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Social Structure of Spotted Hyena (*Crocuta crocuta*) Populations around Mekelle city in Tigray, Ethiopia

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ABSTRACT

Spotted hyenas (*Crocuta crocuta*) show a remarkable social structure compared to most other large carnivores. Clans consist of four to over 90 members and contain several unrelated males and one to more than ten matrilines of adult females and their offspring. The philopatric females and their cubs outrank all adult males in priority of resource access, and males disperse from their natal clan after they become sexually mature. In natural conditions all adult clan members cooperate in defending the territory and hunting. However, in Mekelle, northern Ethiopia, a population of spotted hyenas in a natural prey depleted human dominated landscape switched to a diet consisting almost exclusively of offal and organic waste. Given this remarkable change, the question was raised if this could affect the distinctive social structure of these large carnivores.

In this study, I examined the potential of non-invasive faecal samples as a source of DNA, and I used this DNA in a population genetic analysis to investigate whether these hyenas showed structural differences compared to what is known for conspecific populations. Microsatellite markers appeared to be very useful in revealing genetic structuring: samples from a distant population were successfully distinguished. Surprisingly enough, an impressive population size was found for Mekelle, while (almost) no genetic structuring appeared there. This could indicate that all individuals belong to one and the same clan, and even if not, they likely aggregate during feeding sessions. Both possibilities have been never observed in spotted hyenas before. Furthermore, the sex ratios show to be reversed in comparison with populations elsewhere in Africa, which is again a fascinating result. Potential causes and implications of these findings are discussed. Although the sample size was relatively low, this study reveals clear patterns of high adaptability of a large carnivore in a human-dominated environment, and provides a good foundation for further research.

SUMMARY

Spotted hyenas are large African carnivores that live in close social groups, called clans. Depending on environmental conditions, these clans can vary in size, but the composition is remarkably constant throughout the species' range. Females typically never leave their natal clan, while adult males are known to move to other clans. As a result, a clan usually consists of one to several families of females and their young, and some males that were born in other clans. Despite of their besmirched reputation as scavengers, members of the same clan often hunt together and respect each other's rank in the clan's hierarchy: females and their young always have first access to carcasses. However, in response to the absence of natural prey, a hyena group in Mekelle, northern Ethiopia, changed to a diet of almost entirely offal, living in an environment dominated by humans.

In this study, I collected hyena droppings to look at the DNA of the animals that live around the city of Mekelle. All living creatures possess DNA: molecules which can be seen as a code of instructions that are needed in the owner's development and functioning. As such, the DNA of different species is not the same, and even between individuals of the same species, differences can be found. I looked for these differences with the aim of assessing the structure of the hyena group in Mekelle. Several changes were found compared to what is known for hyenas in natural environments. The group was remarkably larger and there were (almost) no indications for the existence of different clans. Furthermore, there appeared to be more males than females. Although only a part of the droppings collected could be analysed and hence the results are not sure yet, the findings indicate interesting differences in hyena social structure and provide a good basis for further research.

SAMENVATTING

Gevlekte hyena's vertonen een opmerkelijke sociale structuur in vergelijking met de meeste andere grote carnivoren. Clans bestaan uit vier tot meer dan 90 leden en bevatten verschillende niet verwante mannetjes en een tot meer dan tien matrilines van volwassen vrouwtjes en hun nakomelingen. De filopatrische vrouwtjes en hun welpen hebben steeds als eerste toegang tot hulpbronnen en mannetjes verlaten hun geboorteclan wanneer ze geslachtsrijp worden. In hun natuurlijke omgeving nemen alle volwassen individuen deel aan de verdediging van hun territorium en wordt er in groep gejaagd. In Mekelle, Noord-Ethiopië, leeft een populatie gevlekte hyena's echter in een door mensen gedomineerd landschap, waar natuurlijke prooisoorten bijna volledig verdwenen zijn. De roofdieren blijken overgeschakeld te zijn naar een dieet dat haast uitsluitend uit slacht- en organisch afval bestaat, wat de vraag deed rijzen of dit een invloed heeft op hun gekende opmerkelijke sociale structuur.

In deze studie onderzocht ik de mogelijkheden van een non-invasieve staalname van uitwerpselen als een bron van DNA en gebruikte ik dit DNA in een populatiegenetische analyse om te onderzoeken of deze hyena's structurele verschillen vertonen in vergelijking met wat geweten is voor populaties van soortgenoten. Microsatellieten bleken zeer nuttig om genetische structuur aan te tonen: stalen van een veraf gelegen populatie werden succesvol onderscheiden. Verrassend genoeg werd een indrukwekkende populatiegrootte berekend voor Mekelle, terwijl hier (haast) geen genetische structurering gevonden werd. Dit zou kunnen betekenen dat alle individuen tot één en dezelfde clan behoren. Zelfs indien niet, komen ze waarschijnlijk wel samen wanneer ze foerageren. Beide mogelijkheden werden nooit eerder geobserveerd bij gevlekte hyena's. Bovendien werden omgekeerde seksratio's gevonden vergeleken met populaties elders in Afrika, wat opnieuw een intrigerend resultaat is. Potentiële oorzaken en gevolgen van deze bevindingen worden in de discussie besproken. Hoewel de steekproefgrootte relatief klein was, toont deze studie duidelijke patronen aan van een sterk aanpassingsvermogen van grote carnivoren in een omgeving die door mensen wordt gedomineerd. Daarnaast werd ook een goede basis voor verder onderzoek geleverd.

1. INTRODUCTION

Spotted hyenas (*Crocuta crocuta* Erxleben, 1777), hereafter referred to as hyenas, are large carnivores that naturally occur all over sub-Saharan Africa (Mills & Hofer, 1998), where they occupy a wide range of habitats: savannah, deserts, swamps, woodland and montane forest (Holekamp *et al.*, 2012). These gregarious mammals show a remarkable social structure that more closely resembles that of cercopithecine primates than that of other carnivores (Holekamp *et al.*, 2007). The social groups, called 'clans ', consist of four to over 90 members (Holekamp & Dloniak, 2011) and contain several unrelated males and one to more than ten matrilines of adult females and their offspring (Frank, 1986a). This results in a low average relatedness per clan (Van Horn *et al.*, 2004), although both close kin and many unrelated individuals cooperate to hunt (Mills, 1985) and defend their territory (Boydston *et al.*, 2001; Smith *et al.*, 2008). Hyena groups are organised with a strong hierarchical structure, where individuals inherit their social rank from their mother (Holekamp & Smale, 1991, 1993; Engh *et al.*, 2000) and adult males are outranked by adult females and their dependent offspring in priority of resource access (Tilson & Hamilton, 1984; Frank, 1986b; Smale *et al.*, 1993). The female's status is reflected in her masculinized morphology and behaviour: adult female hyenas are larger (Ralls, 1976; Frank *et al.*, 1990) and more aggressive than males, and their genitals form a pseudopenis (Frank, 1997; East & Hofer, 1997; Cunha *et al.*, 2003). Although spotted hyenas have a polygynandric mating system, this gives females virtually complete control over mating (East *et al.*, 1993; East *et al.*, 2003), which drives male dispersal (Höner *et al.*, 2007) after they become reproductively mature (at ± 24 months of age) (Boydston *et al.*, 2005). Female hyenas are philopatric and rear their cubs together in communal dens where up to 20 cubs can reside concurrently (Kruuk, 1972; White, 2006), which is believed to be crucial for social integration of the young into the dominance hierarchy (Holekamp & Smale 1991, 1993; Smale *et al.*, 1993; Engh *et al.* 2000). The sex ratio in spotted hyena clans is usually skewed towards females: it ranges from 1.19 females to one male in the Serengeti, Tanzania (Hofer & East, 1993a), to a bias of 3.29 females to one male in Chobe National Park, Botswana (Cooper, 1989).

Hyenas are mostly observed active at night and around dawn and dusk (Kruuk, 1972; Cooper, 1990; Mills, 1990; Hayward & Hayward, 2006; Kolowski *et al.*, 2007). Despite their reputation as opportunistic scavengers, hyenas are very effective social predators

(Kruuk, 1972; Cooper, 1990; Mills, 1990; Hayward, 2006). In natural habitats of the Maasai Mara ecosystem, Kenya, they hunt 60 to 95% of their food (Smith & Holekamp, 2010). Hyena clans are fission-fusion societies, which permits hyenas to adjust grouping patterns over both short and long time scales in response to local prey abundance and thus feeding competition (Kruuk, 1972; Holekamp *et al.*, 1997; Höner *et al.*, 2005; Smith *et al.*, 2008). They also show high plasticity with respect to their prey preferences: hyenas can derive energy and nutrients from a vast and diverse array of prey, ranging from small insects to the largest herbivores (Hayward, 2006; Holekamp & Dloniak, 2011), and even digest tough body parts such as bones (Smith & Holekamp, 2010). However, in natural reserves they focus on ungulates within a body mass range of 56 to 182 kg (Mills, 1990; Hayward, 2006), whose capture yields the greatest caloric return while demanding the least effort and the fewest risks (Cooper *et al.*, 1999; Holekamp *et al.*, 1997). Hyenas show a variable ranging behaviour, which is again linked to prey abundance. They have been reported to travel from 3.5 to 75 km a night (Eloff, 1964; Hofer & East, 1993b; Kolowski *et al.*, 2007).

Except for tourists and trophy hunters (Croes *et al.*, 2011), spotted hyenas usually are no welcome guests to humans. They are often persecuted because they predate on livestock (Mills, 1990; Mills & Hofer, 1998; Ogada *et al.*, 2003; Kolowski & Holekamp, 2006; Holmern *et al.*, 2007; Kissui, 2008). They are even known to attack and kill people (Løe & Røskaft, 2004; Gade, 2006; Holmern *et al.*, 2007; Abay *et al.*, 2011). Most of the adult hyena mortality in natural reserves has been caused, deliberately or accidentally, by people (Woodroffe & Ginsberg, 1998) and outside protected areas this is the prime source of population decline (Mills & Hofer, 1998). In the Horn of Africa, spotted hyenas are known to depend more on anthropogenic food sources than elsewhere in sub-Saharan Africa (Gade, 2006; Yirga & Bauer, 2010c; Yirga *et al.*, 2013a). One population in a human-dominated landscape around Mekelle, Enderta district, Tigray regional state, northern Ethiopia, even switched to a diet consisting entirely of domestic prey species (Yirga & Bauer, 2010a, 2010b; Abay *et al.*, 2011; Yirga *et al.*, 2013b). However, only 11 and 16% of the consumed biomass originated from depredated livestock in Debri and Aynalem, at 12 and 7 km from the city of Mekelle, respectively (Abay *et al.*, 2011). In this natural prey depleted region, hyenas scavenge on offal and organic waste at rubbish dumps and along the streets (Abay *et al.*, 2011; Yirga *et al.*, 2013a, 2013b). Only during the 55 days of Ethiopian Orthodox Tewahedo Church fasting period (‘lent ‘) where a vast majority of the

people do not consume animal products, hyenas change to active predation, mainly on free ranging donkeys (Yirga *et al.*, 2012). A similar phenomenon is perceived in Wukro, about 47 km from Mekelle (Yirga *et al.*, 2013c). Abundance assessments using calling stations revealed high abundance of hyenas around Mekelle: in 2009 40 individuals responded in Debri and 16 in Aynalem (Yirga *et al.*, 2013b) and more recently 81 and 76 individuals were counted during one session at the rubbish dumps Adi-Kolemay and Kelamino (Yirga *et al.*, 2013b). This is an exceptional example of how well these carnivores can adapt to non-natural conditions without causing a human-wildlife conflict. Although in 2009 70% of the interrogated farmers did not want to conserve spotted hyenas, these carnivores only accounted for an annual livestock loss of about 0.6% (Abay *et al.*, 2011), and so far hyenas have been tolerated in the vicinity of Mekelle city. In this coexistence people also benefit from the presence of hyenas: they are effective sanitation units removing organic waste (Yirga *et al.*, 2012, 2013a, 2013b).

Given the fact that (i) feeding competition constrains social relationships in spotted hyenas (Holekamp *et al.*, 1997; Höner *et al.*, 2005; Smith *et al.*, 2008), and (ii) this population changed to a diet consisting mainly of offal and other waste disposed of by humans (Abay *et al.*, 2011; Yirga *et al.*, 2013a, 2013b), which means the individuals do not rely on cooperative hunting anymore to obtain their food (even for livestock predation 97.5% of the interrogated reported solitary attacks, Yirga & Bauer 2010c), one could wonder if this would affect the social structure of these carnivores. More specifically: is clan structure similar to 'natural' populations, with one to several matrilines with some adult males from other clans, including a female-biased sex ratio? The spotted hyenas around Mekelle are certainly dealing with a different pattern of food availability compared to a completely wild environment, both on a temporal and a spatial scale. Other questions are: where does which clan feed? Do they always go to the same location? Do different clans mix when they feed? This last question is particularly interesting since most studies in natural reserves report foraging trips are restricted to a clan's territory (Kruuk, 1972; Frank, 1986; Cooper, 1989).

The present master thesis is a pilot study which is also exploring the possible use of genetic tools to investigate such questions. Similar methods have been used to study hyena populations elsewhere in a more natural environment (e.g. East *et al.*, 2003; Wilhelm *et al.*, 2003; Höner *et al.*, 2007; Holekamp *et al.*, 2012; Watts *et al.*, 2011). DNA can be extracted

from tissue, hair or blood samples but since a non-invasive and less time-consuming sampling method was preferred, faecal samples were collected for this study. The fact that hyenas use so-called latrines, spots where members of the same clan defecate to mark their territory (Kruuk, 1972; Mills, 1990; Henschel & Skinner, 1991), makes this method even more suitable.

A significant part of this study consisted of the exploration of the potential of the samples and the refinement of the methods and protocols. This includes selection of good quality samples, finding suitable preservation techniques for the samples, decent DNA extraction methods, appropriate markers and PCR conditions. The different techniques that were tested in the lab are briefly discussed in the first part of this thesis. The final protocols have been successfully applied to 55 of the 268 samples collected and are described in more detail. Also properties of the scats that might predict the sample quality were assessed, to be able to select the best samples.

The major objectives of this thesis are:

1. To explore differences in sample quality and protocols for extracting genetic information from scats
2. To investigate the following hypotheses:
    - Spotted hyenas in the anthropogenic environment in Tigray maintain similar population structure as in natural environments elsewhere
    - Spotted hyenas maintain a social system consisting of matrilines with a female-biased sex ratio and little mixing between clans.

2. METHODS

2.1 Sampling procedure

The sampling was carried out at 20 locations within a 12.5 km radius of from the centre of Mekelle, Tigray region, northern Ethiopia (between 12° 13′ - 14° 54′ North and 56° 27′ - 40° 18′ East, Abay *et al.*, 2011). Covering an area of 24 km² at an altitude of 2200 - 2300 m.a.s.l., the regional capital Mekelle has about 200,000 inhabitants (Yirga *et al.*, 2012b), of which 115,000 live in rural areas and are extremely poor (Bureau of Agricultural and Natural Resources Development, 2009 in Abay *et al.*, 2011). Over centuries, the original dry Afromontane forest vegetation (Aerts *et al.*, 2007) has been completely converted into agricultural and grazing lands, except for patchy remnants around most Ethiopian Orthodox Tewahido Churches (Alemayehu, 2007). These dense vegetation spots provide safe resting places for spotted hyenas, since they are protected by religion and tradition (Abay *et al.*, 2011).

Fig. 1: Map of the geographic situation of the city of Mekelle (right) in Enderta district (white) and Kafta Sheraro National Park (KSNP, left) in Kafta Humera district, Tigray region, Ethiopia (Yirga, 2013, edited).

A total of 268 spotted hyena faecal samples were collected between 8:00h and 13:00h on 23 days between September 8th and October 7th, 2014, after the second rainy season. Locations were suggested by my local supervisor, Gidey Yirga, based on earlier work (Yirga & Bauer, 2010a, 2010b; Abay *et al.*, 2011; Yirga *et al.*, 2012b, 2013a, 2013b). Most of the areas where hyenas reside or rest during daytime were covered, together with two sites where hyenas feed at night, viz. a current (Adi-Kolemay) and a former rubbish dump (Shintro-Gereb). These dumps were visited thrice, and all scats were removed after collection in order to be able to recognize fresh droppings during the next collection. All the other locations were only visited once. Scats were also collected from a distant population in Kafta Sheraro National Park (KSNP), about 325 km NW of Mekelle (Fig. 1). At each location, at least one set of geographic coordinates was taken with a Garmin eTrex 10 GPS. The 20 locations where hyena scats were found, can be grouped in six regions: the old rubbish dump (O), the current rubbish dump (C), the Mekelle University campus (U), north of Mekelle (N), east of Mekelle (E) (Fig. 2) and Kafta Sheraro National Park (K) (Fig. 1).

Local farmers and/or their children often helped to locate fresh scats. If available, soft droppings were preferred over dryer ones. The dryer, the older the scats are, and the more the DNA would be degraded (Santini *et al.*, 2007; Vynne *et al.*, 2011; Reddy *et al.*, 2012). For each scat sample the following information was noted: sampling date, location, colour, inside and outside texture, proximity to other scats, hairiness, and any other relevant 'ad hoc' remarks. Since the DNA originating from the individual (rather than from its food) in faeces originates from epithelial gut cells, about 1 g of the surface of each scat was taken and put separately in 97% ethanol in a labelled safe-lock tube (Eppendorf) (see 3.1.1) (Panasci *et al.*, 2011; Watts *et al.*, 2011). Samples were taken at the side of the scats that was protected from direct sunlight (e.g. covered by vegetation or the underside of the scat), and the slimy parts. Also about 1 g of the inside of each scat was taken and transferred to a separate tube with 97% ethanol for later gut parasite and bacteriological investigations (not discussed in this thesis). Special attention was paid to avoid contamination: for each dropping new gloves and blades were used. Helpers were told not to touch the scats before they had been sampled. In order to have back-up material in case of losses during transport, everything was collected in duplo: back-up samples were left at Mekelle University. After collection, all samples were stored and kept at -20 °C, except during travelling back to Belgium.

Fig. 2: Map of the city of Mekelle, Tigray region, Ethiopia (produced with Garmin BaseCamp v4.4.2). The red numbers next to the blue flags indicate the different sampling locations, regions are indicated with their green letter (O = the old rubbish dump, C = the current rubbish dump, U = the Mekelle University campus, N = north of Mekelle, E = east of Mekelle). Kafta Sheraro National Park (location numbers in upper left corner) is not visible on this map. A properly georeferenced map of Mekelle was preferred above more visually attractive ones.

2.2 Labwork

2.2.1 Exploration and refinement of the protocols

Prior to the fieldwork, faecal samples of two male and one female captive spotted hyenas from the Planckendael zoo, Antwerpen, Belgium were used to assess the best preservation and extraction method, and to test the different markers.

2.2.1.1 Preservation

Samples were preserved (i) dry, at room temperature, (ii) in DMSO, at room temperature (Frantzen *et al.*, 1998; Vynne *et al.*, 2011), (ii) dry, after arrival in the lab at -20°C (Taberlet *et al.*, 1996; Sugimoto *et al.*, 2014) and (iv) in 75% ethanol, after arrival in the lab at -20°C (Wasser *et al.*, 1997; Frantzen *et al.*, 1998; Santini *et al.*, 2007; Panasci *et al.*, 2011; Watts *et al.*, 2011, Tende *et al.*, 2014). The effectiveness of each preservation

method was scored by comparing the extract's estimated DNA content and 260/280 results from a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific) (see 2.2.1.2), or the bands on agarose gels after performing a PCR (see 2.2.1.3). It is important to remark that spectrophotometer results only reveal the quantity of the DNA, not the real quality. As such, it is possible to find decent values while actually the DNA is degraded, which makes the sample still not useful in a PCR because the fragments are too short. Furthermore, since the extraction methods do not distinguish between the hyena and the prey DNA present in the faeces, both of them will be in the extract and they will make up the concentration values together. It is thus again possible to obtain decent spectrophotometer results, while there is no hyena DNA in the extract at all.

2.2.1.2 Extractions

Two DNA extraction methods were tested, using a spotted hyena blood sample from the Planckendael zoo as positive control. Initially, a QIAamp DNA Stool Mini Kit (Qiagen) was used (Chaves *et al.*, 2006; Watts *et al.* 2011) according to the manufacturer's protocol.
Secondly, DNA extraction was performed following the protocol of Boom *et al.* (1990) using guanidine thiocyanate (GuSCN) and silica. The GuSCN/silica extraction method has been successfully applied on faecal samples from brown bears (Taberlet *et al.*, 1996), felids (Reddy *et al.*, 2012) and leopards (Sugimoto *et al.*, 2014).
After extraction, the purity of the extracts was assessed using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific).

2.2.1.3 Markers

Because of their high mutation rates and the fact they are codominant and putatively neutral Mendelian markers, microsatellites are particularly suitable for kinship investigations and population genetic analysis (Bowcock *et al.*, 1994; Jarne & Lagoda, 1995; Sunnucks, 2000). In this study, nine species-specific microsatellite markers (Wilhelm *et al.*, 2003) were used to construct consensus genotypes of all samples, to compare them and see if genotypes had been sampled twice, to asses structuring in the data and to check the validity of the assumptions made. In combination with sex (ratio) determination using primers for ZFX/ZFY loci (Schwerin & Pitra, 1994) and mitochondrial DNA (e.g. *cytochrome b*, Rohland *et al.*, 2004, and control region, Wang *et al.*, 2006) to assess different matrilines, it can provide a good idea of the social structure.

The first molecular markers that were tested were microsatellite primers specifically designed for spotted hyenas: *CCr*14 and *CCr*15 (Libants *et al.*, 2000; Van Horn *et al.*, 2004; Watts *et al.*, 2011). This allowed us to confirm whether the DNA in the extracts was not only from prey species. Polymerase chain reactions (PCRs, Mullis & Faloona, 1987) were performed using conditions described in Libants *et al.* (2000) in a Biometra TProfessional Thermocycler (Westburg) using Phusion Hot Start Flex DNA Polymerase (Phusion, NEB). PCR products were separated on 1.3% agarose TBE gels, and visualized with a GeneFlash UV imager (Syngene Bio Imaging). Extractions and amplifications were considered successful if bands around 179-189 bp (*Ccr*14) or 121-131 bp (*Ccr*15) were observed.

Markers for the sex determination were tested following PCR conditions of Schwerin & Pitra (1994) (see 2.2.2.3). The primers P1-5EZ and P2-3EZ are based on a highly conserved region of human and mouse *ZFX*/*ZFY* loci (Aasen & Medrano, 1990). Again the PCR products were separated on 1.3% agarose TBE gels, and visualized with the GeneFlash UV imager. A band around 448 bp indicated a successful extraction and amplification.

Two different mitochondrial DNA (mtDNA) regions were amplified: parts of the cytochrome b gene (*Cyt b*) and a part of the *12S rRNA* gene. Between them lies the highly variable control region (Albert, 2002, Wang *et al.*, 2006), which was the area of particular interest. However, hyena-specific primers of this region have not been developed yet, so it was decided to start with testing flanking regions and to proceed with the control region if these worked well. First, the primers *Cyt b* F1 & R1 and *Cyt b* F2 & R2 (Hofreiter *et al.*, 2004) were tested following Rohland *et al.* (2004) using Phusion Hot Start Flex DNA Polymerase (Phusion, NEB). Successful extractions and amplifications were demonstrated by a band at about 214 bp (*Cyt b* F1 & R1) or 252 bp (*Cyt b* F2 & R2) after separation on agarose gel. If the bands were too weak, the PCR was repeated adding BSA (Bovine Serum Albumine, Farell & Alexandre, 2012). PCR products were sequenced at the Genetic Service Facility (GSF) of the VIB (Vlaams Instituut voor Biotechnologie). BLAST (Basic Local Alignment Search Tool) was used to confirm the amplified DNA was derived from spotted hyena and the sequences were aligned using Geneious 6.0.6 (Biomatters Ltd).

In addition, 394 bp of the mitochondrial *12S rRNA* gene were amplified using the primer pair *12S*-L1091/H1478 (Kocher *et al.*, 1989). Reaction conditions followed Rojas *et al.* (2008), only the annealing temperature was 65 °C instead of 54 °C because of the use of a different DNA polymerase. This region and its flanking primers are conserved fairly well and often used in species determination (Rojas *et al.*, 2008). Together with the fact that mtDNA is more abundant than nuclear DNA and thus easier to amplify, it makes this marker a good option to test the success of extractions and exclude contamination from other species.

Because of the various negative results (see 3.1), more research was done to find other markers of less conserved regions or to create new specific primers for variable regions (e.g. D-loop of the mitochondrial control region, Albert, 2002, Wang *et al.*, 2006). As such, contacts were made with dr. East and dr. Hofer from the IUCN Hyaena Specialist Group. A collaboration was established between the Evolutionary Ecology group of the University of Antwerp and the IZW (Leibniz Institute for Zoo and Wildlife Research) in Berlin, Germany, where dr. East and dr. Hofer are currently based. The next part of the methods and protocols described in this thesis were performed in the lab of the Evolutionary Ecology department at the IZW.

First of all, it was necessary to test if there had been problems with the PCRs or if the extraction method of Boom *et al.* (1990) was responsible for the problems we experienced in the lab in Belgium (see 3.1). A subsample of 34 extracts was taken and a PCR was performed on the *ZFX*/*ZFY* loci (Aasen & Medrano, 1990), applying the protocol described below (see 2.2.2.3) (Schwerin & Pitra, 1994). Because not one of the amplifications of the Ethiopian samples appeared successful, it was decided to determine the 260/280 ratio of four of the extracts with a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific) to be able to exclude PCR error. The average ± standard deviation of 260/280 nm was 1.32 ± 0.16. This indicated a low extract quality, possibly due to the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm (a value of 1.8 for DNA can be considered pure, Thermo Fisher Scientific, 2013). It was concluded that proceeding with the extracts of the GuSCN/silica method (Boom *et al.*, 1990) was not possible.

A total of 16 samples from the six different sampling regions were selected to try another extraction method. DNA was extracted using a NucleoSpin Soil Kit (Macherey & Nagel, Düren, Germany) following the manufacturer's protocol for 250-500 mg sample material. The extract was eluted in a final volume of 50 µL. From eight samples, 1 µL was taken to check the 260/280 nm ratio. This time, the average ± standard deviation was 1.86 ± 0.06, which indicated pure extracts.

A PCR was performed on all 16 extracts with primers for the *ZFX*/*ZFY* loci (Aasen & Medrano, 1990) and the primers L-15162 and H-15915 (Irwin *et al.*, 1991) for a 753 bp sequence of the cytochrome b gene (referred to as *Cyt b II*). The protocols are described in the first paragraph of 2.2.2.3: Sequencing and 2.2.2.4, respectively. Both PCRs appeared successful for at least some samples, when the products were separated on an agarose gel. The nuclear markers did less well than the mtDNA (25% and 50% success on gel, respectively). Samples marked as 'dry ' during collection in Ethiopia (see 2.3.1) did not give a band.

The results of the amplification of another part of the *Cyt b* gene (referred to as *Cyt b I*, see 2.2.2.4) were similar (37.5% success on gel).

Nine microsatellite primers (Wilhelm *et al.*, 2003) were successfully tested (94% success), following the first paragraph of the methods under 2.2.2.2.

The positive PCR products of *Cyt b II* were sequenced following 2.2.2.4, second paragraph. The results were compared with sequences of spotted hyenas from all over Africa (East *et al.*, unpublished data), using MEGA v6.06 (Tamura *et al.*, 2013). Similar to the sequences of *Cyt b* F1 & R1 and *Cyt b* F2 & R2, this mitochondrial marker did not show to be variable (Appendix 1). Also considering the low PCR success, it was decided not to continue with *Cyt b I* and *II*.

The focus of this study was thus put on the microsatellites and the sex markers for the determination of individual identity, population structure, sex ratios and where the hyenas feed.

2.2.2 Final protocol

A total of 55 samples were selected for DNA analysis based on region, location, freshness and proximity to other samples. From samples which had been found closer than 2 m from each other, only one was taken to avoid double sampling.

2.2.2.1 Extractions

Faecal DNA was extracted using a NucleoSpin Soil Kit (Macherey & Nagel, Düren, Germany) following the manufacturer's protocol for 250-500 mg sample material. The extract was eluted in a final volume of 50 µL.

2.2.2.2 Microsatellites

Nine microsatellite loci (*Ccroc*02, *Ccroc*04, *Ccroc*05, *Ccroc*06, *Ccroc*07, *Ccroc*08, *Ccroc*09, *Ccroc*10, *Ccroc*11) were amplified as described previously for the spotted hyena (Wilhelm *et al.*, 2003). PCRs were performed in a peqstar 2x Doppelblock-Thermocycler (Peqlab) using 0.2 ml microreaction tubes (Kisker). DNA was amplified in a 10 µL PCR reaction volume, which contained 5 µL Type-it Multiplex PCR Master Mix (Qiagen), 1 µL of primer mix A (*Ccroc*05, *Ccroc*08, *Ccroc*09, *Ccroc*11) or B (*Ccroc*02, *Ccroc*04, *Ccroc*06, *Ccroc*07, *Ccroc*10), 1 µL 5x Q-Solution (Multiplex PCR Kit Qiagen), 1 µL RNase-free water, and 2 µL of extract with template DNA. Each PCR run was ensured contamination-free by including a DNA-free control (RNase-free water). In case more PCR product was needed for a second amplification, the RNase-free water was replaced with an extra µL of extract with template DNA. The PCR conditions were a variation of Wilhelm *et al.*'s (2003) thermal profile 58. An initial denaturation step of 5 min at 95 °C was followed by 35 cycles of 95 °C for 30 s, annealing at 60 °C for 1 min 30 s, and extension at 72 °C for 30 s. The DNA amplification ended with a final extension of 10 min at 72°C and the PCR products were stored at 8 °C. PCR amplification was confirmed on 1.5% agarose TAE gels in a Mini-Elektrophoresesystem, (Mini Gel II, VWR International). Of each PCR product, 5 µL was mixed with 1 µL loading dye (from 1 mL Ficoll 400, VWR International + 6 µL GelRed 10,000x in DMSO, Biotium) and transferred to a well in the gel. This was run at 125 V for 20-24 min (according to migration speed) together with 2.3 µL of a Hyper Ladder 25bp (Bioline) and visualized with a Gel IX Imager (Intas) to confirm the PCR amplification.

The fragment lengths of the PCR products were determined by a Hitachi 3130 Genetic Analyzer (Applied Biosystems) with an injection time of 3 s. 1.2 µL of each PCR product was pipetted into separate wells of a plate, together with 0.6 µL 500 bp Red DNA Size Standard (NimaGen) and 14 µL HiDi / Super-Di Formamide (NimaGen). If an individual lacked results for a certain locus or a locus was found to be homozygous, the PCR and

fragment analysis were repeated up to three times. Also, individuals with the same genotype were run again together. Amplifications of heterozygotes were not repeated (Watts *et al.*, 2011). To improve the quality of ambiguous results, 1.3 µL PCR product was used, and the injection times were increased to 10 s.

2.2.2.3 Sex determination

For the determination of the sex of the individuals from which a sample was taken, two different methods were applied on a highly conserved region of human and mouse *ZFX*/*ZFY* loci (Aasen & Medrano, 1990). Firstly, the *ZFX*/*ZFY* loci were digested by restriction enzymes for a restriction fragment length polymorphism (RFLP) analysis. From the samples that did not yield a clear result, the *ZFX*/*ZFY* loci were sequenced (Schwerin & Pitra, 1994).

Restriction enzymes

Starting with a PCR, 3 µL extract with template DNA of each sample was added to 12.5 µL Dream Taq Master Mix (Thermo Fisher Scientific), 1.25 µL of both the forward and the reverse primer from a highly conserved region of human and mouse *ZFX*/*ZFY* loci (Aasen & Medrano, 1990) and 7 µL RNase-free water to get a total volume of 25 µL. Reaction conditions were 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 45 °C and 45 s at 72 °C, and a terminal extension step of 10 min at 72 °C and 2 min at 30 °C (modified from Schwerin & Pitra, 1994). 5 µL of the PCR products were run in a 1.5% agarose gel (see 2.2.2.2) to confirm the PCR amplification. Only the PCR products of the samples that showed a band at about 448 bp (Schwerin & Pitra, 1994) were used in the next step.

Based on the strength of the bands, 10 µL (strong) or 20 µL (weak) of PCR volume were digested with each 1 µL of the restriction endonuclease FastDigest PstI (Thermo Fisher Scientific), 2 µL buffer and 17 µL (strong) or 7 µL (weak) RNase-free water to get a total reaction volume of 30 µL. After being incubated for 25 min at 37 °C, the fragments were separated on 3% agarose gels. For strong bands, 8 µL digestion product was used together with 3 µL loading dye (from 1 mL Ficoll 400, VWR International + 6 µL GelRed 10,000x in DMSO, Biotium), for weak bands 15 µL digestion product was used. Digestion products were run at 125 V for about 27 min together with 2.3 µL of Hyper Ladder 25 bp (Bioline).

Given that PstI enzyme digestion yields two fragments (345 and 103 bp) in females and three fragments (448, 345 and 103 bp) in males (the Y locus is not digested), we were able to identify males and females by examining the gel with a Gel IX Imager (Intas) (Schwerin & Pitra, 1994).

Sequencing

A PCR was carried out in a total volume of 10.1 µL containing 5 µL HotStarTaq Plus Master Mix (Qiagen), 0.3 µL of both the forward and the reverse primer, 4 µL of RNase-free water and 0.5 µL extract with template DNA. Initial denaturation was at 94 °C for 15 min, followed by 36 cycles of 94 °C denaturation for 45 s, 54 °C annealing for 30 s and 72 °C extension for 1 min, with a final 72 °C extension of 10 min (modified from Schwerin & Pitra, 1994). Also here, 5 µL of the PCR products were run in a 1.5% agarose gel (see 2.2.2.2) to confirm the PCR amplification. Only the PCR products of the samples that showed a band at about 448 bp (Schwerin & Pitra, 1994) were used in the next step. This time, three categories of band strength were distinguished: strong, medium and weak.

PCR products were cleaned with FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I (Thermo Scientific). 1 µL (strong band), 2 µL (medium band) or 3 µL (weak band) PCR product were added to 5, 4 or 3 µL of Exo/FastAP MasterMix (10 µL exonuclease, 25 µL FastAP and 465 µL distilled water) and digested for 15 minutes at 37 °C. After another 15 min of inactivation at 85 °C, the reaction products were used in the sequencing reaction. 2 µL 5x dilution buffer, 1 µL of the reverse *ZFX/ZFY* primer (Aasen & Medrano, 1990), 0.5 µL BDX64 (enhancing buffer) and 0.5 µL Bright Dye (NimaGen) were added to each tube. Initial denaturation was at 96 °C for 3 min, followed by 30 cycles of 96 °C denaturation for 10 s, 50 °C annealing for 10 s and 60 °C extension for 2 min. Next, a D-Pure^{TM} DyeTerminator Removal kit (NimaGen) was used to remove unincorporated DyeTerminators and salts. Of the resulting liquid, 35 µL was transferred to the wells of the sequencing plate and sequenced using a Hitachi 3130 Genetic Analyzer (Applied Biosystems). The three polymorphisms in the *ZFY* locus, which can be used to discriminate between males and females, are noticeable as two peaks in the sequencing profile of PCR fragments generated from male target DNA where females only have one peak (Schwerin & Pitra, 1994).

2.2.2.4 Cytochrome b

Although the present study could not include mtDNA information, the protocol for the amplification of *Cyt b I* and *II* is provided here, since this will be used in further phylogeographic research on our samples (East *et al.*, unpublished data).

Two mitochondrial cytochrome b markers were amplified: *Cyt b I* (primers L-15162 and H-15915, Irwin *et al.*, 1991) and *Cyt b II* (primers L-14724 and H-15494, Irwin *et al.*, 1991; Koepfli & Wayne, 1998). PCRs were carried out in a total volume of 10.1 µL. 5 µL HotStarTaq Plus Master Mix (Qiagen), was added to 0.3 µL of both the forward and the reverse primer, 4 µL of RNase-free water and 0.5 µL extract with template DNA. Initial denaturation was at 94 °C for 15 min, followed by 36 cycles of 94 °C denaturation for 45 s, 49 °C annealing for 30 s and 72 °C extension for 1 min, with a final 72 °C extension of 10 min (Koepfli *et al.*, 2006, modified). 5 µL of the PCR products were run in a 1.5% agarose gel (see 2.2.2.2) to confirm the PCR amplification. Only the PCR products of the samples that showed a band at about 770 bp (*Cyt b I*) and 753 bp (*Cyt b II*) were used in the next step. Just like for the sex determination, three categories of band strength were distinguished: strong, medium and weak.

PCR products were cleaned with FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I (Thermo Scientific), and the D-Pure^{TM} DyeTerminator Removal kit (NimaGen) was used to remove unincorporated DyeTerminators and salts (see 2.2.2.3: Sequencing). 35 µL of the resulting liquid was transferred to the wells of the sequencing plate and sequenced using a Hitachi 3130 Genetic Analyzer (Applied Biosystems).

2.3 Analysis

2.3.1 Sample quality

RStudio v0.98.1028 was used to assess whether the PCR success rates and/or ADO (allelic dropout: one allele of a heterozygous individual is not amplified during a positive PCR) estimated by GIMLET (see 2.3.2) could be predicted by scat (collection) properties. The confidence intervals were set to 95% and the significance level of the *p*-values was 0.05. ADO and PCR success are both influenced by the degree of degradation and the amount of

DNA (Santini *et al.*, 2007; Vynne *et al.*, 2011; Reddy *et al.*, 2012), and thus can be used to assess sample quality. Of both variables, a modified *box plot* was made to assess the distribution of the values and detect potential outliers. After performing a Shapiro-Wilk test of normality and Bartlett's test for homoscedasticity (in case outliers were detected, the more robust Fligner-Killeen test for homoscedasticity was used following Conover *et al.*, 1981), a correlation test was performed to see whether these two variables were somehow linked. This was not the case (Pearson's $r_{55} = 0.084$, $p = 0.538$), so they both were used in the next step. A multi-factor ANOVA was performed to test which variables were important. Five variables were considered: sample ID (since the fragment analysis was repeated several times for most of the samples, see also 2.2.2.2), time before extraction (three class levels: 143, 133 and 123 days), colour (tested both for six and three class levels), and inside and outside texture (both three class levels), together with their relevant interactions (combinations of colour and texture of inside and outside). The model was simplified omitting non-significant variables. Tukey's HSD (Honestly Significant Difference) test was performed to see which class levels differed from each other.

An overview of the different class levels of the variables is given in Tables 1, 2 and 3. For the texture, the low number of dry samples can be explained by the fact that it was not possible to process all of the samples in the lab, which is why the best available ones per region were selected. From KSNP there were only three samples in total, which were all used. One of them was dry.

Table 1: Overview of the different class levels of the two different colour variables. The last column shows the number (#) of samples that were analysed.

Variable	Class levels	Description	# Samples
6 colours	w	white	9
	g	grey	5
	bl	black	5
	br	brown	13
	w+	whitish	18
	br+	brownish	5
3 colours	light	Light colours	27
	medium	Intermediate colours	10
	dark	Dark colours	18

Table 2: Overview of the different class levels of the variable 'time before extraction ' (represented as three collection time blocks). The mean number (#) of days before extraction was calculated based on the mean date of collection per class level. The last column shows the number (#) of samples that were analysed.

Time block	Collection date	Mean collection date	Mean # days	# Samples
Early	9-15 Sept 2014	13/09/2014	143	30
Middle	16-28 Sept 2014	23/09/2014	133	10
Late	30 Sept. - 7 Oct 2014	3/10/2014	123	15

Table 3: Overview of the different class levels of the two scat texture variables: outside and inside. The number of samples analysed is given.

Texture	Outside	Inside
Soft	42	46
Semi-dry	12	8
Dry	1	1

2.3.2 Marker informativity

To assess the informativity of the different markers used in this study, the allelic frequencies, PCR success rates and PI per locus were estimated using GIMLET 1.3.3 (Valière, 2002).

The different alleles were also compared to the ones known from spotted hyena populations in the Ngorongoro Crater and the Serengeti National Park, Tanzania (East *et al.*, 2003; Wilhelm *et al.*, 2003; Höner *et al.*, 2007; East *et al.*, 2009; Höner *et al.*, 2010).

2.3.3 Consensus genotypes

GIMLET was used to construct consensus genotypes for each sample and to estimate the genotyping error rate from each set of PCR repetitions. The threshold value was set to 1 because in some cases only one PCR had been successful. Also error rates at each locus were estimated using GIMLET. Two kinds of error were examined by comparing the repeated genotypes and the associated consensus genotype for each sample: ADO and false allele (FA: PCR-generated allele as a result of a slippage artefact during the first cycles of the reaction) (Broquet & Petit, 2005).

2.3.4 Population size

GIMLET and CAPTURE (Otis *et al.*, 1978) were run to estimate the population size using both all consensus genotypes (n = 55) and all consensus genotypes excluding the ones from KSNP (n = 52). A mark-recapture approach with constant capture probability was applied. A marked individual was an individual sampled previously and defined by a multilocus genotype. The population was assumed to be closed.

2.3.5 Identical genotypes

To assess whether one genotype had been sampled twice, the regrouping function in GIMLET was used. Missing alleles were treated as any other allele and genotypes were grouped if no differences were found between them. The sampling locations and regions of identical genotypes were then compared, to see if there was information about where individuals from different resting places feed, whether hyenas from different locations mix and if individuals always feed at the same location. Unfortunately this does not necessarily mean that scats found at different latrines are from individuals of different clans. Only if two identical genotypes are found at two different latrines, one might conclude all other genotypes from those latrines belong to the same clan.

To assess the reliability of the assumption that two identical genotypes originated from the same individual, the probability of identity (PI) was computed with GIMLET. I used a less biased equation for correcting for small samples of individuals (Kendall & Stewart, 1977). As the PI also quantifies the power or ability of a molecular marker to resolve between two individuals, it can be used to assess the informativity of the different loci. The total PI for this set of nine markers was calculated by multiplying the PI value over all loci (considering independence of loci).

The second sample of the same genotype was discarded from the dataset before proceeding with the analysis, except for the evaluation of the sample quality.

2.3.6 Kinship

The ‘Kinship‘ function of GIMLET was used to see if it was possible to assess parentage between individuals, incorporating the determined sexes. Again, missing alleles were

considered as any other alleles. An individual was regarded potential parent when at least one allele per locus matched with another individual (the offspring). This is a good basic assumption (Valière, 2002). However, this means with our data it was not possible to tell which of the two individuals was older and thus the potential parent. As such, only possible kinship could be estimated.

2.3.7 Population structure

To evaluate if the different sampling regions correspond to different clans, or, more precisely, if the geographic distribution of the hyenas was reflected in their genetics, a cluster analysis was performed using STRUCTURE 2.3.4 (Pritchard *et al.*, 2000; Waples & Gaggliotti, 2006). A burn-in length of 100,000 was used and 200,000 Markov chain Monte Carlo (MCMC) iterations were run for numbers of *K* from one to six, which was repeated ten times. Assumed was an admixture model with correlated allele frequencies (Falush *et al.*, 2003) without the use of sampling regions as prior information to assist the clustering. Individuals and locations were assigned to clusters based on the highest mean percentage of membership (*Q*) of their samples. To delineate the most likely level of population subdivision, STRUCTURE HARVESTER (Earl *et al.*, 2012) was used to apply the Evanno method for detecting the number of *K* groups that best fit the dataset. Here, the ad hoc statistic *Delta K* (based on the rate of change in the log probability of data between successive *K* values) peaks when the uppermost hierarchical level of structure for the scenarios tested is detected (Evanno *et al.*, 2005). Population subdivision is also indicated by the Dirichlet parameter *Alpha* for degree of admixture, which settles down to be relatively constant once the Markov chain converges (often with a range of < 0.2). If there is no real structure, *Alpha* usually varies greatly during the course of the run (Pritchard *et al.*, 2010).

Because the samples from KSNP were clearly distinguishable, the STRUCTURE analysis was repeated with only the 47 samples collected around Mekelle. The most likely number of clusters in the data (*K*) was this time determined for numbers of *K* from one to five (since there were five sampling regions around Mekelle).

In addition, Arlequin 3.01 (Excoffier *et al.*, 2005) was used to compute population pairwise F_{ST} values (F_{ST} = fixation index) to assess the short-term genetic distance between regions. The allowed level of missing data was set to 0.05 and the number of permutations to 16,000.

2.3.8 Assumptions

The models used to calculate the parameters discussed above are based on certain assumptions. It is important to check these assumptions to be able to assess the reliability of the results.

I tested for linkage disequilibrium (LD = the non-random association of alleles at different loci, Lewontin & Kojima, 1960) for each pair of loci, using Arlequin. The allowed level of missing data was set to 0.05, the number of permutations to 16,000 and the significance level equalled 0.05.

To assess whether populations were in Hardy-Weinberg equilibrium (HWE), Arlequin was used to compute the expected (H_{exp}) and observed (H_{obs}) heterozygosity for each locus. The results of (i) all locations together were compared with (ii) the heterozygosity of all these locations without KSNP. The allowed level of missing data was set at 0.05, the number of steps in the Markov chain was 100,000 and the number of dememorisation steps equalled 3000.

The inbreeding coefficient for Mekelle was calculated following Wright's formula: $F_{is} = (H_{exp} - H_{obs}) / H_{exp}$ (Wright, 1969).

2.3.9 Sex ratios

The results of the sex determination were used to calculate the prevalent sex ratio of each region and the sex ratio of the total population.

3. RESULTS

3.1 Exploration and refinement of the protocols

The results presented in this part, are the ones that were obtained in the lab in Belgium.

3.1.1 Preservation

The best preservation methods turned out to be freezing the samples immediately (dry faeces) or adding ethanol before freezing them at -20 °C (Table 4). This was where the highest DNA concentrations were found. However, in terms of the 260/280 ratio, the purity of the extracts from all preservation methods were very similar. Since in Ethiopia it would be often not possible to freeze the samples soon after collection, it was decided to preserve them in ethanol in the field (Santini *et al.*, 2007; Panasci *et al.*, 2011, Watts *et al.*, 2011, Tende *et al.*, 2014).

Table 4: Results of the spectrophotometer for the different preservation methods after DNA extraction.

Extraction date	Preservation	DNA (ng/µl)	260/280
19/12/2013	Dry	29.3	2.01
	DMSO	35.1	2.13
	-20 °C	45.6	2.05
	Ethanol	87.4	2.08
23/01/2014	Dry	13.2	1.66
	DMSO	20.2	2.14
	-20 °C	129.2	1.96
	Ethanol	126.4	1.91

3.1.2 Extractions and markers

Despite the positive spectrophotometer results (see 3.1.1), both PCRs with P1-5EZ/P2-3EZ and *CCr*14-*CCr*15 primers did not work on the extracts made with the QIAamp DNA Stool Mini Kit. Also when the amount of template DNA was increased (6 µL instead of 3µL extract), no positive PCRs were observed.

The primers for the sex determination as well as the microsatellites did not work on extracts of the GuSCN/silica extraction method either (only the blood sample showed a positive result). However, the GuSCN/silica extraction method did work for the *Cyt b* F1 & R1 and *Cyt b* F2 & R2 primers (Appendix 2), so this method was applied to all the samples from Ethiopia.

Comparisons with BLAST confirmed the amplified DNA originated from spotted hyenas. However, when the extraction and PCRs were applied on five samples from Ethiopia, the alignment of the sequences showed only one bp of variation (Fig. 3). Even when the results of both *Cyt b* F1 & R1 and *Cyt b* F2 & R2 were combined, this was the case. These mtDNA markers did not seem to be useful to estimate matrilines within the same population.

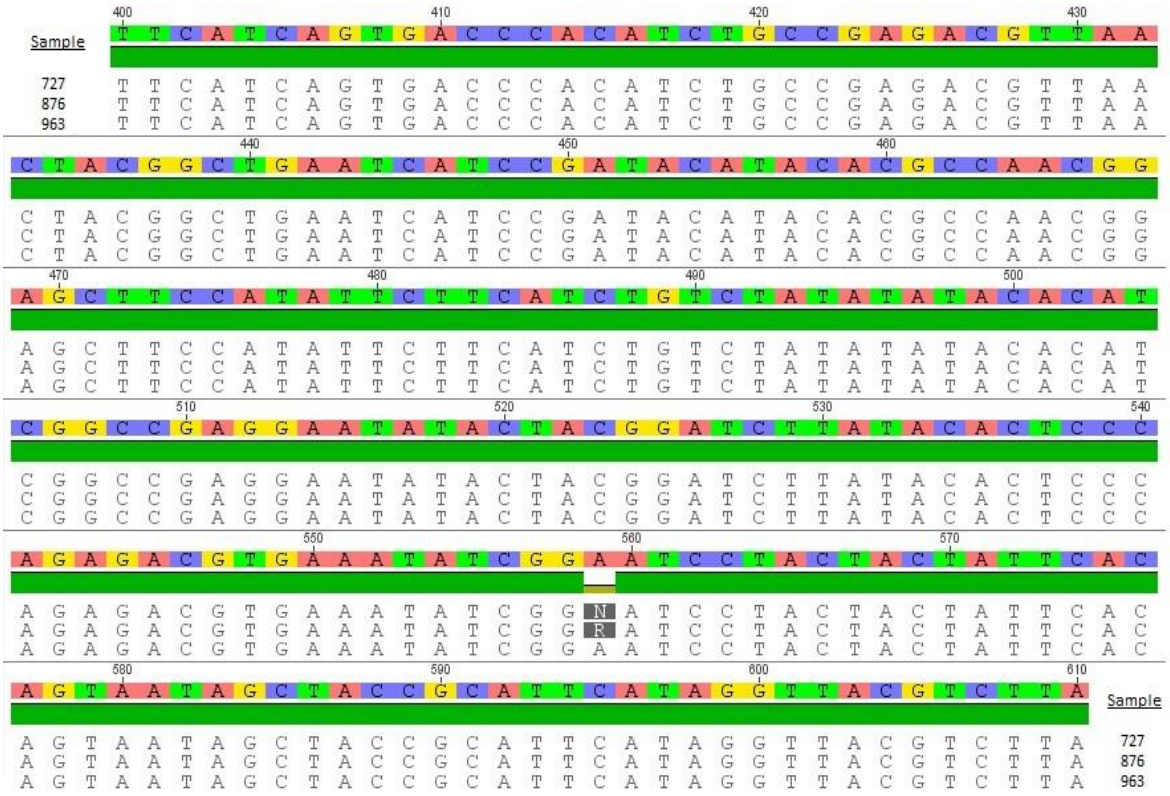

Fig. 3: Alignment of the *Cyt b* sequences of three samples (727, 876 and 963). The sequences obtained by the reverse primer (R2) have been mirrored and added to those of the forward primer (F2) in order to get a better consensus sequence of each sample before combining them. The coloured characters A, C, T, G above represent the nucleobases adenine, cytosine, thymine and guanine that build up the consensus sequence. The green band indicates that the three samples below (grey nucleobases) have the same nucleobase at this position. Only at position 559 the result is not unambiguous (N = any base, R = purine: G/A).

Nevertheless, there were still problems with the PCRs of other samples. Therefore, primers for other parts of the mtDNA were tested. The amplification of a part of the mitochondrial *12S rRNA* gene was only successful for the control (blood) sample. The addition of BSA did not improve the results.
A subsequent quality test with the spectrophotometer of 11 extracts from Ethiopia made with the GuSCN/silica extraction method (Boom *et al.*, 1990) showed low values of DNA purity (average ± standard deviation of 260/280 nm was 1.42 ± 0.14).

As previously stated (see 2.2.1.3), after using the NucleoSpin Soil Kit for extraction at the IZW in Berlin, the PCR yield of mtDNA was still not high enough (only 37.5 and 50% of the *Cyt b I* and *II* PCR products showed a band on agarose gel). Together with the fact that also here the good samples showed no variation (Appendix 1) and that the development of primers for the mitochondrial control region would be too time consuming, it was decided not to continue with mtDNA.
However, the *Cyt b I* and *II* sequences did differ from the ones of spotted hyenas from e.g. Tanzania, South Africa, Nigeria and Kenya. This made the Ethiopian samples valuable in an ongoing study about the phylogeography and genetic diversity of the spotted hyena (East *et al.*, unpublished data). Therefore, aliquots of the samples were left at the IZW for further investigation.

3.2 Sample quality

3.2.1 PCR success

Although the modified box plot of the proportion of PCR success per sample showed one extreme outlier with a value lower than the other samples (sample no. 869), the Shapiro-Wilk test did not indicate deviations from normality of the residual values ($W = 0.917$) and hence the observation was not discarded. Bartlett's test demonstrated that the variances were sufficiently equal ($p = 0.067$). With six colour class levels, not one variable had a significant effect on PCR success. However, colour had a reasonably low p value: $F_{5,38} = 1.696$, $p = 0.159$.
Using only three colour classes, the model's residuals still did not deviate from normality ($W = 0.901$) and a significant effect of scat colour on PCR success was found. There was

no effect of the sample ID (p = 0.685), the texture of the scats ($p_{outside}$ = 0.538, p_{inside} = 0.482), the time before extraction (p = 0.398) or any significant interaction (p > 0.1). After simplification of the model, W equalled 0.914 and the significance of the differences in PCR success between samples of a different colour was indicated by p = 0.011 ($F_{2,52}$ = 4.978). Tukey's HSD test showed that the colours 'medium' and 'light' differed significantly in PCR success (p = 0.007). The other two-by-two comparisons were not significant. The means are plotted in Fig. 4.

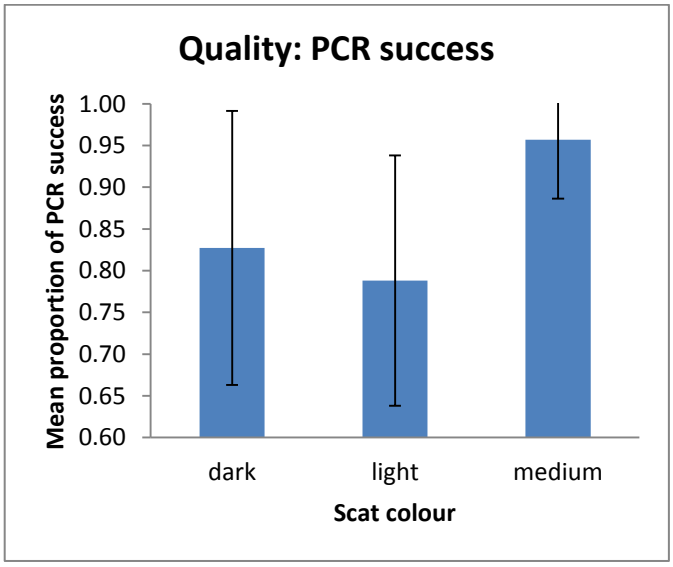

Fig. 4: Average proportion of PCR success for each group of scat colour. Horizontal bars represent standard deviations.

3.2.2 Allelic dropout

Also here, one extreme outlier (sample nr. 869) and two less striking outliers (samples nr. 898 and 948) were detected with the modified box plot of the ADO. Yet, because the residuals of the full model did not deviate from normality (W = 0.952), the observations were kept in the dataset. The Fligner-Killeen test indicated slight deviations from homoscedasticity (p = 0.040) but ANOVA could be considered robust enough to overcome this (Schmider *et al.*, 2010). However, scat colour had no effect when six class levels were used.

With three colour class levels, again ANOVA could be used because the normality assumption was not violated (W = 0.962) and the Fligner-Killeen test only indicated slightly different variances (p = 0.040). Again no significant differences in ADO between samples with a different colour were detected (p = 0.628). After simplification of the

model by omitting this variable, a significant effect of the time before extraction ($F_{2,50}$ = 5.445, p = 0.007) and outside texture ($F_{2,50}$ = 4.8990, p = 0.011) on ADO was found. The interaction of the two was not significant (p = 0.258). Also sample ID (p = 0.058) and inside texture (p = 0.452) did not show an effect on ADO.

Fig. 5 shows the average ADO for each combination of time before extraction and outside texture. Samples that were collected at the end of the fieldwork, showed higher degrees of ADO than those taken at the beginning (p = 0.016) and halfway (p = 0.016). The degree of ADO was significantly higher for dry samples than for soft ones (p = 0.023). However, the statistical power of this test was not high, given the fact that there was only one observation of a dry sample and not every combination of categories was represented (e.g. no 'semi-dry' and 'dry' for time block 'middle').

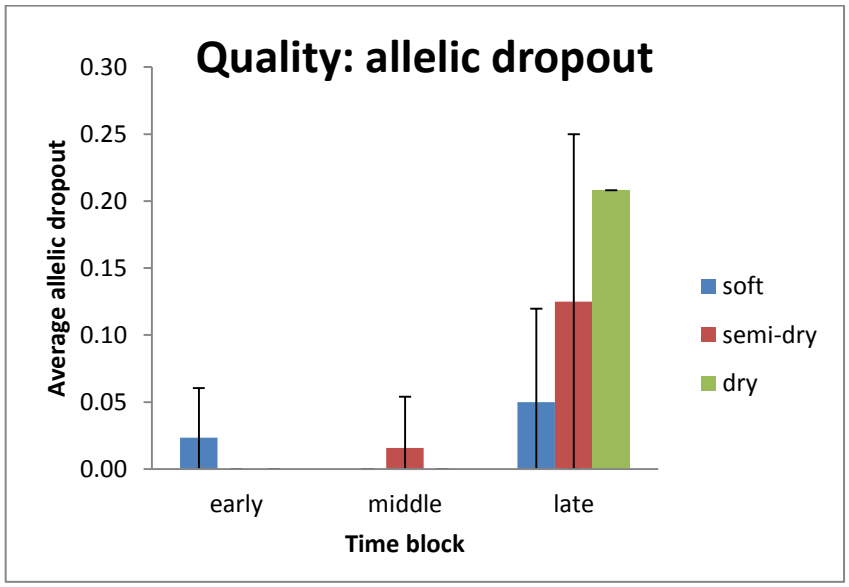

Fig. 5: Average allelic dropout for each combination of time before extraction (represented as three collection time blocks, see Table 2) and outside texture. Horizontal bars represent standard deviations.

3.3 Marker informativity

All nine microsatellite loci analysed were highly polymorphic, with a mean number of alleles per locus of 8.7 ± 2.4 (Table 5). The best performing locus in terms of PCR success was *Ccroc*07 (86% positive PCRs) and the least successful locus was *Ccroc*11 with 73% positive PCR.

With a total of 78 alleles, 14 alleles (18.0%) occurred only as one single copy, six of which (42.9%) were private alleles of KNSP (5.5% of the examined samples). 39.7% of the

alleles had a frequency lower than or equal to 0.05, 65.4% had a frequency lower than or equal to 0.10. The highest allelic frequency was 0.612 for one of the six alleles of *Ccroc*04. The marker with the lowest PI (0.025) was *Ccroc*06. The PI was the highest for *Ccroc*04: 0.216 (Table 5).

Table 5: For each locus, the overall number (#) of alleles, the overall PI value (unbiased), the number of other loci with which the locus shows LD, the degree of ADO, and the overall H_{exp} and H_{obs} (with the *p*-value of their difference) are given.

Locus	# Alleles	$PI_{unbiased}$	# LD	ADO	H_{exp}	H_{obs}	P-value
***Ccroc*02**	7	0.180	4	0.036	0.674	0.600	0.089
***Ccroc*04**	6	0.216	5	0.000	0.604	0.580	0.032
***Ccroc*05**	7	0.084	4	0.021	0.781	0.780	0.645
***Ccroc*06**	13	0.025	8	0.099	0.877	0.760	0.005
***Ccroc*07**	11	0.068	5	0.000	0.800	0.860	0.353
***Ccroc*08**	11	0.033	6	0.071	0.867	0.740	0.012
***Ccroc*09**	7	0.096	4	0.033	0.766	0.760	0.017
***Ccroc*10**	9	0.096	5	0.000	0.767	0.620	0.003
***Ccroc*11**	7	0.132	5	0.000	0.718	0.640	0.010
Total/Mean	8.7	$2.232*10^{-10}$	5	0.029	0.761	0.704	
SD_{mean}	2.4		1		0.087	0.097	

A total of eight new alleles were described for three of the spotted hyena microsatellite loci. For *Ccroc*06, six new alleles were found (240, 244, 248, 310, 316 and 326 bp), *Ccroc*08 and *Ccroc*10 had both one new allele (170 and 222 bp, respectively).

3.4 Consensus genotypes

The samples yielded 55 consensus genotypes. The mean percent of positive PCR was 83 ± 4%. The degree of ADO across loci was 0.029, ranging from 0 to 0.099 (Table 5). GIMLET did not report any case of FA. This is most likely because of the choice of the threshold value. With a threshold of 1, false alleles are always retained because it implies that any allele that occurs at least once is retained (Valière, 2002).

A remark here, is the fact that by only amplifying once in case of an entirely heterozygous genotype (Watts *et al.* 2011), contaminations can remain unnoticed. However, most of the samples (54.5%) have been amplified twice, 40% thrice, one sample (1.8%) even four times and only two samples (3.6%) once. This corresponds closely with the multiple tubes approach used in Taberlet *et al.* (1996). Nevertheless, Navidi *et al.* (1992) claim that for reliable results at least ten tubes (amplifications) per sample have to be applied.

3.5 Population size

The estimated population size for Mekelle was 899 ± 873 individuals (95% confidence interval = 208 - 4522). The probability of capture was estimated at 0.4% (without KSNP samples).

3.6 Identical genotypes

Out of the 55 consensus genotypes, 49 different genotypes were inferred by the regrouping function of GIMLET. Assuming that identical genotypes indicate the samples were from the same individual, six individuals had been sampled twice (Table 6).

Table 6: The six samples of which the genotypes appeared to have been sampled twice, together with their matching samples and details about the location and date of sampling. Regions: O = the old rubbish dump, C = the current rubbish dump, U = the Mekelle University campus, N = north of Mekelle, E = east of Mekelle and K = Kafta Sheraro National Park. A location value of x/y indicates sample a was sampled at location x and sample b at location y.

Sample a	Sample b	Region	Location	Date a	Date b
756	766	O	7	10/09/2014	10/09/2015
776	805	E	11/18	13/09/2014	15/09/2014
778	779	E	11/13	13/09/2014	13/09/2014
808	811	E	18	15/09/2015	15/09/2015
869	963	U/C	30/57	20/09/2015	7/10/2015
897	898	N	43	30/09/2015	30/09/2015

Of these, three had been found at the same location on the same day, two came from the same region but a different location, and one pair was sampled in two different regions (Table 6). However, from the latter pair, sample 869 had 60% missing values in its consensus genotype so therefore, unlike for the other pairs (see 2.3.5), samples 869 and 963 were both kept in the dataset.
The overall PI equalled $2.232*10^{-10}$ (Table 5).

3.7 Kinship

When two input files (genotypes and sex if known) were used for both the one parent and two parents analysis, the kinship analysis did not find a potential parent for any of the individuals, regardless of the number of incompatibilities. When sex was discarded from the input, with only one incompatibility (the number of loci that could not match between an offspring and a potential parent), the one parent analysis already showed a range of possibly related individuals for each genotype (average number of potential parents ± standard deviation = 6.9 ± 5.6). Only samples nr. 895, 896 and 964 were potentially from individuals whose parents were not present in the dataset. The parent pair analysis suggested that 25 out of the 55 genotypes were orphan.

These results are too vague to be of use in this study. As such, it was decided not to work further with them.

3.8 Population structure

The highest *Delta K* value generated by STRUCTURE HARVESTER was 37.35, suggesting two populations (Fig. 6). Plots of the Dirichlet parameter for degree of admixture showed that *Alpha* was relatively constant, with a mean of 0.167. The stagnation indicated that structuring in the population was found, although the range of *Alpha* was > 0.2 (Appendix 3). The low mean value implied that most samples were essentially from one population or another.

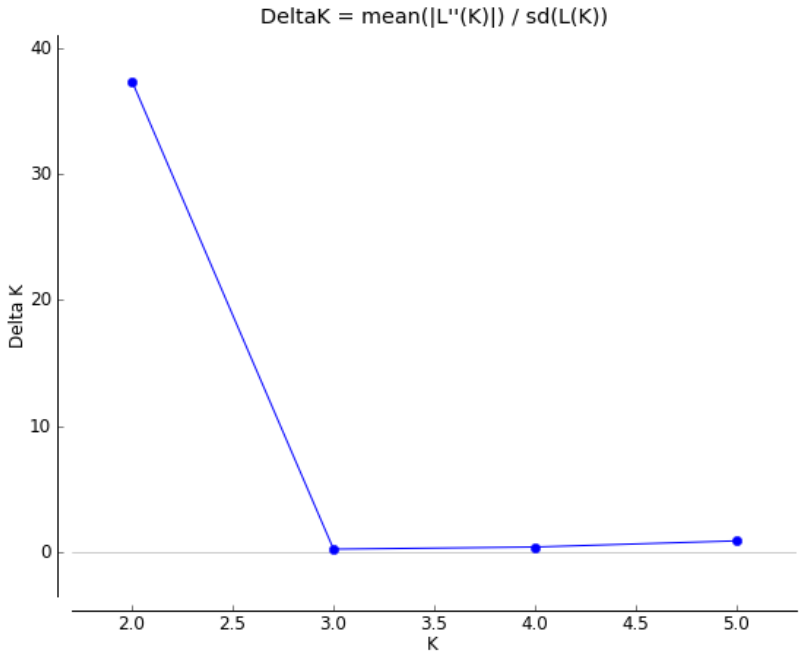

Fig. 6: Graph of *Delta K* for *K* (number of populations) ranging from 1 to 6, for the total number of samples (*n* = 50). *Delta K* peaks for a number of two populations.

Next, the samples from different regions were assigned to the two inferred clusters. It was found that the samples from region K (= KSNP) showed a mean proportion of membership (*Q*) of 0.962 for the second inferred cluster, while most of the Mekelle regions showed greater affinity for cluster 1 (Table 7). However, cluster 2 also corresponded primarily to regions U (the Mekelle University campus: 0.584) and N (north of Mekelle: 0.738), but both regions contained each only two samples.

Table 7: The proportion of membership of each predefined region (including KSNP, *n* = 50) for the *K* = 2 clusters. The last column shows the number (#) of samples that represent the region. Regions: O = the old rubbish dump, E = east of Mekelle U = the Mekelle University campus, K = Kafta Sheraro National Park, N = north of Mekelle, and C = the current rubbish dump.

Region	Cluster 1	Cluster 2	# Samples
O	0.858	0.142	16
E	0.730	0.270	13
U	0.416	0.584	2
K	0.038	0.962	3
N	0.262	0.738	2
C	0.703	0.297	14

The estimated membership coefficients per individual for each cluster are depicted in Fig. 7. The fact that the samples from regions U and N clustered more closely together with the samples from KSNP than those from the other Mekelle regions, can be explained by their mall sample size. For instance, Fig. 7 shows that also in regions O (the old rubbish dump), E (east of Mekelle) and C (the current rubbish dump) some samples corresponded to the second cluster. However, the majority of the other samples from these regions did not, which makes the region as a whole corresponded more to the first cluster.

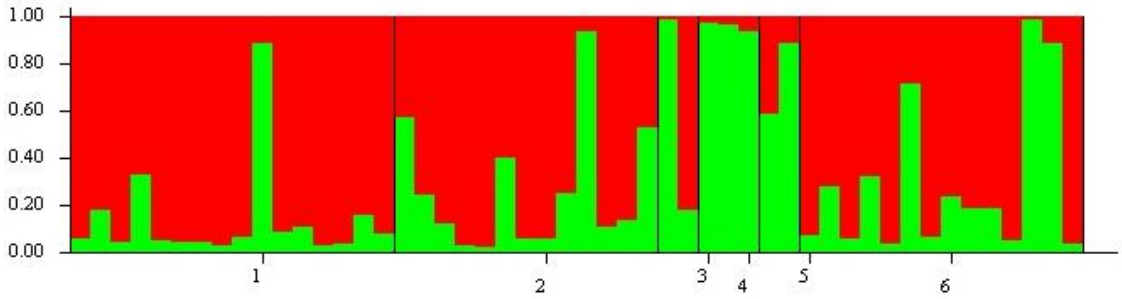

Fig. 7: The individual's estimated membership coefficients (*Q*) (y-axis) for the *K* = 2 clusters. Red = cluster 1, green = cluster2. Each individual is represented by a vertical line segment. Regions (1-6) are indicated on the X-axis: 1 = the old rubbish dump (O), 2 = east of Mekelle (E), 3 = the Mekelle University campus (U), 4 = Kafta Sheraro National Park (K), 5 = north of Mekelle (N), 6 = the current rubbish dump (C).

For the analysis without the samples from KSNP, the optimal number of clusters was again two. However, *Delta K* was low (1.52) and also a number of three clusters had a similar *Delta K* value: 1.27 (Fig. 8). As Pritchard *et al.* (2010) recommended, there was aimed for the smallest value of *K* that captures the major structure in the data: in this case: *K* = 2.

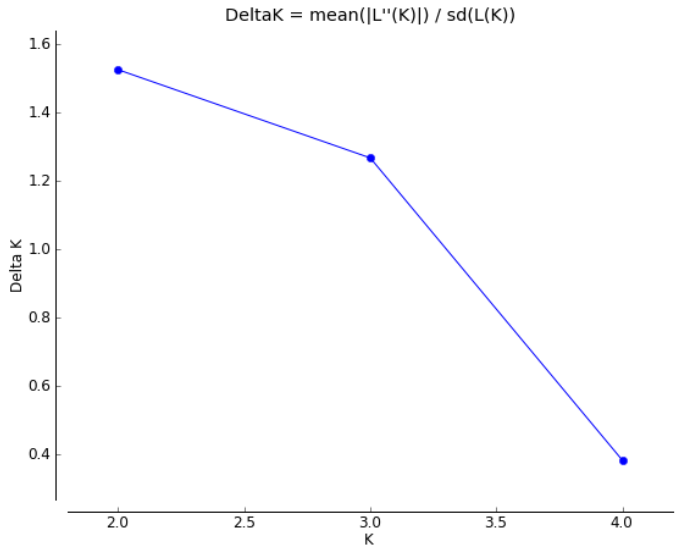

Fig. 8: Graph of *Delta K* for *K* (number of populations) ranging from 1 to 5, for the 47 samples without KSNP. Note that no real peak can be distinguished and that *Delta K* stays low compared to Fig. 6.

Furthermore, *Alpha* was not unambiguous: it seemed to converge but then again it started to oscillate with a mean value of 5.967 (Appendix 4). This could indicate that no real structure existed in the population. As shown in Fig. 9, all individuals had similar proportions of membership (*Q*) for the two clusters. This showed that all individual samples consisted of 50% genetic background from each of both clusters.

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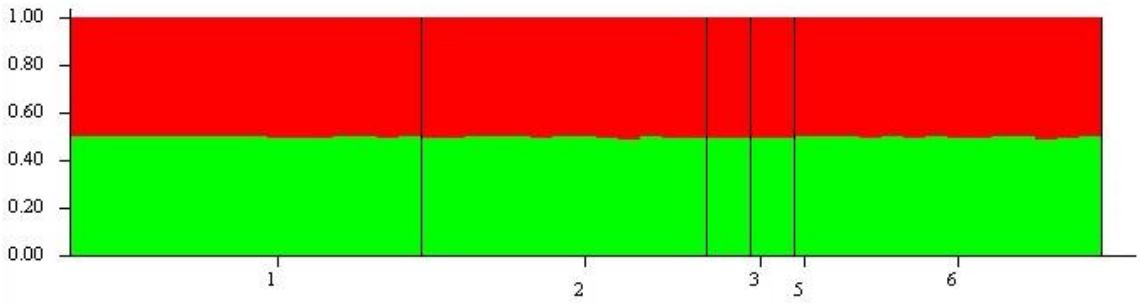

Fig. 9: The individual's estimated membership coefficients (*Q*) (y-axis) for the *K* = 2 clusters. Red = cluster 1, green = cluster2. Each individual is represented by a vertical line segment. Regions (1-6) are indicated on the X-axis: 1 = the old rubbish dump (O), 2 = east of Mekelle (E), 3 = the Mekelle University campus (U), 5 = north of Mekelle (N), 6 = the current rubbish dump (C).

Also for the regions an indication of one panmictic population was found: the mean *Q* ± standard deviation over all five regions for cluster 1 was 0.501 ± 0.002 (Table 8), which again suggested admixture.

Table 8: The proportion of membership of each predefined region (without KSNP, *n* = 47) for the *K* = 2 clusters. The last column shows the number (#) of samples that represent the region. Regions: O = the old rubbish dump, E = east of Mekelle U = the Mekelle University campus, N = north of Mekelle, and C = the current rubbish dump.

Region	Cluster 1	Cluster 2	# Samples
O	0.498	0.502	16
E	0.501	0.499	13
U	0.504	0.496	2
N	0.503	0.497	2
C	0.501	0.499	14

While STRUCTURE showed panmixia for Mekelle, some differences appeared in levels of fixation of alleles between the predefined regions (Table 9). However, the populations with only two samples (the Mekelle University campus and north of Mekelle) are not considered here, because of their low power (see Table 7). The old rubbish dump did not

show any difference in F_{ST} with any of the other regions. The samples from KSNP did show significantly different allele frequencies from the east of Mekelle (F_{ST} = 0.123) and the current rubbish dump (F_{ST} = 0.082). These are normal values for differentiated populations, given the fact that F_{ST} correlates with homozygosity and microsatellite loci are known for their high heterozygosities (Bowcock *et al.*, 1994). However, contradicting the STRUCTURE results, the east of Mekelle was also significantly differentiated from the current rubbish dump (F_{ST} = 0.019).

Table 9: Population pairwise F_{ST} values. * = significant difference (p < 0.05). Regions: O = the old rubbish dump, E = east of Mekelle, U = the Mekelle University campus, K = Kafta Sheraro National Park, N = north of Mekelle and C = the current rubbish dump.

Region	O	E	U	K	N	C
O						
E	0*					
U	0*	0.147				
K	0*	0.123*	0.101			
N	0*	0.113*	0.194	0.149		
C	0	0.019*	0.113	0.082*	0.050	

3.9 Assumptions

The significant linkages (LD) between loci are summarized in Appendix 5. Locus *Ccroc*06 is linked to all other loci, *Ccroc*08 with seven of the others (not with *Ccroc*05 and *Ccroc*11). The loci with the smallest number of linkages are *Ccroc*02, *Ccroc*05 and *Ccroc*09 (each four linkages) (Table 5).

Mean H_{obs} over all loci was 0.69 ± 0.10 for all samples together (Table 5), without the three samples of KSNP the mean was 0.70 ± 0.10 . Mean H_{exp} was slightly higher: 0.73 ± 0.10 with and 0.72 ± 0.09 without KSNP. Three loci did not show significant differences between the overall H_{exp} and H_{obs}: *Ccroc*02, *Ccroc*05 and *Ccroc*07.
The inbreeding coefficient for Mekelle was 0.028.

3.10 Sex ratios

For 30 out of 50 samples the sex was successfully determined (34 out of 55, including the identical genotypes). The overall number of females per male was 0.88, omitting the samples of KSNP the sex ratio equalled 0.87. Table 10 summarizes the number of females and males found in each region.

Table 10: Number of females and males per region, and the number of females per male (Sex ratio). The proportion of samples of which the sex was determined (Sexed) is also given (total number of samples: see Table 7). Regions: C = the current rubbish dump, E = east of Mekelle, K = Kafta Sheraro National Park, N = north of Mekelle, O = the old rubbish dump, and U = the Mekelle University campus.

	Total	**C**	**E**	**K**	**N**	**O**	**U**
Females	**14**	2	4	1	1	6	-
Males	**16**	2	8	1	1	4	-
Sexed	**0.68**	0.29	0.92	0.67	1.00	0.63	0.00
Sex ratio	**0.88**	1.00	0.50	1.00	1.00	1.50	-

4. DISCUSSION

This study showed that scats can be a useful source of DNA to study population genetics of hyenas. However, the success of the extractions depended on the quality of the samples. Freshness was important, although the statistical power of the test was not high given the limited number of samples that could be investigated. Nevertheless, the PCR results of *Cyt b II* at the IZW were also better for soft samples, which confirms the previous conclusion. The colour of the scats was, with higher confidence, a better predictor of how well DNA could be obtained. Brownish scats provided better results than scats with a lighter colour. However, since scat colour is most likely linked with the diet of the animals, preferring scats based on this feature might also cause a bias towards hyenas with a higher social rank. Since those individuals have first access to carcasses, they will produce less light coloured scats than subordinates, who are often left with the bones (Kruuk, 1972; Tilson & Hamilton, 1984; Frank, 1986b; Mills, 1990; Smale *et al.*, 1993). It is therefore highly recommended to sample lighter scats as well, preferably fresh ones (Santini *et al.*, 2007; Vynne *et al.*, 2011).

Unfortunately, no markers for variable parts of the spotted hyena mtDNA could be used because the *Cyt b* regions showed low variability, the PCR success was too low and no primers existed yet for the control region. This means that the population structure could not yet be investigated with the resolution that we had hoped. However, the decent quality data of the nine microsatellite markers and the success with the sex determination primers did allow preliminary investigations of population structure and, although it still concerns rough estimates, the results were quite surprising. Since there still are more samples available than could be analysed in the end here, there are good prospects for further work.
In this study, eight new microsatellite alleles were found compared to what previous studies from the Ngorongoro Crater and the Serengeti National Park, Tanzania (East *et al.*, 2003; Wilhelm *et al.*, 2003; Höner *et al.*, 2007; East *et al.*, 2009; Höner *et al.*, 2010). This offers opportunities for phylogeographic and genetic diversity studies in which all data are combined (East *et al.*, unpublished data). 18% of the alleles occurred as one single copy and presume the risk of loss in future generations (e.g. Castilho *et al.*, 2012; Wright, 1931) This is a high number, but because of their non-coding feature, microsatellites also evolve very fast (Jarne & Lagoda, 1995; Sunnucks, 2000). This means that lost alleles will always be replaced by new ones. Furthermore, because of their neutrality, losing some of these alleles does not affect the phenotype of the individual so this is not of high importance.

As expected, a great proportion of these single alleles were private alleles of a national park more than 300 km away. This was reflected in the results of the population structure analysis, where the samples from this national park were distinguished from the panmixed Mekelle population. This showed this distant population could be truly considered as an 'outgroup' with which social structure results from Mekelle could be compared with. Regrettably, since only three samples were found in the national park, the sample size was not large enough to assess social structure.

Representing the probability that two individuals in the population share the same genotype, the PI can be seen as the informativity of a particular locus (Valière, 2002). Since the total PI is the product of all the separate PI's together, the combination of the used markers gives a good total probability of identity: the chance that two samples with the same genotype originated from a different individual was very low.

Both the structure assessing models and the computation of the PI assume independence of loci. However, significant LD was found between all the different markers. This means that the results reported should be interpreted with care. The overall PI may then be very small, the actual PI (and thus the chance that two samples with the same genotype are not from the same individual) might be higher.

The test for LD on its turn, like other models used in this study, assumes Hardy-Weinberg proportions of genotypes (Excoffier & Slatkin, 1998). The differences between the expected and observed heterozygosity values for all samples together were negligible. Nevertheless, most of the individual loci (66%) showed significantly different values for H_{exp} and H_{obs}. This indicates deviations from HWE. Although the differences were not very large, this is again an indication that the results of the present study should be handled with care.

The population size estimate for spotted hyenas around Mekelle was about 900. This estimate had a large confidence interval, but even at the lower end of that interval, this is a very high number, given the area that was covered in this study was about 490 km², of which a large part is occupied by humans. Even disregarding the human built-up area, the density of the hyenas already reaches a value of 1.84 individuals/km². Previous studies showed that spotted hyenas densities vary greatly throughout the species' range but are generally much smaller: from 0.004 to 0.0085 individuals/km² in the Namib Desert (Skinner & van Aarde, 1991; Tilson & Hamilton, 1984; Tilson & Henschel, 1986), similar to what was found in Kruger National Park, South Africa (Mills, 1985) and

Southern Kalahari (Mills, 1990), over 0.44 individuals/km² in Chobe National Park, Botswana (Cooper, 1989) to about 0.8 individuals/km² in the Maasai Mara National Reserve, Kenya (Frank, 1986a, 1986b), the Serengeti (Hofer & East, 1993a) and the Ngorongoro Crater (Höner *et al.*, 2007, 2005), where in 1972 a density of 1.54 individuals/km² was reported (Kruuk, 1972). More recently, the highest density (1.25 individuals/km²) was recorded in the Hluhluwe-iMfolozi Game Reserves, South Africa (Graf *et al.*, 2009). As such, the Mekelle population seems substantially different from populations in more 'natural' conditions.

Even more surprising was the fact that this whole population did not show genetic structuring, while the samples from a national park more than 300 km away were clearly distinguishable. Only one weak signal indicating two groups was intercepted (see below). Of course, as mentioned before, the resolution was low and therefore it cannot be concluded with full certainty that all the individuals indeed belong to a single clan. Furthermore, clans are not reproductively isolated from each other because of migrating males (Mills, 1985; Frank, 1986a, 1986b; Smale *et al.*, 1993; East & Hofer, 2001; East *et al.*, 2003; Van Horn *et al.*, 2004; Watts *et al.*, 2011). Yet, if this population indeed comprises of one clan, this would be the largest clan size ever reported for spotted hyenas. Even the estimated minimum is more than twice the size of the clan with the most individuals cited in the literature, which is 95 for the Ngorongoro Crater (Kruuk 1972; Höner *et al.*, 2005). The maximum number of individuals belonging to one clan recorded in other studies varied between eight in the Namib Desert (Tilson & Henschel, 1986), 11 in Kruger National Park (Henschel, 1986 in Mills, 1990), 14 for the Southern Kalahari (Mills, 1990) and Hluhluwe-iMfolozi Game Reserves (Whateley & Brooks, 1978; Whateley, 1981), 52 in the Maasai Mara National Reserve (Frank, 1986a), 62 in Chobe National Park (Cooper, 1989) and 80 in the Serengeti, (Frank 1986a; Hofer & East, 1993a). A single clan consisting of many more than 200 individuals, would thus be a fascinating result. It can be questioned whether such group can still be considered a clan, because spotted hyenas in the previously studied clans know every other individual's rank in the dominance hierarchy within the clan (Tilson & Hamilton, 1984; Frank, 1986b; Smale *et al.*, 1993). With such large groups, that seems very unlikely as it would require even stronger developed cognitive abilities than already reported (Holekamp *et al.*, 2007). On the other hand, it can also mean that in this population, the social ranks are of less importance than in 'natural' populations, which again would be a remarkable outcome, but may be linked to the easy access to abundant food sources. Tilson & Hamilton (1984) reported that for spotted

hyenas in the Namib Desert, hierarchies develop in groups of these social hunters, but are expressed only under special environmental circumstances such as the presence of large carcasses. It is possible that around Mekelle, where offal is abundant and hyenas barely hunt (Abay *et al.*, 2011; Yirga *et al.*, 2013a, 2013b), only close kin recognition is needed and hierarchical ranks became blurred. If this is true, the question that can be asked is to what extent we can still speak about a 'clan'.

However, a significant, yet relatively low, pairwise F_{ST} value for samples from the westerly current rubbish dump and the east of Mekelle was found, which suggests some degree of genetic differentiation. These two regions are also geographically poorly connected. The east site lies on a higher plateau with its own village (Quiha) and thus also a small offal source, and the city of Mekelle separates the two regions (Fig. 2). Given the fact that the sample size of both regions was > 10, this could be an indication of the presence of a separate subpopulation or clan. Nevertheless, even if the analysis of more samples would indeed point toward the existence of two clans, this would still be a remarkable result. It would imply that two very large spotted hyena clans coexist in a relatively small, natural prey-depleted area. Furthermore, since the main source of food is the current rubbish dump next to the city, and hyenas are known to be able to travel vast distances for one foraging trip (Eloff, 1964; Hofer & East, 1993b; Kolowski *et al.*, 2007), chances are high individuals of both clans do encounter each other at night during feeding sessions. This is not the case for most other spotted hyena populations, where resources are only shared with other clan members (Tilson & Henschel, 1986; Cooper, 1990; East & Hofer, 1991; Hofer & East, 1993b; Boydston *et al.*, 2001; Trinkel *et al.*, 2004; Smith *et al.*, 2008). Even in areas where spotted hyenas regularly leave their clan's territory to feed on the nearest migratory herds in times of low resident prey abundance ('commuting trips'), residents still respond aggressively to commuters at kills, and engage in prolonged clashes with neighbouring clans (East & Hofer, 1991; Hofer & East, 1993a, 1993b). Perhaps in Mekelle, since the food is abundant and localized, these feeding places are considered neutral ground? Something similar is observed in the Ngorongoro Crater, where eight clans with between 24 and 65 members co-occur within an area of 250 km² (Höner *et al.*, 2005, 2007, 2010). Also here, the clan's foraging is not confined to their own territory and in some cases intruders are tolerated (Höner *et al.*, 2005). In the present study, during a 'call-in' (broadcasting of spotted hyena and gnu distress calls at dusk Ogutu & Dublin, 1998) on the road between Mekelle and the old rubbish dump, about 90 individuals were attracted by the sounds emitted (personal observation, see front cover picture). Three weeks later,

at the current rubbish dump, between 50 and 70 individuals were observed. This not only confirms the very high number of hyenas around Mekelle (in the Maasai Mara, the monthly means of individuals that responded to call-ins ranged between 7 and 17, Ogutu & Dublin, 1998), it also shows their tolerance towards conspecifics, since not one conflict was observed. If the observed hyenas belong to different clans, this certainly is a sign of close coexistence. Alternatively, this could again be seen as an indication that all present individuals belong to the same clan. The fact that they gather at night, could explain why they can sleep separately in more remote places. It is clear that these findings open doors for further research about where individuals that meet at night rest, whether this changes over time, and whether different clans can be distinguished. Also, no matrilines were estimated in this study yet. It is definitely worthwhile to analyse also the remaining 213 samples that were collected but not yet analysed in this study. Do females still stay with their mothers at the age of sexual maturity? Do these matrilines aggregate with other female lineages, accepting only a few unrelated males, like what is known for 'wild' clans (Mills, 1985; Frank, 1986a; Mills, 1990; Smale *et al.*, 1993; Van Horn *et al.*, 2004)? Or are there changes in dispersal patterns, with males becoming more philopatric than in natural environments?

Indeed, the overall sex ratio (0.87 females per male) was considerably lower than what other studies report for spotted hyenas elsewhere in Africa, where females are more abundant than males. In the Serengeti, the sex ratio (females/males) was 1.19 (Hofer & East, 1993a), in the Maasai Mara this was 1.8 (Watts, 2007, in Holekamp & Dloniak, 2011), in the Southern Kalahari 2.5 (Mills, 1990), and even 3.29 females to 1 male were observed in Chobe National Park, Botswana (Cooper, 1989). The current sample size may then not be large, samples were collected at different locations so they do represent the whole Mekelle population, and not one spatially confined part. Furthermore, the region where the largest amount of samples were successfully sexed, east of Mekelle, strikingly showed the most aberrant sex ratio (0.5 females per male). What could be the cause of this atypical abundance of males? One possibility is that females exhibit a different defecation behaviour than males (e.g. defecate less at latrines), which biased the present study's results. However, to present, no evidence in the literature supports this, and female and male samples originated from the same location.

Could the high numbers of males be linked to the large population size? In 'natural' populations, males disperse after they become reproductively mature (Boydston *et al.*, 2005; Höner *et al.*, 2007, 2010). This dispersal is thought to result from an adaptive

response by males to simple female mate-choice rules that have evolved to avoid inbreeding (Höner *et al.*, 2007). Females prefer sires that were born or immigrated into the female's group after the female was born, and by initiating their reproductive careers in groups containing the highest number of young females, males have a higher long-term reproductive success (Höner *et al.,* 2007). As such, it is the male's choice to disperse or to stay. In Mekelle, males could prefer to stay in their natal clan because of the high food abundance and the presence of enough non-related females. East & Hofer (2001) even report that (rare) 'nondispersers' quickly acquired top rank in the male social hierarchy in the Serengeti, which could have facilitated the development of this trend of staying. Of course, this could only have been a trigger, since evidently not all males can obtain the highest rank. In any case, the inbreeding coefficient of the study population was very low, which indicates random mating (Hedrick, 2000). If males do tend to stay, it certainly does not affect the population's microsatellite heterozygosity (Crnokrak & Roff, 1999). The low inbreeding coefficient can also demonstrate that the male biased sex ratio is not the result of non-migrating males.

The results of the current study clearly indicate substantial differences in the population structure of spotted hyenas around Mekelle compared to 'natural' populations. This all shows this carnivore exhibits high adaptability to changing environmental conditions, here changes in food availability and the close presence of humans. The differences are so striking that one could wonder whether they are based on phenotypic plasticity, or whether it concerns genetic changes selected for in this special environment (Savoilainen *et al.*, 2013). It is sure that spotted hyenas possess a certain basis level of behavioural plasticity. This is not only demonstrated by the fact that they can live on various prey (Hayward, 2006; Holekamp & Dloniak, 2011), and survive successfully in a wide range of habitats (Whateley & Brooks, 1978; Whateley, 1981; Frank, 1986a; Tilson & Henschel, 1986; Cooper, 1989; Mills, 1990; Hofer & East, 1993a). Densities and clan sizes also often correlate with prey abundance (Kruuk, 1972; Tilson *et al.*, 1980; Tilson & Hamilton, 1984; Tilson & Henschel, 1986; Mills, 1990; Carbone & Gittleman, 2002; Hayward *et al.*, 2007), spotted hyenas even adjust actively their grouping patterns in response to feeding competition (Smith *et al.*, 2008) and are known to decouple their feeding ranges from territoriality when prey abundances are low (Hofer & East 1993a; Höner *et al.*, 2005). Furthermore, in Wukro, about 50 km from Mekelle, similar conditions as in Mekelle were discovered, with a population estimate (based on call-in counts) of 535 (Yirga *et al.*,

2013c). However, other studies reported consistent avoidance of human settlements by hyenas (Croes *et al.*, 2011). Yet, this definitely will also be affected by behaviour of the inhabitants of the human communities, since hyenas are often persecuted (Mills, 1990; Mills & Hofer, 1998; Ogada *et al.*, 2003; Kolowski & Holekamp, 2006; Holmern *et al.*, 2007; Kissui, 2008), but not so in northern Ethiopia (Abay *et al.*, 2011; Yirga *et al.*, 2012, 2013a, 2013b).

The fact that spotted hyenas show such an adaptability, is good news from a conservation point of view. As the human population keeps increasing, more and more space will be occupied by people and more large carnivores will find themselves living in the vicinity of human settlements and cities. It is of great importance to be able to demonstrate that coexistence with people does not have to necessarily lead to human-wildlife conflicts (which is indeed already observed in Mekelle, Yirga & Bauer, 2010a, 2010b; Abay *et al.*, 2011; Yirga *et al.*, 2013b) and furthermore that the animals can cope with and even benefit from these new conditions. This is valuable information for the decision making for regional planning. In Mekelle for instance, there are plans to convert the half-open rubbish dumps to a closed system (Yirga, 2013). This could be detrimental for the hyena population, and because of the lack of scavengable food, they would be forced to prey on live domestic animals. As such, the famers would also likely suffer more livestock losses, which might impact the human-hyena relationship.

In line with this, also with regard to other species these results can be of significance. Conserving biodiversity, particularly of larger wildlife, often appears incompatible with the environmental changes that are linked to increasing human population densities and welfare (Kerr & Currie, 1995; Forester & Machlis, 1996; Czech *et al.*, 2000; Peterson *et al.*, 2010). Indeed, when settlements develop, large carnivores have difficulties to survive and are often seen as a liability rather than an asset. There are only few examples of where these animals manage to remain present. Lions (*Panthera leo*) attack grazing livestock during the daytime, which makes them vulnerable to direct retaliatory killing (Kissui, 2008), and there are no examples of where lion populations persist close to urbanised settlements. African leopards (*Panthera pardus pardus*) are considered highly adaptable since they are solitary and able to occupy most environments, including village surroundings (Sunquist & Sunquist, 2002), but they suffer from genetic diversity loss due to poor population connectivity by anthropogenic activities (McManus *et al.*, 2015).

The brown bear (*Ursus arctos*), wolf (*Canis lupus*), and Eurasian lynx (*Lynx lynx*) already vanished from all regions of high human activity in Europe because of direct persecution and environmental changes, although they are now gradually coming back (Breitenmoser, 1998). However, just like red foxes (*Vulpes vulpes*) who turned from a shy rural predator to a common species in many cities (Kamler & Ballard, 2002), animals may change their behaviour so as to fit in these ‘unnatural ‘ environments. Conflicts with wolves and American black bears (*Ursus americanus*) still occur in the U. S. A., but American black bears in cities adjust their activity pattern so that they are most active during late night when human activity is minimal (Treves *et al.*, 2004; Merkle *et al.*, 2011). The hyenas of Mekelle are another example of where both animals and people have adapted to each other’s presence with the carnivores having changed their lifestyle substantially and becoming even more abundant than in most natural environments.

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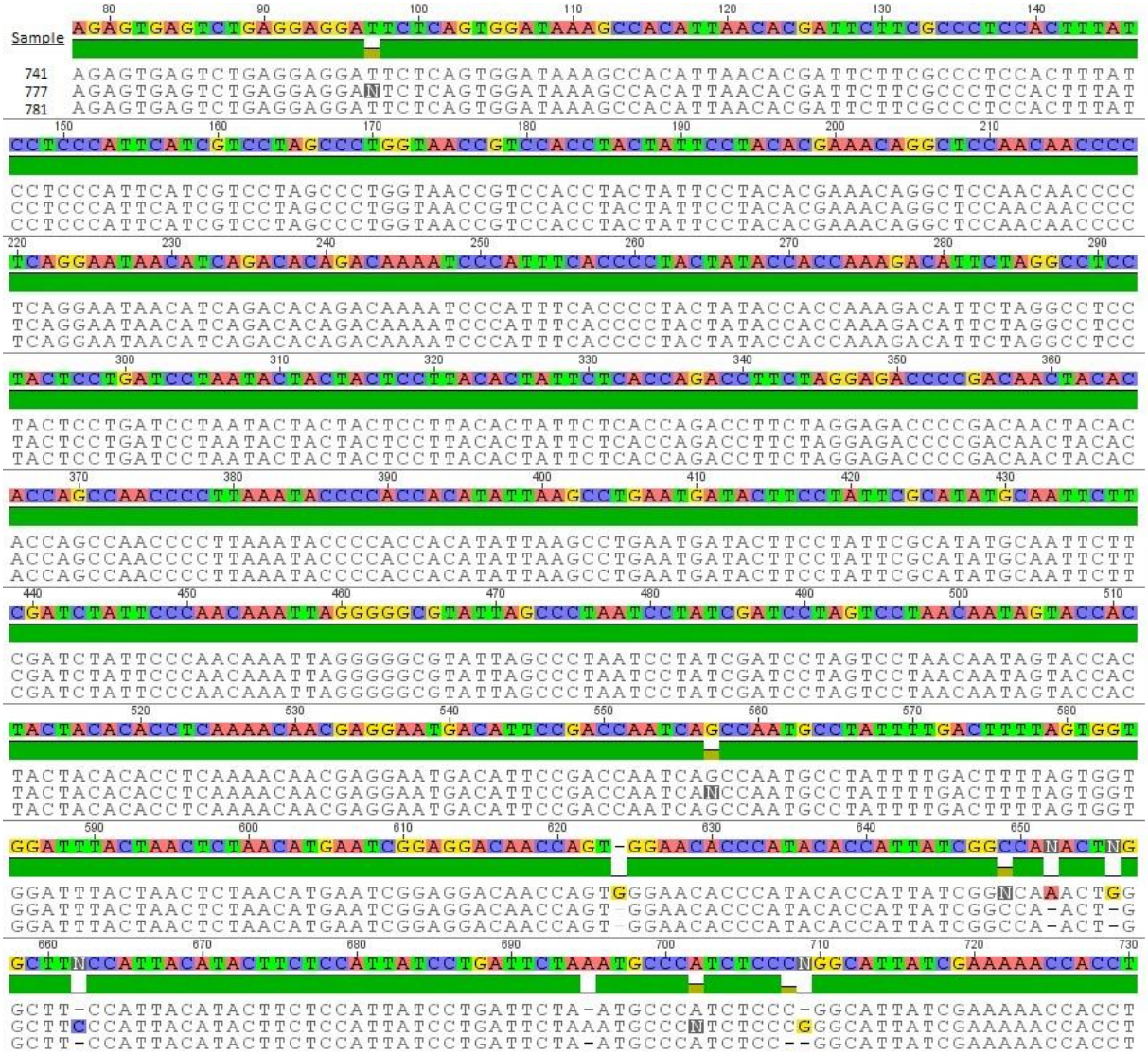
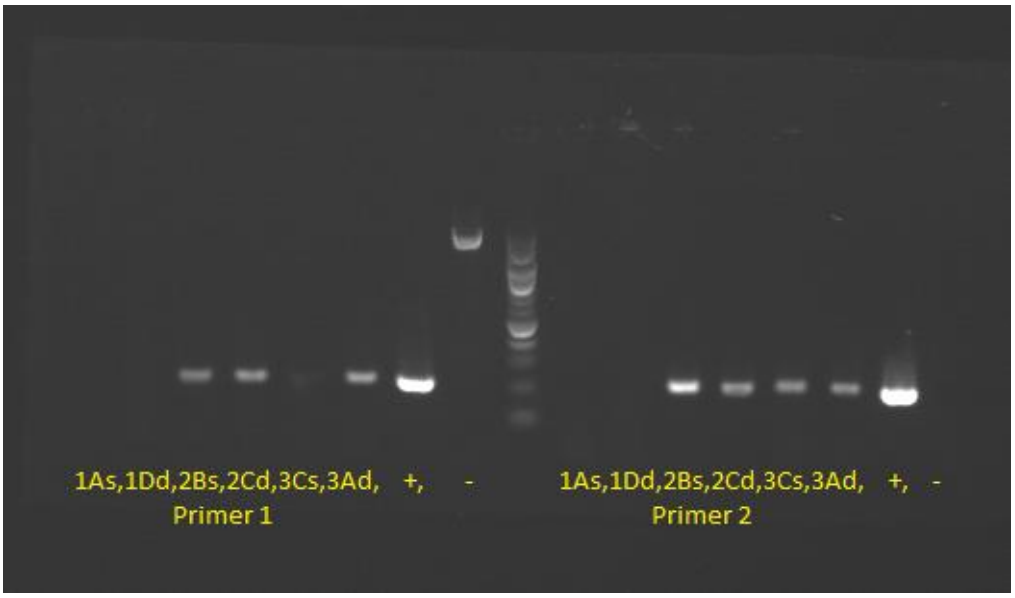
Yekanyele!

Elien Schramme

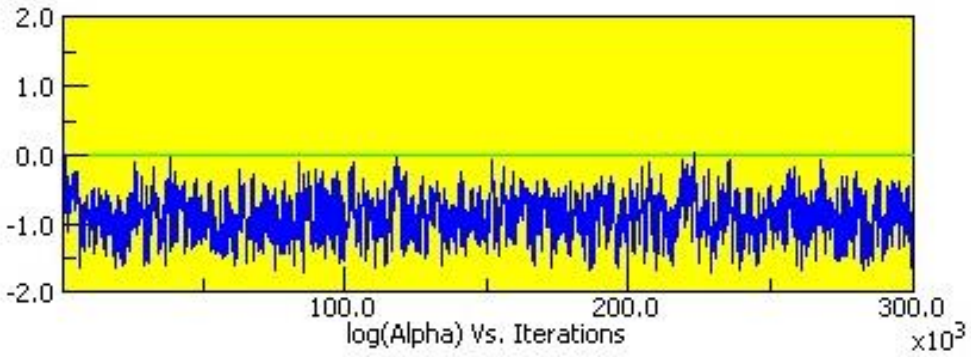
APPENDICES



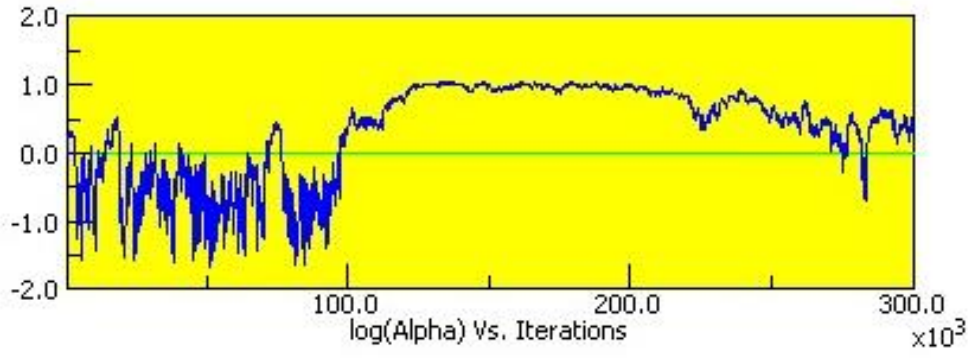
Appendix 1: Alignment of the *Cyt b II* sequences of three samples (741, 777 and 781). The sequences obtained by the reverse primer (H-15915) have been mirrored and added to those of the forward primer (L-15162) in order to get a better consensus sequence of each sample before combining them. The coloured characters A, C, T, G above represent the nucleobases adenine, cytosine, thymine and guanine that build up the consensus sequence. The green band indicates that the three samples below (white background characters) have the same nucleobase at this position. There are only a few positions where the result is not unambiguous (N = any base). The sequences of the samples from the rest of Africa are not depicted because of confidentiality agreements with the involved researchers at the IZW.



Appendix 2: PCR products of *Cyt b* 1&2 (Rohland *et al.*,2004) after separation on an agarose gel. 1-3 = individu, A-D = preservation methods tested (more methods than discussed in the text were tested in this study): A: 75% EtOH, B: 75% EtOh to silica, C: droog, D: silica), s/d = silica/diatom (in protocol Boom *et al.*, 1990). '+ ' stands for positive control (blood), '- ' stands for negative control (H_2O). A 500 bp ladder is visible in the middle. The extraction or PCR of individual 1 was not successful.



Appendix 3: Log(*Alpha*) for *K* = 2 over 200,000 iterations, for the total number of samples (*n* = 50).



Appendix 4: Log(*Alpha*) for *K* = 2 over 200,000 iterations, for the 47 samples without KSNP.

Appendix 5: Overall linkage disequilibrium (LD) between the nine loci examined in this study. A '+ ' stands for a significant association between loci, a '- ' indicates no association.

Locus	*Ccroc*02	*Ccroc*04	*Ccroc*05	*Ccroc*06	*Ccroc*07	*Ccroc*08	*Ccroc*09	*Ccroc*10	*Ccroc*11
***Ccroc*02**									
***Ccroc*04**	-								
***Ccroc*05**	-	+							
***Ccroc*06**	+	+	+						
***Ccroc*07**	-	-	+	+					
***Ccroc*08**	+	+	-	+	+				
***Ccroc*09**	-	+	-	+	+	+			
***Ccroc*10**	+	-	-	+	+	+	-		
***Ccroc*11**	+	+	+	+	-	-	-	+	