuttering, and there was no significant evidence supporting significant drop outs of large alleles. However, low frequencies of null alleles and an excess of homozygotes were documented for loci FH2137 and FH2422 (Table 3 and Table 4). There was no evidence suggesting either scoring error due to stuttering in the alleles, or large allele dropout.

TABLE 3. An analysis produced by Micro-checker to describe homozygosity, scoring error and allele drop out.

Locus	Allele size range	Homozygotes		Homozygote excess	Scoring error due to stuttering	Large allele drop out	Notes
		Expected	Observed				
FH2140	107 – 135	12.56	8	No	No	No	No evidence for null alleles
FH2054	148 – 168	8.21	12	No	No	No	No evidence for null alleles
FH2422	148 – 237	10.84	17	Yes	No	No	Null alleles maybe present at this locus, as is suggested by the general excess of homozygotes for most allele size classes
FH2226	150 – 238	10.80	10	No	No	No	No evidence for null alleles
FH2137	153 – 197	17.11	23	Yes	No	No	Null alleles maybe present at this locus, as is suggested by the general excess of

							homozygotes for most allele size classes
FH2001	118 – 166	14.27	12	No	No	No	No evidence for null alleles
PEZ19	182 – 202	9.14	11	No	No	No	No evidence for null alleles

When four different algorithms implemented in Micro-checker were used to further investigate the presence of null alleles, only FH2137 and FH2422 were consistently characterised by varying degrees of null allele frequencies (Table 4).

TABLE 4. Comparison of the estimated null allele frequencies using the four algorithms (Oosterhout, Chakraborty and Brookfield 1 and 2) implemented in the software Micro-checker.

Locus	Null Alleles	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2
FH2137	Yes	0.12	0.14	0.09	0.20
FH2226	No	-0.01	-0.01	-0.01	0.11
FH2422	Yes	0.09	0.13	0.09	0.30
FH2054	No	0.08	0.09	0.07	0.46
FH2001	No	-0.15	-0.07	-0.05	0.47
PEZ19	No	0.03	0.05	0.04	0.50
FH2140	No	-0.13	-0.10	-0.08	0.30

3.3. Population Analysis

3.3.1. Descriptive Population Overview

TABLE 5. A complete outline of the packs and 43 individuals that amplified with a minimum of four loci.

		Sex			Age			
Pack Name	**Total number of individuals**	**Males**	**Females**	**Unknown**	**Adult**	**Sub adult**	**Juvenile**	**Pup**
Addaa	1	1	0	0	1	0	0	0
Alando	8	6	2	0	6	2	0	0
Darkeena	7	3	4	0	6	1	0	0
Kotera	2	1	1	0	2	0	0	0
Megity	7	3	4	0	7	0	0	0
Mulamo	4	1	2	1	3	0	0	1
Sodota	6	3	2	1	4	0	2	0
Tarura	4	0	4	0	4	0	0	0
Floater	2	0	2	0	2	0	0	0
N/A	1	1	0	0	1	0	0	0
New Sodota	1	0	1	0	0	1	0	0
Total	43	19	22	2	36	4	2	1

There were a higher number of females than males genotyped. Sex was unknown in 100% of pups and 50% of juveniles (van Kesteren 2011). There were more adults sampled

and genotyped than any other age group. Four individuals from Megity did not amplify from the TD-PCRs; two adult males, one juvenile female and one juvenile male. Two individuals from Tarura (one adult male and one sub adult male) and Sodota (one adult female and one sub adult male) did not amplify.

3.3.2. Inbreeding Coefficients and Kinship

TABLE 6. Observed and expected heterozygosities and fixation indices as inferred from Cervus and GenePop on the Web (F_{IS}; Weir and Cockerham 1984).

Locus	H_O	H_E	F_{IS}
FH2137	0.44	0.59	0.26
FH2226	0.76	0.75	-0.01
FH2422	0.55	0.72	0.24
FH2054	0.61	0.75	0.18
FH2001	0.57	0.52	-0.14
PEZ19	0.62	0.70	0.11
FH2140	0.77	0.64	-0.20

F_{IS} values at each loci varied between -0.1983 and 0.2581 (mean 0.0630). Mean F_{IS} was slightly above zero, indicating that there has been a slight deviation in the expectations of random mating. In cases where H_O was lower than the H_E a negative F_{IS} would be observed.

3.3.3. Population Bottlenecks

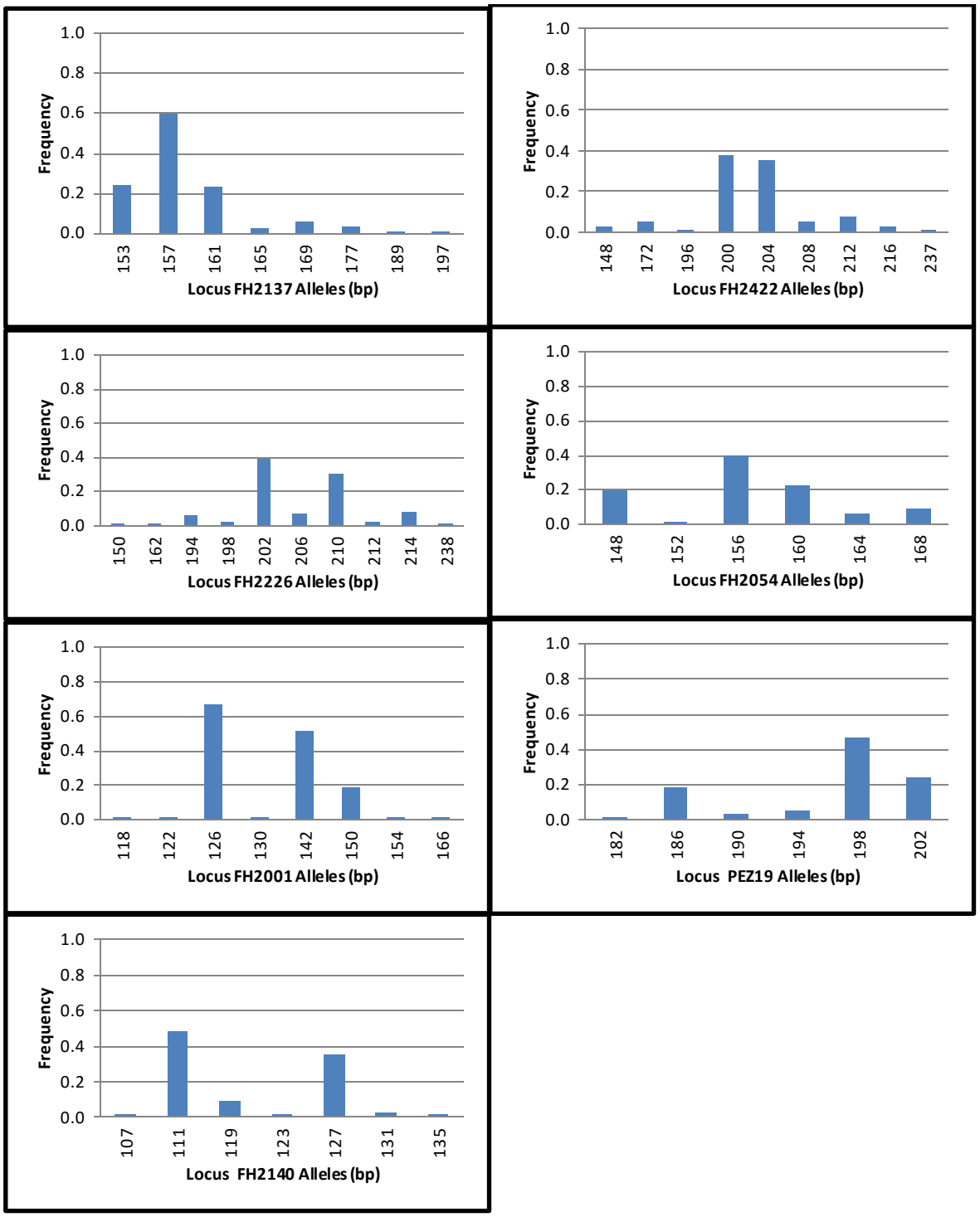

FIGURE 3. Allele frequency histograms for the 7 used microsatellite loci. Alleles are displayed in base pairs (bp) at integers of 4 base pairs due to them loci being composed of tetranucleotide motifs.

Under random mating in large populations and assuming a largely stepwise mutation process of microsatellites, allele frequencies are expected to have a rather symmetric, bell-shaped distribution. If uneven distributions and/or gaps in allelic distributions are present there is an indication that there is high past genetic drift that may have been caused by genetic bottlenecks. Most studied loci are characterised by 2 or 3 common alleles in addition to several alleles at low frequencies (<0.1, Figure 3), with the somewhat uneven distribution of common alleles providing evidence for population processes deviating from idealised populations.

TABLE 7. Results from Wilcoxon tests from Bottleneck software to analyse heterozygosity excess (*P*-values) through S.M.M. The method was applied to produce data for Web Valley 3, for a comparison with findings from Web Valley 1 and 2, extracted from Randall et al (2012).

		Heterozygosity excess (*P*- values)		
Population	**Number of individuals**	**70% S.M.M.**	**80% S.M.M.**	**90% S.M.M.**
Web Valley 1	-	0.09	0.16	0.43
Web Valley 2	61	0.01	0.01	0.04
Web Valley 3	43	0.77	0.85	0.85

TABLE 8. An outline of the pack allele diversity observed from the genotyped data. *N*a: number of alleles present at each locus within a pack, *N*: number of individuals. Missing data indicate amplification failure.

Pack	Private Alleles	*N*	*N*a FH2137	FH2226	FH2422	FH2054	FH2001	PEZ19	FH2140
Alando	5	8	5	4	5	4	3	4	3
Darkeena	0	7	3	3	2	3	3	4	2
Kotera	1	2	2	3	1	2	2	3	4
Megity	1	7	3	6	2	4	2	4	2
Addaa	0	1	2	-	1	-	1	-	2
Mulamo	3	4	2	2	3	2	2	2	2
Sodota	3	7	3	5	5	2	2	3	3
Tarura	5	4	3	5	4	5	2	4	2
Floater	1	2	2	3	2	3	2	2	2
N/A	0	1	1	2	1	2	1	1	2
Total	19	43							

Private alleles were visually detected within the genotyped dataset. The total number of private alleles across all packs was 19, averaging at roughly 2 per pack (Table 8). There is no relation between the number of private alleles and number of individuals. For example, Megity has seven individuals and only one private allele, while Sodota also has seven individuals and three private alleles.

3.4. Population Dynamics and Genetic Structures

3.4.1. Pack Genetic Structure and Diversity

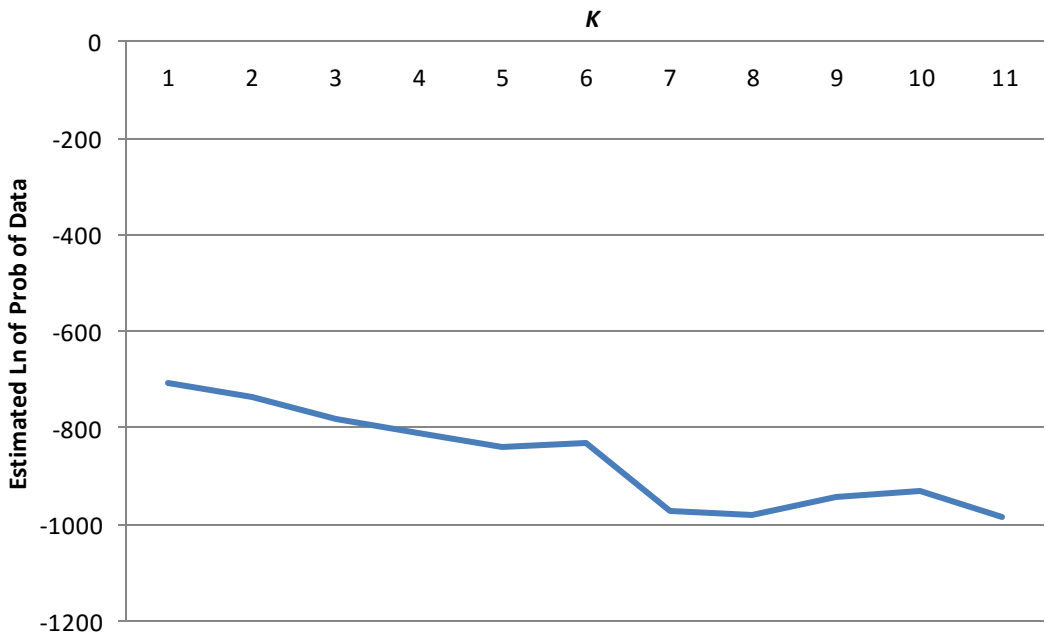

FIGURE 4. Bayesian posterior probabilities assuming between 1 and 11 clusters (*K)* for the genotyped data of all individuals.

The Bayesian clustering method as implemented in the software Structure assigned the highest posterior log likelihood to *K*=1, with a steady decrease in the likelihood with increased *K* (Figure 4). Such a pattern is expected when the algorithm is unable to detect any substructure in a given sample, and Structure is unable to distinguish between packs

or for example clusters of packs based on their locations. This is in line with the observation of regular exchange of individuals between packs, and might also be linked to deviations from Hardy-Weinberg equilibrium within packs. As the number of packs could not be identified the individual from New Sodota was then merged with Sodota. New Sodota was collected at the end of the sampling time. For analysis through genetic variation the individual was included with her original pack.

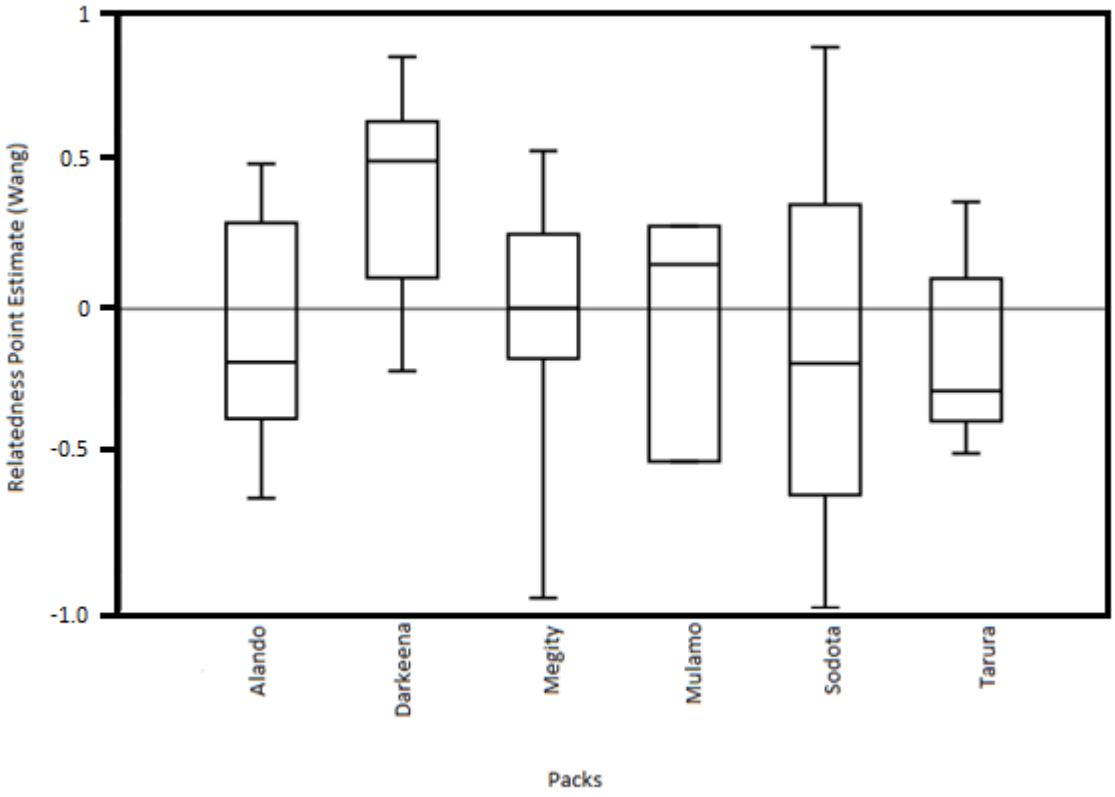

FIGURE 5. A box plot diagram describing the distribution of relatedness coefficients (Wang 2011) within each pack that has more than one individual. Relatedness coefficients vary between -1.0 and 1.0, with zero being the population mean and negative and positive values indicating lower and higher relatedness as expected by chance, respectively.

Relatedness point estimates were calculated separately between each pair of individuals for each pack (Figure 5). The further the mean pairwise relatedness coefficients are from 1.00, the less related pairs of individual are to one another within the pack. Two and three packs had mean coefficients above and below the pairwise population mean, respectively, whereas one pack (Megity) had a mean pairwise relatedness coefficient very close to zero (0.02). This suggests that individuals within packs are not more closely related to each other than individuals between packs. Darkeena has the highest mean relatedness point estimate of all possible pairs of individuals, indicating that all possible pairs of individuals within the pack are closely related. However, Sodota has the largest range of pairwise relatedness coefficients, with a mean relatedness point estimate below 0.

3.4.2. Parentage analysis

TABLE 9. A combination of the data collected in Colony and Coancestry to analyse the parental analysis.

Colony				Coancestry
				Father ID to Mother ID
Posterior		Father		Pairwise Relatedness
Probability	Offspring ID	ID	Mother ID	Coefficient
1.00	54	23	14	0.53
1.00	56	F14	F8	0.27
1.00	68	53	99	0.51
1.00	46	55	9	0.70
1.00	17	F5	F6	-0.39
1.00	F1	F10	F7	-0.71

Colony first found the highest probable mothers and fathers to the six described offspring (sub adults, juveniles and pups) sampled. The whole population was then analysed through Coancestry to determine pairwise relatedness coefficient between every possible pair of individual (according to Wang 2011). The data were adapted into an infographic design to visually display the family relations between mother, father, offspring and packs (Figure 6). All relationships produced a maximum probability of relatedness (1.000) through Colony and therefore were all included into the infographic.

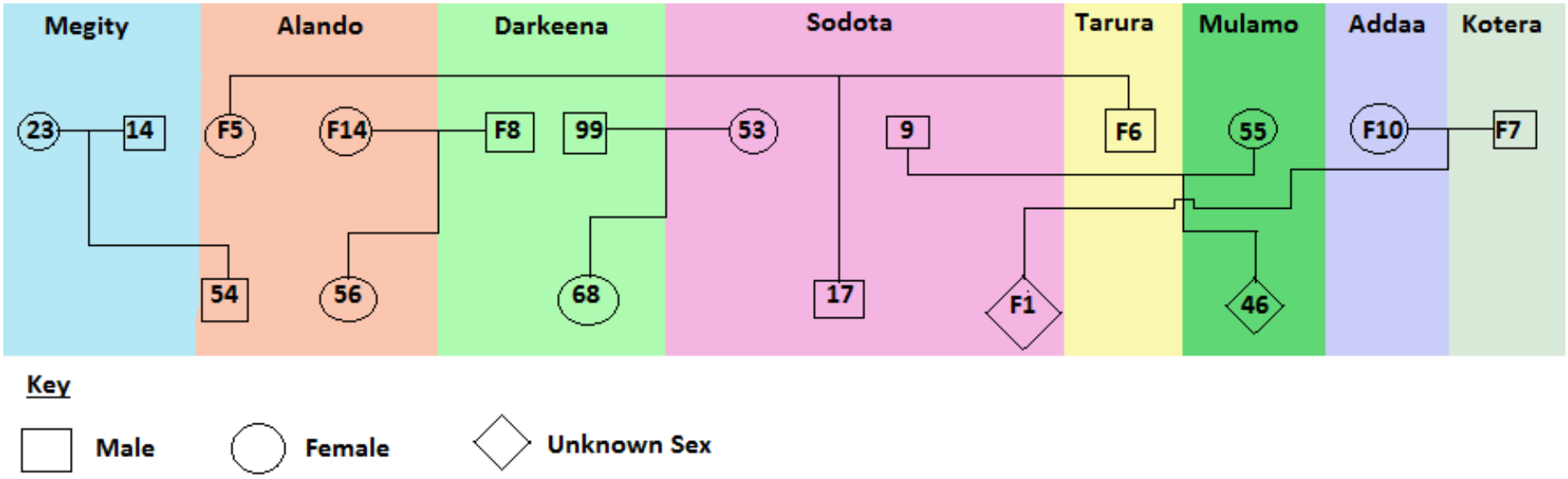

FIGURE 6. An infographic design adapated from the data collected in Colony. Individuals are seperated into packs as indicated by colours. Lines of parents to offsprings are visually decribed including sex of each individual. The full data are shown in Table 9. The infographic is not representative of the geographical locations of the packs.

Chapter Four: Discussion

The present study focuses on a further genetic characterisation of relict populations of the Ethiopian wolf, one of the world's most endangered canids. The main results are threefold. Firstly, the study showed that it was possible to amplify microsatellite DNA from faecal samples collected for hormone analysis and stored for several years. Secondly, the study provided further insights into the standing amount of genetic variation of this charismatic wildlife species. Thirdly, the study used parentage inferences to show that extra-pack matings seem to be a common occurrence, possibly linked to a loss of individuals in specific packs through disease (rabies) outbreaks.

4.1. Genotyping from Non-invasive Samples

Faecal samples were assumed to have been collected for all existing adult wolves in the Web Valley, equal to an estimated total population size of 51 individuals between 2007 and 2010 (van Kesteren et al. 2012; 2013). Individuals were identified through ear tags or individual markings, and any juveniles or pups were sampled once they began to leave the den. However, faecal samples were originally collected for the purpose of testing individual hormone levels, and sampling protocols were therefore not optimised for the purpose of DNA extraction. All samples were stored at -20ºC at the earliest opportunity after sampling, before DNA extractions were conducted. At the time of extraction, samples were between three and seven years of age. Despite this, a high success rate for PCR amplification was achieved: 83% of all faecal samples collected amplified with an average of six out of nine initially tested microsatellite markers. The remaining 17% of samples (nine individuals) did not yield in sufficient quality and quantity of DNA for successful PCR amplification with microsatellite markers.

Nine microsatellites which had been previously found to anneal to Ethiopian wolf DNA were selected based on Randall (2006). However, loci which amplified a product above 300 base pairs in length were unsuccessful in PCRs for more than half of the samples. As samples were not collected through an optimised sampling method for DNA extraction and kept for long periods in freezers, it is possible that degradation of DNA is responsible for PCR failures. In other words, loci amplifying longer strands of DNA were less likely to be successful in situations when template DNA was broken up into small fragments, in addition to small amounts of template being available. This is a common observation when analysing DNA based on degraded samples (e.g. Taberlet et al. 1991). The optimising and genotyping of further loci documented in Randall (2006) was precluded by logistic constraints.

Evidence for null alleles were found at two out of seven loci (FH2137 and FH2422 through the algorithms Oosterhout, Chakraborty and Brookfield 1 and 2 as implemented in the software Micro-checker). These were linked to an observed excess in homozygotes at these two loci. Large amounts of homozygotes are often an indication of low genetic diversity. However null alleles were caused by the absence of a PCR product (on a single strand or on both strands). This was either because it did not amplify in the PCR reaction for technical reasons, or because it can no longer be amplified due to mutations in the primer binding site. Markers FH2422 and FH2137 had seven and six more observed homozygotes than expected, respectively. There was a larger amount of homozygotes in these two loci across most of the allele size classes, suggesting that amplification failure was unrelated to template size without evidence for scoring error due to stuttering. Sequencing of PCR products would reveal whether mutations and polymorphisms in the

primer flanking region have caused the observed allelic dropout, or whether DNA quality and quantity combined with the used PCR conditions have caused the observed homozygote excess. Time and cost constraints have restricted further analysis via sequencing to determine the causes of null alleles. As other loci genotyped for identical individuals were not characterised by an excess of homozygotes, it is unlikely that population demographic processes and/or inbreeding have caused this observation.

4.2. Levels of Genetic Variation, Population Structure and Population History

Observed heterozygotes and total number of alleles as derived in this study were not markedly different from a previous data set produced by Randall et al. (2007; 2009). In these studies, samples were derived from about one decade prior to the present thesis, and collected in multiple regions of the Bale Mountains including the Web Valley (Randall et al. 2007; 2009; Gottelli et al. 2013). While there was no apparent marked decrease in genetic variation over this relatively short time scale, a direct comparison was hampered by different sampling methodologies and only partly overlapping study areas.

The Bayesian clustering method implemented in Structure could not map a signature of genetic substructure to packs or groups of packs. This suggests that all packs descended from one more or less panmictic population, although it is also well documented that for example gradual isolation-by-distance scenarios are not well captured by the algorithm implemented in Structure (Frantz et al. 2009). The lack of apparent population substructure as determined by Structure could also be due the relatively small pack sizes, as the power of the algorithm implemented in Structure decreases with the number of

assumed *K* (Evanno et al. 2005). It could also be a result of deviations from Hardy-Weinberg equilibrium within packs, as they by-and-large represent extended families (Randall et al. 2007). Although pack-specific private alleles were fairly common, they were insufficient for Structure to detect any population subdivision. Gottelli et al. (2013), however working across a larger area investigation, used Structure to successfully assign populations of Ethiopian wolves to three clusters representing mountain ranges on both sides of the Rift Valley. This analysis was also confirmed with sibship analyses to infer recent gene flow, which assigned all putative sibs to the same population (Gottelli et al. 2013).

Genetic bottlenecks occur when populations experience severe, temporary reductions in their effective size, and can dramatically reduce the genetic diversity of populations (Nei et al. 1975; Wright 1986; Lacy 1987; Nature Ed. 2014). For example, a high degree of inbreeding depression is often interpreted as the result of a past population bottleneck (Hedrick & Kalinowski, 2000; Nature Ed. 2014). Traditional measures of genetic diversity (such as heterozygosity and allelic diversity) can be used to infer a past bottleneck. It requires a reference sample either from before the event or from another, non-bottlenecked population. The approach employed in this study is the one implemented in the software Bottleneck, and is based on the idea that bottlenecking gives rise to an excess of heterozygotes compared to the level of heterozygosity expected at mutation-drift equilibrium, because under bottlenecks rare alleles have a higher risk of going extinct than common alleles (Cornuet & Luikart, 1996; Luikart et al., 1998). Depending on the sample size and marker variability, this method can detect heterozygote excess for up to about ten generations after its occurrence (Luikart et al., 1998; 2002). In addition to

the approach implemented in Bottleneck, Garza & Williamson (2001) also develop an approach based on the distribution of allele frequencies across the allelic spectrum.

Despite field observations of regular past rabies outbreaks reducing Ethiopian wolf population sizes, the genetic measures applied in this study failed to detect a demographic signature of such events. These findings are complementary to Randall et al. (2010) who used the same approaches and found significant genetic signature of bottlenecks in some but not all studied populations of Ethiopian wolves, and to Gottelli et al (2012) who, however assuming a slightly different microsatellite mutation model, also failed to detected any evidence for population genetic bottlenecks. Such analyses might however be hampered by the pronounced social structure within Ethiopian wolf populations, leading to deviations from idealised populations, as well as the rather limited number of samples and loci analysed. It should be further noted that the statistical models to detect past bottlenecks are based on the assumptions of demographic recoveries after drastic declines in a population size. The Ethiopian wolf has experienced a continuous decline which becomes intermitted by accelerated troughs as a consequence of disease outbreaks such as rabies (Laurenson 2004; Deressa et al, 2010). Bottlenecks are finally also expected to occur during extinction-recolonization processes in metapopulations (Whitlock & Barton, 1997; Bouzat 2010; England et al., 2003). The present analyses suggest that such dynamic processes appear to be rare or absent in the Ethiopian wolf.

4.3. Parentage and Exchange of Individuals between Packs

The natural boundaries of the study area allowed us to treat the sampled individuals as a confined reproductive community. This was corroborated by the lack of population substructure as inferred through the Structure software. The moderate to high level of genetic variation of the markers used and rather complete field sampling of adults made the microsatellite loci sufficiently powerful for parentage assignments, despite a rather low overall number of loci genotyped. The software Colony, which was employed for the parentage analysis, is generally considered to generate fewer false assignments than other methods that use pairwise relationships (Wang & Santure 2009), further corroborating that the present parentage inferences represent true gene genealogies. For all six sampled offspring for which sufficient genotypic information was available, both parents could be identified within the sampled adults with high certainty (a posterior probability of 1.000 according to the Colony software). This confirms the assumptions that field sampling was exhaustive, and that the genotyping based on the faecal samples yielded in accurate results.

Probably one of the most surprising results from the present study is the apparent high exchange of individuals between packs. Randall et al. (2007) performed a similar analysis, and concluded that about one in three (13/45) pups were sired by extra-pack fathers. Two reasons can account for this difference between the two studies. Firstly, Randall et al. (2007) also considered individual pups in their sample set for which only one parent was known, leading to a proportion of pups which were not counted as being extra-pack sired although they likely had an extra-pack father. Secondly, the studied population was apparently generally characterised by rather unstable packs (see also below for

movements of offspring between packs). This is for example also evidenced by the field observations which assigned a “floater” status to two individuals (van Kesteren et al. 2012; van Kesteren personal communication). While a direct link is difficult to demonstrate, it is plausible to assume that the rabies outbreak shortly before the collection of the samples markedly disturbed the social structure of the studied population. It is possible that reduced pack size and the loss of dominant individuals from given packs was compensated for by matings with extra-pack individuals as well as the exchange of adults between packs. A similar observation of pack disturbance through external factors was also made for European wolves *C. lupus*, when hunted and un-hunted populations were compared (Jędrzejewski et al. 2005) or when studying a rapidly expanding populations after hunting ceased (Caniglia et al. 2014). Based on the existing samples it was however impossible to infer whether multiple paternities occurred within litters (which have previously been shown to be common, Randall et al. 2007), which would provide further insight into mating systems within the studied population. High levels of polygynous and polyandrous matings within and among social groups have also been reported in other canids (for example red fox *Vulpes vulpes*, Baker et al. 2004), although more coherent packs e.g. exist for the African wild dog *Lycaon pictus* (Girman et al. 1997).

Another main result from the parentage inferences is that only two out of the six offspring for which both parents were identified (individuals 46 and 56) were still associated with the packs containing their biological mothers. While field errors cannot be completely excluded, the high confidence of the parentage data makes it very unlikely that genotyping errors have occurred. At the time of sampling, individual 46 was the only

analysed offspring classified as pup, and as expected was found in the same pack as its biological mother. All other offspring was classified as subadults or juveniles (2 males, 2 females, 1 unknown sex). In agreement with relatedness coefficients between inferred parents (see below), this finding suggests that between-pack movements of individuals after weaning seems common (corroborated through field and genetic observations, Sillero-Zubiri et al. 1996; Marino et al. 2006).

The infographic family tree of the data collected from Colony displays how packs are interbreeding. Only half of the offspring sampled were located in the same pack of either of their genetically identified mother or father identified parents. Identified parents male 14 and female 23 from pack Megity produced offspring male 54 in pack Alando. Male 14 and female 23 had a pairwise relatedness coefficient of 0.53, providing evidence for inbreeding. The relocation of offspring from the original family pack to join another pack has been previously observed in the field (Sillero-Zubiri et al. 1996; 1998), and in this case could be adaptive to inbreeding avoidance. Individuals male 99 and female 53 produce female 68 in pack Darkeena. There are two possible senerios in which this could have arisen. The mother 53 may have left the pack to join Sodota while father and offspring continue to reside in Darkeena. The other situation may be that father and offspring have relocated to Darkeena from the Sodota pack, leaving the reproducing female mother. This case provides evidence that more individuals of different ages and sexes are relocating. Mulomo female 55 has been receptive to male 9 from pack Sodota producing offspring 46. Van Kesteren (2012) found that subdominant females will breed out of season with external males, as their receptive hormone levels inducing mating increase. Environmental stresses of disease outbreak could have increased such matings. A further

notable case is that of Alando female F5 and Tarura male F6, who produced the Sodota juvenile male 17. It could be possible that this female was receptive to external breeding from F6, producing a male offspring which then relocated to Sodota. Such dynamic pack structure could be an advantage under situations of declines and losses of indiviudals through disease and habitat reduction.

Despite a limited sample size, this study is also able to draw some inferences on possible overall inbreeding and its avoidance in the study population. Four out of six parental pairs had a positive pairwise relatedness coefficient, suggesting they are outbred with respect to the population average, whereas two parental pairs had a negative coefficient, suggesting a certain degree of inbreeding. This limited evidence suggests that inbreeding avoidance (through mate recognition and/or differential survival of zygotes based on parental genotypes) is not acting as a major force to drive the genetic mating system of the studied Ethiopian wolf population, confirming previous findings by Randall et al. (2007). An increased number of samples and genetic markers would be necessary to draw firm conclusions about inbreeding and its avoidance based on multi-locus genotypes (e.g. Wang 2014).

5. Conclusions

The Ethiopian wolf is endemic to the highlands of Ethiopia, and so long term survival of the species depends on careful management and protection of its habitat. The Web Valley in the Bales Mountain National Park (BMNP) was home to around 51 individuals between 2007 and 2010, from which samples were collected as the basis for this research. This study has documented the consequence of disease outbreaks on pack structure and improved our understanding of the social interaction of the Ethiopian wolf. These findings should be used as the basis of a species management plan which should aim to stabilise the amount of genetic variation in the population.

Designating protected areas such as the BMNP are one of conservation's most basic approaches of protecting wildlife. However such areas are continually being threatened by the expansion of human activity, especially in developing countries such as Ethiopia. To prevent further degradation of the BMNP environment and safeguard the Ethiopian wolf, it has been advised that urgent action to reduce human impacts is needed (Stephens et al. 2001). The rapid decline of individuals which has been shown in this thesis to change social structures and breeding patterns could inadvertently be ensuring the future survival of the species. If habitat fragmentation from human activity continues this interaction between wolf packs may no longer be possible and may have serious negative implications on the species.

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6. Appendix

Table A1: Genotyped allele sizes for each individual sample (in rows). Individuals are further subdivided into 'Packs', displaying the estimated age and sex of each individual provided by Freya van Kesteren. Those which failed to amplify are represented as a dash. ***N***: total number of individuals amplified, ***Na***: the number of alleles observed

Information				Genotypes													
Pack	**Age**	**Sex**	**Sample ID**	**FH2137**		**FH2226**		**FH2422**		**FH2054**		**FH2001**		**PEZ19**		**FH2140**	
Alando	Adult	Male	F14	157	197	206	210	204	238	-	-	-	-	186	198	111	127
Alando	Adult	Male	48	157	157	206	210	204	208	148	160	126	150	186	186	-	-
Alando	Adult	Male	52	157	177	202	206	208	208	-	-	-	-	-	-	111	127
Alando	Subadult	Male	54	157	157	202	210	200	204	-	-	126	150	198	198	111	127
Alando	Subadult	Female	56	157	161	206	210	200	204	156	168	126	126	186	198	111	111
Alando	Adult	Male	F5	161	165	210	210	204	204	-	-	126	150	198	202	111	127
Alando	Adult	Male	85	157	157	202	202	200	204	-	-	-	-	194	198	119	119

Alando	Adult	Female	98	157	161	150	202	208	212	-	-	126	166	-	-	111	127
Darkeena	Adult	Female	F8	161	161	202	210	-	-	156	164	126	154	186	198	111	127
Darkeena	Adult	Female	51	157	169	202	210	200	204	160	164	126	126	194	202	111	127
Darkeena	Subadult	Female	68	157	169	210	214	200	204	156	164	-	-	198	202	111	111
Darkeena	Adult	Male	89	157	157	202	202	200	200	160	160	-	-	-	-	111	127
Darkeena	Adult	Male	95	157	157	202	202	200	204	156	160	126	142	198	202	111	127
Darkeena	Adult	Male	96	157	157	202	210	200	200	156	160	126	126	202	202	111	111
Darkeena	Adult	Female	99	157	169	202	210	200	204	160	164	-	-	198	202	111	127
Floater	Adult	Female	70	161	169	198	202	204	204	148	168	122	126	186	198	-	-
Floater	Adult	Female	94	161	169	202	210	204	212	156	156	-	-	198	198	111	127
Kotera	Adult	Female	F7	157	161	202	206	-	-	-	-	126	142	186	202	127	135

Kotera	Adult	Male	90	157	157	202	210	216	216	156	168	-	-	198	202	119	127
Megity	Adult	Female	14	157	157	202	210	200	204	160	168	126	150	198	198	111	127
Megity	Adult	Female	18	177	177	194	210	-	-	148	148	126	150	-	-	111	127
Megity	Adult	Male	23	157	157	202	210	200	204	148	148	126	150	198	198	111	119
Addaa	Adult	Male	F10	157	161	-	-	172	172	-	-	150	150	-	-	111	131
Megity	Adult	Female	F11	157	157	162	206	-	-	148	156	126	150	186	198	111	111
Megity	Adult	Female	82	-	-	-	-	200	200	156	168	126	150	190	190	111	119
Megity	Adult	Male	91	161	161	202	210	200	200	-	-	126	126	-	-	119	127
Megity	Adult	Male	92	157	157	194	198	200	204	148	148	126	150	194	198	127	127
Mulamo	Pup	unknown	46	157	157	202	202	-	-	156	160	126	126	198	198	123	127
Mulamo	Adult	Female	49	157	157	202	202	204	204	-	-	126	130	198	202	-	-

Mulamo	Adult	Male	55	157	157	202	202	200	212	156	160	-	-	-	-	-	-
Mulamo	Adult	Female	97	153	153	202	214	200	200	156	156	-	-	-	-	-	-
N/A	Adult	Male	34	157	157	202	214	200	200	160	168	126	126	198	198	111	131
Sodota	Adult	Female	87	157	161	194	210	204	204	156	156	-	-	-	-	-	-
Sodota	Adult	Female	9	157	157	202	202	196	200	-	-	126	126	-	-	111	127
Sodota	Adult	Female	12	161	161	194	210	204	204	156	156	-	-	-	-	-	-
Sodota	Juvenile	Male	17	157	161	210	214	200	204	156	156	-	-	-	-	-	-
Sodota	Adult	Male	42	157	165	210	210	212	212	-	-	118	126	-	-	111	111
Sodota	Adult	Male	53	157	157	210	214	200	204	156	160	126	126	198	202	111	111
Sodota	Juvenile	Unknown	F1	-	-	212	212	172	172	156	156	-	-	186	186	107	127
Tarura	Adult	Female	5	157	161	194	210	200	204	148	156	126	126	182	202	111	127

Tarura	Adult	Female	40	161	161	210	214	200	204	148	148	126	126	186	198	111	127
Tarura	Adult	Female	83	157	161	202	238	148	148	160	160	126	126	-	-	-	-
Tarura	Adult	Female	F6	157	189	202	214	200	212	152	156	126	142	202	202	111	127
			Na	4	8	8	7	8	8	4	5	4	6	7	5	6	6
			N		41		41		38		31		29		29		34